A 4 year observation of gastrointestinal nematode egg counts, nemabiomes, and the benzimidazole resistance genotypes of Teladorsagia circumcincta on a Scottish sheep farm.

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**Abstract:** Anthelmintic resistance threatens the sustainability of sheep production globally. Advice regarding strategies to reduce the development of anthelmintic resistance incorporates the outcomes of modelling exercises. Further understanding of gastrointestinal nematode (GIN) species diversity, and population dynamics and genetics (which may vary between species) is required to refine these models; and field studies combining faecal egg outputs, species composition and resistance genetics are needed to calibrate them. In this study, faecal samples were taken from ewes and lambs on a commercial farm in south-east Scotland at approximately 3 to 4 week intervals between spring and autumn over a period of 4 years. Faecal egg counts (FECs) were performed on these samples, and third stage larvae (L3) were collected from pooled coprocultures. Deep amplicon sequencing was used to determine both the species composition of these L3, and the proportions of benzimidazole (BZ) resistant SNPs in the isotype-1 β-tubulin locus of the predominant species, Teladorsagia circumcincta L3. Despite consistent management throughout the study, the results show variation in GIN species composition with time and between age groups, that was potentially associated with weather conditions. The F200Y BZ resistance mutation is close to genetic fixation in the T. circumcincta population on this farm. There was no evidence of variation in isotype-1 β-tubulin SNP frequency between age groups, and no genetic evidence of reversion to BZ susceptibility, despite targeted BZ usage. This study highlights the need to include speciation when investigating GIN epidemiology and anthelmintic resistance, and serves as an example as to how genetic data may be analysed alongside species diversity and FECs, when markers for other anthelmintic classes are identified.
Dear Sir/Madam,

Re: A four year observation of gastrointestinal nematode egg counts, nemabiomes, and the benzimidazole resistance genotypes of *Teladorsagia circumcincta* on a Scottish sheep farm.

We would be grateful if you could consider our work for publication in *International Journal for Parasitology*. In this report we describe variation in gastrointestinal nematode species (the ‘Nemabiome’) on a Scottish sheep farm across a four year period. Analysis of species diversity revealed significant differences between ewes and lambs, and between years. This variation is presented alongside weather data from the farm, which may contribute to the observed variation in species composition. In addition, the frequencies of benzimidazole resistance SNPs in the isotype-1 β-tubulin locus of the *Teladorsagia circumcincta* population were observed across the four year study. This showed no evidence for the reversion to genetic benzimidazole susceptibility in this species, despite targeted use of benzimidazoles on the farm. These findings have significant implications for the future modelling of anthelmintic resistance, and highlight the need for further research into the population genetics of gastrointestinal nematodes, and the selection pressures associated with anthelmintic resistance.

Yours faithfully,

Mike Evans

On behalf of each of the co-authors.
Thank you for your feedback and assistance. The fastq files have been uploaded to SRA and the accession numbers added to the manuscript (lines 27-28 and 268-269).
• Metabarcoded sequencing was used to determine species composition (nemabiome) of GIN larvae.
• Deep amplicon sequencing determined proportions of BZ resistant SNPs in *Teladorsagia circumcincta*.
• Nemabiome varied with time and between ewes and lambs possibly associated with weather.
• The F200Y BZ resistance mutation was close to genetic fixation in *T. circumcincta*.
• There was no genetic evidence of reversion to BZ-anthelmintic susceptibility.
A four year observation of gastrointestinal nematode egg counts, nemabiomes, and the benzimidazole resistance genotypes of *Teladorsagia circumcincta* on a Scottish sheep farm.


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Note: All fastq files were uploaded to SRA (Bioproject accession number: PRJNA669542) and all β-tubulin sequences were uploaded to GenBank (accession numbers: MW081491-MW081536).
Abstract

Anthelmintic resistance threatens the sustainability of sheep production globally. Advice regarding strategies to reduce the development of anthelmintic resistance incorporates the outcomes of modelling exercises. Further understanding of gastrointestinal nematode (GIN) species diversity, and population dynamics and genetics (which may vary between species) is required to refine these models; and field studies combining faecal egg outputs, species composition and resistance genetics are needed to calibrate them. In this study, faecal samples were taken from ewes and lambs on a commercial farm in south-east Scotland at approximately 3 to 4 week intervals between spring and autumn over a period of 4 years. Faecal egg counts (FECs) were performed on these samples, and third stage larvae (L3) were collected from pooled coprocultures. Deep amplicon sequencing was used to determine both the species composition of these L3 and the proportions of benzimidazole (BZ) resistant SNPs in the isotype-1 β-tubulin locus of the predominant species, Teladorsagia circumcincta. Despite consistent management throughout the study, the results show variation in GIN species composition with time and between age groups, that was potentially associated with weather conditions. The F200Y BZ resistance mutation is close to genetic fixation in the T. circumcincta population on this farm. There was no evidence of variation in isotype-1 β-tubulin SNP frequency between age groups, and no genetic evidence of reversion to BZ susceptibility, despite targeted BZ usage. This study highlights the need to include speciation when investigating GIN epidemiology and anthelmintic resistance, and serves as an example as to how genetic data may be analysed alongside species diversity and FECs, when markers for other anthelmintic classes are identified.

Keywords: Sheep; Gastrointestinal nematode; nemabiome; isotype-1 β-tubulin SNPs; modelling anthelmintic resistance
1. Introduction

Gastrointestinal nematode (GIN) infections in sheep have been shown to impact significantly on the outputs of both meat and milk production globally (Mavrot et al., 2015), and modelling suggests that reducing the severity of GIN infection in sheep would result in a linear reduction in the costs of production in Great Britain (Nieuwhof and Bishop, 2005), as well as reducing the carbon footprint of production (Kenyon et al., 2013a). However, the widespread prevalence of anthelmintic resistance threatens the sustainability of sheep production (Kaplan and Vidyashankar, 2012; Rose et al., 2015).

In addition, there have been changes in GIN epidemiology associated with climate change (Kenyon et al., 2009b; Sargison et al., 2012), and modelling suggests that future climate change may impact the sustainability of current management strategies (Rose et al., 2016).

Advice regarding strategies to reduce selection for anthelmintic resistance, whilst avoiding negative impacts on production and animal welfare, are largely based upon maintaining populations of nematodes in refugia, i.e. not exposed to treatment (Van Wyk, 2001; Kenyon et al., 2009a). The impact of such strategies may be predicted by modelling (Cornelius et al., 2016; Park et al., 2015) and monitored phenotypically (Kenyon et al., 2013b; Leathwick et al., 2015). However, there is currently insufficient evidence regarding inheritance of resistance genes, population structuring, and fitness costs to fully incorporate these into model calibration (Hodgkinson et al., 2019). Furthermore, phenotypic monitoring lacks sensitivity at low levels of resistance (Taylor et al., 2002) and is unable to distinguish the relative impact of different GIN species, without time-consuming morphological speciation (McIntyre et al., 2018).

A seasonal pattern of ovine GIN infection has traditionally been described in temperate climates, with overwintering of larvae and a peri-parturient rise in ewes contributing to the infection of lambs, which leads to a progressive rise in pasture contamination through the summer and autumn (Van Dijk et al., 2010). Larval development rates vary between GIN species, associated with soil temperature, rainfall and relative humidity (O’Connor et al., 2006). This gives rise to typical seasonal...
Variation in GIN species in the UK, with *Teladorsagia circumcincta* traditionally predominating in summer, followed by an increased contribution from *Trichostrongylus* species in autumn (Van Dijk et al., 2010). Despite changes in climate and farming practices, a recent observational study on three Scottish farms was consistent with the traditionally described faecal egg count (FEC) profile (Hamer et al., 2018). However, veterinary diagnostic submissions in Northern Ireland suggest that there has been a decrease in the relative seasonality of teladorsagiosis and trichostrongylosis (McMahon et al., 2013). Further investigation of species composition by morphological methods is limited by the requirement for significant skilled labour input; hence the development of a deep-amplicon sequencing approach using the ITS-2 locus to speciate mixed communities of nematodes (the ‘nemabiome’) has provided the opportunity to analyse GIN species diversity at much greater throughput (Avramenko et al., 2015). Redman et al. (2019) reported the validation of this technique for ovine GIN, including the development of correction factors to account for differential efficiency of DNA amplification from L3 of the most common species. It was also suggested that there may be differences in the ‘nemabiome’ between ewes and lambs on the same farms (Redman et al., 2019), although that may also have been affected by the seasons in which the age groups were sampled.

High throughput sequencing techniques present the opportunity to investigate many of the outstanding questions regarding the genetics of anthelmintic resistance (Hodgkinson et al., 2019). Although there are currently no confirmed, specific genetic markers for resistance to levamisole or macrocyclic lactone drugs in GIN, the genetic basis for resistance to benzimidazole (BZ) drugs is characterised by the presence of any of three separate SNPs (at codons 167, 198 and 200) in the isotype-1 β-tubulin locus (Geary et al., 1992; Kwa et al., 1995, 1994; Elard et al., 1996). Deep-amplicon sequencing approaches for this locus have been validated and applied to pooled field samples for multiple ovine GIN (Avramenko et al., 2019) and specifically for *T. circumcincta* (Sargison et al., 2019). However, to the best of our knowledge, there have been no studies assessing variation in isotype-1 1 β-tubulin SNP frequency within a sheep flock, between age groups and with time across multiple years.
This study describes the pattern of faecal GIN egg shedding by ewes and lambs across four years, with varying climate; and applies deep amplicon sequencing techniques to describe variation in both the species compositions and the BZ resistance SNP frequencies within the *T. circumcincta* population. Investigating variation in these factors will determine whether they need to be factored into modelling exercises. This study also serves as a proof of concept for future monitoring of the impact of management and treatment decisions on GIN species diversity and anthelmintic resistance in controlled experiments, or larger observational studies.

2. Materials and Methods

2.1 Description of the study farm

A farm of 150 acres in south-east Scotland (55°52′N, 3°12′W) at an altitude of 175-190m was studied. The breeding flock is comprised of approximately 370 Cheviot Mule ewes, which are crossed with Texel rams in October/November, to give an estimated lambing period from the end of March until the end of April. All ewes lamb in indoor pens and are turned out onto pasture approximately 2 days after lambing. Ewes and lambs co-graze until the lambs are weaned in August (stocking density c.6 ewes plus lambs per acre). After weaning, ewes continue to graze the same pasture, whilst the lambs are moved onto silage aftermaths (stocking density dependent on the date of silaging and rate lambs are drawn for slaughter). Lambs are sold for meat production between August and December, according to their liveweight. Approximately 80 replacement females (22%) are purchased as ewe lambs in October. These join the main ewe flock when aged approximately 18 months, and give birth for the first time at approximately two years of age.

All ewes received oral moxidectin (200µg/kg) between lambing and turn-out, within a few days of lambing. All lambs received two or three treatments with oral albendazole (5mg/kg) during May to July, according to *Nematodirus battus* forecasting (NADIS, 2019) and FEC monitoring results. All
lambs received one or two treatments with oral levamisole (7.5mg/kg) between August and September, according to FEC monitoring results, growth rates and clinical signs of diarrhoea. Lambs were not intentionally moved to clean grazing after the anthelmintic treatments, although the first levamisole treatment in August 2017 coincided with weaning and therefore movement. Replacement ewe lambs were treated with oral monepantel (2.5mg/kg) and an intramuscular injection of doramectin (300µg/kg) on arrival, before being moved to pasture that had been used for lambs within that year.

2.2 Sample Collection

Samples were collected between spring 2016 and autumn 2019. During these four sampling years, 10 freshly voided faecal samples produced by ewes were collected from the ground at approximately three to four week intervals between April and October, with some additional samples taken over the winter of 2016/2017. Ten freshly voided faecal samples produced by lambs were collected from the ground at approximately three to four week intervals between June and October, with some additional sampling points for ad hoc clinical monitoring. Samples were not linked to individual animals. Ethical approval was acquired through Veterinary Ethics Review Committee (VERC) at the University of Edinburgh (reference number VERC 10 16) and consent was given by the farm managers.

2.3 FECs and coprocultures

Individual strongyle FECs were performed on all samples using a cuvette technique with a sensitivity of three eggs per gram (Christie and Jackson, 1982). Approximately equal quantities of remaining faeces from each group were then combined into pooled samples, which were cultured at room temperature of approximately 21°C for 14 days, covered with perforated polythene bags to prevent
The resultant third stage larvae (L₃) were then isolated using a modified Baermann’s technique (MAFF, 1986) and stored at room temperature in 70% ethanol for up to 8 months (DNA lysates were produced after all samples had been collected for each year).

2.4 Genomic DNA extraction

Approximately 1000 L₃ from coprocultures were used for DNA extraction. These were selected by taking an aliquot from the sample, after first estimating the larval density by stereoscopic microscopy. The larvae were washed three times in distilled water, and then centrifuged for two minutes at 7,200 x g and the resulting pellet re-suspended in 50µl of lysis buffer (200 parts Direct PCR lysis reagent (Viagen), 1 part proteinase K solution (Qiagen), and 1 part 1M dithiothreitol (DTT)). This was incubated at 60°C for 2 hours to lyse the larvae followed by 85°C for 15 minutes to inactivate the proteinase K.

2.5 Adapter PCR amplification of rDNA ITS-2 and isotype-1 β-tubulin loci

The 1st round PCR amplification was performed on 321bp fragments of the rDNA ITS-2 region, complementary to the 5.8s and 28s rDNA coding sequences, using sets of universal adapter primers (Avramenko et al., 2015). Simultaneously, 276 bp fragments of T. circumcincta isotype 1 β-tubulin spanning the F200Y, F167Y, and E198L or E198A SNPs were amplified with adapter primers (Sargison et al., 2020). Primers are listed in the online repository (see 2.11). For both rDNA ITS-2 and isotype 1 β-tubulin loci, equal proportions of the four forward and four reverse primers were mixed and used for the adapter PCR with following conditions: 10 µM forward and reverse adapter primers, 10 mM dNTPs, 0.5 U DNA polymerase enzyme, 5X buffer (KAPA Biosystems) and 1 µl of gDNA. Thermocycling conditions were 95°C for 2 min, followed by 35 cycles of 98°C for 20 s, 60°C for 15 s, 72°C for 15 s for ITS-2 and isotype 1 β-tubulin and a final extension of 72°C for 5 min. PCR products
were purified with AMPure XP Magnetic Beads (1X) according to the manufacturer’s instructions (Beckman Coulter).

2.6 Barcoded PCR amplification of rDNA ITS-2 and isotype-1 β-tubulin loci

The 2nd round PCR was performed using 16 forward and 24 reverse barcoded primers (in online repository, see 2.11). Each sample of rDNA ITS-2 and isotype-1 β-tubulin was amplified using a unique combination of barcoded primers. The PCR reaction contained 2 μl of the first round PCR product as a template, 0.5 μl of KAPA HiFi polymerase (KAPA Biosystems), 0.75 μl dNTPs (10mM), 5μl 5X KAPA HiFi Fidelity buffer (KAPA Biosystems), 1.25 μl of each primer (10 μM), and 13.25 μl nuclease-free water. PCR conditions were 98°C for 45 seconds, followed by 7 cycles of 98°C for 20 seconds, 63°C for 20 seconds, and 72°C for 2 minutes. PCR products were purified as described above and further purified by agarose gel electrophoresis, followed by gel extraction using QIAquick Gel Extraction Kit, according to the manufacturer’s instructions (QIAGEN).

2.7 Deep amplicon sequencing and data handling

The purified products from each sample were mixed to prepare a pooled library and measured with the KAPA qPCR library quantification kit (KAPA Biosystems). The library was then run on an Illumina MiSeq sequencer using a 500-cycle pair end reagent kit (MiSeq Reagent Kits v2, MS-103-2003) at a concentration of 15 nM with addition of 10-15% PhiX Control v3 (Illumina, FC-11-2003). During the post-run processing, Mi-Seq splits all sequences by samples using the barcoded indices to produce FASTQ files.

The analysis of both rDNA ITS-2 and isotype-1 β-tubulin FASTQ files were performed in Mothur v1.39.5 software (Schloss et al., 2009), using a modified Command Prompt pipeline (Avramenko et
al., 2015; Sargison et al., 2019) and the standard operating procedures of Illumina Mi-Seq (Kozich et al., 2013).

For the ITS-2 sequence data, paired-end reads were assembled into single contigs and then filtered to remove contigs that were <200bp or >450bp, and pairs that contained any ambiguities. Contigs were then aligned to an ITS-2 rDNA database previously described by Avramenko et al. (2015) and discarded if they did not align to at least 10% of any ITS-2 rDNA amplicon in the database with at least 90% sequence similarity. The remaining sequences were classified by comparing to reference sequences in the database using the k-nearest-neighbour method (k = 3). In order to reduce the impact of potential PCR or sequencing errors, taxonomic levels with fewer than 2000 reads across all samples were removed. Samples with fewer than 2000 reads across all taxonomic levels were then also removed.

In the case of isotype-1 β-tubulin sequence data, paired-ends reads were assembled into single contigs then filtered to remove contigs that were >350bp, and pairs that contained any ambiguities. The sequence data were then aligned with a T. circumcincta reference sequence library previously described by Sargison et al. (2019), and were removed if they did not match with the T. circumcincta isotype 1 β-tubulin locus. Chimeras were removed using VSEARCH (Rognes et al., 2016) and remaining sequences were summarised to generate the T. circumcincta isotype 1 β-tubulin sequences FASTQ file. Once all bulk sequences were classified as T. circumcincta, a count list of the consensus sequences of each population was created. In order to reduce the impact of potential PCR or sequencing errors, haplotypes with fewer than 500 reads across all samples were removed. Samples with fewer than 500 reads across all haplotypes were then also removed. A lower threshold was used than for ITS2 sequences (500 c.f. 2000 reads) to account for potentially low proportions of T. circumcincta within individual coprocultures. The remaining haplotypes were manually examined in Geneious Prime (Biomatters Ltd), and a conservative approach was used, whereby individual SNPs that occurred in just single haplotypes were corrected to the consensus sequence. These haplotypes
were then collapsed using FaBox (Villesen, 2007). The simplified haplotypes were then labelled according to whether the BZ resistance SNPs (F167Y, E198L, F200Y) were present.

2.8 Data processing and presentation

Data were processed and presented using R v3.5.1 in R Studio v1.1.4.5.6 (R Core Team, 2017), utilising ‘cowplot’ (Wilke, 2018) and ‘tidyverse’ (Wickham, 2017) packages. 95% confidence intervals for the arithmetic mean faecal egg count of each sampling event were generated by 500 bootstrap resamples (with replacement) utilising the ‘rsample’ package (Kuhn and Wickham, 2017). Species-specific sequencing biases were corrected for by multiplying the ITS-2 read proportions by correction factors that were previously validated against morphological methods (Redman et al., 2019). These correction factors are also included in the online data repository associated with this paper (see 2.11). Proportional FECs were generated by multiplying corrected species by the arithmetic mean FEC and 95% confidence interval.

2.9 Diversity analysis

Species diversity within each sample (alpha diversity) was assessed using the Inverse Simpson’s Index, calculated in Mothur (Schloss et al., 2009), based on a random subsample of the sequences equal in size to the smallest sample. Differences in the Inverse Simpson’s Index between each year and age group were then assessed using a one-way ANOVA and post hoc Tukey comparisons in R (R Core Team, 2017). Species diversity between the year and age groups (beta diversity) was assessed using the ‘amova’ and ‘metastats’ commands in Mothur (Schloss et al., 2009), based on a random subsample of the sequences equal in size to the smallest sample. A Bonferroni adjustment was used for the AMOVA analysis, dividing the intended alpha of 0.05 by the number of pairwise comparisons. Non-parametric analysis comparing species ranks between years and age groups was performed.
using Kruskal-Wallis Rank Sum tests in R, with a Bonferroni adjustment, followed by post-hoc Dunn’s test using the r package ‘PMCMRplus’ (Pohlert, 2020), with a Bonferroni adjustment, for those species with significant Kruskal-Wallis results.

Cluster analysis was performed in R (R Core Team, 2017) to generate distance matrices based upon the mean proportional FEC for each species, grouped by age group and year, or age group and month. These distances were calculated using the Pearson’s correlation (with transformation [1-r]) and clustering using the UPGMA method using the ‘amap’ package (Lucas, 2018). These distance matrices were then visualised as dendrograms using the ‘ggdendro’ package (de Vries and Ripley, 2016).

2.10 Weather data

Soil temperature, precipitation and relative humidity data were collected by the Centre for Ecology and Hydrology (CEH) at their weather station present within the grounds of the study farm. Soil moisture at a depth of approximately 10-50cm over a 12Ha area on the farm was estimated using a cosmic ray neutron sensor as part of the Cosmos-UK project. Smoothed lines were generated for plots of these data using the LOESS method (span=0.3) (Wickham, 2017).

2.11 Data Accessibility

All parasitological data, fastq files, sequence results, mothur scripts and diversity analysis outputs have been made freely available through Mendeley Data at DOI: ‘10.17632/nfhpswcybc.1’. All fastq files were uploaded to SRA (Bioproject accession number: PRJNA669542) and all β-tubulin sequences were uploaded to GenBank (accession numbers: MW081491-MW081536). All meteorological data and soil moisture data are the property of NERC – Centre for Ecology and Hydrology, who may be contacted directly regarding obtaining raw data for future use.

3. Results
3.1 FECs varied with time and between age groups

Faecal egg counts in the ewes rose around the time of parturition, whilst the FECs of lambs rose in late summer and autumn; and there was variation between years in both the magnitude and the timing of these increases (Fig. 1). These data also illustrate that levamisole treatments of the lambs in 2016, 2018 and 2019 appear to have been effective, although in 2016 and 2018, FECs increased again approximately four weeks post-treatment (Fig. 1). Moxidectin treatment of the ewes in 2016 coincided with a dramatic decrease in FECs, although these rose again approximately three to six weeks after the lambing period ended; there was a similar drop in FECs in the ewes in 2017 and 2019, although in these years the rebound was more rapid (Fig. 1). FECs around the time of the moxidectin treatment of ewes in 2018 were low, although not zero; however, there was no pre-treatment sample from this year.

3.2 Species diversity varied both within and between groups

Visual inspection of the nemabiome suggests that T. circumcincta predominated in the lambs, and there was greater species diversity in the ewes (Fig. 2). The one-way ANOVA of the Inverse Simpson’s Index showed that there were significant differences in the average alpha diversity present in these groups ($F_{(7,50)} = 3.569$, $P = 0.003$), with post hoc analysis indicating significantly higher alpha diversity in the 2016 ewes than the lambs in 2017, 2018 and 2019 ($P = 0.001, 0.044$ and 0.014, respectively).

Beta diversity assessed by AMOVA (Bonferroni $\alpha = 0.002$) showed significant differences in species diversity across all groups ($F_{(7,50)} = 3.062$, $P < 0.001$) and for three pairwise comparisons: 2016 ewes to 2017 lambs ($F_{(1,14)} = 8.389$, $P < 0.001$); 2018 ewes to 2017 lambs ($F_{(1,12)} = 5.494$, $P < 0.001$); and 2019 ewes to 2017 lambs ($F_{(1,12)} = 4.101$, $P = 0.001$). Metastats analysis indicated statistically significant
differences between years and between age groups for *C. curticei*, *T. circumcincta*, *T. axei* and *T. vitrinus* (Tab.1).

Non-parametric analyses were consistent with the metastats analysis, showing significant differences (Bonferroni $\alpha = 0.0083$) in species rank for *C. curticei* ($P = 0.0002$), *T. circumcincta* ($P = 0.0015$), *T. axei* ($P = 0.0002$) and *T. vitrinus* ($P = 0.0082$). *Post hoc* Dunn’s tests showed this variation to be driven by significant differences in species rank between both years and age groups (Tab. 2).

3.3 FECs adjusted for species composition varied with time and between age groups.

Consistent with the species diversity reported above, FEC attributed to each species varied with time and between age groups (Fig. 3A). The peri-parturient rise in FECs contained contributions from multiple species, with *T. circumcincta* predominating, whereas the rebound in FECs towards the end of the lambing period in 2017 and 2019 contained a greater proportion of *Cooperia curticei*.

However, this rise in the *C. curticei* egg output from ewes did not result in a corresponding rise in the samples from lambs. In all four years, egg outputs from lambs were composed predominately of *T. circumcincta*, although there was a rise in the contributions of *Trichostrongylus vitrinus* and *Oesophagostomum venulosum* in the late autumn/winter of 2016, and in these two species plus *C. curticei* in autumn 2019. Although the overview of the ‘nemabiome’ suggests greater species diversity in the lambs of 2018 (Fig. 2), compared to the lambs of 2017, this appears less significant when corrected for FEC (Fig. 3A).

When considering both FEC and species composition, samples taken from the lambs in 2016 were most similar to samples taken from ewes in 2016 (Fig. 3B). Likewise, those from lambs in 2019 were most similar to those from ewes in 2019. Samples from lambs in 2017 and 2018 are clustered together with samples from ewes in 2018, with samples from ewes in 2017 clustered alongside
these three groups. A dendrogram produced after grouping samples by month, year and age group (Fig. S1) did not show clear evidence of clustering according to sample month.

3.4 Isotype-1 β-tubulin SNPs showed little variation

There was little variation in isotype-1 β-tubulin SNP frequency across the four years or between age groups (Fig. 4). The F200Y SNP comprised more than 78% of all reads in all but three samples. Of these three outlier samples, one occurred during the peri-parturient period in the ewes in 2018. The other two occurred around the time of the N. battus-targeted BZ treatments of the lambs in 2017 and 2018, although unfortunately there were no pre-treatment results to compare these with as these samples had low coproculture yields and produced fewer reads than the threshold described in 2.9.

3.5 Over-winter and summer weather patterns varied between years

Soil temperature, relative humidity, soil moisture and rainfall on the farm were documented over the course of the four study years. Winter soil temperatures were lower in 2017/18 than in the other years (Fig. 5A). There was a more prolonged warm period during the summer of 2017, than in 2018 and 2019, and the temperature profile in summer 2016 was between these two extremes (Fig. 5A). The humidity profiles are similar for the four years, although the humidity during the summer/autumn of 2017 was more stable than in the other years, and the 2017/18 winter was more humid than 2016/17 and 2018/19 (Fig. 5B). Soil moisture levels were lower in the winters of 2016/2017 and 2018/2019 than 2017/2018 (Fig. 5C). The soil was also drier during the spring of 2017 and through the spring and summer of 2018 (Fig. 5C). The autumn of 2016 had relatively low rainfall; and the spring/summer of 2017 and 2019 had low rainfall initially, before periods of higher rainfall later in the season (Fig. 5D).
4. Discussion

The FEC results from 2016 were previously presented as Farm 1 in Hamer et al. (2019), alongside data from two nearby farms, demonstrating that patterns of faecal egg production were broadly similar to those traditionally described, despite changes in climate and farm management. In the present study, this profile was similar for both the ewes and lambs in 2017, 2018 and 2019 (although no pre-moxidectin sample was obtained from the ewes in 2018). However, analysis of the ‘nemabiome’ shows that on a single farm with consistent management between years, there were significant differences in species diversity within and between age groups and years. This emphasises the importance of the speciation of the nematodes present within a FEC, and raises questions about the factors driving this variation.

In addition to investigating species diversity, ITS-2 based speciation was previously used to diagnose anthelmintic resistance within the *T. circumcincta* population on this farm, which would have been missed by a traditional FEC reduction test (FECRT) (McIntyre et al., 2018). The present study adds to the evidence that speciation enhances the interpretation of raw FECs: without speciation, the rebound peak in FEC in the ewes in 2017 might suggest anthelmintic resistance; however, given that it is composed predominately of *C. curticei*, this peak may simply reflect pharmacokinetic differences, as moxidectin has been shown to have greater persistence against abomasal than intestinal GIN species in cattle (Eysker and Eilers, 1995), and the datasheet for oral 0.1% moxidectin has no claim of persistence against *C. curticei* (NOAH, 2018). Similarly, the ‘nemabiome’ is at risk of over interpretation if it is not considered in the context of the FECs of the samples.

Redman et al. (2019) demonstrated that within farms there may be differences in GI nematode species composition between ewes at lambing time and lambs at weaning time. This study also demonstrates differences in species composition between ewes and lambs; however, whilst Redman et al. (2019) found *T. circumcincta* to be overrepresented in the samples from ewes, *T. circumcincta*...
was overrepresented in lambs on this farm. In addition, this study suggests that differences between ewes and lambs may be less than those between different years. These differences may have implications for the development of anthelmintic resistance, as during selective treatment events, the within-host refugia sizes of different GIN species may vary with time and between age groups.

Differences in species diversity between ewes and lambs are unsurprising, given their differing life histories and anthelmintic treatments. It is interesting to note that many of the significant pairwise comparisons included the lambs in 2017, the only year when the lambs received two levamisole treatments and ‘dose and move’ was effectively performed due to treatment very close to weaning. However, significant differences were also present between other years, when treatments were extremely similar.

These differences in species diversity described between years could potentially relate to climatic impacts on the overwinter survival of larvae on the pasture. The winters of 2016/17 and 2018/19 were mild and dry compared to the winter of 2017/18. Both these factors would be expected to result in decreased survival of T. circumcincta on pasture (O'Connor et al., 2006; McMahon et al., 2012), yet surprisingly, T. circumcincta predominated in the lambs in 2017. However, previous research has focussed on the climate-driven epidemiology of Haemonchus contortus, Trichostrongylus colubriformis and T. circumcincta, and there is a relative lack of information regarding the other species present in this system. It may be that although fewer T. circumcincta survived in those winters, the relative survival of this species was still greater than that of the other species. The results of the cluster analysis are consistent with this hypothesis, as the samples from ewes and lambs in 2017 and 2018 were clustered together, whereas the years either side were further removed.

Further to any effects on pasture survival, variation in the weather between years is likely to have impacted upon the faecal and pasture microclimates and, therefore, the rate of larval development and translocation. Compared to the other years, the late summer of 2017 had stable relative
humidity; stable, warm soil temperatures; higher soil moisture; and greater precipitation. Similar conditions have previously been shown to favour infective larval availability for *H. contortus* (Wang et al., 2018), and it may be that these conditions gave a selective advantage to *T. circumcincta* relative to the other species present on this farm in 2017. It was not possible to model the impact of the climate data on the results from this study, hence these hypotheses are speculative. However, where possible, data has been made freely available (see 2.11) so that they may be utilised in future modelling.

Significant alterations in species diversity due to the purchase of replacements seems unlikely given the quarantine treatments that were given. In addition, differences between age groups and years may have been affected by the impact of grass growth on ewe and lamb nutrition, with secondary effects on immunity. Alterations in grass growth could have also impacted silage aftermath availability and the rate at which lambs were drawn for slaughter, and therefore the stocking density post-weaning. Differences in host genetics (between age groups and between years) may have also contributed to variation in species-specific immunity, due to the annual replacement of approximately 22% of the breeding flock, and the fact that the lambs are from Texel sires, a breed associated with immunity against GIN (Good et al., 2006).

These results demonstrate the power of the ‘nemabiome’ approach (Avramenko et al., 2015) to investigate variation in different GIN species and contribute to the modelling of GIN infections. However, they also demonstrate the complexity of the systems being studied and emphasise the need to incorporate variation in climatic factors, host factors, and farm management practices into future surveys and models. In addition, this study was impacted by missing data points due to low coproculture yields from some samples. Redman et al. (2019) validated the use of cultures of first-stage larvae (L₁) in addition to the L₃ cultures used in this study. L₁ cultures are less affected by coproculture conditions and are therefore arguably more representative of the eggs shed, but less representative of the larvae that go on to infect the pasture. Further research into how ‘nemabiome’
data correlate with infection levels within hosts and pasture larval composition would therefore be extremely valuable, as would validation of how accurately pooled faecal samples reflect population level variation, and the optimum methods for sampling and preparing these pools.

In order to avoid interpreting PCR or sequencing errors, sequences with low read numbers were rejected prior to analysis and SNPs occurring in single β-tubulin haplotypes were manually corrected to the consensus sequence. However, these conservative methods reduce the sensitivity with which rare alleles may be detected and quantified. Replicated sequencing runs can be used to more reliably identify rare alleles and quantify PCR and sequencing error rates, with Avramenko et al. (2015) reporting variation in species composition of up to 2% between technical triplicate replicates of the same lysates and up to 9% between triplicated lysates derived from the same samples. Such replication can be cost prohibitive in field studies, however these error rates could have significant impacts on parametric analysis and modelling of unreplicated point estimates, particularly when compounded with potential variation between hosts and associated with coproculture conditions.

Similarly, whilst Avramenko et al. (2019) showed very high correlation between deep-amplicon sequencing and pyrosequencing of the β-tubulin locus and an allele detection limit of 0.1%, Sargison et al. (2019) showed imperfect agreement between the expected and observed outcomes of deep-amplicon sequencing of mock pools of laboratory T. circumcincta isolates. The quantitative use of genetic speciation data derived from coprocultures is therefore not perfect, and Francis et al. (2020) utilised a non-parametric approach to compare MT-PCR speciation against morphological identification of cattle GIN. The descriptive results and comparisons between years and ages in this study are supported by the additional non-parametric analyses; however there is a requirement for further studies that quantify the uncertainty around point estimates to support more powerful, parametric use.

Alongside effects on species diversity, variation in survival and infectivity could potentially create evolutionary bottlenecks within GIN species. Such bottlenecks could potentially have significant
effects on the prevalence of anthelmintic resistance genes, reducing their frequency if they are
associated with fitness costs (Leathwick, 2013), or contributing to their fixation if they result in
reduced refugia populations at a time of anthelmintic treatment, as has been reported associated
with droughts (Besier, 1997; Papadopoulos et al., 2001). It would therefore be of value to investigate
the genetic diversity within species in future studies.

In addition to assessing the impact of environmental and management factors on genetic diversity, it
is possible to monitor their impact on anthelmintic resistance more directly using genetic markers.
The use of deep amplicon sequencing to quantify Isotype-1 β-tubulin SNPs in nematode populations
was first described by Avramenko et al. (2019) and Sargison et al. (2019), and this is the first study
(to the authors’ knowledge) that utilises this technique to monitor resistance SNPs in the T.
circumcincta population on a farm across multiple years. Across the four years, there was relatively
little variation in β-tubulin SNP frequency, with the F200Y polymorphism predominating: the high
prevalence of this mutation in the T. circumcincta population on this farm is consistent with the BZ
resistance previously demonstrated in a species-corrected FECRT performed on this farm (McIntyre
et al., 2018).

Previous research in New Zealand showed a non-significant trend towards reversion to phenotypic
BZ susceptibility across seven farms and five years (Leathwick et al., 2015); however, there is no
evidence for progressive genetic reversion to BZ susceptibility on this farm across the four year study
period. This may be due to inadvertent selection pressures placed upon the T. circumcincta
population by the use of BZ to control N. battus infections in early summer, in combination with a
relatively low refugia population at that time of year following the blanket treatment of the ewes
with moxidectin (Leathwick, 2013). Alternatively, it may be that due to the long-term use of BZ on
this farm, resistant polymorphisms have become co-adapted with other fitness traits, removing any
putative fitness costs (Kelly et al., 1978). Given the discussion above, it is interesting to note that the
three outlying values occurred close to anthelmintic treatments, but it is not possible to ascribe
significance using these data. Further field studies on farms with lower levels of resistance, and variation in anthelmintic usage and resistance mitigation techniques would be extremely valuable. Incorporation of speciation into such work would be vital, given the temporal variation in species composition seen in this study, and evidence that anthelmintic resistance selection pressures and optimal resistance mitigation strategies may vary between parasite species (Waller et al., 1989).

Theoretical modelling of the spread of anthelmintic resistance genes within populations suggests that the degree of mixing between treated and untreated subpopulations is likely to have significant impacts on the rate of spread of anthelmintic resistance within a population (Park et al., 2015). However, Hodgkinson et al. (2019) identified that there is a lack of evidence regarding whether population structuring that might prevent such mixing exists. Within the *T. circumcincta* population on this farm, there were no differences in β-tubulin SNP frequencies between years: this may be due to the F200Y mutation already being close to fixation on this farm, but it is also consistent with the findings of Avramenko et al. (2019), which suggested a lack of population structuring. Further research using selectively neutral markers would be of great value for better addressing this outstanding question.

**Conclusions**

This study demonstrates the feasibility of applying deep amplicon sequencing to monitor GIN species diversity and β-tubulin SNP frequency using field samples obtained from a commercial farm. The speciation results show that on a single farm with consistent management between years, there is variation in GIN species diversity with time and between age groups, and that weather patterns may contribute to this variation. In addition, analysis of the ‘nemabiome’ aids in the interpretation of FECs pre- and post-anthelmintic treatment. These findings reiterate the need to include speciation when investigating GIN epidemiology and anthelmintic resistance. Within the *T. circumcincta* population on this farm, the F200Y BZ resistant SNP is close to genetic fixation, and there is no evidence of variation in β-tubulin SNP frequency between age groups. Furthermore, there is no
genetic evidence of reversion to BZ susceptibility across three years, despite the targeting of BZ usage towards N. battus treatment only. This serves as an example as to how genetic data may be analysed alongside species diversity and FECs, when markers for other anthelmintic classes are identified, and re-emphasises the need for further research into the population genetics of GIN and the selective pressures associated with anthelmintic resistance in the field.

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Declarations of conflicts of interest: None

References


Pohlert, T., 2020. PMCMRplus: Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended.


**Figure Legends**

Fig. 1 The arithmetic mean FECs (eggs per gram, epg) of each sampling-point are shown by the points, which are connected by lines to aid interpretation. 95% confidence intervals for the mean FECs (calculated from the 2.5th and 97.5th percentiles of 500 bootstrap resamples) are shown by the shaded areas. Vertical lines show anthelmintic treatments of lambs, with the line type corresponding to the class of treatment. The peri-parturient treatment of ewes (described in 1.1) is illustrated by the shaded vertical band. Colour versions of this figure are available in the online version of this article.

Fig. 2 Stacked bar chart showing each sampling-point and, within it, the proportion of sequence reads assigned to each species, corrected using previously described correction factors (Redman et al., 2019). Some sampling points are not present due to low coproculture yields or sequence read numbers. Colour versions of this figure are available in the online version of this article.

Fig. 3 A: The mean FECs and 95% confidence intervals as shown in Fig. 1, multiplied by the proportion of corrected sequence reads assigned to each species as shown in Fig. 2. Unclassified Trichostrongylus were excluded from this figure, as their corrected FECs were below 3 epg. Some sampling points are not present due to low coproculture yields or sequence read numbers. B: Dendrogram produced using the mean proportional FEC for each species, grouped by year and age group, with distances calculated using the Pearson’s correlation (with transformation [1-r]) and clustering using the UPGMA method. Colour versions of this figure are available in the online version of this article.

Fig. 4 Points show the proportion of sequence reads in each sample, classified according to the presence of the 3 β-tubulin resistance SNPs (E198L, F167Y, F200Y), or the absence of any of these SNPs (susceptible). These points have been connected by lines to aid interpretation. Vertical lines show anthelmintic treatments of lambs, with the line type corresponding to the class of treatment. The peri-parturient treatment of ewes (described in 1.1) is illustrated by the shaded vertical band. Some sampling points are not present due to low coproculture yields or sequence read numbers. It should be noted that where points equal zero, the SNP may either have been completely absent from the sample, or may have been present in haplotypes with fewer than 500 reads and therefore removed during sequence processing (see 2.7). Colour versions of this figure are available in the online version of this article.

Fig. 5 A: Daily mean soil temperature is shown by the red line. B: Daily mean relative humidity is shown by the green line. C: Weekly rainfall is shown by the blue bars. All three subplots are overlain with smoothed lines and 95% confidence intervals, generated by the LOESS method (span = 0.3) (Wickham, 2017). Colour versions of this figure are available in the online version of this article.

**Tables**

Table 1: Beta-diversity for individual gastrointestinal nematode species (mean percentage ± standard error). Statistically significant (p < 0.05) pairwise comparisons between age groups, within a single year are indicated by *. Statistically significant (p < 0.05) pairwise comparisons between years within a single age group are indicated by matching lowercase letters.
<table>
<thead>
<tr>
<th>Species</th>
<th>Significant pairwise comparisons</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. curticei</strong></td>
<td>Ewe 2016 : Lamb 2017</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Ewe 2019 : Lamb 2017</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>T. circumcincta</strong></td>
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<tr>
<td></td>
<td>Ewe 2016 : Lamb 2018</td>
<td>0.0014</td>
</tr>
<tr>
<td><strong>T. axei</strong></td>
<td>Ewe 2016 : Lamb 2017</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Ewe 2016 : Lamb 2018</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Ewe 2017 : Lamb 2017</td>
<td>0.0015</td>
</tr>
<tr>
<td><strong>T. vitrinus</strong></td>
<td>Lamb 2016 : Lamb 2018</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**Table 2: Significant non-parametric pairwise comparisons in species rank between groups (post hoc Dunn’s test), Bonferroni α = 0.0018.** Results of all comparisons are included in the online repository (see 2.11).

**Supplementary Material**

Fig.S1: Dendrogram produced using the mean proportional FEC for each species, grouped by month and age group, with distances calculated using the Pearson’s correlation (with transformation [1-r]) and clustering using the UPGMA method. Labels are in the format ‘Group-Year-Month’.
Figure 5

A

Soil temperature C

2018

2017

2018

2019

B

Relative humidity %

100

90

80

70

60

50

C

Soil Moisture %

50

40

30

20

D

Weekly rainfall mm

100

75

50

25

Click here to access/download:Figure;fig 5.jpeg