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A study of gastrointestinal nematodes co-infecting feral Soay sheep on St Kilda

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ABSTRACT

The unmanaged, feral Soay sheep population on St Kilda has survived for hundreds of years, despite enduring potentially deleterious gastrointestinal nematode co-infections. Co-infections with multiple nematode species are ubiquitous in feral and managed ruminants. Within these mixed burdens, the different species may vary in their pathogenicity, epidemiology and clinical presentation. Elucidating the diversity of different parasite species in a host, rather than studying them as a homogenous group, is a prerequisite to understanding host-parasite interactions. The primary aim of this thesis is to explore and validate non-invasive conventional and next generation molecular parasitological methods to identify and quantify mixed-species infections in feral hosts.

Seasonal patterns of gastrointestinal nematode parasitism in the Soay sheep were investigated by faecal egg counts (FEC). Two FEC datasets were analysed: a large dataset, collected between 1988 and 2014, and counted by a modified McMaster method where each egg count represents 100 eggs per gram (epg); and a smaller dataset collected over one year between April 2015 and April 2016, counted by cuvette salt floatation method with a lower detection limit of 1 epg. FEC generally declined with increasing sheep age, until the animals became geriatric (8 years +). Seasonal FEC patterns in females generally followed a decline over the year starting in Spring. FEC were generally higher in males, regardless of their age category, with little seasonal variation between summer and winter once they became adults (3 years +). Monte Carlo simulations were run in order to compare the effects of different detection limits between the FEC methods detecting eggs in faeces to 100 epg and to 1 epg. The simulations suggest that the method with the detection limit of 50 – 100 epg over-estimates the true egg counts within the samples (at high counts), resulting in data that is highly negatively skewed and with an inflated mean. Despite within-year variation of egg counts, both datasets resulted in overall similar seasonal and host patterns. A shorter study with fewer replicates may benefit from a FEC method with a lower detection threshold.

The eggs of most strongyle nematodes are morphologically similar, hence whilst FEC can identify general trends in parasitology, the method provides limited information about the proportions of mixed gastrointestinal nematode species burdens of the Soay sheep. The development of advanced molecular methods for the in-direct genus or species-specific diagnosis of strongyle infections in ruminants has negated many of the issues arising from traditional parasitological techniques. Chapter 3 compared two molecular methods; a semi-automated multiplex-tandem PCR (AusDiagnostics™) with ITS-2 rDNA next-generation amplicon

sequencing (nemabiome assay), to identify species (presence/absence), and quantify the relative proportion (%) of ovine strongyle species in naturally infected samples. This chapter provides the first, and preliminary, comparison of the sensitivity and quantitative ability of both methods. There was good agreement between both molecular tests in determining the presence/absence of species within a sample, but the correlation in their ability to quantify relative proportions of the species present was moderate (*C. ovina*, $R^2 = 0.6096$) to poor (*Trichostrongylus* spp., $R^2 = 0.2334$). AusDiagnostics™ characterises *a priori* strongyle species present, which partially suits a diagnostic tool for managed ruminant systems. The nemabiome assay proved to be the most effective method for identifying and quantifying previously unknown strongylid species, such as those associated with a feral host.

The sensitivity, bias, and repeatability of the nemabiome assay was tested for the strongylid species identified in the Soay sheep of St Kilda (Chapter 4). A correction factor was calculated for each species in order to reduce potential species-specific sequencing bias. This was subsequently applied to the field data presented in Chapter 5.

Chapter 5 provides an epidemiological survey of the strongyle nematode species co-infecting the Soay sheep of St Kilda. *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus vitrinus*, *Chabertia ovina* and *Bunostomum trigonocephalum* were identified by the nemabiome assay. The study highlights epidemiological trends in the Soay sheep that had not previously been identified using conventional gross and molecular parasitological methods. There were seasonal, age and sex differences in species proportions; whereby trends appeared to correspond with the dynamic life-history of the sheep. Trends included a year round prevalence of all species in lambs (males and females) to the age of one year old, high levels of *T. circumcincta* over summer and winter in females older than 8 years, and no clear seasonality to *T. axei* which persisted, at low levels, over all months in all sex/age groups sampled. However, when relative species proportions were adapted to account for average FEC, the impact of the trends seen was minimised; the adequate representation for individual nematode species relied on a high faecal egg count. This is the first study to use the nemabiome deep amplicon sequencing approach to characterise seasonal patterns in different co-infecting gastrointestinal nematodes in feral sheep. Additionally, it highlights the flexibility of the nemabiome assay as a viable non-invasive tool for parasitological surveys of wild animals.

Better knowledge of the epidemiology of different co-infecting gastrointestinal nematodes in the ancient feral St Kilda Soay sheep population could help to explain the impact of grazing management and anthelmintic drug treatments in managed sheep flocks, aiding in the development of sustainable control strategies.

LAY ABSTRACT

Gastrointestinal roundworms present significant welfare and economic costs to the sheep and goat industries worldwide. Current strategies combining anti-parasitic drug use and grazing management are unsustainable; due to the emergence of multidrug and multi-roundworm species resistance. Co-infections with one or more roundworm species are commonly seen in seen in grazing livestock. Within these mixed burdens, the different species may vary in their pathogenicity, life histories, clinical presentation and resistance to anti-parasitic drugs. A fuller understanding of roundworm interactions based on the accurate characterisation of the parasite communities is needed to inform sustainable parasite control. However, until recently, comparative research on co-infecting roundworms was limited by the challenges associated with accurate morphological identification of species, which requires considerable taxonomical expertise. There is, therefore, a need for an accurate, non-invasive and affordable method of diagnosis of roundworm co-infections of livestock, to investigate the patterns of infection and the biology of the parasites in their environment. Researching the population structures of co-infecting roundworms in unmanaged animals is required to recognise the impact of management decisions on sustainable control. The unmanaged, feral population of Soay sheep on the St Kilda archipelago, 40 miles west of the Scottish Outer Hebrides, in the North Atlantic Ocean, provides an ideal study population. The Soay sheep has survived for thousands of years on an isolated island, despite enduring potentially deleterious roundworm co-infections. The primary aim of this thesis is to explore and validate non-invasive parasitological methods to identify and quantify mixed-species roundworm infections in feral sheep.

The non-invasive, microscopic identification and quantification of eggs within a faecal sample, is an indirect method to measure roundworm burden. Faecal egg counts (FEC) in the Soay sheep age generally declined with age, until they passed their prime (8+ years). Seasonal FEC patterns generally followed a decline over the year. FEC were generally higher in males, regardless of their age category, with little seasonal variation between summer and winter once they came adults (3+ years).

Whilst FEC can identify general trends in parasitology, it is too crude a method to distinguish parasite diversity within the total roundworm population. The development of advanced DNA-based methods to speciate and quantify faecal roundworm parasites has negated many of the issues arising from traditional parasitological methods. This thesis compared two of these methods to identify the presence or absence, and to quantify relative proportion of roundworm species in naturally infected feral sheep faecal samples. The deep amplicon sequencing DNA-

based method was validated for the roundworm species identified in the Soay sheep, ensuring the method is accurate by determining correction factors.

Finally, a survey was undertaken of the gastrointestinal roundworm species infecting the Soay sheep of St Kilda. The study highlights trends in the patterns of co-infection by different roundworm species in the Soay sheep that had not been previously identified using conventional parasitological methods. There were seasonal, age and sex differences in species composition; whereby trends appeared to correspond with the changing annual and seasonal life-history of the sheep. The work described in this thesis will contribute to the understanding of trends in parasite co-infections that is required to inform sustainable gastrointestinal roundworm control strategies in managed grazing livestock.

DECLARATION

I declare that I am the sole author of this thesis. I conducted all the analyses and wrote the thesis under the guidance of my supervisors. All work presented in this thesis is my own, with the following acknowledgements:

The long term data used in this thesis (Chapter 2) had been collected by others as part of the ongoing St Kilda Soay Sheep Project.

In the field, I collected all biological samples with the assistance of Jill Pilkington and Xavier Bal. Additional faecal samples were collected by Soay Sheep Project volunteers on St Kilda on 8-10th of April 2017 (Chapter 4). I counted all FEC except for July 2015, when faeces were sent to the Moredun Research Institute (Scotland) and counted by Dave McBean and Fiona Sargison. This allowed me to stay on St Kilda to collect and process the field samples in August 2015. Jill Pilkington and Moredun Research Institute counted the additional modified McMaster samples for the FEC cross-count (Chapter 2).

I preformed the multiplex-tandem PCR, as presented in Chapter 3, under the direction of Allison Morrison (Moredun Research Institute, Scotland). I preformed the ITS-2 rDNA sequencing, as presented in Chapter 5, under the direction of Libby Redman and Russel Avramenko (University of Calgary, Canada). The correction factor (CF) used for *T. axei* (Chapter 4) was calculated by Redman *et al.*, (2019).

This work has not been submitted for any other degree or processional qualification. All collaborative involvement has been duly acknowledged.

Signed: Date: 31/ 10/ 2019

Alexandra Katherine Chambers
2019

For Mum and Dad

It started with “Snail Polish”

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ABBREVIATIONS

bp	base pair
CF	correction factor
COX	cyclooxygenase
CNV	copy number variation
DNA	deoxyribonucleic acid
DTh	detection threshold
epg	eggs per gram (of faeces)
FCS	faecal consistency score
FEC	faecal egg count
FECRT	faecal egg count reduction test
GI	gastrointestinal
GIN	gastrointestinal nematodes
GLM	generalised linear model
GLMM	generalised linear mixed model
ha	hectare
ITS	internal transcribed spacer
ITS-1	internal transcribed spacer 1
ITS-2	internal transcribed spacer 2
k	kappa
L_1	1^{st} stage larvae
L_2	2^{nd} stage larvae
L_3	3^{rd} stage larvae
L_4	4^{th} stage larvae
Mbp	million base pairs
MT-PCR	multiplexed-tandem polymerase chain reaction
NAO	North Atlantic oscillation
NGS	next generation sequencing
nSSU rRNA	nuclear small subunit ribosomal RNA
PABAK	prevalence-adjusted bias-adjusted kappa

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PK	proteinase K
PPR	periparturient rise
rDNA	ribosomal deoxyribonucleic acid
REML	restricted minimum likelihood
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SG	specific gravity
TWC	total worm count
WAAVP	World Association for the Advancement of Veterinary Parasitology

CHAPTER 1

General Introduction

"They say it is the first step that costs the effort. I do not find it so. I am sure I could write unlimited 'first chapters'. I have indeed written many."

J.R.R. Tolkien
Letter to C. A. Firth (February 1938)

1. 1. INTRODUCTION

As the human population grows, there is increasing demand to produce more animal products and improve global food security (Learmount et al., 2015). Parasitic nematode infections present significant welfare and economic costs to the small ruminant industry worldwide. Losses in milk, meat and fibre production costs the UK up to £84 million annually, while a further £70 million is spent on anthelmintic drugs, with up to USD$3 billion being spent worldwide (Morgan et al., 2013; Nieuwhof and Bishop, 2005; Jackson et al., 2009). Therefore, the sustainable control of nematode parasitism in commercial flocks is essential for cost-effective sheep production. Current strategies combining anthelmintic use and grazing management are unsustainable; over-dependency to anthelmintic drugs has led to the emergence of both multidrug and multi-nematode species resistance, resulting in a greater need for control strategies that minimise the use of anthelmintic drugs (Falzon et al., 2015, Learmount et al., 2015).

Simultaneous infection with multiple gastrointestinal nematode (GIN) parasite species is commonly seen in feral and managed ruminants. Within these mixed burdens, the species can vary in their pathogenicity, epidemiology, clinical presentation, and resistance to anthelmintics. An improved understanding of parasite interactions is a critical component of parasite control, in order to develop sustainable management strategies. Elucidating the diversity of different parasite species in a host, rather than studying them as a homogeneous group, is a prerequisite to understanding host-parasite interactions. The ecology of the major GIN species in agricultural systems is well documented, however, the impact of long-term production strategies on the GIN community structure is unknown. Researching nematode coinfection in the absence of control measures is required to recognise the impact of management decisions on sustainable control. The unmanaged, feral population of Soay sheep on the Scottish archipelago of St Kilda provides an ideal study population.

1. 2. THE PHYUM NEMATODA

Nematoda is an ancient and diverse phylum of organisms, and can be found in most habitats, including within animal and plant hosts. The current classification of the phylum Nematoda is based on ecological and morphological traits, principally the free-living life cycle and/or parasitic phenotypes. Despite the basic anatomy of nematodes, there is a complex diversity of species, with the number of species estimated to be 40, 000 to 100 million (Dorris et al., 1999). Current taxonomy relies on the morphometrics of the worms, including buccal and pharyngeal structure, sense organs and tail shape. Issues arise from errors in identification, morphological overlap between species and the limited number of characteristics that can be measured and

observed in relation to the diversity of the species (Dorris et al., 1999). The taxonomy of Nematoda have been explored for over 20 years (Blaxter et al., 1998). More recent analysis utilised the nuclear small subunit ribosomal RNA gene (nSSU) to supplement the morphological descriptions. The nSSU rRNA gene has enabled the resolution of the deep structures of the Nematoda phylogenetic tree (Blaxter and Koutsovoulos, 2015).

Molecular phylogenetics describes the phylum Nematoda into three subclasses (Enoplia, Chromadoria and Dorylaimia), which are further separated into five major clades; Clade I – Dorylaimia, Clade II – Enoplia, Clade III – Spirurina, Clade VI – Tylenchina and Clade V – Rhabditina. These clades (I, II, III, IV and V) were first defined by Blaxter et al., (1998), and were further revised by Holterman et al., (2006) and van Megen et al., (2009). Clade I is the vertebrate-parasite order Trichocephalida, which includes the ovine parasite species *Trichuris ovis*. The Clade II grouping includes the order Enoplida, and the specie *Capillaria longipes*. Clade III represents a large, novel group of arthropod and vertebrate parasite taxa, including large gut roundworms (Ascaridida) and pinworms (Oxyurida) (Blaxter et al., 1998). The order Rhabditida is a trophically diverse grouping, it is paraphyletic, meaning the taxa is found in two clades – IV and V – both including major parasitic species (Blaxter et al., 1998). A taxonomic group that is paraphyletic is related from a common ancestor but does not include all the descendant groups (Dorris et al, 1999). Clade VI can be split into two subclades, clade IVa and IVb (De Lay and Blaxter, 1999). Clade IVa includes vertebrate-parasitic species such as *Strongyloides papillosus* (Rhabditida, Strongyloididae), whilst clade IVb are mostly invertebrate parasitic and plant parasitic nematodes (e.g. root-cyst nematodes) (Blaxter et al., (1998). *Strongyloides* spp. have traditionally been classified as a rhabditid, and thus a clade V species, however, the nSSU rRNA analysis undoubtedly places it in clade VI, as it is closely related to species that are characterised by complex alternating life cycles (Blaxter, 2001). *Strongyloides* spp. are unusual in that the free-living stage of their life cycle is facultative; depending on the environment, the free-living females can choose to become an infective 3rd-stage larvae (L_3) to infect a host, or become a mature adult (Blaxter, 2001). This highlights the need to supplement life history and morphometric analysis with genetic data to accurately place species within the phylogenetic tree. Clade V includes microbivorous, free-living Diplogasterida and Rhabditida, and the vertebrate-parasitic order Strongylida. Strongylida, or strongyle nematodes, are a diverse group of parasites, and have major ethical and economic importance in human and livestock health.

The structure of the nematode phylogenetic tree is ever evolving; additional tree-placement revisions may still be needed. The nSSU locus provides an ideal gene for deep phylogenetics as it contains highly conserved domains, however, due to its small size (~1800 base pairs) additional

data pertaining to its phylogenetic history is limited. Most analysis, and therefore data, have relied on the single nSSU locus. More recent attempts combining whole-genome-derived and transcriptomics-derived gene sets have been able to replicate findings from previous nSSU analysis, and also resolve conflicts between the different analyses (Blaxter and Koutsovoulos, 2015).

1. 3. STRONGYLE NEMATODES (CLADE V)

Strongyle nematodes, or GIN, have been recognised as a major factor limiting sheep production in Europe, and coinfection with a wide range of species is ubiquitous in grazing sheep (Morgan and van Dijk, 2012). However, not all strongyle species are considered pathogenic, or drug resistant (Crilly and Sargison, 2015; McKenna, 1997). The most important genera in regards to pathology and production-loss are trichostrongylids *Trichostrongylus*, *Teladorsagia*, *Haemonchus* and *Nematodirus*. Other species, such as *Chabertia*, can be considered important in certain circumstances, but usually as part of a mixed species burden (Morgan and van Dijk, 2012). There are some species, such as *Bunostomum*, that are now uncommon in modern sheep farms due to their susceptibility to frequent anthelmintic treatments (discussed further in the Introduction of Chapter 5).

There are varying degrees of pathology caused by the different nematode parasites, which can be altered by the condition of the host, presence of other coinfecting species and environmental conditions. Infections predominant with high burdens of *Teladorsagia* and *Trichostrongylus* usually trigger clinical conditions, such as parasitic gastroenteritis. High burdens can cause weight loss, lethargy, and inflammation of the abomasal wall, resulting in diarrhoea (scouring) (Sutherland, 2009). *Trichostrongylus vitrinus* and *Trichostrongylus axei* develop between the epithelial layer and connective tissue (lamina propria) in the small intestine and abomasum (respectively), and the L_3 actively tunnel to these predilection sites triggering an erosion to the mucosa. *Bunostomum trigonocephalum* adults are hematophagous, and have evolved to efficiently extract and digest host blood. They have a well-developed buccal capsule, which attaches to the mucosal surface to cut out pieces of tissue, potentially causing extensive damage in high burdens by secreting anticoagulant portions these wounds bleed into the lumen of the small intestine (Seguel and Gottdenker, 2017). *Chabertia ovina* are mucosal plug feeders within the large intestine, and high burdens are believed to cause ulcers (Soulsby, 1968; Herd, 1971). The pathogenic importance of *C. ovina* was under dispute for many years, early work regarded the parasite as harmless; it lived off the intestinal contents (Wetzel, 1931). Others observed it causing irritation to the mucus membranes of the large intestine, whilst large infections were noted to cause weight loss (Herd, 1971). To date, it is believed that

C. ovina is not the most harmful of the gastrointestinal nematodes, rather, it worsens the damage caused by other species (Herd, 1971). *Nematodirus* is considered a species prevalent in lambs, and clinical signs of high *Nematodirus* burdens scouring, dehydration and severe weight loss (Craig, 2005). Small erosions on the gut can indicate protein-energy malnutrition through blood-feeding nematodes. Under high numbers, these can contribute to malnutrition by inducing haemorrhaging and anorexia in addition to decreasing the guts ability to absorb nutrients through severe villus atrophy (Gulland, 1992; Barker, 1975). Additionally, it provides the perfect site for secondary bacterial infection (Seguel and Gottdenker, 2017).

Control of GIN is generally achieved through frequent treatments of anthelmintic drugs to kill the adult worms within the gut, and to suppress egg output which subsequently reduces infection pressure by reducing pasture contamination. Species with a short prepatent periods have a selective advantage in re-colonising the host. Studies that administered anthelmintic treatment to a natural population (i.e. wild wood mice, Soay sheep, Bison) showed that whilst drug treatments reduce target parasite species, parasite burdens rapidly reverted back to pre-treated levels. This indicates that the infra-populations are highly stable to perturbation, showing both resistance and resilience to changes in population structure. However, during this time of temporary change, a rapid increase of certain surviving species is noted. Some of these species are relatively harmless when in low numbers, but can increase morbidity within the host when under large numbers (Gulland and Fox, 1992; Rynkiewicz et al., 2015). Work conducted by Budischak et al., (2016) suggests that parasite communities may potentially become more pathogenic during early re-colonisation.

1. 3. 1. Epidemiology

Understanding of the epidemiology of production-limiting nematodes is the foundation upon which strategic parasite control programs are designed. By understanding the life cycle of the parasite, control strategies can be developed to control the adult worm burden as well as the pasture larval populations.

The life cycles of the different nematode species found in the Soay sheep (Table 1. 1) are similar, characteristic of the order Strongylida. These parasites have a predominantly direct lifecycle (i.e. without an intermediate host), which is outlined in this section (Figure 1. 1.). There are some exceptions, such as *Nematodirus* spp., where the larval development occurs inside the egg, and require a frost to hatch (Rober et al., 2014); any other differences are listed in Table 5. 1. (Chapter 5).

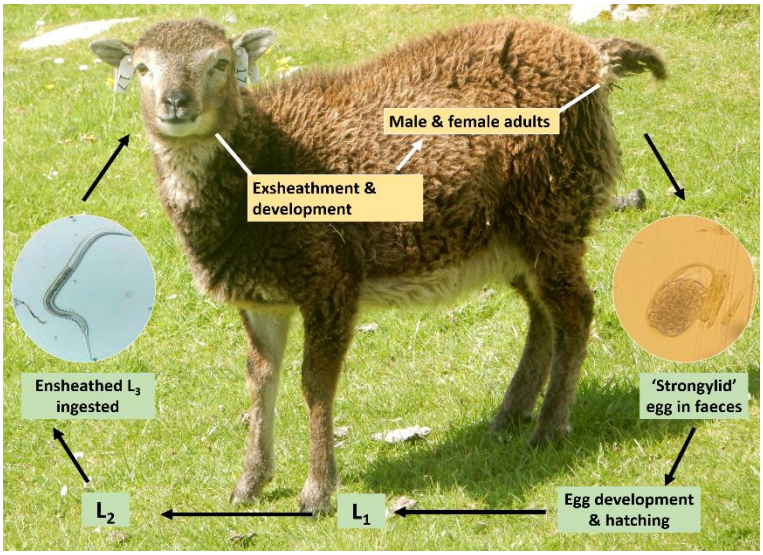


Figure 1. 1. Parasitic and free-living stages of parasites with a direct lifecycle (free living stage – green; infective stage – orange).

1. 3. 1. 1. Free-living stage

Eggs excreted on the pasture hatch and undergo further development within the faecal pat into larvae capable of infecting a host. Moisture and temperature are important factors affecting hatching and development (L_1 to L_3) of the free-living stages of strongyle nematodes of ruminants. These stages are primarily temperature-dependent, though moisture is required to prevent desiccation (Rossanigo and Gruner, 1995). Hatching into 1st stage larvae (L_1) can occur within 24 hours (Perry, 2002). L_1 and L_2 feed on bacteria within the faecal pat. However, as the L_2 sheds into an L_3 it retains the cuticle of the L_2 and therefore cannot feed. This cuticle forms a protective sheath around the larvae, protecting it from environmental conditions as it migrates out of the faecal pat and up onto the herbage. Moisture films on the surrounding vegetation is needed for migration onto the pasture (Sutherland and Scott, 2010). L_3 are limited by their energy stores; they do not migrate far (5cm – Rose, 1961). Rainfall and surface run-off aids in disseminating infective larvae away from the faeces, and has been found to move L_3 up to 90cm (Rose, 1962). L_3 are able to survive in the soil; Waghorn et al., (2011) found they recovered more L_3 from the pasture when they buried the eggs in the soil. However, the activity of earth worms appears to damage the L_3 and in cases where there were earth worms, fewer L_3 were recovered. Other hosts have been seen to transport the larvae across the pasture such as insects (Todd et al., 1971) and *Pilobolus fungi* (Jørgensen et al., 1982). Very high temperatures can lead to desiccation, whilst colder temperatures will slow the development of the larvae. Survival of L_3 on pasture is species-dependent, with *T. circumcincta* and *Nematodirus* spp.

surviving several months, whilst environmentally sensitive *Bunostomum* lasts a few months during spring-summer (see Chapter 5 Introduction for further discussion).

Extrinsic factors such as climate, geography and flora, influence the extent of the L_3 challenge on-pasture (Morgan and van Dijk, 2012). In a diverse environment as St Kilda, pasture larval contamination differs depending on the predominating vegetation, elevation and man-made historical structures (Figure 1. 4. B) (Wilson et al., 2003). This spatial variation is driven by the differences in sheep density between areas (i.e. hefting), and also geographical factors (e.g. flood-prone/ marshy areas, cliff-side areas with high run-off).

1. 3. 1. 2. Infective stage

The parasitic (or infective) stage of the life cycle begins when the infective L_3 is ingested from the pasture, and exsheaths within the proximal gastrointestinal tract of the host. The location of exsheathment is species-dependent, but is usually the rumen for abomasal parasites. This process is a triggered response to sudden changes in environment, which occurs when the parasite reaches the gut (Rogers and Sommerville, 1963). The main factors are believed to be elevated temperature, high levels of carbon dioxide (CO_2), digestive secretions and the pH of the rumen fluid; and that the precise trigger of exsheathment is species-specific depending on where the parasites exsheath (Bekelaar et al., 2019). Increase in temperature to that of the hosts body temperature has been found to be needed to exsheath *T. axei* whereas it's the rapid temperature change that triggers the exsheathment of *Haemonchus contortus* (Bekelaar et al., 2018). Exsheathment can be affected by diet, as diet can alter the rumen. Changing the diet from grass to grain, DeRosa et al (2005) was able to increase the time in which L_3 exsheathed within the rumen. Once exsheathed, the L_3 migrate to their predilection site to further develop to L_4 and L_5 (immature adult); gastric glands for *Teladorsagia* sp. and the epithelial layer and lamina propria for *T. axei* (Balic and Bowles et al., 2000). For *C. ovina*, the L_3 exsheath in the small intestine, penetrating the mucosa to moult to L_4. They then emerge into the cecum, moult to L_5 and migrate to the large intestine to mature (Herd, 1971). *Bunostomum trigonocephalum* is able to infect the host orally and percutaneously (through the feet, legs and soft pallet), the L_3 migrate to the lungs and moult into L_4, they are then coughed up and swallowed where they mature and reproduce in the small intestine (Graham, 1969; Ortlepp, 1939). Hair-loss around the muzzle and local irritation can be an indicator of skin-penetrating parasites, and sheep are known to develop 'stamping' behaviours which is suggestive of parasites invading through the feet (Graham, 1969).

Male and female worms mate and the females begin egg production. The pre-patent period (ingestion of L_3 to egg shedding) and number of eggs shed per day is species specific (listed in Table 5. 1, Chapter 5). Generally, for *Teladorsagia* and *Trichostrongylus* species the prepatent period is around 17 days. In certain circumstances, parasitic development can become arrested at the L_4 stage. Inhibition, or hypobiosis, can occur for several months, allowing the parasites to synchronise with the seasons to enhance over-winter survival. *Teladorsagia* arrest development within the gastric glands. Development usually occurs during spring the following year, which drives infection in susceptible spring lambs (Craig, 2005; Coop and Jackson, 2000; Michel et al 1974). Eggs are released by the female, where they pass through the rest of the gastrointestinal tract before being deposited on pasture.

1. 4. TRADITIONAL PARASITOLOGICAL TECHNIQUES

Current diagnosis for mixed-infections of strongyle nematodes relies mainly on traditional parasitological techniques. These include total worm counts (TWC) from necropsied hosts, and non-invasive (*ante-mortem*) methods that utilise immature stages of the lifecycle (eggs/ larvae) in faeces, such as faecal egg counts (FEC), larval culture and morphological identification of L_3 (Roeber and Kahn, 2014). Generally, these methods are often time-consuming, and/or require a high level of training to perform them correctly.

FEC are the non-invasive, microscopic identification and quantification of eggs within a faecal sample, which are (usually) quantified in eggs per gram of faeces (epg) (MAFF, 1986). FEC are a well-established method in monitoring helminth burdens in both managed and wild systems; and are often the only measure available to estimate parasite intensity in free-living animals. It is an important tool used to indirectly estimate infection intensity (McKenna, 1987), determine pasture contamination with parasitic eggs (Roeber et al., 2014), or assess the efficacy of anthelmintic treatments (Waller et al., 2006), helping to inform management decisions. However, FEC are often pooled for most species (i.e. several strongyle species), and do not allow for the accurate identification of strongylid eggs to species or genus level (see Chapter 2). The fecundity of different GIN varies, and while the diagnostic value of FEC to estimate worm burden has been shown in highly fecund species (i.e. *Haemonchus contortus*), there is a lower correlation for genera with low fecundity (i.e. *Trichostrongylus* and *Teladorsagia*) (Roeber et al., 2014). FEC methodology is discussed further in the Introduction of Chapter 2.

Certain Trichostrongylid species can be differentiated based on egg morphology, however, this is not considered practical in routine diagnostics as it both time consuming and requires each egg to be inspected at high magnification. Additionally, the osmotic pressure of the floatation

solution needed to separate the eggs from the faecal material, such as saturated salt or sugar, can warp the structure of the eggs making them difficult to differentiate (Georgi and McCuoch, 1989; MAFF, 1986). The lectin-binding assay stains *H. contortus* eggs, which allows selective diagnosis of *H. contortus*-positive samples (Palmer and McCombe, 1996; method further developed by Jurasek et al., 2010). However, this method of selectively staining eggs has not been developed to other strongyle species. Therefore, a strongyle FEC is usually the amalgamation of multiple species that have non-morphologically distinct eggs (Table 1. 1).

TWC enables accurate, species-specific quantification of adult nematode burdens within the gut, and is considered the 'gold standard' for assessing nematode infection in livestock by the WAAVP (World Association for the Advancement of Veterinary Parasitology) guidelines, with adult strongylids morphologically identified (Coles et al., 1992). However, the sensitivity of the method that is used can affect results, for example, when only 2-5% of the gut contents are counted, species that occur at low-levels (such as *B. trigonocephalum* within the small intestine) could potentially be below the threshold of detection (Graham, 1969; Craig, 2005). This method also depends of what is being measured, as old or immature worms may be incapable of producing viable eggs that will hatch and survive to the infective stage (Roeber et al., 2014). Additionally, lethal sampling can raise both logistical and ethical challenges, in addition to preventing longitudinal /or multiple sampling of the same individuals over time.

For the identification of different genera in mixed infections, faecal culture is the most commonly used method by veterinary diagnostic laboratories. Coproculture, the incubation of faeces in order to hatch parasite eggs, grow parasites to free-living stages. Based on morphological features, L_1 and L_3 can be identified to genus-level. However, morphology based on the features of L_3 is technically challenging, requires a high level of training, and is often unreliable (Bailey, 2008). There are different protocols described within the literature, which vary in culturing conditions (time, temperature and media) (MAFF, 1986), though temperatures ranging from 21 - 27°C have been recommended (Taylor et al., 2007; Roeber et al., 2014). However, different nematode species require different conditions, such as temperature and humidity, for adequate development (O'Connor et al., 2006). By culturing with specific conditions, it may favour the development of certain species over others, which is an important factor when using larval cultures to estimate the species proportion in mixed infections. 27°C for 7 days is believed to be suitable for most species commonly infecting grazing ruminants (Whitlock, 1956), though *Teladorsagia* develops better at a lower temperature (Dobson et al., 1992). The results obtained from larvae culture can be further skewed by moisture level, pH and oxygen availability (Roberts and O'Sullivan, 1950).

1. 5. MOLECULAR PARASITOLOGICAL TECHNIQUES

The development of DNA-based alternatives for the specific diagnosis, and quantification, of GIN in livestock, has negated many of the issues arising from traditional parasitological techniques. The development of these molecular methods is described in detail in the Introduction of Chapter 3. The first and second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) have provided useful markers for species and genus-specific differentiation, as there is sequence variability between species and low-level variability within species (Gasser, 2006). Amplification of these markers by polymerase reaction (PCR) has enabled the development of methodology that can determine the presence/ absence of (usually) single parasite species. Methods such as multiplex PCR (Bisset et al., 2014), reverse-transcription PCR (RT-PCR) (Roeber et al., 2012) and multiplex tandem PCR (MT-PCR) (Roeber et al., 2012) has broadened the study of ruminant parasites by not only identifying the presence/absence of key infective species in eggs, larvae or directly from faeces, but can provide additional proportional data, negating many of the problems associated with the morphological identification of L_3. See the Introduction of Chapter 3 for further discussion. However, many of these methods are low throughput and may only identify/quantify one specie at a time, or only amplify a small panel of the most production-limiting nematode species found in sheep or cattle. Additionally, these methods can be time consuming; including multiple manual set-ups, individual primer pairs (i.e. identification and quantification of one specie at a time) and/or longer preparation time of larvae. Whilst these methods have broadened the study of GIN of ruminant livestock, they are limited when studying wild hosts where the infecting species may not be known, or some parasite species are either not shared, or are rare, in domestic hosts. The use of specie specific primers has the potential to miss and/or underestimate parasite diversity, which may potentially generate inaccurate results when studying community composition.

Recent developments in high-throughput next-generation sequencing (NGS) has enabled the screening for multiple parasite species, over many samples, concurrently. Molecular barcoding approaches can identify and quantify parasite communities quickly, and enables a large number of samples to be processed within one sequencing run, providing a cost and time efficient alternative to the other molecular methods (Aivelo and Medlar, 2018). A deep amplicon sequencing methodology to study parasite communities was developed by Avramenko et al., (2015), and modified for ovine strongyle species by Redman et al., (2019) for the identification and quantification of mixed-species infections with clade V nematodes. The method is based on deep amplicon next generation sequencing of the ITS-2 locus, and is comparable to 16S rDNA

sequencing of bacterial communities used in microbiome studies. Clade V nematodes are a group of parasites that includes economically significant gastrointestinal species of ruminants (Blaxter et al., 1998), many of which are characterised by morphologically similar eggs (Table 1. 1).

The nemabiome methodology has been developed, and validated, for the identification and quantification of clade V nematode L_3 (Avramenko et al., 2015). Culturing faeces to from eggs to L_3 is preferable in a field environment, as it requires limited equipment, and larvae can be easily extracted from faeces using rudimentary techniques and stored in either water or ethanol for transport from the field site. See Introduction of Chapter 3 for more detail.

However, by culturing faeces to hatch eggs for L_3, some clade V species will not be present to sequence, due to the need for specialist coprocuture and/or Baermannisation (i.e. the extraction of L_3 from faeces into clean water) methods. *Nematodirus* requires a chilled period during culturing to allow eggs to hatch, and as their eggs are morphologically distinct during FEC (Table 1. 1), usually there is no need to include separate cultures, which would require additional faeces and equipment. The lungworms *Dictyocaulus filaria* and *Muellerius capillaris*, are also clade V nematode species. They hatch within the lungs of the host, and exit in faeces as L_1, not as eggs (Table 1. 1) (Urquhart et al., (1996). *M. capillaris* is a protostrongylid lungworm, which requires a snail or slug intermediate host, making culturing L_1 to L_3 *in-vitro* challenging (Urquhart et al., (1996). The L_2 and L_3 stages of *D. filaria* are inactive, therefore they do not migrate through a traditional baermanns filter (Rose, 1962), and would require alternative methods of extraction such as sugar or salt floatation. An alternative method would be an ox bile agar assay (Jorgensen et al., 1975) which has been shown to initiate movement in bovine *Dictyocaulus viviparus* L_3, allowing L_3 extraction from faeces. However, these methods would require additional equipment and time. Adults worms can be identified and counted through post-mortem of the lungs. Alternatively, the L_1 of both lungworm species can be extracted from the faeces by baermanisation, or can be visually counted by some FEC methods. Other nematode species that are not clade V will not be amplified in this nemabiome assay, though many of these species are characterised by morphologically-distinct eggs (*S. papillosus* – IVa, *C. longipes* – II, *T. ovis* – I) therefore their prevalence within a FEC can still be noted (Table 1. 1.)

1. 6. ST KILDA

The isolated archipelago of St Kilda (57° 49'N, 08°34W) is located North West of Scotland (United Kingdom); ~40 miles from the Outer Hebrides and ~110 miles from mainland Scotland (Figure 1. 3). The craggy, fragmented islands of Hirta, Soay, Dun and Boreray (Figure 1. 3) are

eroded remnants of a tertiary volcano (Clutton-Brock and Pemberton, 2004). Located in the North Atlantic Ocean, its temperate oceanic climate is characterised by periods of strong, multidirectional gales and heavy rainfall (Campbell, 1974; Buchanan, 1995); the average monthly air temperature rarely reaching below 0°C and above 22°C (Figure 1. 2). The conditions during winter are variable, snow can fall on the hills but will rarely last, and the changeable winter weather is either wet and windy, or drier and colder, which is dictated by the North Atlantic Oscillation (NAO) (Clutton-Brock and Pemberton, 2004).

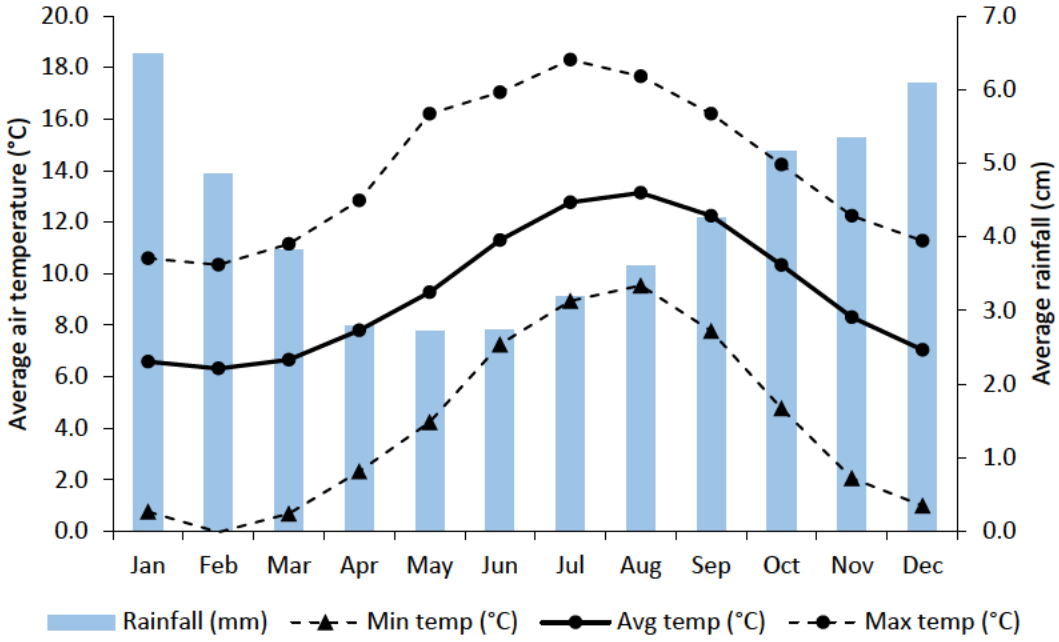


Figure 1. 2. Average seasonal variation in air temperature (minimum, maximum and average) (°C) and rainfall (cm), across different months of 2000-2019. Data averaged from 3 weather stations located on Hirta, across the study system of Village Bay (Briannans, Quarry, Signals).

Hirta (638 ha) is the largest of the islands and has a long history of human occupation reaching back over four thousand years. The final human settlers, originating from the western isles of Scotland, established a small community in Village Bay (Figure 1. 3) (Clutton-Brock and Pemberton (2004). Hirta is covered by several plant community types, including heathland, grassed sea cliffs, fertile grasslands, bog communities and maritime communities (Clutton-Brock and Pemberton (2004). Village Bay was cultivated for crops, and the islanders farmed sheep and cattle to supplement their diet of seabirds. Additionally, they kept cats to maintain the rodent

population; the house mouse (*Mus musculus*), and the St Kilda field mouse (*Apodemus sylvaticus*) which are endemic to St Kilda. However, during the late 19th century the human population began to dwindle until it became unsustainable. In 1930, humans and livestock were fully evacuated. Without humans, the cats died out quickly, along with the house mouse (Harrisson and Moy-Thomas, 1933; Steel, 2011).

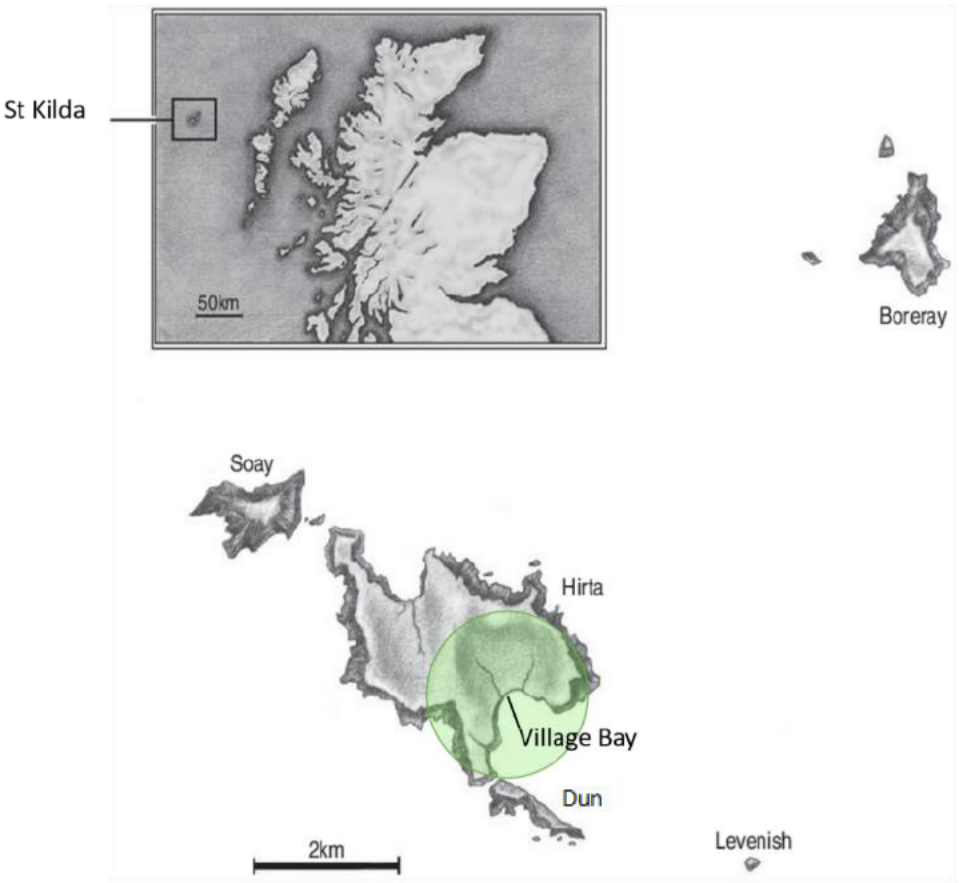


Figure 1. 3. The islands of St Kilda (North West Scotland, UK), with the study area, Village Bay, highlighted (©Becky Holland).

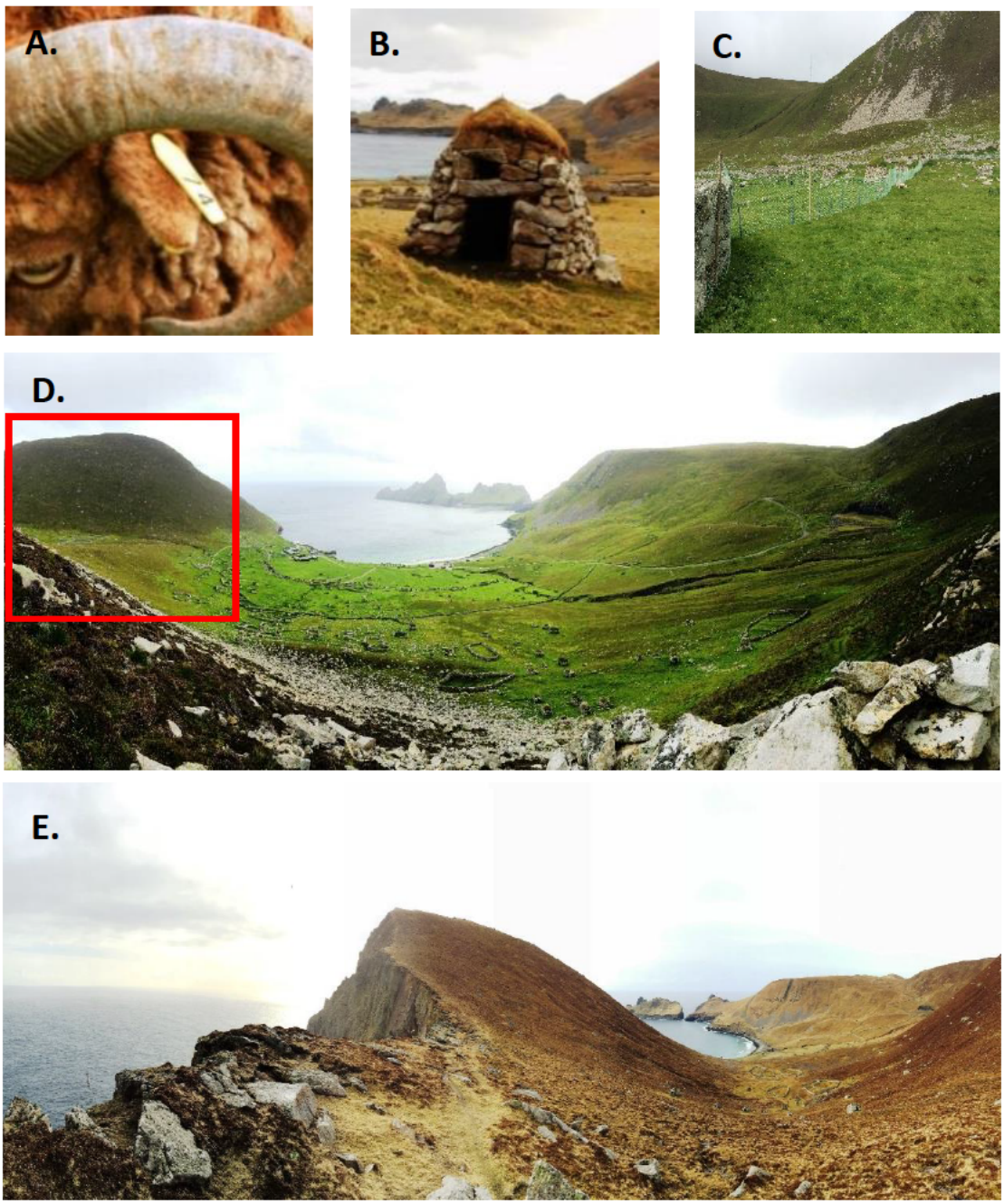


Figure 1. 4. A. Ear tag (example: CY074 – 2012 cohort). B. Over 1000 stone storage chambers, known as cleits, scatter the bay. Originally built by the previous inhabitants of St Kilda, they now form an integral part of the landscape providing shelter for the sheep in the winter months. C. Construction of temporary traps to catch sheep for the annual August data collection conducted by the Soay Sheep Project. D. View of village Bay at the top of the hill (with the island of Dun in the background). E. Upper part of the hill, indicated in the red box (D).

1. 7. SOAY SHEEP

The Soay sheep (*Ovis aries*) of St Kilda are believed to be one of the most primitive breeds of domesticated sheep in Europe, reaching the remote Scottish islands around the Bronze age (Clutton-Brock and Pemberton, 2004). Since then, they have lived feral and unmanaged on the small, remote island of Soay (99 ha) (Figure 1. 3), protected by the steep cliffs making which make the islands difficult and dangerous to access (Clutton-Brock and Pemberton, 2004). The origin of the Soay sheep of St Kilda are further discussed in the introduction of Chapter 5. Soay sheep are small, goat-like, and are usually black or brown (Figure 1. 5. A and B). From 5 years old, adult females average 24 kg and adult males average 38 kg (Clutton-Brock and Pemberton, 2004). In 1932, 107 (20 rams, 22 castrated ram lambs, 44 ewes and 21 ewe lambs) sheep were translocated to the island of Hirta, and have remained a feral, relatively unmanaged, population.

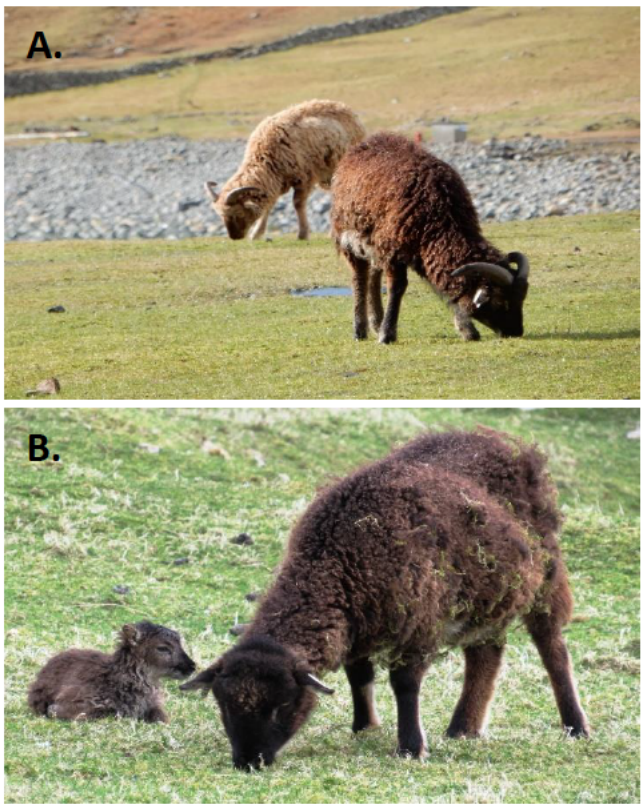


Figure 1. 5. A. Example of the different colouring seen in the sheep with two grazing male Soay sheep. B. Young hornless female Soay with lamb.

Whilst the Soay sheep are free-ranging, they are known to heft and generally will keep to their grazing ranges, hence animals born within Village Bay will tend to stay within the geographical boarders of the bay. Approximately a third of Hirta's population is found within Village Bay (200 – 650 sheep). Hefting, or a heft of sheep, is part of a flock (regardless of sex and age), which will

exclusively graze a section (or sections) of hill and will generally not stray from that area (Morgan et al., 1951). Hefts can consist of smaller cohesive groups of sheep that frequently associate with one another; such as mother-daughter pairs and bachelor groups (i.e. groups of unrelated males of a similar age) (Coulson et al., 1999). Scottish Blackface and Cheviot breeds of hill sheep kept in Scotland also heft (Morgan et al., 1951).

1. 7. 1. Population dynamics

The population dynamic on Hirta is highly unstable, with yearly fluctuations in population size (Figure 1. 6). This has been attributed to the complex interactions of a number of factors including population density and demographic structure, parasitic burden, winter weather and food availability (Hayward *et al*., 2009; Clutton-Brock *et al*., 1996; Craig *et al*., 2006; Gulland and Fox, 1992). Soay females able to reproduce in their first year, and can potentially lamb every year (Figure 1. 5. B). Therefore, the population dynamic is over-compensatory as it reaches and exceeds carrying capacity; subjecting the population to high mortality. During these 'crash' years, the total mortality has been as high as 69%; and is very high in adult males and lambs, reaching up to 86% and 95% respectively (Clutton-Brock et al., 1991; Clutton-Brock et al., 1992). Additionally, individuals that survive a crash year have greater physiological stress which impacts future fitness. Studies that have looked into the future impacts of yearly stress highlighted the importance in the differences between 'chronological' and 'biological' age. Chronological age fails to account for environmental impacts that can affect host parasitism, such as stress during early development and cumulative environmental stress throughout the animals life history, which can impact senescence later in life. This is seen in Hayward et al., (2009), where Soay sheep that have undergone high stress early in life – measured by weight gain and breeding success – are more susceptible to parasitism later in life and have higher prime-age mortality.

1. 7. 2. Parasite diversity

The Soay sheep of St Kilda are a naturally parasitized population. The parasitological investigations began with the population crash in 1964 by Cheyne et al., (1974) and was continued by Gulland (1992), Craig (2005) and Pilkington (unpublished, continued data collection). They provided a comprehensive list of helminth species known to infect the sheep. The Soay sheep are host to a diverse parasitic population comprised of macroparasites (13 nematode species, two cestode species and two arthropod species) and microparasites (13 protozoa species and one trypanosome specie) (Clutton-Brock and Pemberton, 2004). The parasite species found infecting the Soay sheep of St Kilda are similar to that of domesticated hill sheep on mainland Scotland, with a few notable absences. The blow fly *Lucilia sericata*,

sheep tick *Ixodes ricinus*, sheep scab mite *Psoroptes ovis* and the widespread, pathogenic nematode *Haemonchus contortus* are all notably absent from the population. The nematode parasite species found on St Kilda are listed in Table 1. 1, for a more in-depth review see the Introduction of Chapter 5.

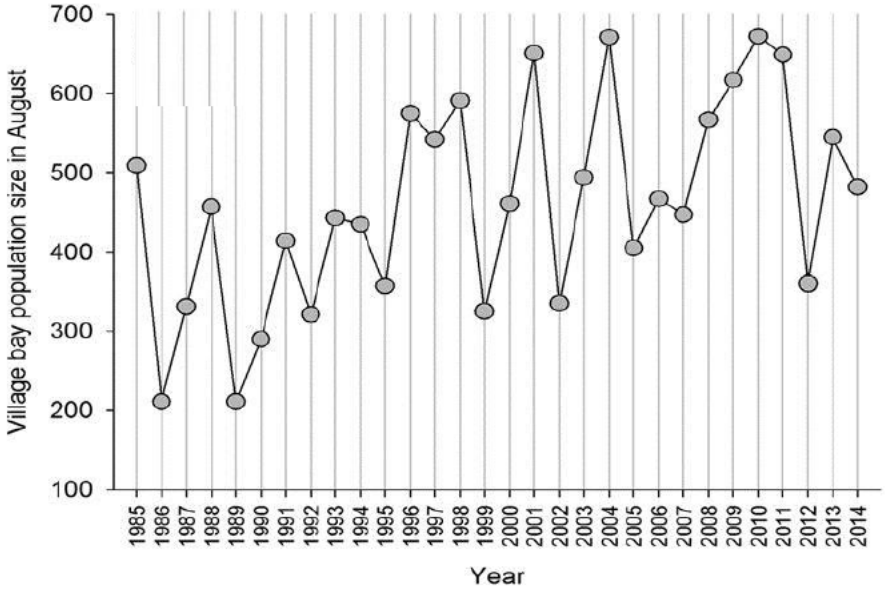


Figure 1. 6. The population of the Village Bay study area (Hirta, St Kilda) from 1985-2014.

Table 1. 1. Nematode species known to infect the Soay sheep of St Kilda, noting organ of infection, clade placement within the phylum Nematoda (based on nSSU rRNA phylogeny) and whether the eggs are morphologically distinguishable (D) or not (ND).

Species[1]	**Infection site**[2]	**Clade**[3&4]	**Eggs**[2]
Dictyocaulus filaria	Lungs	V	L_1***
Muellerius capillaris	Lungs	V	L_1***
Teladorsagia circumcincta *	Abomasum	V	ND
Trichostrongylus axei	Abomasum	V	ND
Trichostrongylus vitrinus	Small intestine	V	ND
Bunostomum trigonocephalum	Small intestine	V	ND
Strongyloides papillosus	Small intestine	IVa	D
Nematodirus battus	Small intestine	V	D
Nematodirus filicollis	Small intestine	V	D
*Nematodirus helvitianus***	Small intestine	V	D
Capillaria longipes	Small intestine	II	D
Trichuris ovis	Large intestine	I	D
Chabertia ovina	Large intestine	V	ND

* Originally described as 3 separate species *(T. circumcinta, T. trifurcata* and *T. davtiani*) but now recognised as only *T. circumcincta* (Grillo et al., 2008); ** Presence not confirmed by Craig (2005) *** Eggs hatch in the lungs and exit host as 1st-stage larvae (L_1)

[1] Clutton-Brock and Pemberton (2004); [2] Urquhart et al., (1996); [3] Blaxter et al., (1998); [4] Mitreva et al., (2005)

1. 7. 3. The Soay Sheep Project

The current study focuses on the sheltered bay (Village Bay ~200 ha) of the most accessible island (Hirta ~638 ha), with the borders of the study system ending at the top of the surrounding hills (Figures 1. 4. D-E). Village Bay covers approximately a third of the islands area, and is home to around a third of the Soay sheep population.

Longitudinal population monitoring began in 1959 until 1968 (Jewell *et al*., 1974). The study of parasites infecting the Soay sheep was initiated by a population crash in 1964 (Cheyne et al., 1974). In 1985, individual-based monitoring began, recording data on many aspects of the sheep population including morphological traits, life-history, genetics and parasitology. A full account of the data-collection process is described by Clutton-Brock and Pemberton (2004).

There are three fieldwork seasons, with two main opportunities to collect samples for parasitological analysis. Spring (March – early May) involves a full census of the study area, identification and potential post-mortem of individuals that had died over the winter, and catching new lambs when they are born. This begins with ten censuses of Village bay to register the survival and location of known individuals. During lambing there is no interference with the birth, lambs are caught a few days later to be tagged and additional samples (blood, tissue, measurements) taken. Every spring, since 1985, lambs within Village Bay are caught and tagged, with the cohort being tagged with a specific colour. Up to 95% of sheep within Village Bay area are tagged, and can be identified by their unique ear tag number/ colour combination (Figure 1. 4. A). The second period is during August, when ~60% of the Village Bay population is caught for blood sampling, weighing, re-tagging and faecal sampling (Figure 1.4.C.). The majority of faecal samples, and therefore parasitological data, is aggregated to this summer collection. Lastly, during November the females go into oestrus and the males compete for females during a rut. During this time new males will visit, from outside the study system, looking for females. Many of these males are sedated, blood sampled, and morphometric data collected.

The two main methods for the thorough, in-direct measure of parasites in the Soay sheep is through ante-mortem (faecal collection) and post-mortem (total worm count) sampling. Faecal samples are processed for FEC by modified McMaster, with a 100 epg detection threshold (MAFF, 1986) in which each egg counted was equivalent to 100 eggs per gram wet of faeces. FEC has provided a non-invasive estimate of parasites numbers. The impact of parasites on the fitness of the Soay sheep have been well-researched through the use of in-direct measures such as FEC; with FEC having a negative association with host weight and over-winter survival (Craig et al., 2008; Coltman et al., 1999; Hayward, 2011). However, since FEC is made up of a mixture

of nematode species (Table 1. 1), it is not known which specie(s) is contributing to this negative effect on the sheep.

1. 8. THESIS OUTLINE

Previous parasitological assessments on St Kilda have been predominantly through opportunistic post-mortem investigations of adult worm burdens during high mortality years. There is no data available for the population in low mortality years, or for those individuals surviving a crash year. FEC have been used as a non-invasive assessment of annual parasite burden, though this provides limited information as the strongyle species that infect the Soay sheep produce morphologically-similar eggs (Table 1. 1), therefore, the mixed-species burdens occur in unknown proportions. The primary aim of this thesis is to explore and validate non-invasive traditional and molecular parasitological methods to identify and quantify mixed-species infections in feral hosts.

1. 5. 1. CHAPTER 2: Effects of age, sex, season and method on faecal egg count patterns of gastrointestinal nematodes in feral Soay sheep

FEC are the non-invasive, microscopic identification and quantification of eggs within a faecal sample, which are (usually) quantified in eggs per gram of faeces. Two FEC databases were compared; a longitudinal study (27 years) with many replicated (12192) counted with a high detection limit (100 epg), and a shorter study (1 year) with 0.07% of the replicates (882) counted with a lower detection threshold (1 epg). Monte Carlo simulations were run in order to compare the effects of different detection limits between the FEC methods (1, 10, 50 and 100 epg). As a proof of concept, the simulations were compared against a direct cross-comparison between modified McMaster (100 epg) and cuvette salt floatation (1 epg).

1. 5. 2. CHAPTER 3: Testing multiplex-tandem PCR and deep amplicon sequencing to detect and differentiate ovine gastrointestinal nematodes

Whilst FEC can identify general trends in parasitology, it is too crude a method to distinguish parasite diversity within the helminth group. The development of molecular methods to identify and quantify mixed-species infections has negated many of the issues arising from routine diagnostics, which typically involve microscopic counting of eggs, and morphological characterisation of L_3. The aim of this chapter was to compare two molecular methods; a semi-automated multiplex-tandem PCR (AusDiagnostics™) with ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay), to identify (presence/absence) and quantify (relative proportion - %) ovine strongylid species in naturally-infected samples.

1. 5. 3. CHAPTER 4: Validation of the nemabiome deep-amplicon sequencing approach for strongyle species identified in the Soay sheep of St Kilda

The sensitivity, bias, and repeatability of the nemabiome assay was tested for the strongylid species identified in the Soay sheep of Hirta. A correction factor was calculated for each species (Chapter 4) in order to reduce potential species-specific sequencing bias.

1. 5. 4. CHAPTER 5: Strongyle nematodes in feral Soay sheep – an epidemiological snapshot

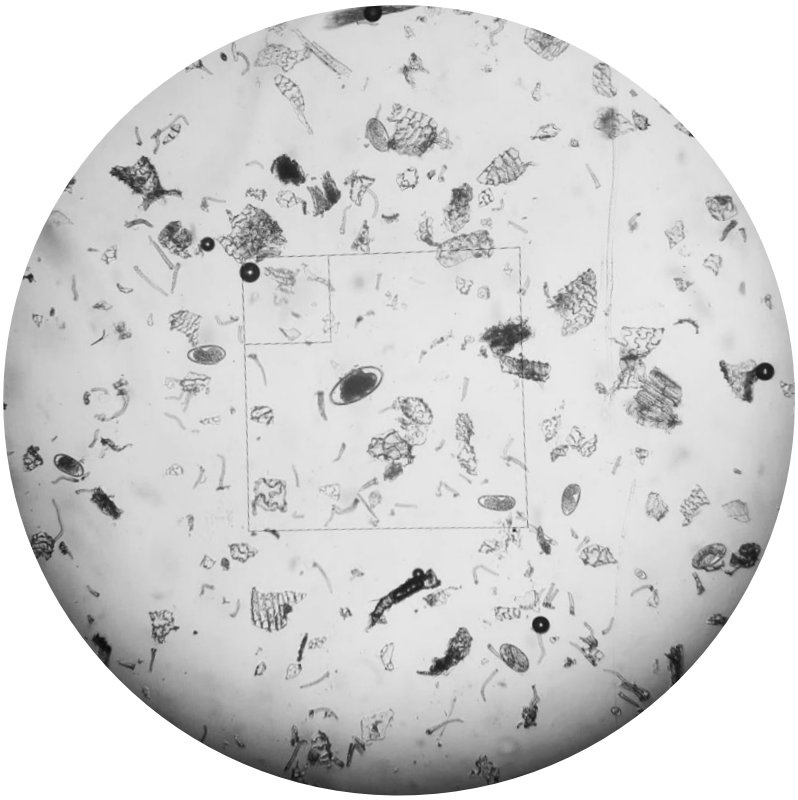
This is the first study to use the next generation 'nemabiome' assay (developed and validated in chapters 2, 3 and 4) to accurately characterise host gastrointestinal nematode burdens and study seasonal patterns in different co-infecting gastrointestinal nematodes in feral sheep. The study highlights epidemiological trends in the Soay sheep that were not previously identified using traditional parasitological methods.

1. 5. 5. CHAPTER 6: General discussion and future directions

In the final chapter the work conducted for the thesis is summarised, the methodology and challenges of working on a remote field site discussed, and potential future research directions considered.

CHAPTER 2

Effects of age, sex, season and method on faecal egg count patterns of gastrointestinal nematodes in feral Soay sheep



2. 1. ABSTRACT

The unmanaged, feral Soay sheep population on St Kilda has survived for hundreds of years, despite enduring the potentially deleterious co-infection of gastrointestinal (GI) nematode. Faecal egg counts (FEC) are the non-invasive measure of parasite burden, and form the basis of the long-term, longitudinal parasitological study of the Soay sheep, which began in 1988.

The objectives of this chapter are to compare two faecal egg count datasets; the large dataset (collected 1988 – 2014) and counted by a modified McMaster method (100 epg), and a smaller dataset collected over 1 year (April 2015 – April 2016) and counted by cuvette salt floatation method with a lower detection limit (up to 1 epg).

Faeces were collected from lambs (4 to 12 months-old), yearlings (13 to 24 months-old), adults (3 to 7 years-old), and geriatrics (8 to 14 years-old), and individual were FEC were performed by modified McMaster and cuvette salt floatation.

The first aim was to correlate the modified McMaster (100 epg) and cuvette salt floatation (up to 1 epg) methods by investigating FEC patterns of season and sex-age. Seasonal and host differences in FEC have been identified, highlighting comparable trends between the two datasets. FEC generally declined with increasing sheep age, until the animals became geriatric (8 years +); lamb > yearling > adult < geriatric. Seasonal FEC patterns in generally followed a decline over the year starting in Spring; Spring > Summer > Winter ($p < 0.0001$). FEC were generally higher in males (<0.0001), regardless of their age category, with little seasonal variation between Summer and Winter once they became adults (3 years +).

Monte Carlo simulations were run in order to compare the effects of different detection limits (1, 10, 50 and 100 epg). A high dilution factor to extrapolate raw strongyle counts to eggs per gram of faeces will magnify Poisson errors, leading to an inflation of the mean and an over estimation of actual egg counts. Consequently, a method that uses a low to no dilution factor has a higher resolution. As a proof of concept, the simulations were compared against a direct cross-comparison between modified McMaster (100 epg) and cuvette salt floatation (1 epg). The results of the cross-count are very similar to that of what was predicted in the simulation (Cross count: $R^2 = 0.452$, simulation: $R^2 = 0.492$).

2. 2. INTRODUCTION

The unmanaged, feral Soay sheep population on St Kilda has survived for hundreds of years, despite enduring the potentially deleterious co-infection of gastrointestinal (GI) nematodes, protozoa and ectoparasite species. Strongyle nematodes are believed to be a major contributor to the high mortality of the Soay sheep, exacerbating the effects of intermittent periods of starvation during winter (Gulland, 1992; Craig, 2005).

The quantitative understanding of parasite population dynamics, and their impact on the host, requires a good estimation of infection prevalence (i.e. proportion of animals infected) and intensity (i.e. mean parasite burden per animal). Since the Soay sheep on St Kilda are protected, it is not possible to assess adult worm burdens by sacrificing healthy animals and post-mortem, therefore, indirect measures are required. Coprological techniques, such as faecal egg counts (FEC), form the basis of GI nematode egg detection, and estimation of parasite abundance within a host. FEC are the non-invasive, microscopic identification and quantification of eggs within a faecal sample, which are (usually) quantified in eggs per gram of faeces (epg) (MAFF, 1986). FEC are a well-established method in monitoring helminth burdens in both managed and wild systems; and are often the only measure available to estimate parasite intensity in free-living animals; routinely used in a range of systems and species (African antelope Vadlejch et al., 2015; reindeer Irvine et al., 2001; wild boar Gassó et al., 2015; red grouse Seivwright et al., 2004; grey mouse lemur Hämäläinen et al., 2015).

The study of parasites infecting the Soay sheep was initiated by a population crash in 1964 (Cheyne et al., (1974). The long-term longitudinal study of the Soay sheep began in 1985 (described in Chapter 1), and the parasitological research includes annual faecal sampling in Summer (which started from 1988) to measure FEC by modified McMaster, and opportunistic post-mortem surveys of naturally-deceased individuals (Gulland,1992; Craig, 2005; Pilkington, *continuous sample collection*). From this longitudinal FEC database, the potential impact of parasitism on Soay sheep life-history have been explored.

The numbers in eggs passaged by the Soay sheep generally decline with age, until they pass prime-age: lamb (0 – 12 months) > yearling (13 – 24 months) > adult (2-7 years) < geriatric (8+ years) (Clutton-Brock and Pemberton, 2004; Hayward et al., 2009). Prime age individuals in the context of this study are the adult groups (2 to 7 years of age), however, within a production system prime breeding animals would most likely range from 2 to 5 years, as after 5 years of age breeding success declines. In both Soay sheep and Scottish hill sheep, lambs first acquire parasites within one to two months of birth, with burdens increasing during the first six months

of life (regardless of sex). Immune competence is not acquired until ≥4 months of age (depending on breed/nematode species challenge) (Miller and Horohov, 2006), which is generally characterised by increasing FEC as they become infected. The parasites establish within their predilection sites, triggering an immune response from the host, which results in a decline in egg output. From 12 months of age, the pattern in FEC in association to age is reversed; the temporal variation in ewe parasitism is dominated by an increase in Spring (April – May) in response to pregnancy. Biological senescence is the age-specific decline in physiological condition of the host, which impacts survival and fecundity of the nematodes (Bonsall, 2006). Research into aging and parasitism in the Soay sheep has found an age-related increase in FEC associated with animals from the age of 8 years old (females) and 5 years old (males) (Hayward et al., 2009). This is believed to be linked with senescence-related changes in parasite resistance, with host immunity response to parasitic infection declining with age. However, these interactions are not linear and are linked to environmental factors, a common factor associated with a variable environment such as St Kida. Defining the impacts of parasitism with changing age needs to account the 'chronological' and 'biological' age of the sheep. Chronological age fails to account for environmental impacts that can affect host parasitism, such as stress during early development and cumulative environmental stress throughout the animals life history, which can impact senescence later in life. This was seen by Hayward et al., (2009), where animals that have undergone high stress early in life – measured by weight gain and breeding success – are more susceptible to parasitism later in life and have higher prime-age mortality.

Seasonal and host egg shedding patterns observed in the Soay sheep are determined by the immunological status of the sheep (age, sex, nutritional-status, hormonal level), temporal trends in pasture larval contamination and biological factors associated with the parasites (arrestment, mortality, fecundity and development). Generally, FEC declines over the year; spring > summer > winter. The relaxation of the ewe's immunity during pregnancy causes an increase in parasite egg output, known as the peri-parturient rise (PPR), which can last up to 8 weeks after parturition. In the Soay sheep on St Kilda, the peak of PPR occurs within ~10 days of parturition, in comparison to managed sheep were it usually occurs two to four weeks after (Wilson et al., 2004). Generally, for all sex-age groups, early spring is characterised by having the highest average FEC, which is due to many factors including PPR, high population density with the arrival of lambs, young lambs yet to develop immunity to parasites, males recovering from the previous mating season and warmer weather, allowing for these eggs to develop successfully and to re-infect in the summer months. There is little seasonal variation seen in the

males, despite a late winter-early spring rise in high density years, in response to low food availability (Gulland and Fox, 1992; Clutton-Brock and Pemberton, 2004).

Parasite prevalence and FEC is generally higher in males, regardless of the age category, and in many vertebrate species a male-biased to a higher parasite burden is the most consistent finding (Schalk and Forbes, 1997; Moore and Wilson, 2002). In mammalian species, these sex-bias increases with sexual size dimorphism; the sex with the larger body mass has a higher adult worm burden (Moore and Wilson, 2002). Previous studies on St Kilda has found that male Soay sheep are susceptible to more intense nematode infections in comparison to females, in addition to a higher diversity of parasite species (Wilson et al., 2008; Craig, 2005). Additionally, the Soay males are subject to strong sexual selection, with an elevation of testosterone during the rutting months. Testosterone has been found to be immunosuppressive while oestrogen can stimulate the immune system (Zuk and Mkean, 1996; Schalk and Forbes, 1997). That being said, during the winter months parasitic larvae in the environment are at their lowest, which means that even though these eggs are being shed onto the pasture at high levels from the males, due to environmental conditions the parasites are not developing into 3rd- stage larvae (L_3) and therefore the other Soay groups are not re-infected and it is the lowest month for FEC in the females.

The examination of faeces for helminth eggs varies from a simple slide smear (Stoll method – Stoll, 1930) in order to detect the presence of parasite eggs, to quantitative methods that can involve floatation solutions, centrifugation and a microscopic-chamber count. A floatation solution is used to separate eggs from heavy faecal debris, and relies on the specific gravity (SG) of the solution for this separation, allowing a cleaner and more accurate count. The SG of a floatation solution is generally chosen for its ability to float ova of specific species, and for its ease in making and disposal. Physical-chemical properties of the solutions, such as viscosity and SG, are important factors in the solutions ability to float strongyle eggs (MAFF, 1986). Saturated sodium chloride (NaCl, SG 1.204) is the most commonly used solution, as it is capable of floating the commonest nematode and cestode species found in the gastrointestinal tract of small ruminants (MAFF, 1971). Multiple species can be pooled during counting due to their morphologically similar eggs (i.e. strongyle-type species).

There are many faecal egg counting methods described in the literature, and they vary in accuracy, precision, sensitivity, specificity, detection limit and technical difficulty (Paras et al., 2018). It is important to understand these differences in order to appropriately interpret the results from different egg counting techniques and their modifications. Accuracy and precision are arguably the most important measures during egg counting, as they relate to the methods

ability to 1. measure close to 'true' egg count (accuracy) and 2. repeatedly detect parasite eggs within the same sample or host (precision) (Levecke et al., 2012; Cringoli et al., 2004). Accuracy can be improved by thorough homogenisation of the faecal suspension prior to subsampling, therefore reducing potential sources of egg loss during processing. Technical (i.e. the egg counting method/s variation – floatation solution) and biological (i.e. host-specific variation – immunology, age, sex) variation may influence the precision of FEC (Levecke et al., 2012). Other qualitative measures such as sensitivity and specificity relate to the qualitative aspects of the faecal egg counting method and are most important when the egg count is low. Sensitivity is the ability of a test to correctly detect egg-positive samples, whilst specificity is the tests ability to correctly identify negative (egg-free) samples (Levecke et al., 2012). The detection limit (also referred to as multiplication or dilution factor) is the smallest egg count detectable with the method, and is particularly pertinent when detecting low numbers of eggs is required, such as a faecal egg count reduction test (FECRT). The detection limit is determined by the weight of faeces, the volume of the flotation solution in which the faeces were homogenised in, and the volume of fluid visualised under a slide/ chamber (Levecke et al., 2012). Methods that have a high detection limit generally require less processing time. The liquid quantities are chosen so the epg can easily be determined by a simple conversion factor. This dilution factor for transformation to epg assumes that the eggs are Poisson distributed; that the eggs are randomly distributed within the floatation solution. Egg counting methods that are based on flotation-dilution principles (as described by Stoll, 1930), make this assumption that eggs are randomly distributed within the flotation solution, and requires the filtrate to be well-mixed before being aliquoted into a counting slide (Dunn and Keymer, 1986).

The McMaster method, and its modifications (Gordon and Whitlock, 1939; Whitlock, 1948), remains the most universally utilised technique for estimating helminth egg number in faeces for humans (Bondarenko et al., 2009), livestock (Coles et al., 1992) and wildlife (Elephants (Lynsdale et al., 2015); wild Buffalo (Budischak et al., 2015); Soay sheep (Gulland, 1991; Craig et al., 2005; Pilkington, unpublished); wild turkeys (Hopkins et al., 1990); Ibex (Lavin et al., 1997)), due to the straightforward methodology (Levecke et al., 2009). The technique is advocated by the World Association for the Advancement of Veterinary Parasitology (WAAVP) for evaluating the efficacy of anthemintic drugs in ruminant livestock, in addition to detecting anthelmintic resistance by the FECRT (Coles et al., 1992).

Within the literature there are many variations of the McMaster method. Adapting the floatation solution, floatation time, sample dilution, additional centrifugation and the size/ number of the McMaster slide counting chambers (i.e. the suspension volume) examined, will

inevitably alter the diagnostic sensitivity of the method (Cringoli et al., 2004; Pereckiene et al., 2007). As in the case of any egg counting method based on the flotation-dilution principle, the modified McMaster is characterised by a low analytic sensitivity (usually a minimum of 10-100 epg), which will affect the interpretation of counts, especially at low egg count numbers (Mes et al., 2001), and thus limited by its sensitivity to detect low egg densities at high detection limits (i.e. at high dilutions).

Several studies have compared the different modifications of the McMaster method (Cringoli et al., 2004; Mes et al., 2001; Mes et al., 2003), to determine which combine the optimal characteristics of accuracy, reliability and practicality. These studies found the adaptation that used a larger weight of faeces and a lower dilution ratio (g of faeces per ml of water) and, subsequently, a lower multiplication factor when converting the raw egg count to epg produced results that were more reliable with improved sensitivity.

The sensitivity of a McMaster count can be improved by counting more than one slide chamber, as it would be assumed that counting a larger volume would result in a smaller multiplication factor (detection limit). Previous studies have concluded that when a larger volume was examined (i.e. both McMasters chambers), the reliability of the technique was improved in comparison to lower volumes, and these lower volumes produced counts that were unreliable overestimates of epg (Cringoli et al., 2004). However, it has been suggested that parasite eggs could potentially aggregate to the centre of the slide, which could explain the high counts seen for the larger slide areas (0.15 ml and 0.30 ml) (Mes, 2003). The detection limit could be reduced further if multiple slides are counted for each sample, but this precision is offset by the increased effort versus only a slight improvement in detection (Lester and Matthews, 2014). Additionally, as in the case for any parasitological method that utilises morphological identification, there will be some human error. When an egg counting method is based on a high detection limit, the margin for human error is compounded. Mite eggs, fungal spores and pollen grains need to be correctly distinguished from the different strongyle egg morphs. When the detection limit is 100 epg, the incorrect diagnosis will increase the result by 100, as 1 egg = 100 epg.

In order to improve the sensitivity of copromicroscopic diagnosis, newer techniques that have a lower detection limit have allowed the identification of strongyle eggs down to 1 epg. They include the Cornell-Wisconsin method (Egwand and Slocombe, 1982), FLOTAC (Cringoli et al., 2010), and cuvette salt-floatation method (Christie and Jackson, 1982). A method that uses a low, or no, multiplication factor to convert the number of eggs seen under a microscopic slide

to an epg estimation, is more sensitive to changes in egg abundance. Methods with a higher sensitivity are generally associated with more steps and specialised equipment, hence are argued to be more time consuming (Lester and Matthews, 2013). The cuvette salt floatation method is a modification of the salt-flotation method and includes a centrifugation step, with the suspension being counted within a cuvette, and has a detection limit of 1 – 9 epg (depending on the proportion of the cuvette that is examined). Many studies have used this method in horses (Lester, 2015), sheep (Hamer et al., 2018) and goats (Patterson et al., 1996).

For this Chapter, two FEC datasets were analysed in order to describe the seasonal and sex-age differences in GI nematode egg shedding patterns in the Soay sheep. Each dataset was used for a different aim. The first dataset was the large database collected by the Soay Sheep Project since 1988 (described in Chapter 1), and had been counted by modified McMaster (100 epg). This data was used for the first initial analysis of seasonal patterns and sex-age differences to inform study design of Chapter 5, which included establishing field-work months and which age-groups to target. The second dataset was created during this project in order to inform and supplement future GI nematode speciation work (Chapter 5). These faecal samples were counted by a method with a lower detection threshold; cuvette salt floatation (1 epg). When analysing both data sets new questions arose: how comparable are the results obtained from modified McMaster (100 epg) and cuvette salt floatation (1 epg)? By lowering the detection limit, would one year of sampling be able to capture the overall seasonal and sex/age trends seen in the large (12192 samples) dataset?

2. 3. METHODS

2. 3. 1. Dataset definitions

Samples collected as part of the Soay Sheep Project monitoring program (described in Chapter 1), over 1988 – 2014, were counted by modified McMaster (100 epg), by Jill Pilkington and volunteers. This dataset will be referred to as the modified McMaster dataset.

Samples collected as part of this thesis, over 2015 – 2016, was counted by cuvette salt floatation (1 epg). This dataset will be referred to as the cuvette salt floatation dataset.

2. 3. 2. Age groups and definitions

Every spring, since 1985, lambs within Village Bay are caught and tagged, with the cohort being tagged with a specific colour. Up to 95% of sheep within Village Bay area are tagged, and can be identified by their unique ear tag number/ colour combination (Figure 1. 3., Chapter 1). The sheep for the study were aged by their cohort year; lambs (4 – 12 months), yearlings (13 – 24 months), 2-year olds, adults (3 – 7 years), and geriatric females (8 – 12 years). A cohort, in the

context of this study, is a group of individuals born within the same year, whilst a group of animals is made up of several individuals of different ages. Lambs and yearlings are a cohort group, whilst adults and geriatrics are groups of sheep born over different years. For the cuvette salt floatation dataset, lambs and yearling were born in 2015 and 2014 (respectively), adults were born between 2008-2012 and geriatrics born between 2003 – 2007. For the modified McMaster dataset, age was determined by year of birth.

Samples collected over 2015-2016 (cuvette salt floatation dataset) did not include certain groups. There were not enough geriatric males in the study system for an additional group. Very young lambs (0 – 4 months) were not sampled to reduce the risk of the mother rejecting the lamb due to prolonged close human proximity. The 2-year olds (2013 cohort) were initially a separate test group but were later removed entirely from the study due to small sample numbers. To keep consistency when comparing the two datasets, the 2-year olds were also removed from the samples collected over 1988-2014 (modified McMaster dataset).

2. 3. 3. Test months/ years

2. 3. 3. 1. *Modified McMaster dataset*

Between 1988 and 2014, 12,192 faecal samples were collected, processed and counted by the Soay Sheep Project. The number of samples collected per season, and per year, varied depending on the work being conducted on the sheep at that time, and many of the samples were collected during opportunistic on-pasture sampling. The majority of faecal samples are aggregated to the summer months (August) (Table 2. 1) when a large number of animals are caught annually and rectally faecal sampled. Faecal samples were collected throughout the year, but due to due to logistical constraints there were no collections during June, July, December and January. To provide consistency in the analysis, these months were combined into 'test seasons': Spring (March – May, excluding June and July), Summer (August) and Winter (September – February, excluding December and January).

Table 2. 1. Number of faecal samples contributing to each test group over the test seasons (Spring, Summer and Winter) from 1988 - 2014. Any animals that were 2-years old at the time of sampling were removed from the dataset. Additionally, 6 castrated males were removed.

Season	Test groups (*n*=)							
	Lambs *4-12 months*		**Yearlings** *13-24 months*		**Adults** *3-7 years*		**Geriatric** *8 -14 years*	
	Females	Males	Females	Males	Females	Males	Females	Males
Spring	882	179	780	71	1686	174	343	5
Summer	1133	1093	550	383	1308	277	618	44
Winter	526	699	139	237	405	522	88	50

2. 3. 3. 2. *Cuvette salt floatation dataset*

Between April 2015 and April 2016, 882 faecal samples were collected (Table 2. 2). Samples were collected over 7 months; Spring 2015 (April), Summer 2015 (August), Winter 2015 (October, November and February) and Spring 2016 (March and April). The precise number of samples collected per season was dependent on the number of animals in the study area, weather, food availability and mortality. During this single sampling year, these two cohorts transitioned from one test group to another (i.e. lambs into yearlings, yearlings into 2-year olds). In order to prevent misleading results, an additional test season was made (Spring 2016).

Table 2. 2. Number of faecal samples contributing to each test group over the test seasons from April 2015 (Spring 2015) - April 2016 (Spring 2016). Individuals from the 2013 cohort (2-year olds at the time of sampling) were removed from the dataset due to low numbers. Lambs < 4 months old were not collected from (Spring 2015).

Season	Test groups (*n*=)						
	Lambs *4-12 months*		**Yearlings** *13-24 months*		**Adults** *3-7 years*		**Geriatrics** *8 -14 years*
	Females	Males	Females	Males	Females	Males	Females
Spring 2015	-	-	31	25	20	28	10
Summer 2015	81	81	12	19	62	11	17
Winter 2015	41	35	25	23	81	31	51
Spring 2016	35	9	22	11	79	24	18

2. 3. 4. Faecal collection and storage

Fresh faecal samples were collected either rectally, or off pasture, from known individuals immediately after direct observation of defecation. Rectally collected faecal samples were collected by a trained technician in guidance with approved ethics. The whole faecal pat was

placed into a zip-lock bag, expelling as much air as possible before sealing, and stored (anaerobically) at 4°C until FEC. Counts occurred within 14 days of collection.

2. 3. 5. Cross-count comparison

Additional faecal samples were collected in order to directly compare results between modified McMaster and cuvette salt floatation; August (104 samples) and November (112 samples) were collected in 2015, modified McMaster was counted by Jill Pilkington. In August 2016, an additional 216 samples were collected by the Soay Sheep Project volunteers and were cross-counted by Jill Pilkington (modified McMaster) and the Moredun Research Institute (Scotland) (cuvette salt floatation). All datasets were combined for a direct cross-count comparison of 432 samples.

2. 3. 5. Faecal egg counting techniques

Strongyle and *Nematodirus* spp. eggs were counted, and the presence/absence of *Moniezia expansa* and *Trichuris ovis* were noted. Strongyles are parasite taxa that have eggs that are morphologically similar and therefore grouped together into a single count; *Trichostrongylus spp., Teladorsagia circumcincta, Chabertia ovina* and *Bunostomum trigonocephalum*. Parasite eggs within a faecal sample are aggregated (Morgan et al., 2005), therefore, prior to weighing faeces for FEC, all faecal samples were well homogenised within their sample bags in an attempt to evenly distribute the eggs.

2. 3. 5. 1. *Modified McMaster method (Gordon and Whitlock, 1939, described in MAFF, 1986)*

In brief, 3 g (wet weight) of faeces were homogenised in 87 ml of saturated sodium chloride solution (NaCl, SG=1.204; MAFF, 1986) and poured over a 1 mm sieve into a beaker, to make a 90 ml total volume. Using a pasteur pipet both chambers of a standard McMaster slide (Figure 2. 1) were filled (1.0 ml). The total number of eggs counted under the grind in both chambers was multiplied by 100 in order to obtain the number of epg, resulting in a method that has a dilution limit of 100 epg of faeces.

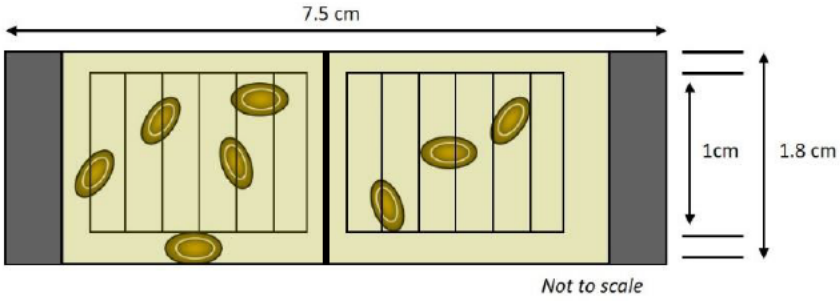


Figure 2. 1. Diagram of a McMaster counting chamber. Volume of each chamber = 0.5 cm^3, volume under grid each side = 0.15 cm^3. Depth of the slide = 0.15 cm, width of each chamber = 1.8 cm, with the width of the counting grid = 1 cm. Eggs located outside of the grid are not counted (adapted from MAFF, 1986).

2. 3. 5. 2. *Cuvette salt floatation method (Christie and Jackson, 1982)*

Faeces were first weighed, and 10 ml of tap water was added per 1 g wet weight of faeces and emulsified thoroughly. A 10 ml subsample (i.e. 1 g of faeces) was passed over a 1 mm sieve into a beaker, washing the retentate with a further 5 ml tap water before discarding. The filtrate was then transferred into a 15 ml polyallomer tube and centrifuged at 203 xg for 2 minutes, and the supernatant was discarded. The faecal pellet was then re-suspended in 10 ml saturated sodium chloride solution (NaCl, SG=1.204) and centrifuged at 203 xg for 2 minutes. The eggs floating at the meniscus of the NaCl solution were collected by clamping the polyallomer tube just below the meniscus with artery forceps and poured into a 2.5 ml cuvette (Sigma-Aldrich®, UK). The cuvette was inverted to ensure even distribution of eggs, topped with NaCl solution until there was a positive meniscus and the lid was attached. The cuvette was then placed horizontally onto an adapted microscope slide and examined under a compound microscope fitted with a Miller Square eyepiece graticule (Graticules Ltd., UK) (x40 magnification, or when the Miller square is one sixth the width of the cuvette) (Figure 2. 2). Strongyle spp. and *Nematodirus* spp. counts, and the presence/ absence of other species, were recorded in an Excel spreadsheet (Microsoft, USA).

Depending on the egg density, all or a portion of the cuvette was counted within the Miller Square (Figure 2. 2). Where there was a low egg density (i.e. ~ < 20 eggs visualised in the whole cuvette) all of the eggs were counted (Figure 2. 3. A) and no multiplication factor was used, resulting in a detection limit of 1 epg. If there was a moderate density of eggs (i.e. ~20 – 50 eggs), two traverses of the cuvette were counted using the large Millar Square (Figure 2. 3. B), resulting in a third of the total cuvette volume being counted. The number of eggs from each

traverse were added together and then multiplied by three to give an epg estimate, resulting in a 3 epg detection limit. If there was a high egg density (i.e. ~>50 eggs), two traverses of the cuvette were counted using the smallest square of the Miller Square (Figure 2. 3. C), which is equal to one ninth of the total cuvette volume. The total number of eggs counted in each traverse was added together and multiplied by nine, resulting in an epg count with a detection limit of 9 epg. To establish the presence/ absence of additional species the whole cuvette was examined, presence was defined as the identification of one, or more, eggs in the whole cuvette.

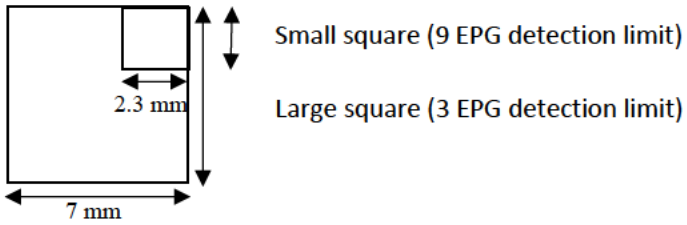


Figure 2. 2. Diagram of the Miller Square. The smallest square is $^1/_3$ the width of the larger square (7 x 7 mm). When there is a moderate egg density the large square is used, resulting in a detection limit (or multiplication factor) of 3 epg. When there is a high egg density, the small square is used, resulting in a detection limit of 9 epg (figure from Lester, 2015).

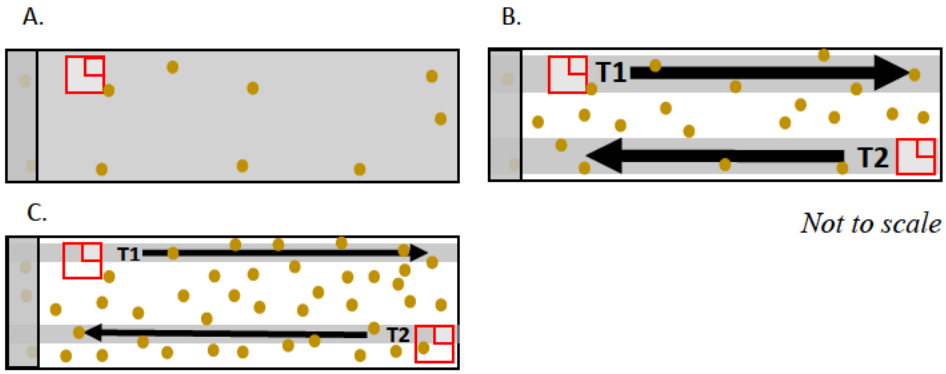


Figure 2. 3. Diagrams illustrating the different areas counted, depending on the density of eggs in the cuvette. A. Low egg density (< ~20 eggs), all the eggs within the cuvette are counted providing an egg count with a sensitivity of 1 epg. B. A medium egg density (~20-50 eggs), the eggs are counted within two traverses (traverse 1 (T1) and 2 (T2)) of the cuvette using the largest square of the Miller Square. Counts are then multiplied by 3 epg. C. A high egg density (>50 eggs) the number of eggs are counted in 2 traverses using the small square of the Miller Square and multiplied by 9 epg (adapted from Bartley and Elsheikha, 2011). Not to scale: the Miller Square is visualised at one sixth of the width of the cuvette. Eggs not counted within the lid.

2. 3. 6. Statistical analyses

The arithmetic mean (epg) (± standard error of the mean, SEM), epg range (min – max) and number (n) of positive samples and proportion (%) of positive samples, for each database, were summarised in tables.

The FEC data were then log-transformed (log (FEC+100) to fit normality assumptions. Strongyle FEC for both modified McMaster and cuvette salt floatation datasets were divided by season, age and sex, and visualised in box/whisker plots. Histograms of FEC before and after log-transformation (log10 (FEC+100) for both datasets were made.

Normalised data were then analysed by univariate generalised linear models (GLM), global tests of each variable (season, age group and sex) were tested for overall significant difference. Generalized linear mixed model (GLMM) post-hoc pairwise comparisons were used when main effects were significant at $p = \leq 0.05$. The modified McMaster dataset had both fixed and random effects. The data had been collected from 1988-2014, therefore, to account for repeated FEC sampling of the same individual between months, and across years, the random effects were the year of sampling and individual ID. The fixed effects were season (3 classes: Spring, Summer and Winter), age group (4 classes: lamb, yearling, adult and geriatric), and sex (2 classes). The cuvette salt floatation dataset was collected over 1 year (2015-2016), therefore no random effects were used. Fixed effects were season (4 classes: Spring 2015, Summer 2015, Winter 2015 and Spring 2016), age group (4 classes) and sex (2 classes). Statistical analyses were conducted using R (version 3. 3. 3. R Development Core Team, 2017), with plots created using the package ggplot2 (Wickham, 2016). Differences of ls means analysis were used for the pair-wise comparisons of the different seasons, by combining Male and female data overall seasonal differences (i.e. the overall pasture contamination between Spring, Summer and Winter) was tested.

2. 2. 6. 1. Comparing dilution factors

Monte Carlo simulation

A FEC relies on the assumption that the eggs are evenly distributed in the floatation solution. If they are evenly distributed then the number of eggs observed in serial subsamples should follow a Poisson distribution. However, dilution from a dilution factor magnifies Poisson errors (Torgerson et al., 2012). In order to investigate the effect of increasing dilution factor on the estimated egg count, four Monte Carlo simulations were run to simulate data assuming underlying Poisson process and different dilution factors, using the package 'eggCounts' (Wang

and Paul, 2016) (version 1.4, http://cran.r-project.org/web/packages/eggCounts/index.html), and the plots were made in R (version 3. 3. 3).

In these simulations the number (n) of 'faecal samples' counted was 1000. A negative binomial distribution is believed to sufficiently model egg count data (Morgan et al., 2005), and an aggregation parameter (or over-dispersion parameter) k (kappa) is used to inform how negatively skewed the data is. By increasing k (>10), the negative binomial distribution of egg counts within a flock will begin to approach a normal distribution. When k is low, the data becomes negatively skewed to the left, which is commonly seen in parasite data. In many studies that are using Monte Carlo simulations in order to test the effect of a parameter (i.e. sample size, dilution factor, egg level, repeated sampling) on egg counts, k=1 is often used as it confers to a high level of egg aggregation across the flock (with a commercial flock varying from 0.2 to 2.3) (Dobson et al., 2009). Four simulations were run, the parameters remained the same for each situation (n=1000, mean=100, k=1) with the exception of the dilution factor f. Dilution factors 1 epg, 10 epg, 50 epg and 100 epg were tested. The simulation generated a table with 3 sets of values; the observed FEC, the true FEC and the master FEC. The master FEC is the number of eggs counted on the McMaster slide before the dilution factor if the mean has a very low value it will be frequently rounded down, creating a negative bias overall. Master FEC differs from the 'true FEC' as it is subject to Poisson variation around the true FEC. The observed FEC is different from the true FEC, due to the variation introduced by the sampling method (i.e. the dilution factor). This variation component was simulated using a Poisson distribution as defined by the expected number of eggs counted (= 'true' host FEC/ dilution factor) (Leveche et al., 2011; Dobson et al., 2009). This comparison of observed and true FEC was simulated for each of the dilution factors and linear regression models were plotted into four graphs.

Cross-count comparison

Linear regression analysis calculated the correlation of the 432 samples that had been cross-counted by modified McMaster and cuvette salt floatation. The values for R^2 (coefficient of determination), b (y-intercept) and m (slope) are shown adjacent to the plot.

2. 4. RESULTS

2. 4. 1. Sex, age and seasonal patterns of faecal egg counts (modified McMaster dataset)

From the global tests of each variable (season, age, and sex) there is an overall statistical difference (p= < 0. 001) between each of the factors (season, age group and sex) (Table 2. 5).

From the GLMM model, Summer (t = -34.114, p < 0.001), Winter (t=-38.417, p < 0.001), lambs (t=53.365, p < 0.001), yearlings (t=23.160, p < 0.001), geriatrics (t=3.154, p < 0.01) and males (t=16.703, p < 0.001) were statistically different (Table 2. 6). When male and female data are combined, pairwise comparisons were made of overall seasonal differences (i.e. the overall pasture contamination between the seasons); FEC during Spring is higher than Summer (t=34.11, p < 0.001) and Winter (t=38.42, p < 0.001), and Summer is higher than Winter (t=10.05, p < 0.001) (Table 2. 7).

2. 3. 1. 1. Females

All female groups (lambs, yearlings, adults and geriatrics) follow the same decline in FEC over the year, starting in Spring; Spring > Summer > Winter (Figure 2. 4). Trends in FEC over the female age groups decline with age, until they reach 8 years old (i.e. the geriatric group), lambs > yearlings > adults < geriatrics (Figure 2. 4). In general, lambs have the highest average egg count over the year; Spring 1628 epg, Summer 615 epg and Winter 489 epg. This is most likely due to the large range (min-max) in egg counts recorded, with some individuals having counts as high as 18,600 (Spring) and 52,000 (Summer). The adult group has the lowest egg counts recorded; Spring 310 epg, Summer 93 epg, Winter 28 epg (18% positively-recorded samples). Average egg counts (±SEM), range and prevalence (i.e. % number of egg-positive samples) are summarised in Table 2. 3.

2. 3. 1. 2. Males

Similar to the females, male lambs and yearlings show a decline in FEC over the year, starting with Spring (Figure 2. 4). However, after 24 months of age, adult males show little seasonality between Spring (325 epg), Summer (373 epg) and Winter (267 epg), with a slight elevation in Summer. Similarly, the geriatric group has the same trend; Spring (302 epg), Summer (363) and Winter (293 epg). However, the male geriatric group must be interpreted with caution as due to small sample size (n=5). Trends in FEC over the male age groups decline with age, until they reach adulthood. (Figure 2. 4). However, as illustrated above, there is little difference between the adults and geriatrics male groups, with a summer rise in FEC. Lambs have the highest average FEC; Spring 1783 epg, Summer 786 epg and Winter 663 epg. Additionally, like the females, there is a large range (min-max) in egg counts from the male lambs, with some individuals producing up to 13,700 epg. Average egg counts (±SEM), range and prevalence (i.e. % number of egg-positive samples) are summarised in Table 2. 4.

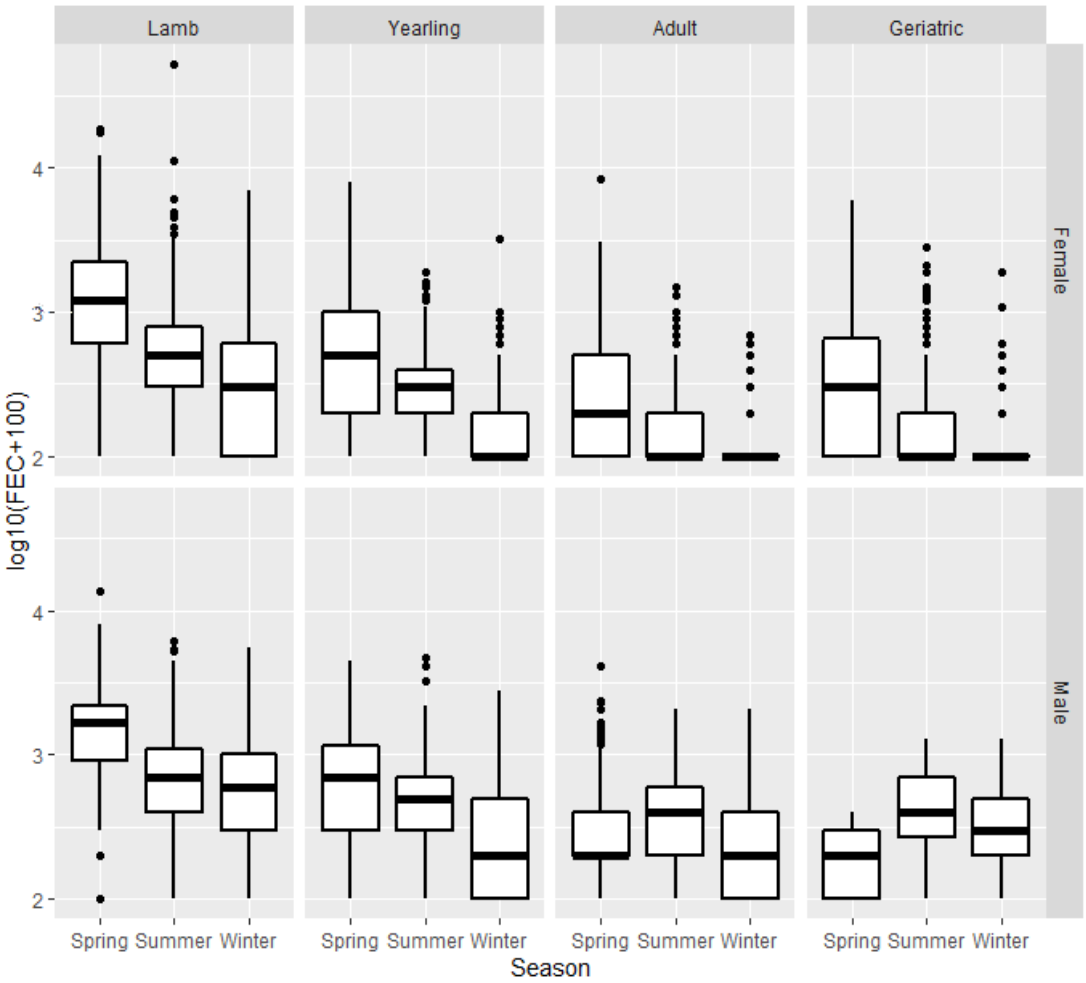


Figure 2. 4. Box and whisker plots of egg count data (log-transformed) collected from 2015 – 2016 and counted by modified McMaster (100 epg). Data divided between age group, sex and season. The data range is shown by the vertical black lines, with the median of each dataset represented by the middle horizontal line within each boxplot and with any outliers shown as points.

Table 2. 3. Average FEC (epg) (± standard error of the mean, SEM) collected from female Soay sheep and counted by modified McMaster method. Collected from 1988 - 2014. n (number of samples), range (epg) and number of positive samples (n) and proportion (%) of positive samples.

Season	Group	n=	Arithmetic mean (epg) ± SEM	Range (epg)	n positive (%)
Spring	Lamb	882	1628 ± 61	0 - 18600	851 (96%)
	Yearling	780	683 ± 32	0 - 7800	638 (82%)
	Adult	1686	310 ± 11	0 - 8400	1096 (65%)
	Geriatric	343	460 ± 64	0 - 5900	233 (68%)
Summer	Lamb	1133	615 ± 50	0 - 52000	1038 (92%)
	Yearling	550	240 ± 12	0 - 1800	416 (76%)
	Adult	1308	93 ± 4	0 - 1400	576 (44%)
	Geriatric	618	125 ± 13	0 - 2700	272 (44%)
Winter	Lamb	526	489 ± 38	0 - 6800	384 (73%)
	Yearling	139	124 ± 26	0 - 3100	67 (48%)
	Adult	405	28 ± 4	0 - 600	73 (18%)
	Geriatric	88	83 ± 33	0 - 1800	20 (23%)

Table 2. 4. Average FEC (epg) (± standard error of the mean, SEM) collected from male Soay sheep and counted by modified McMaster method. Collected from 1988 - 2014. n (number of samples), range (epg) and number of positive samples (n) and proportion (%) of positive samples.

Season	Group	n=	Arithmetic mean (epg) ± SEM	Range (epg)	n positive (%)
Spring	Lamb	179	1783 ± 118	0 - 13700	174 (97%)
	Yearling	71	780 ± 94	0 - 4400	63 (89%)
	Adult	178	325 ± 40	0 - 4000	134 (75%)
	Geriatric	5	302 ± 61	0 - 1000	4 (80%)
Summer	Lamb	1093	786 ± 23	0 - 6000	1054 (96%)
	Yearling	383	470 ± 26	0 - 4600	343 (90%)
	Adult	302	373 ± 19	0 - 2000	259 (86%)
	Geriatric	44	363 ± 70	0 - 1000	39 (89%)
Winter	Lamb	699	663 ± 24	0 - 5400	632 (90%)
	Yearling	237	267 ± 24	0 - 2700	157 (66%)
	Adult	557	210 ± 11	0 - 2000	362 (65%)
	Geriatric	50	293 ± 52	0 - 600	44 (87%)

Table 2. 5. Analysis of variance table for GLM of FEC over season, age group and sex (modified McMaster dataset).

	DF	Sum of square	RSS	F-value	P
Season	2	180.052	90.026	885.20	< 2e-16 ***
Age group	3	297.756	99.252	975.92	< 2e-16 ***
Sex	1	28.372	28.372	278.98	< 2e-16 ***

*** p < 0.001

Table 2. 6. GLMM model summary of level by level comparisons (modified McMaster dataset).

	Estimate	SE	t-value	P
(Intercept)	2.358e+00	2.346e-02	100.548	< 2e-16 ***
Summer	-3.089e-01	9.054e-03	-34.114	< 2e-16 ***
Winter	-4.087e-01	1.064e-02	-38.417	< 2e-16 ***
Geriatric	3.769e-02	1.195e-02	3.154	0.00161**
Lamb	4.702e-01	8.811e-03	53.365	< 2e-16 ***
Yearling	2.277e-01	9.833e-03	23.160	< 2e-16 ***
Male	1.686e-01	1.009e-02	16.703	< 2e-16 ***

** p < 0.01 *** p < 0.001

Table 2. 7. Results of the analysis testing for differences between sampling seasons for data counted by modified McMasters (males and females combined).

Pair-wise comparison	Estimate	SE	DF	t-value	P
Spring - Summer	0.3089	0.0091	11248.9	34.11	***
Spring - Winter	0.4087	0.0106	11977.1	38.42	***
Summer - Winter	0.0998	0.0099	11357.1	10.05	***

Differences of ls means analysis were used for the pair-wise comparisons.
*** p < 0.001

2. 4. 2. Sex, age and season on patterns of faecal egg counts (cuvette salt floatation dataset)

From the global tests of each variable (season, age, and sex) there is an overall statistical difference (p= < 0.001) between each of the factors (Table 2. 10). From the GLMM model, Spring 2016 (t=-4.345, p < 0.001), Summer (t = -12.32, p < 0.001), Winter (t=-11.144, p < 0.001), lambs (t=15.668, p < 0.001), yearlings (t=7.959, p < 0.001) and males (t=4.471, p < 0.001) were statistically different. The geriatric females were not statistically different (geriatrics (t=3.154, p=0.391) (Table. 2. 12).

When male and female data are combined, pairwise comparisons were made of overall seasonal differences (i.e. the overall pasture contamination between spring 2015, summer, winter and spring 2016); FEC during Spring (2015 and 2016) is higher than summer (p < 0.001) and winter (p < 0.001), there was no statistical significant difference between summer 2015 and winter 2015 (p = 0.0934). Spring 2015 was statistically different to Spring 2016 (p < 0.001) (Table 2. 11).

2. 4. 2. 1. Females

Females (lambs and adults) followed a similar trend; an increase in FEC from summer to winter, and then to the following spring (2016) (Figure 2. 5). Female lambs (Summer 133 epg, winter 163 epg, Spring 2016 383 epg) and adults (spring 2015 142 epg, summer 118 epg, winter 115 epg, Spring 2016 324 epg) (Table 2. 8). Yearling females differed in these trends (Figure 2. 5); spring 2015 (656 epg), summer (29 epg), winter (146 epg), and Spring 2016 (372 epg). This reduction in summer average and increase in winter is not noted in the other sex-age groups (male or female), in either dataset. This may be caused by fewer replications in the summer group (n=12). Additionally, only one out of the 12 samples had a negative (0 epg) result. Nevertheless, the range (0-180 epg) is still lower than the winter samples (1-477 epg). Geriatric females had elevated FEC in spring (spring 2015 169.8 epg; spring 2016 386 epg), and, similar to the male groupings, had little seasonal variation between Summer (110 epg) and winter (124 epg). Trends in FEC over the female age groups (generally) decline with age, until they reach 8 years old (i.e. the geriatric group). Average egg counts (±SEM), range and prevalence (i.e. % number of egg-positive samples) are summarised in Table 2. 8.

2. 4. 2. 1. Males

Overall, males have higher FEC than females (estimate = -0.2204654, SE = 0.04930966, df = 874, t=-4.471, p < 0.0001). Males have different seasonal trends to the females. Lambs have an increase from Summer (132 epg), to Winter (144 epg) and then to the following Spring (2016)

394 epg. Following an elevated Spring (2015 and 2016) FEC, there is little seasonal variation between the Summer and Winter counts for the yearling and adult males (Figure 2. 5). Average egg counts (±SEM), range and prevalence (i.e. % number of egg-positive samples) are summarised in Table 2. 9.

Table 2. 8. Average FEC (epg) (± standard error of the mean, SEM) collected from female Soay sheep and counted by cuvette salt floatation method. Collected from 2015 - 2016. n (number of samples), range (epg) and number of positive samples (n) and proportion (%) of positive samples. N/S – not sampled.

Season	Group	n=	Arithmetic mean (epg) ± SEM	Range (epg)	n positive (%)
Spring (2015)	Lamb	N/S			
	Yearling	31	656 ± 173	12 - 5337	31 (100%)
	Adult	20	142 ± 10	2 - 720	20 (100%)
	Geriatric	10	169.8	21 - 459	10 (100%)
Summer (2015)	Lamb	81	133 ± 18	1 - 693	81 (100%)
	Yearling	12	29 ± 15	0 - 180	11 (92%)
	Adult	62	118 ± 29	0 - 486	59 (95%)
	Geriatric	17	110 ± 34	0 - 312	16 (94%)
Winter (2015)	Lamb	41	163 ± 47	3 - 1188	41 (100%)
	Yearling	25	146 ± 59	1 - 477	25 (100%)
	Adult	81	115 ± 39	0 - 396	67 (83%)
	Geriatric	51	124 ± 41	0 - 1233	47 (92%)
Spring (2016)	Lamb	35	383 ± 87	17 - 2430	35 (100%)
	Yearling	22	372 ± 110	7 - 1107	22 (100%)
	Adult	79	324 ± 55	0 - 540	77 (97)
	Geriatric	18	386 ± 127	0 - 585	16 (89%)

Table 2. 9. Average FEC (epg) (± standard error of the mean, SEM) collected from male Soay sheep and counted by cuvette salt floatation method. Collected from 2015 – 2016. n (number of samples), range (epg) and number of positive samples (n) and proportion (%) of positive samples. N/S – not sampled.

Season	Group	n=	Arithmetic mean (epg) ± SEM	Range (epg)	n positive (%)
Spring (2015)	Lamb	N/S			
	Yearling	25	674 ± 197	9 - 2781	25 (100%)
	Adult	28	489 ± 123	27 - 1305	28 (100%)
Summer (2015)	Lamb	81	132 ± 18	0-981	77 (95%)
	Yearling	19	103 ± 33	2 - 459	19 (100%)
	Adult	11	107 ± 26	30 - 318	11 (100%)
Winter (2015)	Lamb	35	144 ± 51	8-3069	35 (100%)
	Yearling	23	154 ± 62	24 - 513	23 (100%)
	Adult	31	139 ± 52	11 - 1647	31 (100%)
Spring (2016)	Lamb	9	393 ± 180	189-1998	9 (100%)
	Yearling	11	351 ± 153	10 - 1305	11 (100%)
	Adult	24	305 ± 95	0 - 396	23 (96%)

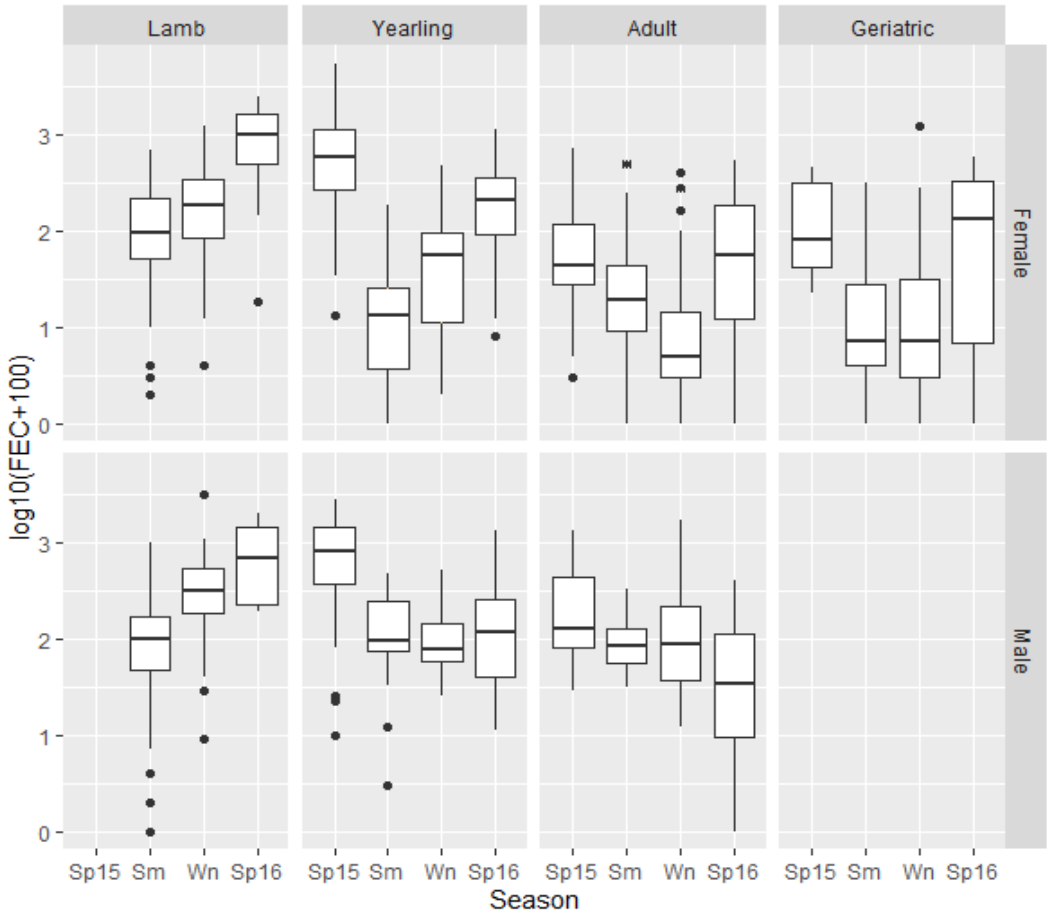


Figure 2. 5. Box and whisker plots of egg count data (log-transformed) collected from 2015 – 2016 and counted by cuvette salt floatation method (1 epg). Data divided between age group, sex and season. The data range is shown by the vertical black lines, with the median of each dataset represented by the middle horizontal line within each boxplot and with any outliers shown as points. Samples collected over spring 2015 (Sp15), summer 2015 (Sm), winter 2015 (Wn) and spring 2016 (Sp16).

Table 2. 10. Analysis of variance table for GLM of FEC over season, age group and sex (cuvette salt floatation dataset).

	DF	Sum of square	RSS	F value	P
Season	3	91.471	463.99	71.537	< 2.2e-16 ***
Age group	3	122.164	494.68	95.541	< 2.2e-16 ***
Sex	1	8.520	381.04	19.990	8.809e-06***

*** p < 0.001

Table 2. 11. GLMM model summary of level by level comparisons (cuvette salt floatation dataset).

Pair-wise comparison	Estimate	SE	DF	t-value	P
Spring15 – Spring16	0.3483387	0.08017536	874	4.345	***
Spring15 – Summer15	0.9769683	0.07988127	874	12.230	***
Spring15 – Winter15	0.8441416	0.07575164	874	11.144	***
Spring16 – Summer15	0.6286296	0.06345913	874	9.906	***
Spring16 – Winter15	0.4958029	0.06102569	874	8.124	***
Summer15 – Winter15	-0.1328267	0.05716113	874	-2.324	0.0934 (NS)

*** p < 0.001; NS – Not significant

Table 2. 12. Results of the analysis testing for differences between sampling seasons for data counted by cuvette salt floatation (males and females combined).

	Estimate	SE	t value	P
(Intercept)	1.99953	0.07264	27.527	< 2e-16 ***
Spring (2016)	-0.34834	0.08018	-4.345	1.56e-05 ***
Summer	-0.97697	0.07988	-12.23	< 2e-16 ***
Winter	-0.84414	0.07575	-11.144	< 2e-16 ***
Geriatric	-0.0665	0.07751	-0.858	0.391 (NS)
Lamb	0.89111	0.05687	15.668	< 2e-16 ***
Yearling	0.50618	0.0636	7.959	5.36e-15 ***
Male	0.22047	0.04931	4.471	8.81e-06***

Differences of ls means analysis were used for the pair-wise comparisons.
*** p < 0.001; NS – Not significant

2. 4. 2. The effect of detection limit on faecal egg count

There was a high degree of correlation between 'true' FEC and the one simulated under Poisson assumption at 1 epg accuracy (Figure 2. 6. D, R^2=0.991), implying that what is counted at a low detection limit (i.e. detection limit of 1 epg), is almost equal to that of the actual number of eggs in the faeces. As the dilution factor increases (i.e. the detection limit increases), the correlation coefficient decreases (Figure 2. 6. C. 10 epg R^2 = 0.941, B. 50 epg R^2 =0.635, A. 100 epg R^2 = 0.492). High dilution factors (Figure 2. 6. A and B) are associated with an over-estimation of true FEC at high egg counts, and underestimation at low egg counts. When log-transformed, data counted with a high dilution (100 epg) fails to conform to a normal distribution (Figure 2. 7), in comparison to a dataset with a lower dilution factor (1 epg) (Figure 2. 8). These comparisons were made under the assumption the eggs were evenly distributed in the faecal sample.

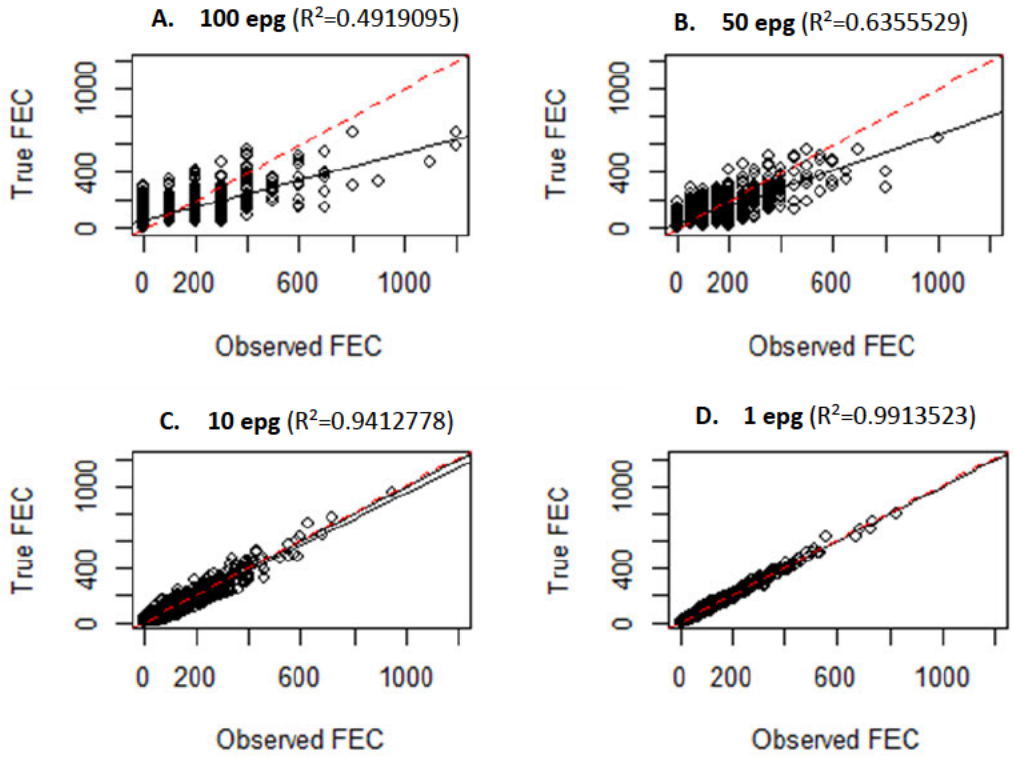


Figure 2. 6. Linear regression analysis of Monte Carlo simulations testing the effect of dilution factor (f) on the number of eggs per gram of faeces counted within a faecal suspension ('True FEC'). Except for the dilution factor (1, 10, 50, 100), all other parameters were kept the same for each simulation (k=1, n=1000, mean = 100). Black line is the line of best fit, fitting both slope and intercept, red line is fitted through the origin (0).

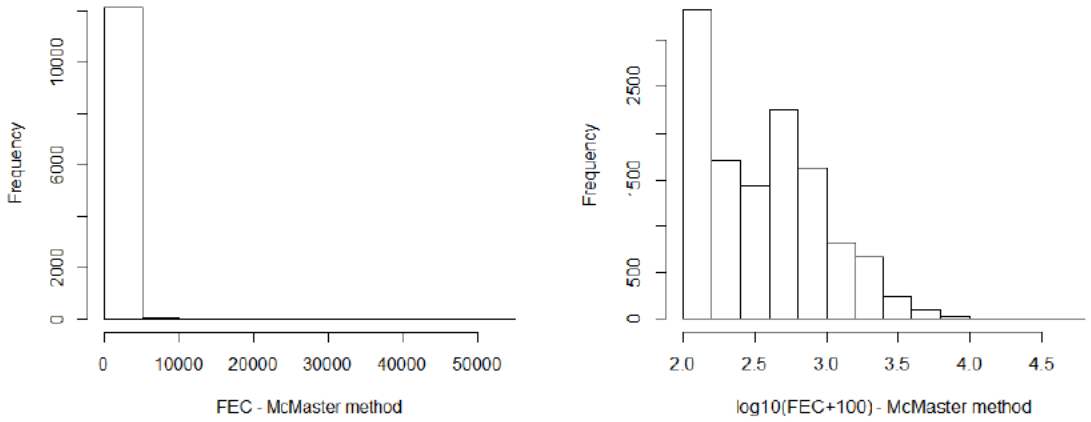


Figure 2. 7. Histogram of strongyle faecal egg counts, measured as eggs per gram of faeces, by modified McMaster (100 epg) before and after log-transformation (FEC+100).

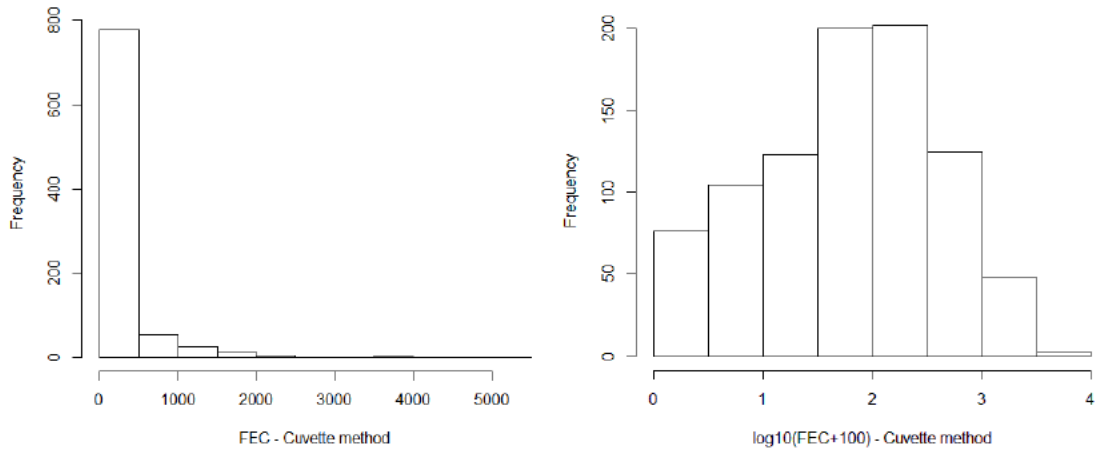


Figure 2. 8. Histogram of strongyle faecal egg counts, measured as eggs per gram of faeces, by cuvette salt floatation method (1epg) before and after log-transformation (FEC+100).

2. 4. 3. Cross count – modified McMaster (100 epg) vs. cuvette salt floatation (1 epg)

As a proof of concept to the theoretical simulations, 432 faecal samples were collected, homogenised and counted using both methods. By comparing modified McMaster (100 epg) to cuvette salt floatation (1 epg) there is a moderate positive correlation across the measures (R^2 = 0.4519). A technique that has a dilution factor of 1 has an egg count similar to that of the actual number of eggs found in the faecal sample when simulated against actual egg number (Figure 2. 6. D) as the R^2 is almost equal to 1. Whilst this comparison is of a technique compared to another technique, rather than for a technique compared to actual egg count, the results of the cross-count are very similar to that of what was predicted in the simulation (Figure 2. 9. A R^2 = 0.4919)

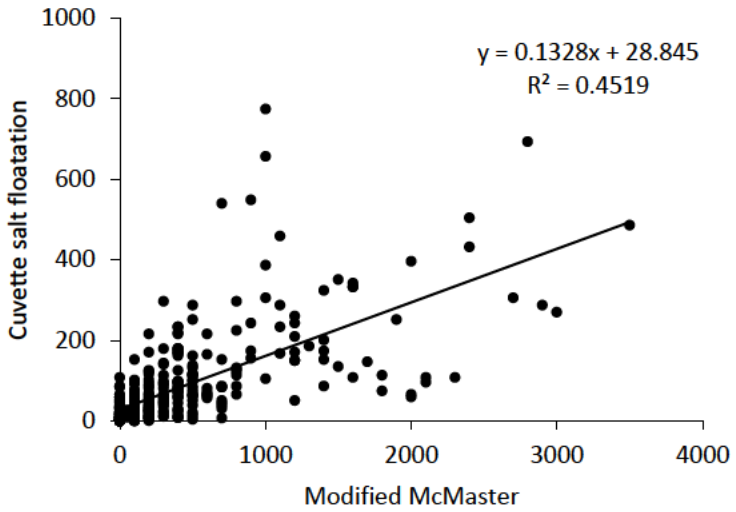


Figure 2. 9. Linear regression analysis of 432 cross-counted faecal samples by cuvette salt floatation- (1 epg detection limit) and modified McMaster (100 epg detection limit). The values for R^2 (coefficient of determination), b (y-intercept) and m (slope) are show adjacent to the plot. If the methods resulted in identical counts the y-intercept = 0, and m = 1.

2. 5. DISCUSSION

FEC are a well-established method in monitoring helminth burdens in both managed and wild systems. The general aim of this chapter was to capture general seasonal and host (sex/age differences) in FEC seen in the Soay sheep, from two datasets counted using different detection limits.

2. 5. 1. Seasonal, host and sex differences

Seasonal and host differences in FEC have been identified, highlighting comparable trends between the two datasets. There is variation in the trends produced by the cuvette salt floatation method, which is most likely due to the effects of within-year variation (which is expected when studying a variable population such as the Soay sheep), and the limited number of replicates in some of the sex/age groups (i.e. yearling females).

Females (4 months – 8+ years) had similar trends, with a seasonal decline in average FEC, starting in Spring. There was a decline in FEC with age, until they reached 8+ years of age (geriatrics); lambs > yearlings > adults < geriatrics. In the cuvette salt floatation dataset, a similar trend is seen between lambs in Spring 2016 and yearlings Spring 2015; at the time of collection they would have been a similar age. It is interesting to note the lamb and yearling females differed in these trends in the cuvette salt floatation dataset, with a Winter increase in FEC. Females are able to reproduce at 11-12 months of age, but they don't usually carry to full-term. The yearlings on the other hand are more likely to carry a lamb to full term, and will have full participation in the mate-guarding activities during the rut. Between December – March they are gestating, March – May they lamb, and June – October they are lactating (Figure 5. 1. Chapter 5). The impact of pregnancy on FEC is seen with the high spring FEC, has been attributed in previous studies to the PPR; a transient loss (or reduction) of immunity to intestinal nematodes within ~10 days of parturition (Wilson et al., 2004). Additionally, ewes are more susceptible to parasite infection during early lactation (Notter et al., 2017). The high egg shedding, contributed from PPR in females and the development of immunity in lambs, will drive the level of infection in the following months. For most GI nematodes in the UK, eggs shed on pasture during early spring can take 10 to 12 weeks to develop, whilst eggs shed during the early summer can take 1 to 2 weeks depending on the species, leading to synchronised development to an infective stage and resulting in a high peak of infectivity during mid-Summer (Soulsby, 1968). The geriatric females are similar to the males (over the age of 13 months), with a high Spring count, and little within-season variation between Summer and Winter. For the cuvette salt floatation dataset the geriatric females were not statistically significant, but this may have been due to limited sample size.

Generally, it is observed across many wild, laboratory and farmed hosts, juveniles and males will be more susceptible to parasitic infection than females and adults (Clutton-Brock and Pemberton, 2004). Male lambs and yearlings follow a similar trend to the females, however, after 24 months, the seasonal differences alter. There is little seasonal variation between summer and winter, with a slight summer elevation in FEC in adult males and geriatrics (modified McMaster dataset), though the male geriatric data must be interpreted with caution due to a small sample size (n=5). The elevation in FEC during summer may be due to a combination of factors, including an increase in pasture contamination from the PPR in adult females and lambs, warmer weather for on-pasture larvae development, and an increase in feed-intake with the growth of new grass. Males gain condition during summer in preparation for the rut in winter, increasing their chances of ingesting newly developed parasites. An elevation in FEC during the winter months may be attributed to restricted feed intake during mating; less faeces produced would concentrate the eggs. However, an elevation in FEC is not truly seen.

By combining groups and sexes, we can consider overall pasture contamination throughout the year. Generally, the trends were Spring > Summer > Winter. The cuvette salt floatation dataset included two Spring samples; 2015 and 2016. Spring 2015 was statistically different to 2016 ($p < 0.0001$), a higher overall Spring 2015 may have been due to a number of temporal, biological and geographical factors, which would require further investigation.

2. 5. 2. Detection limit

It has been reported that the reliability of the McMasters technique decreases with increasing dilution (i.e. increase in detection limit) (Cringoli et al., 2004; Dunn and Keymer, 1986), and using a larger multiplication factor to transform raw counts have been seen to artificially inflate the variance observed in the FEC (Torgerson et al., 2012). The simulations show that when the faecal egg counts are low, there is an underestimation of true faecal egg counts (Figure 2. 6). Therefore, at a dilution factor of 100 epg, you are more likely to not detect any eggs when the real egg count is actually 0 – 99, hence, there is an under-estimation of true values at low egg counts. This means that modified McMaster methods with a detection limit of >50 epg is not sensitive to changes in egg counts around or below the detection threshold, and will most likely result in false-negative results. At high egg counts, the inaccuracy of the method skews towards higher values, therefore there will be an over-estimation of true FEC. Ultimately, this results in a dataset that is negatively skewed and with an inflated mean. Even log-transformed, data counted with a high dilution (100 epg) fails to conform to a normal distribution (Figure 2. 7), in comparison to a dataset with a lower dilution factor (1 epg) (Figure 2. 8). When simulated,

there is a very high level of correlation between true and observed FEC when the dilution factor is low (1-10 epg). When real-world data is compared by the two counting methods, the results of the cross-count are very similar to that of what was predicted in the simulation (Figure 2. 6. A $R^2 = 0.492$).

Whether looking at larvae, adult worms, or eggs within faeces, the parasitological data obtained is usually aggregated over the host population; many individuals having low to zero counts, while a few will have very high counts. Zero-inflation of FEC is common leading to errors and misinterpretation being made during analysis. Faecal egg count data are often characterised by high variability, with frequent zero observations, resulting in data that are skewed with a negative binomial distribution. This right-skewed distribution in egg counts fails to conform to the normal distribution assumed by most commonly-used statistical tests when analysing FEC (i.e. regression analysis, t-tests). The presence of a few 'high shedders' within a population has been noted in horses, where a study assessing >1000 faecal samples found approximately 80% of strongyle egg shedding is contributed by 20% of the population (Relf et al., 2013). Aggregated data is accentuated when the dilution factor is high, as there is a zero-inflation within the dataset; a detection limit of 100 epg results in the lowest positive egg count available being 100 epg, or zero. If the population or test group is characterised by yielding low to zero egg counts, the resulting data is heavily skewed. Conversely, a method that applies no dilution factor (i.e. a dilution factor of 1) has the lowest possible positive count of 1 epg. A dataset that can be log-transformed may improve statistical accuracy.

Collecting more samples, and reducing the dilution factor (or increasing the number of replicates from each sample) will improve the confidence intervals for mean FEC. In many studies that use FEC, a large amount of the variation in the size of the confidence intervals is attributed to the level of egg aggregation, and this can be greatly improved by increasing the number of samples counted (Stringer et al., 2014; Torgerson et al., 2012).

There is also a detection limit difference within the cuvette salt floatation method itself. Depending on the density eggs within the cuvette, all or only a portion of the cuvette is counted. If there is a moderate (~50 eggs) to high (>~50 eggs) egg density one third to one ninth of the cuvette is counted, respectively, and the count multiplied by a multiplication factor (i.e. whole cuvette – 1 epg detection limit, 1/3rd of the cuvette – 3 epg detection limit, 1/9th of the cuvette – 9 epg). From the simulation it would be assumed that the biggest difference in egg counts would be between the 1 epg and 9 epg methods (Figure 2. 6. C). Therefore, as the FEC increase, the methods detection limit decreases. The assumption would be that counts preformed using this method would potentially over-estimate higher counts. Changing

detection limits during counts in response to increasing egg density, rather than keeping the method consistent, could potentially skew the data. A study conducted by Lester (2015) compared the 3 methods of counting a cuvette in 31 horse faecal samples and found there was no statistical difference in the data counted by 1 epg and 9 epg.

A method with a high detection limit (e.g. 100 epg) is not inferior in comparison to one with a low detection limit (1 epg), it all depends on the questions asked of the data. Modified McMasters at 100 epg will still detect the main trends of seasonal and host elevations in FEC, and a test with a high detection limit is less labour intensive and requires no centrifugation step, which is practical in a field-setting. In comparison, the cuvette salt floatation method captures low-level shedding (i.e. between 0 – 99 epg). Very low egg counts still indicate some level of adult worm burden, and these eggs will contribute to the on-pasture larval challenge, and parasite persistence within the flock. For veterinary epidemiological assessments of field cases, this reduced level of sensitivity may not be important. However, when a faecal egg count is required for determining anthelmintic efficacy (FECRT), high sensitivity is very important.

2. 5. 3. Other considerations

Faecal egg counts are affected by complex interaction of factors important to study design. There is not just one factor that can improve the resolution and sensitivity of the method, and decisions on sample size, in addition to detection limit of the methodology, need to be made carefully for the project being run, and to take into account the questions being asked. FEC are inherently error-prone, every step of a faecel faecal egg counting method (regardless of the detection limit) has the potential to introduce variance; from collection through to counting and data interpretation. It is important to understand these limitations, and to appreciate what an egg count really says, or more importantly doesn't say, about the parasite burden. It's becoming increasingly important to better understand the infecting species, and a faecal egg count is now not enough to make informed decisions on management. With issues of anthelmintic resistance, we are now becoming increasingly aware that tools need to be developed in order to not only monitor egg contamination on pasture, but to identify and quantify the specie(s) present.

For example, different trichostrongylid species differ in their pathogenicity and fecundity. *H. contortus* is highly fecund, producing 5000-10000 eggs per female, per day. At high burdens they cause high rates of mortality in any age class. Species of *Trichostrongylus* and *Teladorsagia* contribute to the impact of most infections; causing production losses and morbidity, rather than mortality, but they are less fecund (100-200 eggs per female, per day). Therefore, if a FEC

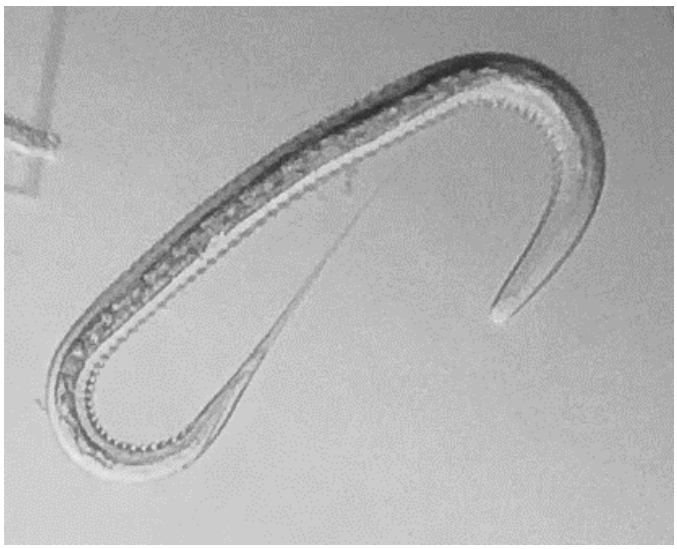
with a detection threshold of 100 (or higher) epg, detected a large contribution of eggs from *Teladorsagia* and/ or *Trichostrongylus*, it would be regarded as higher significant in comparison to *H. contortus*, as it indicates a higher worm burden in comparison to the more fecund species. However, as discussed in the Introduction, FEC is the amalgamation of multiple strongyle species, and whilst it is possible to differentiate certain trichostrongylid parasite species or genera (Georgi and McCulloch, 1988), it is not considered feasible in a diagnostic setting (Roeber and Kahn, 2014). In co-infections, each species may differ in their pathology and epidemiology. Additionally, their pathology, clinical representation, and establishment may alter depending on the burden/s of other co-infecting species (Brundson, 1970). For example, *T. circumcincta* can alter the pH of the abomasum, potentially making it unfavourable to other abomasal species (Rahman et al., 1991). Without additional speciation, the trends in FEC can be measured over many years, but the species composition is unknown. Are these seasonal host/sex trends in FEC influenced by certain parasite species? How do they change over the seasons? And between different sex-age groups? Are the high trends seen in Spring, such as the PPR, linked to certain strongyle species? Are they mixed species or single?

2. 5. 4. Conclusions

Two FEC databases were compared; a longitudinal study (27 years) with many replicated (12192) counted with a high detection limit (100 epg), and a shorter study (1 year) with 0.07% of the replicates (882) counted with a lower detection limit (1 epg). General seasonal and host trends in FEC were similar for both datasets, as the lower detection threshold resulted in higher egg-positive samples. More replicates may be needed for in-depth study of individual host differences, but to supplement the work presented in Chapter 5, we can be confident the trends produced by the cuvette salt floatation method are representative to the previous work conducted on the Soay sheep, and what is usually seen in the sheep. In the following Chapters, we will explore DNA-based approaches for the specific identification and quantification of ovine nematode L_3 in mixed-species burdens (Chapter 3), validate the molecular approach for the species identified in the Soay sheep of St Kilda (Chapter 4), and then explore the seasonal and host differences in species composition over one year (Chapter 5).

CHAPTER 3

Comparison of semi-automated multiplex-tandem PCR with ITS-2 rDNA next-generation amplicon sequencing for the identification and quantification of ovine nematodes



3. 1. ABSTRACT

Concurrent or sequential infection with multiple nematode parasite species is commonly seen in feral and managed ruminants. The accurate, non-invasive characterisation of mixed-species communities is required to investigate the epidemiology and ecology of parasites. The development of molecular methods to identify and quantify mixed-species infections has negated many of the issues arising from routine diagnostics, which typically involve microscopic counting of eggs and morphological characterisation of L_3. The aim of this chapter was to compare two molecular methods; a semi-automated multiplex-tandem PCR (AusDiagnostics™) with ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay), to identify (presence/absence) and quantify (relative proportion - %) ovine strongylid species in naturally-infected samples.

Ausdiagnostic™ identified *Teladorsagia circumcincta*, *Trichostrongylus* spp., and *Chabertia ovina*. In addition to identifying these species, the nemabiome assay was able to speciate the *Trichostrongylus* genus to *T. vitrinus* and *T. axei*, and to identify *Bunostomum trigonocephalum*. The nemabiome bioinformatic pipeline did not identify any 'unknown' sequences (i.e. species not available in the database of reference sequences) indicating what was identified in these field samples are the species present in the study population. This matches previous studies in the Soay sheep that morphologically identified adult worms and L_3.

Whilst there was an excellent agreement between both molecular tests (i.e. identification of species within a sample) (*Trichstrongylus spp*., 100%, *T. circumcinta* = 95% and, *C. ovina* = 90%). There is moderate to poor correlation in their ability to quantify the relative proportions (%) of each species present when compared; *C. ovina* (R^2 = 0.6096), *T. circumcincta* (R^2 = 0.5914), *Trichostrongylus* spp. (R^2 = 0.2334).

There was a high degree of correlation between parasite species proportions as determined by the nemabiome assay compared with those determined on the basis of larval morphology of *C. ovina* (R^2 = 0.9015), and moderate correlation between the AusDiagnostic™ assay and morphological identification of *C. ovina* (R^2 = 0.6496).

This is a proof-of-concept study highlighting the effectiveness of both the semi-automated multiplex-tandem PCR (AusDiagnostics™) with ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay), for the identification and quantification of strongylid nematode larvae in naturally infected sheep. They are advanced methods for the in direct genus/species-specific diagnosis of GIN infections in ruminants. This chapter provides the first, and preliminary, comparison of the sensitivity and quantitative ability of both methods.

3. 2. INTRODUCTION

Concurrent or sequential infection with multiple nematode parasite species is commonly seen in ruminant livestock. Within these mixed burdens, the species can vary in their pathogenicity, epidemiology and resistance to anthelmintics (Bisset et al., 2014). Fuller understanding of parasite interactions is a critical component of parasite control that will depend on the accurate characterisation of the parasite communities. However, until recently, comparative research on nematode parasite species was limited by the challenges associated with accurate morphological identification of species, which required extensive taxonomical expertise. Therefore, there is a need for a quick, accurate, non-invasive and affordable method of diagnosis of gastrointestinal (GI) infections of ruminants, to investigate the epidemiology and ecology of parasites.

Current diagnosis for mixed-infections of strongyle nematodes relies mainly on traditional parasitological techniques. These include total worm counts (TWC) from necropsied hosts, and non-invasive (*ante-mortem*) methods that utilise immature stages of the life cycle (eggs/ larvae) in faeces, such as faecal egg counts (FEC) and larval culture (Roeber and Kahn., 2014). Generally, these methods are often time-consuming, and/or require a high level of training to perform them correctly.

For many years, larval cultures had been the main method for speciating strongylids. Larval cultures are a means of growing 3rd stage larvae (L_3) from eggs (usually within 7-14 days, depending on the conditions), and species can be identified by the length of the tail-sheath extension, shape of larval tail, cell number, head shape and the presence/ absence of additional features (i.e. refractile bodies) (MAFF, 1986; van Wyk and Mayhew, 2013). In mixed-species infections (which are most commonly seen), different trichostrongylid species develop at different rates when exposed to a common culture protocol (Berrie et al., 1988; Roeber and Kahn, 2014), as changes such as faecal moisture and temperature can influence their development. Identifying L_3 by their morphological characteristics is technically demanding and subjective, and whilst larval identification keys are available for the most common species found in ruminant livestock (MAFF, 1986; van Wyk and Mayhew, 2013), this method is limited when applied to wild hosts as novel/rare species will not be found in such keys. Additionally, accurate identification and differentiation can be difficult due to overlapping morphological features of some commonly seen genera (*Trichostrongylus* vs. *Teladorsagia*) and species (*Oesophagostomum venulosum* vs. *Chabertia ovina*) in farmed ruminants (van Wyk and Mayhew, 2013). Larval cultures are important in the diagnosis of anthelmintic resistance by determining the efficacy of anthelmintic groups on different strongyle species. *T. circumcincta*

and *T. colubriformis* have been reported to be resistant to the three classes of broad-spectrum anthelmintics, and due to their overlapping morphological characteristics the true and accurate diagnosis of anthelmintic resistance on-farm can be often inaccurate resulting inappropriate advice being provided by animal health advisors, unless TWC are preformed (Waghorn et al., 2014; Roeber and Kahn, 2014).

To overcome the limitations of traditional methods of diagnosis, DNA technology has provided alternative strategies for parasite identification. These advances have enabled new and sensitive diagnostic methods, and by allowing for the specific identification of parasites (regardless of life cycle stage – eggs, faeces, larvae, adult worms) have provided new avenues of study in population genetics and epidemiology. The first and second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) have provided useful markers for species and genus-specific differentiation, having sequence variability between species and low-level variability within species (usually <1.5%) (Gasser, 1999; Bisset et al., 2014; Gasser, 2006). Additionally, due to multiple copies present in the genomes of most organisms, rDNA amplification by polymerase chain reactions (PCR) is usually straightforward (Hung et al., 1999). Many studies have used rDNA markers in PCR assays in order to determine the presence/absence of (usually) single parasite species in eggs or larvae of wild and domesticated ruminants (Gasser and Monti, 1997; Campbell et al., 1995; Wimmer et al., 2004; Bisset et al., 2014). However, when studying wild or novel hosts, where the infecting species are not known, or some parasite species are either not shared, or are rare, in domestic hosts, the use of species-specific primers has the potential to miss and/or underestimate parasite diversity and is potentially inaccurate when representing community composition. An example of this is a study conducted on an intervention-free flock of sheep, where a chance post-mortem found an additional rare species (*B*. *trigonocephalum)* which was not part of the PCR assay, and required additional species-specific PCR targeting (Sinclair et al., 2016). The first attempt to characterise strongyle species that infect the Soay sheep on St Kilda by ITS-2 PCR (Wimmer et al., 2004) required in-depth traditional parasitological studies (TWC and L_3 morphological identification) being performed previously to inform primer choice (Craig, 2005; Gulland, 1991; Pilkington, *unpublished*). Wimmer et al., (2004) found that in a comparison between morphological and molecular identification, the molecular method provided quicker and more efficient species-level identification, though this had (at the time) limited sensitivity.

Cryptic species (parasites that are morphologically distinct but genetically similar) have proven to be difficult to identify morphologically and require PCR-speciation. In the context of St Kilda, through morphological identification, *Teladorsagia* had been identified as three separate

species; *T. circumcincta, T. trifurcata* and *T. davtiani*. However, nuclear and mitochondrial sequence analysis of the St Kilda *Teladorsagia* population suggests that the three species cannot be genetically differentiated (Grillo et al., 2008). Previous ITS-2 sequence analysis (Stevenson et al., 1996), mtDNA sequence diversity studies (Grillo et al., 2007) and microsatellite genotyping (Grillo et al., 2008) support this, finding no detectable genetic differentiation between UK populations, concluding that they are all morphotypes of the same species. Further population genetic analysis of European strains suggests that parasites currently identified through morphological identification as *T. circumcincta* may be a cryptic species (Grillo et al., 2007). Similar findings have been described in *Haemonchus* (Sargison et al., 2019[a]). Nevertheless, species identification on St Kilda generally just recognises the presence of *T. circumcincta*. The presence of cryptic species highlights the need to include additional molecular methodology, utilising next generation sequencing, to verify traditional parasitological techniques.

Recent developments of molecular methods have both allowed diagnostic applications (i.e. presence/ absence) of eggs and larvae, and enabled quantification of key livestock species in order to give additional proportional data. A multiplex PCR method (Bisset et al., 2014) has overcome the limitations arisen from simple PCR, and has quickened the process allowing the identification of multiple strongylid species in a single PCR run. Methods such as these also can cover many strongylid species seen in cattle, sheep and deer. In addition to allowing the processing of multiple species in multiple samples simultaneously, at reasonable efficiency, it has been used in different research applications, including geographical species distribution and the development of anthelmintic resistance (see Oliver et al., 2014; Waghorn et al., 2014). However, whilst multiplex PCR has quickened the diagnosis within the PCR-set up (i.e. removing the need for individual primer pairs and multiple manual set-ups), the method developed by Bisset et al., (2014) requires single L_3 to be individually picked into 96-well plates, which adds further processing time. Additionally, picking 96 L_3 from a culture may bias the proportional results, as species at low-levels may be missed.

Reverse transcription PCR (RT-PCR) was developed in the 1990 (Higuchi et al., 1992), and the main advantage of RT-PCR over conventional PCR is that it provides high-throughput analysis, without requiring any additional steps (i.e. gel electrophoresis). The limitation of RT-PCR (as developed for parasites by Roeber et al., (2012)) is that it uses individual primer pairs for the detection for individual species/genera, therefore it requires many steps.

The development of a semi-automated multiplex tandem PCR (MT-PCR) (AusDiagnostics™) has enabled genus - or species-specific, high-throughput diagnosis of mixed-infections in eggs, larvae and faeces (Roeber et al., 2012), and has been validated for both sheep (Roeber et al.,

2017[a]) and cattle (Roeber et al., 2017[b]) species. The method is based on the detection and amplification of species-specific markers of nuclear DNA. Species specific primers (AusDiagnostics Pty. Ltd) were design to the internal regions of the ITS-2 sequences of the targeted species, in order to produce amplicons of 100-200 bp (depending on the species) in the second phase of MT-PCR (Figure 3. 1) ((Roeber et al., 2017[a]). However, this assay has been developed for identification and quantification of production-limiting species, and currently has several commercial kits that will identify six/seven specific nematode species commonly found in European and Australian livestock (https://www.ausdiagnostics.com/production-animals.html).

Recent developments in high-throughput next-generation sequencing (NGS), has allowed screening for multiple parasite species concurrently. Molecular barcoding, or 'metabarcoding', approaches can identify and quantify parasite communities quickly, and can process a large number of samples (Aivelo and Medlar, 2018). Whilst metabarcoding is a relatively new approach to the study of parasite communities, it has been used for the study of strongylids and nematodes in both livestock (cattle Arvamenko et al., 2015), captive wildlife (buffalo Budischak et al., 2015; red kangaroo Lott et al., 2015) and wild-culled wildlife (wild rats Tanaka et al., 2014). A longitudinal study investigating nematode populations in wild mouse lemurs is the only other study, to date, that uses this methodology on free-living animals (Aivelo et al., 2017; Aivelo et al., 2015).

The development of a deep amplicon sequencing methodology to study parasite nematode communities was developed by Avramenko et al., (2015), and modified for ovine strongyle species by Redman et al., (2019) for the identification and quantification of mixed-species infection with clade V parasites (see Chapter 1). Clade V nematodes are a group of parasites that includes economically significant gastrointestinal species of ruminants (Blaxter et al., 1998). The method is based on deep amplicon next generation sequencing of the ITS-2 rDNA locus and is comparable to 16S rDNA sequencing of bacterial communities used in microbiome studies. The assay has been further developed to identify strongyle species in bison (Avramenko et al., 2018), domestic sheep (Redman et al., 2019; Hamer et al., 2019), dairy heifers (Scott et al., 2019), domestic horses (Mitchell et al., 2019; Chaudhry and Sargison, *unpublished*), feral Sable island horses (Gilleard et al., *unpublished*) and deer (Gilleard et al, *unpublished*).

With all of these different traditional parasitological methods and molecular platforms, validation of diagnostic tests is important, in order to ensure accurate identification, quantification and repeatability. Validation of a diagnostic test requires the determination of several parameters; the sensitivity, specificity, accuracy, agreement, and reliability (Roeber et

al., 2013). The sensitivity of a method measures the proportion of positive samples which test positive, and the specificity measures the proportion of negative samples which test positive. Determination of these parameters requires an independent 'gold standard' to define true status of the samples. Accuracy is the consistency between test results and the actual status of the sample. Accuracy is dependent on the number of false-negatives and false-positives in comparison to the results acquired by the validated 'gold standard' method. The Agreement between two diagnostic tests is usually measured by the Kappa test, which measures the proportion of agreement that may be expected by chance. Finally, Reliability measures how consistent results are between repeated experiments.

The aim of this chapter was to compare two molecular methods; multiplex-tandem PCR (AusDiagnostics™) with deep amplicon sequencing (nemabiome assay), to identify and quantify ovine strongylid species in naturally infected samples collected from the feral sheep population on St Kilda.

3. 3. METHODS

All sample collection and traditional parasitology outlined in this chapter was done by the author, unless explicitly stated in text. The Illumina sequencing techniques were completed by the author under the direction of Libby Redman and Russel Avramenko.

3. 3. 1. Faecal collection

Between March 2015 – March 2016 faecal samples were collected from Soay sheep on St Kilda (full description in Chapter 5). Faecal samples were collected off pasture after direct observation of defecation, without handling the animals. Samples were collected within 2 minutes of being voided to minimise potential animal misidentification or contamination by free-living nematodes. The faeces were collected in individual zip-lock bags, with the air fully expelled. Once collected, the faecal samples were homogenised in their collection bags and weighed out for L_3 culture on the day of collection. All faecal cultures were prepared and incubated whilst on St Kilda.

3. 3. 2. Conventional parasitology

3. 3. 2. 1. Coproculture and Baermannisation

Coproculture is a means of hatching nematode eggs within faeces, and growing them to the L_3-stage. Faeces were weighed into a large container, mixed with vermiculite into a crumbing consistency. A detailed description of coproculture preparation is described in Chapter 5. The

container was then covered and periodically sprayed with water, and incubated at ~20°C for >14 days. The cultures were stirred every few days to break-up fungal growth and aerate.

The Baermann technique is a means of harvesting L_3, whereby the larvae migrate through a filter in order to isolate them from the faecal material, and is the most practical method for larval isolation under field conditions. After flooding the coproculture container with tepid water and incubating for 4 hours at 20°C, the supernatant was then poured through an assembled Baermann apparatus (MAFF, 1987). The Baermann apparatus was made up of a sieve made from single-ply tissue suspended over a beaker of water. It was left overnight to allow larvae to migrate through the filter into clean, warm tap water. The larvae were left for 24 hours at 4°C to settle, and the supernatant was carefully siphoned. The larvae were stored in 50ml tissue culture flasks (Sarstedt, Germany) in 10ml of tap water. The flask was set on its side with the lid slightly open to allow aeration and stored at 4°C. The culture flasks were temporarily closed and sealed when the larvae were transported off St Kilda.

3. 3. 2. 2. Preparing larvae

For each culture the total number of larvae recovered was estimated. The tissue culture flask lid was closed and the flask was inverted several times to evenly suspend the larvae, and the number of larvae present in a 10µl aliquot was counted under a compound microscope 5 times. The average was used to calculate total larvae number in a 10ml suspension. Multiple aliquots of ~1000 larvae were pipetted into 2ml screw-top microcentrifuge tubes (Sigma-Aldrich , USA), made up to a 2ml total volume to a total concentration of 70% ethanol, and stored at -80°C until molecular analysis. There were 40 aliquots (2 from each culture) of 1000-2000 L_3 were made, 20 to be processed by AusDiagnostics™ and 20 by the nemabiome assay. The remaining larvae were stored in water, at 4°C, for a maximum of 4 months, until morphological identification.

Aliquots of 1000 larvae were used for AusDiagnostics™. However, the nemabiome assay was conducted at the University of Calgary, prior to molecular analysis the larval aliquots were vortexed thoroughly, and halved to aliquots of approximately ~500 L_3, to allow a contingency if lysates needed to be remade.

The total number of larvae between the aliquots will vary, as it is not possible to produce a completely homogeneous larval suspension. In an attempt to minimise variability, the larvae were cooled before counting, to slow their movements and reduce clumping.

3. 3. 2. 3. Microscopic identification and counting

Due to the nature of this method of classification, larvae were identified to genus-level, and *Teladorsagia* and *Trichostrongylus* species were grouped together. Full morphological identification was attempted, however, the morphological differentiation between *T. circumcincta* and *Trichostrongylus* species is notoriously unreliable, therefore, these species were removed from the morphological comparison with the molecular methods. Additionally, *B. trigonocephalum* was removed from this comparison study as I was unhappy with the quality of the data. The morphological data presented in this chapter is based on the length of the tail sheath extension. The length of a tail sheath extension denotes species that have short (*Teladorsagia* sp., *Trichostrongylus* sp.), medium (*Cooperia* sp.) and long (*Chabertia* sp.) tail lengths.

A 10µl aliquot of water-suspended larvae was pipetted onto a microscope slide, fixed with 2µl of 5% Lugol's helminthological iodine and covered with a coverslip. Under a light microscope at a 40x magnification, the tail sheath extension was measured against the smallest eyepiece graticule square of the Miller Square eyepiece graticule (Graticules Ltd., UK) (Chapter 2, Figure 2. 2). Per 50-100 larvae (depending on how many larvae were available), the tail lengths were counted to establish the proportion of small, medium and long tails per culture.

3. 3. 3. Molecular parasitology

3. 3. 3. 1. Semi-automated multiplex tandem PCR (MT-PCR) (AusDiagnostics™)

3. 3. 3. 1. A. *Parasitic material preparation & DNA extraction*

Aliquots of 1000 L_3 were vortexed briefly and centrifuged at 1000 xg for 1 minute. The ethanol supernatant was then removed. The pellet was re-suspend in 2 ml 1X Phosphate-buffered saline (PBS) (800 ml distilled H_20, 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, pH 7.4, distilled H_20 to 1L total volume), and incubated at room temperature for ~15 minutes to rehydrate the L_3. The aliquot was centrifuged at 1000 xg for 1 minute, and the supernatant removed. Using a wide-bore pipette tip, the whole L_3 pellet was transferred into a PowerBead Tube (MO-BIO Laboratories Inc., USA). Parasitic DNA was extracted using the MO-BIO PowerSoil® DNA Isolation Kit (MO-BIO Laboratories Inc., USA), following manufactures protocol. DNA was eluted in 100µL MO-BIO elution buffer and stored at -20°C.

A single L_3 is expected to produce 25-100ng/ul of DNA (Roeber et al., 2015), therefore the total concentration of DNA in a pool of 1000 larvae exceeds the total needed for this assay. A 1:100 dilutions were made with molecular-grade water. These were stored at -20°C until use.

3. 3. 3. 1. *B. Robotic reaction setup and MT-PCR assays*

MT-PCR comprises of two amplification phases: a 'target enrichment' phase using multiplexed primer sets, and a subsequent analytical amplification phase involving the targeted amplification (in tandem rather than multiplex) of each genetic locus of the species being investigated, as specific nested primers. The amplification phase is limited to prevent amplification bias by reducing interactions between multiplexed primer sets (for MT-PCR), reduces competition or the generation of artefactual products.

MT-PCR was performed using the Easy-Plex system (AusDiagnostics Pty. Ltd., Australia), consisting of a Rotor-Gene 6000 real-time PCR thermocycler (Qiagen, Germany), and a Gene-Plex CAS1212 liquid handling robot (AusDiagnostics Pty. Ltd.). For a breakdown of the methods for this step see Figure 3. 1. The MT-PCR test panel was, at the time, the only available assay and was designed for six production-limiting species of sheep. The robotic reaction and MT-PCR setup followed protocols outlined in Roeber et al., (2015), including details of the MasterMix, PCR setup and the nematode-specific olingonucleotide primers. Six specific primer pairs (AusDiagnostics Pty. Ltd.) were used in separate reactions for the specific amplification within the second internal transcribed spacer of nuclear ribosomal DNA (ITS-2 rDNA) of *Teladorsagia circumcincta, Trichostrongylus* spp*., Chabertia ovina, Cooperia curteci, Oesophagostomum venulosum* and *Haemonchus contortus.* A positive control ('Pan-Nematode') was run alongside, which is an assay developed by AusDiagnostics specific to the ITS-2 srequences of the nematodes, and is included as a positive control. In the primary amplification, 5µl of diluted DNA representing each sample (n = 9) was loaded into 0.2 ml PCR strips and placed into a 24-well thermocycling block within the Easy-Plex robotic platform. Following the loading of each sample into the robot, and initial PCR, the remainder of the set-up and analysis of the MT-PCR was semi-automated, utilising the program 'Easy-Plex Assay Setup' (AusDiagnostics Pty. Ltd.)

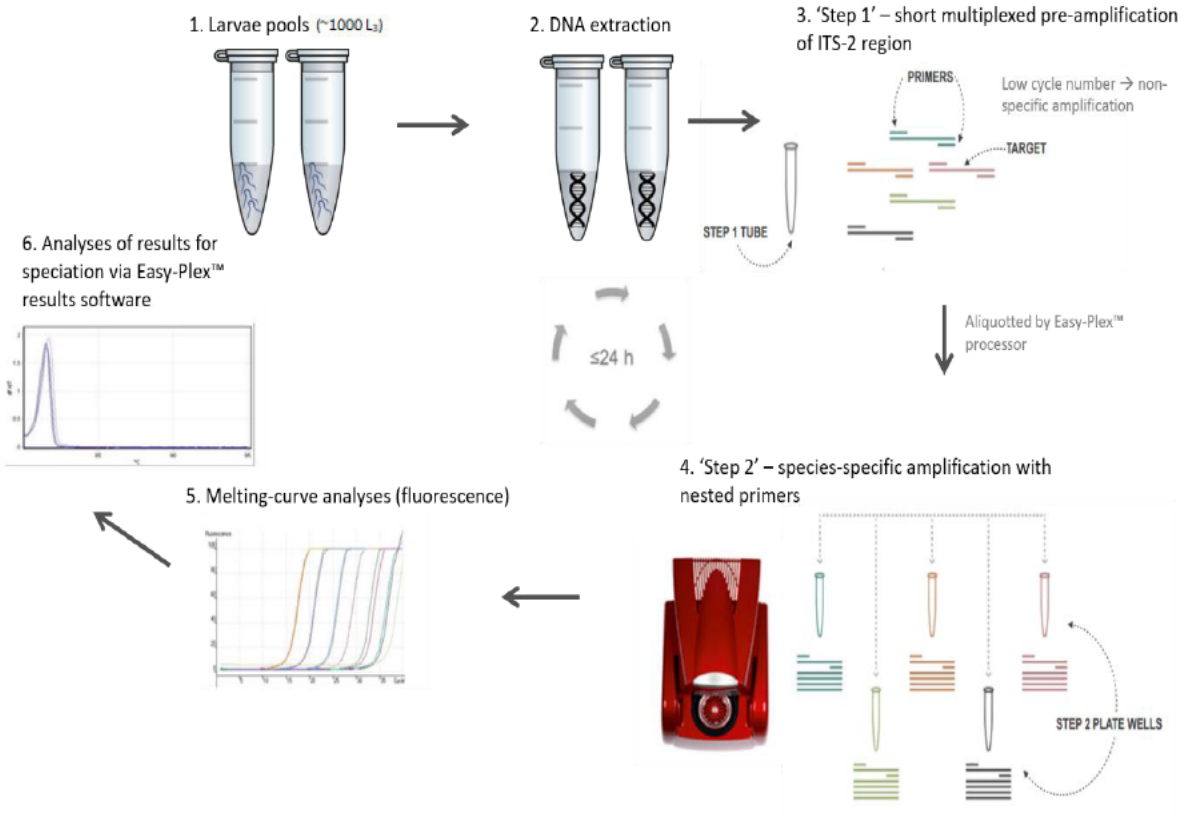


Figure 3. 1. Laboratory workflow for the AusDiagnostics™ kit for identifying and quantifying 6 ovine nematode species in mixed-infection pools (adapted from Roeber et al., 2015). AusDiagnostics™ uses an Easy-Plex™ system which incorporates Multiplexed Tandem Polymerase Chain Reaction (MTPCR) in order to speciate nematode parasites in a partially automated diagnostic system. MT-PCR involves two sequential PCR steps referred to as Step 1 and Step 2.

1. Pools of mixed-species larvae (~1000 L_3) stored in 70% ethanol. 2. Larvae DNA extracted by PowerSoil® DNA Isolation Kit (MO BIO) in a separate tube per sample (x2 tubes in figure illustrative). 3. DNA, mastermix, water and oil are loaded into the Easy-Plex robotic platform, and Step 1 – a short multiplexed pre-amplification reaction – is run in the platform. The reaction uses universal ITS-2 primers in order to target all 6 species. Each pair of nested primers are specific to one of the species. The amplified DNA for each sample is then aliquoted out into a plate via the robotic system, 6 wells (for 6 species) per sample. The plate is then covered, centrifuged and transferred to a Rotor-Gene 6000 real-time PCR thermocycler (Qiagen). The primers for Step 2 are designed to be nested inside the Step 1 primers, thus increasing specificity and sensitivity of the assay, and reducing the chance of cross-reaction. 5. The samples are amplified and the thermocycler monitors the levels of fluorescence. The dye present in the step 2 reaction will fluorescence in the presence of double-stranded DNA. As the fluorescence increases, the built-in analyser will analyse the level of the fluorescence. 6. Once the run is complete, the Easy-Plex™ Results Software automatically analyse all samples, and will report the presence/ absence of a species, in addition to the percentage presence of the species.

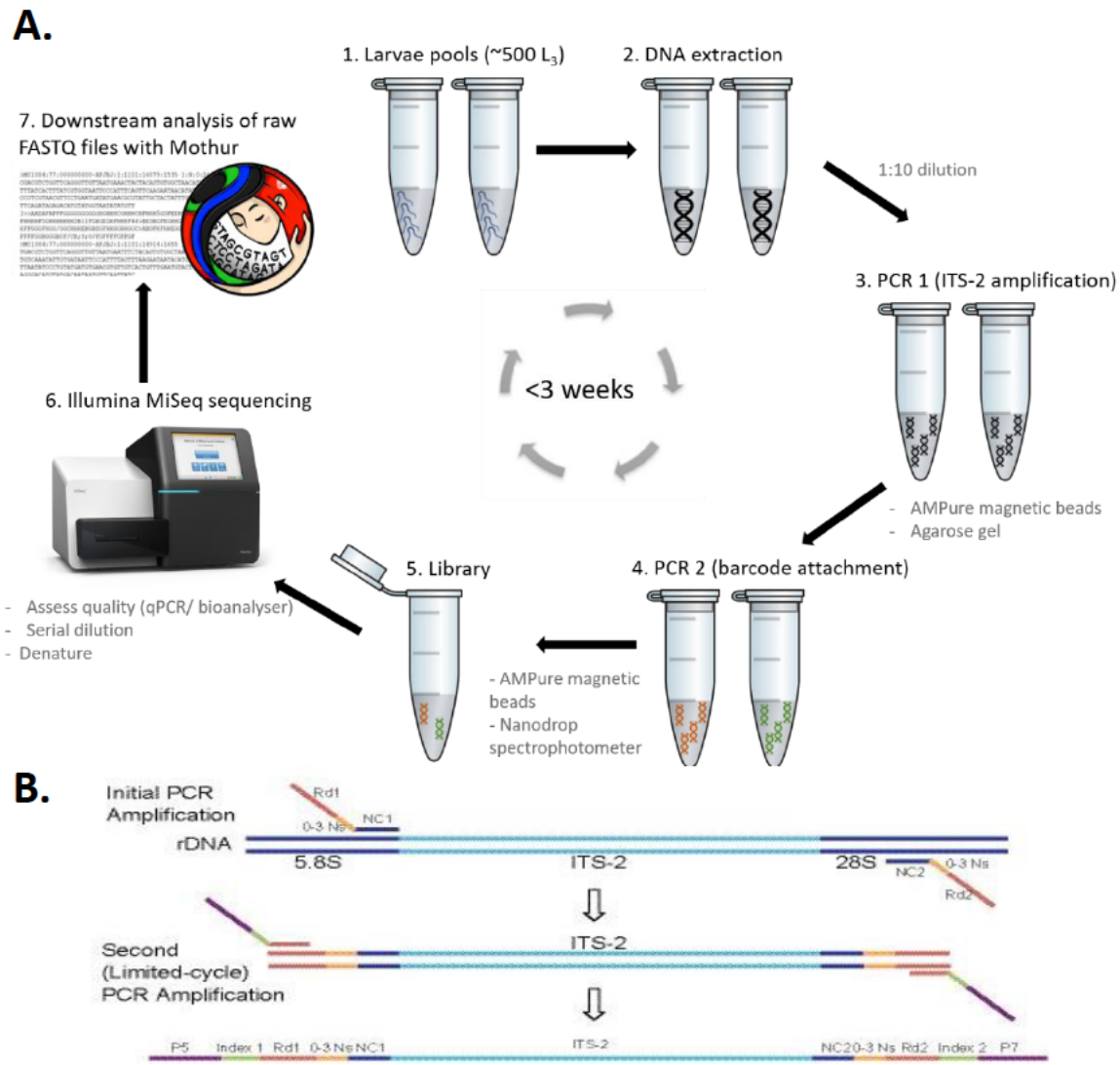


Figure 3. 2. A. Laboratory workflow for the nemabiome approach to identifying and quantifying clade V nematode species in mixed-infection pools. B. Diagram illustrating the preparation of the library for the Illumina sequencing (taken from Avramenko et al., 2015).

1. Pools of mixed-species larvae (~500 L_3) stored in 70% ethanol. 2. Lysates made and diluted (1:10). 3. 1st PCR - Amplification of ITS2 rDNA region (clade V specific), using overhanging NC1 and NC2 primers (developed by Gasser 1993). Primers adapted by Russell et al., (2015) to include 0-3 random nucleotides (0-3Ns) and Illumina adaptor sequencing tags (Rd1 & Rd2), providing a binding site for additional primers. Rd1 & Rd2 are offset to increase diversity during sequencing and to prevent oversaturation of the MiSeq sequencing channels. 4. 2nd PCR – Addition of barcoded primers (Index 1 & Index 2) and Illumina Flow Cell binding sites (P5 & P7). Cycles kept low to limit potential biases. Index 1 & Index 2 – each forward and reverse primer combination unique for each pool of larvae, so the sample can be identified by Illumina MiSeq. 5. Create a master sequencing library from equal amounts of DNA (ng) from each sample. 6. Denature library to singled stranded DNA for sequencing, serial dilute and load into Illumina MiSeq sequencer. 7. Set the sequencer to produce raw FASTQ files with no further analysis. Downstream analysis of raw reads with Mothur, with realigned and trimmed reads being transferred into Excel (Microsoft) to be analysed.

3. 3. 3. 2. ITS-2 rDNA next-generation amplicon sequencing ('nemabiome assay')

2. 3. 3. 2. A. *Genomic DNA lysate preparation*

The aliquots of 1000 L_3 were vortexed thoroughly before being halved (~500 L_3). The method of lysate preparation of sheep L_3 larvae was developed by Avramenko et al (2015) and adapted for sheep larvae by Redman et al., (2019). Ethanol-fixed larvae were initially washed 3 times by centrifugation in 1ml of lysis buffer (2.5mL 1M KCl, 500µL 1M Tris (pH 8.3), 125µL 1M $MgCl_2$, 225µL 0.45% Nonidet p-40, 225µL 0.45% Tween-20, 250uL 2% gelatin, dH_20 to 50mL) at 1000 xg/ 4 minutes, and resuspended in a final volume of 2ml. To break open the larval-sheath, the larvae were heated at 95°C for 15 minutes, shaking at 1000rpm and then frozen at -80°C for a minimum of 60 minutes . 9µL of proteinase K (PK) (Promega, USA) was added, vortex briefly and heated at 60°C for 98 minutes, shaking at 700 rpm to lyse the larvae. The PK was inactivated by heating at 94°C for 15 minutes, continuing to shake at 700rpm. A 1:10 dilution was made with molecular-grade water, and stored at -20°C until 1st round PCR.

2. 3. 3. 2. B. *Deep amplicon sequencing of the clade-V-specific rDNA ITS-2*

The rDNA ITS-2 primers NC1 and NC2, originally described in Gasser et al (1993), were subsequently modified by Avramenko et al (2015). These primers amplified an approximate 311-331bp region and are clade-V nematode-specific. The modifications included adaptors to allow for subsequent annealing of primers, and 0-3 random nucleotides to increase amplicon diversity (Table 3.1, Appendix). The multiple forward and reverse primers where mixed in equal proportions and the mastermix was made up: 5µL KAPA HIFI HotStart Fidelity Buffer (X5) (KAPA Biosystems, USA), 0.75µL dNTP (10mM), 0.5µL KAPA HiFi Polymerase (0.5U), 0.75µL NC1 + Adapter Primer (10µL), 0.75µL NC2 + Adapter Primer, 13.25 dH_20 and 4µL of 1:10 lystate. The PCR program was the following: 95°C for 3 minutes, followed by 35 cycles of 98°C for 20 seconds, 62°C for 15 seconds and 72°C for 15 seconds, ending with 72°C for 2 minutes. This was then held at 10°C. The number of cycles were increased from 25X, as used in Avramenko et al (2015), to 35X to account for the smaller number of larvae (500 vs. 2000). The potential of between-well cross-contamination by air-borne contaminates was minimised by using a PCR-hood pre-treated with DNAZap™ (Thermo Fisher Scientific), filter pipette tips and easy release PCR cover Microseal 'A' Films (Bio-Rad MSA5001) during the PCR mastermix setup and the 1st and 2nd PCR.

Products were purified using Agencourt AMPure XP magnetic bead kit (1X) (Beckman Coulter, UK) following manufactures recommended protocol, with elution in 40µl dH_2O and a final volume of 32µl. Template was then visualised by gel electrophoresis to ensure amplification; 5µl

of cleaned product and 2.5µl of loading buffer (SYBR Green, Invitrogen, USA) were loaded onto a 1.2% agarose gel, with 1kb ladder (Invitrogen, USA). Negative sample made with molecular-grade water.

Illumina barcode indices and P5/P7 sequencing tags were then added to the rDNA ITS-2 amplicons by limited-cycle PCR amplification. Eight index 1 (i7) and 16 index 2 (i5) adapters, from the Nextera XT Index Kit (v2), were combined to make 61 unique barcode combinations. The mastermix was made up: 5µL KAPA HiFi Fidelity Buffer (5x), 1.25µl forward primer (N501-506) (10µM), 1.25µL reverse Primer (N701-712) (10µM), 0.75µL dNTPs (10mM), 0.5µL KAPA HiFi Polymerase (0.5U), 13.25 ddH_20 and 4µL purified first round product. Reagents were covered with easy release PCR cover Microseal 'A' Film (Bio-Rad MSA5001) and ran (preheat lid to seal): 98°C for 45 seconds and 7 cycles of 98°C for 20 seconds, 63°C for 20 seconds and 72°C for 2 minutes, and held at 10°C. All primers used in the library preparation are listed in Table A3. 1 (appendix).

Following a second purification with Ampure XP magnetic beads (1X) (following manufacturers protocol, see above), the tagged amplicons were quantified and assessed for purity by NanoDrop™ Spectrophotometer (Thermo Scientific™, USA), and then pooled in equimolar concentrations of 125ng to create a master sequencing library. The library's final concentration was determined by KAPA qPCR Library Quantification Kit (KAPA Biosystems, USA) following the manufactures protocol. The pooled library was run on an Ilumina MiSeq Desktop Sequencer using a 500-cylcle pair-end reagent kit (MiSeq Reagent Kits v2, MS-103-2003) at a concentration of 12.5nM with a 25% PhiX Control v3 (Illumina, FC-110-3001). The libraries were sequenced using the MiSeq Reagent Kit v2 50 bp. The FASTQ files generated had no post-run analysis, and the sequences were automatically separated by sample during post-run processing by indices-recognition. All protocols followed Illunina's standard MiSeq operating protocol. For a breakdown of the methods see Figure 3. 2.

3. 3. 4. Bioinformatics

3. 3. 4. 1. AusDiagnostics™

The quantitative results were calculated by the 'Easy-Plex Analysis' software (AusDiagnostics Pty.Ltd.), and displayed as percentages for the individual parasite species. These were transferred to an Excel spreadsheet (Microsoft Corporation, USA).

3. 3. 4. 2. Nemabiome assay

Samples were analysed using the Mothur bioinformatics tool (versions 1.37.6 and 1.39.5, Schloss et al., 2009) using Windows Command Prompt (Microsoft Corporation, USA). The FASTQ

files were unzipped, raw pair-end reads were assembled to create single contigs and the reads were then filtered and removed if they were <200 bp or >450 bp, or if there were ambiguities between the overlapping paired-end reads. The bespoke taxonomy database was created by Avramenko et al., (2015), containing available nematode ITS-2 sequences created from the public databases and with their own reference samples. This was updated to include ovine strongyle species known to infect the Soay sheep and common European ovine species (i.e. *Bunostomum trigonocephalum, Chabertia ovina*), sequences obtained from a BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), sequences that had >99% identity was added to the taxonomy file. The reads were aligned to the ITS-2 database, if they did not align to at least 10% of any ITS-2 amplicon with at least 90% sequence similarity it was discarded. The remaining sequences were classified against the database reference sequences; by using the k-nearest-neighbour method (k = 3) if the three nearest matches did not map to a single species, were therefore assessed at a higher taxonomic level until it is correctly classified. The raw reads, divided between species identified per sample, were then inputted into Excel (Microsoft Corporation, USA).

Species-specific biases during sequencing were identified in Chapter 4, these were then used to calculate a Correction Factor (CF), which can be then multiplied by the sequencing reads prior to analysis to account for these species-specific differences. These CFs were calculated for each species identified on St Kilda (Chapter 4); *B. trigonocephalum* = 0.865676, *C. ovina* = 2.044679, *T. circumcincta* = 0.840703 and *T. vitrinus* = 0.857427. A CF could not be calculated for *T. axei*, therefore the correction factor calculated by Redman et al., (2019) was used (*T. axei* = 1.278952).

Species prevalence below <0.05% would relate to fewer than one worm per sample, with these results probably being due to contamination or misidentification of reads (Avramenko et al., 2015). If a species resulted in a proportion <0.05% it was removed. The total number of reads for the sample was then adapted by removing the low-burden species reads, and the percentages for the other species were recalculated. The percentage species composition of each sample was calculated by: (the corrected number of raw reads per species/ the total number of reads per sample) x 100.

3. 3. 5. Statistics

The resulting proportional data (%) for both methods were visualised in stacked bar graphs made in Excel (Microsoft Excel, 2010).

Due to each methodology identifying grouped species, the results of each platform were adapted in order to compare the differences in identification and quantification of three main species: *C. ovina*, *T. circumcincta* and *Trichostrongylus* spp. The nemabiome assay results were adapted by adding together the number of reads for each species to calculate the total number of reads for that pooled species (i.e. *T. vitrinus* and *T. axei* reads combined to result in '*Trichostrongylus'* spp). The result was then divided by the total number of reads for that sample and multiplied by 100.

The agreement of positive versus negative results between both methods was calculated. AusDiagnostics™ and nemabiome assay data were converted into binary data based on presence or absence of nematode genera/species, and frequency tables (2 x 2) were constructed for each genera/species of strongylids. To determine the level of agreement and Cohen's kappa values were calculated by using an established method (Conraths and Schares, 2006) using the WinEpiscope 2.0 online tool (http://www.winepi.net/). The interpretation of kappa results done according to Conraths and Schares (2006). Kappa measures the proportion of agreement (%) and its values (listed below) can be applied to compare results generated from different tests from one set of samples (i.e. one culture, in this case). Kappa has a range from -1 to 1. A benchmark can be used arbitrarily to interpret kappa values as 0: poor agreement; 0–0.20: slight agreement; 0.21–0.40: fair agreement; 0.41–0.60: moderate agreement; 0.61–0.80: substantial agreement; ≥ 0.81: almost perfect agreement (Conraths and Schares, 2006). Kappa were adjusted for prevalence and bias (PABAK, prevalence-adjusted bias-adjusted kappa) according to Byrt et al., (1993) with a score interpretation -1 to 1 as described above for the kappa values.

Linear regression analysis was performed to assess the correlation between the percentage composition of *C. ovina*, *T. circumcincta* and *Trichostrongylus* spp in each of the 20 mixed-species samples as determined by AusDiagnostics™ platform and the nemabiome assay. The values for R^2 (coefficient of determination), b(y-intercept) and m(slope) are shown adjacent to the plot. The plots were produced in Excel (Microsoft, USA).

3. 4. RESULTS

3. 4. 1. Strongyle species identified

3. 4. 1. 1. AusDiagnostics™

The species panel used for AusDiagnostics™ included *Teladorsagia circumcincta, Trichostrongylus* spp*., Chabertia ovina, Cooperia curteci, Oesophagostomum venulosum* and

Haemonchus contortus. The species identified in this study were *C. ovina, T. circumcincta* and *Trichostrongylus spp.* (Figure 3. 3).

3. 4. 1. 2. Nemabiome assay

The species identified by the nemabiome assay were *T. circumcincta, T. axei, T. vitrinus, C. ovina* and *B. trigonocephalum,* with no new novel species (Figure 3. 3).

3. 4. 1. 3. Tail lengths

By looking at tail-lengths, species are grouped, and whilst allowing for assessing general genus composition it lacks the sensitivity of speciation of the other two methods. The results were grouped into short (*Teladorsagia* spp. and *Trichostrongylus* spp.), medium (*Cooperia* spp.) and long (*Chabertia* spp.) tail lengths (Table 3. 1), in addition to noting species that had a novel morphology (*B. trigonocephalum*). *C. ovina* is the only species identifiable by a long tail, and no other long-tailed species (i.e. *Oesophagostomum*) was identified by the molecular methods. Therefore, L_3 identified with a long tail were presumed to be *C. ovina* and will be compared with the other methods (nemabiome assay and AusDiagnostics™).

Cooperia sp. was thought to have been identified at low levels in one culture (2 out of 100 L_3); *Cooperia* are the only species that are identified by a medium tail. Upon identification of a medium tail, the head was examined at a higher magnification and potential refractile-bodies were identified. However, *Cooperia cuteci* and *Cooperia* species were not identified using both AusDiagnostics™ platform and nemabiome assay, despite both methodologies have been proven to not only identify these species, but also identify L_3 at low-levels in mixed species pools (Avramenko et al., 2015; Roeber et al., 2017[a]; Roeber et al., 2017[b]). Therefore, it was decided that this was a misidentification and the *Cooperia* sp. results were removed from the dataset and the proportion (%) of the other species identified were recalculated.

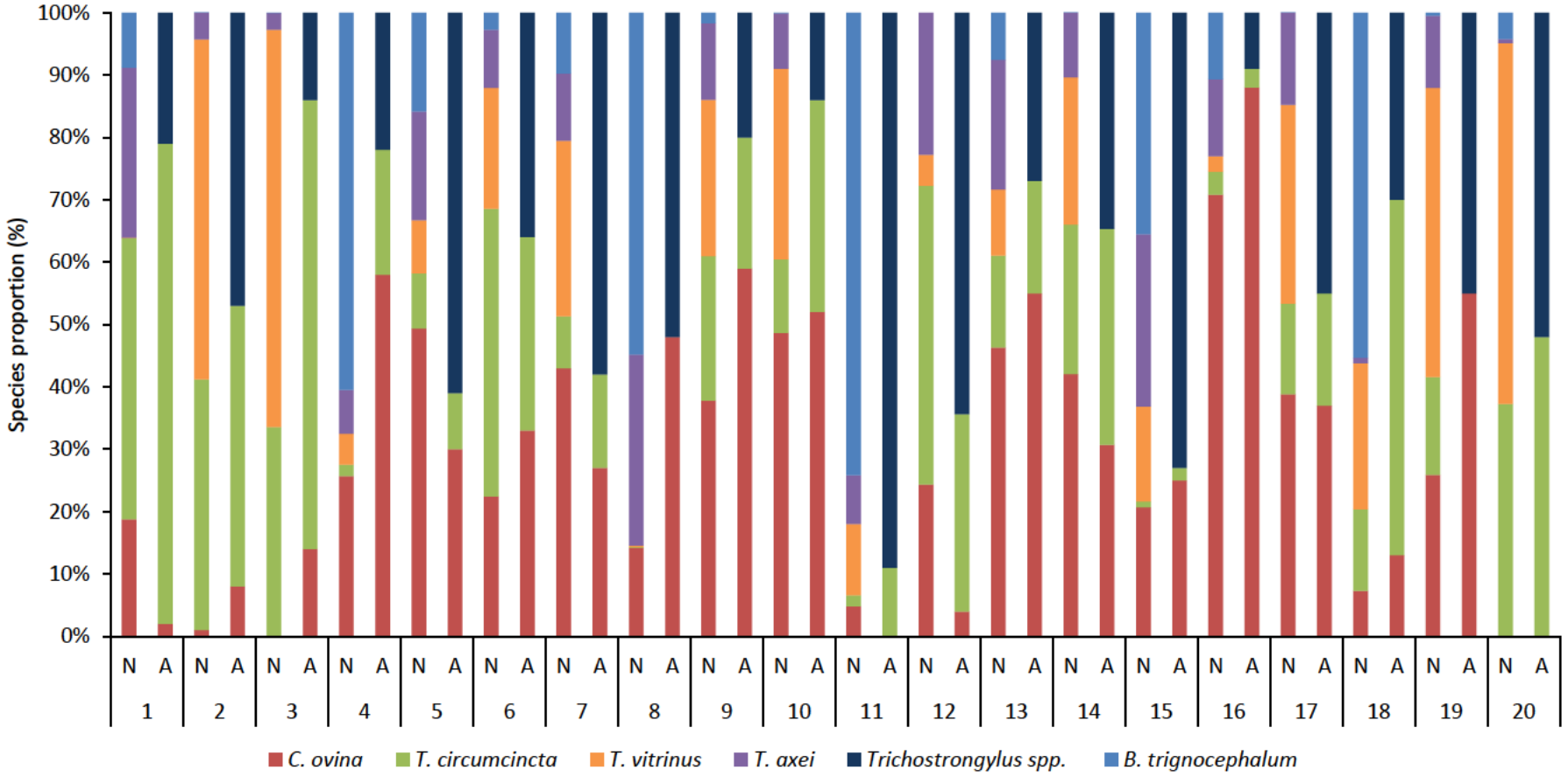


Figure 3. 3. Comparison of the proportional species (%) data generated by the nemabiome assay (N) (corrected reads) with AusDiagnostics™ (A) between 20 field samples.

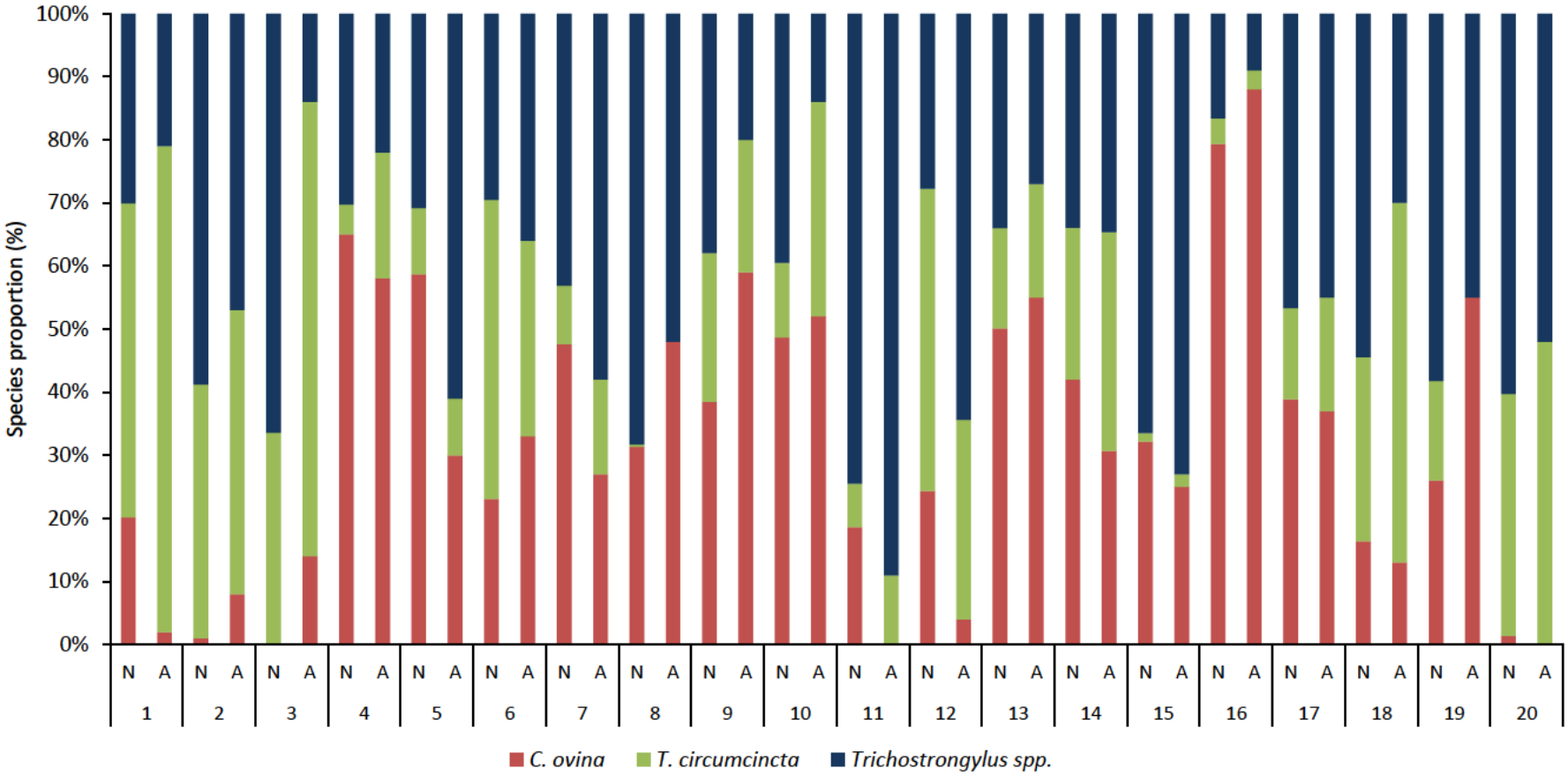


Figure 3. 4. Comparison of the proportional species data generated by the nemabiome assay (N) with AusDiagnostics™ (A) between 20 field samples. Nemabiome assay results have been adapted to remove *B. trigonocephalum* and combine *T. vitrinus* and *T. axei* reads (‘*Trichostrongylus* spp.’) in order to compare with AusDiagnostics™.

3. 4. 2. Comparison of semi-automated multiplex-tandem PCR (AusDiagnostics™) with ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay) for the identification and quantification of strongylid species in mixed infections

Due to each methodology identifying different species, the results of each platform were adapted in order to compare the differences in identification and quantification of three main species: *C. ovina*, *T. circumcincta* and *Trichostrongylus* spp. (Figure 3. 4). *C. ovina* is the only species identifiable by a long tail, therefore, L_3 identified with a long tail was presumed to be *C. ovina.*

In many samples, the results of both molecular tests consistently determined the species/genus present at the highest proportion. *Trichostrongylus* sp. was consistently identified by both methods to have the highest proportion in samples 2, 8, 11, 15, 17, 19 and 20 (Figure 3. 3). *C. ovina* was identified by both methods to have the highest proportion in sample 4, 19, 10, 13 and 16. Only one sample had *T. circumcincta* predominating (sample 1). Other samples (3, 5, 6, 7, 12, 14 and 18) had some disagreement between the results of the two molecular methods, with different species predominating.

The agreement of positive vs. negative results between the nemabiome assay and AusDiagnostics™ was highest for *Trichostrongylus* spp., with a calculated agreement of 100%, PABAK 1.0 (perfect agreement), followed by *T. circumcincta* with 95% agreement and PABAK of 0.9 (almost perfect agreement), and *C. ovina* with 90% agreement and PABAK of 0.8 (almost perfect agreement). The agreement of positive vs. negative results for nemabiome assay/AusDiagnostics™ and morphological identification of *C. ovina*, with a calculated agreement of 95% and PABAK of 0.9 (almost perfect agreement). In general, the agreement between the results of nemabiome assay vs. AusDiagnotics™ was higher than the agreement between nemabiome assay/AusDiagnostics™ and morphological identification of *C. ovina* (Table 3. 1.).

The kappa agreements are very low, due to the observer agreement paradox; a high agreement (90 – 100%) but low k score. When the observer agreement is high (i.e. 90 - 100% in this trial), the agreed responses fall into a single nominal category (i.e. both tests resulted in parasite-positive result - ++). Therefore, the 'observed' disagreement is very low (0 -10%) resulting in a very low to no reliability statistic (Table 3. 1). Therefore, the test will interpret these results as -1 to 0; the result occurring purely due to chance. Hence why prevalence-adjusted bias-adjusted kappa (PABAK) is used.

Table. 3. 1. Table showing agreement (%) of tests (positive/negative) beyond chance for the nemabiome assay vs. AusDiagnostics™, nemabiome assay vs. morphological ID (Morph.), and AusDiagnostics™ vs. morphological ID (Morph.) Number of samples (n=) tested positive (+) and negative (-) by each method, the kappa values (k), adjusted kappa (PABAK), and standard error (SE).

	Species	**n=**	**+, +**	**+, -**	**-, +**	**-, -**	**Agreement (%)**	**Kappa (k)**	**PABAK**	**SE**
Nemabiome	AusDiagnostics									
	C. ovina	20	18	2	0	0	90.00	0.000	0.8	0
	T. circumcincta	20	18	1	0	1	95.00	0.643	0.9	0.325
	Trichostrongylus spp.	20	20	0	0	0	100.00	-1.000	1.0	0
Nemabiome	Morph.									
	C. ovina	20	19	1	0	0	95.00	0.000	0.9	0
AusDiagnostics	Morph.									
	C. ovina	20	18	0	1	1	95.00	0.643	0.9	0.209

3. 4. 2. 1. *Chabertia ovina*

There was a high level of correlation between the nemabiome sequencing assay and the morphological identification (Figure 3. 5. 1) (R^2=0.9015). There is a similar, moderate correlation between the results generated by AusDiagnostics™ platform and the morphological identification (R^2=0.6496) and nemabiome assay and AusDiagnostics™ (R^2=0.6096) (Figure 3. 5. 2, Figure 3. 5. 3.)

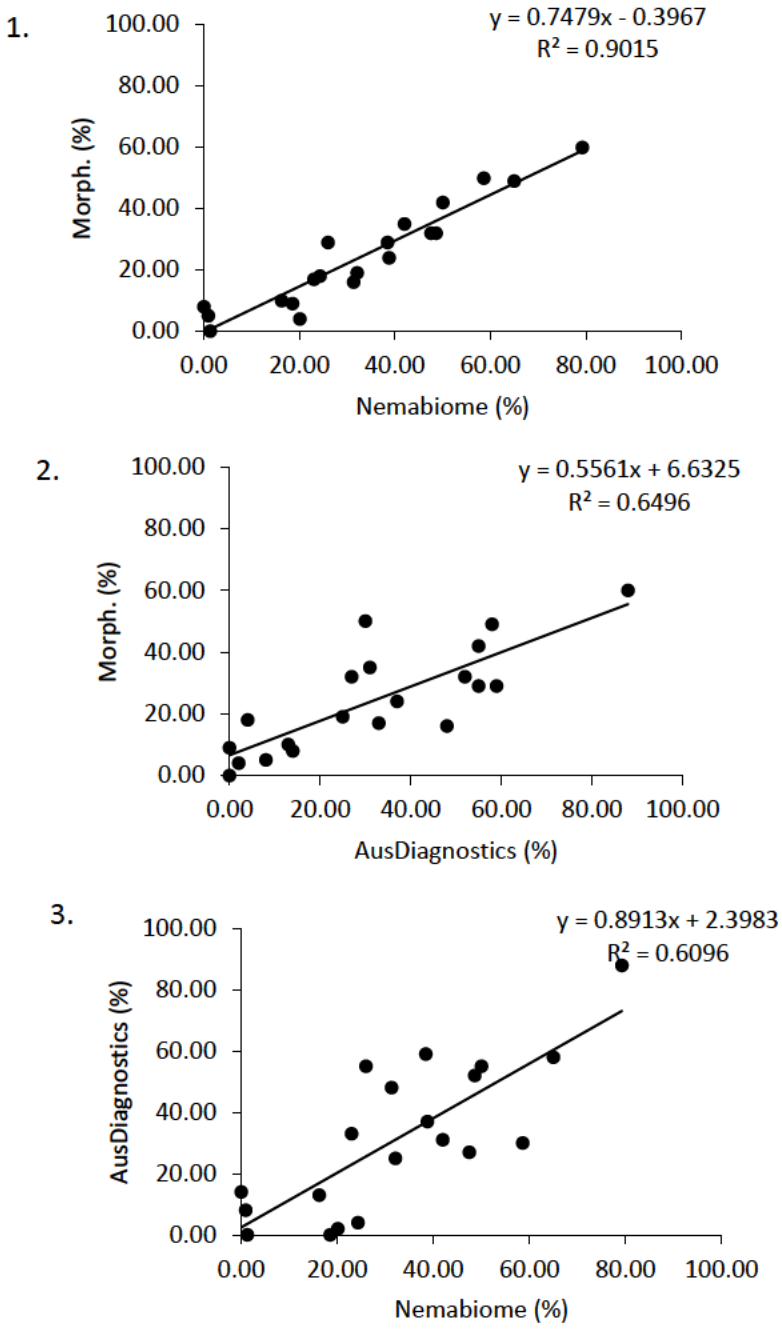


Figure 3. 5. Linear regression analysis to assess the correlation between the percentage composition of *C. ovina* from 20 field samples, as determined by multiplex-tandem PCR

(AusDiagnostics™), ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay) (corrected reads) and morphological identification (Morph.). Cross-comparisons were made between the methods to test the methods ability to detect and quantify *C. ovina*; 1. Morphological identification (Morph.) and nemabiome assay, 2. Morphological identification (Morph.) and AusDiagnostics™, 3. AusDiagnostics™ and nemabiome assay.

3. 4. 2. 2. *Teladorsagia circumcincta*

There was a moderate correlation between the AusDiagnostics™ and nemabiome assay when identifying *T. circumcincta* in mixed-species pools (Figure 3. 6, R^2=0.5917).

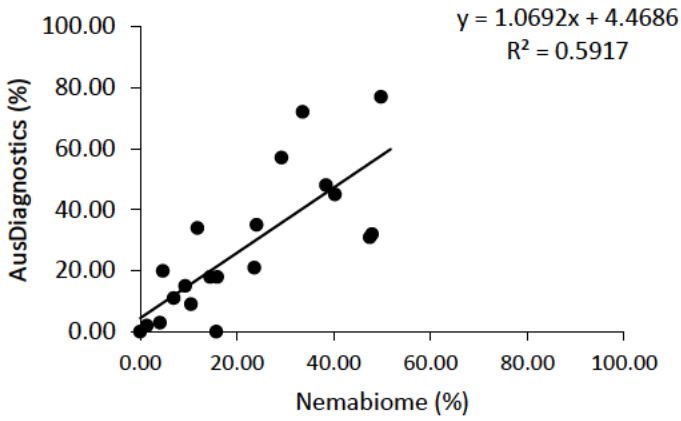


Figure 3. 6. Linear regression analysis to assess the correlation between the percentage composition of *T. circumcincta* from 20 field samples, as determined by multiplex-tandem PCR (AusDiagnostics™), ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay) (corrected reads).

3. 4. 2. 3. *Trichostrongylus* spp.

There was a poor correlation between the AusDiagnostics™ and nemabiome assay when identifying *Trichostrongylus spp.* in mixed-species pools (Figure 3. 7, R^2=0.2334). If the reads generated by the nemabiome assay data were not combined (i.e. combining *T. axei* and *T. vitrinus*), and the two species of *Trichostrongylus* tested separate against the AusDiagnostics™ *Trichostrongylus spp.* , the correlation is very poor between the two methods (*T. axei* v.s *Trichostrongylus* R^2 = 0.0508, *T. vitrinus* vs. *Trichostrongylus* R^2 = 0.0076).

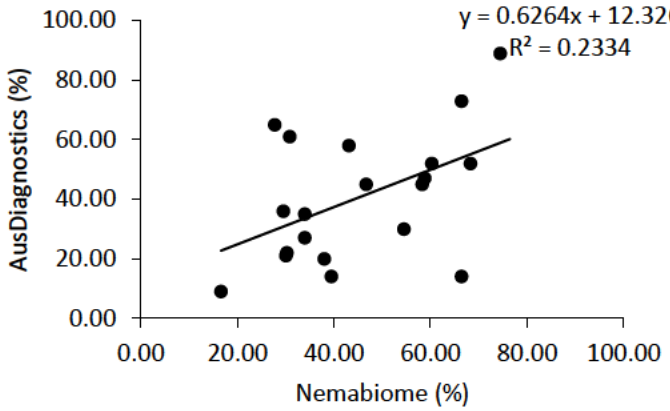


Figure 3. 7. Linear regression analysis to assess the correlation between the percentage composition of *Trichostrongylus spp.* from 20 field samples, as determined by multiplex-tandem PCR (AusDiagnostics™), ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay) (corrected reads).

3. 5. DISCUSSION

Simultaneous infection with multiple nematode parasite species is commonly seen in feral and managed ruminants. The development of molecular methods to identify and quantify mixed-species infections has negated many of the issues arising from traditional parasitological techniques. The aim of this chapter was to compare two molecular methods; multiplex-tandem PCR (AusDiagnostics) with deep amplicon sequencing (Nemabiome), to identify and quantify ovine strongylid species in naturally-infected samples.

3. 5. 1. Species identified

The species identified fit the parameters of the method; AusDiagnotics™ identified 3 out of the 6 species/genus in its diagnostic panel (*T. circumcincta, C. ovina* and *Trichostrongylus spp.*), whilst Nemabiome was able to identify 5 strongylid species (*T. circumcincta, C. ovina*, *T. axei,* T. *vitrinus* and *B. trigonocephalum*). The nemabiome assay did not identify any 'unknown' sequences (i.e. species not available in the database of reference sequences generated by Avramenko et al., (2015)), indicating what was identified in these field samples represents the species present in the study population. This matches previous studies in the Soay sheep that morphologically identified adult worms (Craig, 2005; Gulland, 1991), and it is highly unlikely there is an additional species present within the gut that was not picked up by Craig (2005), Wimmer (2004) and this present study in the isolated sheep population.

The drawback of the AusDiagnostics™ platform is its inability to differentiate the *Trichostrongylus* genus to species-level. This is a drawback, for example, because different species within the genus *Trichostrongylus* may differ in their pathogenicity and resistance to anthelmintics, a factor that may underpin the need to speciate L_3. The identification of *Trichostrongylus* spp. between the two methods had excellent agreement (100%), meaning all samples had the same negative/positive result. In these 20 samples, all had some level of *Trichostrongylus* spp. present. However, the correlation of both methods was very poor indicating both methods are similar in their ability to identify species present, but have significant differences in their quantification results. The excellent agreement between both samples is most likely due to the level at which this genus was present in the cultures (nemabiome assay range = 16.59 – 74.47%, AusDiagnostics™ range = 9 – 89%). The presence of two species within the *Trichostrongylus* spp. genus is probably the cause of the poor correlation between the two methods; combining reads generated by the nemabiome assay may not have been accurate (though correlation did not improve when both species were correlated separately). Furthermore, there may be a mis-match between the primers for both methods (discussed below).

T. circumcincta and *C. ovina* have a good agreement (95% and 90%, respectively), and good correlation between the methods (R^2 = 0.5917 and R^2 = 0.6096, respectively).

3. 5. 2. Method discussion

The molecular methods are different in their approach to identifying and, most importantly, quantifying parasite species in a mixed-species infections. Whilst there was good agreement between both molecular tests (i.e. identification of species within a sample) (Table 3. 2) there is moderate to poor correlation in their ability to quantify the species present; *C. ovina* (R^2 = 0.6096), *T. circumcincta* (R^2 = 0.5917), *Trichostrongylus* spp. (R^2 = 0.2334).

Because both methodologies were in their development stages when this work was conducted, this is the first comparison made between the two molecular methods, and further work is needed to fully understand and identify the reasons as to the differences between the quantitative data generated by the different methods. However, there are many potential contributing factors that could lead to these results; DNA extraction method (MO-BIO DNA extraction kit vs. lysate preparation), larval aliquoting, amplification and primer-type (multiplex-tandem PCR vs. deep amplicon sequencing of the rDNA ITS-2) and bioinformatics to determine quantitative data (melt-curve analysis vs. read count).

3. 5. 2. 1. Primers and amplification

The ITS-2 region of parasitic nematodes within the order Strongylida provides an accurate genetic marker for diagnostic PCR-based methods. The target region is short (<800 bp) (Gasser, 2006), which determines efficiency and specificity of any PCR amplification method (Gasser, 2006; Roeber et al., 2014).

The nemabiome assay used a 311-331 bp fragment encompassing the rDNA ITS-2 sequence (using the NC1 and NC2 primers as described by Gasser et al (1993)). The primers are adapted to accommodate the barcoding region. The ITS-2 region is amplified by PCR, and the barcodes attached by cycle-limited PCR (Figure 3. 2). Deep-amplicon sequencing generates sequences, or reads, per sample. A bioinformatics tool will screen sequences and assign each sequence to a species reference sequence. It is from the number of reads per species divided by the total number of reads generated for the sample which creates the proportional data.

In comparison, AusDiagnostics™ is a multiplex-tandem PCR, using smaller parasite-specific primers within the ITS-2 region; *T. circumcincta* = 218 bp, *Trichostrongylus* spp. = 267-268 bp and *C. ovina* =162 bp (Roeber et al., 2012). By using species-specific primers, there may be a potential PCR biases if primers have differing reaction efficiencies. Multiplex-tandem PCR comprises of two amplification phases: a 'target enrichment' phase using multiplexed primer sets, and a subsequent analytical amplification phase involving the targeted amplification (in tandem rather than multiplex) of each genetic locus of the species being investigated, as specific nested primers. The amplification phase is limited to prevent amplification bias by reducing interactions between multiplexed primer sets, reduces competition or the generation of artefactual products. The real time PCR involves the incorporation of a specific fluorescent dye into the PCR, binding to double-stranded DNA and allows the detection of accumulating DNA (Roeber et al., 2015). By using florescent dyes, multiplex-tandem PCR is able to establish the relative or absolute quantification of a species by allowing the identification of the cycle threshold (C_t) at which amplification begins. This is because the disadvantage of using fluorescent dyes is it binds to all double-stranded molecules, including non-specific targets (e.g. primer dimers). Therefore, the melting point of an amplicon is linked to the length and composition of a nucleotide, which can be used to detect sequence variation in a sample (i.e. melting-curve analysis) which can then be used to speciate a sample. Quantification occurs through melt-curve analysis (C_t) through a commercial, pre-validated system ('Easy-Plex Analysis' software (AusDiagnostics Pty.Ltd)).

To my knowledge, no one has tested the differences between parasite species quantification between samples generated by number of sequenced reads and melt-curve analysis.

3. 5. 2. 2. Larval preparation and DNA extraction method

In order to fully understand the differences between the two molecular methodologies – samples of known species proportions need to be run to test correlation and repeatability. Bias between the techniques may have occurred due to different subsamples of L_3 being taken from the same culture to carry out the different methods. Additionally, 500 L_3 were tested by the nemabiome assay and ~1000 L_3 by AusDiagnotics™. Therefore, potential diagnostic differences between the methods will occur before they are even sequenced, leading to a potentially poorer representation of species in one method over another. Avramenko et al., (2015) tested biological replication (i.e. multiple pools of the same culture) and found no significant difference in species proportion, which is an important observation, as a fully homogeneous culture is not possible when pipetting out aliquots from a bulk culture of L_3. However, species at low levels within the culture may be skewed due to these difference and may be responsible for the observed discrepancies in some of the samples when a species is at low levels within the culture.

As described in Chapter 4, the nemabiome assay was based on microbiome sequencing, the advantage of working with nematode species is it is easier to test for, and correct, biases within the sequencing data, as mock populations of known species proportions can be tested. This sentiment is also applicable to comparison studies between molecular methods. Future work should include testing mock populations of known proportions, in order to accurately establish the quantitative accuracy and/or difference between both methods. Additional factors could be tested for, including detection threshold and repeatability.

DNA extraction may also be a factor influencing the results of the method comparison. Many studies that sequence L_3 favour an initial mechanical breakdown step during extraction, such as a bead breakdown (AusDiagnostics™). The nemabiome assay relies on rapid temperature change (freeze-thawing), in order to break the protective cuticle. For some species this may not be enough to fully release the gut cells for digestion. The differences in DNA extraction methods (i.e. bead beating vs. freeze thaw) are not known, and would ideally need to be tested before method comparison.

3. 5. 2. 3. Larval culture and morphological identification

There was a high degree of correlation between parasite species proportions as determined by the nemabiome assay compared with those determined on the basis of larval morphology of *C.*

ovina (R^2 = 0.9015), suggesting what is being sequenced by the nemabiome assay is what is seen in the cultures, which is similar to the morphological comparisons made by Avramenko et al (2015) with other species (*Cooperia, Haemonchus*). There was moderate correlation between the AusDiagnostic™ assay and morphological identification of *C. ovina* (R^2=0.6496). Morphological identification of L_3 takes time and skill. The morphological differentiation between *T. circumcincta* and *Trichostrongylus* species is notoriously unreliable, therefore, these species were removed from the morphological comparison with the molecular methods. Additionally, B. *trigonocephalum* was removed from this comparison study, because it is a rare parasite on the mainland, and the morphological identification requires an expert eye. However, I successfully identified this species for the validation work in Chapter 4.

3. 5. CONCLUSION

This is a proof-of-concept study highlighting the effectiveness of multiplex-tandem PCR (AusDiagnostics™) and ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay) for the identification and quantification of strongylid nematode larvae in naturally infected sheep. Whilst there was good agreement between both molecular tests (i.e. presence/absence of specific species within a sample) there is moderate to poor correlation in their ability to quantify the species present. Considering this is the first study to compare both molecular methods, the results generated indicate good detection for all species, but further work is required in comparing the quantitative abilities of both methods. A semi-diagnostic tool, such as AusDiagnostics™, provides a relatively quick method to identify strongyle species present *a priori,* which suits a diagnostic tool for managed ruminant systems. The nemabiome deep amplicon sequencing assay proved to be the most effective method for identifying and quantifying the strongylid species associated with a feral host. The sensitivity, bias, and repeatability of this method will be verified for the strongylid species identified in the Soay sheep in the following chapter (Chapter 4).

CHAPTER 4

Validation of the nemabiome deep-amplicon sequencing approach for strongyle species identified in the Soay sheep of St Kilda

4. 1. ABSTRACT

Simultaneous infection with multiple nematode parasite species is commonly seen in feral and managed ruminants. Within these mixed burdens, the species can vary in their pathogenicity, epidemiology and resistance to anthelmintics. Quick and accurate diagnosis of gastrointestinal infections of livestock is a critical component for monitoring anthelmintic resistance, and assessing parasite interactions. The development of the nemabiome deep-amplicon sequencing method has negated many of the issues arising from traditional parasitological techniques. Whilst the nemabiome method was based on microbiome sequencing, the advantage of working with nematode species is it is easier to test for, and correct, biases within the sequencing data.

The main aim of this chapter is to validate the nemabiome deep-amplicon sequencing assay (i.e. the nemabiome method) by testing artificially-made pools of infective 3rd-stage larvae (L_3) for biases that may be introduced by DNA extraction efficiency, species-specific biases during ITS-2 amplification and within-sequencing repeatability.

The first objective was to establish the quantitative accuracy of the assay, to identify potential species amplification biases for the species identified in the Soay sheep, as discussed in Chapter 3; *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus vitrinus*, *Bunostomum trigonocephalum* and *Chabertia ovina*. Then, to calculate a 'Correction Factor' (CF) for each species that can then be applied to the field data (Chapter 5) in order to reduce potential species-specific sequencing bias within the data. A Correction Factor (CF) for *B. trigonocephalum* (0.865676); *C. ovina* (2.044679); *T. circumcincta* (0.840703) and *T. vitrinus* (0.857427) was calculated and will be used for all nemabiome sequencing results in this thesis (Chapter 3 and Chapter 5). A CF for *T. axei* could not be established, but due to the consistency in methodology between this study and other studies, and the flexibility of the assay, the CF of a previous published work will be used (*T. axei* = 1.278952).

The next objective was to test the assays detection threshold in order to identify low-level infections of *B. trigonocephalum* and *C. ovina* in artificially-made mixed-species pools. This chapter provides a preliminary assessment of the assays detection threshold for *B. trigonocephalum* and *C. ovina* when they are present at very low numbers within large, mixed-species pools, however, further work is required.

The final objective was to test within-sequencing repeatability of both artificially made populations and field-collected populations. Within-sequencing is highly repeatable for both artificially made populations and field-collected populations.

The sensitivity, bias, and repeatability of the nemabiome assay was tested for the strongylid species identified in the Soay sheep of Hirta. A correction factor was calculated from these biases, for each species identified from the Soay sheep, in order to reduce potential species-specific sequencing bias, and was subsequently applied to the analysis of the field data (Chapter 5). Further work is required to validate this methodology.

4. 2. INTRODUCTION

The identification of a species by sequencing a marker gene in a DNA barcoded template, has provided a means to identify diversity in both environmental (water, soil, air, faeces) and biological (parasitological, vertebrate, plants) samples. The danger of blindly assigning taxonomic identity directly to generated metabarcoded sequences is the production of data that is inaccurate and unreliable, notably when species are rare or cryptic, or when the species populations being investigated are unknown (Cowart et al., 2015; Aivelo and Medlar, 2018). This is especially true for the nemabiome deep-amplicon sequencing approach (i.e. identification and quantification of nematodes in a mixed-species pool by metabarcoding sequencing), as the downstream speciation of reads relies on a pre-made, bespoke library of well-defined sequences (Avramenko et al., 2015; Redman et al., 2019).

Nematode speciation by deep amplicon sequencing is a relatively novel approach and, as with most meta-barcoding methods, relies on species-specific primers and the direct use of databases from GenBank. In recent studies, there has been high agreement between molecular sequencing and morphological identification in order to speciate and quantify mixed species of 3rd-stage larvae (L_3) (Roeber et al., 2017$^{a\&b}$; Avramenko et al., 2015; Bisset et al., 2014; Ji et al., 2013). The benefit of studying the helminth parasite burden in the Soay sheep of St Kilda is the wealth of parasitological work that precedes it, which was initiated by the population crash of 1964 by Cheyne et al (1974), and continued by Gulland (1992), Craig (2005) and Pilkington (*unpublished*, *ongoing*). This work has provided a list of expected species, which has informed both the downstream bioinformatics (Chapter 3) and also the validation work for the metabarcoding in this study. This information, in addition to the data generated by the AusDiagnostics™ results (Chapter 3), has provided a species list of clade V helminth parasites present in the Soay sheep on St Kilda; *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus vitrinus*, *Bunostomum trigonocephalum* and *Chabertia ovina*. In line with previous work, *Teladorsagia daviani* and *Teladorsagia trifurcata* were not identified, and are

considered simple (or cryptic) species with morphological polymorphism and no genetic discrimination between morphs (Craig, 2005).

An advantage of working with nematode communities, in comparison to microbial or protozoan populations, is that it is easier to test for, and correct, biases within the sequencing data (Avramenko et al., 2015; Redman et al., 2019). In comparison to bacteria, 3rd-stage infective nematode larvae (L_3) of farmed ruminants can cultured and morphologically identified. A pool of L_3 made up of known species composition can then be sequenced, and then directly compared to the results generated by the sequencing methodology. By creating mock populations from pure laboratory-grown strains, the assay sensitivity, repeatability and potential biases can be tested for, and corrected. This is rarely done for microbiome studies, due to the complexity of the bacterial communities, and the difficulty of growing many of the species *in vitro*. Possible sources of species representation bias may include species-specific differences in copy number of the ITS2-rDNA target, species-specific differences in DNA content (i.e. differences in larval size and cell number), and differences in sheath-thickness affecting digestion efficiency (i.e. DNA extraction).

Copy number variations (CNV) are defined as insertions, deletions, and duplications of genomic sequence between two individuals of the same species, and can range from 50 base pairs (bp) to 5 million base pairs (Mbp) (Yang et al., 2017). CNV are widespread in eukaryotic genomes, ranging from yeast (Maleszka and Clark-Walker, 1993), to humans (Conrad et al., 2010), and to ruminants (Yang et al., 2017). CNV have also been suggested for free-living nematodes (Bik et al., 2013), and have been studied in malaria (*Plasmodium falciparum*) (Cheeseman et al., 2016), *Leishmania* spp. (Marie-Claude et al., 2016) and helminth spp. (Samson-Himmelstjerna et al., 2002). Differences in amplicon efficiency could be due to species-specific sequence variation in the amplicon, but CNV of the rDNA cistron is most likely the main cause as it is exists as a multi-copy tandem repeat (Avramenko et al., 2015). Additional factors, such as sequence polymorphism in the primer binding sites are unlikely, as this method of sequencing is clade V-specific (i.e. using a highly-conserved region) therefore the binding sites are highly conserved.

The species identified in the Soay sheep differ in morphological characteristics of their L_3 (Table 4. 1), some are larger with more cells (i.e. *C. ovina*) whilst others differ in physical size but contain a similar cell number (*Trichostrongylus spp., Teladorsagia spp.,* and *Bunostomum sp.),* which would imply, but not confirm, similar DNA content (i.e. larger cells or nuclei do not infer higher DNA content). When the L_2 moults into an L_3 (Chapter 1) they will retain the cuticle from the previous moult, forming a protective sheath. This sheath enables the larvae to survive in the environment on pasture, providing resistance to desiccation. Different species have differing

survival rates; *T. axei* has a wide climatic distribution whilst *T. vitrinus* is commonly found in more temperate regions. *B. trigonocephalum* is the most susceptible and will only survive a short while on pasture (O' connor et al., 2006). An environmentally resistant species would imply a tougher sheath, which could potentially be difficult to break down during DNA extraction, resulting in differences in DNA lysis efficiency between species.

Table 4. 1. Morphological characteristics of 3rd-stage larvae (L_3) (adapted from MAFF (1986), larvae lengths from Soulsby, (1968), tail sheath lengths from van Wyk et al., (2004)).

Genus	Intestinal cell number	Head characteristic	Mean tail sheath length (μm)	Estimated length* (μm)**–
Teladorsagia (Ostertagia)	16	Square	65	797 – 907
Trichostrongylus	16	Tapered	29	560 – 796
Bunostomum	16	Tapered	101	560 – 637
Chabertia	32	Broad/ round	152	650 – 789
Cooperia	16	Square	45	666 – 956

* Length of larvae used for illustrative purposes only – length differs within species depending on the environmental factors they developed (heat/ temperatures) and are therefore is not generally used for morphological speciation.
** excluding tail sheath

It has been established in previous studies applying meta-barcoding methods to assay gut nematode community structure in ruminants, that biases are consistent between replicated assays, with little variance between repeated samples. This means, though whilst interesting, it is not essential to locate the source/s of bias, but be aware of them as we validate the assay for each new study system and host (Avramenko et al., 2015; Redman et al., 2019). Therefore, if a standardised protocol is used, the inherent species-biases can be corrected for, and has been done for work in cattle (Avramenko et al., 2015; Avramenko et al., 2017), Bison (Avramenko et al., 2018) and sheep (Redman et al., 2019) parasites. This is the first study using next generation sequencing (NGS) methods to study the parasite communities on St Kilda, it will contribute to the first set of validations for sheep parasites, and create a bespoke validation for the species found in the Soay sheep.

4. 2. METHODS

The traditional parasitology and sequencing techniques outlined in this chapter was performed by the author, unless explicitly stated in text.

4. 2. 1. Parasite material

T. circumcincta, T. axei and *T. vitrinus* were obtained from stock monocultures provided by the Moredun Research Institute (Scotland, UK), that were derived from experimentally grown and characterised isolates. They were stored in water in tissue culture flasks at 4°C until use, and were < 6 months old.

No laboratory-grown stains of *B. trigonocephalum* and *C. ovina* were available, and were grown from St Kilda-collected faecal samples. *C. ovina* is not considered an important parasite of sheep (in comparison to *Teladorsagia* or *Trichostrongylus*), therefore no monocultures were available. *B. trigonocephalum* is very difficult to establish in sheep in a laboratory environment, potentially due to its ability to infect both orally and percutaneously. The Moredun Research Institute (Scotland) has attempted to artificially infect sheep with *B. trigonocephalum* with limited success (*Fiona Kenyon, private comm*.)

24 faecal samples were collected on-pasture by volunteers from the Soay sheep on St Kilda on 8-10th of April 2017. These were partially sealed to allow aeration during transportation, and kept at room temperature before being sent to the laboratory (Moredun Research Institute, Scotland) within 7 days of collection. As these faecal samples were required as a source of nematodes, all faeces were pooled into a composite culture, regardless of host sex/age group. The culture was mixed in medium-grade vermiculite and incubated for a further 7 days at 23°C. The L_3 were extracted and stored in water, by methods described in (Chapter 3). Individual larvae were identified and picked from this mixed-infection culture. *C. ovina* is the only strongyle species that infects the Soay sheep whose L_3 is identifiable by a long filamentous tail, broad rounded head and 32 intestinal cells (Maff, 1986) (Table 4. 1). *B. trigonocephalum* also has a tail characteristic of a long tailed species, but it has the smallest L_3 encountered (of any strongyle species) with 16 ill-defined gut cells and a short filamentous tail (Figure 4. 1.) (Table 4. 1).

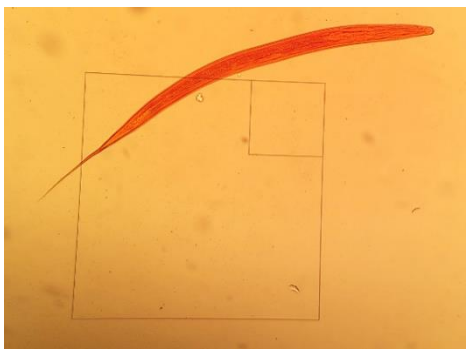


Figure 4. 1. *Bunostomum trigonocephalum* stained with Lugol's Iodine identified on a compound microscope (100x).

All larvae were individually picked using filter-pipettes to minimise cross-contamination. Stock larvae were poured into individual glass petri-dishes and picked individually under a dissection microscope, into 1.5 ml Eppendorf (Eppendorf, Germany) tubes to make different mock populations (Table 4. 2). *B. trigonocephalum* and *C. ovina*, which required morphological identification (Figure 4. 1), were picked individually from a glass slide under a compound microscope. The larvae were pooled live, and were not fixed with Lugol's iodine as its effects on PCR amplification were not known. The mock populations were then fixed in a total concentration of 70% ethanol and stored at -80°C until DNA extraction.

4. 2. 2. Mock populations

Most ovine strongyle species L_3 are morphologically distinguishable by a trained technician. The nemabiome sequencing assay can be tested and validated by sequencing samples with known numbers and types of L_3, the sequencing results can then be compared with the known starting composition. This forms the basis of the work presented here and the species mixes made to test against the nemabiome assay is listed in Table 4. 2.
The prevalence of *B*. *trigonocephalum* was very low in the composite culture, therefore the number of L_3 used had to be reduced to ensure all tests could be run.

4. 2. 2. A. Confirming pure larvae cultures

Separate pools of *T. circumcincta*, *T. axei*, *T. vitrinus, C. ovina* and *B. trigonocephalum* L_3 were stored separately, in water, to be used to create the mock populations listed in Table 4. 2. To ensure the larvae were correctly speciated, and not contaminated with other nematode species, 10 L_3 were picked into separate 1.5 ml Eppendorf tubes, per species, and fixed with 70%, stored at -20°C prior to sequencing (Table 4. 2).

B. trigonocephalum is a rare parasite in managed systems within the United Kingdom due to its susceptibility to anthelmintics and long prepatent period. Therefore, an accurate morphological identification of the species within a mixed-specie pool took a substantial amount of time, which was further hindered by a low prevalence within the culture, therefore this step was especially important to confirm the identification of *B. trigonocephalum*. Due to the very low yield of *B. trigonocephalum,* 10 larvae were collected from additional faeces collected on St Kilda by volunteers, and was sequenced separately in a different sequencing run to what is presented in this chapter. However, the sequencing conditions were kept consistent.

4. 2. 2. B. Correction Factor (CF) pool

In order to assess quantitative accuracy, and identify potential sequence representation bias for the different species identified in the Soay sheep, a mock sample was prepared by individually

picking 10 L_3 of each of the five species (*B. trigonocephalum, C. ovina, T. circumcincta, T. axei* and *T. vitrinus*) into a 1.5 ml Eppendorf tube for a single pool of 50 larvae (Table 4. 2). Larvae were fixed with 70%, stored at -20°C prior to sequencing (Table 4. 2).

Table 4. 2. Number of 3rd-stage larvae (L_3) picked individually into 1.5 ml Eppendorf's (samples 1-15) to make mock populations.
Samples 1 – 5: 5 Eppendorf's containing 10 larvae of a single specie, sequenced to ensure no contamination with other nematode species.
Sample 6 ('Correction Factor'): a pool of 50 L_3 made up of equal proportions (i.e. 10 each) *B. trigonocephalum*, C*. ovina, T. circumcincta, T. axei* and *T. vitrinus.*
Samples 7 – 10: a pool of L_3 containing equal numbers (167) of *T. circumcincta, T. axei* and *T. vitrinus* (501 total) and spiked with increasing numbers of *B. trigonocephalum: 0, 1, 3, and 6 L_3.*
Sample 11: a pool of L_3 containing equal numbers (50) of *C. ovina, T. circumcincta, T. axei* and *T. vitrinus* and spiked with 2 *B. trigonocephalum*
Samples 12 – 15: a pool of L_3 containing equal numbers (167) of *T. circumcincta, T. axei* and *T. vitrinus* (501 total) and spiked with increasing numbers of *c. ovina: 0, 1, 5, and 10 L_3.*

Sample number	Mock population	Number of L_3				
		B. trigonocephalum	*C. ovina*	*T. circumcincta*	*T. axei*	*T. vitrinus*
1	*T. circumcincta*			10		
2	*T. axei*				10	
3	*T. vitrinus*					10
4	*C. ovina*		10			
5	*B. trigonocephalum**	10				
6	Correction factor	10	10	10	10	10
7	*Bunostomum* detection threshold #0	0		167	167	167
8	*Bunostomum* detection threshold #1	1		167	167	167
9	*Bunostomum* detection threshold #3	3		167	167	167
10	*Bunostomum* detection threshold #6	6		167	167	167
11	Additional *Bunostomum* detection	2	50	50	50	50
12	*Chabertia* detection threshold #0		0	167	167	167
13	*Chabertia* detection threshold #1		1	167	167	167
14	*Chabertia* detection threshold #5		5	167	167	167
15	*Chabertia* detection threshold #10		10	167	167	167

* separate sequencing run

4. 2. 2. C. Detection Threshold (DTh) pools

The detection threshold of the assay was tested for species when present at low levels in mixed populations. Due to the nature of the field samples, mock populations could only be made from laboratory passaged isolates and then spiked with rare, field-grown species. Therefore, only two

species could be tested; *B. trigonocephalum* and *C. ovina*. The prevalence of *B. trigonocephalum* was very low in the composite culture, therefore the number of spiked L_3 was reduced to ensure all tests could be run. Eight separate pools of 501 larvae were made from equal proportions of *T. vitrinus, T. axei* and *T. circumcincta,* and the remaining absent species were then added in increasing numbers. 0, 1, 5 and 10 for *C. ovina* and 0, 1, 3 and 6 for *B. trigonocephalum*. The numbers of spiked larvae were determined by the very low availability of *C. ovina* and *B. trigonocephalum,* and the lowest possible level of detection was of interest. In order to run a test that included all five species, an additional pool of 200 larvae was made up of equal proportions of *T. vitrinus, T. axei*, *T. circumcincta.* and *C. ovina,* which was spiked with two *B. trigonocephalum* (Table 4. 2). Larvae were fixed with 70%, stored at -20°C prior to sequencing (Table 4. 2).

4. 2. 3. Repeatability of amplicon sequencing – field isolates

Having validated this assay on artificially created pools of larvae, the assay was then tested on field samples (i.e. samples collected on St Kilda for Chapter 5). Within-sequencing repeatability was tested on aliquots of 500 L_3 from 6 randomly-selected samples from the 2015 collection on St Kilda (described in Chapter 5) and each sample sequenced in triplicate. Because method-efficiency was being tested, each field sample was given a number (1-6), as the month/ test group was not investigated in this chapter.

4. 2. 4. DNA extraction, library preparation and deep amplicon sequencing

Lysate preparation, DNA extraction, DNA amplification and purification, master sequencing library preparation and Illumina sequencing followed the protocols outlined in Chapter 3. A DNA lysate was made for each of the mock pools (Table 4. 2). The Correction Factor pool (section 4. 2. 2. B) and 6 field sample (section 4. 2. 3.) lysates were sequenced in triplicate. Barcoded primers are listed in Table A4. 1 (Appendix).

A 125ng of PCR product was pooled per sample into a final master sequencing library (Figure 3. 2. Chapter 3). A 1:2 dilution with DNA/RNA-free water (ThermoFisher Scientific, USA) was made with the completed library and then sent to Edinburgh Genomics (http://genomics.ed.ac.uk/) to be sequenced by Illumina MiSeq (v2 250 pair-end read (PE)). Initial quality control of the library was done by Edinburgh Genomics (Qbit, Bioanalyser and qPCR) (Figure A4. 4, Appendix). The library was only sequenced on satisfactory quality control results as advised by Edinburgh Genomics.

4. 2. 5. Bioinformatics

FASTQ files were created with no additional analysis, and sent from Edinburgh Genomics via Aspera software (Aspera Inc., IBM, USA). The FASTQ files were then analysed by Mothur by the parameters described in Chapter 3. Corrected raw reads were then transferred to Excel (Microsoft, USA).

4. 2. 6. Analysis

Species proportions (%) were calculated by: Number of reads for the species/ Total number of reads in the sample) x 100. If species resulted in a proportion <0.05% it was removed, the total number of reads for the sample were adapted by removing the low-burden species reads, and the percentages for the other species were recalculated. Species with a prevalence below <0.05% relates to fewer than one worm per sample, with these results probably relating to either contamination or misidentification of reads (Avramenko et al., 2015).

4. 2. 6. A. Calculating correction factor

The CF, as previously described in Avramenko et al (2015), was calculated for each species by dividing the true species proportion (based on the proportion of counted larvae in the Correction Factor Pool (section 4. 2. 2. B)), each L_3 from five species make up a proportion of 20%) by the percentage proportion of Illumina sequence reads (% observed), calculated from the mean number of reads from the 3 lysates run in triplicate. The correction factor was then multiplied to the number of sequencing reads prior to calculating species proportions (%).

Correction factor (CF) = %Actual/ %Observed

A non-parametric Chi-square test calculated the differences between the actual species proportion (20%) and observed (or sequenced) proportions before and after the application of the correction factor to see whether there was a statistical difference for each species. Corrections, species proportions and all charts were calculated in Microsoft Excel (Microsoft Corporation, USA).

4. 3. RESULTS

4. 3. 1. Confirming pure larvae cultures

10 L_3 of each of the four species (*C. ovina, T. circumcincta, T. axei* and *T. vitrinus*) were sequenced in separate pools to confirm whether the larvae used to make the mock populations outlined in Table 4. 2. were not contaminated with other nematode species. *B. trigonocephalum* was sequenced in a separate run (see section 4. 2. 2. A.) and were identified correctly.

The *T. axei* monocultures were contaminated with *T. circumcincta* (54% and 46%, respectively) (Figure 4. 2). There is no contamination in the negative control for the ITS-2 PCR (Appendix, Figure A4. 1), indicating a method error, rather than sequencing contamination. The underestimation of *T. axei* in the uncorrected mock population (Appendix, Figure A4. 2) would indicate larval contamination, rather than an assay error.

T. axei reads were removed from the subsequent datasets described in this chapter (CF and DTh). The larvae originated from the same stock culture that made the other mock pools presented in this chapter (Table 4. 2), and it cannot be confirmed where the source of contamination originated from; either occurring from the stock culture itself or when the larvae were pipetted into the mock pools.

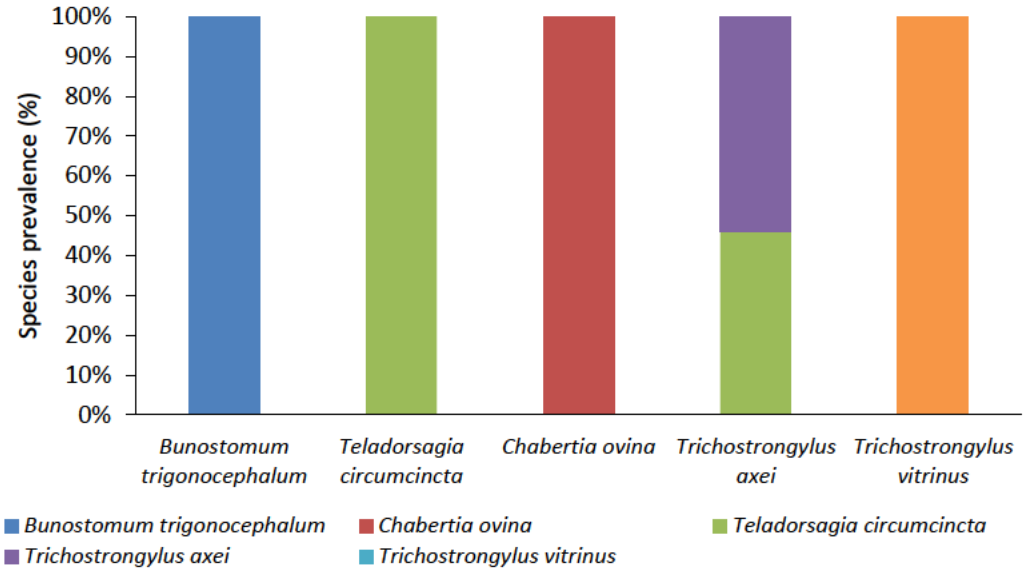


Figure 4. 2. Pools of 10 individual L_3 were sequenced in order to test whether the starting cultures were not contaminated with other nematode species. *T. axei* was not a pure culture. *B. trigonocephalum* sequenced on a separate run.

4. 3. 2. Correction factor

Due to the contamination of *T. axei* with *T. circumcincta* (Figure 4. 2), the *T. axei* reads were removed from the analysis of the CF (data including *T. axei* presented in Figure A4. 2.).

To assess the quantitative accuracy of the assay, in addition to determining any sequence representation bias for the different species, a mock population was made where each species represented 25% of the total population (i.e. equal proportions of 4 species – excluding *T. axei*) (Figure 4. 3). Each species was either underrepresented or overrepresented by the actual number of sequences generated in comparison to the true proportions within the mock pool

(i.e. 25%). *B. trigonocephalum*, *T. circumcincta* and *T. vitrinus* overrepresented similarly by 2-5%, whilst there was an underrepresentation of *C. ovina* of 12%. However, the biases are consistent between the triplicates, allowing for a Correction Factor to be calculated: *B. trigonocephalum* = 0.865676644; *C. ovina* = 2.04467986; *T. circumcincta* = 0.840703051; *T. vitrinus* = 0.857427396.

Before the application of the correction factor (Figure 4. 3. A) there was a statistical difference between the four species (p = 0.026, p= 0.039 and p = 0.038), despite the same starting proportion (25%). By applying the CF (section 4. 2. 6. A), it removed the statistical differences between the four species (Figure 4. 3. B) (p = 0.995, p = 0.986, p = 0.998).

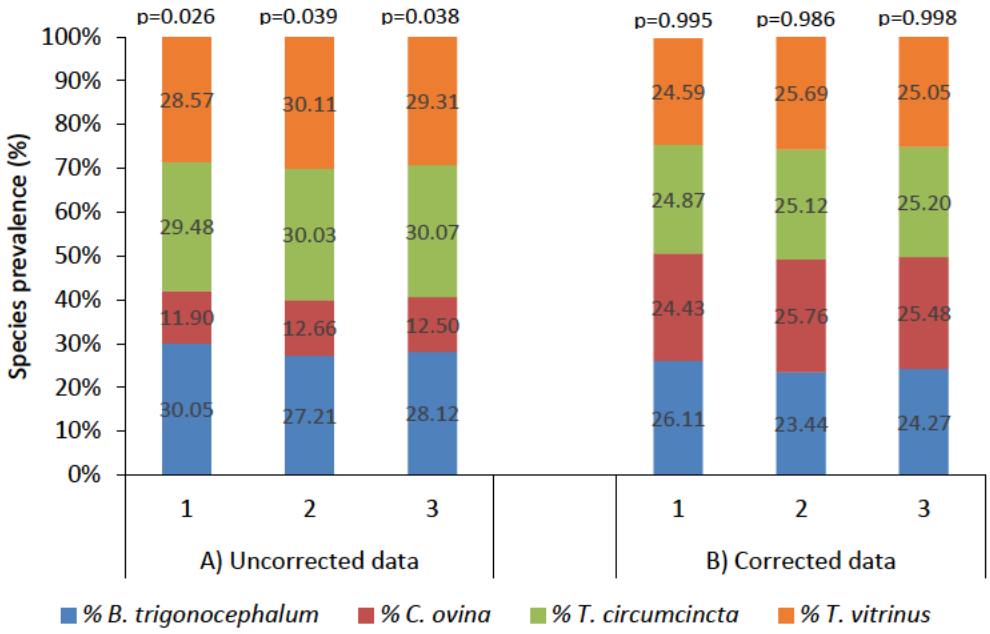
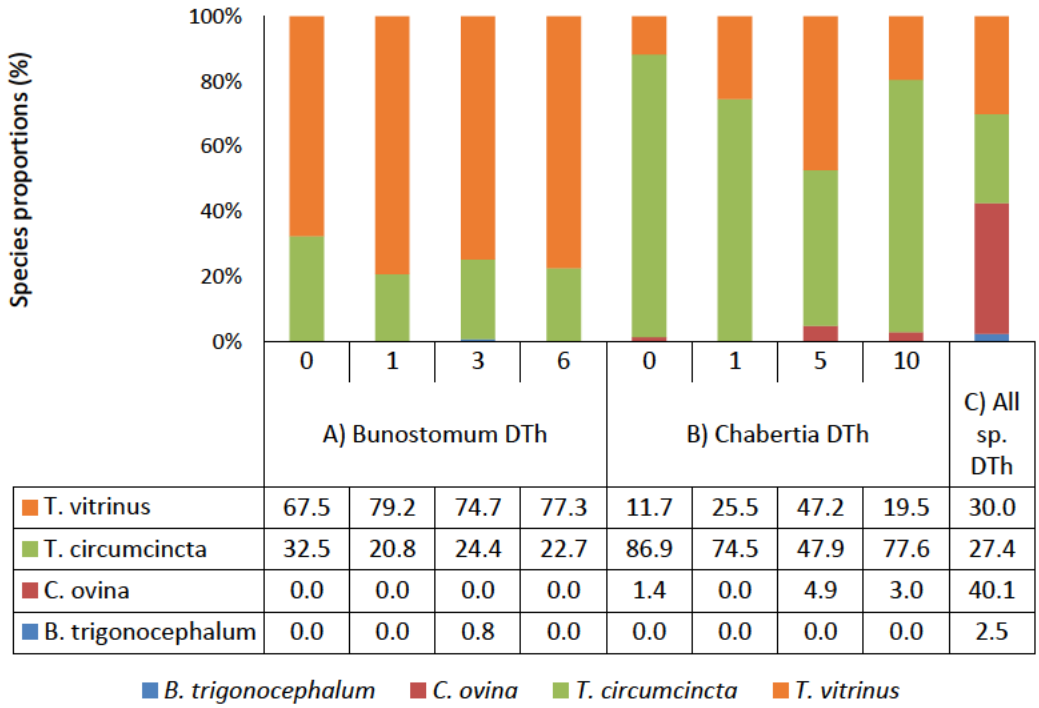


Figure 4. 3. Assessing sequence representation bias and the determination of the species correction factors for the amplicon sequencing assay. Three replicates of each sample were analysed (1-3). A) The chart shows the species proportions as determined by the actual number of ITS-2 rDNA sequences generated by the amplicon sequencing assay. B) The chart shows the same data as in graph (A) but following the application of a correction factor for each species to the sequence number. Numbers in both graphs are the percentage proportions of each species within the mock pool. The p-values above each column indicate whether the species proportions, as determined by the uncorrected (A) and corrected (B) numbers of ITS-2 rNDA sequences generated, are statistically different from the actual proportions of larvae in the pool (25%).

4. 3. 3. Detection threshold of *Bunostomum trigonocephalum* and *Chabertia ovina*

The starting mock population of 167 *T. vitrinus* and 167 *T. circumcincta* L_3 resulted in large differences across the samples, when the two species should have sequenced consistently in equal proportions (Figure 4. 4). Low level *B. trigonocephalum* was identified (3 L_3 = 0.8%) but was not consistent, as it was not identified with double the number of larvae (6 L_3). 5 (4.9%) and 10 (3%) *C. ovina* larvae were identified in the mixed-species pool. However, *C. ovina* was also identified in the negative (i.e. 0 *C. ovina*) sample (1.4%). This is most likely due to not including a read analysis step during sequencing analysis, whereby samples with low-level reads are removed (discussed in section 4.4.4). The detection of low-level infection of *B. trigonocephalum* and *C. ovina* in mixed species pools was attempted, however, from this data we can not reliably detect low infection levels and further work is required.



	A) Bunostomum DTh				B) Chabertia DTh				C) All sp. DTh
	0	1	3	6	0	1	5	10	
T. vitrinus	67.5	79.2	74.7	77.3	11.7	25.5	47.2	19.5	30.0
T. circumcincta	32.5	20.8	24.4	22.7	86.9	74.5	47.9	77.6	27.4
C. ovina	0.0	0.0	0.0	0.0	1.4	0.0	4.9	3.0	40.1
B. trigonocephalum	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0	2.5

Figure 4. 4. Detection threshold (DTh) tests for A) *B. trigonocephalum*, B*) C. ovina* and C) All species, with the actual species proportions for each stacked bar chart tabled below. A) Pools made up of equal proportions of *T. circumcincta* and *T. vitrinus* were then spiked with 0, 1, 3 and 6 *B. trigonocephalum* larvae. B) Pools made up of equal proportions of *T. circumcincta* and *T. vitrinus* were then spiked with 0, 1, 5 and 10 C. ovina L_3. C) Equal proportions of *T. circumcincta, T. vitrinus* and *C. ovina* were spiked with 2 *B. trigonocephalum* L_3. For all 3 datasets, T. axei was removed from the analysis.

4. 3. 4. Repeatability of amplicon sequencing

Having ran the nemabiome assay on artificially created pools of larvae (Figure 4. 3 and 4. 4.), the assay was then tested on field samples. There was a high level of reproducibility for the three technical replicates of each DNA lysate, with the range for each species being typically within 1-6%. There was an exception with Field 6, replicate 2 and 3, that identified low-level *B. trigonocephalum* in the population (1.24% and 0.17% respectively), but not identified in replicate 1. Additionally, *C. ovina* is being over-amplified in replicate 1/ Field 4 (35.98%, 16.51% and 15.69% respectively). This doesn't change with the application of the correction factor, as the changes to the species quantified are proportional. This demonstrates that preforming replicates of the same sample, and taking a mean average of reads, should minimise any difference in representation produced by the assay.

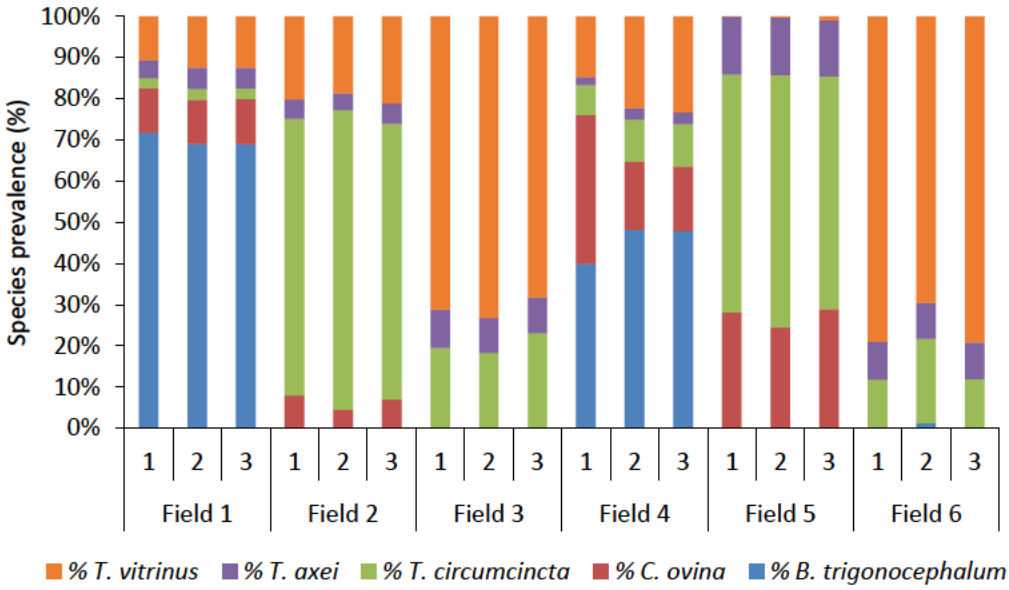


Figure 4. 5. Testing within-run repeatability of the assay on triplicated DNA lysates from 6 field isolates (~500 mixed-species L_3).

4. 4. DISCUSSION

Simultaneous infection with multiple nematode parasite species is commonly seen in feral and managed ruminants. Whilst the nemabiome method was based on microbiome sequencing, the advantage of working with nematode species is it is easier to test for, and correct, biases within the sequencing data. The aim of this chapter was to validate the deep amplicon sequencing assay for the accurate identification and quantification of *Bunostomum trigonocephalum*, *Chabertia ovina*, *Teladorsagia circumcincta*, *Trichostrongylus axei* and *Trichostrongylus vitrinus* in mixed-infection pools of 3rd-stage larvae. A CF was calculated for *B. trigonocephalum, C.*

ovina, T. circumcincta and *T. vitrinus*, to account for potential species-specific biases whilst sequencing, which can be applied to the field data presented in Chapter 5. The detection threshold was tested for low-level presences of *C. ovina* and *B. trigonocephalum*. Within run sequencing was highly repeatable between technical replicates of artificially made populations and field-collected populations.

4. 4. 1. Contamination of cultures

To ensure 100% accuracy when creating mock populations, 10 larvae of each species were sequenced separately. There was a cross-contamination of *T. axei* with *T. circumcincta* (Figure 4. 2) in the mock populations, with almost 50:50 proportion of both species. This is probably due to a technical error rather than an issue with the sequencing technique itself. Due to there being no contamination in the negative control for the PCR (Appendix, Figure A4. 1), and the wells were not located next to one another in the 96-well plate, the cross-contamination of *T. axei* and *T. circumcincta* is most likely due to either pipetting error during the mock population set-up, or a contamination in the laboratory-passed isolates, rather than sequencing contamination. The underestimation of *T. axei* in the uncorrected mock population (Appendix, Figure A4. 2) would, again, indicate a larvae error, rather than a sequencing error. It could be argued that *T. axei* under-represents in the sequencing assay, but other validation studies calculated the CF for *T. axei* to be 1.296071 (Avramenko et al., 2015) and 1.278952 (Redman et al., 2019) rather than 3.137827 in this study. The detection of trace amplicon contamination may have occurred; either from handling the material (cross-well contamination) or during the first PCR amplification. However, this trace contamination would not affect the interpretation of species proportion because the number of sequences corresponding to those generated from a single L_3 is far higher than that of minor contamination. As found by Avramenko et al., (2015), the number of reads never exceeded 0.05% of the total read count for a species when it was absent from a pool. It has been well-established that accurate and repeatable morphological identification of *Teladorsagia* and *Trichostrongylus* species is difficult due to many overlapping characteristics, including similarities in shape and size of the tail sheath extension. When exsheathed, the L_3 of *T. axei* has a characteristic smooth tail, similar to other ovine *Teladorsagia* species. *T. axei* and *T. circumcincta* do differ in total mean length (720 µm and 820 µm, respectively), however, determining species identity of L_3 by total length is considered an inaccurate method as length is dependent on culturing conditions (moisture and temperature) (Van Wyk and Mayhew, 2013). For the validation work, time and larvae availability were very much limiting factors and whilst every care was taken to limit contamination, additional tests could not be run as the sequencing could not be repeated. In future studies, it would be ideal to

run preliminary ITS-2 PCRs to ensure a pure monoculture of each species before setting up mock populations.

4. 4. 2. Correction Factor (CF)

Due to the cross-contamination, a CF for *T. axei* could not be established. The CF calculated by Redman et al., (2019) for *T. axei* will be used (1.278952). Avramenko et al., (2015) tested whether adapting additional factors, such as number of amplification cycles, would affect the proportional representation of the species. It did not, and this consistency and flexibility of the assay ensures that using a previously validated correction factor from a different study should not be detrimental to the results, providing the methodology was kept consistent. Recently, CFs were calculated for ovine helminth species (Redman et al., 2019) and are similar to that of this study, demonstrating the flexibility of this methodology between laboratories and parasitic isolates; Redman et al., (2019) - *C. ovina* = 2.499072, *T. circumcincta* = 0.837043, *T. vitrinus* = 0.810258, and this thesis - *C. ovina* = 2.04467, *T. circumcincta* = 0.840703, *T. vitrinus* = 0.857427. *B. trigonocephalum* (0.865676) is a new species to add to the ovine helminths list. Redman et al., (2019) has recently furthered this validation, by comparing corrected independent field samples to 'known' proportions (as determined by individual PCR of 90 larvae), and found no statistical difference between the two methods.

4. 4. 3. Detection threshold

For this series of work, the results for the detection threshold of *B. trigonocephalum* and *C. ovina* are inconclusive, and would require further investigation. Individual larvae were pooled into a mock population of known proportions, however, these pools did not result in the expected proportions (i.e. equal amounts, spiked with low levels of either *B. trigonocephalum* or *C. ovina*). Rather than take aliquots from cultures to form these pools, individual larvae were picked from a plate and placed into the Eppendorf. This is where the error may have occurred. In retrospect, a hair or thin needle to pick the larvae (as described by Bisset et al., 2014) would have been more accurate than a pipette tip as L_3 can stick to the inside of the pipette (especially when pipetting such small volumes of water).

Avramenko et al (2015) was able to establish the diagnostic sensitivity of this method with other strongyle species, but in our hands the method is not useful for detection at extremely low burdens (0.2 – 1.2% of the culture, 1 – 6 L_3 in a pool of 500 L_3), but was able to detect 10 L3 in a pool of 500 (2%), which is probably representative of 1-2 adult female worm/s. Whilst this level of sensitivity is important in anthelmintic drench tests, it is not required for the descriptive work presented in Chapter 5.

4. 4. 4. Technical discussion

Additional laboratory factors, such as larval collection/ storage, DNA extraction method, amplification efficiency and sequencing repeatability can alter the proportional results.

The majority of the samples collected for this thesis were collected off-pasture. As a consequence, many cultures were contaminated with free-living nematodes which are picked up from the environment (vegetation and soil). If the sample is retrieved quickly after voiding, free-living nematodes are unavoidable. Even rectally-collected faecal samples can become contaminated from the animal's tail (*Tania Waghorn, private comm*.). For other molecular methods of larval identification, such as picking out individual L_3 to PCR, the free-living larvae pose little importance. However, for methods that require pools of larvae straight from culture, there will be free-living nematodes. It was noted that some of the field samples collected during the course of the project had almost 50:50 composition of L_3 to free-living nematodes. Free-living nematodes will not be amplified by the NC1/NC2 primers during the first PCR, though the effect of their DNA at high levels within a lysate during that first amplification PCR is not known, and would be worthy of investigation. Another negative result of many free-living nematodes was it made the identification of the L_3 time consuming, especially when the L_3 were at low-levels within the culture. Free-living nematodes can be removed by drying the larvae on filter paper, which kills the free-living nematodes. The L_3 can then be rehydrated, and usually survive the method (MAFF, 1986), and could be used in future studies.

In this study, larvae were not fixed in iodine, however, the standard method of preparing L_3 for morphological identification is to 'fix' them in helminthological iodine. Fixing larvae using helminological iodine aids in identification, as it kills the larvae (allowing for inspection at higher magnifications), and would have made the identification of *B. trigonocephalum* easier, quicker and potentially more accurate (especially in future cases when large numbers of L_3 are required). However, further validation is needed to ensure that helminological iodine does not affect DNA extraction and amplification for this assay. It most likely doesn't, but in order for the CF to work accurately, samples need to be processed consistently, and any deviation from the method needs to be validated. Other studies using PCR to speciate L_3 have undergone this validation and found that this additional step of fixing L_3 in helminological iodine does not affect amplification (*Tania Waghorn, private comm*.)

As established by Avramenko et al., (2015), providing the methods of DNA extraction, amplification and sequencing are kept consistent, any errors of under/over estimation of any of the species can be accounted for. Many studies that sequence L_3 favour an initial mechanical

step during DNA extraction, such as a bead breakdown (AusDiagnostics™, Chapter 3). The nemabiome method relies on rapid temperature change (freeze-thawing), in order to break the protective cuticle. For some species this may not be enough to fully release the gut contents for digestion. Exact reasons for species differences are unknown and would require further investigation.

There is very good within-run repeatability between technical replicates of DNA lysates, which is consistent with other studies (Avramenko et al., 2015), and gives some indication that well location (within the 96 well plate) during PCR amplification does not ultimately affect the proportional results. Standard measures of correlation assume the data are independent, which is not true of proportional (%) data, therefore the presentation of the technical repeatability within this chapter remains descriptive. Investigating between-run repeatability with the same lysate would be the next logical step, to ensure not just repeatability within the sequencing run, but between sequencing runs. Avramenko et al., (2015) tested biological replication (i.e. multiple pools of the same culture) and found no significant difference in species proportion, which is an important observation, as a fully homogeneous culture is not possible when pipetting out aliquots from a bulk culture of L_3. An additional test could be to test the amplification efficiency at low diversity (such as these Soay sheep samples) and high diversity (Avramenko et al., 2018 Bison samples), to see whether the number and type of strongyle species present has any effect on the amplification efficiency.

C. ovina, appears to be underestimated in the assay, whilst the other three species presented quite similarly with a slight overestimation (Figure 4. 3. A). By applying the CF for each species, the variations can be normalised (Figure 4. 3. B). Differences in amplification efficiency may be due to sequence variation, which may be expected as amplification efficiency depends highly on the secondary structure and GC content of the sequence target. For some species in this study, there is sequence variation in the ITS-2 rDNA (Avramenko et al., 2015). However, if this was a big influence, the biases would be emphasised with increased amplification cycles of the first PCR, however, Avramenko et al., (2015) did not find this.

The number of reads generated per species, per sample, will be variable between runs and cannot (currently) be corrected for. Using number of reads as a parameter for analysing data would potentially bias speciation at low burdens when the overall total read number is low. This threshold for 'low' species levels will change depending on the total number of reads generated for each sample. Proportion of total reads assigned to each species, in comparison to total read numbers, is repeatable between replicates/ triplicates within the same run; as the number of reads may change, the change is proportional (Appendix, Figure A4. 3.). Care needs to be taken

that determination of species identity at low burdens is not biased when the total read number for the sequencing is low. Avramenko et al., (2015) included a parameter during analysis that removed species and/or samples that resulted in 2000 reads or less. I chose not to do this for the work presented in this thesis, and chose to remove species with a prevalence < 0.05%. Due to low DNA yield for some of the samples in Chapter 4 (most likely due to small numbers of larvae). However, as a result, some species may occur at low levels in the results due to these low-level reads not being removed (*C. ovina* – Figure 4. 4).

This method requires consistency if proportions are to be compared to one another, and for validation parameters to be set; from collection, storage, amplification, sequencing to the bioinformatic parameters. This is in line with many of the methods used in this thesis, including the traditional parasitological techniques. Error is inherent in every method, but if the methods are kept consistent and methodical, this becomes a consistent error which can be accounted for and is the same for every sample collected and processed.

The main aim of this chapter is to validate the nemabiome deep-amplicon sequencing assay for the ovine helminth species identified in the Soay sheep of St Kilda. The assay has good within-run repeatability, and has the potential to identify L_3 when present at low levels within the mixed-species cultures. Additionally, a correction factor can be now applied to the field data presented in Chapter 5, which reduces species representation bias within the sequencing assay.

CHAPTER 5

Strongyle nematodes in feral Soay sheep – an epidemiological snapshot

'…the unique breed had lived on Soay, untended and undomesticated since time immemorial.'

Tom Steel
The life and death of St Kilda

5. 2. ABSTRACT

The Soay sheep on St Kilda have ancient origins, dating back to animals first being left on the island of Soay, up to 3,000 years ago. The population has since been rigorously isolated, providing a unique resource for the study of gastrointestinal nematode parasites in unmanaged, feral animals. Seasonal patterns of gastrointestinal nematode parasitism have been investigated, supporting conventional faecal egg counts by post-mortem worm identification and pasture larval counting methods; albeit these methods may not represent the situation in live animals. Understanding co-infection is important in gaining an insight into parasite transmission, host-parasite interactions, and their impact on host survival.

Between April 2015 and March 2016, a total of 918 individual on-pasture faecal samples (607 female and 311 male) were collected, from lambs (4 to 12 months-old), yearlings (13 to 24 months-old), adults (3 to 7 years-old), and geriatric females (8 to 14 years-old). Individual strongyle/ *Nematodirus* faecal egg counts were conducted by a cuvette salt floatation method (validated and analysed in Chapter 2). The faeces were then pooled into composite coprocultures (per sex/age/season) and incubated to grow third stage larvae (L_3). Pools of 500 L_3 were speciated and quantified into relative proportions by next generation sequencing (Illumina MiSeq) using the nemabiome assay. The reads were corrected by previously calculated correction factors to account for potential species-specific bias in amplification (as described in Chapter 4).

Only *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus vitrinus, Chabertia ovina* and *Bunostomum trigonocephalum* were identified, presumably reflecting those species that were originally present on the island of Soay, and survived movement of their hosts onto Hirta in 1932. There were seasonal, age and sex differences in species composition; trends appeared to correspond with the dynamic life-history of the sheep, including a spring peri-parturient rise in pregnant females of *C. ovina* and *T. vitrinus* (yearlings) and *T. circumcincta* and *T. vitrinus* (adults).

With the exception of *B. trigonocephalum*, each of the nematode species identified had a year-round prevalence in lambs to the age of one year old. Subsequent trends showed a sequential rise and decline in different species. In the yearling and adult female and male sheep: *T. vitrinus* predominated during the spring; *B. trigonocephalum* predominated during the late spring and early winter; *C. ovina* predominated during the late summer and early winter; and *T. circumcincta* predominated during the winter. While *B. trigonocephalum* had a strong association with spring/summer, it was also identified at low levels in February. This trend then

deviated when the females were older than 8 years, with high levels of *T. circumcincta* during the summer and winter. There appeared to be no clear seasonality to *T. axei* in comparison to the other species; it persisted, at low levels, throughout all months sampled in all age groups for both sexes.

When relative species proportions were adapted to account for average FEC, the trends seen were minimised. The adequate representation for individual nematode species appears to rely on a high faecal egg count; and when the egg count is low, for example, in yearling and adult females during the summer, autumn and winter, the impact of the trends seen was minimised. Further work is required to explore alternative methods to analyse and present speciation data.

This is the first study to use the nemabiome assay (validated in chapters 2, 3 and 4) to accurately characterise host gastrointestinal nematode burdens and study seasonal patterns in different co-infecting gastrointestinal nematodes in feral sheep. The study highlights epidemiological trends in the Soay sheep that were not previously identified using conventional parasitological methods, and highlights the flexibility of the nemabiome assay as a viable non-invasive tool for parasitological surveys of wild animals.

5. 2. INTRODUCTION

The Soay sheep (*Ovis aries*) of St Kilda are believed to be one of the most primitive breeds of domestic sheep in Europe. Restricted to the small island of Soay (~99 ha) since the Bronze Age, 107 sheep were relocated to Hirta in 1932, following the 1930 evacuation of all humans, livestock (cattle and sheep) and domestic animals. From this focal population, numbers increased rapidly, and by 1952 the total island population reached 1,114 (Paterson et al., 1998; Gulland and Fox, 1992). The population was estimated to be 1,401 in 2018.

Infection with multiple gastrointestinal nematode (GIN) parasite species is commonly seen in both wild and managed ruminants, and the Soay sheep on the island of Hirta in the St Kilda archipelago are host to a diverse parasitic population comprised of macroparasites (nematodes, cestodes and arthropod species) and microparasites (protozoa species) (Table 1. 1. Chapter 1) (Clutton-Brock and Pemberton, 2004). However, some nematode species (*Haemonchus contortus, Cooperia sp*., and *Oesophagostomum sp*), which are commonly seen in sheep flocks across the United Kingdom and are considered economically important, are notably absent from St Kilda.

Hosts that have been introduced to a new ecosystem tend to have a lower species diversity of parasites in comparison to endemic hosts (Aivelo[b] et al., 2018), which suggests that once introduced, one (or more) of their original infecting parasite species may be lost (Aivelo et al., 2018[b]; MacLeod et al., 2010). This is potentially due to many factors, including the size and demographic of the relocated population, and environmental factors such as climate and presence/absence of intermediate hosts, which would impact parasite transmission and survival. Introduced populations are generally made up of a very small subset of the original native population, thus reducing the probability of introducing certain parasite species. This is especially true for species that are more commonly associated with a particular age or sex (Torchin et al., 2003). In the context of the Soay sheep, the parasite species present would have been influenced by the movement of sheep from Soay to Hirta, or from their original founding population when they were left on Soay more than three thousand years ago. The transfer of parasite species would have been dependent on the original species present, the number of sheep moved, the sex and age groups of the founding population, and the time of year. Additionally, the introduced parasites needed to be able to survive the harsh, unsheltered environment of St Kilda. Little is known about the true origins of the Soay sheep on the island of Soay, therefore not much is known about the original parasites. There is evidence to suggest that the Soay-type of sheep (i.e. European short-tailed sheep) was widespread in Britain during the prehistoric times, for example, Neolithic bones of a Soay-type being similar to that of

modern Soay sheep were excavated at Skara Brae (Orkney, Scotland), which was a vibrant community some 3,000 years ago. There is some anecdotal evidence that the Soay sheep were brought to Soay by the Vikings, and sheep skeletons recovered from settlements around Greenland were similar to that of Lille Dimon sheep (Faroe Islands, Denmark) (Clutton-Borck and Pemberton, 2004; Ryder, 1981). With no other mammalian species on Soay, and restricted access to the island, it can be assumed that once established, the sheep parasite diversity remained relatively constant.

The original Hirta founding population of 107 sheep was made up of 20 rams, 22 castrated ram lambs, 44 ewes and 21 ewe lambs. Access to the island of Soay is restricted, so an accurate whole-island census has not been possible. In 1697 500 sheep were estimated, however, a more recent estimate (from the sea) was 360 individuals. Similar to Hirta, the Soay sheep population on Soay has been observed to fluctuate. The sheep removed from Soay could have been an estimated 21-30% of the total island population (Clutton-Brock and Pemberton, 2004). As discussed in Chapter 2, parasites generally are aggregated over the host population; many individuals having a low worm burden whilst a few having very high burdens. By moving a subpopulation of sheep from Soay, there is the risk that not all the parasite species were moved onto Hirta. Additionally, younger animals (generally) have a higher faecal GI nematode egg count (FEC), which is required to propagate the parasite species into the environment. If the sheep were transferred to Hirta during a time in the year when certain species are residing in the hosts at very low levels, once moved to clean pastures, there may not be enough of the founding parasite population to establish in a complex community of other infecting species. There may be competition between different species. For example, *H. contortus* infection elevates the pH of the hosts abomasum, by inhibiting gastric acid production, which creates an environment unfavourable to other abomasal parasites (Li et al., 2016). Alternatively, if the parasite species has a complex life cycle, it may potentially limit the establishment of the introduced parasites. If the host/s for the intermediate stages are not present the parasite cannot establish, which may explain why species such as *Fasciola hepatica,* that requires the intermediate mud snail *Galba truncatula*, are not present. No parasitological work has been conducted on the Soay sheep population of Soay, due to the island being difficult to access; so the parasite burden of the original founding population of Hirta is unknown.

A previous study investigating heminth diversity in an unmanaged Soay flock on mainland Scotland identified 14 species of Strongyle nematodes, some of which are thought to have originated from rabbits and deer (Sinclair et al., 2016). Apart from visiting humans and the St Kilda field mouse *(Apodemus sylvaticus hirtensis),* there are no other mammalian species on

Hirta or Soay, and no other grazing ruminants in which to share infecting species and contribute to the helminth parasite diversity in the Hirta Soay sheep population. This trend of isolated populations harbouring fewer parasite species is also seen in the Arctic Svalbard reindeer, with just two helminth species (Stien et al., 2002), in comparison to multiple species in farmed sheep (Taylor et al., 2012). The Svalbard reindeer are non-migratory and exist under high population densities, and extreme environmental conditions (Irvine et al., 1999). Whilst they may harbour fewer infecting species, hosts from isolated island habitats have significantly higher parasite loads and greater prevalence of parasitism, resulting from high host population density and low host genetic variability (Fornberg, 2017). By comparison, the geographically restricted Soay sheep population on Hirta maintain genetic diversity through density-associated mortality (Clutton-Brock and Pemberton, 2004).

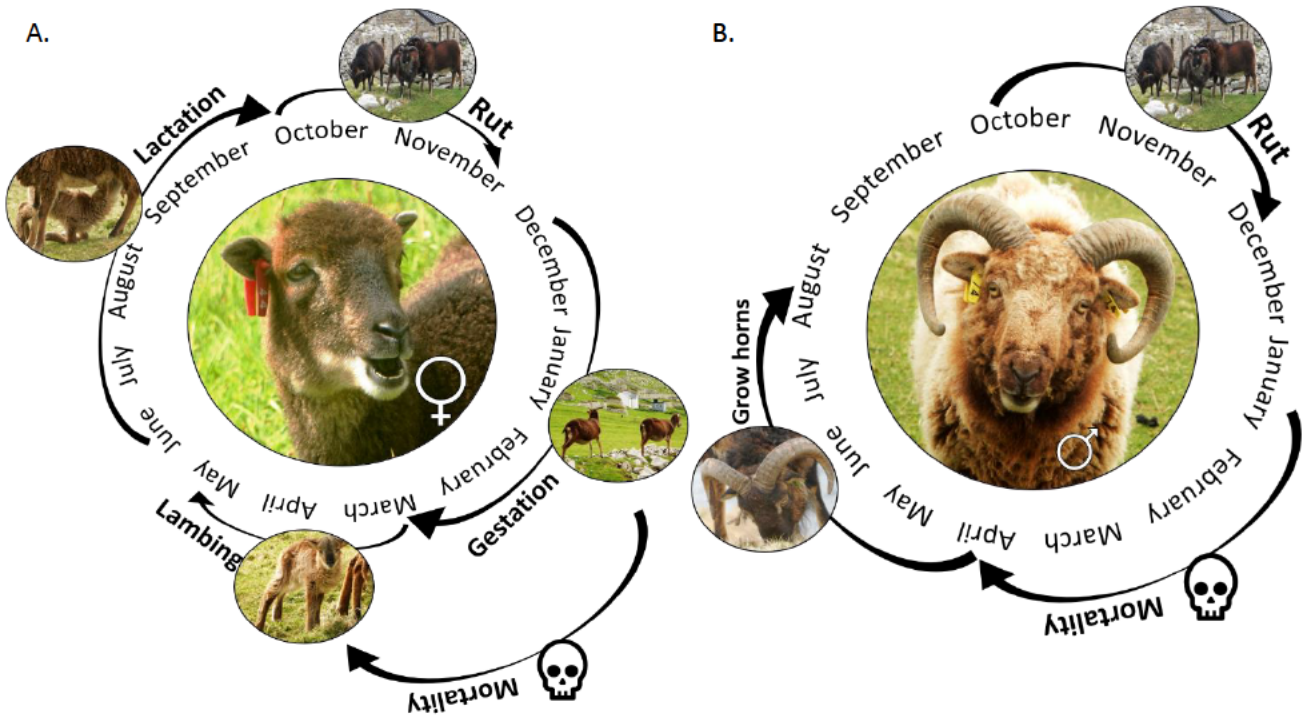


Figure 5. 1. Stylised life-history for Soay sheep.

A. **Female** Soay sheep; December – March: Gestation, March – May: lambing, June – October: Lactation, October – November: Rut, late January – April: period of high mortality.

B. **Male** Soay sheep; late January – April: period of high mortality, April – August: horn growth, October – November: Rut.

The Soay population dynamic on Hirta is highly unstable, with yearly fluctuations in population size. This has been attributed to the complex interactions of a number of factors including population density and demographic structure, parasitic burden, winter weather and food availability (Hayward *et al*., 2009; Clutton-Brock *et al*., 1996; Craig *et al*., 2006; Gulland and Fox, 1992). Soay females are able to reproduce in their first year (Figure 5. 1. A) and can potentially lamb every year. Therefore, the population dynamic is over-compensatory as it reaches and exceeds carrying capacity; subjecting the population to high mortality when combined with parasite burden, adverse weather and poor food availability. During these 'crash' years, the total death-toll (mortality) has been as high as 69%; this is highest in adult males and lambs, reaching up to 86% and 95% respectively (Clutton-Brock et al., 1991/1992). To examine the impact of gastrointestinal parasitism during population crashes, a few small-scale anthelmintic bolus and drench treatment experiments have been conducted (Gulland, 1992; Gulland et al., 1993; Jones et al., 2006; Craig et al., 2009). They demonstrated reduced overwinter mortality in animals treated with anthelmintic, and that parasite susceptibility is both sex and age dependent. Hence, infections with low numbers of GI nematodes is predictive of survival in young and adult male animals. Subsequent studies have shown a strong selection for individuals that are most genetically resilient to the effect of parasitism throughout the year (Gulland, 1992; Hayward et al., 2009).

It was initially presumed that *Teladorsagia* sp. (originally described as *T. circumcincta, T. trifurata* and *T. davtiani*) was the predominating nematode species in all age classes of Soay sheep of Hirta. However, a large-scale necropsy study (Craig, 2005) and molecular PCR speciation of nematode eggs in faeces (Wimmer et al., 2004) revealed a more complex situation, with not one nematode species predominating through the life of the Soay sheep. *T. circumcincta, Trichostrongylus axei* and *Trichostrongylus vitrinus* were the most prevalent species in the Soay sheep, which is similar to the study in of mainland hill sheep flocks in northern UK (Craig et al., 2006). *T. circumcincta* was the most predominant species in the older Soay sheep age classes, comprising up to 75% of the GI burden. This species appears to persist throughout the life of the sheep hosts, as it is the only parasite recorded to have 100% prevalence in adults more than 2 years old (Craig, 2005). *T. axei* and *T. vitrinus* predominated in younger sheep, with higher burdens in males than females (Craig et al., 2005; Craig et al., 2006).

On a farm, the diversity of infecting species is controlled by management including the use of anthelmintic drugs, pasture management and grazing strategies. These practices may favour GI nematode species that have short prepatent periods and can survive well in the environment (Budischak et al., 2016). In managed sheep flocks it is common to find a lower species diversity

in comparison to the diverse co-infections ubiquitous in wildlife. It is common to identify fewer than four GI nematode species in mainland flocks, with seasonal predominance of one or two (Burgess et al., 2012), resulting in clinical disease relating to these mono-infections (i.e. teladorsagiosis, trichostrongylosis) (Sargison et al., 2007).

There are species present on Hirta that are now considered to be uncommon in managed sheep populations on mainland Britain. *Nematodirus helvetianus, Bunostomum trigonocephalum* and *Chabertia ovina* are rare in mainland UK sheep due to their susceptibility to frequent anthelmintic treatments (Rehbein et al., 2000; Braisher et al., 2004). *B. trigonocephalum* feeds on host blood and had originally been noted to be an economically-important parasite in hill country sheep in New Zealand (Graham, 1969), Scotland (Morgan et al., 1951; Wilson et al., 1953; Parnell et al., 1954) and Russia (Sarimsakov, 1959). It is assumed that the advent of broad-spectrum anthelmintic drug use, starting with the benzimidazole class, lead to the reduction in the prevalence of *B. trigonocephalum* and *C. ovina.* On the mainland, *N. helvetianus* was commonly found in cattle, but known to infect sheep in small numbers under natural conditions (Rehbein et al., 2000). However, it is now rarely seen in the UK, presumably due to anthelmintic treatments and management strategies aimed at controlling *Ostertagia ostertagi* in cattle (Sargison et al., 2010). *Nematodirus* spp. eggs are adapted for long survival in the environment (Table 5. 1), therefore, the cattle-species may be a remnant from the previous inhabitants of Hirta, who kept dairy cattle. The presence of *N. helvetianus* (Wilson et al., 2004) was not confirmed by the in-depth parasitological study by Craig (2005), this may have been because it was in such low levels within the naturally-deceased animals to be detected, or it was originally misidentified.

Seasonal patterns of parasitism in the St Kilda Soay sheep have previously been measured by strongyle FEC or by post-mortem of naturally-deceased animals. Seasonal variations in FEC are determined by temporal patterns in infective larvae (L_3) stages on pasture and survival, and egg shedding by adult stages which is influenced by the immunological status of the host sheep. Pastures become contaminated if there are eggs present, but only become infective if the eggs have successfully developed to L_3 and there are suitable climatic conditions in order for the L_3 to survive and migrate onto herbage to be ingested (O'Connor et al., 2006). Temperature, weather and stocking density will influence the level of infectivity of the pasture. High rainfall will aid in the movement of L_3 from the faecal pellets onto the surrounding grass, whilst temperature affects the development of the larvae from egg to L_3 (O'Connor et al., 2006). For most GI nematodes in the UK, eggs shed on pasture during early spring can take 10 to 12 weeks to develop, whilst eggs shed during the early summer can take 1 to 2 weeks depending on the

species, leading to synchronised development to an infective stage and resulting in a high peak of infectivity during mid-summer (see Chapter 1 for more detail). Development and survival times on pasture, and development from ingestion to egg laying adults (pre-patent period) is species-specific (Table 5. 1.). *Nematodirus battus* eggs are able to survive longer in cold temperatures, and may require a frost before hatching. L_3 develop within the eggs and mass hatching occurs, usually after spring temperature increases, resulting in high challenge on pasture at a time when young lambs are also present (Roeber et al., 2013).

Strongyle nematodes have evolved species-specific mechanisms enabling them to overcome environmental and host constraints, allowing successful survival and reproduction (Table 5. 1). These include differential larval environmental resistance, seasonally-increased fecundity of adults, resilience to host immune responses and hyobiosis. Hypobiosis is the prolonged but temporary developmental arrestment of 4th-stage larvae (L_4). This is characterised by no egg shedding and enables the parasites to synchronise favourably to host and environmental conditions (Gibbs, 1982; O'Connor et al., 2006). Along with overwintered L_3, adult GI nematodes developing from hypobiotic larvae in ewes are a source of pasture contamination in the following Spring (Wilson et al., 2008).

Table 5. 1. List of clade V GI nematode species identified in the Soay sheep on Hirta, including adult worm infection site ('A' – abomasum, 'SI' – small intestine, LI – large intestine); infection route ('O' - oral, 'P' - percutaneous); pre-patent period ('PPP'); average L_3 pasture survival time ('LPST'); approximate number of eggs shed per-day by a healthy adult worm; whether eggs are morphologically distinguishable to genus or species level ('D') or not ('ND') during faecal egg counting (FEC) (adapted from Clutton-Brock and Pemberton (2004) and Soulsby (1968) unless stated).

Species	Organ	Inf.	PPP	LPST	Eggs shed	Eggs
Teladorsagia circumcincta^	A	O	~17d	14m*	100-200	ND
Trichostrongylus axei	A	O	~17d	6m	100-200	ND
Trichostrongylus vitrinus	SI	O	~17d	6m	100-200	ND
Bunostomum trigonocephalum	SI	O; P	~54-60d[1]	2m[1]	2579-10,480[1]	ND
Nematodirus battus	SI	O	~15-26d	10m**/***	50-100	D
Nematodirus filicollis	SI	O	~15-26d	10m*/ **	50-100	D
Nematodirus helvetianus^^	SI	O	~15-26d	10m*/ **	50-100	D
Chabertia ovina	LI	O	~48-54d	10m*	5000-10000	ND

KEY: * L_3 are able to 'over-winter' meaning they have the potential to survive longer ** Able to survive up to 2 years on-pasture ***Survives on-pasture as eggs, not L_3. ^ Originally described as *T. circumcincta, T. davtiai* and *T. trifurcate*. ^^ Presence **not** confirmed by Craig (2005). [1]Graham (1969)

On Hirta, there are two main peaks in infective larvae on the pasture (measured by pasture larval counts), firstly during the spring (April-May), mainly caused by the migration of over-wintered L_3 onto the sward. The second peak in summer (August) is density-dependent, and is linked to high lambing years and originates from the peri-parturient ewes shedding strongyle eggs in faeces (see Chapter 2) that then develop into L_3 (Wilson et al., 2003; Clutton-Brock and Pemberton, 2004).

Extrinsic factors such as climate, geography and flora, influence the extent of the L_3 challenge on-pasture (Waghorn et al., 2011). Within the study system, Village Bay , there are differences in pasture larval counts (Wilson et al., 2003). This spatial variation is driven by the differences in sheep density between areas (i.e. hefting), and also geographical factors (e.g. flood-prone/ marshy areas, cliff-side areas with high run-off). Whilst the Soay sheep are free-ranging, they are known to heft and generally will keep to their grazing ranges, hence animals born within Village Bay will tend to stay within the geographical boarders of the study system (Chapter 1).

Accurate characterisation of the GI nematodes parasite community is required to fully understand the epidemiology and influences of co-infections. Faecal egg counts (FEC) will only provide information on a group of parasites, not individual species, due to the limited morphological differentiation of species possible (Table 5. 1). Previous studies on Hirta based on post-mortem examination of naturally deceased Soay sheep are biased by studying diseased or weak animals, confounding effects of the months (and years) of high mortality. Additionally, by only counting 1 or 10% of the whole gut burden, there is the danger of underestimating or completely missing some species that reside in the host at low-levels. Speciation based on morphological characterisation of adult male worms is subjective and may lack accuracy in every case.

By using methods compared and validated in this thesis, this will be the first study to use next-generation sequencing in order to identify species and quantify parasites in the Soay sheep of St Kilda, to provide an epidemiological snapshot of the parasites present in healthy animals, and to demonstrate how these change between different ages, sexes and seasons.

5. 3. METHODS

5. 3. 1. Age groups and definitions

The sheep for the study were identified by their ear tags (Chapter 1), and were aged by their cohort year; lambs (4 – 12 months), yearlings (13 – 24 months), 2-year olds, adults (3 – 7 years), and geriatric females (8 – 12 years). A cohort, in the context of this study, is a group of individuals born within the same year, whilst a group of animals is made up of several individuals of different ages. Lambs, yearlings and 2-year-olds are a cohort group (born in 2015, 2014 and 2013, respectively), whilst adults (2008-2012) and geriatrics (2003 – 2007) are groups of sheep born over different years. There were not enough geriatric males in the study system for an additional group. Very young lambs (0 – 4 months) were not sampled to reduce the risk of the mother rejecting the lamb due to prolonged close human proximity.

5. 3. 2. Test months/ years

Samples were collected over Spring 2015 (April), Summer 2015 (July-August), Winter 2015 (October, November and February 2016), and Spring 2016 (March). Due to logistical constraints, no samples were collected during June, December and January (Table 5. 2).

5. 3. 3. Faecal collection and storage

Hefting behaviour is observed in the sheep; hence it is assumed there will be some within-test group variation in parasite burdens due to the specific areas in which the sheep choose to graze. These areas will inevitably differ in on-pasture larval counts, vegetation-type, shelter and elevation. The aim of the study was to capture a representative cross-section of the parasite composition for each sheep sex/age group in Village Bay, across different seasons. Samples were collected from across the whole Village Bay area, including collecting from the animals that favoured grazing ranges on the periphery of the study system. See Chapter 1 for a description of the study system.

Between March 2015 – March 2016, a total of 918 individual faecal samples (607 female and 311 male) were collected (Table 5. 2.). Due to the logistics of collecting faecal samples from the Soay sheep grazing over a large area in Village Bay (i.e. not all samples could be collected on one day) faecal collection occurred over a of two weeks. Animal tags were identified using binoculars (Figure 1. 3. A, Chapter 1), and faecal samples were collected off pasture after direct observation of defecation, without handling the animals. Samples were collected within 2 minutes of being voided to minimise potential animal misidentification or contamination by free-living nematodes. The faeces were collected in individual zip-lock bags, with the air fully expelled. Tag number, date, initials of collector and collection location were noted on the bag,

with these details being noted onto a spreadsheet later. Faecal samples were collected from 20 separate geographical locations across Village Bay. The tag number was double checked in the Soay sheep database before the faecal sample was separated into aliquots for coproculture and FEC within 24 hours of collection.

The precise number of samples collected per season was dependent on the number of animals in the study area, weather, mortality and any work being conducted on the sheep at that time. Bad weather in winter 2015 (November and February) limited the collections available on the hill, and sampling could not occur when the sheep were sheltering in the cleits (Figure 1. 3. B, see Chapter 1). Many of the faecal samples were aggregated to August (Table 5. 3), when a large number of animals are caught annually and rectally sampled as part of the Soay Sheep Project's ongoing research (Figure 1. 3. C., see Chapter 1). During this sampling period, large faecal samples were split so they could be part of this study. They were collected by a trained technician in guidance with approved ethics.

Once collected, the faecal samples were homogenised in their collection bags and 2-5g removed for FEC, and different weights for a bulk coproculture (see Table 5. 2), on the day of collection. Faeces for FEC were stored anaerobically in labelled zip lock bags at 4°C, and were counted within 14 days of collection. Processing of the faecal samples was dependent on the weight of faeces collected, which was limited in the younger animals and during the autumn/winter months. In times when there was a small weight of faeces, FEC took priority over coproculture.

5. 3. 4. Conventional parasitology

5. 2. 4. 1. Faecal egg count

All faecal egg counts (FEC) were counted onsite on St Kilda, except for July. July samples were sent off island in a portable refrigerator, to be counted at the Moredun Research Institute.

Individual faecal egg counts were prepared by cuvette salt floatation (Christie and Jackson, 1982), which has an egg detection limit as low as 1 egg per gram of faeces (epg). The method is described, in full, in Chapter 2. Strongyle-type and *Nematodirus* eggs were counted and recorded into an Excel spreadsheet (Microsoft, USA).

5. 3. 4. 2. Coproculture and Baermannisation

Coproculture is a means of hatching nematode eggs within faeces, and growing them to L_3. Faeces from each individual within a sex/age group were pooled, to obtain a representative composite sample per group for subsequent coproculture. To ensure that the parasite species composition of the pooled coproculture was not disproportionally influenced by that of any one

individual sheep, equal weights of faeces per animal was used (Table 5. 2). The weight of faeces per individual per month depended on the total amount that was collected, which differed between different test groups (lambs vs. adults) and seasons. During seasons with lower rainfall, or reduced food availability, the weight of faeces that contributed to the coproculture had to be reduced.

Faeces were weighed and pooled per sex/age into a container. Faeces were mixed with vermiculite into a crumbing consistency before pooling. The container was then covered, periodically sprayed with water, and incubated at ~20°C for >14 days. The cultures were stirred every few days to break-up fungal growth and aerate. Faecal collection occurred over the course of (max.) two weeks, with faeces being continually added to the cultures. The coproculture was incubated for a further 14 days after the last sample was added.

The Baermann technique is a means of harvesting L_3, whereby the larvae migrate through a filter in order to isolate them from the faecal material, and is the most practical method for larvae isolation under field conditions. After flooding the coproculture container with tepid water and incubating for 4 hours at 20°C, the supernatant was poured through an assembled Baermann apparatus (MAFF, 1968). The Baermann apparatus was a sieve made from one sheet of single-ply tissue suspended over a beaker of water. It was incubated overnight at room temperature to allow larvae to migrate through the filter into clean, warm tap water. The larvae were left for 24 hours at 4°C to settle, and the supernatant was carefully removed by siphon. The larvae were stored in 50ml tissue culture flasks (Sarstedt, Germany) in 10ml of tap water. The flask was set on its side with the lid slightly open to allow aeration and stored at 4°C. The culture flasks were closed and sealed when the larvae were transported off St Kilda. Due to the restrictions of ethanol on the helicopter, the larvae could not be fixed before transport.

Table 5. 2. Summary of faecal collection for coproculture. Number of individual animals collected per group (n=) along with the weight of faeces per animal that was pooled for coproculture (g). Faecal samples were collected over 7 sampling months to make 61 aliquots of 1000 larvae. X – indicates groups that were not sampled that month.

Sampling season	**Sampling month**	**Test group (n=/ g)**								
		Lambs *4-12 months*		**Yearlings** *13-23 months*		**2-year olds** *24-35 months*		**Adults** *3-7 years*		**Geriatric** *8-13 years*
		Females	Males	Females	Males	Females	Males	Females	Males	Females
Spring 2015	Apr-15	X	X	n=30/ 5g	n=22/ 5g	X	X	n=20/ 8g	n=28/ 8g	n=10/ 8g
Summer 2015	Jul-15	n=26/ 2g	n=32/ 2g	n=14/ 6g	n=11/ 6g	n=14/ 8g	n=5/ 5g	n=52/ 6g	n=17/ 8g	n=12/ 8g
	Aug-15	n=43/ 2g	n=42/ 2g	n=12/ 6g	n=8/ 5g	n=10/ 8g	n=3/ 5g	n=62/ 6g	n=11/ 6g	n=17/ 6g
Winter 2015	Oct-15	n=14/ 1g	n=14/ 2g	n=6/ 2g	n=9/ 2g	n=8/ 5g	n=5/ 5g	n=19/ 5g	n=13/ 5g	n= 17/ 5g
	Nov-15	n=16/ 1g	n=20/ 1g	n=13/ 2g	n=11/ 2g	n=10/ 5g	n=11/ 5g	n=26/ 5g	n=15/ 5g	n=19/ 5g
	Feb-16	n=11/ 1g	n=1/ 10g	n=6/ 2g	n=3/ 2g	n=7/ 5g	n=2/ 4g	n=34/ 4g	n=3/ 6g	n=15/ 6g
Spring 2016	Mar-16	n=17/ 1g	n=5/ 1g	n=10/ 2g	n=6/ 2g	n=5/ 8g	n=5/4g	n=27/ 4g	n=14/ 5g	n=5/ 8g

5. 3. 4. 3. Preparing larvae

For each culture the total number of larvae cultured was estimated. The tissue culture flask lid was closed and the flask was inverted several times to evenly suspend the larvae, and the number of larvae present in a 10µl aliquot was counted under a compound microscope 5 times. The average was used to calculate total larvae number in the 10ml suspension. Aliquots of ~1000 larvae were pipetted into 2ml screw-top microcentrifuge tubes (Sigma-Aldrich , USA), made up to a 2ml total volume to a total concentration of 70% ethanol, and stored at -80°C until molecular analysis. The total number of larvae between the aliquots will vary, as it is not possible to produce a completely homogeneous larval suspension. In an attempt to minimise variability, the larvae were cooled before counting, to slow their movements and reduce clumping. This resulted in 59 aliquots of ~1000 L_3, collected over 7 sampling months for 7 different sex/age groups (Table 5. 2).

Some groups yielded low numbers of L_3, which meant that for some sex/age groups on particular months there was only one tube of ~1000 L_3. Prior to molecular analysis, the larval aliquots were vortexed thoroughly, they were then halved to aliquots of approximately ~500 L_3, to allow a contingency if any failures incurred.

5. 3. 5. Molecular parasitology – The nemabiome assay

The methods for L_3 DNA extraction (lysates), PCR reations, gel electrophoresis, library preparation and deep amplicon sequencing are fully described in Chapter 3. The bioinformatics and read analysis (to calculate species proportion - %) is described in Chapter 3. For the nemabiome assay workflow summary diagram see Figure 3. 2 (Chapter 3).

5. 3. 5. 1. Correction Factor (CF)

Species-specific biases during sequencing were identified in Chapter 4, these were then used to calculate a Correction Factor (CF), which can be then multiplied by the sequencing reads per prior to analysis to account for these species-specific differences. These CFs were calculated for each species identified on St Kilda (Chapter 4); *B. trigonocephalum* = 0.865676, *C. ovina* = 2.044679, *T. circumcincta* = 0.840703 and *T. vitrinus* = 0.857427. A CF could not be calculated for *T. axei*, therefore the correction factor calculated by Redman et al., (2019) was used (*T. axei* = 1.278952).

5. 3. 6. Statistical analysis

For each sampling month and sex/age group the arithmetic mean FEC was calculated. The proportion of eggs allocated to each species was calculated by: average monthly FEC * (% species proportion / 100).

Proportional data (%) were analysed using a Linear Mixed Model by Restricted maximum likelihood (REML) for the response variable proportion (% species) using species (*T. circumcincta, T. vitrinus, B. trigonocephalum, C. ovina*) nested within the age class of the animal (lambs, yearlings, adults and geriatrics) and month (April 2015, July 2015, August 2015, October 2015, November 2015, February 2016 and March 2016). The data for *T. axei* showed the least change over time so it was not included in the analysis as only 4 species are independent due to the proportions adding to 100. The analysis of the composition of different samples (and thus different species), can prove challenging. Standard measures of correlation assume the data are independent, which is not true of proportional (%) data. If *T. axei* was to be included in the analysis, the data would not conform to the assumptions made by the test. Proportional data is not independent; if one increases, the others must decrease. A t-test was used to determine that there was no significant difference between the 2 two sexes (p=0.25), so sex was not included as a factor in the model, which provided two replicate values for all combinations except geriatric class for which there was no male data. Analysis was conducted using GenStat 18th edition (VSN International Ltd).

5. 4. RESULTS

5. 4. 1. Simultaneous detection and identification of GI nematode species in multiple pools of mixed-species L_3

Five species of GI nematodes (clade V) were identified in the Soay sheep of Hirta (St Kilda); *B. trigonocephalum*, *C. ovina*, *T. circumcincta*, *T. axei*, and *T. vitrinus*. Other strongyle species such as *H. contortus*, *Cooperia* spp., and *O. venulosum*, that are highly prevalent in managed Scottish flocks, were absent.

Despite the isolation of the study system and novelty of the sequencing method, 59 of 61 samples amplified. Some samples were lost during culturing, transport from the field site, sequencing and bioinformatics (Table 5. 3). Sample 24 (yearling males/August) and 36 (adult females/ November) did not amplify (Figure 5. 2). The ITS-2 amplification was re-run twice more on these samples with 12µl of lysate (1:10 dilution). They did not amplify and were removed from the project. Sample 43 (lamb males/ November), failed to run successfully through the bioinformatics as the index number did not reach the threshold (i.e. did not produce useable number of reads for the analysis) and was therefore excluded from the statistics. In October four samples (adult males, 2-year old males, lamb males and geriatric females) lost their identifying labels during transit resulting in four 'unknown' samples, three of which were

sequenced anyway (samples 33, 34 and 35). However, they were ultimately not included. Lamb males (February) yielded insufficient L_3 for sequencing (Table 5. 3).

Figure 5. 2. Gel electrophoresis (1.2% agarose gel) of amplified field samples (NC1/NC2 primer amplification) and barcode addition. Sample numbers correspond to Table A5. 1. (Appendix) Sample 24 and 36 absent. 115V/40-45 minutes, negative control (C) (molecular-grade water dH_20). L = 1KB DNA ladder (Invitrogen, USA).

Table 5. 3. Summary of all the samples collected during the project, sample number corresponding to gel image (Figure 5. 2). March (2015) and the 2-year old groups were ultimately removed from the analysis due to insufficient sample number, and number of individuals sampled for the group.

Age group	Sex	Sample month							
		Mar (15)	Apr (15)	Jul (15)	Aug (15)	Oct (15)	Nov (15)	Feb (16)	Mar (16)
Adult	Female	1	5	10	19	28	**36**[1]	45	53
	Male	2	6	11	20	*	37	46	54
2 year	Female	X	X	12	21	29	38	47	55
	Male	X	X	13	22	*	39	48	56
Yearling	Female	3	7	14	23	30	40	49	57
	Male	4	8	15	**24**[1]	31	41	50	58
Lamb	Female	X	X	16	25	32	42	51	59
	Male	X	X	17	26	*	**43**[2]	^	60
Geriatric	Female	X	9	18	27	*	44	52	61
Unknown (*)	1					33			
	2					34			
	3					35			

X were not collected, [1]Failed to amplify, [2]Failed bioinformatics, *Lost labels during transport resulting in 4 'unknown' samples, 3 of which were run anyway but not included in the final analysis, ^insufficient larvae cultured.

5. 4. 2. Detection and molecular quantification of GI nematode species in coprocultured L_3 composite pools from different sex/age groups

The species pools of L_3 were identified by next generation sequencing from a composite pool of ~500 L_3 per sex/age group/month. Percentage proportions of each species (%), as determined by the sequencing assay, were corrected by previously validated CFs to account for species-specific biases which can occur during sequencing. The nemabiome species results were adjusted to the average (arithmetic mean) FEC, in an attempt to give an estimate of the species contaminating pasture.

5. 4. 2. 1. Lambs (4 – 12 months)

Collection began in July 2015, and as the year progresses the single cohort reaches their first birthday in March 2016. The females by spring 2016 will most likely be pregnant (Figure 5. 1. A), whilst the males will have participated in their first rut in November 2015 (Figure 5. 1. B).

Females

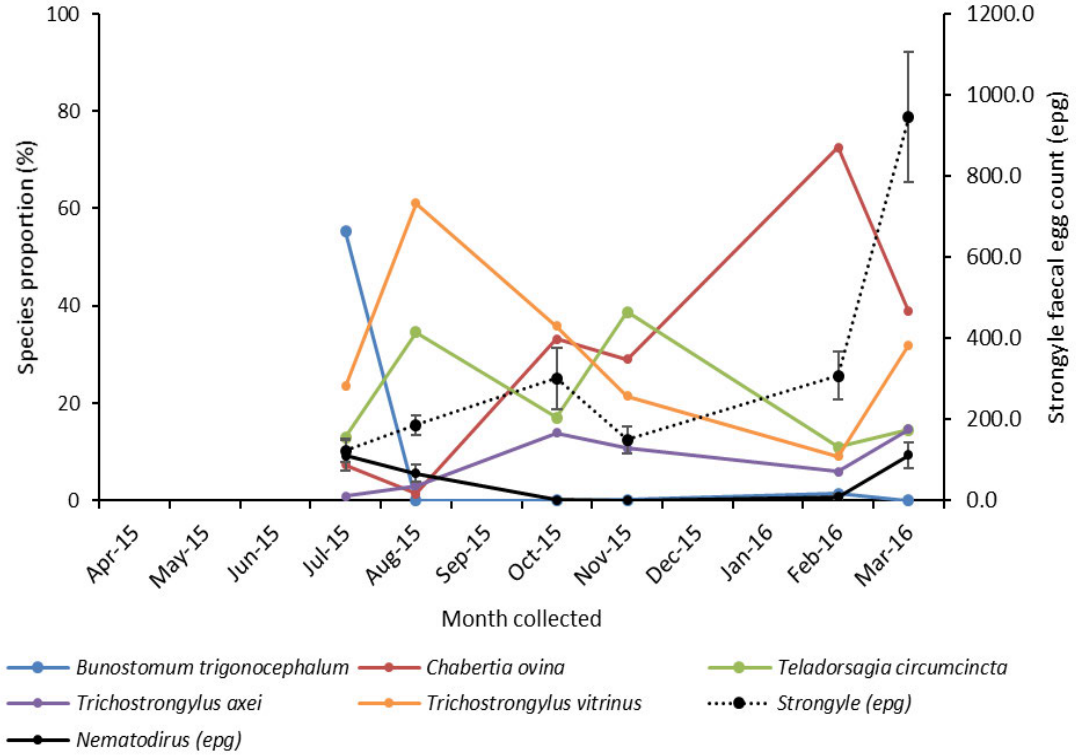
T. circumcincta (range = 11-39%), *T. axei* (range = 1-15%), *T. vitrinus* (range = 9-61%) and *C. ovina* (range = 1-73%) were identified in all months sampled, whilst *B. trigonocephalum* was identified in July (55%) and February 2016 (1%). *T. vitrinus* and *T. circumcincta* are the

predominating species in the female lambs during summer months (61% and 35%, respectively), with a slightly greater proportion of *T. circumcincta* during the early winter (39%). *C. ovina* predominated during later winter (73%) and spring 2016 (39%), when the FECs were highest (308 epg and 945 epg, respectively) (Figure 5. 3. A.) When the average FEC is taken into account, the number of eggs (epg) allocated to each species identified within the culture is small, indicating low levels of pasture contamination, with a main peak seen in March 2016 the following year as the female lambs go into their first lambing season with *C. ovina* and *T. vitrinus* predominating (Figure 5. 3. B.). *Nematodirus* counts were highest in July 2015 (110 epg) and March 2016 (112 epg) (Table 5. 5).

Males

There is less confidence in the data from the male lambs, as there are few data points (n=4). However, it is interesting to note that there are similarities to the female lambs; *T. circumcincta* (range = 16 – 37%), *T. axei* (range = 1-14%), *T. vitrinus* (range = 21-57%) and *C. ovina* (range = 1-26%) were identified in all months sampled, whilst *B. trigonocephalum* was identified in July (4%) and November (20%). *T. vitrinus* L_3 dominates the coproculture in summer 2015 (57% for July and August), when the FECs, representing the strongyle nematode burden, are at their lowest (112 epg and 172 epg, respectively). *T. vitrinus* also dominate in spring 2016 when the FECs are highest (46%) (391 epg). (Figure 5. 4. A). When average faecal egg count is taken into account, there are no clear changes in species composition with time, but the proportions remain consistent (Figure 5. 4. B). *Nematodirus* counts were highest in July (143 epg) and August (67 epg) (Table 5. 6).

A



B

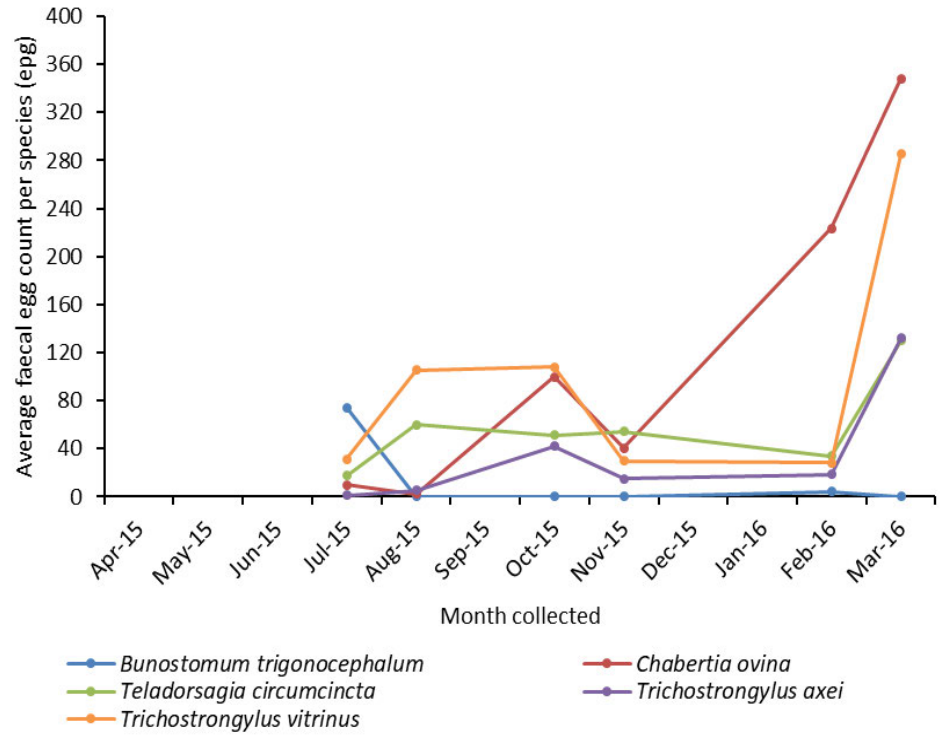


Figure 5. 3. A) Monthly differences in L_3 output for **female lambs**, presented as species proportion (%). Strongyle and *Nematodirus* faecal egg counts presented as mean (arithmetic) eggs per gram (epg) Error bars ±SEM. Data points indicate time sampled. B) Proportion of eggs (epg) allocated to each species identified within the culture.

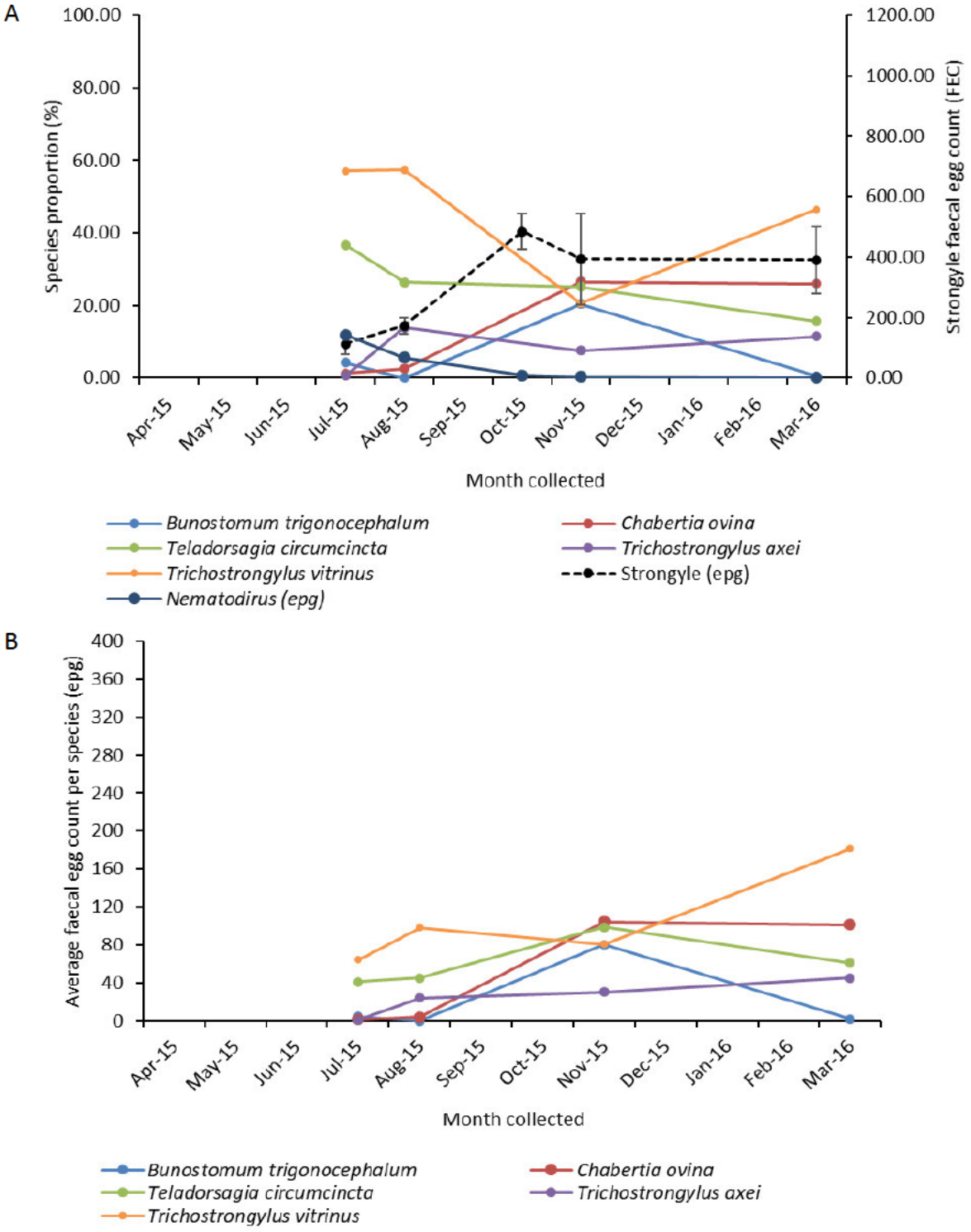


Figure 5. 4. A) Monthly differences in L_3 output for **male lambs**, presented as species proportion (%). Strongyle and *Nematodirus* faecal egg counts presented as mean (arithmetic) eggs per gram (epg). Error bars ±SEM. Data points indicate time sampled. B) Proportion of eggs (epg) allocated to each species identified within the culture.

5. 4. 2. 2. Yearlings (13 – 24 months)

The yearlings consisted of a single year of age cohort, from their first birthday in March and April 2015 to their first lambing season in Spring 2016.

Females

C. ovina (range = 5 – 74%) and *T. axei* (range = 2 – 22%) had 100% prevalence in all months sampled. *B. trigonocephalum* (range = 0 – 81%) was not identified in April and February, *T. circumcincta* (range = 0 – 48%) was not identified in August and *T. vitrinus* (range = 0 -21%) was not identified in the August and February cultures. There is a high summer peak of *B. trigonocephalum* during July (74%) and August (81%), preceded and followed by very low parasite levels. *C. ovina* and *T. circumcincta* predominate between November and February (Figure 5. 5. A). When average FEC for each sampling month is taken into consideration, the trends seen are less clear; as the average FEC decreases, the separation between the infecting species are not as clear. From August to March the following year, there are no clear changes in species composition with time, but the proportions remain consistent (Figure 5. 5. B). During the time of the PPR (April), the species are dominated by *C. ovina* and *T. vitrinus*, with a high average strongyle FEC (1076 epg) in addition to a *Nematodirus* count (92 epg) (Table 5. 5).

Males

Trends for the male yearlings are less clear than those of the females due to the critical absence of data points during August and February. Nevertheless, the data throughout most of the year are similar to those for the female group, with the exception of Spring 2016, when the FECs are much lower than those of the females (strongyle FEC: females 945 epg, males 390 epg; Nematodirus FEC: females 112 epg, males 0 epg), and *C. ovina* predominates (Table 5. 6). When average FEC is taken into account, trends seen are similar to those of the yearling females (Figure 5. 6. A/B).

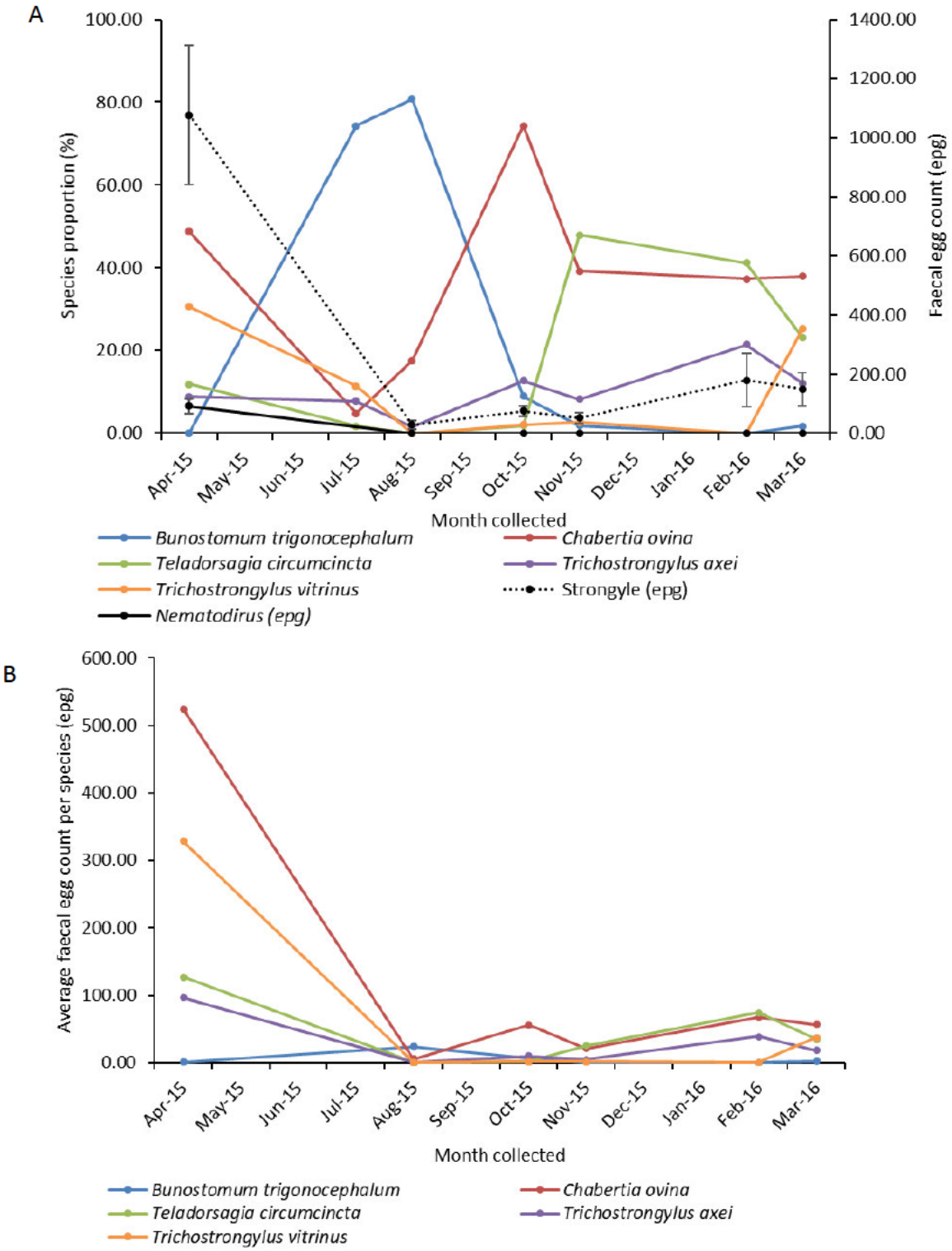
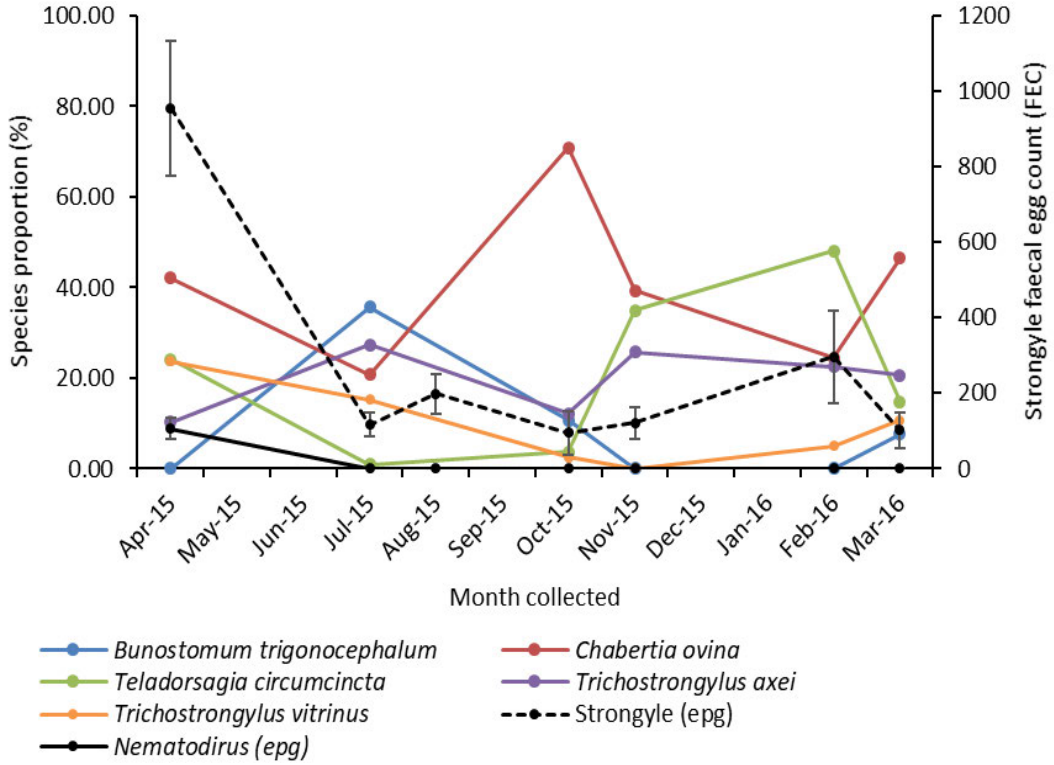


Figure 5. 5. A) Monthly differences in L_3 output for **female yearlings**, presented as species proportion (%). Strongyle and *Nematodirus* faecal egg counts presented as mean (arithmetic) eggs per gram (epg).). Error bars ±SEM. Data points indicate time sampled. B) Proportion of eggs (epg) allocated to each species identified within the culture. No July FEC datapoint.

A



B

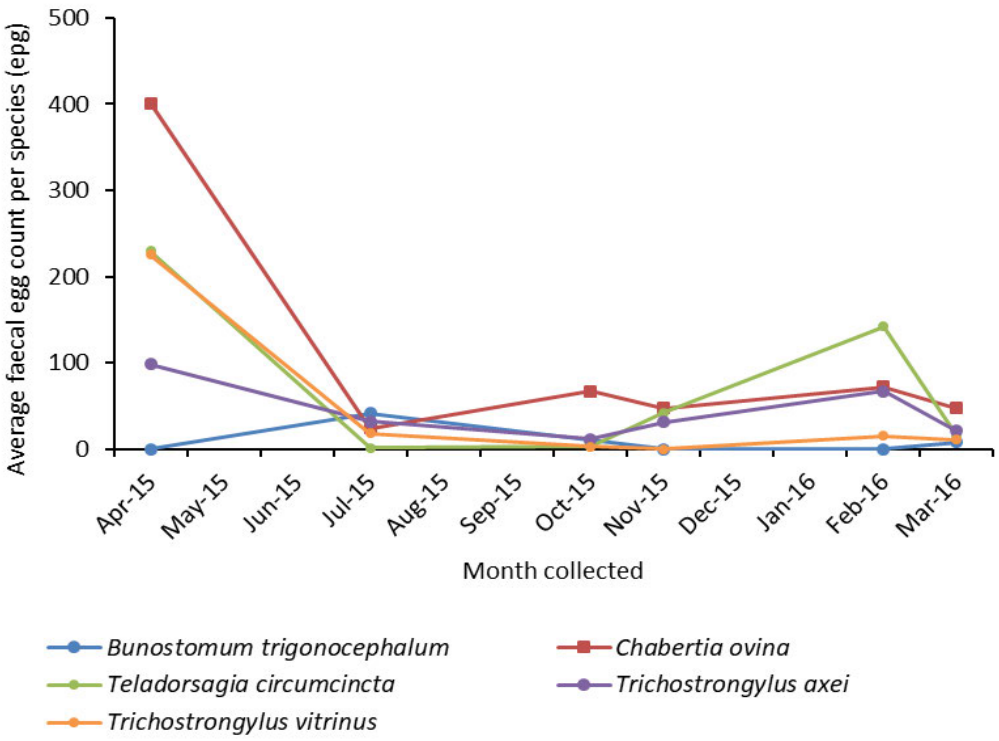


Figure 5. 6. A) Monthly differences in L_3 output for **male yearlings**, presented as species proportion (%). Strongyle and *Nematodirus* faecal egg counts presented as mean (arithmetic) eggs per gram (epg). Error bars ±SEM. Data points indicate time sampled. B) Proportion of eggs (epg) allocated to each species identified within the culture.

5. 4. 2. 3. Adults (3 – 7 years)

Females

T. circumcincta (range = 1-45%) and *T. axei* (range = 3-27%) were identified in all the months sampled. *C. ovina* (range = 0 – 49%) was not identified in April, *T. vitrinus* (range = 0 – 64%) was not identified in August or February 2016, and *B. trigonocephalum* (range = 0 – 60%) was not identified in April 2015 and March 2016 (Figure 5. 7. A). Similar to the yearling females, when proportion of eggs are allocated to each species, the clear sequential peaking *of B. trigonocephalum, C. ovina* and *T. circumcincta* is not as clear. The species seen in April 2015 (*T. vitrinus, T. circumcincta* and *T. axei*) are similar to the proportions in March 2016 (Figure 5. 7. B). During the time of the PPR (April), the species are dominated by *C. ovina* and *T. circumcincta*, with a high average strongyle FEC (115 epg) in addition to a small *Nematodirus* count (1.8 epg) (Table 5. 5). The *Nematodirus* count in adults was unexpected, and whilst the average count is low, 50% of the samples counted for April had a *Nematodirus* count.

Males

The male adults differed to the adult females; *B. trigonocephalum* (range = 3 – 71%), *T. axei* (range = 2 - 31%) and *C. ovina* (range = 2 - 43%) were identified in all months sampled. *T. vitrinus* (range = 0 - 28%) was not identified in July or February 2016, and *T. circumcincta* (range = 0 – 75%) was not identified in July or August (Figure 5. 8. A). When the relative species proportions were adapted to account for egg count, *T. circumcincta* predominates (Figure 5. 8. B). Similarly to the adult females, the males had a very low, but unexpected, *Nematodirus* count in April (4 epg) with 32% of the samples counting *Nematodirus*.

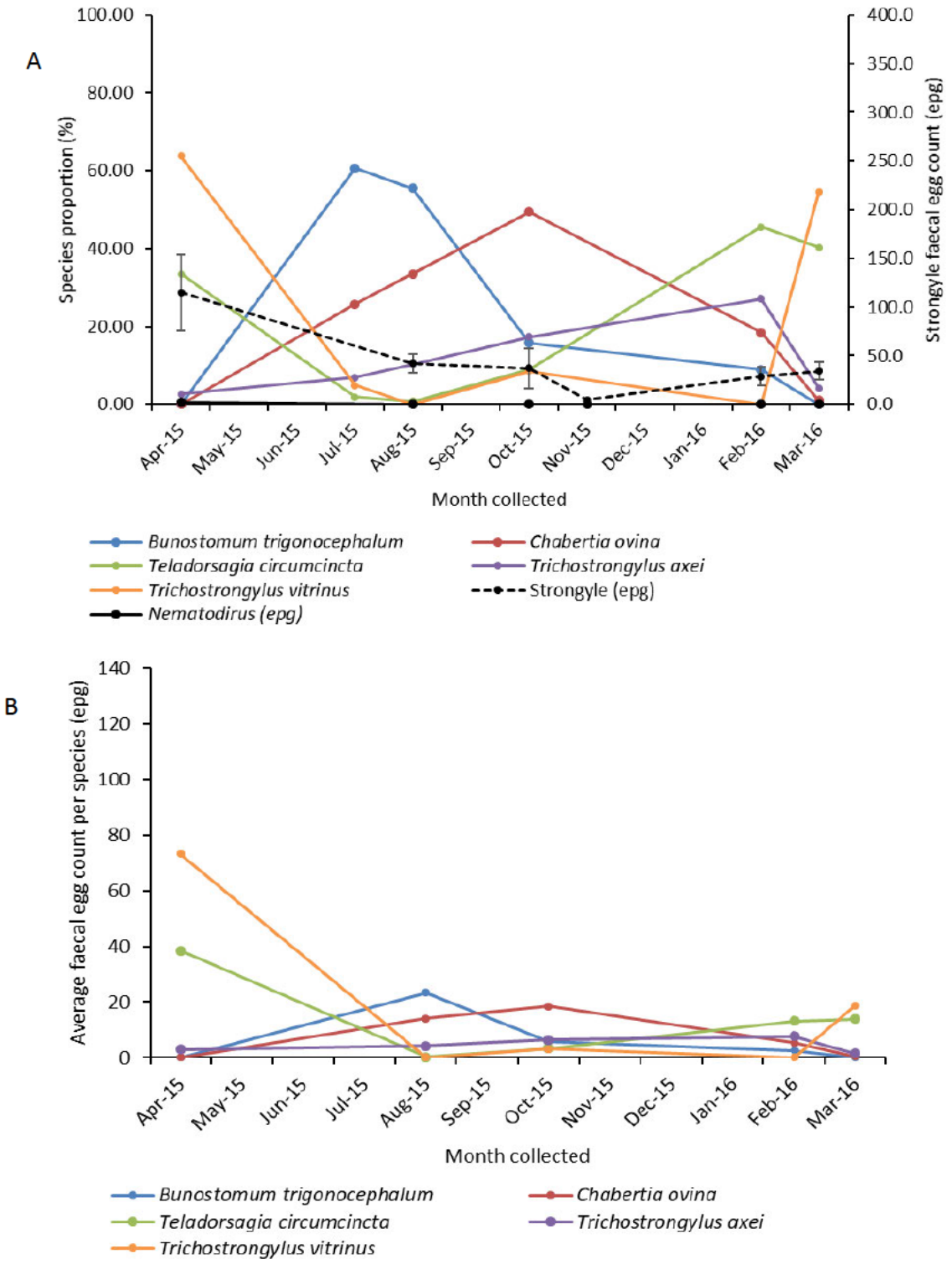


Figure 5. 7. A) Monthly differences in L_3 output for **female adults**, presented as species proportion (%). Strongyle and *Nematodirus* faecal egg counts presented as mean (arithmetic) eggs per gram (epg). Error bars ±SEM. Data points indicate time sampled. B) Proportion of eggs (epg) allocated to each species identified within the culture. No July FEC datapoint.

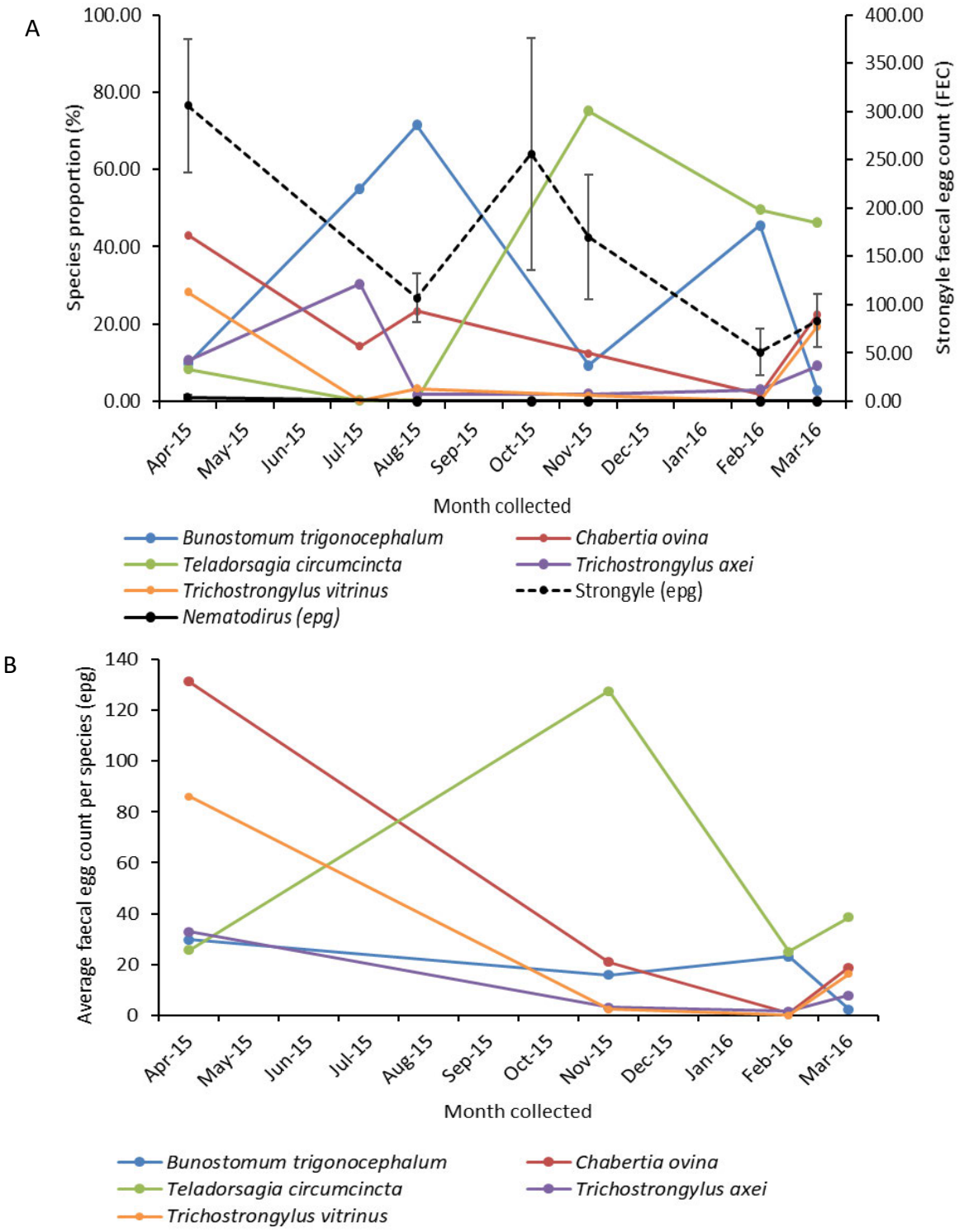


Figure 5. 8. A) Monthly differences in L_3 output for **male adults**, presented as species proportion (%). Strongyle and *Nematodirus* faecal egg counts presented as mean (arithmetic) eggs per gram (epg). Error bars ±SEM. Data points indicate time sampled. B) Proportion of eggs (epg) allocated to each species identified within the culture. No July FEC datapoint.

5. 4. 2. 4. Geriatric females (8+ years)

There is are only 5 data points for the geriatric group, set against a background of low FEC representing low either a GI nematode burden, or low/ suppressed worm fecundity, therefore care needs to be taken in the interpretation of the data. *T. circumcincta* (range = 1 – 88%), *T. axei* (range = 3 – 5%), and *C. ovina* (range = 4 – 35%) were identified in all months sampled, whilst *B. trigonocephalum* (range = 0 – 58%) was not identified in February, and *T. vitrinus* (range = 0 – 32%) was not identified in the cultures in August and November 2015 (Figure 5. 9. A). When the average FEC is taken into account, the number of eggs (epg) allocated to *T. circumcincta* in November is substantially reduced and a new peak is seen in February the following year in response to an increase in FEC (139.6 epg) (Figure 5. 9. B). Similarly to the adults, there was an unexpected, but low, *Nematodirus* count in April (3 epg) with 40% of the samples counted in April infected with *Nematodirus* (Table 5. 5).

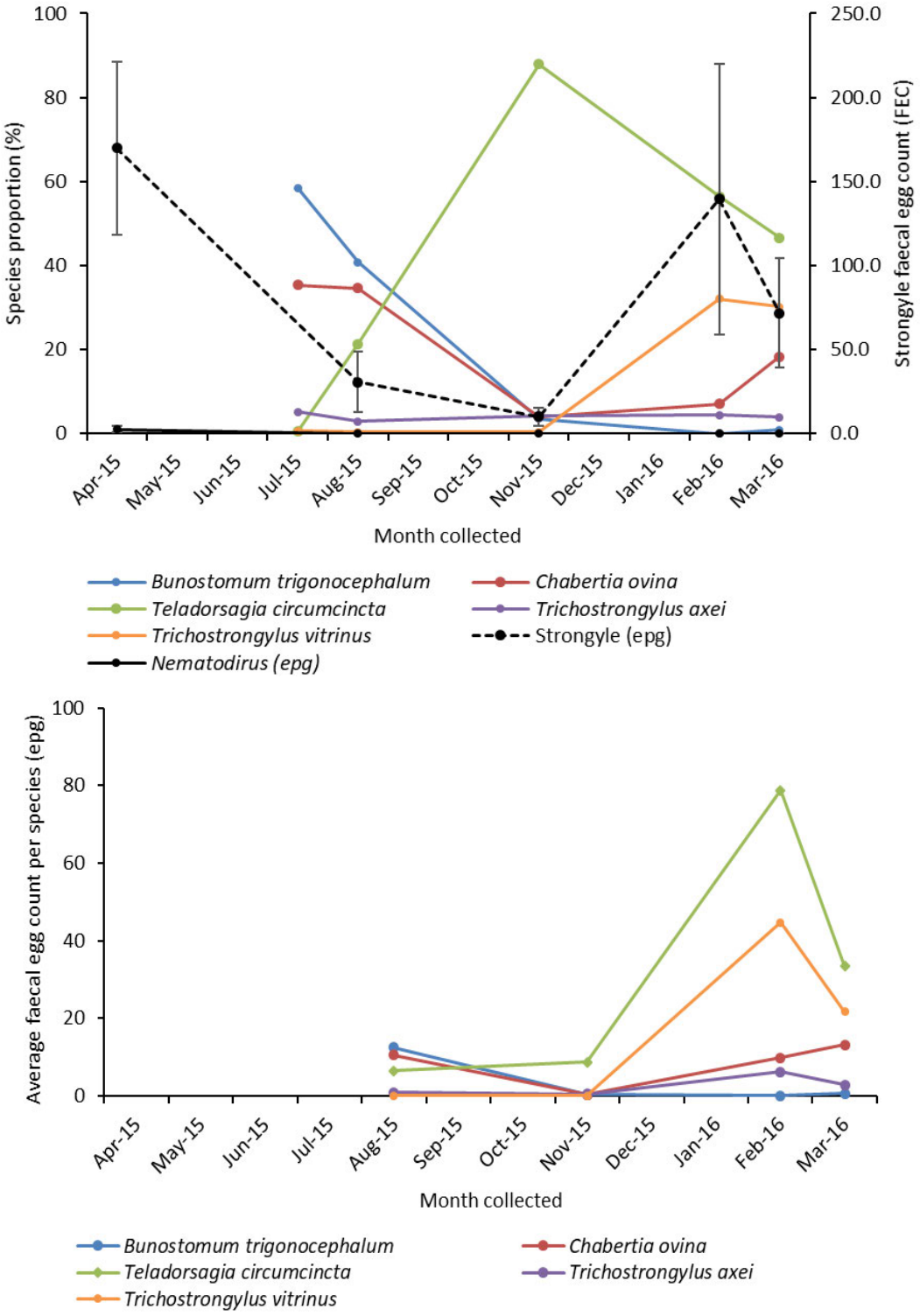


Figure 5. 9. A) Monthly differences in L_3 output for **female geriatrics**, presented as species proportion (%). Strongyle and *Nematodirus* faecal egg counts presented as mean (arithmetic) eggs per gram (epg). Error bars ±SEM.Data points indicate time sampled. B) Proportion of eggs (epg) allocated to each species identified within the culture. No July FEC datapoint.

5. 4. 3. Seasonal trends

Many of the species identified are highly seasonal, driven by their survivability on pasture, development time and interactions with the host. Presented in this section is the relative proportional species identification data (%) divided between each species, and presented in radar plots. These plots present the pure nemabiome data, and have not been adjusted for FEC.

5. 4. 3. 1. *Bunostomum trigonocephalum*

For the females, *B. trigonocephalum* shows the same pattern in each group, with the highest percentage recovered from culture in late spring to summer; July (range = 55 – 74%) and August (range = 0 – 81%). Additionally, B. *trigonocephalum* was identified in cultures at the end of winter/ beginning of spring (February - March) for male (45%) and female (9%) adults (Figure 5. 10).

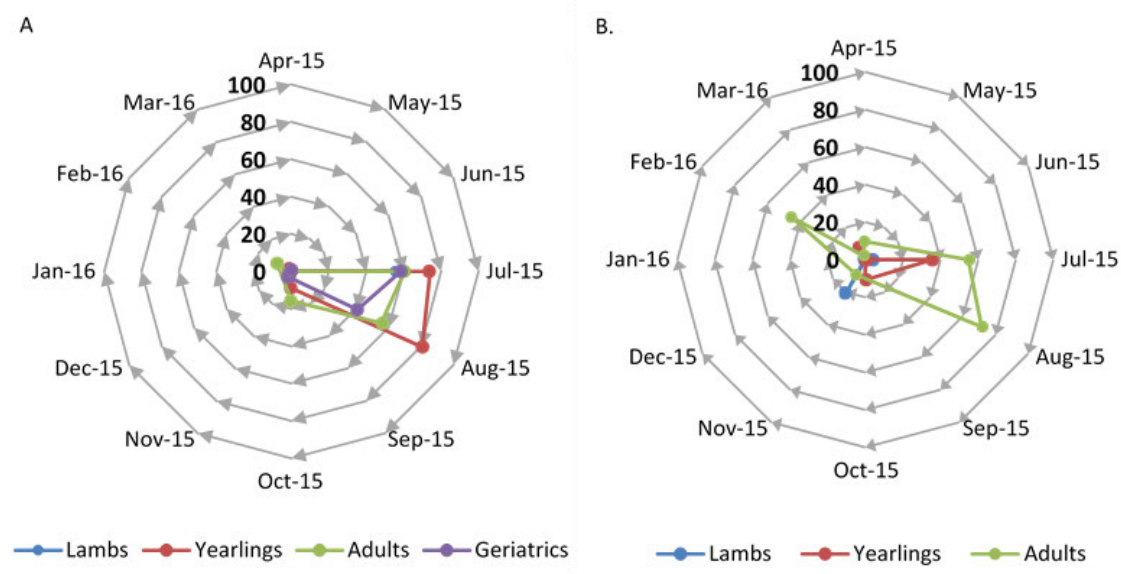


Figure 5. 10. Radar charts showing the seasonal proportion (%) of *B. trigonocephalum* in A. **Female**, and B. **Male** Soay, for each of the age groups (lambs, yearlings, adults and geriatrics (females)). Data collected from April 2015 (Apr-15) to March 2016 (Mar-16). Points indicate time sampled. Radar plot read clockwise (indicated by arrows), each interval = 20%.

5. 4. 3. 2. *Chabertia ovina*

C. ovina appears to be more associated with the winter months for both sexes, with high shedding seen in the yearling groups notably in October (Figure 5. 11).

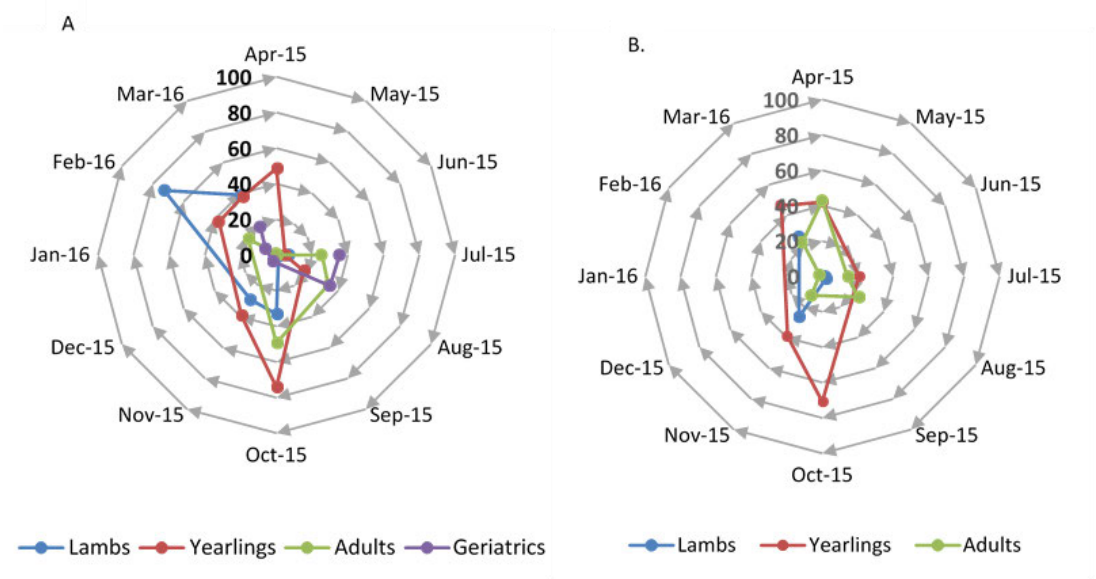


Figure 5. 11. Radar charts showing the seasonal proportion (%) of *C. ovina* in A. **Female**, and B. **Male** Soay, for each of the age groups (lambs, yearlings, adults and geriatrics (females)). Data collected from April 2015 (Apr-15) to March 2016 (Mar-16). Points indicate time sampled. Radar plot read clockwise (indicated by arrows), each interval = 20%.

5. 4. 3. 3. *Teladorsagia circumcincta*

T. circumcincta has similar trends in both females and males (Figure 5. 12. A. and Figure 5. 12. B), in comparison to the lambs where it appears late spring to summer as well. The similarities between the geriatric females and adult males is interesting, with a large increase in winter (November: females = 88%, males = 75%; February: females = 56%, males = 50%) and spring the following year (March: females = 47%, males = 46%).

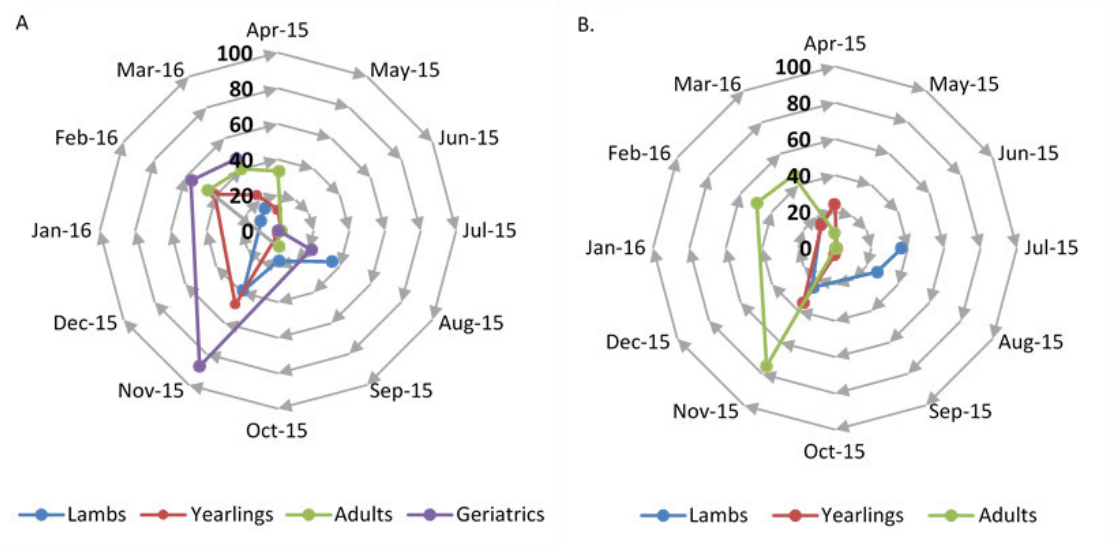


Figure 5. 12. Radar charts showing the seasonal proportion (%) of *T. circumcincta* in A. **Female**, and B. **Male** Soay, for each of the age groups (lambs, yearlings, adults and geriatrics (females)). Data collected from April 2015 (Apr-15) to March 2016 (Mar-16). Points indicate time sampled. Radar plot read clockwise (indicated by arrows), each interval = 20%.

5. 4. 3. 4. *Trichostrongylus vitrinus*

T. vitrinus is present during spring – summer months in both male (Figure 5. 12. B) and female (Figure 5. 12. A) yearlings, adults and geriatrics whilst there is are high spring proportions in lambs.

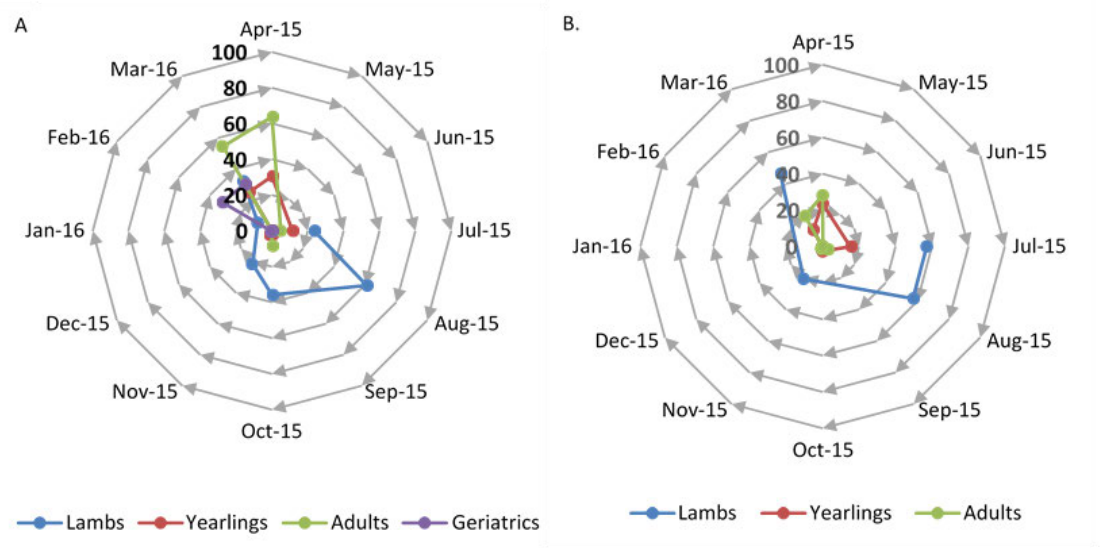


Figure 5. 13. Radar charts showing the seasonal proportion (%) of *T. vitrinus* in A. **Female**, and B. **Male** Soay, for each of the age groups (lambs, yearlings, adults and geriatrics (females)). Data collected from April 2015 (Apr-15) to March 2016 (Mar-16). Points indicate time sampled. Radar plot read clockwise (indicated by arrows), each interval = 20%.

5. 4. 3. 5. *Trichostrongylus axei*

Out of all the parasites identified, *T. axei* presents within the culture consistently low (no more than 31% in the males, and 27% in the females). Additionally, it is the only parasite to have 100% prevalence in all the months sampled for both sexes; there is consistent low-level *T. axei* egg shedding in all age classes in both sexes, regardless of season. The only group to show a slightly, and consistent, elevated *T. axei* output is the yearling (13-24 months) males (Figure 5. 14).

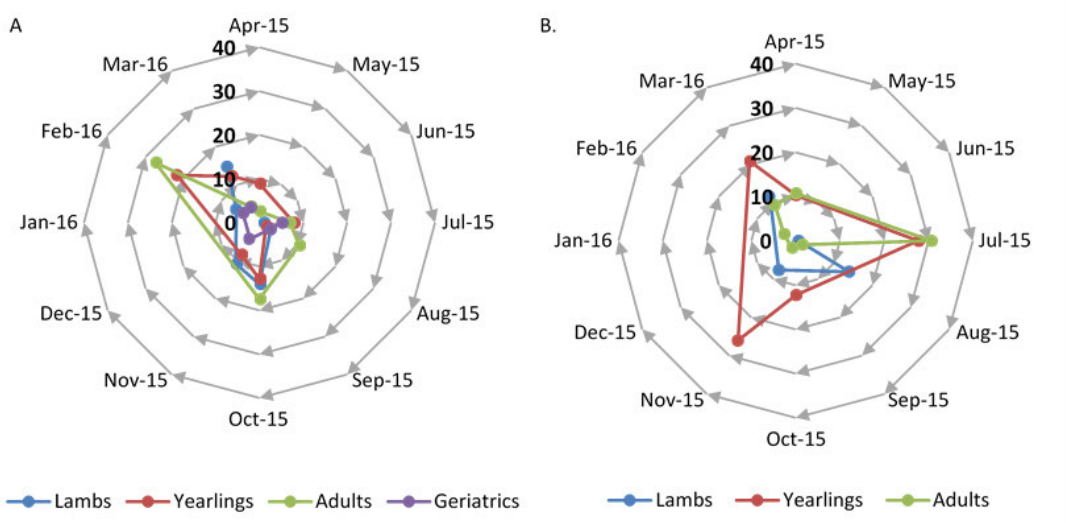


Figure 5. 14. Radar charts showing the seasonal proportion (%) of *T. axei* in A. **Female**, and B. **Male** Soay, for each of the age groups (lambs, yearlings, adults and geriatrics (females)). Data collected from April 2015 (Apr-15) to March 2016 (Mar-16). Points indicate time sampled. Radar plot read clockwise (indicated by arrows), each interval = 10%. Note: total proportion (%) = 40% (not 100%).

The seasonal and host data presented in the previous two sections show some interesting trends. However, the analysis of the data is challenging; due to the study design, the data requires a more circumspect interpretation which limits the application of thorough statistics. The study design of the project lends itself to the limitations of working in a remote field location and using novel molecular sequencing methods; this resulted in missing data points in both sex groups, just one sampling year and no replication. Nevertheless, it is important to attempt some statistical analysis. REML (linear mixed effects model) variance components analysis was used and found a statistically significant difference in the species proportions between different months and between the age groups ($p < 0.001$, Table 5. 4).

Table 5. 4. Testing for a statistical difference between class (lamb, yearling, adult, geriatric), month [year] (April 2015, July 2015, August 2016, October 2015, November 2015, February 2016, March 2016) and species (*B. trigonocephalum, C. ovina, T. circumcincta, T. vitrinus*).

Fixed Term	**Wald statistic**	**n.d.f.**	**F statistic**	**d.d.f.**	**F pr**
Class	0.73	3	0.24	56.0	0.865
Class.Month[year]	1.64	21	0.08	56.0	1.000
Class.Month[year].Species	**483.07**	**75**	**6.44**	**56.0**	**<0.001**

n.d.f contains the degrees of freedom for the Wald statistic; d.d.f. – contains the denominator degrees of freedom for the F statistic; Fpr – p -value for F statistics

Additional statistical analysis was performed on the data; Fishers least significant differences were used for a post hoc test, comparing species proportions over sampling months and between the sexes. However, due to missing corresponding data points in both sex groups, it encouraged the inaccurate inference of trends and without a full dataset a robust comparison of the monthly proportional data cannot be made. Therefore, this was excluded from this analysis.

Table 5. 5. Full summary of **female** Soay sheep speciation and relative proportional data, with strongyle and *Nematodirus* faecal egg count (FEC) as eggs per gram of faeces (epg).

Season	Month	Group	n	Strongyle FEC (epg) (±SEM)	*Nematodirus* FEC (epg) (±SEM)	Relative species proportion (%)				
						B. trigonocephalum	*C. ovina*	*T. circumcincta*	*T. axei*	*T. vitrinus*
Spring 2015	April	Lamb	N/S							
		Yearling	30	1076.6 (±234.9)	91.7 (±24.7)	0.07	48.65	11.82	8.93	30.53
		Adult	20	114.8 (±39.0)	1.8 (±0.83)	0.06	0.06	33.52	2.62	63.74
		Geriatric	10	169.8 (±51.5)	2.5 (±1.77)	N/D	N/D	N/D	N/D	N/D
Summer 2015	July	Lamb	26	122.9 (±28.6)	110.8 (±37.59)	55.32	7.30	13.03	0.90	23.45
		Yearling	14	N/D		74.15	4.81	1.79	7.85	11.41
		Adult	52	N/D		60.49	25.70	1.85	7.02	4.94
		Geriatric	12	N/D		58.39	35.32	0.51	5.20	0.58
	August	Lamb	43	185.4 (±24.4)	66.5 (±20.80)	0.03	1.34	34.58	2.99	61.06
		Yearling	12	29.2 (±14.9)	0.0 (±0.00)	80.71	17.58	0.00	1.70	0.01
		Adult	62	42.1 (±9.6)	0.0 (±0.00)	55.40	33.44	0.75	10.40	0.01
		Geriatric	17	30.5 (±18.0)	0.0 (±0.00)	40.82	34.64	21.34	2.88	0.33
Winter 2015	October	Lamb	14	300.9 (±76.8)	0.8 (±1.12)	0.00	33.17	17.00	14.01	35.82
		Yearling	6	75.0 (±17.4)	0.0 (±0.00)	8.95	74.17	1.89	12.82	2.17
		Adult	19	37.2 (±20.7)	0.0 (±0.00)	15.83	49.38	8.85	17.46	8.48
		Geriatric	17	16.4 (±5.3)	0.0 (±0.00)	N/D	N/D	N/D	N/D	N/D
	November	Lamb	16	149.3 (±32.9)	0.1 (±0.36)	0.11	29.00	38.64	10.83	21.42
		Yearling	13	52.9 (±18.5)	0.0 (±0.00)	1.88	39.20	47.82	8.38	2.72
		Adult	26	4.4 (±1.0)	0.0 (±0.00)	N/D	N/D	N/D	N/D	N/D
		Geriatric	19	10.0 (±2.8)	0.0 (±0.00)	3.46	4.05	87.89	4.28	0.33
	February	Lamb	11	307.5 (±60.0)	9.5 (±16.24)	1.40	72.53	10.91	6.04	9.12
		Yearling	6	180.2 (±89.5)	0.0 (±0.00)	0.00	37.22	41.10	21.68	0.01
		Adult	34	28.7 (±9.8)	0.0 (±0.00)	8.85	18.38	45.38	27.38	0.01
		Geriatric	15	139.6 (±80.6)	0.0 (±0.00)	0.03	7.09	56.42	4.43	32.03

Spring 2016	March	Lamb	17	944.8 (±161.2)	111.7 (±31.66)	0.01	38.84	14.48	14.76	31.91
		Yearling	10	149.6 (±57.2)	0.0 (±0.00)	1.71	37.82	23.15	12.22	25.10
		Adult	27	34.3 (±9.3)	0.0 (±0.00)	0.01	1.00	40.21	4.24	54.54
		Geriatric	5	71.8 (±32.3)	0.0 (±0.00)	0.90	18.34	46.63	4.00	30.13

N/S – group not sampled from. N/D – No speciation data available.

Table 5. 6. Full summary of male Soay sheep speciation and relative proportional data, with strongyle and *Nematodirus* faecal egg count (FEC) as eggs per gram of faeces (epg).

Season	Month	Group	n	Strongyle FEC (epg) (±SEM)	*Nematodirus* FEC (epg) (±SEM)	Relative species proportion (%)				
						B. trigonocephalum	*C. ovina*	*T. circumcincta*	*T. axei*	*T. vitrinus*
Spring 2015	April	Lamb	N/S							
		Yearling	22	955.1 (±179.0)	105.9 (±27.64)	0.02	42.07	24.07	10.20	23.64
		Adult	28	305.9 (±68.9)	4.0 (±2.21)	9.76	43.01	8.40	10.63	28.21
Summer 2015	July	Lamb	32	121.3 (±33.6)	143.1 (±28.05)	4.17	1.30	36.76	0.62	57.16
		Yearling	11	116.8 (±30.8)	0.1 (±0.09)	35.67	20.79	0.91	27.36	15.27
		Adult	17	N/D		55.05	14.23	0.15	30.37	0.21
	August	Lamb	42	171.8 (±26.3)	67.3 (±14.23)	0.01	2.46	26.42	13.80	57.32
		Yearling	8	197.8 (±52.8)	0.1 (±0.13)	N/D	N/D	N/D	N/D	N/D
		Adult	11	106.9 (±25.6)	0.0 (±0.0)	71.42	23.32	0.34	1.71	3.21
Winter 2015	October	Lamb	14	484.7 (±57.7)	9.1 (±3.734)	N/D	N/D	N/D	N/D	N/D
		Yearling	9	94.7 (±15.7)	0.0 (±0.0)	10.72	70.93	3.67	12.12	2.56
		Adult	13	N/D	0.0 (±0.0)	N/D	N/D	N/D	N/D	N/D
	November	Lamb	20	394.0 (±151.2)	3.9 (±11.77)	20.39	26.48	25.08	7.53	20.53
		Yearling	11	120.8 (±41.3)	0.0 (±0.0)	0.00	39.27	34.95	25.76	0.01
		Adult	15	169.9 (±65.0)	0.0 (±0.0)	9.35	12.38	75.07	1.81	1.39
	February	Lamb	1	N/D		N/D	N/D	N/D	N/D	N/D
		Yearling	3	296.0 (±123.8)	0.0 (±0.0)	0.00	24.41	48.07	22.50	5.01
		Adult	3	50.7 (±24.7)	0.0 (±0.0)	45.46	1.83	49.64	3.06	0.01
Spring 2016	March	Lamb	5	390.6 (±110.8)	0.0 (±0.0)	0.45	25.94	15.68	11.47	46.45
		Yearling	6	101.3 (±46.1)	0.0 (±0.0)	7.57	46.44	14.78	20.58	10.63
		Adult	14	83.5 (±28.0)	0.0 (±0.0)	2.76	22.45	46.18	9.19	19.41

N/S – group not sampled from. N/D – No speciation data available.

5. 5. DISCUSSION

This is the first study to look at the seasonal parasitology of the Soay sheep in this way, and one of the first to use the nemabiome assay in sheep, and in a non-production system. This chapter offers a novel parasitological snapshot of seasonal and host trends in healthy Soay sheep, and highlights the future applications of this sequencing platform to indirectly study parasites in faeces.

Due to the isolation of the study system and novelty of the sequencing method, samples were lost during culturing, transport, sequencing and bioinformatics. With incomplete datasets, the inaccurate inference of trends may arise. Therefore, the aim is to keep this study mainly descriptive. The analysis of the composition of different samples (and thus different species), can prove challenging. Standard measures of correlation assume the data are independent, which is not true of proportional (%) data. Even in the studies with more robust data, the danger of spurious correlations resulting from the analysis of proportional data (predominantly seen in microbiome studies) can occur, and the development of more robust tools of analysis is still being developed (Di Bella, 2013). As discussed in Chapter 3, inconsistencies in species proportions may be derived from external factors such as culturing methods, larvae storage, host-differences, and sequencing, but as all samples were handled in a similar way any potential biases are consistent throughout the study. However, many of the seasonal trends seen in this study conform to the previously known epidemiology of the parasites, which is encouraging. This study highlights some very important trends not previously studied in the Soay sheep, which merits further research in order to understand the epidemiology of GI nematodes in feral animals.

The quantitative data produced by the nemabiome assay can be adapted to show the estimated number of eggs (epg) allocated to each species. This method was originally developed for pre- and post-drench tests. FEC provides little information on the worm species present, and undifferentiated FECRT has the potential to produce results that are misleading. The development or potential reversion to resistance may be missed, therefore, the quantitative morphological identification of L_3 from larval culture were adapted to average FEC. This method of interpreting speciation results relies on the assumption all species are producing the same number of eggs, which, as highlighted in Table 5. 1., is not the case. However, this method is used in order to interpret and visualise the trends generated (McKenna, 1996; McKenna, 1990), and illustrates that adequate representation for individual nematode generally relies on a high egg count, and when the egg count is low, the differences in species proportions are not as impactful (McKenna, 1996). This is seen in many of the sex/age groups in this study; there are

no changes in the species composition with time, but the proportions remain consistent, notably in the male and female yearlings, where there is a low-level shedding of eggs over many sampling months. A trend not commonly seen in managed sheep farms.The presentation and interpretation of the nemabiome depends on the question being asked of the data. By converting the data to egg count, you are illustrating the level of pasture contamination, and which species are contributing to it. Whereas relative proportion describes which species are leaving the animal, and what relative proportions are associated with a sex/age group (Section 5. 4. 2) or overall seasonal trends associated with the parasite (Section 5. 4. 3). By understanding the epidemiology of the free-living stages of each species, trends within the host population can be inferred. Future analysis of this type of data should consider taking average FEC into account, as the trends seen when FEC is low, the trends may not be as pertinent to those months of high FEC.

Whilst the Soay sheep of Hirta provide a relatively simple study population in terms of the parasite species present (and no other grazing ruminants), the mechanisms which control the Soay sheep population numbers are not. This implies the trends in actual adult worm burden and egg output between the different sex/age groups are complex and subject to yearly change depending on the demographic structure and environmental factors of the island. Therefore, we must bear in mind that these samples may only represent a transitory, seasonal, parasitological snapshot of what is going on in the Soay sheep, and the monthly cross-sectional evaluation presented in this chapter requires careful interpretation. The only data that comparisons available are from naturally-deceased (weak/ sick) animals, additionally, this study is unlikely to be representative for the seasonality of the Soay sheep adult worm burden within the gut. Many factors contribute to the fecundity of an adult female worm, such as age and density-dependent fecundity. Arrested development may also halt egg production and may be dependent on both environmental and parasite-related factors (Schad, 1997). Density-dependent fecundity is the suppression of egg output in response to an increase in parasite burden (Keymer, 1982). Research in the Soay sheep suggests there is no density-dependent fecundity (Clutton-Brock and Pemberton, 2004). However, different parasite species appear to respond differently to increasing density, several studies indicate an absence of density dependent effects on fecundity in helminth species; *H. contortus* in sheep (Coyne et al., 1991) and *Trichostrongylus tenuis* in red grouse (Shaw and Moss, 1989). *B. trigonocephalum* is a hookworm, and many species of human and canine hookworms have been reported to have density-depended fecundity (Kotze and Kopp, 2008).

However, what the data does present is an interesting snapshot of the species infecting the sheep at the given time of sampling, and what is contributing to pasture contamination. This dataset suggest that there may be consistent trends between years when species proportions are compared between April 2015 and March 2016 for many of the age classes past 12 months (males and females). For example, the main species predominating in the cultures from adult females was *T. circumcincta* (April=34%, March 2016=40%) and *T. vitrinus* (April 2015=64%, March 2016=55%) (Figure 5. 7).. Future longitudinal data collection over many years would provide a clearer yearly picture of parasite dynamics. Combined with known knowledge of parasite and Soay sheep epidemiology and population demographics, we can start understanding what drives the parasitism on the island, with future predictions for the following year.

Seasonal variation in FEC is determined by temporal trends in the immunological status of the sheep (age, sex, nutritional status) as well as the number of viable L_3 available on pasture. Temporal trends in pasture contamination is determined by species-specific trends in larval development; time taken from egg to fully reproducing adult, each step can take hours or weeks and can be halted depending on the external environment. The Soay sheep have a dynamic life history (Figure 5. 1), which present periods of high physiological and energetic stress, which may impact parasitic resistance. The Soay males are subject to strong sexual selection, with an elevation of testosterone during the rutting months (October – December) (Figure 5. 1. B). During this time *T. circumcincta* and *C. ovina* predominate in the cultures in males over the age of 1 year. Females of *T. circumcincta* are less fecund than *C. ovina*, with an average egg production of 100 – 200 eggs per female, per day, in comparison to 5000-10000 eggs. With large differences in fecundity, the high prevalence of the low-shedding *T. circumcincta* is of note. Testosterone has been found to be immunosuppressive while oestrogen can stimulate the immune system (Zuk and Mkean, 1996; Schalk and Forbes, 1997). Previous studies of wild ruminants have found a strong positive correlation between testosterone levels and FEC (Decristophoris et al., 2007). The immunocompetence handicap hypothesis means males face the evolutionary trade-off between sex hormones and immune defence against parasites. Additionally, stress can lead to greater disease susceptibility (Schalk and Forbes, 1997). That being said, during the months of elevated testosterone is the time when parasitic larvae in the environment are at their lowest, additionally, males feed less due to rut-related activities such as mate-guarding, which would suggest the winter increase in *T. circumcincta* eggs is linked to hypobiotic larvae. Or, due to an reduction in feed intake, less faeces would be produced, potentially concentrating the eggs.

Periods of high annual mortality occur late winter to early spring (January – May) (Figure 5. 1. A&B). There is no clear species trend seen during the early part of the year, though more data is required during December – February. In lambs, all species (except for *B. trigonocephalum*) are present with the proportions remaining consistent. For the other groups the trends differ, though *T. circumcincta* and *C. ovina* are commonly seen in males and females. Individually, *C. ovina* has low pathogenicity, but may contribute to parasitic gastroenteritis (Roeber et a., 2014). It is interesting to note that the trends seen in the adult males are similar to that of the geriatric females; with a reduction of all species other than *T. circumcincta* between August 2015 and March 2016. *T. circumcincta* does not feed on blood, the larval stages cause the most pathogenic effects (Roeber et al., 2014). Biologically, the Soay males age faster than females and die younger, which is why a geriatric male group is absent from the study. *T. circumcincta* is believed to be a predictor of mortality in the Soays, as it was always found in high burdens in animals that had died during a crash (Craig, 2005; Gulland; 1990). During late winter-early spring there is high mortality within the population (Chapter 1).

Generally, for all sex-age groups, early spring (March – June) is characterised by having the highest average FEC (Chapter 2), which is due to many factors including periparturient rise (PPR), high population density with the arrival of the lambs, young lambs yet to develop immunity to parasites, males recovering from the previous mating season and warmer weather, allowing eggs to develop successfully and to re-infect. Immune competence is not acquired until ≥4 months of age (depending on breed/nematode species challenge) (Miller and Horohov, 2006), which is generally characterised by increasing FEC as they become infected, the parasites establish, triggering an immune response. The relaxation of the ewe's immunity during this time causes an increase in parasite egg output, known as the PPR, which can last up to 8 weeks after parturition. In the Soay sheep on St Kilda, the peak of PPR occurs within ~10 days of parturition (Wilson et al., 2004). The decrease of immunity increases the survival and egg production of parasites within the gut, in addition to increasing the hosts susceptibility to new infection, and contributes to high pasture contamination when the lambs begin to graze. During the PPR (April) yearling females had high *C. ovina* and *T. vitrinus* whilst the adult females were shedding predominantly *T. vitrinus* and *T. circumcincta*.

5. 5. 1. *Teladorsagia circumcincta* and *Trichostrongylus axei*

For both males and females, when the animals reach 24 months they shed fewer *T. axei* eggs and higher numbers of *T. circumcincta*. Both species are present in all months sampled for both males and females (except yearling females in August 2015). *T. circumcincta* appears to be associated with autumn and winter months (Figure 5. 12), whilst *T. axei* is prevalent at low

levels all through the year with no apparent seasonality (Figure 5. 14). In previous work, *T. axei* was associated with lambs (Craig, 2009), however, it is the only group to show a slightly, and consistent, elevated *T. axei* output is the yearling (13-24 months) males and adult females.

In previous studies, with increasing age there was a significant increase in the intensity of *Teladorsagia* spp. adult worm counts, with a corresponding decrease in *T. axei* intensity (though no significant correlation was found between the two) (Craig, 2005). Despite *T. circumcincta* and *T. axei* sharing the same environment within the host (abomasum), L_3 and L_4 develop in different micro-environmental niches. *T. axei* larvae invade the epithelial layer and lamina propria, whilst *Teladorsagia* spp. develop within the gastric glands (Balic and Bowles et al., 2000), this difference in invasion-sites may explain the differing host-age responses to infection, with a stronger acquired immunological response to the species that cause more damage. Turner et al., (1962) used experimental coinfections to measure the interactions between *T. circumcincta* and *T. axei* and found that whilst *T. circumcincta* intensity was strongly affected by *T. axei, T. axei* may actually be enhanced by *T. circumcincta* infections. The regulatory effects of acquired immunity are seen for both species; with *T. axei* intensity decreasing with host age (Gibson, 1952), and a weaker effect against *T. circumcincta* burdens. However, there is evidence of separate mechanisms of control in lambs (Wailer and Thomas, 1981). A reduction in *T. axei* infection only occurs after a threshold intensity is reached, whereas *T. circumcincta* has a more continual density-dependant turnover in control where adult worms are continually removed and replaced (Craig, 2005). *Teladorsagia* larval development occurs within the gastric glands, causing nodule formation in the abomasal mucosa and damage to parietal cells, resulting in reduced hydrochloric acid production and a reduction in abomasal pH, which can impact the establishment of other abomasal nematode species.

On-farm infections with *T. circumcincta* can present as Type I and Type II infections. Type I is triggered by the ingestion of large numbers of L_3 by lambs in late summer as there is a high level of larvae on pasture, contributed from both the peri-parturient females and young lambs developing their immunity. Type II is a chronic form of teladorsagiosis and is seen late winter to early spring, triggered by a mass emergence of hypobiotic larvae. The type I infection appears to become an important source of infection for both geriatric females and adult males; whilst the egg count is low over the winter months for both groups, *T. circumcincta* is predominating in the cultures. Previous work looking at the development survival of *T. circumcincta* on pasture found that the parasite survives well, and is capable of developing to L_3 during most times of the year. This suggests *T. circumcincta* overwinters better than other species, and that the eggs deposited onto the pasture over winter (namely from the rutting males and geriatric females)

will survive, and develop successfully in early spring (Waghorn et al., 2011). The age of the host has been shown to affect the hatchability and development success of *T. circumcincta*; eggs passed from lambs develop better than those by older sheep, where they are under continual pressure from the immune system (Jorgensen et al., 1998). Which this in mind, the true level of *T. circumcincta* within the geriatric cultures may be even higher.

5. 5. 2. *Bunostomum trigonocephalum*

B. trigonocephalum has the longest prepatent period of all the strongyle species infecting the Soay sheep; it has two routes of infection – oral and percutaneous. As it can take up to 9 weeks to establish and reproduce within the host, it would be expected that eggs were not shed by very young lambs, however, B. trigonocephalum was identified from both males and female lambs in this study in July, when the lambs were (approx.) 3 months old. The trends in this work mirrored the whole worm counts (WWC) conducted by Craig (2005); in her work B. *trigonocephalum* significantly increased with age ($p=0.001$). However, it was higher in males, and whilst the bias was not significant it was high ($p=0.022$). Additionally, she found no worms in lambs and yearlings. The positive association with host age implies there is no development of an acquired immunity resistance, despite a continual exposure to larval invasion via the gut and skin.

There is a peak seen in July (female and male lambs). Lambs born early March may be infected by the L_3 shed onto pasture by the adult males in February. As the parasite can infect percutaneously, they could infect the lambs through their belly or soft hooves. Also lambs have been observed nibbling vegetation before full weaning.

Bunostomum sp. (generally) resides in the host at low levels, and only takes a few hundred to cause pathology within the host (Craig, 2005; Graham., 1969), and therefore could be underestimated in previous post-mortem studies when only 1-5% of the gut is investigated. Additionally, like all data collected from morphological identification of adult worms, there may be a sex bias. As males are the ones identified morphologically, a sex-bias may skew gut-burden estimates.

B. trigonocephalum does not survive well on pasture, surviving around 2 months, additionally it arrests development at temperatures below 15°C entirely (Graham, 1969; Wimmer et al., 2004). Therefore, this species has a very narrow window of opportunity to develop to L_3, migrate from the faecal pellet and locate/ infect a host. The parasite is aided by having two routes of infection, and adult females that are highly fecund . These patterns are seen clearly in the males and females, with the short infection month between July and August – implying infection with

viable L_3 occurred April/May (or as early as March), a time when the lambs are born. Hence, it could be possible the lambs are infected to produce eggs in July. There is a small peak for both sexes in February, increasing eggs at the end of winter suggests the resumed development of inhibited larvae and increased fecundity by the remaining female worm population, contributing to pasture recontamination and continuity of the nematode life cycles.

5. 5. 3. *Trichostrongylus vitrinus*

From 24 months of age until 8 years of age, in both males and females, *T. vitrinus* only occurs at low levels within the cultures, with a slight spring rise. Even then, *T. vitrinus* is not as prevalent within the cultures as the other species. This is similar to the trends found by Craig (2005), who found a negative association between prevalence and host age, with a male bias in infection. There is an increase in *T. vitrinus* in the geriatric females, and like the lambs, may be due to insufficient immunity. Previous work done in the Soay found senescence-linked increase in faecal egg counts in animals over the age of 8 (Chapter 2).

5. 5. 4. *Chabertia ovina*

Similar to that of *B. trigonocephalum*, a single *C. ovina* adult female can produce 5000-10,000 eggs a day, therefore there is the potential for *C. ovina* to 'flood' the coproculture when the females start to shed eggs. C. ovina may be associated with the PPR in young animals (yearling females). Additionally, it appears to shed the highest during winter, during a time when other species have either in hypobiosis or halted egg production. The significance of this finding is it indicates the levels of pasture contamination will be dominated by C. ovina, but its survival during extreme cold temperatures is limited.

5. 5. 5. *Nematodirus*

Nematodirus spp. are considered a parasite of lambs, with severe infection commonly seen in Spring. High burdens are often associated with sudden scouring and weightless. Severe larval challenge can result in death (Urquhart et al., 1996). It is not commonly identified in adult sheep. During this study, *Nematodirus* was identified in all sex/age groups in Spring 2015 (Table 5. 5. And Table 5. 6). The detection of this low level infection may have occurred due to the specificity of the faecal egg counting methodology, that had a detection limit of 5 epg, which would have been able to pick up low-level infection.

5. 5. 6. Concluding remarks

Through the identification of nematode species from faeces, we were able to reveal more about how different species challenge hosts during different seasons. We were able to demonstrate

how this changes between different ages, sexes and seasons, and is one of the first in-depth studies looking at healthy animals as they transition between different life-events and seasons.

Potential differences from the patterns of co-infecting parasites seen in managed sheep populations are intriguing. For example: the presence and seasonal patterns of *C. ovina* and *B. trigonocephalum*, which are now considered rare in managed flocks; and the different seasonal predominance of *T. circumcincta*. These different seasonal trends in the predominance of individual gastrointestinal nematode species will be driven by: temperature, moisture and physical characteristics of the biomes occupied by the parasites' free-living stages; immunologically mediated host responses; and regulatory influences of one nematode species upon another. The manner whereby factors might have act independently and, or, interactively to determine the impact of the burden of particular parasitic nematode species on their sheep hosts is unknown and merits further research.

CHAPTER 6

General discussion and future directions

6. 1. THESIS SUMMARY

Co-infection with one or more gastrointestinal nematode species is ubiquitous in feral and managed ruminants. Within these mixed burdens, the different species can vary in their pathogenicity, epidemiology and clinical presentation. Elucidating the diversity of different parasite species in a host, rather than studying them as a homogenous group, is a prerequisite to understanding host-parasite interactions. Previous parasitological assessments on St Kilda have been predominantly through post-mortem investigations of adult worm burdens during high mortality years. There are no data available for the population in low mortality years, or for those individuals surviving a crash year. FEC have been used as a non-invasive assessment of annual parasite burden, though this provides limited information as the predominating strongyle species that infect the Soay sheep produce morphologically-similar eggs, therefore the mixed-species burdens occur in unknown proportions. The primary aim of this thesis was to explore and validate non-invasive conventional and molecular parasitological methods to identify and quantify mixed-species infections in feral sheep hosts.

Seasonal patterns of gastrointestinal nematode parasitism in the Soay sheep were investigated by faecal egg counts (FEC). Two FEC databases were compared; a longitudinal study (27 years) with many replicates (12192) counted by modified McMaster, with a high detection limit (100 epg), and a shorter study (1 year) with 0.07% of the samples (882) counted by cuvette salt floatation, with a lower detection limit (1 epg). FEC generally declined with increasing sheep age, until the animals became geriatric (8 years +). Seasonal FEC patterns in females generally followed a decline over the year starting in Spring. FEC were generally higher in males, regardless of their age category, with little seasonal variation between summer and winter once they became adults (3 years +). Monte Carlo simulations were run in order to compare the effects of different detection limits (1, 10, 50, 100 epg). The simulations suggest that the method with the detection limit of 50 – 100 epg potentially underestimates low-level counts, and over-estimates the true egg counts within the samples at high counts, resulting in data that is highly negatively skewed and with an inflated mean. Despite within-year variation of egg counts seen in the cuvette salt floatation dataset, general seasonal and host trends in FEC were similar for both datasets, as the lower detection threshold (1 epg) resulted in more egg-positive samples. Therefore, a shorter study with fewer replicates may benefit from a FEC method with a lower detection threshold.

Whilst FEC can identify general trends in parasitology, it is too crude a method to distinguish parasite diversity within the helminth group. The development of advanced molecular methods for the genus/species-specific diagnosis of GIN infections in ruminants has negated many of the

issues arising from traditional parasitological techniques, such as FEC and L_3 morphological identification. Chapter 3 compared two molecular methods; the semi-automated multiplex-tandem PCR (AusDiagnostics™) with ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay), to identify (presence/absence) and quantify (% proportion) ovine strongylid species in naturally infected samples collected from the Soay sheep. The Ausdiagnostics™ platform identified *Teladorsagia circumcincta*, *Chabertia ovina* and *Trichostrongylus* spp. In addition to identifying these species, the nemabiome assay identified *Bunostomum trigonocephalum*, and was able to speciate the *Trichostrongylus* genus to *T. vitrinus* and *T. axei*, thus proving to be the most specific method for identifying the novel strongylid species associated with a feral host.

The sensitivity, bias, and repeatability of the nemabiome assay was tested for the strongylid species identified in the Soay sheep of Hirta (Chapter 4). The advantage of working with L_3 is it is easier to test for, and correct, biases within the sequencing data. The main aim of this chapter is to validate the nemabiome assay by testing artificially-made pools of L_3 for biases that may be introduced by DNA extraction efficiency, within-sequencing repeatability and species-specific biases during ITS-2 amplification. A correction factor was calculated from these biases, for each species identified from the Soay sheep, in order to reduce potential species-specific sequencing bias, and was subsequently applied to the analysis of the field data (Chapter 5).

By using methods compared and validated in this thesis, Chapter 5 provides an epidemiological survey of the strongyle nematode species infecting the Soay sheep of Hirta, St Kilda. Between April 2015 and March 2016, a total of 918 individual on-pasture faecal samples (607 female and 311 male) were collected, from lambs (4 to 12 months-old), yearlings (13 to 24 months-old), adults (3 to 7 years-old), and geriatric females (8 to 14 years-old). Individual strongyle/ *Nematodirus* faecal egg counts were conducted by a cuvette salt floatation method (validated and analysed in Chapter 2). The faeces were then pooled into composite coprocultures (per sex/age/season) and incubated to grow third stage larvae (L_3). Pools of 500 L_3 were speciated and quantified into relative proportions by using the nemabiome assay. The reads were corrected by previously calculated correction factors to account for potential species-specific bias in amplification (as described in Chapter 4). This is the first study to use the nemabiome assay to accurately characterise host gastrointestinal nematode burdens and study seasonal patterns in different co-infecting gastrointestinal nematodes in feral sheep.

6. 2. GENERAL DISCUSSION

6. 2. 1. General parasite trends

Previous parasitological assessments on St Kilda have been predominantly through opportunistic post-mortem investigations of adult worm burdens during high mortality years. There is no data available for the population in low mortality years, or for those individuals surviving a crash year due to the ethical constraints of culling healthy animals. FEC have been used as a non-invasive assessment of seasonal parasite burden on St Kilda, and it is a well-established method to indirectly estimate infection intensity (McKenna, 1987), and to determine pasture contamination with parasitic eggs. However, FEC provides limited information as the strongyle species that infect the Soay sheep produce morphologically-similar eggs, therefore, the mixed-species burdens occur in unknown proportions. The fecundity of different GIN varies, and while the diagnostic value of FEC to estimate worm burden has been shown in highly fecund species (i.e. *Haemonchus contortus, C. ovina*), there is a lower correlation for genera with low fecundity (i.e. *Teladorsagia*, *Trichostrongylus*) (Roeber and Kahn, 2014). A large contribution of eggs from *Teladorsagia* and/ or *Trichostrongylus*, would be regarded as highly significant in comparison to *H. contortus* or *C. ovina*, as it indicates a higher worm burden in comparison to the more fecund species (Roeber and Kahn, 2014). As presented in Chapter 2, FEC trends in the Soay sheep generally declined with increasing sheep age, until the animals became geriatric (8 years +); lamb > yearling > adult < geriatric. Seasonal FEC patterns in generally followed a decline over the year starting in spring; spring > summer > winter. FEC were generally higher in males, regardless of their age category, with little seasonal variation between summer and winter once they became adults.

Both male and female lambs appear to have no true parasite trends, and shed parasite eggs from all species throughout the year. These trends may be due to the establishment of the parasite coinfection community within the gut, and the development of host immunity. As female lambs reach their first birthday (March 2016), many will be pregnant, therefore the increase in FEC in spring (mostly contributed by *C. ovina* and *T. vitrinus*) may be due to the periparturient rise (PPR) (Figure 5. 3, Chapter 5). Similarly, this trend is mirrored by the yearling females in April 2015 (Figure 5. 5. Chapter 5). However, the PPR in adults and geriatrics are contributed largely from *T. vitrinus* and *T. circumcincta* (Figure 5. 7. Chapter 5). According to the FEC data, males will generally have a higher FEC in comparison to females. In the adult males this may be driven by *T. circumcincta* the highly fecund *C. ovina*. The high prevalence of *T. circumcincta* in the adult male cultures during winter (November 2015) is of note, and links to other studies on the Soay sheep linking winter mortality to high burdens of *T. circumcincta*

(Craig, 2005). Additionally, *T. circumcincta* appears to be an important parasite of geriatric females (8+ years) (Figure 5. 9) though more data is required. In comparison, the other Trichostrongylid specie, *T. axei* was identified in all samples, and appear consistently at low levels throughout the year. It is thought *T. axei* infection declines with host age (Gibson, 1952) through the regulatory effects of acquired immunity. However, in the Soay sheep they may be maintaining a low-level population, regardless of sex or age.

These coinfection trends are driven by complex interactions of many biological factors. This thesis provides a preliminary investigation, and future research will need to include many more animals sampled over many sampling months/ years.

6. 2. 2. Molecular parasitology and an isolated field site

Wimmer et al., (2004) was the first to speciate the parasites of St Kilda through DNA amplification and was able to obtain the prevalence of nine helminth species living in the Soay sheep through differentiation of eggs concentrated from faecal sample. The method was qualitative, providing a presence/ absence of infecting species, and provided the first species assessment in a healthy Soay sheep. However, purification of eggs from faecal samples is time-consuming and the phenols and other inhibiting substances within the faeces can mean amplification can fail. Additionally, eggs from the moment they are shed from the host onto the pasture will start to develop which has implications when quantifying relative species proportions.

During this project, both the semi-automated multiplex-tandem PCR (AusDiagnostics™) and ITS-2 rDNA next-generation amplicon sequencing (nemabiome assay) were in development. There was no previous work applying these methods to a wild or feral population, or any validated studies on ovine parasites, which presented an exciting opportunity to use brand new methods in a novel system. At the time that the St Kilda samples were sequenced (2016), there were two concurrent studies developing the nemabiome methodology; thone developing the Nemabiome for bovine strongylids (Avramenko et al., 2015) and another validating the nemabiome method for European ovine parasites (Redman et al., 2019). The method has now been developed to identify strongyle species in bison (Avramenko et al., 2018), domestic sheep (Redman et al., 2019; Hamer et al., 2019), dairy heifers (Scott et al., 2019), domestic horses (Mitchell et al., 2019; Chaudhry and Sargison, *unpublished*), feral Sable island horses (Gilleard et al., *unpublished*) and deer (Gilleard et al, *unpublished*). Additionally, the nemabiome assay has been recently validated against standard PCR, eggs, L_1 and L_3, and morphological identification of a plethora of ovine strongyle species (Redman et al., 2019). L_3 are ideal for validation studies,

as they can be morphologically identified, mock populations could be easily tested for biases in amplification and limits to detection in mixed-species pools (Chapter 4).

However, regardless of how rudimentary a coproculture is, in a field-setting on St Kilda there were logistical constraints in terms of equipment, maintaining constant ambient temperatures for culture, and transport of live larvae. Additionally, the Baermann equipment for extracting larvae from faeces to clean water had to be adapted for the field. It is well-known that the culturing conditions can affect the species proportions grown through differential die-off (Chapter 3). To account for this, the conditions were kept consistent throughout the study, so if there were errors they were consistent errors throughout the collections. Recent research compared the ITS-2 rDNA amplicon sequencing data generated from harvested eggs, L_1 and L_3 from faecal culture identified no statistical significant differences for the more abundant parasite species (Redman et al., 2019). This could provide exciting new avenues of research in the Soay sheep. To help avoid issues of PCR inhibition by contaminants from the faeces, eggs extracted after a faecal egg count could be washed and then fixed in ethanol or frozen. The cuvette salt floatation method is able to extract the eggs from a faecal sample at high sensitivity (1 egg per gram) (Chapter 2), following an egg count the suspension liquid could be sieved and the eggs pooled into an Eppendorf. This provides more flexibility in the field, allowing for more individual-collected data to be analysed. Annual faecal collection and FEC occurs on St Kilda, eggs could be extracted, and a bank of samples collected. Illumina MiSeq is high-throughput; it is capable of sequencing 450 individual pools or mixed-species larvae or eggs without compromising the sequencing coverage (i.e. number of reads per sample). Additionally, by analysing eggs, other species known to infect the Soay sheep can be studied.

Other clade-V species, such as *Dictyocaulus* and *Nematodirus*, were not identified by the nemabiome approach due to the need for specialist coproculture and/or Baermannisation methods. *Nematodirus* requires a chilled period during culturing to allow eggs to hatch, and due to their eggs being morphologically distinct during the FEC, there was no need to culture these separately. However, the *Nematodirus* sp. counts are mixed-species; two species of *Nematodirus* have been shown to infect the Soay sheep (*N. battus* and *N. fillicollis*). Studies into the interaction between these two species would be of interest, testing whether one is more prevalent than the other. The lungworm *Dictyocaulus filaria* has previously been identified in the Soay sheep by morphological identification of the adults in the airways (Gulland et al., 1992), morphological identification of the L_1 within the faeces (Craig, 2005) and through molecular speciation of the L_1 (Wimmer et al., 2004). *D. filara* L_3 were not identified in the coprocultures as *Dictyocaulus* sp. have an inactive L_3 stage, so they do not migrate through a

traditional Baermanns filter and would require either a sugar/salt floatation. Additionally, they are a parasite commonly seen in autumn/ winter, and culture best at lower temperatures (around 16°C) (Rose et al., 1962). To my knowledge, no attempt to quantify *Nematodirus* eggs or *Dictyocaulus* L_1 has been made, but recent advancements into adapting the nemabiome assay to other parasitic species, including beyond clade-V species, (piroplasm haemoprotozoa (Chaudhry et al., 2019); *Calicophoron daubneyi* (Sargison et al., 2019[b])) means this development is in the near future. Both *Nematodirus* and *Dictyocaulus* are associated with young animals, and high burdens may have detrimental impacts on growth and survival. In order to fully understand host-parasite interactions, ideally all the parasites would be studied.

6. 2. 3. *B. trigonocephalum* and *C. ovina* – neglected species?

Prior to the research presented in this thesis, parasitological research on the Soay sheep of St Kilda relied upon FEC and morphological identification of adult worms, extracted from animals that died naturally, most notably during years of high morality. These methods do not allow for speciation data to be collected for those individuals surviving the crash years, or in living hosts in low mortality years.

The parasitological research conducted on the Soay sheep has developed with the available methodology. It was initially presumed that *Teladorsagia* sp. was the predominating nematode species in all age classes of Soay sheep of Hirta (Gulland, 1992; Wilson et al., 2004). However, a large-scale necropsy study (Craig, 2005) and molecular PCR speciation of nematode eggs in faeces (Wimmer et al., 2004) revealed a more complex situation, with not one nematode species predominating through the life of the Soay sheep. *T. circumcincta, Trichostrongylus axei* and *Trichostrongylus vitrinus* were the most prevalent species in the Soay sheep, (Craig et al., 2006). The work conducted in this thesis indicates a large contribution of *C. ovina* and *B. trigonocephalum* to the species proportions for all sex/age groups across the different seasons.

C. ovina was identified, at some level, in all sex/age groups across all seasons. Infection with *B. trigonocephalum* appears to increase with host age until they reach prime age (8+ years), though this would needed to be confirmed with further data collection. Previous studies may have missed burdens as infection may have occurred below the level of detection. Craig (2005) calculated the percentage of adult strongyle-egg producing *B. trigonocephalum* females and found 0.06% in male lambs, and no higher than 2.9% found in male adults. Therefore, despite a very low gut burdens the 0-2.9% of fecund *B. trigonocephalum* females, those eggs contribute highly to pasture contaminations seen in July-August; 55-71% of cultures in adult males and 55-60% of cultures in adult females (Chapter 5).

B. trigonocephalum is a hookworm of sheep, but has been identified in a number of wild and feral hosts; Iberian ibex (Perez et al., 1996), European bison (Karbowiak et al., 2014) and fallow, red and roe deer (Pav et al., 1975; Zalewska-Schonthaler and Szpakiewicz, 1986; Demiaskiewicz et al., 2002). These blood feeding nematodes are highly pathogenic in domestic, wild and human hosts. It has been reported in domesticated sheep that only a small number of adult worms cause haemorrhagic enteritis, anaemia and death (Graham, 1969). Despite its known pathology in domesticated ruminants, there is little evidence of the pathological impacts of *B. trigonocephalum* in wild hosts and research in this field, to date, is rudimentary (Seguel and Gottenker, 2017). Importantly, in the context of the Soay sheep, a parasite that is detected very low levels through conventional methods, may be an important species to study in terms of parasite-host co-infections.

Hookworms are associated with density dependent depression of parasite fecundity. Hookworm biomass (prevalence, burden and worm size) within a population of other co-infecting parasites within the small intestine, drives the pathogenic impacts of the hookworm (Seguel and Gottenker, 2017). By utilising molecular parasitological methods by in directly measuring burden from faeces now has provided tools in which these factors can be measured.

6. 3. FUTURE DIRECTIONS

6. 3. 1. Group vs. individual parasitism

The aim of this thesis was to attempt to measure 'group' parasitism, or the representative burden of a demographic group, therefore, faeces were pooled together per sex/age group into composite. By pooling faeces, we were ensuring we had enough parasitic DNA in order to accurately speciate L_3, as at the time of study planning, the diagnostic sensitivity of the nemabiome assay in sheep or in the field (Chapter 4) was not known. In order to mitigate any bias, and to ensure that the parasite species composition of the pooled coproculture was not disproportionally influenced by that of any one individual sheep, equal weights of faeces per animal was used.

However, as discussed in Chapter 2, a main feature of host-parasite interactions is the wide variation in infection and infectiousness (Lass et al., 2013). Parasites are usually aggregated over the host population; many individuals having low to zero counts, while a few will have very high counts and are responsible for a large proportion of the pasture contamination. Whilst the main population has a natural resistance to large burdens of parasites and do not typically expresses disease, some individuals have the potential to be highly infectious and are referred to as 'super-shedders'. There is a major challenge to identify the underlying mechanisms which may

generate super-shedders, and one of the drivers may be interactions with a secondary infection (bacterial or viral), or co-infection through the simultaneous infection with two or more parasitic species (Lass et al., 2013). Are certain parasitic species associated with super-shedders? Which species are contributing to these large pasture contaminations? Are they mixed species or mono-specific? Additionally, this raises the question as to how different parasite diversity arises within individuals of the same demographic group, and what implications these differences have on their respective reproductive success and survival.

Investigating within-host differences at key life-stages of the Soay sheep would give a unique insight into the development of the parasitic biome in an unmanaged system. By following lambs, we could investigate development of immunity and the associated development and establishment of the parasite community. In comparison, the senescence-linked depression in immunity seen in geriatrics could provide a unique insight into the parasite community structure associated with aging. The results in this thesis indicate something interesting which warrants further investigation; lambs over their first year appear to shed eggs from all species whilst geriatric females appear to have high mono-specific burdens of *T. circumcincta*.

6. 3. 2. Temporal dynamics of pasture contamination – future considerations

During this thesis, the aim of culturing and speciating L_3 was to monitor and identify sources of pasture contamination. A better understanding of the epidemiology of the nematodes includes unravelling the dynamics of the free-living stages (eggs - L_3) on pasture. When the environmental aspect of the life cycles are better understood, the dynamics of the free-living stages on pasture can be linked to the Soay sheep population density, food availability and climatic conditions. There have been studies on St Kilda studying the temporal changes (seasonal patterns associated with weather), spatial variation (distribution, on pasture, across Village Bay), and development and survival of L_3 (Gulland, 1992), see chapter 1 and 5 for discussion. However, none of these studies were able to speciate the infecting larvae. Some species can survive longer on pasture then others (Table 5. 1), therefore, if there is a high pasture larval count in, for example, in winter, but the specie/s present do not survive well in cold climates, the elevation in counts may not be that impactful in re-infecting sheep. Molecular methods have been developed to identify larvae extracted from pasture (Bisset et al, 2014; Sweeny et al., 2012), and the nemabiome could be developed for pasture larval counts. While an understanding of the seasonality of egg hatching, development and survival of L_3 is likely to improve our understanding of the seasonality of the different nematode species, recent studies have indicated that the dynamics of parasites on pasture may be more complex. L_3 have been recovered from both faeces and soil. The presence of large numbers of L_3 in microhabitats other

than on herbage (where they are ingested), means they could be present without the livestock ingesting them. Additionally, if they can move between these environments, the pasture larval contamination could potentially change quickly as the larvae migrate up onto the grass when conditions are favourable, potentially triggering an outbreak in infection (Waghorn et al., 2011; Waghorn et al., 2002; Leathwick et al., 2011). Additionally, the role of earthworms ingesting faeces and introducing parasites to the soil has been noted, and would require further investigation on St Kilda(Waghorn et al., 2011). Faeces that have been experimentally buried under the soil yielded greater number of L_3 than what was on the soil surface (Waghorn et al., 2011).

6. 3. 3. Expanding co-infection studies: Exploring infracommunity and the microbiome

The study of co-infection and interspecific parasite interactions is becoming increasingly important; infection with multiple species is the norm, and the tools of assessment are becoming readily available. The application of high-throughput metabarcoded DNA sequencing methods, has revolutionised our understanding of microbial and parasitic diversity, allowing for a broader investigation of entire communities (Aivelo et al., 2018[b]). Co-infection can be defined as the process whereby multiple species, or genera, infect a single host. In the case of the clade V parasitic nematodes, this involves the independent production of eggs or $L_{1,}$ which can be used to in-directly measure internal parasitism (May and Nowak, 1995). Many gastrointestinal parasite populations coexist within a single host, collectively these parasitic populations can be referred to as an infracommunity (Bush and Holmes, 1986). The structure, dissemination and size of these infracommunities are restricted by the space within the host and limited to the hosts lifespan. These communities can be made up of different parasitic species and genera, and will either interact, remain isolated or compete with one another (isolationist vs. interactive parasitic communities) (Pugachev, 2000). Competitive interactions amongst parasites can be beneficial to the host, they may result in lower fecundity, slower growth and/or reduced pathogenicity and lifespan of individual species. Competition between different parasitic species is usually unbalanced; some species may be largely affected whilst to others it is minimal (Knowles et al., 2013). The susceptibility of the host to parasitic infection can be modulated, either directly or indirectly, by existing parasites (Aivelo, 2015). For example, one of the characteristic traits of parasitism by *Haemonchus contortus* and *Teladorsagia circumcincta* is the elevation of abomasal pH, which creates an environment unfavourable to other abomasal parasites, which can prevent or limit the establishment of other abomasal species (Li et al., 2016). Furthermore, *H. contorus* egg production is correlated to abomasum pH (Honde and Bueno, 1982). Therefore, by expanding research beyond a single species we can improve our

understanding of these interactions. By studying wild host populations, such as the Soay sheep of St Kilda, we can study these interactions in the absence of management interactions; allowing parasite infections to occur naturally without the aid of artificial infections, or the need for intervention once the infection occurs.

The research presented in this thesis has focused mainly on the impacts of strongyle nematode species (clade V) on the St Kilda Soay sheep. However, they are also host to protozoa, fly, louse and tapeworm species, including at least 11 *Eimeria* species, *Giardia duodenalis, Crytosporidium parvum, Melophagus ovinus, Bovicola ovis* (formerly *Damalinia ovis), Moniezia expansa and Taenia hydatigena* (Clutton-Brock and Pemberton., 2004). Whilst ectoparasites can be easy to identify, internal endoparasites require additional methods. In order to fully understand co-infection within the host, research will need to be broadened to include more than just clade V nematodes (strongyles) in co-infection studies.

Eimeria species, develop within the small and large intestine causing coccidiosis, which can be an important disease of young lambs. Infection is associated with high stocking density, and is characterised by diarrhoea, poor body condition and weight loss. Past experiments have provided evidence of negative interactions between *Eimeria* and gastrointestinal nematodes; following anthelmintic treatments fo gastrointestinal nematodes, *Eimeria* parasites increased (Fenton et al., 2014). *Eimeria* infection is usually diagnosed by coproscopical examination, by floating oocysts from faecal material with a saturated saline flotation solution (usually a McMasters technique). This is quick and does not rely on technical equipment, however, oocyst counts may not be indicative of true burden as these parasites can multiply within the host. However, identification and speciation of *Eimeria* requires a high level of training, and is usually made on the basis of the morphological criteria of the oocysts after sporulation (Chartier and Paraud, 2012). The metabarcoding approach through amplification of the 18S rDNA has been applied to identify *Eimeria* species in fish (Scheifler et al., 2019) and poultry (Hinsu et al., 2018). Whilst this method was able to identify the presence of several species, further work is needed to ensure accurate detection, as Hinsu et al., (2018) noted potential false positives. Lastly, further work is needed in order to characterise novel species, which highlights an important caveat to this type of methodology: in order to accurately identify infective species, a reliable reference sequence is required. There are coccidea of sheep with uncertain taxonomic status, such as *Eimeria (Globidium) gilruthi* (Maratea and Miller, 2007). Reference sequences rely on the accurate morphological characterisation of the parasite and for some Eimeria species, these morphological keys are either rare, or do not exist.

Microbiotia play a pivotal rose in host nutrition, health, in addition to supporting host immune development (Li et al., 2016.) Infection with helminth parasites has been found to prompt significant change in the function and structure of the host gut microbiome. In humans, those with helminth infections had greater microbial diversity, though future work is required to fully understand these relationships (Lee et al., 2014). Mockler et al., (2018) found that the microbiome can have an impact on the parasite community. Even subtle changes to the microbiome structure in bumble bees had significant impacts on its ability to defend against *Crithidia bombi*, though the characteristics of the microbiome responsible for this interaction has not yet been identified. Abomasal nematodes can elevate the pH of the hosts abomasum, by inhibiting gastric acid production. These changes trigger an increase in anaerobic bacteria in the abomasum, suggesting parasite infection can alter and change the structure and function of the microbiome. Again, further work is required to fully understand these relationships, as the microbiomes of ruminants are complex (Li et al., 2016). Based on mouse studies Harp et al., (1992) found that normal intestinal flora decreased susceptibility to infection by *C. parvum*, whilst the presences of microbiota increased the pathogenicity of different *Eimeria* species (Visco and Burns, 1972; Berrilli et al., 2012). With several of these studies, many of these interactions, whilst identified, are not well understood, and raises questions on what their ultimate impact on the host actually is (i.e. survival, reproduction, mortality). The next stage is to characterise the microbiome structure in order to understand these complex interactions between parasites and microbiome.

As with any population study, either microbiome, nemabiome or even the sheep themselves, the interpretation of results is important in the context of a biological system. There must be a clear understanding between differences of causation and effect. Parasite interactions are variable and context-dependent, which adds a layer of complexity. It may be that interactions are only significant under specific conditions (dependent on the timing of co-infection), or significant within a small proportion of the host population (e.g. certain age, sex or those that are immune-compromised). Additionally, these studies will be skewed and biased by the methods in which they are measured. These new methods of microbiome and nemabiome study are usually an in-direct measure of internal interactions (i.e. they measure microbiota and parasites in faeces). How different is this from the microflora directly from the gut (abomasum, small intestine) fluid? How does this impact our understanding of the data? In the context of the Soay sheep, the bulk of the study conducted on the population is indirect, and any internal examinations made will only occur after they die naturally, therefore skew the data. This is further confounded by the fact different studies, to date, will often use different statistical

approaches to infer interactions, therefore, it is not always clear whether reported trends or differences have resulted from actual biological systems, or differences in the methods used.

6. 4. CONCLUDING REMARKS

Quantifying L_3 from different gastrointestinal nematode species has revealed a unique insight into the parasitism of the Soay sheep of St Kilda, revealing a complex community where no single species predominates through the life of the host. New molecular parasitological methods have provided tools with which to in-directly measure co-infections. This work can now be developed to start exploring theories regarding associations with host genetic variation and survival, providing a unique study opportunity to explore and understand the evolutionary consequences of co-infection in wild hosts.

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A.3. APPENDIX – Chapter 3

Supplemental data for Chapter 3.

Table A3. 1. ITS-2 Primers with Illumina Adapters (from Avramenko et al., 2015).

Primer Name	Primer Sequence (5' - 3')*
NC1	ACGTCTGGTTCAGGGTTGTT
NC2	TTAGTTTCTTTTCCTCCGCT
NC1Adp	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACGTCTGGTTCAGGGTTGTT**
NC1Adp1N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGN**ACGTCTGGTTCAGGGTTGTT**
NC1Adp2N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNN**ACGTCTGGTTCAGGGTTGTT**
NC1Adp3N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNN**ACGTCTGGTTCAGGGTTGTT**
NC2Adp	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TTAGTTTCTTTTCCTCCGCT**
NC2Adp1N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGN**TTAGTTTCTTTTCCTCCGCT**
NC2Adp2N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNN**TTAGTTTCTTTTCCTCCGCT**
NC2Adp3N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNN**TTAGTTTCTTTTCCTCCGCT**

Table A3. 2. A. Forward barcoded primers (Illumina Sequencing) (from Avramenko et al., 2015).

Primer Name	Primer Sequence (5' - 3')*
N501 i5	AATGATACGGCGACCACCGAGATCTACAC**TAGATCGC**TCGTCGGCAGCGTC
N502 i5	AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**TCGTCGGCAGCGTC
N503 i5	AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**TCGTCGGCAGCGTC
N504 i5	AATGATACGGCGACCACCGAGATCTACAC**AGAGTAGA**TCGTCGGCAGCGTC
N505 i5	AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**TCGTCGGCAGCGTC
N506 i5	AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**TCGTCGGCAGCGTC
N507 i5	AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**TCGTCGGCAGCGTC
N508 i5	AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**TCGTCGGCAGCGTC

Table A3. 2. B. Reverse barcoded primers (Illumina Sequencing) (from Avramenko et al., 2015).

Primer Name	Primer Sequence (5' - 3')*
N701 i7	CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTCTCGTGGGCTCGG-
N702 i7	CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTCTCGTGGGCTCGG
N703 i7	CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTCTCGTGGGCTCGG
N704 i7	CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTCTCGTGGGCTCGG
N705 i7	CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGG
N706 i7	CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTCTCGTGGGCTCGG
N707 i7	CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTCTCGTGGGCTCGG
N708 i7	CAAGCAGAAGACGGCATACGAGAT**CCTCTCTG**GTCTCGTGGGCTCGG
N709 i7	CAAGCAGAAGACGGCATACGAGAT**AGCGTAGC**GTCTCGTGGGCTCGG
N710 i7	CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTCTCGTGGGCTCGG
N711 i7	CAAGCAGAAGACGGCATACGAGAT**TGCCTCTT**GTCTCGTGGGCTCGG
N712 i7	CAAGCAGAAGACGGCATACGAGAT**TCCTCTAC**GTCTCGTGGGCTCGG

Table A3. 3. Raw relative proportional data (%) from Figure 3. 3. (Nem – Nemabiome assay, AusD - AusDiagnostics™). *T. vitrinus, T. axei* and *B. trigonocephalum* not identified by AusDiagnostics™.

Sample ID	Sample #	*C. ovina*		*T. circumcincta*		T. *vitrinus*		*T. axei*		*Trichostrongylus spp.*		*B. trignocephalum*	
		Nem.	*AusD*	*Nem.*	*AusD*	*Nem.*	*AusD*	*Nem.*	*AusD*	*Nem.*	*AusD*	*Nem.*	*AusD*
Adult/Female/Feb(16)	1	18.85	2.00	45.38	77.00	0.01		27.38			21.00	8.85	
Adult/Female/Mar(16)	2	1.00	8.00	40.21	45.00	54.54		4.24			47.00	0.01	
Adult/Female/Apr(15)	3	0.06	14.00	33.52	72.00	63.74		2.62			14.00	0.06	
Adult/Female/Jul(15)	4	25.7	58.00	1.85	20.00	4.94		7.02			22.00	60.49	
Adult/Female/Oct(15	5	49.38	30.00	8.85	9.00	8.48		17.46			61.00	15.83	
Adult/Male/Mar(16)	6	22.42	33.00	46.13	31.00	19.39		9.30			36.00	2.76	
Adiult/Male/Apr(15)	7	42.95	27.00	8.38	15.00	28.17		10.76			58.00	9.74	
Adult/Male/Jul(15)	8	14.17	48.00	0.15	0.00	0.21		30.65			52.00	54.82	
Yearling/Female/Mar(16)	9	37.82	59.00	23.15	21.00	25.10		12.22			20.00	1.71	
Yearling/Female/Apr(15)	10	48.65	52.00	11.82	34.00	30.53		8.93			14.00	0.07	
Yearling/Female/Jul(15)	11	4.81	0.00	1.79	11.00	11.41		7.85			89.00	74.15	
Yearling/Male/Feb(16)	12	24.34	4	47.93	32	5.00		22.74			65	0.00	
Yearling/Male/Mar(16)	13	46.31	55	14.74	18	10.60		20.80			27	7.55	
Yearling/Male/Apr(15)	14	42.01	31	24.04	35	23.61		10.32			35	0.02	
Yearling/Male/Jul(15)	15	20.72	25	0.90	2	15.22		27.63			73	35.54	
Yearling/Male/Oct(15)	16	70.82	88	3.67	3	2.55		12.26			9	10.70	
Lamb/Female/Mar(16)	17	38.84	37.00	14.48	18.00	31.91		14.76			45.00	0.01	
Lamb/Female/Jul(15)	18	7.30	13.00	13.03	57.00	23.45		0.90			30.00	55.32	
Lamb/Male/Mar(16)	19	25.90	55	15.66	0	46.38		11.60			45	0.45	
Lamb/Male/Jul(15)	20	1.30	0	36.76	48	57.15		0.63			52	4.17	

Table A3. 4. Raw relative proportional data (%) from Figure 3. 4. (Nem – Nemabiome assay, AusD - AusDiagnostics™, Morpho. – morphological ID as determined by tail length). Nemabiome assay results have been adapted to remove *B. trigonocephalum* and combine *T. vitrinus* and *T. axei* reads ('*Trichostrongylus* spp.') in order to compare with AusDiagnostics™.

Sample ID	Sample #	Species proportion						
		C. ovina			*T. circumcincta*		*Trichostrongylus spp.*	
		Nem.	*AusD*	Morpho.	*Nem.*	*AusD*	*Nem.*	*AusD*
Adult/Female/Feb(16)	1	20.16	2.00	4.00	49.78	77.00	30.06	21.00
Adult/Female/Mar(16)	2	1.00	8.00	5.00	40.22	45.00	58.78	47.00
Adult/Female/Apr(15)	3	0.06	14.00	8.00	33.54	72.00	66.40	14.00
Adult/Female/Jul(15)	4	65.05	58.00	49.00	4.68	20.00	30.27	22.00
Adult/Female/Oct(15	5	58.67	30.00	50.00	10.51	9.00	30.82	61.00
Adult/Male/Mar(16)	6	23.06	33.00	17.00	47.44	31.00	29.51	36.00
Adiult/Male/Apr(15)	7	47.58	27.00	32.00	9.29	15.00	43.13	58.00
Adult/Male/Jul(15)	8	31.36	48.00	16.00	0.00	0.00	68.31	52.00
Yearling/Female/Mar(16)	9	38.47	59.00	29.00	23.56	21.00	37.97	20.00
Yearling/Female/Apr(15)	10	48.68	52.00	32.00	11.83	34.00	39.48	14.00
Yearling/Female/Jul(15)	11	18.60	0.00	9.00	6.93	11.00	74.47	89.00
Yearling/Male/Feb(16)	12	24.34	4.00	18.00	47.93	32.00	27.73	65.00
Yearling/Male/Mar(16)	13	50.09	55.00	42.00	15.94	18.00	33.97	27.00
Yearling/Male/Apr(15)	14	42.02	31.00	35.00	24.05	35.00	33.94	35.00
Yearling/Male/Jul(15)	15	32.14	25.00	19.00	1.40	2.00	66.45	73.00
Yearling/Male/Oct(15)	16	79.30	88.00	60.00	4.11	3.00	16.59	9.00
Lamb/Female/Mar(16)	17	38.84	37.00	24.00	14.48	18.00	46.68	45.00
Lamb/Female/Jul(15)	18	16.34	13.00	10.00	29.17	57.00	54.49	30.00
Lamb/Male/Mar(16)	19	26.02	55.00	29.00	15.73	0.00	58.25	45.00
Lamb/Male/Jul(15)	20	1.35	0.00	0.00	38.36	48.00	60.29	52.00

A.4. APPENDIX – Chapter 4

Supplemental data for Chapter 4.

Table A4. 1. Nextera DNA Index kit index 1 (i7) and index 2 (i5) adapters for library preparation of validation samples.

i7	
Index name	**Bases in adapter**
N701	TCGCCTTA
N702	CTAGTACG
N703	TTCTGCCT
N704	GCTCAGGA
N705	AGGAGTCC
N706	CATGCCTA
N707	GTAGAGAG
N708	CCTCTCTG
N709	AGCGTAGC
N710	CAGCCTCG
N711	TGCCTCTT
N712	TCCTCTAC

i5	
Index name	**Bases in adapter**
N501	TAGATCGC
N502	CTCTCTAT
N503	TATCCTCT
N504	AGAGTAGA

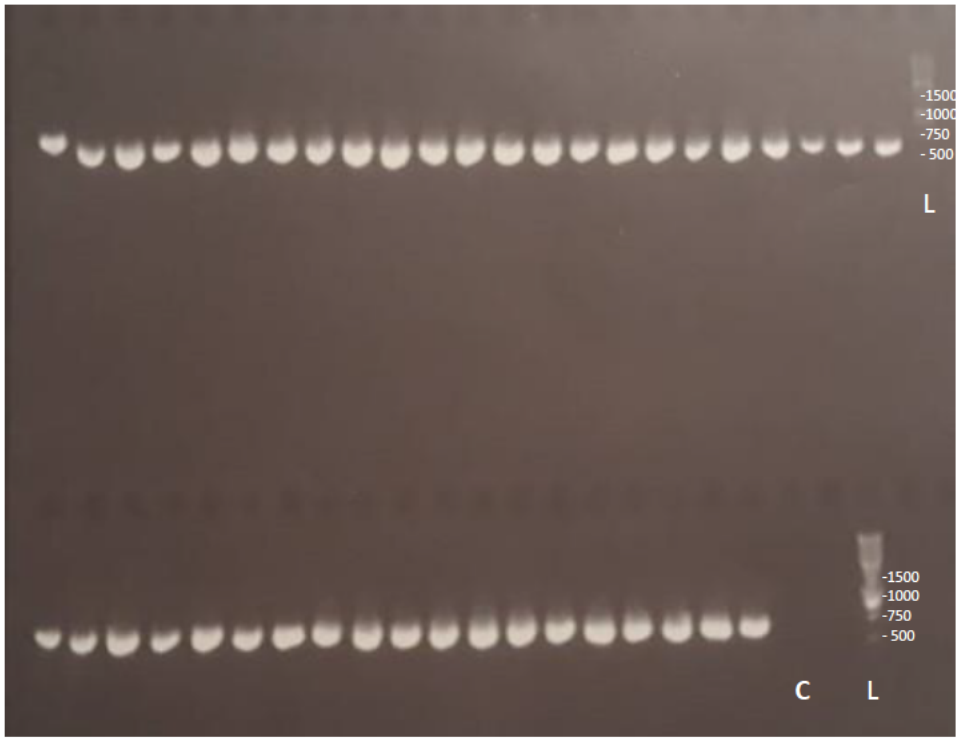


Figure A4. 1 . ITS-2 amplification visualised on an 1.8% agarose gel (90V/ 40min). Samples used in the validations in this Chapter. C = negative control (molecular-grade water). L = 1 kb DNA ladder (base pairs indicated).

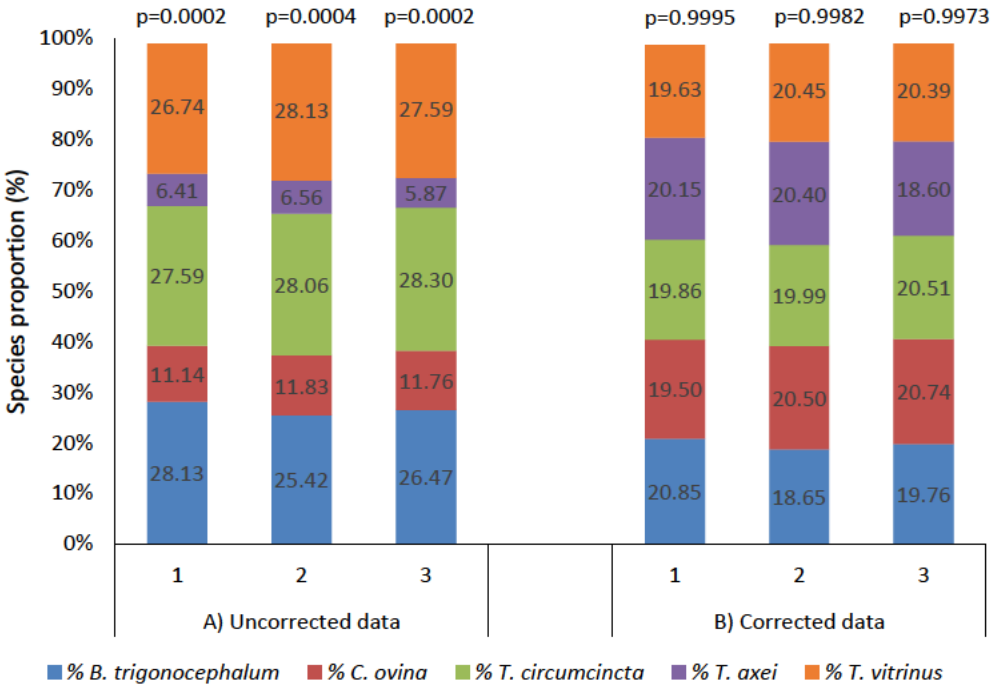


Figure A4. 2. Assessing sequence representation bias and the determination of the species correction factors for the amplicon sequencing assay including *T. axei*. The lysate was ran in triplicate (1-3). A) The chart shows the species proportions as determined by the actual number of ITS-2 rDNA sequences generated by the amplicon sequencing assay. B) The chart shows the same data as in graph (A) but following the application of a correction factor for each species to the sequence number. Numbers in both graphs are the percentage proportions of each species within the mock pool. The p-values above each column indicate whether the species proportions, as determined by the uncorrected (A) and corrected (B) numbers of ITS-2 rNDA sequences generated, are statistically different from the actual proportions of larvae in the pool (20%).

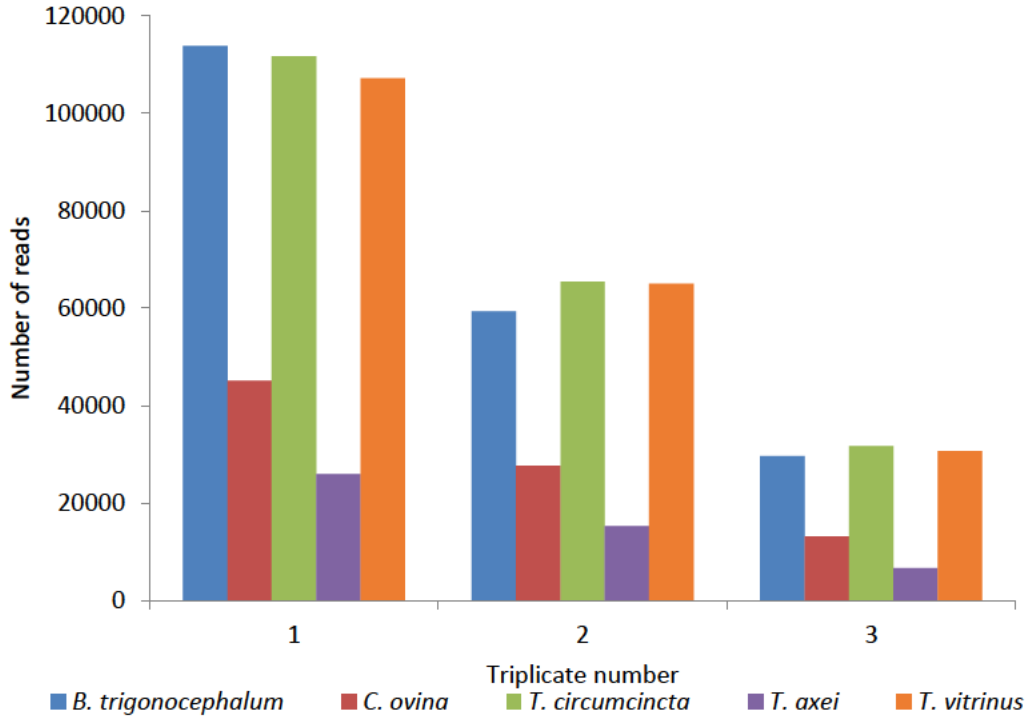


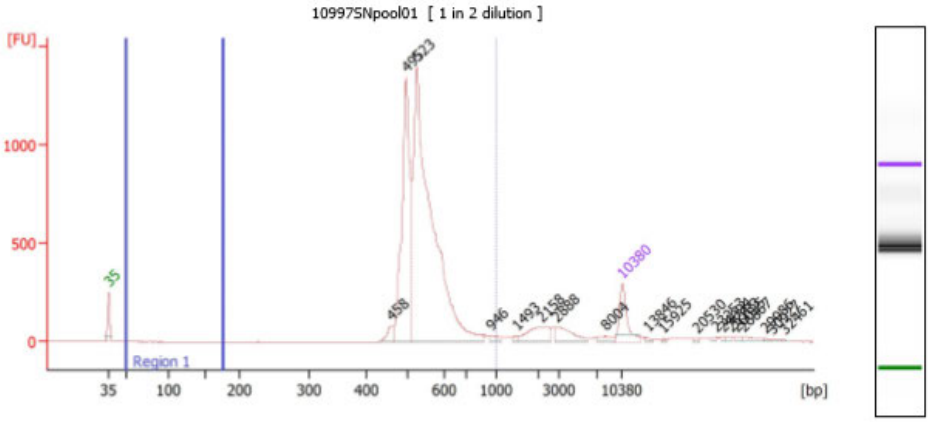
Figure A4. 3 . Number of reads, rather than species proportion (%) of technical triplicates of the uncorrected data presented in Figure 4. 3

2100 expert_High Sensitivity DNA Assay_DE04105522_2017-07-26_14-28-42.xad Page 1 of 1

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04105522_2017-07-26_14-28-42.xad

Created: 7/26/2017 2:28:42 PM
Modified: 7/26/2017 2:51:35 PM

Electropherogram Summary



Overall Results for sample 1 : 10997SNpool01

Number of peaks found:	19	Corr. Area 1:	1.5
Noise:	0.5	Corr. Area 2:	6,981.0

Peak table for sample 1 : 10997SNpool01

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	458	31.79	105.1	
3	495	686.11	2,101.4	
4	523	1,514.36	4,386.2	
5	946	14.98	24.0	
6	1,493	5.94	6.0	
7	2,158	63.16	44.3	
8	2,888	51.03	26.8	
9	8,004	13.66	2.6	
10	10,380	75.00	10.9	Upper Marker
11	13,846	0.00	0.0	
12	15,925	0.00	0.0	
13	20,530	0.00	0.0	
14	23,253	0.00	0.0	
15	24,094	0.00	0.0	
16	25,183	0.00	0.0	
17	26,025	0.00	0.0	
18	26,867	0.00	0.0	
19	29,986	0.00	0.0	
20	30,927	0.00	0.0	
21	32,461	0.00	0.0	

Region table for sample 1 : 10997SNpool01

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
50	175	1.5	0	58	16.8	0.72	18.5	
175	1,000	6,981.0	88	545	12.1	2,162.99	6,101.0	

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Figure A4. 4. Bioanalyzer summery prior to sequencing (validation sequencing).