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1 Development of the larval migration inhibition test for comparative  
2 analysis of ivermectin sensitivity in cyathostomin populations

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20

21 *Keywords*

22 Cyathostomins; Anthelmintic resistance; Ivermectin; Larval migration inhibition test

23

24

25 ABSTRACT

26 Cyathostomins are the most prevalent parasitic pathogens of equids worldwide. These  
27 nematodes have been controlled using broad-spectrum anthelmintics; however, cyathostomin  
28 resistance to each anthelmintic class has been reported and populations insensitive to more  
29 than one class are relatively commonplace. The faecal egg count reduction test (FECRT) is  
30 considered the most suitable method for screening anthelmintic sensitivity in horses, but is  
31 subject to variation and is relatively time-consuming to perform. Here, we describe a larval  
32 migration inhibition test (LMIT) to assess ivermectin (IVM) sensitivity in cyathostomin  
33 populations. This test measures the paralysing effect of IVM on the ability of third stage  
34 larvae (L3) to migrate through a pore mesh. When L3 from a single faecal sample were  
35 examined on multiple occasions, variation in migration was observed: this was associated  
36 with the length of time that the L3 had been stored before testing but the association was not  
37 significant. Half maximal effective concentration (EC50) values were then obtained for  
38 cyathostomin L3 from six populations of horses or donkeys that showed varying sensitivity  
39 to IVM in previous FECRTs. Larvae from populations indicated as IVM resistant by FECRT  
40 displayed significantly higher EC50 values in the LMIT than L3 from populations classified  
41 as IVM sensitive or L3 from populations that had not been previously exposed to IVM or had  
42 limited prior exposure. The analysis also showed that EC50 values obtained using L3 from  
43 animals in which IVM faecal egg count reduction (FECR) levels had been recorded as <95%  
44 were significantly higher than EC50 values obtained using L3 from animals for which FECR  
45 was measured as >95%. For one of the populations, time that had elapsed since IVM  
46 administration had an effect on the EC50 value obtained, with a longer time since treatment  
47 associated with lower EC50 values. These results indicate that the LMIT has value in  
48 discriminating IVM sensitivity amongst cyathostomin populations, but several factors were  
49 identified that need to be taken into account when executing the test and interpreting the  
50 derived data.

51

## 52 **1. Introduction**

53 Cyathostomins are highly prevalent and potentially pathogenic parasitic nematodes found in  
54 the large intestine of horses and other equids worldwide. The cyathostomin group comprises  
55 around 50 species (Lichtenfels et al., 2008); however, little is known of the ecology of the  
56 individual species or how they interact with one another in the host or in external environment.  
57 Anthelmintic resistance (AR) is a major issue in this group of nematodes: resistance to  
58 benzimidazoles (BZ) is widespread and, in some areas, resistance to pyrantel, a member of the  
59 tetrahydropyrimidine (THP) class, is highly prevalent (Kaplan, 2002). Reduced sensitivity to  
60 the macrocyclic lactone (ML) anthelmintics, ivermectin (IVM) and moxidectin (MOX), has also  
61 been recorded in cyathostomin populations (Trawford et al., 2005; Trawford and Burden, 2009;  
62 Molento et al., 2008; Traversa et al., 2012; Relf et al., 2014). Multi-class resistance in single  
63 populations to BZ and THP class anthelmintics is also commonly reported (Kaplan et al., 2004;  
64 Canever et al., 2013; Lester et al., 2013). As no new anthelmintic classes are being developed  
65 for use in horses in the short to medium term, and reversion to anthelmintic sensitivity does not  
66 seem to readily occur in resistant nematode populations (Jackson and Coop, 2000), it is essential  
67 to preserve efficacy of the currently effective products. For these reasons, tests that facilitate  
68 decisions regarding anthelmintic treatment in horses will play an increasingly important role in  
69 control (Matthews, 2014). In this context, it is important to identify AR as soon as practically  
70 possible so that measures can be taken to prevent its spread (Tandon and Kaplan, 2004). The  
71 faecal egg count reduction test (FECRT) is currently the ‘gold standard’ non-invasive test for  
72 assessing anthelmintic efficacy in horses (Vidyashankar et al., 2012). This test is relatively  
73 labour intensive to implement: faecal samples for analysis need to be obtained on at least two  
74 occasions and it is often a challenge to obtain adequate numbers of horses with a faecal egg  
75 count (FEC) of sufficient magnitude to perform the test with high accuracy. The non-uniform  
76 distribution of eggs within and between faecal samples further complicates data analysis  
77 (Denwood et al., 2010). For these reasons, AR detection methods that are more efficient to

78 perform and subject to less variability need to be investigated. For cyathostomins, such tests  
79 should focus on ML anthelmintics. This is because these products hold the major market-share  
80 worldwide and, as the prevalence of ML resistance is currently less advanced than with BZ and  
81 THP anthelmintics (Molento et al., 2012), and hence tests that inform on sensitivity to ML are  
82 likely to have most impact on mitigating the spread of resistance. Molecular mechanisms  
83 leading to ML resistance in cyathostomins remain to be defined, so there are no molecular tests  
84 available, leaving bench-based in vitro tests as the remaining option.

85 The larval development test has been investigated for measuring anthelmintic sensitivity in  
86 cyathostomins; however, this test has not proven particularly informative in defining ML  
87 sensitivity levels (Tandon and Kaplan, 2004; Lind et al., 2005; Matthews et al., 2012). An  
88 alternative test that has been investigated for assessing anthelmintic sensitivity in ruminant  
89 nematode (Demeler et al., 2010; 2012; 2013) and in cyathostomin (van Doorn et al., 2010)  
90 populations is the larval migration inhibition test (LMIT). Here, we assessed the potential of the  
91 LMIT for measuring IVM sensitivity in cyathostomin larvae derived from different equine  
92 populations. This test was deemed appropriate for purpose because the major targets of IVM  
93 are ligand-gated chloride channels, which, when bound, result in nematode paralysis (Shoop et  
94 al., 1995) and hence will affect the ability of larvae to migrate through small pores of a filter.  
95 The cyathostomin populations examined in the current study were derived from groups of  
96 donkeys demonstrated previously, by FECRT, to exhibit differing levels of sensitivity to IVM in  
97 vivo (Trawford and Burden, 2009), or from equids administered with minimal or no ML  
98 treatments (Wood et al., 2013). The proportion of L3 that migrated through a pore filter at  
99 increasing concentrations of IVM were measured and the data compared amongst individual  
100 equids and between populations to inform on the value of this test.

101

102

103

104 **2. Materials and methods**

105

106 *2.1. Populations*

107 Parasites from six equine populations were used: four consisted of donkeys and two of  
108 ponies. Three donkey herds (Populations A-C) were based at the UK Donkey Sanctuary  
109 (Devon, England, UK). The three populations were grazed separately on geographically  
110 distinct farms. Donkeys in Populations A and B had been demonstrated previously, by  
111 FECRT, to harbour cyathostomins that exhibited reduced sensitivity to IVM and MOX  
112 (Table 1, Trawford et al., 2005; Trawford and Burden, 2009). In Population C, the  
113 cyathostomins were deemed IVM sensitive as indicated by the finding of a mean FECR of  
114 >95% in treated animals 14 days after IVM administration (Table 1); however, the strongyle  
115 egg reappearance period for some of the donkeys in this population was below the standard  
116 egg suppression period of IVM and MOX (F. Burden, pers comm.), described previously as 8  
117 weeks (Lyons et al., 1992, Bello, 1996) and 13 weeks (DiPietro et al., 1997), respectively.  
118 These observations are generally accepted as an early indicator of AR (Molento et al., 2012).  
119 In all three Donkey Sanctuary populations, IVM or MOX subcutaneous injection  
120 preparations registered for use in cattle had previously been administered orally (F. Burden,  
121 pers comm.). This may have predisposed the nematodes in these populations to reduced  
122 sensitivity to ML anthelmintics. Population D comprised a privately owned herd of donkeys  
123 grazed in Cheshire, UK. These donkeys had not received IVM in the 5 years preceding this  
124 study and had only received MOX and anti-cestode treatments during this time. No  
125 anthelmintic had been administered in the preceding 12 months. As part of this study,  
126 Population D was subjected to an IVM FECRT following World Association for the  
127 Advancement of Veterinary Parasitology guidelines (Coles et al., 1992) using a double  
128 centrifugation FEC technique sensitive down to 1 egg per gram (Christie and Jackson, 1982).  
129 In Population D, IVM FECR was found to be 100% in all donkeys tested. Populations E and

130 F comprised two groups of ponies used for conservation purposes in the Fens of East Anglia,  
131 South East England (Wood et al., 2013). Population E comprised Dartmoor ponies grazed on  
132 grassland fen. At the start of the study, all ponies had grazed the Fens for two years. During  
133 this time, these ponies were not administered with ML products. Population F consisted of  
134 Konik ponies grazed on grassland fen since the mid-1990s. These animals did not receive  
135 ML products during this time and were unlikely to have ever received this class of  
136 anthelmintic. Due to potential eco-toxicological risks (McKellar, 1997), IVM FECRT were  
137 not performed at the sites grazed by Populations E and F, as the use of ML products is not  
138 permitted. Given the lack of ML treatments in these populations, it is assumed that the  
139 resident cyathostomins in Populations E and F are highly sensitive to IVM and MOX.

140

## 141 *2.2. Preparation of third stage larvae for LMIT analysis*

142 Freshly voided faecal samples were collected from identified individuals and immediately  
143 placed into labelled plastic bags, which were sealed to exclude as much air as possible to  
144 retain anaerobic conditions. All samples were sent on the day of collection. Each sample  
145 was weighed, homogenised thoroughly and a 10 g sub-sample removed for FEC analysis  
146 (Christie and Jackson, 1982). The remainder was cultured under aerobic conditions by  
147 transferring faeces to plastic trays, which were placed inside perforated plastic bags. Faeces  
148 were incubated at 15°C for up to 22 days, after which, the trays were flooded with lukewarm  
149 tap water for 4 h. The supernatants, containing strongyle L3, were poured over a Baermann  
150 filter (MAFF, 1986) and the filter placed in the neck of a jam jar filled with tap water. The  
151 filter was left overnight, removed the next morning and the remaining volume reduced to 20  
152 ml. The L3 were transferred to culture flasks and enumerated in 10 x 10 µl aliquots and  
153 classified as small or large strongyle L3 on the basis of gut cell morphology (Thienpont et al.,  
154 1986). No large strongyles were observed in any samples from Populations A-E. Very low  
155 numbers of large strongyle larvae (<1%) were observed in samples from Population F. A

156 complete lack of ivermectin or moxidectin treatments in Population F are likely to explain  
157 why this was the only population that was identified as positive for large strongyle larvae.  
158 The L3 were stored in tap water in vented flasks at 4°C at a maximum concentration of 2,500  
159 L3 ml<sup>-1</sup> for up to 60 days. At this time point, viable L3, as assessed by motility and the  
160 presence of intact gut cells, were observed. The water was replenished weekly.

161

### 162 *2.3. Larval migration inhibition test*

163 The LMIT described here is an adaptation of the method developed for assessing  
164 anthelmintic sensitivity of ruminant nematode L3 by Demeler et al. (2010; 2012; 2013). This  
165 protocol utilises a migration system that enables physical separation of motile from non-  
166 motile L3 through a filter mesh (Nytal mesh). The pore diameter allows active larvae, but  
167 not dead larvae, to pass through the mesh. For each test, approximately 2,500 L3 were  
168 removed from each culture derived from an individual animal. The L3 were exsheathed in  
169 700 µl, 2% w/v sodium hypochlorite solution for 3.5 min at room temperature and washed  
170 thoroughly three times by centrifugation for 2 min at 203 x g in phosphate buffered saline,  
171 pH 7.4 (PBS: 150 mM sodium chloride, 150 mM sodium phosphate). Exsheathed L3 were  
172 subjected to Baermannisation for 2 h at 26°C immediately before the test was run. The L3  
173 were collected by centrifugation, recounted and re-suspended in 1,200 µl PBS. As IVM had  
174 to be dissolved in 100% dimethyl sulphoxide (DMSO) for the test, the effect of DMSO on L3  
175 motility was tested over a range of concentrations (0, 5, 6, 7, 8, 9, 10% w/v DMSO/PBS).  
176 Each dilution was assessed in duplicate using L3 from Population C and the experiment  
177 repeated on three occasions. The derived data indicated that 5% DMSO w/v PBS was the  
178 highest concentration at which < 2% adverse effect was observed on migration compared to  
179 PBS-only control wells (data not shown). The impact of the diameter of the pores within the  
180 mesh was then assessed. This was performed by killing the L3 by incubation at 70°C for 20  
181 min, then adding them in PBS-only or 5% DMSO w/v PBS to the upper side of filters of pore



182 diameter 25 and 28  $\mu\text{m}$ . After 2 h, the underside of the filters was examined to assess if L3  
183 ‘fell through’ by gravity: L3 were not observed on the underside of filters of pore diameter 25  
184  $\mu\text{m}$  or 28  $\mu\text{m}$ . Subsequently, 25  $\mu\text{m}$  pore diameter filters were selected for use in the test.  
185 For assessment of the effect of IVM on L3 migration, analytical grade IVM (Sigma Aldrich,  
186 cat. no: I8898) was dissolved in 100% DMSO to give a stock solution of 3,000  $\mu\text{gml}^{-1}$  IVM.  
187 Before each batch of tests, this stock was serially diluted in PBS/DMSO to give working  
188 dilutions of 5, 20, 60, 300, 3,000  $\mu\text{gml}^{-1}$  IVM/5% DMSO w/v PBS (final molarity used in the  
189 test ranged from  $1.12 \times 10^{-5}$  -  $6.73 \times 10^{-3}$  M). In all tests, L3 migration was assessed in a  
190 positive control well containing only 5% DMSO w/v PBS and IVM test concentrations and  
191 controls were set up in duplicate (approximately 100 L3 analysed per well). The L3 were  
192 pre-incubated at 26°C for 2 h in the dark in 10  $\mu\text{l}$  IVM at each test concentration in 5%  
193 DMSO w/v PBS. After this, L3 in IVM solution were transferred to the upper side of filters  
194 in corresponding duplicate wells on a migration plate, containing 1,910  $\mu\text{l}$  of each test  
195 concentration. The samples were incubated for 2 h at 26°C in the dark. After this, migration  
196 chambers were lifted out and 600  $\mu\text{l}$  PBS used to wash the outside of the chambers so that  
197 any adhering (but migrated) L3 were washed into the corresponding well. The upper  
198 chamber was inverted and, using 2 x 1,000  $\mu\text{l}$  PBS, L3 that had not migrated were washed  
199 into the corresponding well in the row below. The effect of IVM on the ability of the L3 to  
200 migrate through the mesh pores was confirmed by observations, prior to fixing, that the  
201 worms that had come through the mesh were motile and moving in classical sinusoidal  
202 movements, whilst those retained above the mesh moved slowly or not at all, or assumed  
203 angular postures and performed jerky movements of the head and tail regions. The L3 were  
204 fixed with 200  $\mu\text{l}$ , 100% molecular grade ethanol and migrated and non-migrated L3  
205 enumerated at x100 magnification using an inverted stereomicroscope. To study  
206 repeatability of the test and the potential effect of L3 storage time on migration in the LMIT,  
207 L3 derived from a single culture from a donkey in Population B were analysed on seven

208 separate occasions. This donkey had not received IVM in the 780 days preceding sample  
209 collection. The test was run using L3 that had been stored from 22 to 55 days at 4°C.  
210 Finally, to compare the value of the LMIT in defining IVM sensitivity among cyathostomin  
211 populations of varying sensitivity to the anthelmintic in vivo, L3 derived from single time  
212 point faecal samples from each Population: A (n=4), B (n=5), C (n=6), D (n=5), E (n=6) and  
213 F (n=6) were assessed. All samples were tested at each IVM concentration in duplicate. Only  
214 when (un-scaled) mean migration of the L3 in the two positive control wells exceeded 70%,  
215 was migration in the presence of IVM subjected to further data analysis.

216

#### 217 *2.4. Statistical analysis and modelling*

218 For data analysis, the percentage migration was calculated for each replicate, with  
219 migration in the PBS/DMSO-only well scaled to 100%. For data exploration, dose-response  
220 curves (DRC) of the proportion of L3 migrating versus  $\text{Log}_{10}(\text{IVM conc} + 0.01)$  were  
221 plotted for each sample. The small additive component, 0.01, allowed the inclusion of data at  
222 zero IVM concentration and was found empirically to have minimal impact on estimated  
223 EC50 values. The DRC were statistically modelled using a four-parameter logistic dose  
224 response model on the natural logarithm of IVM concentration (Demeler et al., 2010; 2012)  
225 permitting estimation of EC50. Summary EC50 values were compared using the Kruskal  
226 Wallis test with post-hoc analysis using the method of Siegel and Castellan (1988). The  
227 association between EC50 and time since last IVM treatment was tested with a linear model  
228 predicting EC50 from time since last IVM treatment and population membership. DRC  
229 modelling, hypothesis testing and post-hoc analysis were performed in the R statistical  
230 system (R Development Core Team, 2012) using the packages “drc”, “lme4” and “pgirmess”.

231

232

233

234 **3. Results**

235

236 *3.1. Value of the LMIT results in defining IVM sensitivity amongst cyathostomin populations*

237 When the test was run on seven occasions on different days using L3 derived from a  
238 single donkey, it was found that L3 stored for shorter periods in culture generally exhibited  
239 higher migration in the presence of IVM, but the differences observed between storage time  
240 points were not significant. The L3 migration values were then compared amongst the six  
241 cyathostomin populations. The percentage of times that < 70% migration in the control wells  
242 was observed was 10%. When < 70% migration was observed, the test was repeated with L3  
243 from the same individual; however, < 70% migration was achieved in all subsequent tests  
244 with these samples, so the LMIT data from these L3 were not used in subsequent analyses  
245 and are not included in the numbers quoted for each equid population, above. The range of  
246 EC50 values obtained for each population (A-F) is shown in Table 2 and the derived DRC's  
247 are depicted in Figure 1. Resistance ratios were generated by dividing single EC50 estimates  
248 from data for each Population (A-E) by the EC50 value obtained from data from Population  
249 F (Table 2). Highest EC50 values were obtained using cyathostomin L3 from populations A  
250 and B, which had been demonstrated to be resistant to IVM in vivo. The next highest EC50  
251 value was obtained using L3 from the population for which a reduced ERP had been  
252 demonstrated following IVM and MOX treatment (Population C). Lower EC50 values were  
253 obtained using L3 from populations shown to be sensitive to IVM by FECRT (Population D)  
254 or populations in which IVM treatments had been minimal or non-existent (Populations E  
255 and F).

256 The predictive value of the LMIT for assessing relative IVM sensitivity was further  
257 considered by comparing EC50 values obtained using L3 derived from populations  
258 demonstrated to be IVM resistant (IVM-R) on the basis of mean FECR <95% after IVM  
259 treatment (Populations A and B), with those values obtained using L3 from the population

260 (C) for which the mean FECR was reported as >95%, but the ERP was reduced following  
261 IVM administration (IVM-RERP), and with populations that were highly sensitive to IVM  
262 (i.e. FECR 100%) or had received no IVM treatments (Populations D, E and F), combined  
263 here as IVM-sensitive (IVM-S, Figure 2). The data analysis indicated that EC50 values  
264 obtained with IVM-R L3 were significantly higher than EC50 values obtained using IVM-S  
265 L3 ( $p < 0.05$ ), but not the EC50 values obtained using IVM-RERP L3. In addition, EC50  
266 values obtained using L3 from the IVM-RERP population were significantly higher than  
267 those obtained with L3 from the IVM-S populations ( $p < 0.05$ ).

268

269 *3.2. Association between derived EC50 values and data derived from IVM-FECRT results in*  
270 *individual animals*

271 EC50 values obtained using L3 from Populations A, B and C (i.e. those populations that  
272 had been subjected previously to IVM FECRT analysis) were used to investigate the  
273 hypothesis that there would be a negative association between the percentage reduction in  
274 FEC observed 2 weeks after IVM administration and the EC50 values obtained in the IVM-  
275 LMIT (Figure 3). The analysis indicated that there is indeed a relationship, with individuals  
276 measured as having a FECR of >95% having lower EC50 values in the IVM-LMIT than  
277 those for which FECR was <95% (two-way ANOVA, EC50 and FECR>95%,  $p(\text{FECR})$   
278  $p=0.000284$ ).

279

280 *3.3. Analysis of IVM sensitivity in single populations over time since last IVM treatment*

281 The EC50 values obtained using L3 from Populations A, B and C (i.e. those populations  
282 that had a relatively recent IVM treatment) were used to examine if there was a relationship  
283 between the proportion of L3 that migrated in the presence of IVM with the number of days  
284 since the last recorded IVM administration (Figure 4). The time since IVM treatment for  
285 each population ranged as follows: Population A (n=4) – 40-102 days (mean, 57.5 days),

286 Population B (n=5) – 41-780 days (mean, 250.25 days), and Population C (n=5, one animal  
287 of the original 6 animals was not treated with IVM) – 65-194 days (mean, 143.50 days). The  
288 analysis indicated that there is a relationship, with higher proportions of migration observed  
289 with L3 derived from samples obtained nearer to IVM treatment: i.e. the EC50 value  
290 obtained was negatively associated with time since last IVM treatment. This association was  
291 significant in the case of the L3 that were derived from Population C (p=0.028)

292

#### 293 **4. Discussion**

294

295 Macrocytic lactone anthelmintics, such as IVM, that paralyse nematode somatic muscles,  
296 among other modes of action, have been assessed in vitro via their effect on larval motility or  
297 migration. Such tests are potential options for detecting anthelmintic resistance because they  
298 are cheap, relatively quick to perform, preclude host influences and, as they can be run over a  
299 concentration range, may provide reproducible parameters with which to measure phenotype  
300 (Demeler et al., 2013). Several studies utilising ruminant parasitic nematodes have indicated  
301 that motility and migration tests are useful tools for informing on the ML sensitivity of single  
302 species populations (Martin and Le Jambre, 1979; Folz et al., 1987; Sangster et al., 1988;  
303 Demeler et al., 2010, 2012, 2013). Here, we examined the value of the LMIT for use with  
304 cyathostomin larvae obtained by culture from equine faeces. We assessed utility of the test  
305 for informing on IVM sensitivity of cyathostomins obtained from populations for which  
306 FECRT data was available or populations in which IVM treatments had not been applied or  
307 had been limited. Here, we used cyathostomin larvae from donkeys in a comparison with  
308 larvae derived from horses because it is problematic to obtain populations of small strongyles  
309 from horses for which an IVM FECR of <95% has been demonstrated. For example,  
310 shortened IVM ERP has been identified several times in cyathostomins in horse populations  
311 (for example, Relf et al., 2013), but populations exhibiting a mean FECR of less than 95%

312 have been reported only sporadically in horses and primarily in South America (Canever et  
313 al., 2013). In our comparisons, the LMIT was found to discriminate IVM sensitivity amongst  
314 cyathostomin populations in agreement with results that had been previously generated using  
315 the FECRT. For example, the derived EC50 values obtained with L3 from IVM-R  
316 (Populations A and B) and IVM-RERP (Population C) populations were significantly higher  
317 than EC50 values obtained using L3 from all IVM-S populations (D, E and F). These results  
318 concur with studies on *Haemonchus contortus*, where correlations were identified between  
319 the results of the LMIT and the in vivo anthelmintic resistance status (Gill and Lacey, 1998).  
320 In the *Haemonchus* study, the association was found to vary depending on how the nematode  
321 strains were selected, with no correlations found when using strains that had been selected  
322 experimentally using sub-optimal doses of anthelmintic. In agreement with the current study,  
323 though, good correlations between migration in the test and the results of prior FECRT  
324 analysis were found when resistant strains isolated from the field (i.e. selected with  
325 therapeutic doses of anthelmintic) were compared.

326 One observation from the current study was that although there was a significant  
327 difference observed in EC50 values between IVM-R or IVM-RERP and IVM-S populations,  
328 there was variation within each population in the EC50 value obtained using L3 derived from  
329 individual animals (Table 2). This was particularly noticeable in the IVM-R isolates.  
330 Although this concurs with variation in ML FECR levels obtained by FECRT in the IVM  
331 resistant populations here, this level of variability could affect the value of this test if pooled  
332 samples were to be assessed from a given population in the field, where it could be  
333 impractical to run the test on many individuals. Two further confounding factors were  
334 identified. One of these was the length of time that L3 had been stored in the laboratory  
335 before the test. An effect of culture age on migration was observed, even though L3 were  
336 Baermannised just prior to running the test. In previous publications using ruminant  
337 nematode larvae, the effect of L3 storage time was not detailed: for example, Demeler et al.

338 (2010) used L3 from sheep faecal cultures stored for ‘up to 3 months’, but the impact of  
339 culture period was not specifically addressed. Other studies (for example, Sangster et al.,  
340 1988) do not mention the length of time that L3 were stored for prior to use in the LMIT. In  
341 others, the effect of L3 storage time has been assessed: for example, using the LMIT, Molan  
342 et al. (2000) compared the sensitivity to condensed tannins of *Trichostrongylus colubriformis*  
343 larvae stored for 1 month versus larvae stored for 7 months. Similar to the findings here,  
344 these authors found that the *T. colubriformis* L3 stored for longer periods in the laboratory  
345 were more sensitive to the xenobiotic tested than larvae stored for shorter periods ( $p < 0.001$ ).  
346 The differences observed in the current study were not significant; however, larvae were  
347 stored up to only 55 days as opposed to 7 months. On the basis of the results here and the  
348 observations made in other nematode species, it is recommended that L3 be used as fresh as  
349 possible when assessing IVM sensitivity in the LMIT.

350 The analysis also indicated that the time that elapsed between last IVM treatment and  
351 when the faecal samples were obtained for processing had an effect on the derived EC50  
352 values in the LMIT. The effect was only found to be significant for Population C: this may  
353 have been because this population had the widest range in days since last IVM treatment in  
354 the donkeys that were selected for supply of L3 for the LMIT. This observation could be  
355 explained by the fact that the nearer to IVM treatment that the L3 are tested in the LMIT, the  
356 more likely it is that the parasites used are derived from nematodes that may have survived  
357 treatment. This is particularly problematical to investigate in cyathostomins because the  
358 exact length of the life cycle of different individual species is unknown and these parasites  
359 can undergo a variable period of encystment in the large intestinal wall (Love et al., 1999).  
360 Because of the effect of time since last anthelmintic treatment observed here, it is  
361 recommended that the impact of this parameter be assessed further in future.

362 Despite these various caveats, the results here showed clear differences in EC50 values  
363 measured amongst the cyathostomin populations that were tested. This was observed even

364 although the L3 samples tested were likely to comprise mixed cyathostomin species. It has  
365 been indicated in preliminary studies that different cyathostomin species may vary in their  
366 sensitivity to IVM in the LMIT (van Doorn et al., 2010). Also, in ruminant nematode studies  
367 it has been observed that different species differ in ML sensitivity in the LMIT, which cannot  
368 always be predicted from their relative sensitivity to MLs in vivo (Demeler et al., 2012). In  
369 the van Doorn et al., (2010) study, where cyathostomin L3 were rendered more tolerant in  
370 vitro through iterative selection by several cycles of migration in the presence of IVM, it was  
371 identified that *Cyathostomin catinatum* became the predominant species in the two  
372 populations that were tested. Furthermore, cyathostomin species composition in donkeys can  
373 be different from species composition in horses (Matthee et al., 2004). For these reasons, the  
374 authors will now examine the species of cyathostomin present in these populations using L3  
375 recovered from the LMIT utilising species specific DNA probes that they have developed  
376 based on intergenic spacer region nucleotide sequences (Cwiklinski et al., 2012).

377 Although the results here indicate that the LMIT has value in providing information on the  
378 IVM sensitivity status of a cyathostomin population, it cannot be assumed that the  
379 ‘resistance’ mechanisms that affect the ability of the L3 to migrate in the LMIT are the same  
380 as those present in parasitic stages that operate to allow these stages to survive treatment in  
381 the host. As such, the LMIT provides only a gauge on the relative IVM sensitivity of a  
382 population. There is some indication from the work of van Doorn et al., (2010), that the  
383 mechanism at play in the LMIT involves glutamate, but this requires further study. Despite  
384 the aforementioned limitations, significant differences in LMIT EC50 values were observed  
385 here between cyathostomin larvae derived from IVM-R or IVM-RERP populations and  
386 larvae obtained from populations that were shown to be, or assumed to be, IVM sensitive.  
387 Moreover, at the individual equid level, a direct correlation was identified between the  
388 percentage reduction in FEC measured in vivo using the IVM-FECRT and the LMIT EC50  
389 value obtained using L3 from the same animal. The parasite isolates used here may be at the



390 extremes of IVM sensitivity and resistance (as indicated by the high resistance ratios  
391 generated for populations A and B when compared to population F) than may be found  
392 generally, and work now needs to be performed using samples derived from a wider range of  
393 populations for which the in vivo resistance phenotype is not so obvious, for example,  
394 cyathostomin populations for which IVM FECR is > 95% but the strongyle ERP is reduced.

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#### 397 **Authors' contributions**

398 JM, IH and CM designed the study. CM and AR performed the LMIT. FB and JH provided  
399 some of the faecal samples. CM and IH performed the data analysis. JM and CM drafted the  
400 manuscript, IH generated the figures and the other authors provided comments to the  
401 manuscript and approved the final version.

402

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#### 409 **Conflict of interest**

410 None of the authors have an actual or potential conflict of interest, including financial,  
411 personal or other relationship that could inappropriately influence, or be perceived to  
412 influence, the work presented in this manuscript.

413

414 **References**

415

416 Bello, T.R., 1996. Alternative antiparasitic treatment of horses with pyrantel pamoate  
417 suspension and ivermectin oral solution compared with horses treated only with ivermectin  
418 oral solution. *J. Eq. Vet. Sci.* 16, 18-21.

419 Canever, R.J., Braga, P.R., Boeckh, A., Grycajuck, M., Bier, D., Molento, M.B., 2013. Lack of  
420 cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. *Vet. Parasitol.*  
421 194, 35-39.

422 Christie, M., Jackson, F., 1982. Specific identification of strongyle eggs in small samples of  
423 sheep faeces. *Res. Vet. Sci.* 32, 113-117.

424 Coles, G.C., Bauer, C., Borgsteede, F.H., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J.,  
425 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.)  
426 methods for the detection of anthelmintic resistance in nematodes of veterinary importance.  
427 *Vet. Parasitol.* 44, 35-44.

428 Cwiklinski, K., Kooyman, F.N., Van Doorn, D.C., Matthews, J.B., Hodgkinson JE. 2012. New  
429 insights into sequence variation in the IGS region of 21 cyathostomin species and the  
430 implication for molecular identification. *Parasitology.* 139,1063-1073.

431 Demeler, J. Küttler, U., El-Abdellati, A., Stafford, K., Rydzik, A., Varaday, M., Kenyon, F.,  
432 Jackson, F., Vercruyse, J., von Samson-Himmelstjerna, G., 2010. Standardization of the  
433 larval migration inhibition test for the detection of resistance of ivermectin in  
434 gastrointestinal nematodes of ruminants. *Vet. Parasitol.* 174, 58-64.

435 Demeler, J., Kleinschmidt, N., Küttler, U., Koopmann, R., von Samson-Himmelstjerna, G.,  
436 2012. Evaluation of the egg hatch assay and the larval migration inhibition assay to detect  
437 anthelmintic resistance in cattle parasitic nematodes on farms. *Parasitol. Int.* 61, 614-618.

438 Demeler, J., Gill, J.H., von Samson-Himmelstjerna, G., Sangster, N.C. 2013. The in vitro assay  
439 profile of macrocyclic lactone resistance in three species of sheep trichostrongyloids. Int J  
440 Parasitol Drugs Drug Resist. 3, 109-118.

441 Denwood, M.J., Reid, S.W., Love, S., Nielsen, M.K., Matthews, L., McKendrick, I.J., Innocent,  
442 G.T., 2010. Comparison of three alternative methods for analysis of equine Faecal Egg  
443 Count Reduction Test data. Prev. Vet. Med. 93, 316-323.

444 DiPietro, J.A., Hutchens D.E., Lockc, T.F., Walkerd, K., Paul, A.J., Shipleyc, C., Rullie, D.,  
445 1997. Clinical trial of moxidectin oral gel in horses. Vet. Parasitol. 72, 167-177.

446 Folz, S.D., Pax, R.A., Thomas, E.M., Bennet, J.L., Lee, B.L., Conder, G.A., 1987.  
447 Development and validation of an in vitro *Trichostrongylus colubriformis* motility assay.  
448 Int.J. Parasitol. 17, 1441-1444.

449 Gill, J.H., Lacey, E., 1998. Avermectin/milbemycin resistance in trichostrongyloid nematodes.  
450 Int J Parasitol. 28:863-877.

451 Jackson, F., Coop, R.L., 2000. The development of anthelmintic resistance in sheep nematodes.  
452 Parasitology, 120 Suppl, S95-S107.

453 Kaplan, R.M., 2002. Anthelmintic resistance in nematodes of horses. Vet Res. 33, 491-507.

454 Kaplan, R.M., Klei, T.R., Lyons, E.T., Lester, G., Courtney, C.H., French, D.D., Tolliver, S.C.,  
455 Vidyashankar, A.N., Zhao, Y., 2004. Prevalence of anthelmintic resistant cyathostomes on  
456 horse farms. J. Am. Vet. Med. Assoc. 225, 903-910.

457 Lester, H.E., Spanton, J., Stratford, C.A., Bartley, D.J., Morgan, E.R., Hodgkinson, J.E.,  
458 Coumbe, K., Mair, T., Swane, B., Lemone, G., Cookson, R., Matthews, J.B., 2013.  
459 Anthelmintic efficacy against cyathostomins in horses in England. Vet. Parasitol. 197, 189-  
460 196.

461 Lichtenfels, J.R., Kharchenko, V.A., Dvojnjos, G.M., 2008. Illustrated identification keys to  
462 strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae). Vet.  
463 Parasitol. 156, 4-161.

464 Lind, E.O., Ugglå, A., Waller, P., Höglund, J., 2005. Larval development assay for detection of  
465 anthelmintic resistance in cyathostomins of Swedish horses. *Vet. Parasitol.* 128, 261-269.

466 Lyons, E.T., Drudge, J.H., Tolliver, S.C., Granstrom, D.E., Stamper, S., 1992. Evaluation of  
467 exclusive use of ivermectin vs. alternation of antiparasitic compounds for control of internal  
468 parasites of horses. *Am. J. Vet. Res.* 52, 97-104.

469 Martin, P.J., Le Jambre, L.F., 1979. Larval paralysis as an in vitro assay of levamisole and  
470 morantel tartrate resistance in *Ostertagia*. *Vet. Sci. Comms.* 3, 159-164.

471 Matthee, S., Krecek, R.C., McGeoch, M.A. 2004. A comparison of the intestinal helminth  
472 communities of Equidae in Southern Africa. *J Parasitol.* 90, 1263-1273.

473 Matthews, J.B., 2014. Anthelmintic resistance in equine nematodes. *Int. J. Parasit. Drugs and*  
474 *Drug Resistance.* In Press. <http://dx.doi.org/10.1016/j.ijpddr.2014.10.003>.

475 Matthews, J.B., McArthur, C., Robinson, A., Jackson, F., 2012. The in vitro diagnosis of  
476 anthelmintic resistance in cyathostomins. *Vet. Parasitol.* 185, 25-31.

477 McKellar QA., 1997. Ecotoxicology and residues of anthelmintic compounds. *Vet. Parasitol.* 72,  
478 413-426.

479 Molan, A.L., Waghorn, G.C., Min, B.R., McNabb, W. C., 2000. The effect of condensed  
480 tannins from seven herbage on *Trichostrongylus colubriformis* larval migration in vitro.  
481 *Folia Parasitologica* 47, 39-44.

482 Molento, M.B., Antunes, J., Bentes, R.N., Coles, G.C., 2008. Anthelmintic resistant nematodes  
483 in Brazilian horses. *Vet. Rec.* 162, 384-385.

484 Molento, M.B., Nielsen, M.K., Kaplan, R.M., 2012. Resistance to avermectin/milbemycin  
485 anthelmintics in equine cyathostomins - current situation. *Vet. Parasitol.* 185, 16-24.

486 Love S, Murphy D, Mellor D. 1999. Pathogenicity of cyathostome infection. *Vet Parasitol.*  
487 85:113-121.

488 R Development Core Team. 2012. R: A language and environment for statistical computing. R  
489 Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL  
490 <http://www.R-project.org>.

491 Relf, V.E., Lester, H.E., Morgan, E.R., Hodgkinson, J.E., Matthews, J.B., 2014. Anthelmintic  
492 efficacy on UK Thoroughbred stud farms. *Int. J. Parasitol.*, 44, 507-514.

493 Sangster, N.C., Riley, F.L., Collins, G.H., 1988. Investigation of the mechanism of levamisole  
494 resistance in trichostrongylid nematodes of sheep. *Int. J. Parasitol.* 18, 813-818.

495 Shoop, W.L., Mrozik, H., Fisher, M.H., 1995. Structure and activity of avermectins and  
496 milbemycins in animal health. *Vet. Parasitol.* 59, 139-156.

497 Siegel, S., Castellan, N. J. Jr., 1988. Nonparametric statistics for the behavioural sciences. In:  
498 McGraw-Hill International Editions, New York, N.Y., 2nd ed., pp 213-214.

499 Tandon, R., Kaplan, R.M., 2004. Evaluation of a larval development assay (DrenchRite®) for  
500 the detection of anthelmintic resistance in cyathostomin nematodes of horses. *Vet. Parasitol.*  
501 121, 125-142.

502 Thienpont, D., Rochette, F., Vanparijs, O.F., 1986. Diagnosing helminthiasis by coprological  
503 examination. 2<sup>nd</sup> ed. Janssen Research Foundation, Beerse, Belgium, 85 pp.

504 Traversa, D., Castagna, G., von Samson-Himmelstjerna, G., Meloni, S., Bartolini, R., Geurden,  
505 T., Pearce, M.C., Woringer, E., Besognet, B., Milillo, P., D'Espois, M. 2012. Efficacy of  
506 major anthelmintics against horse cyathostomins in France. *Vet. Parasitol.* 188, 294-300.

507 Trawford A.F., Burden F., Hodgkinson J., 2005. Suspected moxidectin resistance in two donkey  
508 herds at The Donkey Sanctuary. Proceedings of the 20<sup>th</sup> International Conference of the  
509 WAAVP, Christchurch, New Zealand.

510 Trawford, A.F., Burden, F., 2009. Ivermectin resistance in cyathostomes in four donkeys herds  
511 at the Donkey Sanctuary, UK. Proceedings of the 22<sup>nd</sup> International Conference of the  
512 WAAVP, Calgary, Canada.

513 van Doorn, D.C., Kooyman, F.N., Eysker, M., Hodgkinson, J.E., Wagenaar, J.A., Ploeger,  
514 H.W., 2010. In vitro selection and differentiation of ivermectin resistant cyathostomin  
515 larvae. *Vet. Parasitol.* 174, 292-299.

516 Vidyashankar, A.N., Hanlon, B.M., Kaplan, R.M., 2012. Statistical and biological  
517 considerations in evaluating drug efficacy in equine strongyle parasites using fecal egg  
518 count data. *Vet. Parasitol.* 185, 45-56.

519 Wood, E.L., Matthews, J.B., Stephenson, S., Slote, M., Nussey, D.H., 2013. Variation in fecal  
520 egg counts in horses managed for conservation purposes: individual egg shedding  
521 consistency, age effects and seasonal variation. *Parasitology* 140, 115-128.

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**Table 1.** Details of the equid populations used to provide L3 for the larval migration inhibition test and their relative sensitivity to ivermectin as indicated by faecal egg count reduction tests. Details for population moxidectin sensitivity is also indicated where the data is available. ML; macrocyclic lactone, IVM; ivermectin, MOX; moxidectin, ERP; egg reappearance period, FECR; faecal egg count reduction, L3; third stage larvae.

<b>Population name</b>	<b>Host species</b>	<b>Location</b>	<b>ML sensitivity or treatment history</b>	<b>Mean IVM-FECR measured in population from which L3 samples were derived<sup>1</sup> (lower confidence limits)</b>
<b>A</b>	Donkey	South west England	IVM resistant [MOX resistant]	91% (0%)
<b>B</b>	Donkey	South west England	IVM resistant [MOX resistant]	82% (0%)
<b>C</b>	Donkey	South west England	IVM reduced ERP [MOX reduced ERP]	>95% (>90%)
<b>D</b>	Donkey	North west England	IVM sensitive	100%
<b>E</b>	Horse	South east England	No ML in last 2 years	ND <sup>2</sup>
<b>F</b>	Horse	South east England	ML not administered	ND <sup>2</sup>

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<sup>1</sup> Where indicated, resident nematode populations were tested for ivermectin sensitivity using a faecal egg count reduction test (FECRT) method based on World Association for the Advancement of Veterinary Parasitology guidelines for ruminants (Coles et al. 1992). The mean reduction in faecal egg count at 14 days after administration is indicated here. <sup>2</sup> FECRT not performed, as macrocyclic lactone use was not permitted as the horses are graze on natural conservation sites.

541 **Table 2.** EC50 value ranges obtained in the larval migration inhibition test for L3 from  
542 individual equids in each population. IVM: ivermectin, R: resistant, S: sensitive. A: IVM  
543 FECR < 95%, B: IVM FECR <95%, C: reduced strongyle egg reappearance period after  
544 IVM and MOX treatment, D: IVM FECRT >95%, E: IVM not administered in previous two  
545 years, F: IVM never administered. Resistance ratios were generated by dividing the EC50  
546 estimate for each population (using all dose response data) by the EC50 estimate for  
547 Population F.

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<b>Population IVM sensitivity status</b>	<b>EC50 range (µg/ml) obtained using L3 from individual equids</b>	<b>EC50 (µg/ml) estimate for each population using all dose response data for each to fit a single best curve: [95% upper and lower confidence intervals]</b>	<b>Resistance ratio</b>
A IVM-R	3.07-13.19	6.24 [8.66, 3.81]	56.7
B IVM-R	1.33 - 6.14	2.31 [2.98, 1.65]	21.0
C ML-RERP	0.48 - 2.30	1.28 [1.60, 0.96]	11.6
D IVM-S	0.06 - 0.26	0.11 [0.13, 0.08]	1.0
E IVM-S	0.15-0.48	0.25 [0.33, 0.16]	2.3
F IVM-S	0.09-0.14	0.11 [0.15, 0.07]	-

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559 **Legends to figures**

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561 **Figure 1.** Dose response curves (% migration against concentration [log  
562 10(concentration+0.01] in µg/ml) generated for each population (A-F) in the larval migration  
563 inhibition test. A: IVM-R, B: IVM-R, C: ML-RERP, D: IVM-S, E: IVM-S, F: IVM-S.

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565 **Figure 2.** Box plots of derived EC50 values from the ivermectin larval migration inhibition  
566 test using L3 from equine populations grouped as harbouring cyathostomins that were  
567 ivermectin resistant as assessed by FECRT (IVM-R), displayed a reduced strongyle egg  
568 reappearance period post ivermectin treatment (IVM-RERP) or were sensitive to ivermectin  
569 as assessed by FECRT or had limited or no treatments of ivermectin in the preceding decade  
570 (IVM-S).

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572 **Figure 3.** Comparison of EC50 values obtained using L3 from those individuals for which  
573 ivermectin faecal egg count reduction test analysis had been performed. Individual equids  
574 are separated into two groups: those for which a faecal egg count reduction of <95% (upper  
575 chart) was recorded and those for which a faecal egg count reduction of >95% (lower chart)  
576 had been obtained. Note that there was variation within populations in the level of  
577 ivermectin faecal egg count reduction when the test was applied and hence one individual  
578 from Population A (IVM-R) had an ivermectin faecal egg count reduction of > 95%. The y-  
579 axis shows the frequency of individuals over the range of EC50 values that were measured.  
580 The x-axis depicts the EC50 value obtained using L3 from individual equids.

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582 **Figure 4.** Percentage migration of L3 in the larval migration inhibition test, comparing time  
583 since last ivermectin administration for larvae derived from Populations A, B and C. The

584 mean EC50 value obtained at each log concentration for each set of time to sample data is  
585 shown.







