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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★Note: All fastq files were uploaded to Sequence Read Archive (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-eastern Scotland at approximately 3 t- 4 week intervals between spring and autumn over a period of 4 years. Faecal egg counts (FECs) were performed on these samples, and L_3 were collected from pooled coprocultures. Deep amplicon sequencing was used to determine both the species composition of these L_3 and the proportions of benzimidazole (BZ)-resistant single nucleotide polymorphisms (SNPs) in the isotype-1 β-tubulin locus of the predominant species, *Teladorsagia circumcincta* L_3. 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 of 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 (Park et al., 2015; Cornelius et al., 2016) 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 and are 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* spp. 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 internal transcribed spacer (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 L_3 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 Single Nucleotide Polymorphisms (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 GINs (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 4 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

[Check that suppliers are mentioned for chemicals/supplies/equipment and on first mention of each supplier, include the country of origin of supplies.]

2.1. Description of the study farm

A farm of 150 acres in south-eastern Scotland (55°52'N, 3°12'W) at an altitude of 175-190 m 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 is dependent on the date of silaging and the 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 2 years of age.

All ewes received oral moxidectin (200 μg/kg) between lambing and turn-out, within a few days of lambing. All lambs received two or three treatments with oral albendazole (5 mg/kg) during May to July, according to *Nematodirus battus* forecasting (NADIS, http://www.nadis.org.uk/parasite-forecast/) and FEC monitoring results. All lambs received one or two treatments with oral levamisole (7.5 mg/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 (2.5 mg/kg) and an i.m. injection of doramectin (300 μg/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 3 - 4 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 3 - 4 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, Scotland (reference number VERC 10 16), and consent was given by the farm managers.

147 *2.3. FECs and coprocultures*

148 Individual strongyle FECs were performed on all samples using a cuvette technique with a
149 sensitivity of three eggs per gram (epg; Christie and Jackson, 1982). Approximately equal quantities
150 of remaining faeces from each group were then combined into pooled samples, which were cultured
151 at room temperature of approximately 21°C for 14 days, covered with perforated polythene bags to
152 prevent desiccation. The resultant L_3 were then isolated using a modified Baermann's technique
153 (MAFF, 1986) and stored at room temperature in 70% ethanol for up to 8 months (DNA lysates were
154 produced after all samples had been collected for each year).

155

156 *2.4. Genomic DNA extraction*

157 Approximately 1000 L_3 from coprocultures were used for DNA extraction. These were
158 selected by taking an aliquot from the sample, after first estimating the larval density by
159 stereoscopic microscopy. The larvae were washed three times in distilled water, and then
160 centrifuged for 2 min at 7,200 *g* and the resulting pellet re-suspended in 50 µl of lysis buffer (200
161 parts Direct PCR lysis reagent (Viagen Biotech, USA), 1 part proteinase K solution (Qiagen, UK), and 1
162 part 1 M DTT). This was incubated at 60°C for 2 h to lyse the larvae followed by 85°C for 15 min to
163 inactivate the proteinase K.

164

165 *2.5. Adapter PCR amplification of rDNA ITS-2 and isotype-1 β-tubulin loci*

166 The first round PCR amplification was performed on 321 bp fragments of the rDNA ITS-2
167 region, complementary to the 5.8S and 28S rDNA coding sequences, using sets of universal adapter
168 primers (Avramenko et al., 2015). Simultaneously, 276 bp fragments of *T. circumcincta* isotype 1 β-
169 tubulin spanning the F200Y, F167Y and E198L or E198A SNPs were amplified with adapter primers
170 (Sargison et al., 2020). Primers are listed in the online Mendeley repository (see section 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 genomic DNA. 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, USA).

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

The second round PCR was performed using 16 forward and 24 reverse barcoded primers (in the online Mendeley repository, see section 2.11). Each sample of rDNA ITS-2 and isotype-1 β-tubulin was amplified using a unique combination of barcoded primers. The PCR contained 2 µl of the first round PCR product as a template, 0.5 µl of KAPA HiFi polymerase (KAPA Biosystems), 0.75 µl of dNTPs (10 mM), 5 µl of 5X KAPA HiFi Fidelity buffer (KAPA Biosystems), 1.25 µl of each primer (10 µM), and 13.25 µl of nuclease-free water. PCR conditions were 98°C for 45 s, followed by seven cycles of 98°C for 20 s, 63°C for 20 s, and 72°C for 2 min. PCR products were purified as described above and further purified by agarose gel electrophoresis, followed by gel extraction using a 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 quantitative PCR library quantification kit (KAPA Biosystems, USA). 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, Illumina, USA) at a concentration of 15 nM with addition of 10-15%

Phix Control v3 (FC-11-2003, Illumina). 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 <200 bp or >450 bp, 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 amplicons 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 istoype-1 β-tubulin sequence data, paired-ends reads were assembled into single contigs, then filtered to remove contigs that were >350 bp, 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 (submitted to Sequence Read Archive (SRA), see section 2.11). 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, New Zealand), 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, Austria), utilising 'cowplot' (Wilke, 2018) and 'tidyverse' (Wickham, 2017) packages. Ninety-five percent confidence intervals for the arithmetic mean FEC 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 Mendeley online data repository associated with this paper (see section2.11). 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 (α = 0.05) in R (R Core Team). Species diversity between the year and age groups (beta diversity) was assessed using the 'amova' and 'metastats' commands (α = 0.05) 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 analysis of molecular Variance (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 (α = 0.05), with a Bonferroni adjustment, followed by post-hoc Dunn's test (α = 0.05) 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) 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 unweighted pair group method with arithmetic mean (UPGMA) method using the 'amap' pacakage (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), UK at their weather station present within the grounds of the study farm. Soil moisture at a depth of approximately 10-50 cm over a 12 Ha 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 Locally Weighted Scatterplot Smooting (LOESS) method (span=0.3) (Wickham, 2017).

2.11. Data accessibility

All parasitological data, primer sequences, 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 Sequence Read Archive (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 Natural Environment Research Council (NERC) – Centre for Ecology and Hydrology, UK who may be contacted directly regarding obtaining raw data for future use.

3. Results

3.1. FECs varied with time and between age groups

FECs 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 4 weeks post-treatment (Fig. 1). Moxidectin treatment of the ewes in 2016 coincided with a dramatic decrease in FECs, although these rose again approximately 3 - 6 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

291 Visual inspection of the nemabiome suggests that *T. circumcincta* predominated in the
292 lambs, and there was greater species diversity in the ewes (Fig. 2). The one-way ANOVA of the
293 Inverse Simpson's Index showed that there were significant differences in the average alpha
294 diversity present in these groups ($F_{(7,50)}$= 3.569, P = 0.003), with post hoc analysis indicating
295 significantly higher alpha diversity in the 2016 ewes than the lambs in 2017, 2018 and 2019 (P =
296 0.001, 0.044 and 0.014, respectively).

297 Beta diversity assessed by AMOVA (Bonferroni α = 0.002) showed significant differences in
298 species diversity across all groups ($F_{(7, 50)}$ = 3.062, P < 0.001) and for three pairwise comparisons:
299 2016 ewes to 2017 lambs ($F_{(1,14)}$= 8.389, P<0.001); 2018 ewes to 2017 lambs ($F_{(1,12)}$ = 5.494, P <
300 0.001); and 2019 ewes to 2017 lambs ($F_{(1,12)}$ = 4.101, P = 0.001). Metastats analysis indicated
301 statistically significant differences between years and between age groups for *Cooperia curticei*,
302 *Teladorsagia circumcincta*, *Trichostrongylus axei* and *Trichostrongylus vitrinus* (Table 1).

303 Non-parametric analyses were consistent with the metastats analysis, showing significant
304 differences (Bonferroni α = 0.0083) in species rank for *C. curticei* (P = 0.0002), *T. circumcincta* (P =
305 0.0015), *T. axei* (P = 0.0002) and *T. vitrinus* (P = 0.0082). Post hoc Dunn's tests showed this variation
306 to be driven by significant differences in species rank between both years and age groups (Table 2).

307

308 *3.3. FECs adjusted for species composition varied with time and between age groups*

309 Consistent with the species diversity reported above, the FEC attributed to each species
310 varied with time and between age groups (Fig. 3A). The peri-parturient rise in FECs contained
311 contributions from multiple species, with *T. circumcincta* predominating, whereas the rebound in
312 FECs towards the end of the lambing period in 2017 and 2019 contained a greater proportion of *C.*
313 *curticei*. However, this rise in the *C. curticei* egg output from ewes did not result in a corresponding
314 rise in the samples from lambs. In all 4 years, egg outputs from lambs were composed

predominately of *T. circumcincta*, although there was a rise in the contributions of *T. 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 with 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). Similarly, 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 (Supplementary 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 4 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 section 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 4 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), together with 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 nematode species in cattle (Eysker and Eilers, 1995), and the datasheet for oral 0.1% moxidectin has no claim of persistence against *C. curticei* (NOAH, http://www.noahcompendium.co.uk/datasheets). 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 GIN 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 over-represented in the samples from ewes, *T. circumcincta* was over-represented 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 with 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 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 with 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 section 2.11) so that those 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

414 replacement of approximately 22% of the breeding flock, and the fact that the lambs are from Texel
415 sires, a breed associated with immunity against GINs (Good et al., 2006).

416 These results demonstrate the power of the nemabiome approach (Avramenko et al., 2015)
417 to investigate variation in different GIN species and contribute to the modelling of GIN infections.
418 However, they also demonstrate the complexity of the systems being studied and emphasise the
419 need to incorporate variation in climatic factors, host factors, and farm management practices into
420 future surveys and models. In addition, this study was impacted by missing data points due to low
421 coproculture yields from some samples. Redman et al. (2019) validated the use of cultures of L_1 in
422 addition to the L_3 cultures used in this study. L_1 cultures are less affected by coproculture conditions
423 and are therefore arguably more representative of the eggs shed, but less representative of the
424 larvae that go on to infect the pasture. Further research into how nemabiome data correlate with
425 infection levels within hosts and pasture larval composition would therefore be extremely valuable,
426 as would validation of how accurately pooled faecal samples reflect population level variation, and
427 the optimum methods for sampling and preparing these pools.

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

Together with 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, R.B., 1997. Ecological selection for anthelmintic resistance: Re-evaluation of sheep worm control programs. In: Van Wyk J and Van Schalkwyk PC (eds) Managing Anthelmintic Resistance in Endoparasites. Workshop held at the 16th International Conference of the World Association for the Advancement of Veterinary Parasitology, Sun City, South Africa, 10–15 August 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, to our knowledge, this is the first study that utilises this technique to monitor resistance SNPs in the *T.*

circumcincta population on a farm across multiple years. Across the 4 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 faecal egg count reduction test (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 5 years (Leathwick et al., 2015); however, there is no evidence for progressive genetic reversion to BZ susceptibility on this farm across the 4 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.

In conclusion, 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 3 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 GINs and the selective pressures associated with anthelmintic resistance in the field.

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Figures

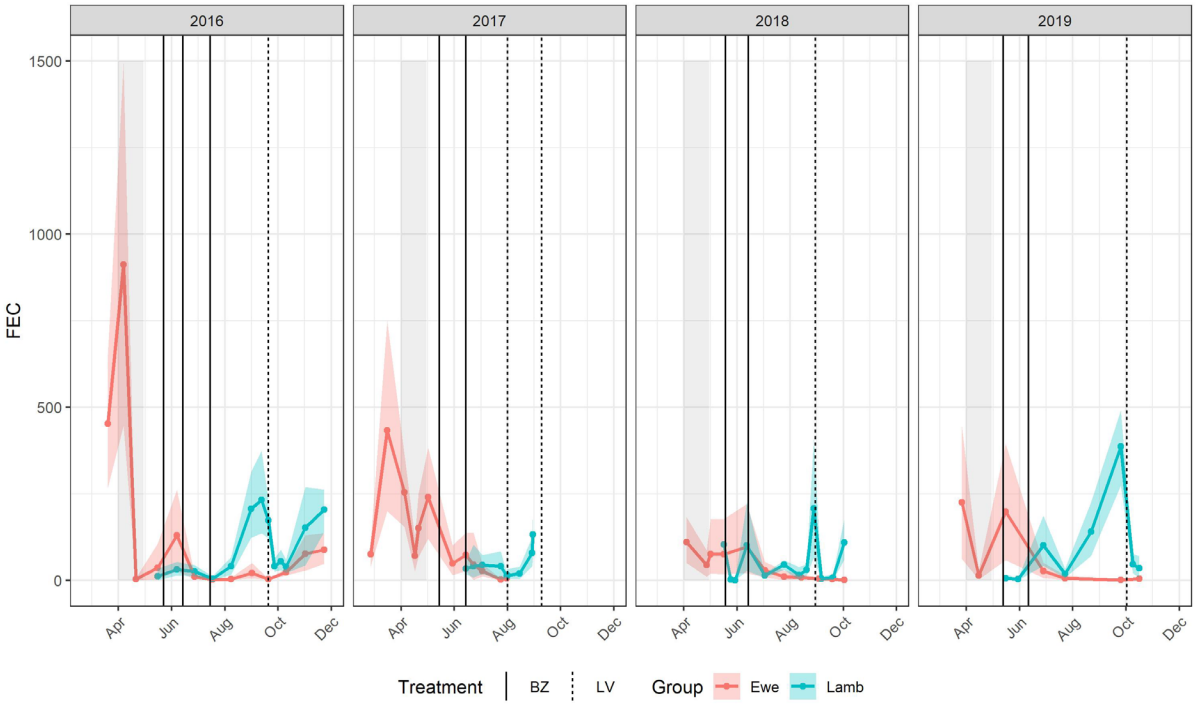
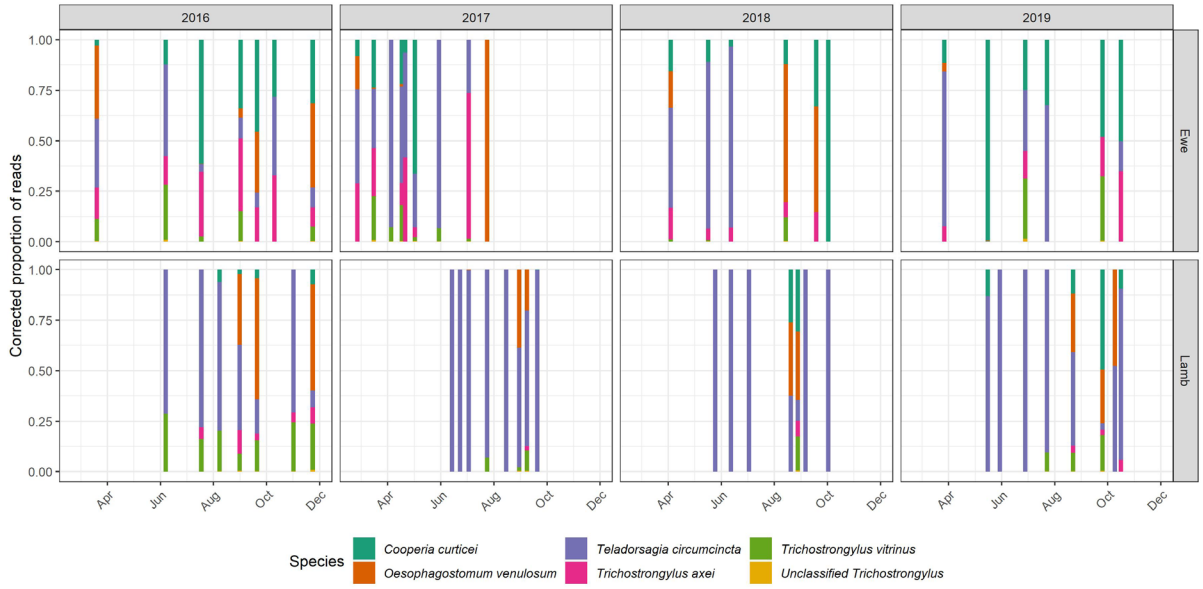
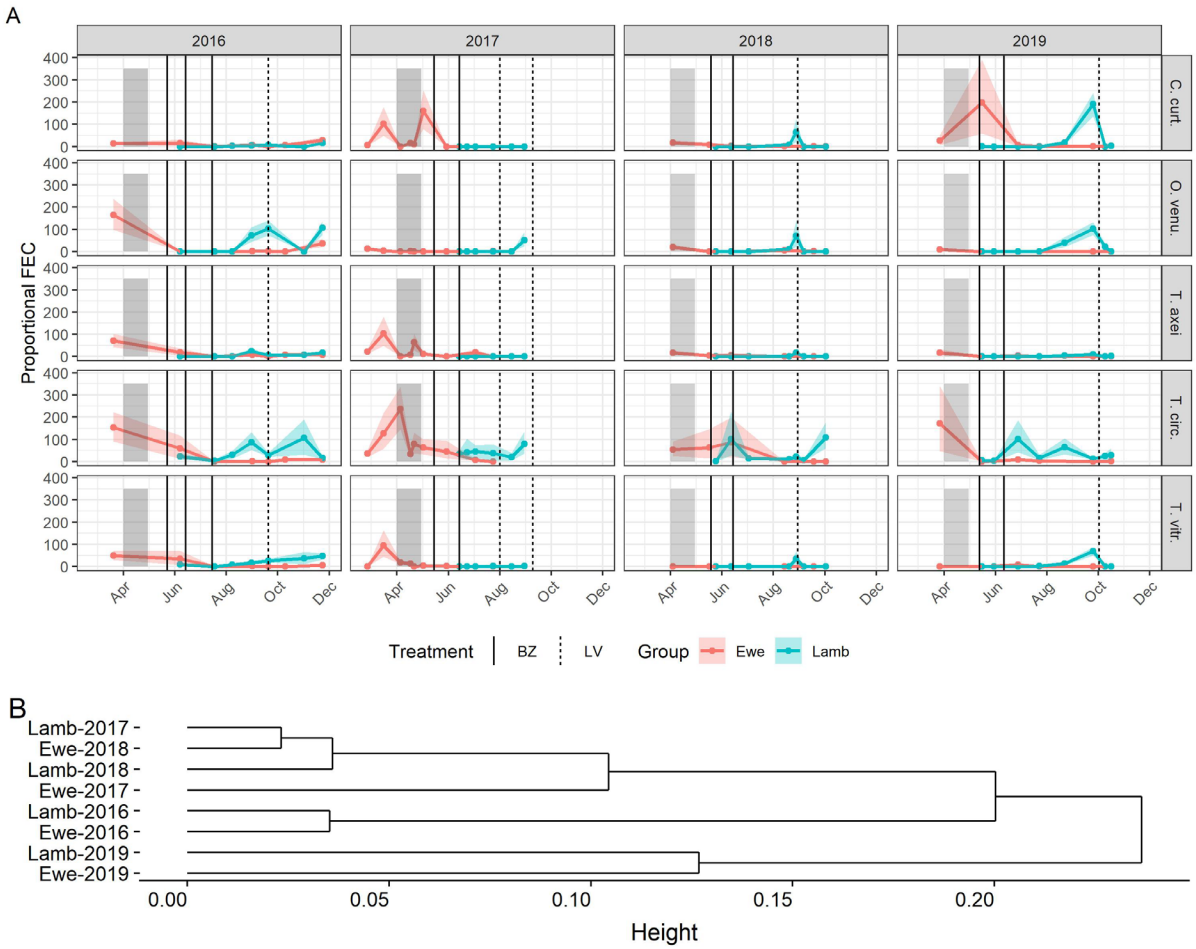


Fig. 1. The arithmetic mean Faecal Egg Counts (FECs) (eggs per gram, epg) of each sampling point are shown by the points, which are connected by lines to aid interpretation. Ninety-five percent confidence intervals for the mean FECs (calculated from the 2.5^{th} and 97.5^{th} percentiles of 500 bootstrap resamples) are shown by the shaded areas. Vertical lines show anthelmintic treatments of lambs, with the line type corresponding to the class of treatment (benzimidazole (BZ) or levamisole (LV)). The peri-parturient treatment of ewes (described in section 2.1) is illustrated by the shaded vertical band. Colour versions of this figure are available in the online version of this article.



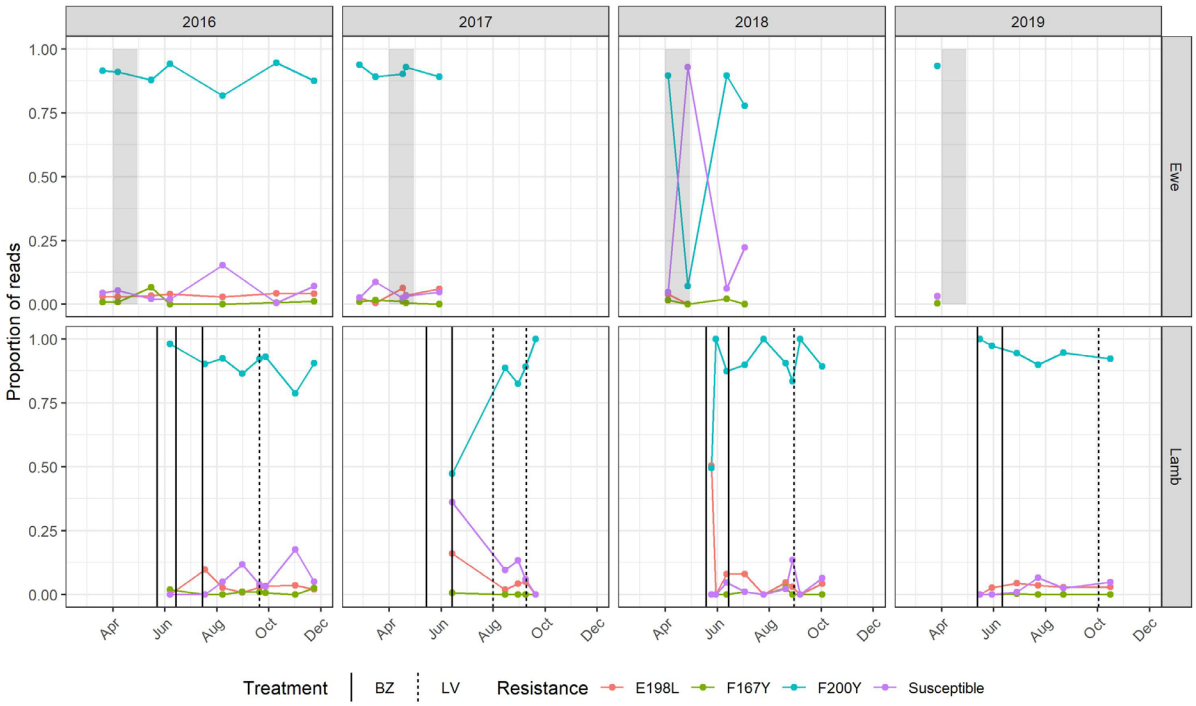
662

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



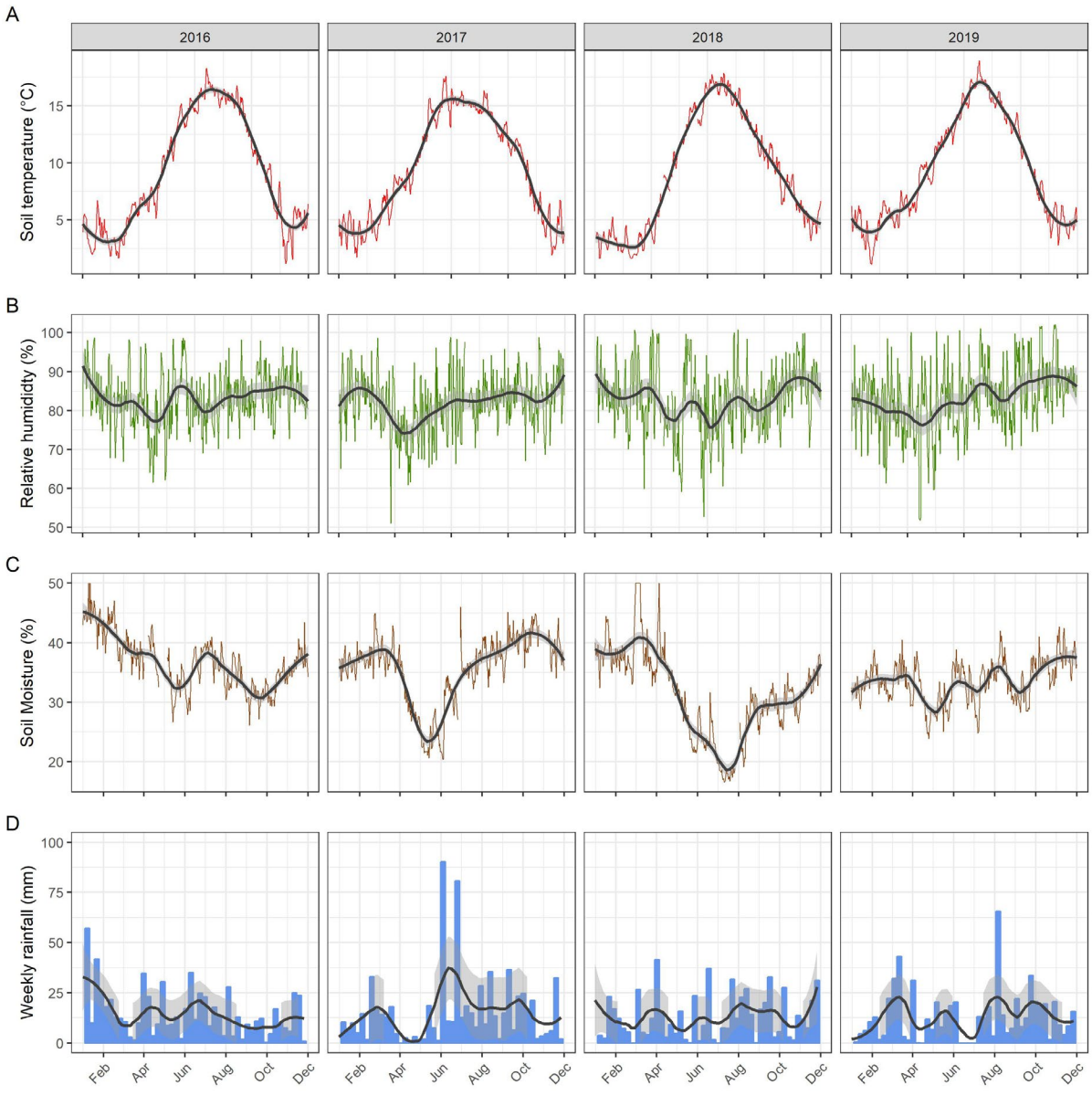
667

668 Fig. 3. Proportional Faecal Egg Counts (FECs) with time and between age groups. (A) The mean FECs
669 (eggs per gram, epg) and 95% confidence intervals as shown in Fig. 1, multiplied by the proportion of
670 corrected sequence reads assigned to each species as shown in Fig. 2. Vertical lines show
671 anthelmintic treatments of lambs, with the line type corresponding to the class of treatment
672 (benzimidazole (BZ) or levamisole (LV)). The peri-parturient treatment of ewes (described in section
673 2.1) is illustrated by the shaded vertical band. Unclassified *Trichostrongylus* were excluded from this
674 figure, as their corrected FECs were below 3 epg. Some sampling points are not present due to low
675 coproculture yields or sequence read numbers. (B) Dendrogram produced using the mean
676 proportional FEC for each species, grouped by year and age group, with distances calculated using
677 the Pearson's correlation (with transformation [1-r]) and clustering using the unweighted pair group
678 method with arithmetic mean (UPGMA) method. Colour versions of this figure are available in the
679 online version of this article.



680

681 Fig. 4. Points show the proportion of sequence reads in each sample, classified according to the
682 presence of the three β-tubulin resistance single nucleotide polymorphisms (SNPs) (E198L, F167Y,
683 F200Y), or the absence of any of these SNPs (susceptible). These points have been connected by
684 lines to aid interpretation. Vertical lines show anthelmintic treatments of lambs, with the line type
685 corresponding to the class of treatment (benzimidazole (BZ) or levamisole (LV)). The peri-parturient
686 treatment of ewes (described in section 2.1?) is illustrated by the shaded vertical band. Some
687 sampling points are not present due to low coproculture yields or sequence read numbers. It should
688 be noted that where points equal zero, the SNP may either have been completely absent from the
689 sample, or may have been present in haplotypes with fewer than 500 reads and therefore removed
690 during sequence processing (see section 2.7). Colour versions of this figure are available in the online
691 version of this article.

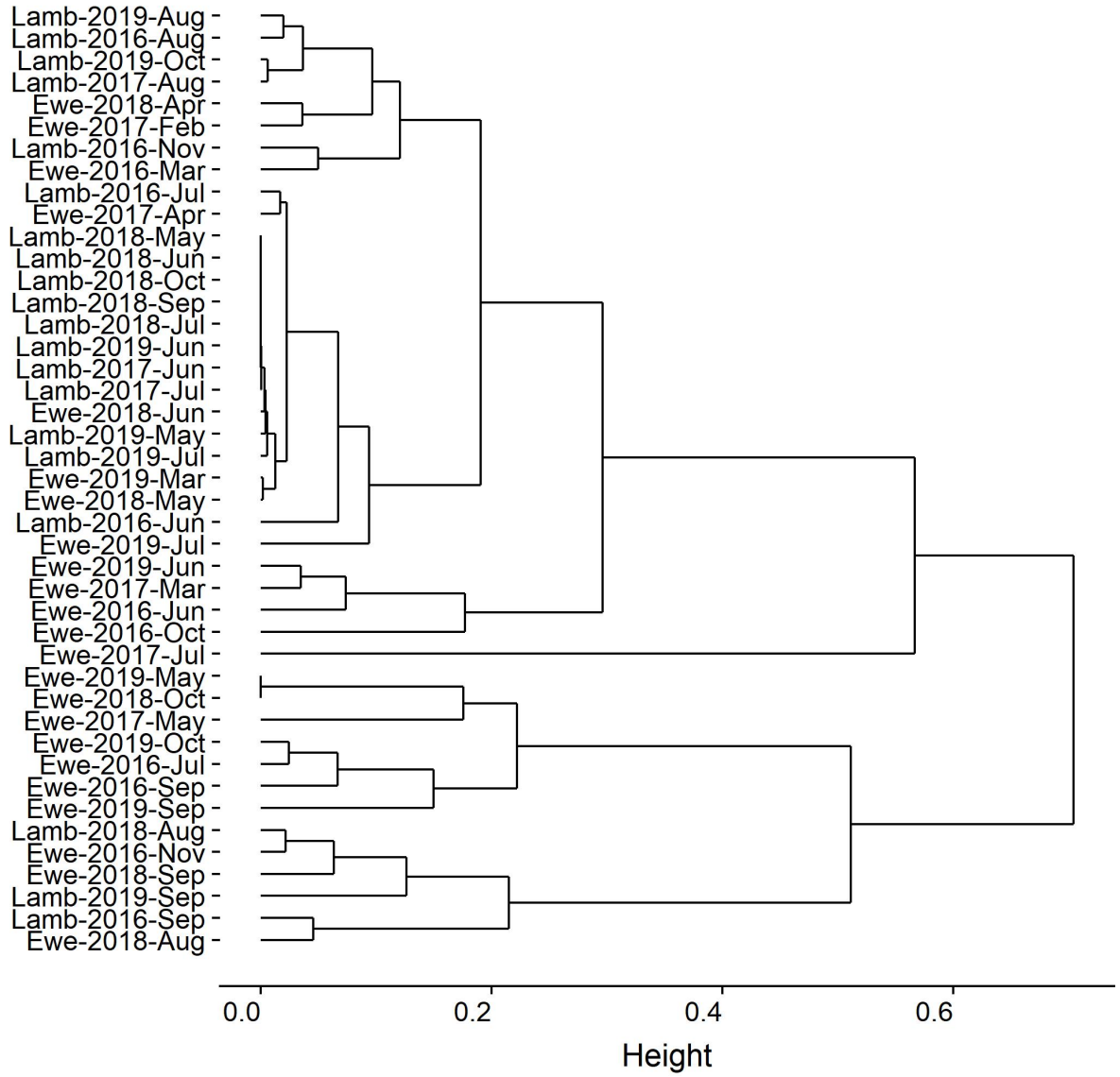


692

693 Fig. 5. Weather data plotted against time. (A) Daily mean soil temperature is shown by the finer line
694 (red in colour version). (B) Daily mean relative humidity is shown by the finer line (green in colour
695 version). (C) Soil moisture is shown by the finer line (brown in colour version). (D) Weekly rainfall is
696 shown by the bars (blue in colour version). All four subplots are overlain with thicker smoothed lines
697 and shaded 95% confidence intervals, generated by the Locally Weighted Scatterplot Smoothing
698 (LOESS) method (span = 0.3) (Wickham, 2017). Colour versions of this figure are available in the
699 online version of this article.

700

Supplementary figure



Supplementary Fig. S1. Dendrogram produced using the mean proportional faecal egg count (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 unweighted pair group method with arithmetic mean (UPGMA) method. Labels are in the format 'Group-Year-Month'.

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 [a]. Statistically significant (P< 0.05) pairwise comparisons between years within a single age group are indicated by matching lowercase letters.

Species	Lamb				Ewe			
	2016	**2017**	**2018**	**2019**	**2016**	**2017**	**2018**	**2019**
Cooperia curticei	2.7 ± 1.2 [a] a	0.0 ± 0.0 [a] ab	8.4 ± 5.4	9.6 ± 6.2 [a] b	30.5 ± 8.0 [a]	11.3 ± 6.1 [a] c	31.7 ± 15.3	38.9 ± 13.3 [a] c
Oesophagostomum venulosum	8.3 ± 4.2	2.2 ± 1.5	3.6 ± 2.3	4.4 ± 2.3	6.1 ± 2.8a	11.7 ± 11.0	11.7 ± 6.7	0.2 ± 0.2a
Teladorsagia circumcincta	58.3 ± 9.2 [a] a	94.7 ± 2.8 [a] a	82.5±12 .2	78.1 ± 11.1 [a]	29.1 ± 7.7 [a]	52.5 ± 9.9 [a]	41.5 ± 18.9	36.4 ± 15.2 [a]
Trichostrongylus axei	4.5 ± 1.6 [a] abc	0.2 ± 0. 2 [a] a	1.2 ± 1.2 [a] b	1.2 ± 0.6c	21.0 ± 3.4 [a]	16.8 ± 7.0 [a]	8.5 ± 2.8 [a]	10.6 ± 5.0
Trichostrongylus vitrinus	25.8 ± 4.0 [a] abc	2.7 ± 1.6a	4.2 ± 4.2b	6.7 ± 3.9c	12.6 ± 4.5 [a]	7.4 ± 3.3	5.5 ± 5.0	13.2 ± 8.3
Unclassified	0.2 ± 0.0 [a] a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0a	0.6 ± 0.2 [a]	0.3 ± 0.1	1.1 ± 0.8	0.4 ± 0.2
Trichostrongylus								

Table 2. Significant non-parametric pairwise comparisons in species rank between groups (post hoc Dunn's test), Bonferroni $\alpha = 0.0018$. Results of all comparisons are included in the Mendeley online repository (see section 2.11).

Species	Significant pairwise comparisons	*P* value
Cooperia	Ewe 2016 : Lamb 2017	0.0002
Curticei	Ewe 2019 : Lamb 2017	<0.0001
Teladorsagia	Ewe 2016 : Lamb 2017	0.0067
Circumcincta	Ewe 2016 : Lamb 2018	0.0014
Trichostrongylus	Ewe 2016 : Lamb 2017	<0.0001
axei	Ewe 2016 : Lamb 2018	0.0002
	Ewe 2017 : Lamb 2017	0.0015
Trichostrongylus vitrinus	Lamb 2016 : Lamb 2018	<0.0001