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Mating barriers between genetically divergent strains of the parasitic nematode *Haemonchus contortus* suggest incipient speciation.

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ABSTRACT

Haemonchus contortus, in common with many nematode species, has extremely high levels of genetic variation within and between field populations derived from distant geographical locations. MHco10(CAVR), MHco3(ISE) and MHco4(WRS) are genetically divergent *H. contortus* strains, originally derived from Australia, Kenya and South Africa, respectively, that have been maintained by numerous rounds of *in vivo* experimental infection of sheep. In order to explore potential pre-zygotic competition or post-zygotic incompatibility between the strains, we have investigated the ability of MHco10(CAVR) to interbreed with either MHco3(ISE) or MHco4(WRS) during dual strain co-infections. Sheep were experimentally co-infected with 4,000 infective larvae (L_3) *per os* of the MHco10(CAVR) strain and an equal number of either the MHco3(ISE) or the MHco4(WRS) strain L_3. The adult worm establishement rates and the proportions of F_1 progeny resulting from intra- and inter-strain mating events were determined by admixture analysis of microsatellite multi-locus genotypes. Although there was no difference in adult worm establishment rates, the proportions of F_1 progeny of both the MHco10(CAVR) x MHco3(ISE) and MHco10(CAVR) x MHco4(WRS) dual strain co-infections departed from Mendelian expectations. The proportions of inter-strain hybrid F_1 progeny were lower than the expected 50%, suggesting either pre-zygotic competition or post-zygotic incompatibility between the co-infecting strains. To investigate this further, both eggs and hatched first stage larvae (L_1) of broods from single adult female worms recovered from each dual co-infection were genotyped. Unhatched eggs from the broods revealed no inter-strain hybrid genotype deficit, suggesting there is no pre-zygotic competition between the strains. In contrast, there was a deficit in L_1 inter-strain hybrid genotypes in the broods derived from MHco3(ISE) or MHco4(WRS) maternal parents, but not from MHco10(CAVR) maternal parents. This suggests that hybrid progeny of MHco10(CAVR) paternal parents have reduced post-zygotic development and/or viability consistent with incipient speciation of the MHco10(CAVR) strain. The presence of mating barriers between allopatric *H. contortus* strains has important implications for parasite ecology, including the ability of newly introduced anthelmintic resistant parasite populations to compete and interbreed with populations already established in a region.

Keywords or phrases: *Haemonchus contortus*; genetic divergence; dual-strain infections; interbreeding; hybridisation.

1. Introduction

There has been little investigation of relative fitness, competition and mating compatibility between field populations or laboratory strains in parasitic nematode species. This is an important knowledge gap, as such differences are likely to impact many aspects of parasite biology, epidemiology and experimentation. For example, the establishment of parasites introduced into a new geographical location will depend on their ability to mate, and potentially compete with individuals that are already established in the parasite community. This could have major practical implications for the consolidation and spread of anthelmintic resistant parasites. The ability to undertake and appropriately analyse, genetic crossing experiments depends on the extent to which different strains can interbreed. To our knowledge, there are no previous experimental studies directly comparing relative fitness, competition and mating compatability of different strains or populations of any parasitic nematode species. Consequently, we have chosen to investigate this using the blood-feeding gastrointestinal nematode of small ruminants, *Haemonchus contortus*.

H. contortus is an important model for anthelmintic resistance research, and has amongst the best genomic resources for any parasitic nematode (Gilleard 2013; Laing et al., 2013, 2016). The parasite is well suited to investigate questions of inter-strain competition and mating compatibility for a number of reasons. Firstly, there is an increasing number of well characterised strains, primarily derived from anthelmintic resistance studies. The Consortium for Anthelmintic Resistance and Susceptibility (CARS) database lists a total of 21 *H. contortus* isolates originating from different countries and regions around the world that are currently actively maintained for use by the wider research community (http://vbc.med.monash.edu.au/cars/cars.py/). Secondly, there is a high level of genetic differentiation between some of these laboratory passaged strains and between field isolates (Redman et al., 2008). In some cases, the level of genetic differentiation between different *H. contortus* strains is higher than seen between cryptic species of other parasites such as the closely related gastrointestinal nematode, *Teladorsagia circumcincta* (Grillo et al., 2006). This leads us

to question whether significant differences occur between phenotypic and life history traits of some of these *H. contortus* strains. Thirdly, there are well characterised microsatellite marker panels available for *H. contortus*, enabling individual worms to be unambiguously assigned to specific strains based on their multi-locus genotypes (Redman et al., 2008). This allows reliable strain assignment of parental worms and their progeny to be tracked during experimental co-infections of multiple strains.

In this work we used three independently derived and genetically divergent *H. contortus* strains that are maintained at the Moredun Research Institute by *in vivo* passage in sheep and larval coproculture: designated MHco3(ISE); MHco4(WRS); and MHco10(CAVR) (Redman et al., 2008). These strains are known to be capabale of interbreeding through genetic crosses (Redman et el., 2012) but direct phenotypic comparison, or investigation of their behaviour during co-infection has not yet been undertaken. The inbred *H. contortus* SE (ISE) strain, which is susceptible to the benzimidazole, macrocyclic lactone and imidazothiazole anthelmintic drug classes, was derived from a highly heterogeneous population thought to have originated in Kenya (Otsen et al., 2001). Inbreeding was achieved by 15 rounds of serial experimental infections using the broods of single adult female worms at each generation (Roos et al., 2004). The White River (WRS) strain *H. contortus* originated from the Transvaal in South Africa, as a field population that was resistant to benzimidazole, ivermectin and salicylanilide anthelmintics (Van Wyk and Malan, 1988). The WRS strain has subsequently been characterised using *in vitro* and *in vivo* methods, confirming its resistance to multiple anthelmintic groups benzimidazole, ivermectin and salicylanilide anthelmintics (Van Wyk et al., 1989) and susceptibility to levamisole and moxidectin (Oosthuizen and Erasmus, 1993, Gill et al., 1995, Jeannin et al., 1990, Le Jambre et al., 1995). The Chiswick Avermectin Resistant strain (CAVR) arose as a serendipitous, extraneous, ivermectin resistant contaminant of a laboratory passaged *T. colubriformis* strain in Armidale, New South Wales, Australia (Le Jambre, 1993) and has subsequently been characterised as being resistant to ivermectin, but susceptible to benzimidazoles, levamisole and salicylanilide anthelmintics (Le Jambre et al., 1995).

In this paper we describe a detailed comparison of adult worm morphology of the MHco10(CAVR), MHco4(WRS) and MHco3(ISE) *H. contortus* strains and the investigation of the ability of the MHco10(CAVR) to interbreed with the other two strains. This was undertaken to identify any competitive incompatibility between the strains, which could have significant

implications in the field. For example, a competitive disadvantage of an anthelmintic resistant or susceptible strain, when introduced into a genetically divergent population of parasites could have a major impact on the spread of anthelmintic resistant parasites, or suggest strategies by which populations might revert to anthelmintic susceptibility, respectively.

2. Materials and Methods

2.1. H. contortus *strains*

The three genetically distinct strains of *H. contortus* with diverse histories and geographic origins used in this study were MHco3(ISE), MHco4(WRS) and MHco10(CAVR).

2.2. Morphometric strain characterisation

Ten male and 10 female adult worms for each strain were preserved in 2% formalin and examined and measured using established protocols (Lichtenfels et al., 1994) including vulval morphology and the percentage of the body covered by longitudinal ridges (synlope), described in Supplementary Tables S1 and S2. Univariate analysis of the variance of each parameter was performed with strain as a factor. When strain was not considered as a statistically significant factor in the model, but there were observable differences between the strains independent t-tests were used to test for statistical significance between any two mean estimates (SPSS Statistics 18).

2.3. Single-strain infections

Experimental infections were undertaken by oral administration of 5,000 L_3 per animal to three controlled efficacy test treatment groups of five, parasite naïve 7 month-old lambs for each of the three strains (n = 15 lambs per strain in total) as described previously (Redman et al., 2012). The lambs had been reared and maintained indoors under conditions designed to eliminate the risk of trichostrongylid nematode infection and treated sequentially with 5 mg/kg of fenbendazole (Panacur 2.5%; Intervet) and 7.5 mg/kg of levamisole (Levacide 3%; Norbrook) 14 days before oral infection with L_3. Faecal worm egg counts (FWECs) were determined for each

lamb just prior to infection (day 0), using a salt floatation method with a potential sensitivity of one egg per gram (epg) (Christie and Jackson, 1982), to confirm their parasitic nematode-free status. This was repeated on days 14, 16, 18, 21, 28, 29, 30 and 36 pi, to allow the duration of the pre-patent period (PPP) of each of the three strains to be determined. Lambs were euthanased on day 36, enabling the enumeration, sexing and staging (only adults were seen) of *H. contortus* worm burdens in abomasal saline washings and digests using 2% subsamples (MAFF, 1986; Wood et al., 1995). Worm fecundity was estimated by dividing the egg count on day 36 pi by the adult female worm burden.

2.4. Dual-strain co-infections and brood production

Five month-old lambs were infected with equal numbers of two different strains (4,000 L_3 of each strain per animal) to set up two different dual strain co-infections: MHco10(CAVR) with MHco3(ISE) and MHco10(CAVR) with MHco4(WRS). The experiment was repeated providing two bio-replicates of both dual infections. The lambs had been reared, maintained and treated with anthelmintics, and FWECs were estimated exactly as described in section 2.3. FWECs were estimated exactly as described in section 2.3. In addition, approximately 100g of faeces were collected from each lamb on day 28 pi and day 36 pi and eggs extracted, the eggs were allowed to develop and hatch in water at 20˚C for 48 hours to produce L_1 from which individual worm DNA lysates were prepared using previously described techniques (Redman et al., 2008). Populations consisting of 30 F_1 progeny L_1 were generated for both time points and DNA lysates of these were prepared and genotyped with microsatellite markers. The co-infected lambs were euthanased on day 36 pi and their total *H. contortus* burdens estimated. A 10% sub-sample of the abomasal washings and digests was counted, sexed and staged to estimate the overall and sex-ratio establishment rates of each of the strains within the dual-infections. DNA lysates were made from individual adult worms from a second 10% sub-sample.

In the case of the second bio-replicates for dual co-infections of MHco10(CAVR) with MHco3(ISE) and MHco10(CAVR) with MHco4(WRS), following necropsy 24 fresh adult female *H. contortus* were individually placed into 1ml of pre-warmed RPMI 1640 medium plus 2.5ug/mL amphotericin B, 100U/mL penicillin and 100ug/mL streptomycin (1:10 Fungizone™) in wells of a 24-well plate and incubated for 6 hours at 37ºC and 5% CO_2 to prepare egg and L_1

broods. For each female, a sub-sample of eggs was harvested prior to hatching with the remainder left for 48 hours to produce L_1. For each adult female worm, DNA lysates were prepared from the head of the worm and from individual broods comprising sixteen unhatched eggs and sixteen L_1.

2.5. Microsatellite markers and PCR amplification

DNA lysates were diluted in double distilled water (ddH_2O) for use in PCR to avoid reaction inhibition: 1µl of a 1:20 dilution (egg, L_1, L_3 and female head lysates) and 1µl of a 1:40 dilution (adult male lysates) were used as PCR template. Dilutions of several aliquots of lysate buffer, made in parallel, were included as negative controls for all PCR amplification experiments. Microsatellites used to genotype the populations were chosen for their ability to successfully differentiate between the parental strains of the two respective dual co-infections. In the case of the MHco10(CAVR) and MHco3(ISE) co-infections, five microsatellite markers (Hcms36, 8a20, Hc3086, Hc22193 and Hc53265) were used. In the case of the MHco10(CAVR) and MHco4(WRS) co-infections, seven microsatellite markers (Hcms27, Hcms36, Hcms40, Hc3086, Hc22193, Hc44104 and Hc53265) were used. The primers for loci Hcms27, Hcms36, and Hcms40 (Otsen et al., 2000), Hcms8a20 (Redman et al., 2008), and Hc3086, Hc53265, Hc22193 (Redman et al., 2015) have been previously published. The primers for locus Hc44104, which have not been previously described, were F: TCGGCTGCTTTCATAGAC, R: GGTATCGACCAAGATTCAG. The same reaction conditions were used for all microsatellite loci as previously reported (Redman et al., 2008). Capillary electrophoresis was performed using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) for the accurate sizing of microsatellite PCR products. The forward primer of each microsatellite primer pair was 5'-end labeled with FAM, HEX, or NED fluorescent dyes (MWG) and electrophoresed with GeneScan ROX 400 (Applied Biosystems) internal size standard. Individual chromatograms were analyzed using Genemapper Software Version 4.0 (Applied Biosystems). For each individual worm or larvae, a multilocus microsatellite genotype (MLG) was generated by combining the data for each marker.

2.6. Admixture analyses

The ancestry of individual worms from each of the respective parental strains used in the dual co-infections was determined from their multi-locus microsatellite genotypes. A Bayesian clustering procedure implemented in STRUCTURE 2.1 (Pritchard et al., 2000) was used to assign individual progeny as the products of inter- or intra- strain mating and establish the predicted and observed frequency of these events by first identifying the number of genetically distinct clusters (K) that maximise the likelihood of the data, and second assigning individuals to these clusters using only genotype information. Five repetitions of $9x10^4$ Markov chain Monte Carlo (MCMC) iterations were used following a burn-in period of 10^4 iterations. Admixture analysis was performed with the 'admixture' model which allows each individual to have ancestry from more than one parental population. The value of K (the number of clusters/populations), was determined as the value that maximised the increase in the posterior probability of the data (Garnier et al., 2004). The proportion membership (*Qi*) of each individual worm to the inferred clusters was then estimated.

2.7. Analysis of the proportion of hybrids in individual adult worm broods

The parental genotype of each of the 24 adult female worms (bioreplicate 2) was determined using the admixture model in order to identify four female worms for each parental strain for each of the co-infections. L_1 from the brood of each of these adult worms were then genotyped with the same microsatellite markers. Validation for the admixture analysis of each individual brood required the generation of separate simulated F_1 populations using the software HYBRIDLAB, using the genotype of the female worm for one side of the cross and actual genotype data for the remaining parental strain in the dual infection. The admixture analysis of the parental and simulated F_1 genotypes was used to validate a threshold value for *Qi* of 0.8. The genotype data for each brood in turn were analysed alongside their corresponding parental and simulated F_1 genotypes and the proportions of hybrid and parental genotypes were estimated in each brood.

Populations of eggs from the same brood female worms were also genotyped prior to egg hatch to investigate the possibility that the deficits in hybrid genotypes of the L_1 were due to reduced development of eggs.

2.8. Statistical analyses

Both the establishment rate and fecundity data were modelled assuming a normal error distribution, using the generalised linear modelling facility of SPSS Statistics 18 (IBM®). The relative proportions of parental and hybrid genotypes identified in F_1 populations were analysed using the Chi-squared statistic against the relative proportions of parental and hybrids expected from a dual infection. The data for the proportion of hybrids identified within each brood were first logit transformed (logit = ln(p/1-p)), where p = proportion of hybrids) and then modelled assuming a binary error structure using the generalised linear modelling facility of SPSS Statistics 18 (IBM®). Deviations were adjusted via the scale parameter to allow for over dispersion of the data.

2.8. Ethics statement

All experimental procedures described in this manuscript were examined and approved by the Moredun Research Institute Experiments and Ethics Committee and were conducted under approved UK Home Office licenses in accordance with the Animals (Scientific Procedures) Act of 1986. The Home Office license numbers are PPL 60/03223 and PPL 60/03899 and experimental IDs for these studies were E06/58. E06/75 and E09/36.

3. Results

3.1. Morphometric strain characterisation

A comprehensive set of parameters was measured using 10 male and 10 female worms of each strain and the data are summarised in Supplementary Tables S1 and S2.

Examination of the vulval process of adult females revealed that there were differences in the proportions of each of the main morphotypes present between the strains: MHco3(ISE) and MHc4(WRS) were predominately linguiform; whereas MHco10(CAVR) was predominately smooth (Figure 1A and Supplementary Figure S1). Adult female MHco4(WRS) worms were wider than both MHco3(ISE) and MHco10(CAVR) worms at the oesophageal-intestinal junction (EIJ) ($F_{2,30} = 6.318$, $p = 0.006$) and possessed a wider oesophagus than MHco3(ISE) worms ($t_{18} = 2.189$, $p = 0.042$). The position of the nerve ring ($t_{18} = 2.101$, $p = 0.05$) and excretory pore (t_{18}

263 = 2.21, p = 0.04) for female MHco10(CAVR) worms was further from the anterior than for
264 MHco4(WRS) worms and the end of the synlophe was further from the anterior for MHco3(ISE)
265 worms than for MHco4(WRS) (t_{18} = 2.354, p = 0.03). There were statistical differences between
266 the three strains in the percentage synlophe ($F_{2,30}$ = 16.946, p < 0.0001; Figure 1B).

267 The level of inter-strain variation proved considerably higher for adult male than for
268 female worms with observable differences for 11 of the 14 different male characters measured
269 compared to just 6 of the 20 female characters (Supplementary Table S3). As with the female
270 worms the percentage of the body covered with synlophe was found to vary considerably
271 between strains (Figure 1B; $F_{2,30}$ = 10.04, p = 0.001), although the pairwise comparison between
272 MHco3(ISE) and MHco10(CAVR) worms was not significant. The two male characters for
273 which statistical significant differences were observed between all three strains were male
274 spicule length ($F_{2,30}$ = 20.81, p < 0.0001) and length of oesophagus ($F_{2,30}$ = 12.14, p < 0.0001),
275 but the ranges for all of the characters overlapped (Supplementary Table S3).

276

277 *3.2. Characterisation of life history traits of the three strains using single-strain infections*

278 The numbers of adult worms recovered at necropsy on Day 36 post-experimental
279 infection revealed no statistical differences in establishment rates between the three strains as
280 measured by adult worm establishment ($F_{2,14}$ = 1.12, p = 0.36, Figure 2A). In the absence of daily
281 sampling and data points, it was not possible to pinpoint the exact time when egg production
282 started, but it is clear that there was no egg shedding by day 16 pi for all three strains, but eggs
283 were being produced by day 18 pi. At this point, 12 of 15 animals infected with MHco3(ISE)
284 worms had started to shed eggs, while only 4 of 15 animals infected with MHco4(WRS) and 5 of
285 15 animals infected with MHco10(CAVR) had begun egg shedding. This variability in the onset
286 of egg production was also reflected by significantly higher FECs for MHco3(ISE) than for
287 MHco4(WRS) or MHco10(CAVR) (Figure 2B and Supplementary Table S4) on day 18 pi.
288 Therefore, the prepatent period of MHco3(ISE) appears to be 24 to 48 hours shorter than that of
289 either MHco4(WRS) or MHco10(CAVR). However, by day 21pi there was no significant
290 difference between the FEC of the three strains (Figure 2B and Supplementary Table S4), and so
291 the effect of this small difference in pre-patent period appeared to be temporary during the initial
292 establishment and did not result in any residual differences in egg production between the three

strains. The three *H. contortus* strains were found to be equally fecund when dividing the number of eggs produced on day 36 pi (epg) by the total female worm burden at necropsy ($F_{2,15} = 0.33$, $p = 0.73$).

3.3. Establishment rates of dual-infections

The adult worm establishment rates of the dual infections was similar to that observed in single-strain infections (Table 1). Approximately equal proportions of each strain were identified in the 10% sub-samples of the adults collected from dual strain co-infected hosts at necropsy, indicating that the establishment rates of the strains were the same in dual infections as in a single-strain infection. There were equal ratios of male and female adult worms in each dual infection (Table 1). There was therefore no evidence of one strain out-competing the other, in terms of the establishment rate of adult worms, following the oral infection of the mixture of infective L_3.

3.4. Genetic differentiation between parental strains

There was a high level of genetic differentiation between the adult worms of each parental population revealed by the separate clustering, by strain, of their multi-locus genotypes on PCA analysis (based on genotyping 30 individual adult worms for each strain) (Figure 3A). Importantly, there was no overlap between the parental clusters, indicating that the panels of microsatellite markers will allow subsequent unambiguous assignment of F_1 progeny either as the product of selfing of either parental strain, or as hybrids of inter-strain mating. The pairwise F_{ST} values are slightly higher than previous reports (Redman et al., 2008).

3.5. Simulation of admixture analysis to assess the reliability of identification of parental and hybrid genotypes

The power of admixture analysis to correctly identify individuals from the three parental strains without any prior population information was assessed. The multi-locus genotypes of the MHco4(WRS) and MHco10(CAVR) parental strains (based on genotyping 30 individual adults) were analysed to determine a threshold value of proportion membership at which all individuals

correctly clustered according to their *a priori* parental population (Figure 3 B). A similar analysis was carried-out for the MHco3(ISE) and MHco10(CAVR) parental populations. A threshold of *Qi*=0.8 was established for both analyses. In order to assess the power of admixture analysis to correctly identify F_1 hybrids, simulated hybrid populations were generated (Figure 3B). Genotypes from parental populations were used to generate 30 simulated F_1 genotypes with the software HYBRIDLAB (Nielsen et al., 2001). Hybrid genotypes were created by random sampling the frequency distributions of the parental populations assuming random mating, neutrality and linkage equilibrium. Both analyses of the parental strains were repeated with the inclusion of a simulated F_1 population. The same admixture model was used with no prior population information. The threshold value (*Qi*) at which these "blind" genotypes could be successfully recognised as of either parental or of hybrid origin was estimated. A threshold of *Qi*=0.8 was established as the discriminatory value at which all parental and simulated hybrid genotypes could be attributed to their correct *a priori* populations for both types of co-infections (MHco3(ISE) and MHco10(CAVR), or MHco4(WRS) and MHco10(CAVR). The assignment of parental genotypes was unambiguous, allowing the threshold for admixture analysis to be determined and validating the method to determine the parental origins of F_1 hybrid genotypes. This methodology was then used to interpret the actual genotypes of the progeny of the dual infections.

3.6. Parental and hybrid identification in F_1 progeny

In a freely interbreeding 1:1 dual infection of two equally infective and fecund strains, where equal numbers of each strain establish within a single host, the F_1 progeny would consist of 50% hybrid, 25% 1st parental and 25% 2nd parental genotypes. Significant deviations from these ratios could indicate bias in the mating efficiencies between the two co-infecting strains. To test this, 30 F_1 progeny from each dual infection were genotyped and their multi-locus genotypes analysed, with the admixture model, alongside their corresponding parental populations and the simulated F_1 populations. For each individual worm, the admixture analysis produces values of proportional membership of the two parental populations (when K=2) that when summed total one. These data can be visualised with a bar-graph that plots each individual worm (x-axis) against proportion membership (y-axis). Figure 3B shows just a small portion of

the data in this format for illustrative purposes. The proportions of parental and hybrid genotypes represented in the F_1 populations were established using the discriminatory threshold value of *Qi*=0.8.

The Chi-squared statistic was used to compare the proportions of hybrid and parental genotypes observed in F_1 progeny resulting from a dual infection with those expected if there was equally efficient mating within and between strains. For the MHco3(ISE) and MHco10(CAVR) co-infection, the frequency distribution of genotypes in the F_1 on day 28 pi was significantly different from the expected ratio (Table 2A; $\chi^2_2 = 10.2$, $p = 0.006$). The proportion of hydrids present (0.3) was lower than the expected proportion of 0.5. Although the parental MHco10(CAVR) genotypes were represented in the F_1 progeny at approximately the expected proportion (0.2 compared to the expected 0.25), the MHco3(ISE) parental genotypes were over represented in the F_1 progeny (0.5 compared to the expected 0.25). The second bio-replicate of the same MHco3(ISE) and MHco10(CAVR) dual co-infection (Table 2A; bio-replicate 2) revealed similar proportions of the different genotypes in the F_1 population at both day 28 pi and at an additional second time point taken at day 35 pi. This over-representation of one of the parental strains and under-representation of hybrids also occurred in the distributions of F_1 progeny genotypes resulting from the dual co-infection of the MHco4(WRS) and MHco10(CAVR) strains (Table 2B). Hydrids were present at a proportion of 0.276 (expected proportion of 0.5) and the MHco4(WRS) parental genotype was present at a proportion of 0.517 (expected proportion of 0.25). In contrast, the MHco10(CAVR) genotypes were present at a similar level as predicted by Mendelian genetics (0.207 compared to 0.25; Table 2B). Similar genotype proportions were also seen in the progeny produced later in the infection at day 35 pi and in the progeny of a second MHco4(WRS) and MHco10(CAVR) dual co-infected sheep (Table 2B; bio-replicate 2).

3.7. Proportion of hybrids within progeny broods of individual adult worms

There is a variety of explanations for the deviations from the expected proportions of parental and hybrid genotypes in the F_1 progeny of the dual strain co-infections. In the illustrative example presented in Figure 4A, the lack of hybrids may be the result of limited hybridisation between MHco10(CAVR) males and MHco3(ISE) females, or alternatively

between MHco10(CAVR) females and MHco3(ISE) males (Figure 4A). To investigate this, the proportion of hybrids produced from individual female worms of known strain identity from both strain combinations of dual infections was determined. A total of sixteen individual female worm broods were analysed: four MHco3(ISE) female broods and four MHco10(CAVR) females broods of the MHco3(ISE) and MHco10(CAVR) dual species co-infection, and four MHco4(WRS) female broods and four MHco10(CAVR) females broods of the MHco4(WRS) and MHco10(CAVR) dual species co-infection.

From the MHco3(ISE) and MHco10(CAVR) dual infection, the mean proportion of hybrid F_1 genotypes per brood for the MHco3(ISE) adult females was 0.2 and for the MHco10(CAVR) adult females it was 0.54 (Figure 4B). This suggests that MHco10(CAVR) female worms are equally likely to produce L_1 progeny resulting from mating with MHco10(CAVR) males or with MHco3(ISE) males; but in contrast the MHco3(ISE) female worms are significantly less likely to produce L_1 progeny resulting from mating with MHco10(CAVR) males than mating with MHco3(ISE) males. A similar result was obtained for the broods of adult female worms recovered from the MHco4(WRS) and MHco10(CAVR) dual infections; female MHco4(WRS) worms produced significantly fewer hybrid progeny than the female MHco10(CAVR) worms (Figure 4B; MHco3(ISE)/ MHco10(CAVR); $\chi^2_1 = 16.11$, $P < 0.0001$ and MHco4(WRS)/ MHco10(CAVR); $\chi^2_1 = 4.70$, $P = 0.03$).

Admixture analysis identified hybrid genotypes of unhatched eggs at approximately 0.5 for both strains in both types of dual infections (Figure 4B), indicating that deficits in hybrid genotypes of the L_1 were due to reduced development of eggs. The data suggest that the eggs resulting from MHco10(CAVR) male x MHco3(ISE) female or MHco10(CAVR) male x MHco4(ISE) matings have reduced viability or developmental capacity than those resulting from intra-strain matings.

4. Discussion

The relatively simple anatomy of nematodes limits the amount of easily visible phenotypic variation and belies the high levels of genetic variation that occurs within many species. A large amount of genetic variation exists within and between field populations, and laboratory strains, of many nematode species, particularly those with large effective population

sizes (Gilleard and Redman, 2016). However, there has been little investigation of potential reproductive barriers that could occur between genetically divergent strains for any nematode species. *H. contortus* is arguably the parasitic nematode in which genetic variation has been most intensively studied (Gilleard and Redman, 2016). This parasite species has extremely high levels of intra-specific genetic variation and although genetic differentiation can be low within a local geographical region, it can be extremely high over larger geographical scales (Troell et al., 2006; Redman et al., 2008; Redman et al., 2012; Gilleard and Redman, 2016). MHco3(ISE), MHco4(WRS) and MHc10(CAVR) are *H. contortus* strains that were originally derived from different continents and subsequently been maintained by numerous rounds of *in vivo* experimental infection of sheep. Consistent with this history, these strains are highly genetically divergent, with pairwise Fst values in the range of 0.16 and 0.27 based on microsatellite marker genotyping (Redman et al 2008), and similar values based on genome wide SNP analysis (Doyle et al., 2018a). This high level of genetic differentiation between strains is of a similar scale to that found between cryptic species of other stronglyid nematodes (Grillo et al., 2006). However, we do know that these *H. contortus* strains do not represent completely separate or full cryptic species since they can mate to produce viable, fertile progeny during experimental crosses performed to genetically map a major ivermectin resistance locus (Redman et al., 2012, Doyle et al., 2018b). Nevertheless, the fact that adult males of one strain can successfully mate, and produce fertile offspring, with adult females of another following direct transplantation into the host abomasum, does not necessarily mean that they freely interbreed during co-infections. Significant mating barriers could still exist between such strains. Consequently, in this work, we have investigated the extent to which the MHco10(CAVR) strain, the most genetically divergent of the three strains used in our study, can interbreed with either the MHco3(ISE) or MHco4(WRS) strains in dual strain experimental co-infections. Our results clearly demonstrate that, although the co-infecting strains do interbreed to some extent, there is a significant mating barrier between MHco10(CAVR) and the other two strains suggesting that MHco10(CAVR) is an incipient species based on the biological species concept.

We first investigated inter-strain morphological and morphometric variation and found significant differences in both male and female adult worms between the three strains. Variation in temperature tolerance in relation to different vulval morphology has led to speculation regarding sub-speciation within H. contortus (Crofton et al., 1965; Le Jambre and Royal, 1977).

There were just two female characteristics that differed between the three strains, the vulval morphotype (Le Jambre, 1977) and the synlophe; longitudinal cuticular ridges in the anterior portion of the worm that have been previously used to differentiate between different nematode species (Lichtenfels et al., 1994). There were statistically significant differences in the frequency of the vulval process morphotypes between the strains, with the MHco10(CAVR) being the most divergent, and in the percentage of the body covered by the synlophe. There were also statistically significant differences between mean estimates for a number of adult male characters including the extent of the synlophe, spicule length and morphometric features of the oesophagus. We also compared a number of life history traits between the strains. There were no statistically significant differences between the establishment rates of the three strains, as determined by the number of adults collected at necropsy on day 36 pi relative to the number of L_3 used to infect the donor animals. There was a trend towards the MHco3(ISE) strain commencing egg production earlier than the other two strains. However, this effect was only temporary during the initial establishment stage of infection, before day 21 pi. This is consistent with previous work undertaking genetic crosses between the same three strains, where daily egg production was measured and consistently observed to commence one to two days earlier in MHco3(ISE) donor lambs (Sargison, 2009). There was no difference in the estimated fecundity of the strains in single strain infections, calculated by dividing the number of eggs produced (epg) by the total number of female worms recovered postmortem on day 36 pi. This contrasts with previous work that showed differences in fecundity of the Australian Wallangra 2003, Kirby 1981, Gold Coast 2004 and McMaster 1931 *H. contortus* strains (Hunt et al., 2008). Consequently, overall there were only a limited number of subtle morphological differences between the strains and no major differences in life history characters.

The primary aim of our study was to investigate if there was any detectable mating barrier between the strains; specifically between the most genetically divergent of the three strains, MHco10(CAVR), and the other two strains. The experimental design was to orally co-infect a recipient sheep with 4,000 larvae of MHco10(CAVR) and 4,000 larvae of either the MHco3(ISE) or the MHco4(WRS) strain to establish adult worm populations and then use genetic analysis to determine the proportions of L_1 in the F_1 progeny that were derived from intra-strain compared to inter-strain matings. We used an admixture model to assign strain identity to the adult parental worms collected from the co-infected hosts at necropsy and found

474 equal proportions from each of the two strains in each dual infection. This showed that neither
475 strain out-competed the other in terms of the number of adult worms establishing following oral
476 co-infection with a mixed population of L_3. Consequently, any deviation in the expected
477 Mendelian proportions of intra-strain and inter-strain hybrid genotypes in the F_1 progeny must
478 result from differences in the mating outcomes of the parental worms, or in differential
479 development and survival of F_1 progeny.

480 The admixture analysis of the L_1 (F_1 progeny) multilocus genotypes revealed that the
481 proportions of parental and hybrid F_1 progeny departed from Mendelian expectations for both
482 bio-replicates of both the MHco10(CAVR) x MHco3(ISE) and MHco10(CAVR) x
483 MHco4(WRS) dual strain co-infections. The proportions of inter-strain hybrid F_1 progeny were
484 significantly lower than the expected 50% from both the MHco10(CAVR) x MHco3(ISE) and
485 MHco10(CAVR) x MHco4(WRS) dual strain co-infections. This suggests that there is either
486 pre-zygotic competition between the strains such as assortive mating (mate preferences) or sperm
487 competition, or a post-zygotic reduction of development and survival of the inter-strain hybrid
488 genotypes. In order to investigate this, we genotyped both unhatched eggs and hatched L_1 of the
489 F_1 progeny from broods of a number individual adult female worms recovered at necropsy from
490 dual strain co-infected infected lambs. The genotyping of unhatched eggs from the broods
491 revealed no deficit of hybrid genotypes suggesting that there is no pre-zygotic competiton
492 between the strains. In contrast, the genotyping of L_1 from same broods revealed a deficit in the
493 inter-strain hybrid progeny from the MHco3(ISE) and MHco4(WRS) maternal parents, but not
494 from the MHco10(CAVR) maternal parents. This suggests that hybrid progeny derived from
495 MHco10(CAVR) paternal parents have reduced post-zygotic development and/or viability. The
496 mechanisms for this are not known but there are a variety of possibilities based on recent
497 research in free-living nematode species. Hybrid fitness deficits in nematode genetic crosses
498 have been proposed to be a result of inherited factors from one parental population (Lamelza and
499 Ailion, 2017). For example, studies in the model nematode organism, *Caenorhabditis elegans*,
500 suggest that these factors may sometimes involve incompatibilities between maternally inherited
501 small RNAs regulating gene expression in developing embryos (Han et al., 2009), or
502 mitochondrial DNA (Dean et al., 2014) and the nuclear genome. Alternatively, the reduced
503 proportion of inter-strain hybrid progeny from the MHco3(ISE) or MHco4(WRS) maternal
504 parents and MHco10(CAVR) paternal parents might be due to the presence of maternal selfish

genetic elements (Hurst and Werren, 2001), whereby the MHCo10(CAVR) males lack a zygotically expressed antidote. Both paternal and maternal genetic elements have been described in *Caenorhabditis elegans*, consisting of tightly linked genes coding for sperm (Sinkins, 2011), or embryos (Ben-David et al., 2017) that can deliver toxins and zygotically expressed antidotes. These elements have been proposed as mechanisms of subverting the laws of Mendelian segregation to promote their own transmission; by causing developmental arrest or mortality in filial progeny that do not inherit the zygotically expressed antidote. In arthropods, selfish genetic elements such as those regulating the inheritance of intracellular *Wolbachia* bacteria from mothers to eggs can give infected females a competitive advantage over uninfected females (Landmann et al., 2009). Further work will be needed to test these potential mechanisms of reduced hybrid post-zygotic viability in the hybrid progeny with MHco10(CAVR) paternity.

The reduced proportion of F_1 hybrid genotypes discussed above would be expected to be accompanied by an equal increase in the proportions of the intra-strain homozygous progeny for each of the two parental strains in a co-infection. However, in each case, MHco10(CAVR) intra-strain F_1 progeny, were under represented. Given that based on the brood analysis, 50% of eggs from MHco10(CAVR) adult maternal parents were intra-strain progeny, this suggests that the total egg production of MHco10(CAVR) maternal parents is less than that of the maternal parent of the second MHco3(ISE) or MHco4(WRS) co-infecting strain. The reason for this is unclear, but given that it has been previously shown that *H. contortus* is polyandrous (Redman 2008; Doyle et al., 2018b), it is possible that there is a reduction in overall fecundity in those MHco10(CAVR) females that mate with males of more than one strain as will often occur in co-infections.

The identification a degree of post-zygotic incompatibility between genetically divergent strains of a parasitic nematode species has implications for the behavior and ecology of gastrointestinal nematode parasites in the field. For example, the ability of gastrointestinal nematode strains, translocated by animal movement, to establish and thrive in a new region will depend on successful competition and mating with the locally established parasite population. In the case of *H. contortus*, mating incompatibilities have specific practical implications when considering the population genetics of anthelmintic resistance conferring alleles. This in turn could have a major impact on the spread of anthelmintic resistant parasites, or suggest strategies by which populations could be reverted to anthelmintic susceptibility.

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Figure legends

Figure 1 Frequency distribution and photographs of common vulvar processes represented in different *H. contortus* strains (A). These were Smooth, Knobbed, Linguiform A with a singular cuticular inflation that varied considerably in size between individuals, Linguiform B with no cuticular inflation and Linguiform C with two cuticular inflations. Scale bars = 100μm.

Percentage of body covered in synlophe (±2SE) for females and males (B).

Figure 2 Establishment rates of *H. contortus* strains estimated from, mean (± s.e.) female, male and total worm burden (A). Characterisation of the reproductive capacity of *H. contortus* strains. The only significant differences in FEC of the strains was in evidence on day 18pi, when sheep infected with MHco3(ISE) had a significantly higher FEC than either MHco4(WRS) or MHco10(CAVR) infected animals, (B). This higher FEC at the start of the infection was directly correlated with a differential prepatent period (PPP) of the *H. contortus* strains, with MHco3(ISE)-infected animals starting egg production sooner than animals infected with either MHco4(WRS) and MHco10(CAVR).

Figure 3 Principal component analysis (using GenAlEx) showing the relationship of multi-locus genotypes of 30 individual worms of the different *H. contortus* parental strains. (A). Genetic differentiation between the strains MHco3ISE and MHco10CAVR (5 microsatellite markers) and between the strains MHco4WRS and MHco10CAVR (7 microsatellite markers). X and Y axes represent first and second factors of variability. STRUCTURE analyses (B) performed assuming two distinct genetic clusters (K=2), of the observed parental genotypes in dual infection, F_1 progeny genotypes produced 28 days p.i. and simulated F_1 progeny genotypes that were generated by HYBRIDLAB. Hybrid analyses of progeny of dual infection of MHco3ISE and MHco10CAVR and of MHco4WRS and MHco10CAVR. Admixed genotypes at threshold q_i = 0.8 are evidenced.

Figure 4 Potential crosses occurring within a dual infection and the resulting expected proportions of genotypes within F_1 generation. Crosses that produce hybrids have been shaded. Proportion of F_1 hybrids produced by individual MHco3(ISE) and MHco10(CAVR) female worms following dual infection and proportion of F_1 hybrids produced by individual MHco4(WRS) and MHco10(CAVR) female worms following dual infection (B). A deficiency of crosses between MHco3(ISE) females and MHco10(CAVR) males appears to explain observed distribution of genotypes in F_1.

Table 1

The establishment (proportion of L_3 that established as adults on day 36 post infection) and contribution of each parental strain to overall worm burden (estimated from the number of adults collected at necropsy). Single strain infections were established by oral dosing of donor lambs with 5,000 L_3 (section 2.3.) and dual strain infections by oral dosing of donor lambs with 4,000 L_3 of each strain (section 2.4.). The proportions of each strain recovered on postmortem were determined by multilocus genotyping of adult worms (numbers shown in brackets).

Type of infection	Single strain	Dual strain bioreplicate 1			Dual strain bioreplicate 2		
	Infectivity	Infectivity	Proportion of strain	Sex ratio (♂:♀)	Infectivity	Proportion of strain	Sex ratio (♂:♀)
MHco3(ISE) + MHco10(CAVR)	0.55	0.652	0.45 (38)	1.0	0.623	0.4 (40)	0.9
	0.544		0.55 (38)	1.0		0.6 (40)	1.0
MHco4(WRS) + MHco10(CAVR)	0.79	0.721	0.55 (40)	1.2	0.575	0.5 (40)	1.0
	0.544		0.45 (40)	0.9		0.5 (40)	1.2

Table 2

Expected and observed proportions of parental and hybrid genotypes in F_1 progeny populations with Chi-squared statistic and p-value.

A	F_1 progeny of MHco10(CAVR) x MHco3(ISE) dual infection				
		Bioreplicate 1		Bioreplicate 2	
Progeny type	Expected	Day 28 pi	Day 35 pi	Day 28 pi	Day 35 pi
MHco10(CAVR)	0.25	0.2	na	0.1818	0.2444
MHco3(ISE)	0.25	0.5	na	0.5	0.5111
F_1 hybrids	0.5	0.3	na	0.3182	0.2444
N		30		44	45
χ^2_2		10.2		14.73	18.156
		(p=0.006)		(p=0.001)	(p=0.000)
B	F_1 progeny of MHco10(CAVR) x MHco4(WRS) dual infection				
		Bioreplicate 1		Bioreplicate 2	
Progeny type	Expected	Day 28 pi	Day 35 pi	Day 28 pi	Day 35 pi
MHco10(CAVR)	0.25	0.2069	0.2580	0.2414	0.2188
MHco4(WRS)	0.25	0.5172	0.4194	0.5172	0.4375
F_1 hybrids	0.5	0.2758	0.3226	0.2414	0.3125
N		29	31	29	32
χ^2_2		11.414	5.516	12.17	7.065
		(p=0.003)	(p=0.0063)	(p=0.002)	(p=0.029)

na: DNA lysates of insufficient quality to allow robust microsatellite amplification

Contributions

JSG, FJ and NDS conceived the experimental design, overall methodology and analytical approach. NDS, AAM and DJB undertook the animal work and gross parasitology. ER and NDS undertook the molecular work and analysis of the data. NDS, ER, and JSG wrote the paper, with input from each of the co-authors.