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1 **A 4 year observation of gastrointestinal nematode egg counts, nemabiomes and the**
2 **benzimidazole resistance genotypes of *Teladorsagia circumcincta* on a Scottish sheep**
3 **farm✭**

4

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6

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21 ✭Note: All fastq files were uploaded to Sequence Read Archive (SRA) (Bioproject accession number:
22 **PRJNA669542**) and all β-tubulin sequences were uploaded to GenBank (accession numbers:
23 **MW081491-MW081536**).

24

25 **Abstract**

26 Anthelmintic resistance threatens the sustainability of sheep production globally. Advice regarding
27 strategies to reduce the development of anthelmintic resistance incorporates the outcomes of
28 modelling exercises. Further understanding of gastrointestinal nematode (GIN) species diversity, and
29 population dynamics and genetics (which may vary between species) is required to refine these
30 models; and field studies combining faecal egg outputs, species composition and resistance genetics
31 are needed to calibrate them. In this study, faecal samples were taken from ewes and lambs on a
32 commercial farm in south-eastern Scotland at approximately 3 t- 4 week intervals between spring
33 and autumn over a period of 4 years. Faecal egg counts (FECs) were performed on these samples,
34 and L_3 were collected from pooled coprocultures. Deep amplicon sequencing was used to determine
35 both the species composition of these L_3 and the proportions of benzimidazole (BZ)-resistant single
36 nucleotide polymorphisms (SNPs) in the isotype-1 β-tubulin locus of the predominant species,
37 *Teladorsagia circumcincta* L_3. Despite consistent management throughout the study, the results
38 show variation in GIN species composition with time and between age groups, that was potentially
39 associated with weather conditions. The F200Y BZ resistance mutation is close to genetic fixation in
40 the *T. circumcincta* population on this farm. There was no evidence of variation in isotype-1 β-
41 tubulin SNP frequency between age groups, and no genetic evidence of reversion to BZ
42 susceptibility, despite targeted BZ usage. This study highlights the need to include speciation when
43 investigating GIN epidemiology and anthelmintic resistance, and serves as an example of how
44 genetic data may be analysed alongside species diversity and FECs, when markers for other
45 anthelmintic classes are identified.

46

47 *Keywords*: Sheep; Gastrointestinal nematode; Nemabiome; Isotype-1 β-tubulin SNPs; Modelling
48 anthelmintic resistance

49

50 **1. Introduction**

51 Gastrointestinal nematode (GIN) infections in sheep have been shown to impact significantly
52 on the outputs of both meat and milk production globally (Mavrot et al., 2015), and modelling
53 suggests that reducing the severity of GIN infection in sheep would result in a linear reduction in the
54 costs of production in Great Britain (Nieuwhof and Bishop, 2005), as well as reducing the carbon
55 footprint of production (Kenyon et al., 2013a). However, the widespread prevalence of anthelmintic
56 resistance threatens the sustainability of sheep production (Kaplan and Vidyashankar, 2012; Rose et
57 al., 2015). In addition, there have been changes in GIN epidemiology associated with climate change
58 (Kenyon et al., 2009b; Sargison et al., 2012), and modelling suggests that future climate change may
59 impact the sustainability of current management strategies (Rose et al., 2016).

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

70 A seasonal pattern of ovine GIN infection has traditionally been described in temperate
71 climates, with overwintering of larvae and a peri-parturient rise in ewes contributing to the infection
72 of lambs, which leads to a progressive rise in pasture contamination through the summer and
73 autumn (Van Dijk et al., 2010). Larval development rates vary between GIN species and are
74 associated with soil temperature, rainfall and relative humidity (O'Connor et al., 2006). This gives

75 rise to typical seasonal variation in GIN species in the UK, with *Teladorsagia circumcincta*
76 traditionally predominating in summer, followed by an increased contribution from *Trichostrongylus*
77 spp. in autumn (Van Dijk et al., 2010). Despite changes in climate and farming practices, a recent
78 observational study on three Scottish farms was consistent with the traditionally described faecal
79 egg count (FEC) profile (Hamer et al., 2018). However, veterinary diagnostic submissions in Northern
80 Ireland suggest that there has been a decrease in the relative seasonality of teladorsagiosis and
81 trichostrongylosis (McMahon et al., 2013). Further investigation of species composition by
82 morphological methods is limited by the requirement for significant skilled labour input; hence the
83 development of a deep-amplicon sequencing approach using the internal transcribed spacer (ITS_-2
84 locus to speciate mixed communities of nematodes (the 'nemabiome') has provided the opportunity
85 to analyse GIN species diversity at much greater throughput (Avramenko et al., 2015). Redman et al.
86 (2019) reported the validation of this technique for ovine GIN, including the development of
87 correction factors to account for differential efficiency of DNA amplification from L_3 of the most
88 common species. It was also suggested that there may be differences in the nemabiome between
89 ewes and lambs on the same farms (Redman et al., 2019), although that may also have been
90 affected by the seasons in which the age groups were sampled.

91 High throughput sequencing techniques present the opportunity to investigate many of the
92 outstanding questions regarding the genetics of anthelmintic resistance (Hodgkinson et al., 2019).
93 Although there are currently no confirmed, specific genetic markers for resistance to levamisole or
94 macrocyclic lactone drugs in GIN, the genetic basis for resistance to benzimidazole (BZ) drugs is
95 characterised by the presence of any of three separate Single Nucleotide Polymorphisms (SNPs) (at
96 codons 167, 198 and 200) in the isotype-1 β-tubulin locus (Geary et al., 1992; Kwa et al., 1995, 1994;
97 Elard et al., 1996). Deep-amplicon sequencing approaches for this locus have been validated and
98 applied to pooled field samples for multiple ovine GINs (Avramenko et al., 2019) and specifically for
99 *T. circumcincta* (Sargison et al., 2019). However, to the best of our knowledge, there have been no

100 studies assessing variation in isotype-1 1 β-tubulin SNP frequency within a sheep flock, between age
101 groups and with time across multiple years.

102 This study describes the pattern of faecal GIN egg shedding by ewes and lambs across 4
103 years, with varying climate; and applies deep amplicon sequencing techniques to describe variation
104 in both the species compositions and the BZ resistance SNP frequencies within the *T. circumcincta*
105 population. Investigating variation in these factors will determine whether they need to be factored
106 into modelling exercises. This study also serves as a proof of concept for future monitoring of the
107 impact of management and treatment decisions on GIN species diversity and anthelmintic resistance
108 in controlled experiments, or larger observational studies.

109

110 **2. Materials and methods**

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

113 *2.1. Description of the study farm*

114 A farm of 150 acres in south-eastern Scotland (55°52'N, 3°12'W) at an altitude of 175-190 m
115 was studied. The breeding flock is comprised of approximately 370 Cheviot Mule ewes, which are
116 crossed with Texel rams in October/November, to give an estimated lambing period from the end of
117 March until the end of April. All ewes lamb in indoor pens and are turned out onto pasture
118 approximately 2 days after lambing. Ewes and lambs co-graze until the lambs are weaned in August
119 (stocking density c. 6 ewes plus lambs per acre). After weaning, ewes continue to graze the same
120 pasture, whilst the lambs are moved onto silage aftermaths (stocking density is dependent on the
121 date of silaging and the rate lambs are drawn for slaughter). Lambs are sold for meat production
122 between August and December, according to their liveweight. Approximately 80 replacement

123 females (22%) are purchased as ewe lambs in October. These join the main ewe flock when aged
124 approximately 18 months, and give birth for the first time at approximately 2 years of age.

125 All ewes received oral moxidectin (200 μg/kg) between lambing and turn-out, within a few
126 days of lambing. All lambs received two or three treatments with oral albendazole (5 mg/kg) during
127 May to July, according to *Nematodirus battus* forecasting (NADIS, http://www.nadis.org.uk/parasite-
128 forecast/) and FEC monitoring results. All lambs received one or two treatments with oral levamisole
129 (7.5 mg/kg) between August and September, according to FEC monitoring results, growth rates and
130 clinical signs of diarrhoea. Lambs were not intentionally moved to clean grazing after the
131 anthelmintic treatments, although the first levamisole treatment in August 2017 coincided with
132 weaning and therefore movement. Replacement ewe lambs were treated with oral monepantel (2.5
133 mg/kg) and an i.m. injection of doramectin (300 μg/kg) on arrival, before being moved to pasture
134 that had been used for lambs within that year.

135

136 *2.2. Sample collection*

137 Samples were collected between spring 2016 and autumn 2019. During these four sampling
138 years, 10 freshly voided faecal samples produced by ewes were collected from the ground at
139 approximately 3 - 4 week intervals between April and October, with some additional samples taken
140 over the winter of 2016/2017. Ten freshly voided faecal samples produced by lambs were collected
141 from the ground at approximately 3 - 4 week intervals between June and October, with some
142 additional sampling points for ad hoc clinical monitoring. Samples were not linked to individual
143 animals. Ethical approval was acquired through Veterinary Ethics Review Committee (VERC) at the
144 University of Edinburgh, Scotland (reference number VERC 10 16), and consent was given by the
145 farm managers.

146

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

171 both rDNA ITS-2 and isotype 1 β-tubulin loci, equal proportions of the four forward and four reverse
172 primers were mixed and used for the adapter PCR with following conditions: 10 µM forward and
173 reverse adapter primers, 10 mM dNTPs, 0.5 U DNA polymerase enzyme, 5X buffer (KAPA
174 Biosystems) and 1 µl of genomic DNA. Thermocycling conditions were 95°C for 2 min, followed by 35
175 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
176 extension of 72°C for 5 min. PCR products were purified with AMPure XP Magnetic Beads (1X)
177 according to the manufacturer's instructions (Beckman Coulter, USA).

178

179 *2.6. Barcoded PCR amplification of rDNA ITS-2 and isotype-1 β-tubulin loci*

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

189

190 *2.7. Deep amplicon sequencing and data handling*

191 The purified products from each sample were mixed to prepare a pooled library and
192 measured with the KAPA quantitative PCR library quantification kit (KAPA Biosystems, USA). The
193 library was then run on an Illumina MiSeq sequencer using a 500-cycle pair end reagent kit (MiSeq
194 Reagent Kits v2, MS-103-2003, Illumina, USA) at a concentration of 15 nM with addition of 10-15%

195 Phix Control v3 (FC-11-2003, Illumina). During the post-run processing, Mi-Seq splits all sequences by
196 samples using the barcoded indices to produce FASTQ files.

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

201 For the ITS-2 sequence data, paired-end reads were assembled into single contigs and then
202 filtered to remove contigs that were <200 bp or >450 bp, and pairs that contained any ambiguities.
203 Contigs were then aligned to an ITS-2 rDNA database previously described by Avramenko et al.
204 (2015) and discarded if they did not align to at least 10% of any ITS-2 rDNA amplicons in the
205 database with at least 90% sequence similarity. The remaining sequences were classified by
206 comparing to reference sequences in the database using the k-nearest-neighbour method (k = 3). In
207 order to reduce the impact of potential PCR or sequencing errors, taxonomic levels with fewer than
208 2000 reads across all samples were removed. Samples with fewer than 2000 reads across all
209 taxonomic levels were then also removed.

210 In the case of istoype-1 β-tubulin sequence data, paired-ends reads were assembled into
211 single contigs, then filtered to remove contigs that were >350 bp, and pairs that contained any
212 ambiguities. The sequence data were then aligned with a *T. circumcincta* reference sequence library
213 previously described by Sargison et al. (2019), and were removed if they did not match with the *T.*
214 *circumcincta* isotype 1 β-tubulin locus. Chimeras were removed using VSEARCH (Rognes et al., 2016)
215 and remaining sequences were summarised to generate the *T. circumcincta* isotype 1 β-tubulin
216 sequences FASTQ file (submitted to Sequence Read Archive (SRA), see section 2.11). Once all bulk
217 sequences were classified as *T. circumcincta*, a count list of the consensus sequences of each
218 population was created. In order to reduce the impact of potential PCR or sequencing errors,
219 haplotypes with fewer than 500 reads across all samples were removed. Samples with fewer than

220 500 reads across all haplotypes were then also removed. A lower threshold was used than for ITS2
221 sequences (500 c.f. 2000 reads) to account for potentially low proportions of *T. circumcincta* within
222 individual coprocultures. The remaining haplotypes were manually examined in Geneious Prime
223 (Biomatters Ltd, New Zealand), and a conservative approach was used, whereby individual SNPs that
224 occurred in just single haplotypes were corrected to the consensus sequence. These haplotypes
225 were then collapsed using FaBox (Villesen, 2007). The simplified haplotypes were then labelled
226 according to whether the BZ resistance SNPs (F167Y, E198L, F200Y) were present.

227

228 *2.8. Data processing and presentation*

229 Data were processed and presented using R v3.5.1 in R Studio v1.1.4.5.6 (R Core Team,
230 Austria), utilising 'cowplot' (Wilke, 2018) and 'tidyverse' (Wickham, 2017) packages. Ninety-five
231 percent confidence intervals for the arithmetic mean FEC of each sampling event were generated by
232 500 bootstrap resamples (with replacement) utilising the 'rsample' package (Kuhn and Wickham,
233 2017). Species-specific sequencing biases were corrected for by multiplying the ITS-2 read
234 proportions by correction factors that were previously validated against morphological methods
235 (Redman et al., 2019). These correction factors are also included in the Mendeley online data
236 repository associated with this paper (see section2.11). Proportional FECs were generated by
237 multiplying corrected species by the arithmetic mean FEC and 95% confidence interval.

238

239 *2.9. Diversity analysis*

240 Species diversity within each sample (alpha diversity) was assessed using the Inverse
241 Simpson's Index, calculated in Mothur (Schloss et al., 2009), based on a random subsample of the
242 sequences equal in size to the smallest sample. Differences in the Inverse Simpson's Index between
243 each year and age group were then assessed using a one-way ANOVA and post hoc Tukey

244 comparisons (α = 0.05) in R (R Core Team). Species diversity between the year and age groups (beta
245 diversity) was assessed using the 'amova' and 'metastats' commands (α = 0.05) in Mothur (Schloss et
246 al., 2009), based on a random subsample of the sequences equal in size to the smallest sample. A
247 Bonferroni adjustment was used for the analysis of molecular Variance (AMOVA) analysis, dividing
248 the intended alpha of 0.05 by the number of pairwise comparisons. Non-parametric analysis
249 comparing species ranks between years and age groups was performed using Kruskal-Wallis Rank
250 Sum tests in R (α = 0.05), with a Bonferroni adjustment, followed by post-hoc Dunn's test (α = 0.05)
251 using the r package 'PMCMRplus` (Pohlert, 2020), with a Bonferroni adjustment, for those species
252 with significant Kruskal-Wallis results.

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

259

260 *2.10. Weather data*

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

267

268 *2.11. Data accessibility*

269 All parasitological data, primer sequences, fastq files, sequence results, mothur scripts and
270 diversity analysis outputs have been made freely available through Mendeley Data at DOI:
271 10.17632/nfhpswcybc.1. All fastq files were uploaded to Sequence Read Archive (SRA) (Bioproject
272 accession number: **PRJNA669542**) and all β-tubulin sequences were uploaded to GenBank (accession
273 numbers: **MW081491-MW081536**). All meteorological data and soil moisture data are the property
274 of Natural Environment Research Council (NERC) – Centre for Ecology and Hydrology, UK who may
275 be contacted directly regarding obtaining raw data for future use.

276

277 **3. Results**

278 *3.1. FECs varied with time and between age groups*

279 FECs in the ewes rose around the time of parturition, whilst the FECs of lambs rose in late
280 summer and autumn; and there was variation between years in both the magnitude and the timing
281 of these increases (Fig. 1). These data also illustrate that levamisole treatments of the lambs in 2016,
282 2018 and 2019 appear to have been effective, although in 2016 and 2018, FECs increased again
283 approximately 4 weeks post-treatment (Fig. 1). Moxidectin treatment of the ewes in 2016 coincided
284 with a dramatic decrease in FECs, although these rose again approximately 3 - 6 weeks after the
285 lambing period ended; there was a similar drop in FECs in the ewes in 2017 and 2019, although in
286 these years the rebound was more rapid (Fig. 1). FECs around the time of the moxidectin treatment
287 of ewes in 2018 were low, although not zero; however, there was no pre-treatment sample from this
288 year.

289

290 *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

315 predominately of *T. circumcincta*, although there was a rise in the contributions of *T. vitrinus* and
316 *Oesophagostomum venulosum* in the late autumn/winter of 2016, and in these two species plus *C.*
317 *curticei* in autumn 2019. Although the overview of the nemabiome suggests greater species diversity
318 in the lambs of 2018 (Fig. 2), compared with the lambs of 2017, this appears less significant when
319 corrected for FEC (Fig. 3A).

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

326

327 *3.4. Isotype-1 β-tubulin SNPs showed little variation*

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

335

336 *3.5. Over-winter and summer weather patterns varied between years*

337 Soil temperature, relative humidity, soil moisture and rainfall on the farm were documented
338 over the course of the four study years. Winter soil temperatures were lower in 2017/18 than in the

339 other years (Fig. 5A). There was a more prolonged warm period during the summer of 2017 than in
340 2018 and 2019, and the temperature profile in summer 2016 was between these two extremes (Fig.
341 5A). The humidity profiles are similar for the 4 years, although the humidity during the
342 summer/autumn of 2017 was more stable than in the other years, and the 2017/18 winter was more
343 humid than 2016/17 and 2018/19 (Fig. 5B). Soil moisture levels were lower in the winters of
344 2016/2017 and 2018/2019 than 2017/2018 (Fig. 5C). The soil was also drier during the spring of
345 2017 and through the spring and summer of 2018 (Fig. 5C). The autumn of 2016 had relatively low
346 rainfall; and the spring/summer of 2017 and 2019 had low rainfall initially, before periods of higher
347 rainfall later in the season (Fig. 5D).

348

349 **4. Discussion**

350 The FEC results from 2016 were previously presented as Farm 1 in Hamer et al. (2019),
351 together with data from two nearby farms, demonstrating that patterns of faecal egg production
352 were broadly similar to those traditionally described, despite changes in climate and farm
353 management. In the present study, this profile was similar for both the ewes and lambs in 2017,
354 2018 and 2019 (although no pre-moxidectin sample was obtained from the ewes in 2018). However,
355 analysis of the nemabiome shows that on a single farm with consistent management between years,
356 there were significant differences in species diversity within and between age groups and years. This
357 emphasises the importance of the speciation of the nematodes present within a FEC, and raises
358 questions about the factors driving this variation.

359 In addition to investigating species diversity, ITS-2 based speciation was previously used to
360 diagnose anthelmintic resistance within the *T. circumcincta* population on this farm, which would
361 have been missed by a traditional FEC reduction test (FECRT) (McIntyre et al., 2018). The present
362 study adds to the evidence that speciation enhances the interpretation of raw FECs: without
363 speciation; the rebound peak in FEC in the ewes in 2017 might suggest anthelmintic resistance,

364 however, given that it is composed predominately of *C. curticei,* this peak may simply reflect
365 pharmacokinetic differences, as moxidectin has been shown to have greater persistence against
366 abomasal than intestinal nematode species in cattle (Eysker and Eilers, 1995), and the datasheet for
367 oral 0.1% moxidectin has no claim of persistence against *C. curticei* (NOAH,
368 http://www.noahcompendium.co.uk/datasheets). Similarly, the nemabiome is at risk of over-
369 interpretation if it is not considered in the context of the FECs of the samples.

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

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

385 These differences in species diversity described between years could potentially relate to
386 climatic impacts on the overwinter survival of larvae on the pasture. The winters of 2016/17 and
387 2018/19 were mild and dry compared with the winter of 2017/18. Both these factors would be
388 expected to result in decreased survival of *T. circumcincta* on pasture (O'Connor et al., 2006;

389 McMahon et al., 2012), yet surprisingly, *T. circumcincta* predominated in the lambs in 2017.
390 However, previous research focussed on the climate-driven epidemiology of *Haemonchus contortus*,
391 *Trichostrongylus colubriformis* and *T. circumcincta*, and there is a relative lack of information
392 regarding the other species present in this system. It may be that although fewer *T. circumcincta*
393 survived in those winters, the relative survival of this species was still greater than that of the other
394 species. The results of the cluster analysis are consistent with this hypothesis, as the samples from
395 ewes and lambs in 2017 and 2018 were clustered together, whereas the years either side were
396 further removed.

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

407 Significant alterations in species diversity due to the purchase of replacements seems
408 unlikely given the quarantine treatments that were given. In addition, differences between age
409 groups and years may have been affected by the impact of grass growth on ewe and lamb nutrition,
410 with secondary effects on immunity. Alterations in grass growth could have also impacted silage
411 aftermath availability and the rate at which lambs were drawn for slaughter, and therefore the
412 stocking density post-weaning. Differences in host genetics (between age groups and between
413 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

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

448 Together with effects on species diversity, variation in survival and infectivity could
449 potentially create evolutionary bottlenecks within GIN species. Such bottlenecks could potentially
450 have significant effects on the prevalence of anthelmintic resistance genes, reducing their frequency
451 if they are associated with fitness costs (Leathwick, 2013), or contributing to their fixation if they
452 result in reduced refugia populations at a time of anthelmintic treatment, as has been reported
453 associated with droughts (Besier, R.B., 1997. Ecological selection for anthelmintic resistance: Re-
454 evaluation of sheep worm control programs. In: Van Wyk J and Van Schalkwyk PC (eds) Managing
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456 the World Association for the Advancement of Veterinary Parasitology, Sun City, South Africa, 10–15
457 August 1997; Papadopoulos et al., 2001). It would therefore be of value to investigate the genetic
458 diversity within species in future studies.

459 In addition to assessing the impact of environmental and management factors on genetic
460 diversity, it is possible to monitor their impact on anthelmintic resistance more directly using genetic
461 markers. The use of deep amplicon sequencing to quantify Isotype-1 β-tubulin SNPs in nematode
462 populations was first described by Avramenko et al. (2019) and Sargison et al. (2019) and, to our
463 knowledge, this is the first study that utilises this technique to monitor resistance SNPs in the *T.*

464 *circumcincta* population on a farm across multiple years. Across the 4 years, there was relatively
465 little variation in β-tubulin SNP frequency, with the F200Y polymorphism predominating: the high
466 prevalence of this mutation in the *T. circumcincta* population on this farm is consistent with the BZ
467 resistance previously demonstrated in a species-corrected faecal egg count reduction test (FECRT)
468 performed on this farm (McIntyre et al., 2018).

469 Previous research in New Zealand showed a non-significant trend towards reversion to
470 phenotypic BZ susceptibility across seven farms and 5 years (Leathwick et al., 2015); however, there
471 is no evidence for progressive genetic reversion to BZ susceptibility on this farm across the 4 year
472 study period. This may be due to inadvertent selection pressures placed upon the *T. circumcincta*
473 population by the use of BZ to control *N. battus* infections in early summer, in combination with a
474 relatively low refugia population at that time of year following the blanket treatment of the ewes
475 with moxidectin (Leathwick, 2013). Alternatively, it may be that due to the long-term use of BZ on
476 this farm, resistant polymorphisms have become co-adapted with other fitness traits, removing any
477 putative fitness costs (Kelly et al., 1978). Given the discussion above, it is interesting to note that the
478 three outlying values occurred close to anthelmintic treatments, but it is not possible to ascribe
479 significance using these data. Further field studies on farms with lower levels of resistance, and
480 variation in anthelmintic usage and resistance mitigation techniques would be extremely valuable.
481 Incorporation of speciation into such work would be vital, given the temporal variation in species
482 composition seen in this study, and evidence that anthelmintic resistance selection pressures and
483 optimal resistance mitigation strategies may vary between parasite species (Waller et al., 1989).

484 Theoretical modelling of the spread of anthelmintic resistance genes within populations
485 suggests that the degree of mixing between treated and untreated subpopulations is likely to have
486 significant impacts on the rate of spread of anthelmintic resistance within a population (Park et al.,
487 2015). However, Hodgkinson et al. (2019) identified that there is a lack of evidence regarding
488 whether population structuring that might prevent such mixing exists. Within the *T. circumcincta*

489 population on this farm, there were no differences in β-tubulin SNP frequencies between years; this
490 may be due to the F200Y mutation already being close to fixation on this farm, but it is also
491 consistent with the findings of Avramenko et al. (2019), which suggested a lack of population
492 structuring. Further research using selectively neutral markers would be of great value for better
493 addressing this outstanding question.

494 In conclusion, this study demonstrates the feasibility of applying deep amplicon sequencing
495 to monitor GIN species diversity and β-tubulin SNP frequency using field samples obtained from a
496 commercial farm. The speciation results show that on a single farm with consistent management
497 between years, there is variation in GIN species diversity with time and between age groups, and
498 that weather patterns may contribute to this variation. In addition, analysis of the nemabiome aids
499 in the interpretation of FECs pre- and post-anthelmintic treatment. These findings reiterate the need
500 to include speciation when investigating GIN epidemiology and anthelmintic resistance. Within the
501 *T. circumcincta* population on this farm, the F200Y BZ-resistant SNP is close to genetic fixation, and
502 there is no evidence of variation in β-tubulin SNP frequency between age groups. Furthermore,
503 there is no genetic evidence of reversion to BZ susceptibility across 3 years, despite the targeting of
504 BZ usage towards *N. battus* treatment only. This serves as an example as to how genetic data may be
505 analysed alongside species diversity and FECs, when markers for other anthelmintic classes are
506 identified, and re-emphasises the need for further research into the population genetics of GINs and
507 the selective pressures associated with anthelmintic resistance in the field.

508

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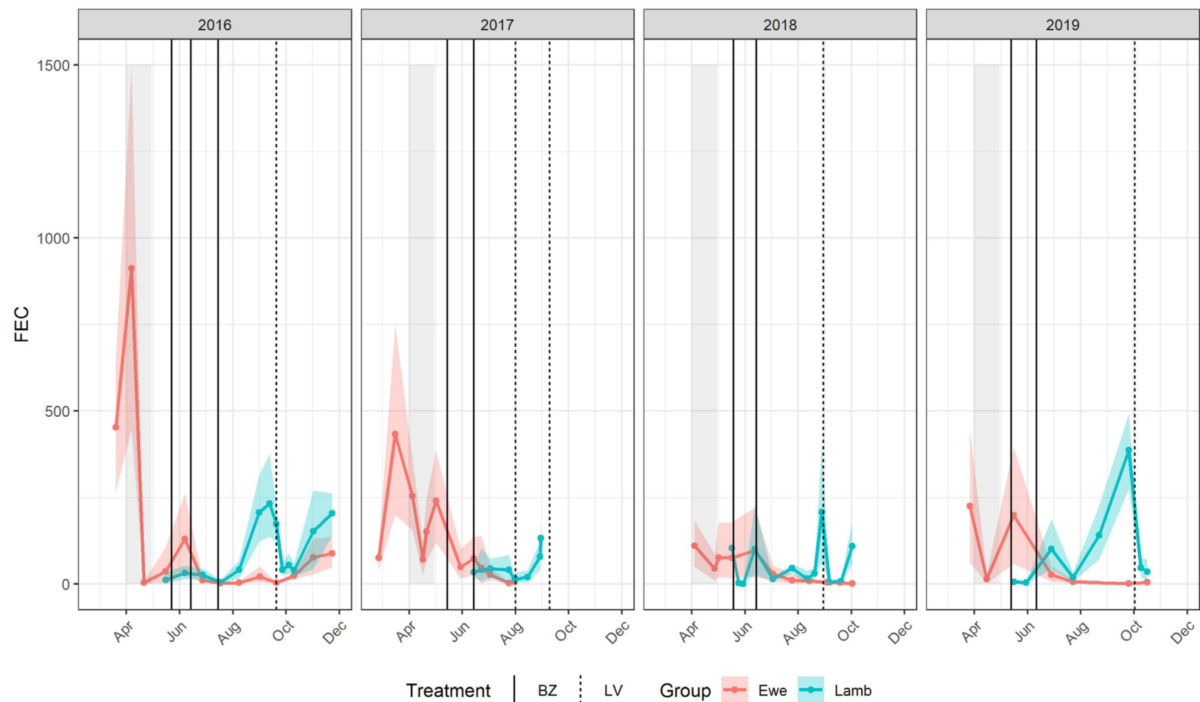
653 **Figures**



655 Fig. 1. The arithmetic mean Faecal Egg Counts (FECs) (eggs per gram, epg) of each sampling point are

656 shown by the points, which are connected by lines to aid interpretation. Ninety-five percent

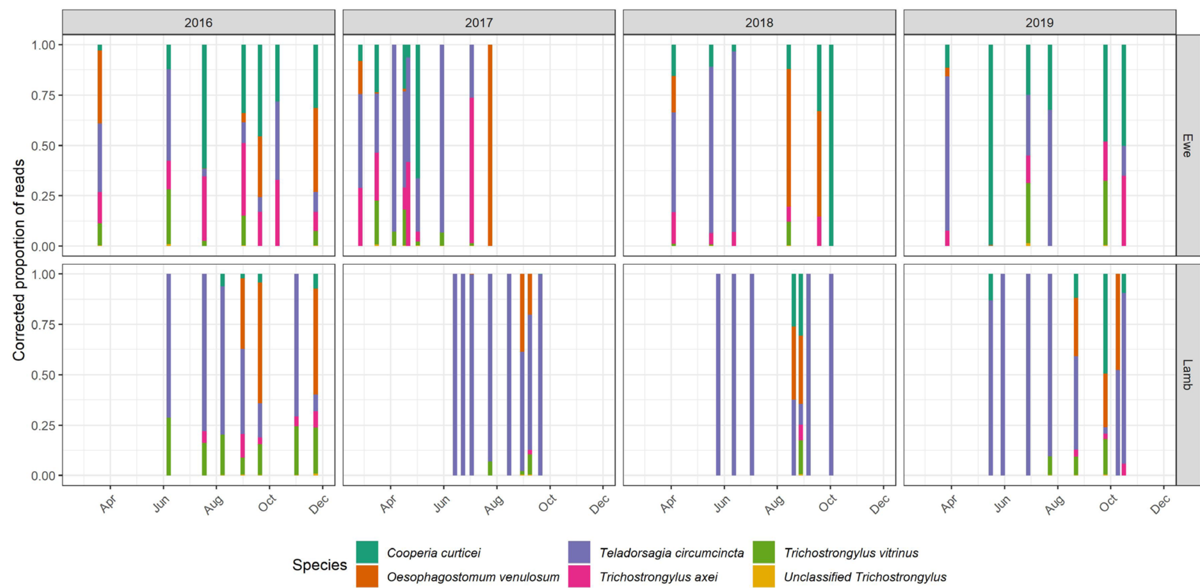
657 confidence intervals for the mean FECs (calculated from the 2.5th and 97.5th percentiles of 500

658 bootstrap resamples) are shown by the shaded areas. Vertical lines show anthelmintic treatments of

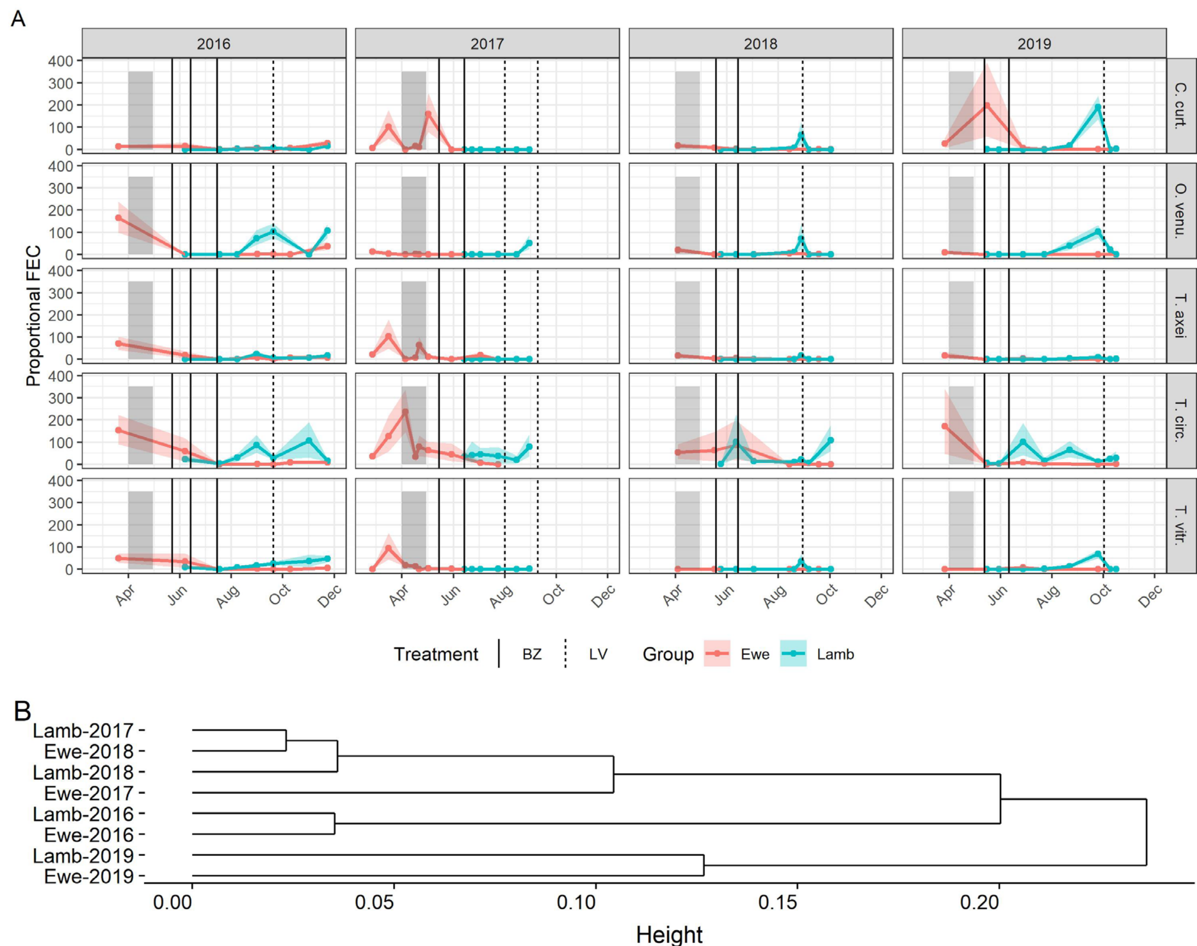
659 lambs, with the line type corresponding to the class of treatment (benzimidazole (BZ) or levamisole

660 (LV)). The peri-parturient treatment of ewes (described in section 2.1) is illustrated by the shaded

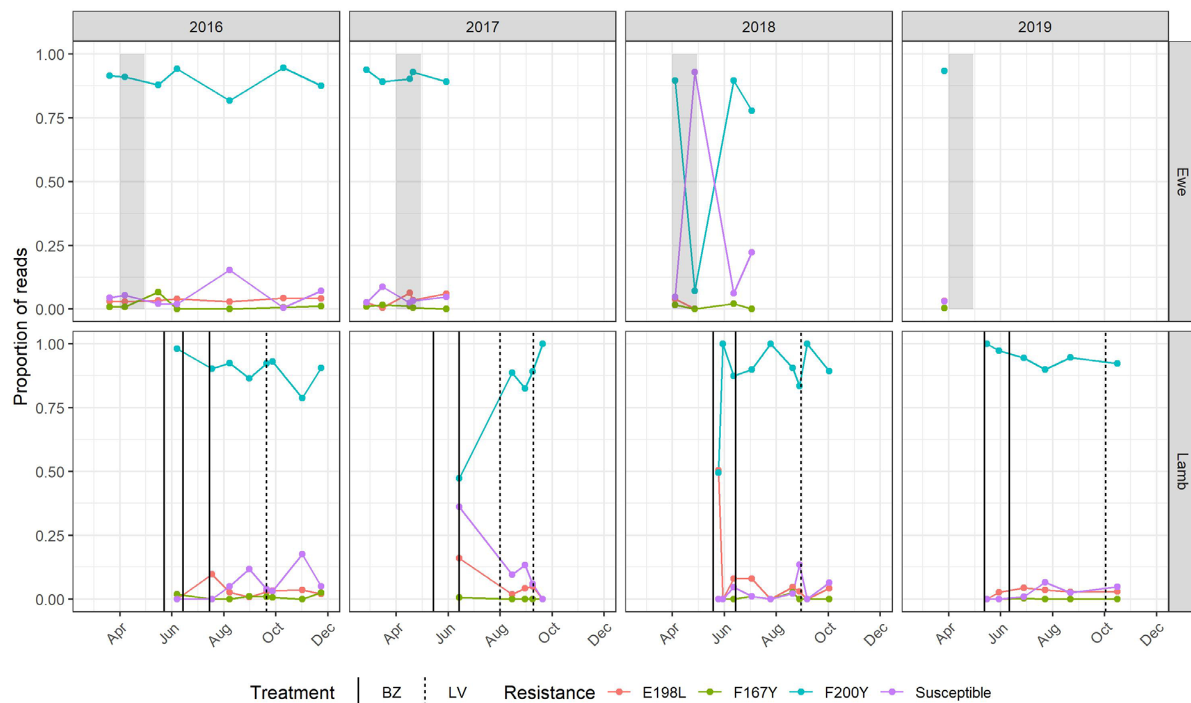
661 vertical band. Colour versions of this figure are available in the online version of this article.



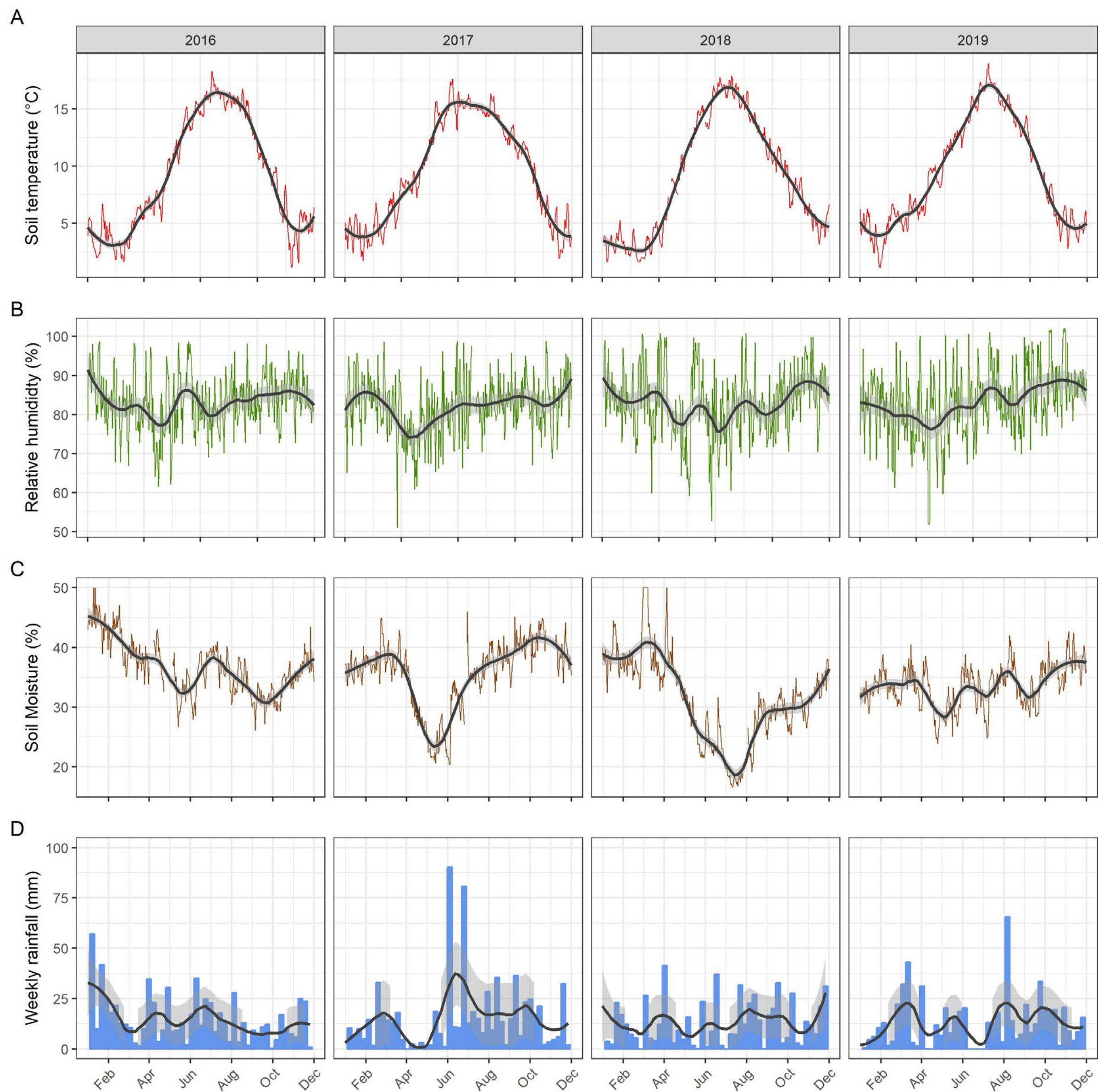
663 Fig. 2. Stacked bar chart showing each sampling-point and, within it, the proportion of sequence
664 reads assigned to each species, corrected using previously described correction factors (Redman et
665 al., 2019). Some sampling points are not present due to low coproculture yields or sequence read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



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.



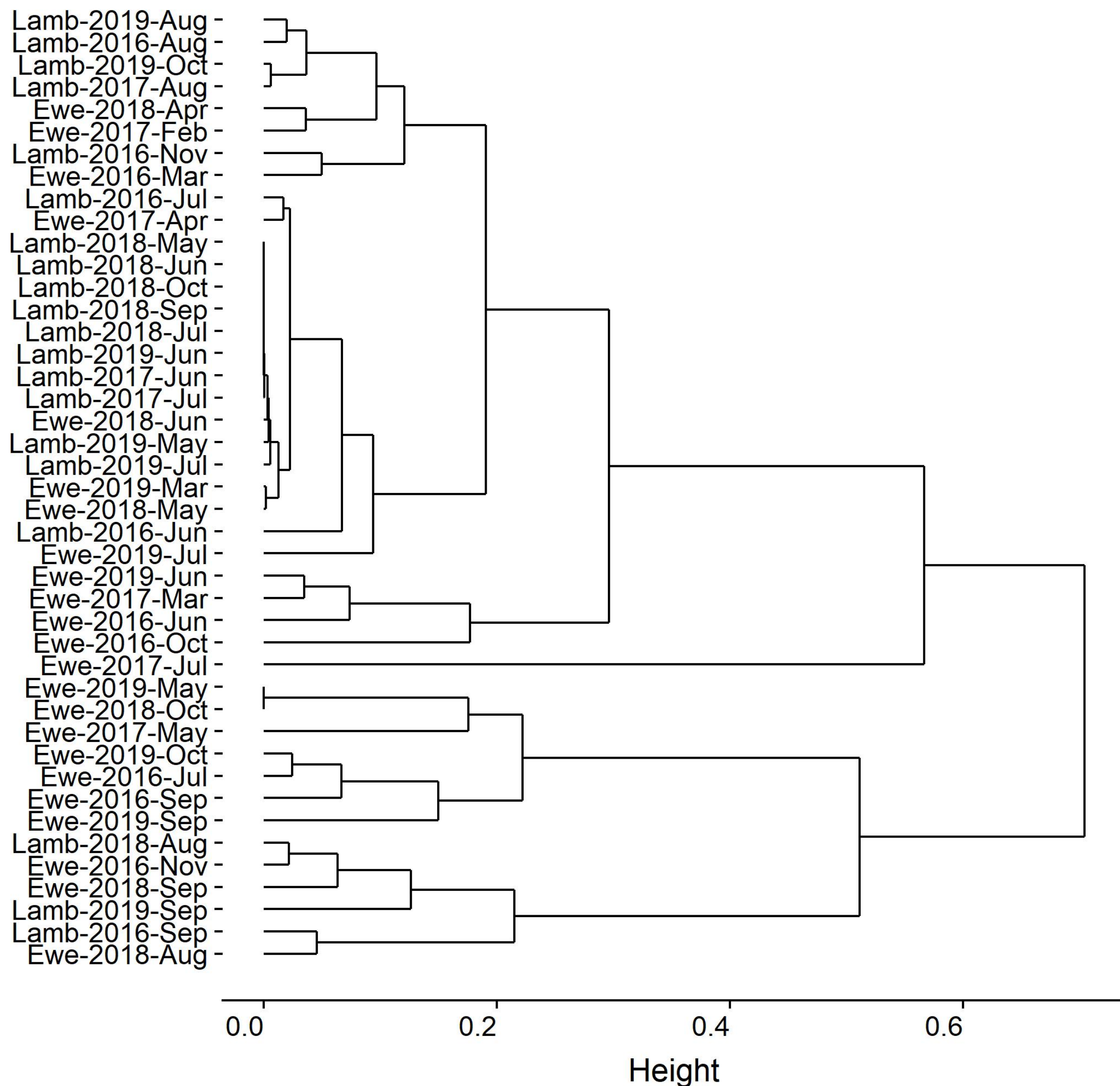
681 Fig. 4. Points show the proportion of sequence reads in each sample, classified according to the
682 presence of the three β-tubulin resistance single nucleotide polymorphisms (SNPs) (E198L, F167Y,
683 F200Y), or the absence of any of these SNPs (susceptible). These points have been connected by
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.



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

701 **Supplementary figure**



703 Supplementary Fig. S1. Dendrogram produced using the mean proportional faecal egg count (FEC)
704 for each species, grouped by month and age group, with distances calculated using the Pearson's
705 correlation (with transformation [1-r]) and clustering using the unweighted pair group method with
706 arithmetic mean (UPGMA) method. Labels are in the format 'Group-Year-Month'.

707

708

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 *Trichostrongylus*	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

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 Mendeley online repository (see section 2.11).

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