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Rona Sinclair[a*], Lynsey Melville[b*], Fiona Sargison[b], Fiona Kenyon[b], Dan Nussey[a], Kathryn Watt[a] and Neil Sargison[c¥].

a. University of Edinburgh, Institute of Evolutionary Biology.

b. The Moredun Research Institute.

c. University of Edinburgh, Royal (Dick) School of Veterinary Studies.

* Contributed equally.

¥ Corresponding author. Tel.: +44 131 650 6229

E-mail address: neil.sargison@ed.ac.uk (N.D. Sargison)

ABSTRACT

Molecular methods based on ITS2 sequence analysis were used to identify strongylid parasites and describe their diversity in a management intervention and anthelmintic drug treatment-free sheep flock. Fourteen different nematode parasite species were identified in the flock and the results showed a greater level of nematode species diversity than is normally reported in managed farmed flocks, with the presence of parasites such as *Bunostomum trigonocephalum, Ostertagia leptospicularis*, *Spiculopteragia houdemeri* and *Trichostrongylus retortaeformis* that are considered to be absent or rare in sheep kept in comparable localities. The implied prevalences of *Haemonchus contortus* in lambs, and of *Trichostrongylus axei* in lambs, ewes and rams, were higher than those in farmed sheep kept in similar regions, while those of *Teladorsagia circumcincta* and *Trichostrongylus vitrinus* were lower. Comparison of the patterns of nematode parasite infection between the summer and autumn sampling

periods showed differences from the scenarios that are commonplace in comparable managed flocks; with *T. vitrinus* burdens of the lambs being higher in the summer than in the winter, and *Oesophagostomum venulosum* being the predominant nematode species in the adult sheep during the summer, while more-or-less absent from these groups during the winter. Rams played an important role in the epidemiology of certain parasitic nematode species. The relatively non-pathogenic *O. venulosum* was the only parasitic nematode species to predominate in any group during the study. This preliminary characterisation of the nematode parasite burdens of sheep extensively grazed on diverse unimproved pastures will aid in the understanding of the parasitological consequences of intensive grazing management and of the manner in which modern agriculture upsets the equilibrium between parasites and their hosts. These factors must be accounted for when defining the concept of sustainable parasite control and informing sustainability with reference to commercially efficient sheep farming.

1. Introduction

Sheep are potential hosts to numerous genera and species of gastrointestinal nematode parasites. The relationship between nematode parasites and their hosts has evolved over millions of years, but has been upset in relatively recent times by domestication and farm management practices that either inadvertently select for more susceptible hosts, or create environments that enable the differential establishment of larger numbers of free-living stages of the parasites (Sutherland and Scott, 2010). Consequently, *Teladorsagia circumcincta*, *Haemonchus contortus*, *Trichostrongylus vitrinus* (or *colubriformis* in warmer regions) and *Nematodirus battus* have become the major production limiting species affecting managed, improved sheep in temperate climates.

Most studies of sheep parasitic nematodes have been conducted in managed flocks, which are grazed on improved grass pastures that are conducive to differentially high rates of free-living stage larval development and survival and are treated with anthelmintic drugs with differential efficacy or

49 persistence. In these situations, most hosts are infected with just a few nematode species, often
50 showing sequential variation in the predominance of just one or two (Boag and Thomas, 1977; Paton et
51 al., 1984). A seasonal trend in the predominance of individual major species is driven by temperature,
52 moisture and physical characteristics of the biomes occupied by the parasites' free-living stages
53 (Stromberg, 1997; Wang Tong et al., 2014), immunologically mediated host responses (Stewart, 1955)
54 and regulatory influences of one nematode species upon another (Coop and Field, 1983). The manner
55 whereby factors might act independently and, or, interactively to determine the impact of the burden
56 of particular parasitic nematode species on their sheep hosts is poorly defined. Better understanding of
57 these trends and interactions is required to inform sustainable control strategies, and depends upon
58 accurate characterisation of the hosts' nematode parasite burdens.

59 Extrinsic factors such as climate, management systems and the influence of geography and flora on the
60 ecological niche occupied by free-living stages of the gastrointestinal parasitic nematodes, clearly exert
61 a large influence on the size of the infective larval challenge (Jackson et al., 1992) and its potential to
62 limit animal productivity (Morgan, 2013). The manner in which these factors might also influence the
63 seasonal prevalence of different species, contributing to the temporal development of heavy mono-
64 specific burdens in sheep grazed on managed grass and clover pastures is poorly understood.
65 Conversely, it is unclear if these factors might in part allow for the development of lower burdens of a
66 larger number of gastrointestinal parasitic nematode species, causing less severe production loss, in
67 sheep grazed in more diverse natural environments. Preliminary characterisation of the nematode
68 parasite burdens of sheep extensively grazed on diverse unimproved pastures will therefore aid in the
69 understanding of the parasitological consequences of intensive grazing management.

70 The manner in which modern agriculture upsets the equilibrium between parasites and their hosts must
71 be accounted for when defining the concept of sustainable parasite control (Morgan et al., 2012).
72 Neither naturally unmanaged grazing, nor planned evasive nematode management strategies are
73 sustainable, and the prerequisite understanding of conditions influencing the biotopes of different

74 parasite populations is inadequate with regards to the development of sustainable systems (Morgan et
75 al., 2013). Dependence on pharmaceutical control of nematode parasites inevitably selects for
76 anthelmintic resistance, hence is also unsustainable (Kaplan and Vidyashankar, 2012).

77 This study provided an insight to the parasitic nematode species diversity in unimproved Soay sheep
78 kept in a natural environment without active management or pharmaceutical nematode control
79 measures. The aim was to improve general understanding of the impact of management on nematode
80 parasite diversity and interactions, as a prerequisite for the development of sustainable control (Lello et
81 al., 2004) of gastrointestinal nematode parasites in commercially-managed sheep.

82

83 **2. Materials and Methods**

84 *2.1. Study group and farm*

85 The closed Soay sheep population of 11 lambs, 23 ewes and 10 rams (during the study period) was co-
86 grazed with six native ponies on 30 ha of natural rough grazing, historically improved pastures and
87 woodland, between about 25 and 100 m above sea level in coastal Argyll, Scotland. There had been
88 neither animal or grazing management intervention, nor modern broad-spectrum anthelmintic drug
89 treatments given since the establishment of the flock, about 12 years previously. Individual animals
90 were uniquely identified using pictures and a panel of phenotypic descriptive indices. Thus, the flock
91 afforded a unique opportunity to observe domesticated livestock under conditions that reflected those
92 applying prior to the implementation of modern management systems.

93 *2.2. Parasitological methods*

94 Faecal samples voided by every animal in the Soay sheep flock were collected immediately off the
95 ground during the summer (August 2013) and winter (January 2014), without handling the animals. This
96 was done by watching each animal in turn. Samples were collected within one minute of being voided

97 to minimise potential contamination by free living nematodes or environmental stages of
98 unrepresentative parasitic nematodes. Faecal nematode egg counts (FECs) were performed using a
99 modified McMaster method (MAFF 1986) where one egg counted represented 50 eggs per gram (epg)
100 (conducted on farm in summer), and a salt floatation and cuvette method (Christie and Jackson, 1982)
101 with a potential sensitivity of 3 epg (conducted in the laboratory in winter). The different methods were
102 used for practical reasons, and while the results cannot be compared at less than 100 epg due to the
103 differences in sensitivities, we have shown the two methods have been shown to give practically
104 comparable results at higher egg counts (data on file). *Nematodirus* spp. eggs were enumerated and
105 recorded separately from those of other genera. Approximately 5g of faeces from each animal was
106 pooled for lambs, rams and ewes and incubated for 72 hours at 20°C (±2°C) to promote egg hatching.
107 Hatched L_1 were recovered by Baermannisation and fixed in ethanol (final concentration ~70% ethanol).
108 Hatching was monitored in subsamples of extracted eggs to ensure that more than 95% of eggs,
109 excluding those of *Nematodirus* spp. developed to the larval stage. This was done to account for
110 potential species-proportional differences in egg hatching rates.

111 Ethanol-fixed samples were bathed in 1x phosphate buffered solution (PBS) for 30 minutes to rehydrate
112 the larvae (1/100 v/v). 88 individual L_1 were transferred into individual wells of 96 well plates (Axygen),
113 containing 50µl of lysis buffer (50 mM KCl; 10 mM Tris [pH8.3]; 2.5 mM $MgCl_2$; 0.45% Nonidet P-40;
114 0.45% Tween-20; 0.01% gelatine and 0.1mg/ml proteinase K (Bioline)). Plates were placed at -20 °C
115 overnight and incubated at 56 °C for a minimum of 4 hours. The lysates were then heated to 95 °C for
116 15 minutes to deactivate proteinase K. DNA was precipitated by adding 100µl ethanol to each well;
117 plates were kept at -20°C overnight and centrifuged (4000G, 40minutes at 4°C). The supernatant was
118 removed and plates were air-dried briefly. Extracted DNA was then re-suspended in 50µl of DNA-free
119 water.

120 *2.3 Species identification by PCR*

Species identification was carried out following the method developed by the Moredun Research Institute (Melville et al., 2016). Briefly, a published multiplex PCR assay was used to identify five nematode species most commonly found in the UK; *T. circumcincta*, *H. contortus* and the *Trichostrongylus axei*, *T. colubriformis* and *T. vitrinus* in a single reaction (Bisset et al., 2014). Amplification of each species results in a unique product length for each gastrointestinal nematode species. Positive controls were included in each PCR reaction using genomic DNA extracted from single adult nematodes of each the five species tested following morphological species identification (MAFF, 1986) to verify results and ensure that band size were accurately and specifically analysed. Multiplex PCR products were analysed using QIAxcel advanced capillary electrophoresis using the QIAxcel DNA High resolution kit (Qiagen) following the manufacturers' protocol. The Qiaxcel advanced capillary system used provides a much more accurate measure of PCR product size compared to conventional agarose gel electrophoresis. Analysis was completed using QIAxcel ScreenGel software (Qiagen).

Generic pan-nematode primers (ITS2GF and ITS2GR) (Bisset et al., 2014) were also included in the multiplex PCR. Detection of a PCR product from the pan-nematode primer set but not from species specific primers indicated the presence of nematode larvae DNA of a species not included in the test. As all of PCR speciation methods used were previously validated and published, sequencing of all individual samples was not considered necessary or feasible for this proof of concept study. Larvae not identified by the multiplex PCR were further tested by species specific PCR targeting *Oesophagostomum venulosum* and *Chaberia ovina* (Bott et al., 2009), and *Cooperia curticei* (Burgess et al., 2012). A species specific PCR targeting *Bunostomum trigonocephalum* was also conducted using primers described by Wimmer et al. (2004). *B. trigonocephalum* PCRs were performed using NovaTaq Hot Start master mix (Merck) in 10µl volumes containing 5µl 2x buffer, 1mM $MgCl_2$, 0.2mM of each primer, 1µl target DNA and ddH_20. Reactions were incubated at 94°C for 10 minutes followed by 35 cycles of 94 °C for 30 seconds, 52°C for 30 seconds and 72 °C for 30 seconds, followed by a final extension phase at 72 °C for 10 minutes. PCR products were run on a 2% agarose gel stained with gel red (Bioline) and visualised under UV illumination.

147 Larval DNA samples for which species were not identified by multiplex or species specific PCR were
148 amplified using generic ITS2 PCR primers described as NC1 and NC2 by Gasser et al. (1996). PCR
149 products were purified using QIAquick DNA purification kits (Qiagen) following the manufacturer's
150 protocol and the DNA was sequenced by MWG Eurofins. Sequences obtained were compared to
151 reference sequences in Gen-Bank using BLASTn at the European Bioinformatics Institute website
152 (http://www.ebi.ac.uk/).

153 *2.4. Statistical analysis*

154 FECs of the ewes and rams were compared by an independent 2-group Mann-Whitney U test. FECs of
155 the lambs, ewes and rams could not be compared between the summer and winter sampling periods
156 due to the use of different egg counting methods. Statistical analyses were run within R, version 3.0.3
157 (R. C. Team. R: A Language and Environment for Statistical Computing, 2014).

158

159 **3. Results**

160 *3.1. FECs*

161 The arithmetic mean trichostrongylloidea (excluding *Nematodirus* spp.), strongyloidea and
162 ancylostomatoidea FECs are shown in Table 1. In addition, *N. battus* and *N. filicolis* eggs, identified and
163 recorded separately according to their morphology, were seen in most groups, with the highest mean
164 *Nematodirus* spp. counts of 73 (±23 SEM) epg in the lamb faecal samples collected during January 2014.

165 *3.2. Gastrointestinal parasitic nematode species diversity*

166 The species identity was determined for 62, 75 and 78 Clade V nematode L_1 recovered from bulk faecal
167 samples collected from the groups of lambs, ewes and rams, respectively during August 2013 and for
168 145, 81 and 74 nematodes recovered from samples from lambs, ewes and rams, respectively during
169 January 2014. (The larger number of identified nematode L_1 reported in the lamb samples collected in

January arose due to failure to identify sufficient Clade V nematode DNA in the first 96 well plate to be examined and consequent need to prepare and examine a second plate.) *Ostertagia leptospicularis*, *Spiculopteragia houdemeri* and *Trichostrongylus retortaeformis* were identified following BLASTn comparison of their ITS2 DNA sequences to reference sequences in Gen-Bank (maximum coverage/identity: 93%/99%, 93%/97% and 99%/99%, respectively). The proportions of the different Clade V nematode species that were identified are shown in Fig. 1. It is noteworthy that: i) *H. contortus* accounted for 9.7% and 1.4% of the larvae in lamb samples taken in summer and winter, respectively, but was not identified in larvae recovered from ewe or ram faeces; ii) *Trichostrongylus axei* accounted for between 3.2% and 11.5% of the larvae recovered from each group; iii) *Bunostomum trigonocephalum* accounted for between 1.6% and 35.8% of the larvae recovered from each group; iv) *T. circumcincta* only accounted for 29% and 12.4% of the larvae identified from lamb faecal samples taken in summer and winter, respectively; v) a single *O. leptospicularis* larva was identified in the faecal samples collected from the ewes during the winter; vi) two larvae of the deer-adapted parasitic nematode species, *S. houdemeri*, were identified in faecal samples collected from the lambs during the winter; and vii) several larvae of the rabbit-adapted parasitic nematode species, *T. retortaeformis* were identified in the faeces collected from lambs and a single larva was identified in the faeces collected from rams during the winter.

The molecular identification of 8 and 12 different known Clade V nematode parasite species in the flock during the summer and winter, respectively, in addition to the morphological differentiation of eggs of two *Nematodirus* spp. imply differences from the parasitic nematode species diversity that is usually reported in managed sheep flocks kept in similar environments. The lowest levels of parasitic nematode species diversity were seen in the ewes and rams sampled during the summer, owing to the predominance of *O. venulosum* in the ewes in particular.

3.3. Seasonal differences in gastrointestinal parasitic nematode prevalence

194 Subjective interpretation of the species composition of the larvae identified in bulk faecal samples
195 (combining the FEC data in Table 1 with the species composition data in Fig. 1) collected from the
196 groups of lambs, ewes and rams highlighted potentially intriguing epidemiological trends. For example:
197 i) the prevalence of the proximal small intestinal parasite, *T. vitrinus*, was higher in the lambs during the
198 summer than during the winter, while the parasite was only identified in the rams during the winter,
199 and its prevalence in the ewes was very low; ii) the seasonal prevalence of the abomasal parasite, *T.*
200 *circumcincta*, in the lambs, ewes and rams was similar to that of *T. vitrinus*, being highest in the lambs
201 during the summer; iii) the prevalence of the abomasal parasite, *H. contortus*, in the lambs was higher in
202 the summer than during the winter, while the parasite was not identified in the other groups; iv) there
203 was no clear difference in the seasonal prevalence of the abomasal parasite, *T. axei*, being slightly
204 higher in the lambs during the winter and slightly higher in the ewes and rams during the summer; v)
205 the prevalence of the small intestinal parasite, *O. venulosum*, was highest during the summer, being
206 zero and very low in the ewes and rams, respectively, during the winter; vi) there was a tendency
207 towards a higher prevalence of the distal small intestinal parasite, *B. trigonocephalum*, during the
208 winter, in particular in the rams; vii) the presence of the rabbit small intestinal parasite, *T.*
209 *retortaeformis* was highest in the lambs during the winter; and viii) the prevalences of the other
210 parasitic nematodes that were identified were too low to allow for comment on seasonal variation.

211

212 **4. Discussion**

213 The PCR-based method to identify strongylid parasite larvae recovered from ovine faecal cultures has
214 been shown to be useful and unambiguous (Gasser, 2001; Wimmer et al., 2004), and the multiplex
215 method used here was practical. A limitation of this approach, using primers targeted towards species
216 specific rDNA ITS2 sequence is that it can only identify those parasites for which primers are chosen.
217 This was overcome by also using primers targeting highly conserved rDNA ITS2 sequence, and
218 sequencing products from those lysates that did not amplify using the species specific primers (Bisset et

al., 2014). *Nematodirus* spp. were evaluated separately, based on their morphologically distinctive eggs, because larvae do not hatch under the standard conditions that were used. A proportion of the samples collected from each group of animals were negative, in that there was no amplification of the pan-nematode ITS2 sequence. This is probably due to non-confounding factors such as L_1 inadvertently not placed into the well, or the larvae not being strongylid nematodes, perhaps due to occasional transfer of free-living nematodes, inadvertently picked up from the ground during faecal collection.

The preliminary study of nematode parasite diversity in this group of Soay sheep showed the presence of species that are uncommon in sheep flocks kept in similar environments. The consistent identification of *B. trigonocephalum* represents a novel finding, as the presence of this parasite has not been reliably reported in mainland UK sheep flocks for several decades. Its disappearance probably coincided with the introduction of modern broad spectrum benzimidazole drugs during the 1970s, and its long prepatent period rendering populations particularly susceptible to anthelmintic treatments. The last reports of the hookworm parasite in UK sheep originated from the St Kilda archipelago in intervention-free, feral Soay sheep (Gulland and Fox, 1992; Craig et al., 2006). *B. trigonocephalum* isolated from the study flock of Soay sheep and knowledge of the sequential burdens of the lambs, ewes and rams, therefore, provides a potentially useful resource for further studies of hookworm parasite epidemiology. The detection of *H. contortus* in the Soay lambs during the summer and failure to identify the parasite in the ewes and rams was intriguing with reference to the generally perceived scenario in equivalent groups in managed sheep flocks kept in similar environments. The prevalence of *H. contortus* in managed sheep flocks is generally either very low (Burgess et al., 2012), or very high (Sargison et al., 2007a; Falzon et al., 2013), as a putative consequence of interactions, or absence of interactions with other parasitic nematode species, respectively. The results support the need for further epidemiological studies of haemonchosis in a range of environments and farming systems.

The identification of *O. leptospicularis*, *S. houdemeri* and *T. retortaeformis* in the study flock of Soay sheep was novel. These parasites might not have been identified by conventional coproculture and

244 third stage larval morphology methods (Van Wyk and Mayhew, 2013), or by the morphological
245 examination of a subsample of adult nematodes recovered at necropsy (MAFF, 1986), owing to the
246 imprecision of these methods (Wimmer et al., 2004) in all bar highly experienced hands (Bisset et al.,
247 2014). *O. leptospicularis* and *S. houdemeri* are primarily abomasal parasites of roe, sika and red deer,
248 which are reported to be seen periodically on the study farm. *O. leptospicularis*, has rarely been
249 reported in sheep (Mayo et al., 2013; Bisset et al., 2014) and cattle, in which it may hybridise with
250 *Ostertagia ostertagi* (Suarez et al., 1993). *T. retortaeformis* is a lagomorph intestinal parasite that has
251 rarely been reported in sheep, showing the potential for cross-transmission of parasites between
252 wildlife and sheep in the natural environment (Tai et al., 2013). The possibility that the L_1 of these novel
253 species had hatched from eggs inadvertently collected off the ground during faecal sampling cannot be
254 discounted, albeit the risk was minimized by the methods used and the scenario is unlikely.
255 Furthermore, the more robust method of collection of faeces from the rectum is not possible in the
256 study of wild, feral or unmanaged animal populations.

257 The large number of nematode parasite species that was identified in the unimproved Soay sheep
258 grazed on natural pastures, without active management intervention, differed from the situation that
259 usually pertains in improved sheep flocks kept on intensively managed grass and clover pastures and
260 subject to active helminth parasite control. In managed flocks, it is commonplace to identify as few as
261 four parasitic nematode species, with seasonal predominance of just one or two (Parnell et al., 1954;
262 Barger, 1985; Boag and Thomas, 1971; Boag and Thomas, 1977) giving rise to diseases referred to as
263 teladorsagiosis, nematodirosis, haemonchosis, or trichostongylosis (Sargison et al., 2007a). By contrast,
264 co-infection with a larger and more diverse range of nematode parasites is pervasive in wildlife (Petney
265 and Andrews, 1998; Lello et al., 2004) and has been shown in feral Soay sheep (Wimmer et al., 2004;
266 Craig et al., 2006), which have not been subject to intensification and management. In addition to
267 those species such as *T. circumcincta* and *T. vitrinus* that are common in managed sheep flocks, in the
268 study flock of Soay sheep there was a relatively high abundance of parasitic nematode species such as *T.*
269 *axei* and *B. trigonocephalum* that have become rare elsewhere. Livestock management practices create

niches that are suited to the development and survival of free-living stages of helminth parasites; enhance sheep exposure to infective stages of the parasites; and alter the host innate or adaptive immune responses to infection (Shaw and Dobson, 1995). Better understanding is needed of the manner whereby these factors may upset the equilibrium between different nematode species or populations, allowing some to predominate and potentially become pathogenic, while limiting others, thereby reducing nematode parasite species diversity, and allowing for sequential variation in the predominance of individual species. Thus, consideration of those factors that allowed for the diversity and seasonal abundance of individual nematode parasite species in the study flock of Soay sheep is pertinent.

Grazing management creates environments that are more or less conducive towards the development, survival and availability of free-living stages of certain parasitic nematode species. High stocking densities can influence levels of pasture contamination that may upset the natural sustainable evolutionary balance between gastrointestinal nematode parasites and their sheep hosts, with reference to the principle that the parasites do not compromise their hosts to an extent that will threaten the survival of their future generations, especially when climatic and environmental conditions are optimal for the development of contaminating eggs. It is intriguing that biodiverse environments such as that grazed by the study flock of Soay sheep may provide a variety of microhabitats that favour the build-up of infective larval challenge of some parasitic nematode species, while restricting the development or contamination with others as a consequence of differential optimal temperature and moisture requirements, or susceptibilities to plant secondary metabolites (Githiori et al., 2006; Rios de Alvarez et al., 2012). Selective grazing or browsing of flora with bioactive anthelmintic properties, enabled by extensive management, but prevented by intensification, may also have differential regulatory effects on nematode parasite species abundance.

The seasonal prevalence and predominance of individual parasitic nematode species is influenced by the onset of acquired immunity in lambs and its loss in ewes during the periparturient period (Salisbury

295 and Arundel, 1970). Periparturient ewes of improved sheep breeds kept in managed flocks are less
296 susceptible to *T. vitrinus* than to *T. circumcincta* (Jackson et al., 1988), hence the extent of the
297 periparturient relaxation of immunity contributes to the seasonal prevalence of each species. The
298 extent of the periparturient rise is influenced by the availability and partitioning of protein nutrition and
299 by the ewe's reproductive effort (Houdijk et al., 2001). These factors will undoubtedly differ between
300 native sheep breeds kept in natural environments and prolific commercial sheep kept on improved
301 pastures and offered supplementary feeding, and could potentially and in part account for some of the
302 nematode parasite species diversity seen in the study flock of Soay sheep. The rams in the study flock
303 also appeared to play an important role in the epidemiology of the different parasitic nematode species.
304 This is consistent with previous observations of higher FEC in male than in female Soay sheep on St Kilda
305 (Coltman et al., 2001; Craig et al., 2006). The total FECs of the rams were as high as those of the ewes in
306 both summer and winter, with *O. venulosum* accounting for the majority of their summer egg output.
307 Thus, consideration of the impact of differential host immune responses to a diverse range of parasitic
308 nematode species in shaping the overall parasite community (Grafen and Woolhouse, 1993) is pertinent
309 with regards to implications of consequential levels of infective larval challenge to parasitic nematode
310 control strategies. This is particularly pertinent in the face of emerging constraints to the sustainability
311 of current control methods (Dalton et al., 2003).

312 A clearly defined seasonal progression from predominance of *T. circumcincta* in the summer to *T.*
313 *vitrinus* in the winter that is seen in naïve lambs in managed flocks was not seen in the Soay lambs
314 (Coop et al., 1988; Jackson et al., 1992). However, the study was only conducted over a period of one
315 year, with only two sampling periods, hence this observation must be interpreted with caution, not
316 least due to likely annual variation in the prevalence and intensity of nematode infections (Stear et al.,
317 1998). The early stage establishment of *T. circumcincta* physiologically mediates the subsequent
318 establishment of *T. vitrinus* perhaps by altering the pH in the abomasum and proximal small intestine
319 (Jackson et al., 1992), allowing heavy mono-specific burdens of *T. circumcincta* to accumulate during the
320 spring and summer, and establishment of heavy *Trichostrongylus* spp. burdens in the autumn and

winter, only once host acquired immune responses modulate the *T. circumcincta* burdens. Similar interactions driven by differential early stage larval challenge and the onset of protective immunity have been reported between *T. colubriformis* and *Nematodirus spathiger* (Dineen et al., 1977), *H. contortus* and *T. axei* (Reinecke et al., 1980), *T. axei* and *T. circumcincta* (Reinecke et al., 1982), and in cattle between *Ostertagia ostertagi* and *Cooperia oncophora* (Bairden et al., 1992). Not all such interactions operate to the detriment of the species involved, for example severity of *N. battus* infections may be increased by prior or simultaneous infection with coccidian protozoa (Christensen et al., 1987; Catchpole and Harris, 1989), or the establishment of *Dictyocaulus viviparus* may be enhanced by prior infections with *O. ostertagi* and *C. oncophora* (Kloosterman et al., 1990). Thus, regulatory influences of the large number of nematode parasite species on each other in the study Soay sheep flock could have contributed to the less clear pattern of sequential variation in predominance of individual species than is seen in intensively managed flocks. These interactions could have had implications on the dynamics of the parasitic nematode community (Lello et al., 2004), preventing the establishment of production limiting mono-specific parasitic nematode challenge or burdens. Conversely, the efficacy of nematode parasite management could be unsustainable if such potential interactions are not taken into account (Lello et al., 2004).

Anthelmintic drug treatments favour survival of parasitic stages of those species against which the drug is least efficacious, and of species in which the frequency of nematodes with alleles conferring anthelmintic resistance is high (Kaplan and Vidyashankar, 2012). Frequent anthelmintic treatments have a greater effect on the total population size of parasite species such as *B. trigonocephalum* and *O. venulosum*, which have longer prepatent periods, than of species such as *T. circumcinca* or *T. vitrinus*, which have prepatent periods that are shorter than the frequency of treatment with non-persistent acting drugs, thereby favouring re-establishment of host infections of the latter. Anthelmintic drug treatments vary between different gastrointestinal parasitic nematode species in their efficacy and persistence of protection against reinfection. These effects are most pronounced for the macrocyclic lactone drugs, in particular moxidectin, due to their potency and lipophilicity, hence prolonged

residence time (Alvinerie, 1998). Moxidectin formulations afford a longer period of protection against reinfection with *T. circumcincta* than with *T. vitrinus* (Demeler et al., 2013), which has anecdotally been associated with summer trichostrongylosis in treated lambs. The use of macrocyclic lactone drugs in beef calves has effectively controlled *Ostertagia ostertagi*, but allowed the establishment of pathogenic burdens of *Cooperia* spp. and *Nematodirus helvetianus* (Sargison et al., 2010), for which the efficacy is lower and period of persistence of protection against reinfection is shorter. Thus, the history of no anthelmintic drug treatments having been administered to the study flock of Soay sheep might account for the high abundance of parasites such as *O. venulosum* and *B. trigonocephalum*, which have long pre-patent periods, and for the relatively low abundance of *T. circumcincta*, which commonly survives anthelmintic drug treatment in managed flocks, as a consequence of a high level of selection for anthelmintic resistance (Sargison et al., 2007b).

Production limiting disease in sheep is usually associated with exposure to high levels of monospecific infective larval challenge or parasitic burdens arising as a consequence of intensive management of genetically susceptible animals (Sykes, 1994; Stear et al., 1998). None of the FECs attributed to individual nematode parasite species in the study flock of Soay sheep was indicative of levels of infection generally associated with disease, or with the implied high burdens in feral Soay sheep on St Kilda (Gulland, 1992; Craig et al., 2006). Hence, the high level of parasitic nematode species biodiversity associated with natural grazing management of genetically unimproved sheep may be a more sustainable scenario with regards to parasite control, albeit the production efficiency of the system is economically unsustainable. Further study is needed to investigate the concept of sustainability within the context of parasite biodiversity. Nematode parasites, such as *H. contortus* and *B. trigonocephalum* limit sheep production, due to the direct effects of their blood feeding behaviour, while the pathogenic effects of most gastrointestinal parasitic nematodes arise as a consequence of host innate and adaptive immune responses (Fox, 1977; Greer et al., 2005; Greer, 2008) damaging the absorptive lining of the gastrointestinal tract. The net pathophysiological effects of these activities are inefficient feed utilisation, inducing a state of relative protein deficiency, fluid and electrolyte or macroelement

373 imbalances and anaemia, leading to clinical signs, such as reduced appetite, poor weight gains,
374 diarrhoea and death (Coop, 1979; Coop and Field, 1983). Overall, the greatest economic importance of
375 nematode parasites is suboptimal productivity arising from continuous low-level exposure to infective
376 larvae (Coop et al., 1982). Nematode parasitism also causes production loss due to the considerable
377 cost incurred by its treatment and management (Nieuwhof and Bishop, 2005). The only parasitic
378 nematode species to predominate in the study flock of Soay sheep, *O. venulosum* is considered to be
379 relatively non-pathogenic, therefore, consideration of the factors favouring this species could help to
380 inform sustainability with reference to commercially efficient sheep farming.

381 The greater strongylid nematode species diversity shown in this study compared to that seen under
382 modern sheep management systems provides an insight to potential interactions between species, and
383 to the seasonal roles of different sheep classes in the epidemiology of parasitic nematodes. If
384 supported by evidence from experimental studies, the concept that encouraging higher levels of
385 parasite species diversity might result in lower and less pathogenic burdens of certain strongylid
386 nematodes, could have practical relevance to sustainable roundworm control. The work demonstrates
387 the potential for further development of molecular nematode speciation techniques and their
388 application to provide a basis for applied investigations into the putative mechanisms and
389 consequences of regulatory or competitive nematode species interactions.

390

391 **Contributors**

392 RS, NS, KW and DN conceived and designed the study. RS conducted most of the field work. RS and NS
393 undertook most of the conventional parasitology, while LM, FS and FK undertook most of the molecular
394 parasitology. NS prepared the manuscript with substantial input from each of the co-authors.

395

396 **Acknowledgments**

The field work was conducted under the auspices of a University of Edinburgh ecology BSc Honours project. Access to the farm and an insight into its ecology and management was provided by Mr and Mrs Sinclair. The Moredun Research Institute receives funding from the Scottish Government.

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555

556

557 **Table legends**

558 **Table 1**

559 The arithmetic mean total trichostrongylloidea (excluding *Nematodirus* spp.), strongyloidea and

560 ancylostomatoidea FECs (±SEM) during the summer and winter sampling periods.

561 **Table 1**

	Summer (epg)	Winter (epg)
Lambs (n=11)	345 (±82)	163 (±38)
Ewes (n=23)	72 (±16)	15 (±5)
Rams (n=10)	90 (±21)	74 (±23)
Significance of differences between ewe and ram FEC (independent 2-group Mann-Whitney U test)	p-value = 0.43	p-value = 0.0002

562

563

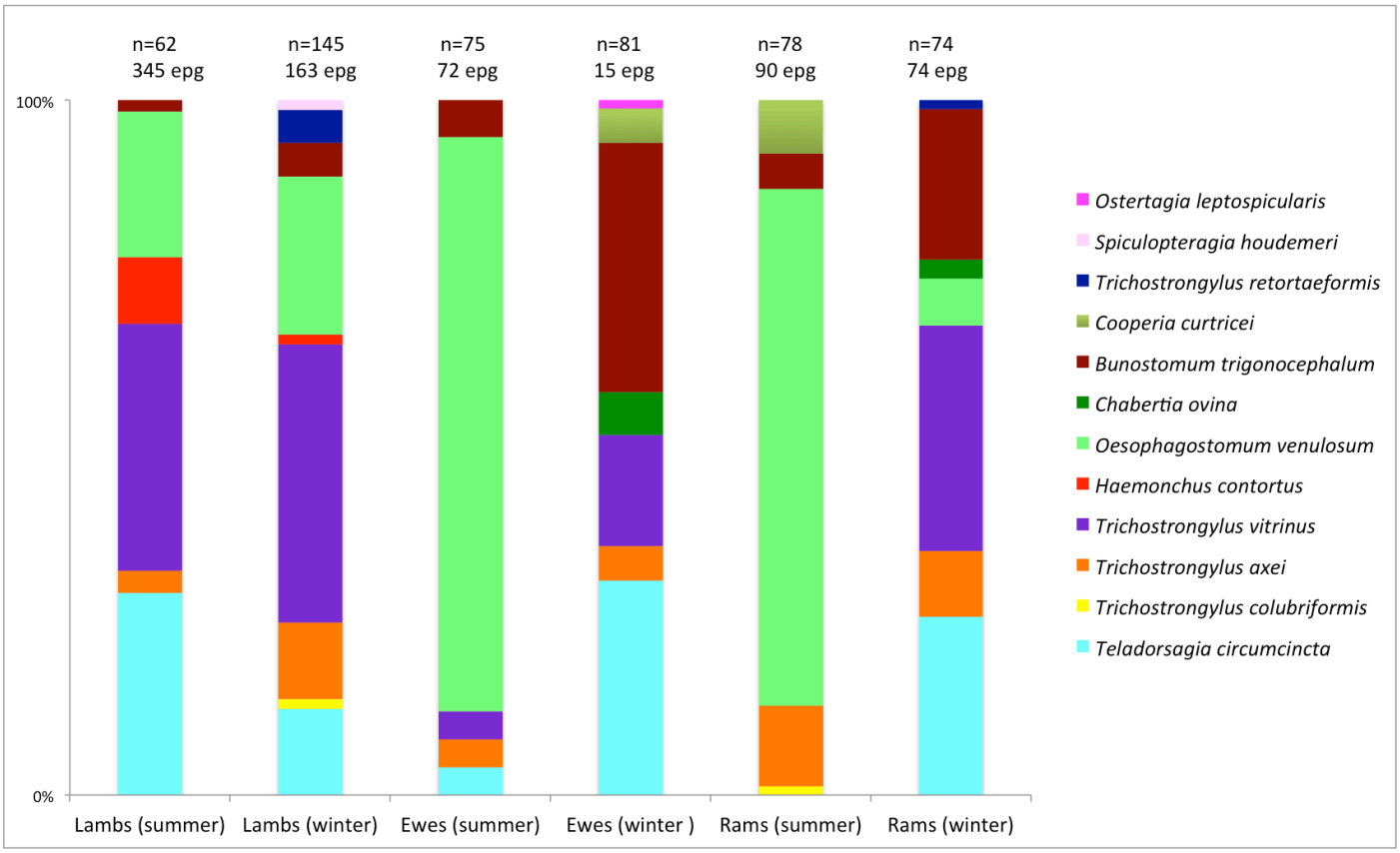
564 **Figure legends**

565 **Fig. 1.** Faecal egg counts and species composition of L_1 hatched from eggs shed by lambs, ewes and

566 rams during August 2013 and January 2014. *Nematodirus* spp. could not be included because the eggs

567 do not hatch under the conditions used in the study.

Fig. 1


n=62
345 epg
n=145
163 epg
n=75
72 epg
n=81
15 epg
n=78
90 epg
n=74
74 epg
100%
0%
Lambs (summer)
Lambs (winter)
Ewes (summer)
Ewes (winter)
Rams (summer)
Rams (winter)
Ostertagia leptospicularis
Spiculopteragia houdemeri
Trichostrongylus retortaeformis
Cooperia curtricei
Bunostomum trigonocephalum
Chabertia ovina
Oesophagostomum venulosum
Haemonchus contortus
Trichostrongylus vitrinus
Trichostrongylus axei
Trichostrongylus colubriformis
Teladorsagia circumcincta