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Title: Transcriptional profiling of the ovine abomasal lymph node reveals a role for timing of the immune response in gastrointestinal nematode resistance

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1 **Transcriptional profiling of the ovine abomasal lymph**  
2 **node reveals a role for timing of the immune response**  
3 **in gastrointestinal nematode resistance**

4

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Nematode resistant and susceptible lambs were identified using a previously developed model

Resistant lambs had more immature and shorter worms

Genes involved in the inflammatory response, attraction of T lymphocytes and binding of leukocytes were more highly expressed in resistant animals at 7 dpi and in susceptible animals at 14 dpi indicating that resistant lambs appear to generate an earlier immune response

No SNP in differentially expressed genes were significantly associated with nematode resistance but SNP in 2 genes (*SLC30A2* and *ALB*) were suggestively associated

## 26 **Abstract**

27 Gastrointestinal nematodes are a serious cause of morbidity and mortality in grazing  
28 ruminants. The major ovine defence mechanism is acquired immunity, with protective  
29 immunity developing over time in response to infection. Nematode resistance varies  
30 both within and between breeds and is moderately heritable. A detailed understanding  
31 of the genes and mechanisms involved in protective immunity, and the factors that  
32 regulate this response, is required to aid both future breeding strategies and the  
33 development of effective and sustainable nematode control methods. The aim of this  
34 study was to compare the abomasal lymph node transcriptome of resistant and  
35 susceptible lambs in order to determine biological processes differentially expressed  
36 between resistant and susceptible individuals.

37 Scottish Blackface lambs, with divergent phenotypes for resistance, were  
38 challenged with 30,000 *Teladorsagia circumcincta* larvae (L3), and abomasal lymph  
39 nodes recovered at 7 and 14 days post-infection (dpi). High-throughput sequencing of  
40 cDNA from the abomasal lymph node was used to quantitatively sample the  
41 transcriptome with an average of 32 million reads per sample. A total of 194 and 144  
42 genes were differentially expressed between resistant and susceptible lambs at 7 and  
43 14 dpi respectively. Differentially expressed networks and biological processes were  
44 identified using Ingenuity Pathway Analysis. Genes involved in the inflammatory  
45 response, attraction of T lymphocytes and binding of leukocytes were more highly  
46 expressed in resistant animals at 7 dpi and in susceptible animals at 14 dpi indicating  
47 that resistant animals respond to infection earlier than susceptible animals. Twenty-  
48 four Single Nucleotide Polymorphisms (SNP) within 11 differentially expressed  
49 genes, were tested for association with gastrointestinal nematode resistance in the

50 Scottish Blackface lambs. Four SNP, in 2 genes (*SLC30A2* and *ALB*), were  
51 suggestively associated with faecal egg count.

52 In conclusion, a large number of genes were differentially expressed in the abomasal  
53 lymph node of resistant and susceptible lambs responding to gastrointestinal  
54 nematode challenge. Resistant Scottish Blackface lambs appear to generate an earlier  
55 immune response to *T. circumcineta*. In susceptible lambs this response appears to be  
56 delayed. SNP in 2 differentially expressed genes were suggestively associated with  
57 faecal egg count indicating that differentially expressed genes may be considered  
58 candidate loci for mediating nematode resistance.

## 59 **Keywords**

60 Scottish Blackface; Sheep; *Teladorsagia circumcineta*; Host-parasite interaction;  
61 Transcriptome.

62

## 63 1. Introduction

64 Gastrointestinal nematodes (GIN) are a serious cause of morbidity and mortality in  
65 grazing ruminants. Infected lambs have a reduced ability to absorb nutrients from the  
66 gastrointestinal tract, resulting in ill-thrift and, occasionally, death. Sub-clinical  
67 infection adds to the production losses in the form of reduced growth rate and light,  
68 under-finished carcasses. Anthelmintic drenching has been the method of choice for  
69 nematode control for the last 50 years; however, consumer concerns about food  
70 products from animals subjected to chemical treatment, combined with the inevitable  
71 evolution of anthelmintic resistant nematodes, means alternative, sustainable methods  
72 of parasitic nematode control are required.

73 Resistance to GIN is moderately heritable ( $h^2 \sim 0.3$ ) (Bishop and Morris,  
74 2007; Safari et al., 2005), therefore a sustainable method of nematode control is to  
75 select for genetically resistant individuals (Kemper et al., 2009). Selection using  
76 phenotypic traits, such as faecal egg count (FEC), requires prior exposure to GIN,  
77 whereas selection could be simplified through the identification of molecular markers.  
78 A detailed understanding of the genes and mechanisms involved in expressing a  
79 resistant phenotype and the factors that regulate this response would facilitate the  
80 identification of candidate markers.

81 Transcriptome analysis is a powerful method for the identification and  
82 quantification of genes expressed during a physiological perturbation. A number of  
83 previous studies have been undertaken to characterise the duodenal (Diez-Tascon et  
84 al., 2005; Keane et al., 2007; Keane et al., 2006), abomasal mucosal (Knight et al.,  
85 2011; Rowe et al., 2009) and lymph node and lymph fluid transcriptome (Andronicos  
86 et al., 2010; Gossner et al., 2013; Knight et al., 2010; MacKinnon et al., 2009) and

87 have led to the identification of genes and biological processes associated with the  
88 host response to GIN. As a result of these studies, a number of pathways have been  
89 postulated to be involved in the development of a resistant phenotype; however, no  
90 clear consensus has emerged. In Perendale selection lines, susceptible lambs were  
91 found to have increased intestinal mucosal expression of genes involved in the stress  
92 response, while resistant animals had increased expression of Major  
93 Histocompatibility Complex (MHC) class II, free radical scavenging and fatty acid  
94 metabolism genes (Keane et al., 2007; Keane et al., 2006). Transcriptomic analysis of  
95 the abomasal lymph node of Texel (resistant) and Suffolk (susceptible) lambs  
96 suggested that a balanced T helper (Th) cell response was associated with resistance  
97 (Ahmed, 2013). A comparison of the abomasal lymph node transcriptome of resistant  
98 and susceptible Scottish Blackface lambs also identified Th cell differentiation and  
99 polarisation as important in the development of a resistant phenotype (Gossner et al.,  
100 2013). Differences between the studies may reflect biological or technical variation in  
101 the experimental design such as tissue sampled, lamb age, nematode exposure history,  
102 the magnitude and species of the nematode challenge, or the transcriptomic platform.  
103 Alternatively, the differences may reflect physiological differences between breeds  
104 and individuals in how they develop resistance.

105 Resistance to GIN may be manifested by controlling worm burden, worm  
106 fecundity or a combination of both (Stear et al., 1996b). The majority of previous  
107 studies concerning gene expression in resistant and susceptible animals have been  
108 based on a model where resistant and susceptible animals differ significantly in worm  
109 burden (Ahmed, 2013; Gossner et al., 2013; Keane et al., 2007; Keane et al., 2006;  
110 Pernthaner et al., 2005; Zaros et al., 2014). However, the genes and pathways  
111 involved in regulating worm fecundity may differ from those involved in controlling

112 worm burden. We previously described a method to reliably identify repeatable  
113 within-breed variation in the ability of Scottish Blackface lambs to resist GIN  
114 infection (McRae et al., 2014). Resistant lambs were found to display lower FEC,  
115 lower worm fecundity and a higher level of anti-nematode IgA in both serum and  
116 mucosa. The physiological response to infection, as indicated by anti-nematode  
117 antibody levels, haematology and pepsinogen, was most pronounced at 7 and 14 days  
118 post-infection (dpi), although the phenotype (reduced FEC) was not yet evident at this  
119 time point.

120         The aim of the present study was to use high-throughput sequencing of cDNA  
121 to sample the transcriptome of the abomasal lymph node of Scottish Blackface lambs  
122 with divergent phenotypes for GIN resistance in order to identify genes and biological  
123 processes associated with the ability to express resistance. In this breed, repeatable  
124 differences among individuals in FEC, were positively associated with both increased  
125 worm burden and increased worm fecundity (McRae et al., 2014; Stear et al., 1995).  
126 Differentially expressed (DE) genes were considered candidate genes for mediating  
127 resistance and markers in these genes were tested for association with FEC in a larger  
128 Scottish Blackface cohort.

129



## 130 2. Materials and Methods

### 131 2.1 Ethical approval

132 The animal procedures described in this study were conducted under experimental  
133 licence from the Irish Department of Health in accordance with the Cruelty to  
134 Animals Act 1876 and the European Communities (Amendments of the Cruelty to  
135 Animals Act 1976) Regulations, 1994.

### 136 2.2 Animals

137 Purebred male Scottish Blackface lambs ( $n = 92$ ) were sourced from the flock at the  
138 Teagasc Hill Sheep Farm, Leenane, Co. Mayo in 2010. Lambs were managed from  
139 birth on improved lowland pasture where the major nematode species is *Teladorsagia*  
140 *circumcincta* (B. Good, unpublished data). All lambs received an oral benzimidazole  
141 anthelmintic treatment at 5 weeks of age to control *Nematodirus battus* infection.

142 Flock FEC (eggs per gram (epg)) was monitored weekly, from when lambs  
143 were approximately 8 weeks of age, using the FECPAK method (Fecpak). Eggs were  
144 distinguished as *Nematodirus* spp. (FEC<sub>NEM</sub>) and ‘other trichostrongyles’ spp  
145 (FEC<sub>OT</sub>). When FEC<sub>OT</sub> reached approximately 600 epg the lambs were individually  
146 sampled twice (FEC1A and FEC1B), 1 week apart, and FEC was determined for each  
147 sample using the modified McMaster method (Anon, 1986). FEC1A<sub>OT</sub> and FEC1B<sub>OT</sub>  
148 were averaged to give FEC1<sub>OT</sub>, the first phenotypic measurement of resistance.  
149 Following FEC1B the lambs were treated with a non-persistent macrocyclic lactone  
150 (ML, Oramec, Merial Animal Health Ltd) in accordance with manufacturer’s  
151 recommendations. Flock FEC was again monitored weekly until FEC<sub>OT</sub> reached  
152 approximately 600 epg when 2 more FEC (1 week apart) per individual were

153 completed (FEC2A and FEC2B) and the average computed to generate FEC2<sub>OT</sub>, the  
154 second phenotypic measurement of resistance. This cohort of animals constituted  
155 grazing group 1. This process was replicated in 2011 with male (n = 76) and female  
156 (n = 90) lambs in grazing groups 2 and 3, resulting in 2 phenotypic FEC  
157 measurements from 258 animals which were used for genetic association studies.

### 158 *2.3 Experimental infection*

159 For the animals born in 2010 (n = 92), individual animal values for  $\ln(\text{FEC}_{\text{OT}} + 25)$   
160 were used to identify the most resistant (subsequently known as “LowFEC”) and  
161 susceptible (subsequently known as “HighFEC”) lambs, using mixed model  
162 procedures (SAS® v9.1). Data for each natural infection (FEC1<sub>OT</sub> and FEC2<sub>OT</sub>) were  
163 analysed separately using a model that included rearing type (single or twin) and  
164 sample date (A or B sample of round) as fixed effects and animal as a random term.  
165 To get the selection differential for each animal, the estimated animal effect from each  
166 round was scaled by the standard error of prediction and averaged across rounds.  
167 These differentials were used to select 10 HighFEC and 10 LowFEC animals. Five  
168 sires were used in the flock; all 5 had progeny selected as HighFEC while 3 sires had  
169 progeny selected as LowFEC.

170 The selected lambs (n = 20) were cleared of helminth infection with a non-  
171 persistent ML (Oramec, Merial Animal Health Ltd), in accordance with  
172 manufacturer’s recommendations, and housed on straw bedding until slaughter, with  
173 free access to water and 600 g commercial lamb ration per head per day. All lambs  
174 were free of helminth infection for a minimum of 5 weeks prior to the experimental  
175 infection (based on FEC measurements on 3 consecutive days). All lambs received an  
176 oral challenge of approximately 30,000 *T. circumcincta* larvae (L3) at 31 (range 29 –

177 32) weeks of age. Lambs (5 per phenotype) were slaughtered at 7 and 14 dpi by  
178 electrical stunning followed immediately by exsanguination. The experimental design  
179 is summarised in Figure 1.

#### 180 *2.4 Phenotypic measurements and analysis*

181 Worm burden, female worm length, haematology variables and anti-nematode  
182 antibody level in both serum and abomasal mucosa were determined from samples  
183 taken at slaughter, as previously described (McRae et al., 2014). As all worms  
184 recovered at 7 dpi were immature and could not be sexed, worm length was measured  
185 at 14 dpi only. For one animal (LowFEC) all worms recovered at 14 dpi were  
186 immature, for the remaining animals the mean number of female worms measured  
187 was 77 (range 51 – 135). Log transformations were performed on worm burden data  
188 ( $\ln(X + 25)$ ) to stabilise the variance. Data were analysed using the Proc MIXED of  
189 SAS® (v9.1) to fit a model that had effects for phenotype (HighFEC or LowFEC), dpi  
190 and their interaction.

#### 191 *2.5 Tissue collection and RNA extraction*

192 Abomasal lymph node tissue, recovered at slaughter, was immediately cut into pieces,  
193 approximately 0.5 cm<sup>3</sup>, and submerged in 10 volumes of RNAlater® (Ambion). This  
194 was stored overnight at 4 °C followed by long-term storage at -80 °C. Total RNA was  
195 extracted from the tissue using Sigma TRI Reagent® (Sigma Aldrich, UK) according  
196 to the manufacturer's instructions. Small RNAs (<200 nucleotides) and residual  
197 genomic DNA were removed with the RNeasy Mini Kit (Qiagen, Germany) and an  
198 in-solution DNase digestion (RNase-free DNase set; Qiagen, Germany) according to  
199 the manufacturer's instructions. RNA quality was assessed using an Agilent® RNA  
200 6000 Nano Assay on a 2100 Bioanalyzer, and total RNA was quantified using the

201 NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, UK). All  
202 samples had a RIN value  $\geq 8.5$  and a 28S:18S ratio of  $>1.5$ .

### 203 *2.6 Library preparation and sequencing*

204 Illumina TruSeq™ libraries were prepared following the TruSeq™ RNA sample  
205 preparation v2 guide for total RNA (Part #15026495 Rev. B) with the following  
206 modifications: (i) the number of PCR cycles was reduced to 10 to minimise  
207 overcycling and (ii) the PCR products were purified using a Qiagen MinElute column  
208 rather than AMPure XP beads to avoid bead contamination. Libraries were visualised  
209 using an Agilent® DNA 1000 assay on a 2100 Bioanalyzer, and quantified using the  
210 Qubit® dsDNA BR assay (Invitrogen, UK) according to the manufacturer's  
211 instructions. The indexed cDNA libraries containing the specific Illumina TruSeq  
212 adapters were sent to GATC Biotech (Kontanz, Germany), where they were  
213 sequenced on an Illumina HiSeq2000 with 50 bp paired-end reads.

### 214 *2.7 Bioinformatics analysis*

215 Trim Galore (v0.3.3) (TrimGalore), which utilises Cutadapt (v1.2.1), was applied to  
216 the RNAseq reads using the default settings for paired-end data. Reads with a median  
217 Phred-scaled quality score below 20 were removed. Trimmed reads were mapped to  
218 the ovine genome (OARv3.1) (Jiang et al., 2014) using STAR (v2.3) (Dobin et al.,  
219 2013), with the Ensembl *Ovis aries* transcriptome annotation (release 74) supplied.  
220 Only uniquely mapped reads with a maximum of 2 mismatches to the reference  
221 genome were retained for expression analysis. The mapped reads were used to  
222 estimate raw counts per gene using HTSeq (v0.5.3p3) (HTSeq) with the union overlap  
223 resolution mode. The between group analysis (BGA) function from the Bioconductor  
224 package MADE4 (v1.42.0) (Culhane et al., 2005) was used to visualize the samples

225 based on transcriptomic profiles. The Bioconductor package EdgeR (v3.0.8)  
226 (Robinson et al., 2010) was run within R software (v3.0.2) to analyse differential  
227 expression of read counts. Comparisons were made between HighFEC and LowFEC  
228 animals at either 7 or 14 dpi, or within phenotype over time between 7 and 14 dpi.  
229 Low expression tags were filtered, keeping only genes that achieved at least 1 count  
230 per million in at least 5 samples. Trimmed mean of M-values normalisation  
231 (Robinson and Oshlack, 2010) was used to account for differences in RNA  
232 composition between samples. Data were analysed using both common and tagwise  
233 dispersions. To account for multiple testing, genes were filtered using a Benjamini  
234 and Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995) of 0.1 or  
235 0.05 for tagwise and common dispersion analyses, respectively. All genes identified  
236 as DE using common dispersion estimates were included in pathway analysis. Pre-  
237 calculated 1-to-1 Human orthologs (Ensembl release 74) were obtained using  
238 Ensembl's Biomart tool (Biomart). Ingenuity® Systems Pathway Analysis (IPA;  
239 Ingenuity Systems, Redwood City, CA, USA; v18841524) was used to identify the  
240 top networks, canonical pathways, diseases and functions from DE genes.

#### 241 *2.8 cDNA synthesis and RT-qPCR*

242 First-strand cDNA synthesis from 1.5 µg of total RNA was carried out using the High  
243 Capacity cDNA reverse transcription kit (Applied Biosystems) according to the  
244 manufacturer's instructions. Quantitative PCR was performed in triplicate in 20 µl  
245 reactions with 10 µl 2 X Fast Sybr Green Master Mix (Applied Biosystems), 1 µl of  
246 forward and reverse primer (300 nM each) and 1 µl of cDNA and reverse  
247 transcriptase in an Applied Biosystems Fast 7500 instrument. Reactions were  
248 denatured at 95 °C for 20 s then cycled 40 times at 95 °C for 3 s and 60 °C for 30 s.

249 A dissociation analysis was carried out at the end of the reaction to ensure a single  
250 product was generated. Reaction efficiencies for each primer pair were calculated  
251 using a 1:2 dilution series over 5 points and only those between 0.9 and 1.1 were  
252 retained for analysis. Variation in PCR efficiency was corrected for and expression  
253 levels were normalised to that of the reference genes *GAPDH*, *H3F3A* and *YWHAZ*  
254 using GenEx (v.3.6.170). RNASeq and qPCR data were compared by calculating the  
255 correlation coefficient for each gene. Primer sequences and correlations can be found  
256 in Supplementary file 1.

### 257 *2.9 SNP genotyping and analysis*

258 Whole blood was collected from 258 Scottish Blackface lambs for which FEC  
259 phenotypes were recorded (Figure 1). Genomic DNA was extracted from 9 mL of  
260 blood using the high salt method (Montgomery and Sise, 1990). In some cases blood  
261 was frozen, which results in lysis of the white blood cells; for these samples genomic  
262 DNA was extracted from 400  $\mu$ L of blood using the Maxwell® 16 Research System  
263 (Promega, UK), according to the manufacturer's instructions. DNA could not be  
264 extracted from 21 samples. Consequently, DNA was available for 237 Scottish  
265 Blackface lambs.

266 SNP information derived from the Ovine Infinium® HD SNP BeadChip was  
267 extracted for 11 of the 13 genes DE between HighFEC and LowFEC animals using  
268 tagwise dispersion (Supplementary File 2). The remaining 2 genes were on scaffolds  
269 or contigs, so their genomic location was unknown. These were discarded from  
270 further analysis. SNP were further classified as in coding or non-coding regions. SNP  
271 were prioritised for genotyping using the following criteria: 1) at least one SNP in

272 each gene of interest, 2) SNP in a coding region of the gene of interest, and 3) SNP  
273 polymorphic in the Scottish Blackface RNA-Seq data set.

274 Twenty-four SNP in 11 DE genes were genotyped in 237 Scottish Blackface  
275 animals by Sequenom GmbH (Germany) using the MassARRAY® system. All  
276 genotyped SNP had a minor allele frequency >0.02 and a call rate >90%, and as a  
277 consequence were included in downstream analysis. No SNP deviated from Hardy  
278 Weinberg equilibrium.

279 SNP effects for FEC traits (FEC1 and FEC2 for *Nematodirus* and for ‘other  
280 trichostrongyles’) were estimated in ASReml (Gilmour et al., 2009) by fitting the  
281 SNP, one at a time, as fixed effects. The model also included sex and grazing group as  
282 fixed effects. After Bonferroni correction, the significance level thresholds were  $P <$   
283  $2.08 \times 10^{-3}$  and  $P < 4.17 \times 10^{-2}$  for genome-wide significance ( $P < 0.05$ ) and  
284 suggestive significance (that is, one false positive per genome scan), respectively.

## 285 3. Results

### 286 3.1 Phenotypic measurements

287 Scottish Blackface lambs at the extremes of the distribution of parasite resistance and  
288 susceptibility were identified. The selected lambs, chosen for high ( $n = 10$ ) or low ( $n$   
289  $= 10$ ) FEC had an average (range) FEC of 1,373 (1,000-1,661) and 216 (85-267) epg,  
290 respectively (Figure 2A). This selection method has previously been demonstrated to  
291 reliably identify resistant and susceptible individuals (McRae et al., 2014) and the  
292 difference in selection differential between High and LowFEC lambs was 3.18  
293 standard deviation units.

294 After a controlled challenge with *T. circumcincta* there was no significant  
295 difference between HighFEC and LowFEC animals in worm burden, at either 7 or 14  
296 dpi. This was expected, as resistance in this flock primarily manifests as reduced  
297 worm fecundity in resistant individuals, although worm burden may also be a  
298 contributing factor (McRae et al., 2014). In total, 75% of worms recovered from the  
299 abomasum of HighFEC lambs had developed to the L5 stage by 14 dpi with the  
300 remainder at L4. This compared to 54% L5 in the LowFEC lambs; however, the  
301 difference in adults as a proportion of total worms was not significant (Mann-Whitney  
302 U test,  $P = 0.15$ ). Excluding the LowFEC lamb which carried only immature larvae,  
303 the mean (s.e) length of female *T. circumcincta* in HighFEC and LowFEC animals  
304 was 6.38 (0.25) and 5.59 (0.27) mm, respectively ( $P = 0.07$ ). In agreement with our  
305 previous study, the number of circulating basophils was higher in LowFEC animals in  
306 comparison to HighFEC animals (Figure 2B;  $P = 0.03$ ). Phenotypic differences for  
307 other haematology measurements were not significant. The weight of the abomasal  
308 lymph nodes was also higher in LowFEC lambs than in HighFEC lambs (means of



309 4.86 g and 3.65 g respectively;  $P = 0.048$ ) while the level of IgA specific for *T.*  
310 *circumcincta* was significantly higher at 7 dpi in both serum (Figure 2C;  $P = 0.002$ )  
311 and mucosa (Figure 2D;  $P = 0.002$ ).

### 312 *3.2 RNA Sequencing*

313 High throughput sequencing of the 20 ovine abomasal lymph node RNA samples  
314 resulted in 790,415,623 paired-end reads. Approximately 1% of reads were excluded  
315 from downstream analysis due to low quality (Phred score  $< 20$ ). An average of  
316 32,573,191 reads per sample (83%) mapped to a unique region of the ovine genome,  
317 in excess of the 20 to 25 million mappable reads recommended by the ENCODE  
318 Consortium for gene expression analysis (ENCODE). Of the uniquely mapped reads,  
319 an average of 18,040,721 (55%) aligned to a known feature and an average of  
320 14,205,416 (44%) did not align to a known gene. The remaining 1% of reads could  
321 have been assigned to more than one feature, and were thus classified as ambiguous.  
322 A between-group analysis (BGA) plot, based on correspondence analysis of overall  
323 gene expression values, is shown in Supplementary File 3.

### 324 *3.3 Differential gene expression*

325 The number of DE genes detected is shown in Table 1. Genes detected as DE using  
326 tagwise dispersion are reported in Table 2 while the complete list of genes identified  
327 as DE using common dispersion are in Supplementary File 4. Tagwise dispersion  
328 ranks genes more highly when counts are consistent between individuals within a  
329 group, while common dispersion is more likely to rank genes as DE even when they  
330 are highly variable within a group.

### 331 *3.4 Genes DE between HighFEC and LowFEC animals*

332 A total of 13 genes were identified as DE between HighFEC and LowFEC animals  
333 using tagwise dispersion - 7 DE at 7 dpi and 6 DE at 14 dpi (Table 1); 5 (38%) were  
334 novel protein coding genes and 3 (23%) had been identified in previous studies (Table  
335 2). These percentages are in line with those identified using the common dispersion  
336 analysis where 126 DE genes (37%) were novel protein-coding genes and 91 DE  
337 genes (27%) had been identified in previous studies. Human 1-to-1 orthologs were  
338 found for 60% of the common dispersion DE genes (Supplementary File 4) and these  
339 orthologs were used as input for IPA analysis.

340 The top network for genes DE between HighFEC and LowFEC animals at 7  
341 dpi was 'Cell-To-Cell Signalling and Interaction, Cellular Movement, Immune Cell  
342 Trafficking' while the top network at 14 dpi related to 'Cellular Assembly and  
343 Organisation, Lipid Metabolism and Small Molecule Biochemistry' (Table 3). Two  
344 canonical pathways were also significant in the HighFEC vs LowFEC comparison at  
345 7 dpi (Table 4). These pathways included a number of chemokine (C-X-C motif)  
346 ligands (CCL and CXCL) and receptors (CCR) that were more highly expressed in  
347 LowFEC animals at 7 dpi.

### 348 *3.5 Immune response over time*

349 Changes in the immune response to GIN over time post-infection were examined  
350 within phenotype (HighFEC or LowFEC) by looking at the transcriptional profiles of  
351 animals slaughtered at 7 dpi compared to those slaughtered at 14 dpi. A total of 21 DE  
352 genes were detected using tagwise dispersion, 7 were DE in HighFEC animals and 14  
353 in LowFEC animals. Of these genes, 13 (61%) were novel protein coding genes while  
354 5 (24%) had been identified in previous studies (Table 2). Of the 387 DE genes found  
355 using common dispersion estimates, 138 (36%) were novel protein-coding genes and  
356 110 (28%) had been identified in previous studies.

357 The networks identified by IPA using genes DE in HighFEC or LowFEC  
358 animals over time are given in Table 3. The network ‘Cell-To-Cell Signalling and  
359 Interaction, Cellular Movement, Immune Cell Trafficking’ was the second highest  
360 network for LowFEC animals. Six canonical pathways were significantly DE between  
361 7 and 14 dpi in LowFEC animals (Table 4). This was once again primarily due to the  
362 increased expression of a number of chemokine ligands and receptors in the LowFEC  
363 animals at 7 dpi.

### 364 *3.6 Visualisation across multiple analyses using Ingenuity Pathway Analysis*

365 The Comparison Analysis in IPA was used to compare results from the HighFEC vs  
366 LowFEC analyses at both 7 and 14 dpi. The top diseases and biological functions  
367 (Figure 3) and the top upstream regulators (Figure 4) were compared. At 7 dpi,  
368 functions including ‘inflammatory response’, ‘attraction of T lymphocytes’ and  
369 ‘synthesis of reactive oxygen species’ were increased in LowFEC animals (Figure 3).  
370 In contrast, genes related to ‘cancer’ were more highly expressed in HighFEC  
371 animals. By 14 dpi, genes relating to ‘inflammatory response’ and ‘synthesis of  
372 reactive oxygen species’ were more highly expressed in HighFEC lambs. At 7 dpi,  
373 expression of genes downstream from Tumour Necrosis Factor (TNF) and a number  
374 of members of the interferon (IFN) group of signalling proteins were increased in  
375 LowFEC animals. Genes downstream of these cytokines were not increased in  
376 HighFEC animals until 14 dpi.

### 377 *3.7 Validation of expression*

378 In order to validate the results of the RNA sequencing, a panel of 8 DE genes were  
379 chosen for validation by RT-qPCR. These were *ALB*, *ASZI*, *CXCL11*, *GABBR2*,  
380 *GSDMA*, *LYVE1*, *MF12* and *STPG1*. The gene expression pattern, in terms of  
381 direction and magnitude of 7 of the 8 genes was reproducible by qPCR

382 (Supplementary file 1). For one gene (*LYVE1*) the direction of change was the same  
383 for the RNASeq and qPCR but the correlation was not significant.

### 384 3.8 SNP analysis

385 The results of an association analysis between SNP in DE genes and FEC are shown  
386 in Table 5. The results yielded a range of significance for each individual SNP;  
387 however, no single SNP reached significance after Bonferroni correction for multiple  
388 comparisons (Abdi, 2007). Four SNP were, however, suggestively associated with  
389  $FEC_{2OT}$ : 2 coding SNP within *SLC30A2*, and 2 non-coding SNP within *ALB*. Both  
390 SNP within *SLC30A2* code for missense variants; however, SNP OAR2\_239929582  
391 is classified as 'tolerated' by the SIFT algorithm (score = 1) (Kumar et al., 2009),  
392 whereas SNP OAR2\_239931409 is classified as 'deleterious' (score = 0). The latter  
393 SNP has a minor allele (A) frequency of 0.03 within the set of individuals studied,  
394 with only 14 out of 237 animals heterozygous and no animal homozygous for this  
395 allele. However, the allele was not out of Hardy-Weinberg equilibrium ( $P = 0.90$ ).

396

## 397 **4. Discussion**

### 398 *4.1 Phenotypic differences between resistant and susceptible lambs*

399 We have previously described a method to reliably identify repeatable within-breed  
400 variation in the ability of Scottish Blackface lambs to resist GIN infection (McRae et  
401 al., 2014). Resistant lambs had lower FEC, primarily due to lower worm fecundity.  
402 This method was used to generate the HighFEC and LowFEC lambs for the present  
403 study. As the host response to infection was most pronounced in the first 2 weeks of  
404 infection (McRae et al., 2014), the lambs were euthanized before the infection became  
405 patent meaning we could not measure FEC in the lambs; however, resistant lambs  
406 tended to have more immature larvae and shorter worms in addition to a higher  
407 number of circulating basophils. This is consistent with our previous study, and  
408 induction of basophils is known to be a feature of the anti-helminth response (Allen  
409 and Maizels, 2011). Recent studies indicate that basophils play a role in regulating  
410 acquired immunity by initiating Th2 cell differentiation, as well as in amplifying the  
411 humoral memory response (Karasuyama et al., 2011). The weight of the abomasal  
412 lymph node was also higher in resistant lambs, which may reflect increased cellular  
413 recruitment to the lymph node.

### 414 *4.2 Response to GIN infection in phenotypes divergent for resistance*

415 At 7 dpi *ALB* was more highly expressed in the HighFEC group. This gene has been  
416 associated with GIN infection in 4 separate studies. Serum albumin, the main protein  
417 of plasma, is a carrier protein for steroids, fatty acids and thyroid hormones, and  
418 functions as a regulator of the colloidal osmotic pressure of blood. *ALB* is a negative  
419 acute phase protein, which has been shown to decline in response to internal

420 challenges, such as infection, inflammation or stress (Murata et al., 2004). *ALB* has  
421 been reported to be more highly expressed in the duodenum of helminth-naïve  
422 genetically-resistant animals compared to susceptible individuals (Keane et al., 2006)  
423 and to decline in the abomasum of sheep during the course of repeated truncated  
424 immunising infections with *Trichostrongylus colubriformis* larvae (Knight et al.,  
425 2010). *ALB* levels were also increased in the abomasal mucosa of resistant selection-  
426 line animals compared to their susceptible counterparts 3 days after experimental  
427 challenge with *Haemonchus contortus* (Nagaraj et al., 2012). In a separate study on  
428 changes in abomasal protein expression following trickle infection with *T.*  
429 *circumcincta*, *ALB* was relatively highly expressed in the mucosa of helminth-naïve  
430 animals in comparison to both their immune and immune-waning counterparts  
431 (Pemberton et al., 2012). In the same study serum albumin was significantly lower in  
432 GIN immune sheep compared to naïve controls. Pemberton et al. hypothesised that  
433 albumin may be constitutively released into the gastric mucus and may therefore play  
434 an innate protective role. In this study, the response to GIN infection in the HighFEC  
435 animals appears to be similar to that previously observed in naïve animals, suggesting  
436 that the HighFEC animals may not be generating a timely immune response to GIN  
437 infection. Expression of the Solute Carrier Family 30 (Zinc Transporter), Member 2  
438 (*SLC30A2*) was also increased in HighFEC animals. *SLC30A2* is involved in the  
439 essential maintenance of cellular  $Zn^{2+}$  (Huang and Tepasamordech, 2013). Studies  
440 using zinc-deficient nematode-infected mouse models have shown that parasites are  
441 better able to survive in zinc-deficient hosts compared to well-nourished hosts, with  
442 the function of T cells and antigen-presenting cells impaired by zinc deficiency (Scott  
443 and Koski, 2000).

444 At 14 dpi, 6 genes were identified as DE between HighFEC and LowFEC animals  
445 based on tagwise dispersion. Of note is *ECTL2* (epithelial cell transforming sequence  
446 2 oncogene-like), expression of which was higher in LowFEC than HighFEC animals.  
447 Recurrent somatic mutations in *ECT2L* have been associated with early T-cell  
448 precursor acute lymphoblastic leukaemia (Zhang et al., 2012). In contrast to the  
449 findings from this study, expression of *ECT2L* has been reported to be increased in  
450 Suffolk (relatively susceptible) when compared to Texel (relatively resistant) lambs  
451 over the course of a controlled challenge with *T. circumcincta* (Ahmed, 2013) and the  
452 reason for these opposing results remains to be resolved. Expression of a putative  
453 MHC class I antigen (ENSOARG00000001701) was also increased in LowFEC  
454 animals. Class I antigens have previously been associated with reduced FEC in  
455 Scottish Blackface lambs (Buitkamp et al., 1996; Stear et al., 1996a), and with genetic  
456 susceptibility in naïve sheep (Keane et al., 2006).

457 *4.3 Temporal changes in gene expression in the abomasal lymph node of lambs*  
458 *challenged with T. circumcincta*

459 In the HighFEC animals, expression of *ALB* and *MX2* (Myxovirus resistance 2) were  
460 significantly higher at 7 dpi compared to 14 dpi. The down-regulation of *ALB* by 14  
461 dpi was by far the most significant change, with a fold change of 166 (FDR =  $8.5 \times 10^{-24}$ ).  
462 Expression of *MX2* is strongly induced by IFN- $\gamma$  (Kane et al., 2013; Melén et al.,  
463 1996), and the *MX2* protein shows antiviral activity (Sasaki et al., 2014). Despite the  
464 association between this gene and viral infections, it has previously been shown to be  
465 increased at both the mRNA and protein levels in GIN susceptible animals (Ahmed,  
466 2013; Nagaraj et al., 2012). The genes *LYVE1* (Lymphatic vessel endothelial  
467 hyaluronan receptor 1) and *CHI3L2* (chitinase 3-like 2) were more highly expressed

468 in HighFEC animals at 14 compared to 7 dpi. *LYVE1* is a major receptor for  
469 hyaluronan on the lymph vessel wall (Banerji et al., 1999). Hyaluronan is an abundant  
470 component of skin and mesenchymal tissues, where it facilitates cell migration during  
471 wound healing, inflammation, and embryonic morphogenesis. Chitinase-like proteins  
472 such as *CHI3L2* also have a role in inflammation, tissue remodelling and injury (Lee  
473 et al., 2011). Increased expression of *CHI3L2* has been observed in the abomasum of  
474 18 and 21 week old steers exposed to *Ostertagia ostertagi* and the abomasal lymph  
475 node of resistant and susceptible Scottish Blackface lambs infected with *T.*  
476 *circumcincta* in comparison to sham-infected controls (Gossner et al., 2013). In  
477 human macrophages, *CHI3L2* has been found to be up-regulated by IL-4 and TGF-  
478 (Gratchev et al., 2008). The increased expression of these genes indicates that  
479 HighFEC animals are mounting an inflammatory response at approximately 14 dpi.

480         Like their HighFEC counterparts, LowFEC animals expressed higher levels of  
481 *MX2* at 7 dpi than at 14 dpi. While all of the DE genes that were increased in  
482 LowFEC animals at 14 dpi are currently annotated as “novel protein coding” genes in  
483 Ensembl (Ensembl Release 78), several are orthologous to *Bos taurus* and *Homo*  
484 *sapiens* MHC genes. ENSOARG000000016098 is a 1-to-1 ortholog of *BOLA-DRB3*.  
485 ENSOARG000000002985 and ENSOARG000000015866 are one-to-many orthologs of  
486 *BOLA-DQA1* and *BOLA-DQB*, respectively. ENSOARG000000010572 is a many-to-  
487 many ortholog of multiple human MHC class I genes. The MHC is involved in the  
488 induction and regulation of the immune response, and associations between the MHC  
489 genes and both resistance and susceptibility to GIN have been found in multiple  
490 studies reviewed by Venturina et al. (2013) (Venturina et al., 2013).



491 *4.4 Pathway analysis*

492 Biological processes involving ‘inflammatory response’, ‘attraction of T  
493 lymphocytes’ and ‘binding of leucocytes’ were more highly expressed in resistant  
494 animals at 7 dpi. Therefore, these animals appear to be generating an earlier immune  
495 response to infection than susceptible animals, through an increase in migration of  
496 cells involved in the response to pathogens. Increased cellular recruitment to the  
497 lymph node in resistant animals was also indicated by the increased weight of the  
498 abomasal lymph node tissue. Upstream regulators of the genes involved in these  
499 processes included TNF, IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$ . The pro-inflammatory cytokine  
500 TNF is produced in the gastric mucosa during inflammation and can promote  
501 gastrointestinal homeostasis, although excess TNF production can contribute to  
502 gastric mucosal inflammation and injury (Wallace and Ma, 2001), and has been  
503 implicated in the intestinal pathology of nematode infections (Lawrence et al., 1998).  
504 Following deliberate infection with *T. circumcincta*, elevated *TNF* expression has  
505 been observed in both the abomasal lymph node of challenged compared to  
506 unchallenged lambs at 5 days post infection (Craig et al., 2007), and in the abomasal  
507 mucosa of *DRB1\*1101* carrier lambs compared to their non-carrier counterparts at 3  
508 days post infection (Hassan et al., 2011). The type I IFNs (  $\alpha$  and  $\beta$  ) can be produced  
509 by almost every cell type, including leukocytes. While IFNs are best known for their  
510 role in the cellular response to viral infections, they also possess immunomodulatory  
511 activities (González-Navajas et al., 2012). The connection between type I IFNs and  
512 several human autoimmune and inflammatory disorders is well known, with several  
513 inflammatory syndromes shown to benefit from the administration of type I IFNs  
514 (González-Navajas et al., 2012). While the antiviral capability of type III IFNs,  
515 including IFN- $\omega$ , is not as highly studied as that of the type I IFNs, they have their

516 own profile of immunomodulatory functions, specifically at the immune/epithelial  
517 interface (Gallagher et al., 2010).

518 Taken together, this indicates that the LowFEC animals are mounting a  
519 response that involves inflammatory cytokines, immune cell recruitment and the  
520 synthesis of reactive oxygen species by 7 dpi. By approximately 14 dpi, genes  
521 downstream from these regulators were no longer up-regulated in the LowFEC  
522 animals; however, they were activated in the HighFEC animals. This indicates that the  
523 susceptible animals may have a delayed immune response to infection compared to  
524 resistant lambs.

#### 525 *4.5 Comparison with other studies*

526 Intestinal smooth muscle contractility (Diez-Tascon et al., 2005), pathogen  
527 recognition, via MHC class II molecules, and T helper cell polarisation (Gill et al.,  
528 2000; Hassan et al., 2011; Hein et al., 2004) have all been previously suggested as  
529 processes that control the response to GIN (Keane et al., 2007; Pernthaner et al.,  
530 2005). Of these, T helper cell polarisation has probably the most support (Hassan et  
531 al., 2011; Muñoz-Guzmán et al., 2012; Pemberton et al., 2011). However, these  
532 pathways were primarily identified in studies that compared animals that differed in  
533 worm burden. Worm burden may influence gene expression directly and some of the  
534 biological processes detected may therefore be a consequence of the differences in  
535 worm burden rather than the cause. We examined gene expression in a breed that  
536 primarily manifests resistance by regulating worm fecundity, although regulation of  
537 worm burden may also contribute (McRae et al., 2014; Stear et al., 1995; Stear et al.,  
538 1996b). Despite the differences between the present study and previous studies, many  
539 of the DE genes observed in this study were previously reported in other studies

540 examining resistance to GIN in sheep via regulation of worm burden, including those  
541 investigating the transcriptome (Ahmed, 2013; Gossner et al., 2013) and the proteome  
542 (Nagaraj et al., 2012). Only 1 DE gene (*IL13*) was common with Salle et al (2014), a  
543 study which examined gene expression differences in lambs that controlled worm  
544 fecundity, although this may be due to the fact that they examined a limited number of  
545 genes, at a different time-point, and in lambs infected with a different parasite species  
546 (Salle et al., 2014). A systems genetics study based on data from multiple QTL and  
547 gene expression studies, led to the identification of common pathways between genes  
548 in QTL associated with genetic resistance to GIN in various populations (Sayre and  
549 Harris, 2012). This suggests that, despite the difference mechanisms of GIN resistance  
550 between breeds, there may be some common pathways associated with GIN resistance  
551 across breeds of sheep and GIN species.

552         The number of genes identified as DE using tagwise dispersion estimates was  
553 relatively low when compared to other RNA-Seq studies of the same tissue (Ahmed,  
554 2013; Gossner et al., 2013; Pemberton et al., 2011). However, it must be noted that  
555 the methods for identifying DE genes varied between the studies. Gossner et al. used  
556 the Limma package, which was developed and optimised for array data, while Ahmed  
557 used EdgeR with common dispersion estimates. When using common dispersion  
558 estimates in this study, the number of DE genes was similar to that reported by  
559 Ahmed. Another possibility is that there may be variation among animals in how they  
560 manifest resistance and the timing of manifestation. In this scenario common  
561 dispersion may be a more appropriate technique for identifying differential  
562 expression. Indeed, a larger number of significantly DE genes were found using this  
563 method.

#### 564 4.6 SNP analysis

565 We hypothesised that markers in DE genes may be associated with resistance. The  
566 goal was not to identify causative mutations *per se* but to test markers which may be  
567 in linkage disequilibrium with causative mutations. As DE genes detected by tagwise  
568 dispersion were more consistent between animals within a group, markers in these  
569 genes were tested for association with FEC. While a number of SNP in *ALB* and  
570 *SLC30A2* were suggestively associated with FEC<sub>2OT</sub> they were not significant after  
571 correction for multiple testing. The present study is limited by the number of animals  
572 (237) available for association analysis. Previous studies have involved between 752  
573 and 1275 individuals (Riggio et al., 2013; Sall et al., 2012) for GWAS. Future work  
574 on validating the SNP of interest from this study would require a larger number of  
575 Scottish Blackface animals. As noted in reports on multiple studies, host resistance to  
576 GIN appears to be mediated by many genes, each with a relatively small effect  
577 (Kemper et al., 2011; Riggio et al., 2014). The candidate gene approach is therefore  
578 unlikely to capture all of the variation underlying known phenotypic differences;  
579 future work on identifying SNP to be used in selection programmes will likely focus  
580 on utilising a panel of SNP in addition to searching for individual causative mutations.  
581 Despite these limitations, suggestive associations between FEC<sub>2OT</sub> and multiple SNP  
582 within the same gene indicate that genotyping SNP within DE genes may be a valid  
583 way of discovering polymorphisms associated with GIN resistance.

## 584 5. Conclusions

585 Transcriptional profiling of the abomasal lymph node during a controlled challenge  
586 with *T. circumcineta* indicated that in resistant (LowFEC) Scottish Blackface lambs,  
587 pathways relating to the inflammatory response, migration of T lymphocytes and

588 synthesis of reactive oxygen species were more highly expressed at 7 dpi. In their  
589 susceptible (HighFEC) counterparts this response was delayed until ~14 dpi  
590 indicating that resistant animals are generating an earlier immune response to *T.*  
591 *circumcincta*. SNP in 2 DE genes (*SLC30A2* and *ALB*), were suggestively associated  
592 with FEC.  
593

594 **Conflict of Interest Statement**

595 The authors declare that they have no conflict of interests.

596

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606

626 **Figures**

627 **Figure 1 – Experimental design.**

628 Purebred Scottish Blackface lambs were used in the study. Flock faecal count (FEC)  
629 was monitored weekly from when lambs were approximately 8 weeks of age. Once  
630 this reached ~600 eggs per gram (epg) lambs were individually sampled twice, 1  
631 week apart, to give FEC1. Lambs were then dosed with an anthelmintic, and returned  
632 to pasture where the process was repeated for the second natural infection (FEC2).  
633 Blood was collected from all animals for DNA extraction. FEC1 and FEC2 values for  
634 other trichostrongyles from the cohort of 92 2010-born male Scottish Blackface  
635 lambs<sup>a</sup> were used to select the 10 most resistant (LowFEC) and 10 most susceptible  
636 (HighFEC) lambs for gene expression studies. Phenotypic measurement was repeated  
637 in 2011 on both male and female lambs<sup>b</sup> (n = 166), resulting in FEC1 and FEC2  
638 measurements from 258 lambs<sup>a,b</sup>, which were used for the genetic association study.

639

640



641 **Figure 2 – Response of susceptible (HighFEC) and resistant (LowFEC) lambs**  
642 **to natural and artificial challenge GIN infection.**

643 Mean ( $\pm$ s.e) faecal egg count of HighFEC (n = 10) and LowFEC (n = 10) individuals

644 during natural challenge (A). Mean ( $\pm$ s.e) circulating basophil numbers (B), Mean

645 ( $\pm$ s.e) serum anti-*Teladorsagia circumcincta* IgA (C) and Mean ( $\pm$ s.e) mucosa anti-*T.*

646 *circumcincta* IgA (D) in HighFEC and LowFEC animals during a controlled

647 challenge with  $3 \times 10^4$  *T. circumcincta* L3 larvae.

648

649 **Figure 3 - Top 20 DE diseases and biological functions.**

650 Heat map of the top 20 IPA-derived diseases and biological functions from genes DE

651 between HighFEC and LowFEC animals at 7 or 14 dpi, sorted by P value.

652

653 **Figure 4 - Top 10 IPA-derived upstream regulators.**  
654 Heat map of the top 10 IPA-derived upstream regulators from genes DE between  
655 HighFEC and LowFEC animals at 7 or 14 dpi, sorted by P value.  
656

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**Table 1 – Number of genes differentially expressed using common and tagwise dispersion analyses**

<b>Comparison</b>		<b>Common dispersion</b>	<b>Tagwise dispersion</b>
		<i>FDR &lt;0.05</i>	<i>FDR &lt;0.1</i>
<b>HighFEC vs LowFEC</b>	7 dpi	194	7
	14 dpi	144	6
<b>7 vs 14 dpi</b>	HighFEC	224	7
	LowFEC	163	14

FDR = False discovery rate.

**Table 2 – Genes identified as differentially expressed in abomasal lymph node using tagwise dispersion**

Comparison	Up in	Ensembl ID	Gene	Description	FC <sup>1</sup>	CPM <sup>2</sup>	FDR <sup>3</sup>
7 dpi	HighFEC	ENSOARG00000013782	<i>ALB</i>	Albumin	166.3	1.7	<0.01
		ENSOARG00000020224	<i>COL9A2</i>	Collagen, type IX, alpha 2	6.4	17.1	0.01
		ENSOARG00000011275	-	Uncharacterized protein	4.2	1.7	0.01
		ENSOARG00000003000	-	Uncharacterized protein	0.6	12.5	0.01
		ENSOARG00000005312	-	-	0.7	35.2	0.01
		ENSOARG00000001778	<i>OSBPL5</i>	Oxysterol binding protein-like 5	0.4	2.1	0.02
		ENSOARG00000005490	<i>SLC30A2</i>	Solute carrier family 30 (zinc transporter), member 2	0.5	46.1	0.03
14 dpi	HighFEC	ENSOARG00000020373	<i>MF12</i>	Antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	3.7	19.3	<0.01
		ENSOARG00000011529	<i>ZFR2</i>	Zinc finger RNA binding protein 2	2.5	99.6	0.05
	LowFEC	ENSOARG00000005549	-	40S ribosomal protein S3a	2.9	9.7	<0.01
		ENSOARG00000001701	-	Uncharacterized protein	2.1	1136.4	<0.01
		ENSOARG00000004858	-	Uncharacterized protein	3.7	6.6	0.04
		ENSOARG00000000928	<i>ECT2L</i>	Epithelial cell transforming 2 like	0.2	166.9	0.04
HighFEC	7 dpi	ENSOARG00000013782	<i>ALB</i>	Albumin	0.2	202.5	<0.01
		ENSOARG00000010231	<i>MX2</i>	Myxovirus (influenza virus) resistance 2 (mouse)	0.4	59.5	0.04
		ENSOARG00000004858	-	Uncharacterized protein	0.3	74.8	0.08
	14 dpi	ENSOARG00000010111	-	Uncharacterized protein	0.4	1.7	0.03
		ENSOARG00000011072	<i>LYVE1</i>	Lymphatic vessel endothelial hyaluronan receptor 1	0.3	28.1	0.07
		ENSOARG00000019641	<i>LSAMP</i>	Limbic system-associated membrane protein	0.7	151.9	0.08
		ENSOARG00000019517	<i>CHI3L2</i>	Chitinase 3-like 2	0.5	38.4	0.08
		ENSOARG000000010231	<i>MX2</i>	Myxovirus (influenza virus) resistance 2 (mouse)	0.5	8.2	<0.01
LowFEC	7 dpi	ENSOARG00000020801	-	Uncharacterized protein	0.2	1.9	0.02
		ENSOARG00000020811	-	Uncharacterized protein	0.4	3.3	0.02
		ENSOARG00000020789	-	Uncharacterized protein	0.1	3.8	0.03
		ENSOARG00000020194	<i>MAB21L3</i>	Mab-21-like 3 ( <i>C. elegans</i> )	0.4	5.4	0.04
		ENSOARG00000016098	-	Uncharacterized protein	0.6	12.8	<0.01
	14 dpi	ENSOARG00000002985	-	Uncharacterized protein	0.4	5.2	0.02
		ENSOARG00000010572	-	Uncharacterized protein	0.4	2.1	0.03
		ENSOARG00000020792	-	Uncharacterized protein	0.3	2.0	0.03
		ENSOARG00000001720	-	Uncharacterized protein	0.3	4.4	0.03

ENSOARG00000000058	-	Uncharacterized protein	4.7	63.6	0.03
ENSOARG00000015866	-	Uncharacterized protein	1.8	352.1	0.03
ENSOARG00000001279	-	Galectin-14 (LOC443162)	3.6	1.6	0.09
ENSOARG000000005126	-	Uncharacterized protein	5.2	3.6	0.10

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<sup>1</sup>FC = fold-change. <sup>2</sup>CPM = counts-per-million. <sup>3</sup>FDR = false discovery rate.

**Table 3 – Top 5 networks for each comparison identified by Ingenuity Pathway Analysis using DE genes.**

Comparison	Network	IPA Score <sup>1</sup>	Genes
<b>7 dpi HighFEC vs LowFEC</b>	Cell-To-Cell Signaling and Interaction, Cellular Movement, Immune Cell Trafficking	32	17
	Amino Acid Metabolism, Cardiovascular Disease, Hematological Disease	25	14
	Developmental Disorder, Hereditary Disorder, Metabolic Disease	25	14
	Cardiovascular System Development and Function, Organismal Development, Visual System Development and Function	22	13
	Energy Production, Cellular Development, Connective Tissue Development and Function	20	12
<b>14 dpi HighFEC vs LowFEC</b>	Cellular Assembly and Organization, Lipid Metabolism, Small Molecule Biochemistry	41	19
	Cell Signaling, Molecular Transport, Nucleic Acid Metabolism	23	12
	Gene Expression, Organ Morphology, Cell Morphology	20	11
	Developmental Disorder, Endocrine System Disorders, Organismal Injury and Abnormalities	16	9
	Hematological Disease, Respiratory Disease, Hematological System Development and Function	16	9
<b>HighFEC 7 vs 14 dpi</b>	Cancer, Cellular Movement, Connective Tissue Development and Function	45	23
	Carbohydrate Metabolism, Small Molecule Biochemistry, Energy Production	35	19
	Developmental Disorder, Drug Metabolism, Energy Production	35	19
	Infectious Disease, Inflammatory Disease, Neurological Disease	30	17
	Cellular Compromise, Hereditary Disorder, Skeletal and Muscular Disorders	21	13
<b>LowFEC 7 vs 14 dpi</b>	Cardiovascular System Development and Function, Tissue Development, Organismal Development	33	17
	Cell-To-Cell Signaling and Interaction, Cellular Movement, Immune Cell Trafficking	28	15
	Connective Tissue Disorders, Hereditary Disorder, Metabolic Disease	24	13
	Neurological Disease, Developmental Disorder, Endocrine System Disorders	24	13
	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	24	13

<sup>1</sup>IPA network score is expressed as the  $-\log(\text{Fisher's exact test p-value})$ .

**Table 4 – Significant canonical pathways identified by Ingenuity Pathway Analysis using differentially expressed (DE) genes.**

Comparison	Ingenuity Canonical Pathways	BH P value <sup>1</sup>	Ratio <sup>2</sup>	Genes
<b>7 dpi</b>	Pathogenesis of Multiple Sclerosis	0.00002	0.44	<b><i>CXCL10, CXCL11, CCR5, CXCL9</i></b>
<b>HighFEC vs LowFEC</b> <sup>3</sup>	Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.01	0.04	<i>CCR5, COL13A1, LAMA1, LBP, CXCL9, COL9A2, MMP9</i>
<b>LowFEC</b>	Pathogenesis of Multiple Sclerosis	0.000007	0.44	<b><i>CXCL10, CXCL11, CCL5, CXCL9</i></b>
<b>7 vs 14 dpi</b> <sup>4</sup>	IL-17A Signaling in Gastric Cells	0.01	0.12	<b><i>CXCL10, CXCL11, CCL5</i></b>
	Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde	0.02	0.25	<i>KMO, IDO1</i>
	Granulocyte Adhesion and Diapedesis	0.04	0.03	<b><i>CXCL10, CXCL11, CCL22, CCL5, CXCL9</i></b>
	Agranulocyte Adhesion and Diapedesis	0.04	0.03	<b><i>CXCL10, CXCL11, CCL22, CCL5, CXCL9</i></b>
	NAD biosynthesis II (from tryptophan)	0.04	0.13	<i>KMO, IDO1</i>

<sup>1</sup>BH P value = Benjamini-Hochberg corrected P value. <sup>2</sup>The ratio is calculated by taking the number of DE genes that participate in a Canonical Pathway, and dividing it by the total number of genes in that Canonical Pathway. <sup>3</sup>Genes more highly expressed in LowFEC animals are in bold, while genes more highly expressed in HighFEC animals are normal typeface. <sup>4</sup>Genes more highly expressed at 7 dpi are in bold, while genes more highly expressed at 14 dpi are normal typeface.

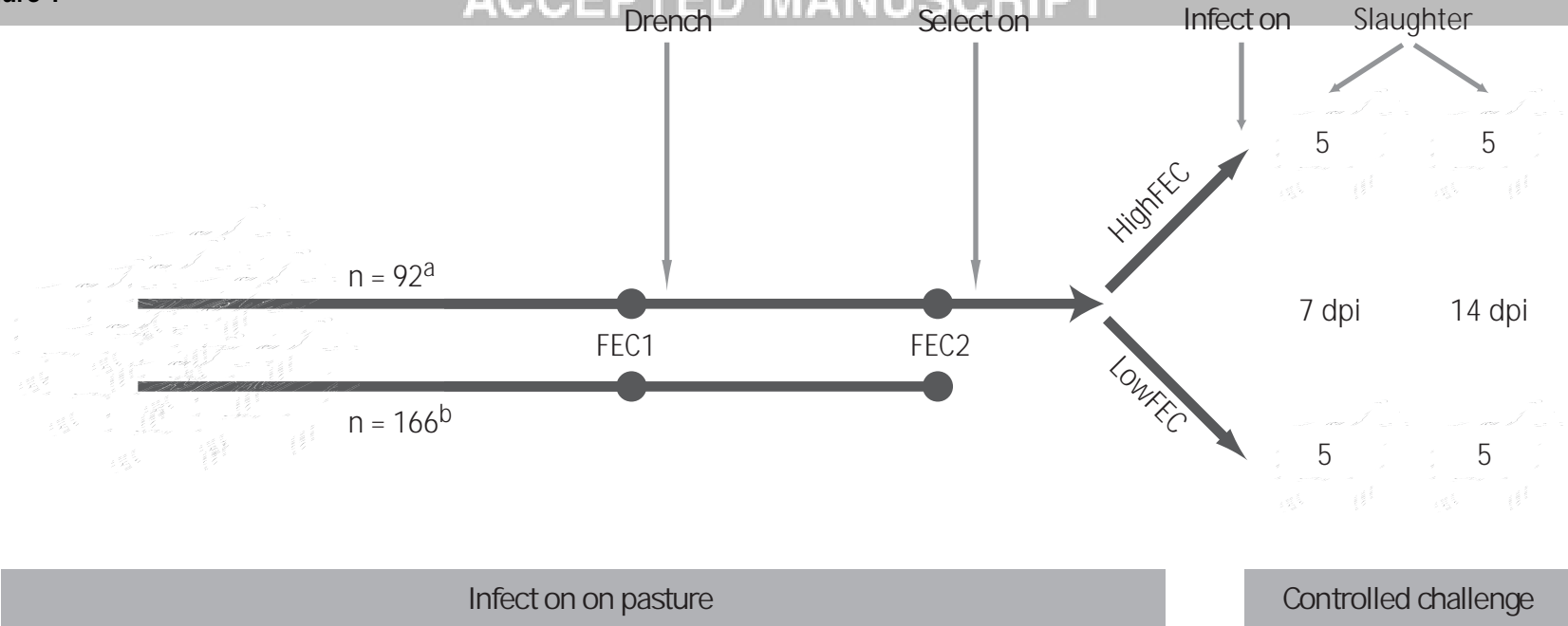
**Table 5 – Association between SNP in differentially expressed genes and gastrointestinal nematode resistance traits in Scottish Blackface lambs**

Ensembl gene ID	Name	OAR <sup>1</sup>	Position	SNP ID <sup>2</sup>	Consequence <sup>3</sup>	SIFT <sup>4</sup>	MAF <sup>5</sup>	Alleles <sup>6</sup>	n <sup>7</sup>	P value <sup>8</sup>			
										<i>FEC1</i>	<i>FEC1</i>	<i>FEC2<sub>O</sub></i>	<i>FEC2<sub>N</sub></i>
										<i>OT</i>	<i>NEM</i>	<i>T</i>	<i>EM</i>
ENSOARG00000020224	<i>COL9A2</i>	1	14688520	rs425322992	Missense	0.45	0.38	C/T	237	0.67	0.63	0.40	0.45
		1	14691551	rs402057753	N/A	-	0.38	G/A	237	0.67	0.63	0.40	0.45
ENSOARG00000020373	<i>MFI2</i>	1	189728511	rs405258217	N/A	-	0.11	T/C	237	0.40	0.08	0.77	0.75
		1	189741476	rs424822608	N/A	-	0.21	A/G	237	0.99	0.99	0.39	0.83
ENSOARG00000005490	<i>SLC30A2</i>	2	239929582	rs408045395	Missense	1	0.27	G/T	237	0.97	0.93	<b>0.03</b>	0.35
		2	239931409	rs414806237	Missense	0	0.03	T/A	237	0.64	0.48	<b>0.03</b>	0.47
		2	239932238	rs426472558	Synonymous	-	0.47	T/C	237	0.97	0.38	0.25	0.84
ENSOARG00000011529	<i>ZFR2</i>	5	17723806	rs414694841	Missense	1	0.46	G/A	237	0.87	0.44	0.50	0.20
		5	17725481	rs406633004	Missense	0.81	0.08	G/A	237	0.67	0.21	0.69	0.90
		5	17734892	rs409768412	N/A	-	0.45	A/A	237	0.90	0.62	0.41	0.27
ENSOARG00000013782	<i>ALB</i>	6	88140673	rs399322137	N/A	-	0.39	G/A	237	0.23	0.58	<b>0.03</b>	0.70
		6	88146314	rs412988422	Missense	0.9	0.27	A/C	237	0.45	0.81	0.79	0.99
		6	88151153	rs398193652	N/A	-	0.16	G/T	235	0.30	0.08	<b>0.03</b>	0.59
ENSOARG00000000928	<i>ECT2L</i>	8	63609947	rs427698166	N/A	-	0.12	C/A	236	0.22	0.40	0.19	0.96
		8	63613802	rs402663145	N/A	-	0.12	G/A	237	0.20	0.43	0.24	0.94
		8	63636645	rs160248153	Missense	0.1	0.17	G/T	236	0.41	0.29	0.61	0.75
		8	63639731	rs411594326	Missense	1	0.17	C/T	237	0.42	0.35	0.58	0.73
ENSOARG00000005312	-	14	46056292	rs429181932	Missense	0.36	0.15	G/A	237	0.47	0.30	0.47	0.15
		14	46070690	rs160946903	Synonymous	-	0.28	C/A	236	0.84	0.81	0.30	0.34
		14	46070771	rs162105426	Missense	0.81	0.25	C/T	237	0.08	0.25	0.18	0.41
ENSOARG00000005549	-	17	6550163	rs405054724	N/A	-	0.17	A/G	237	0.28	0.99	0.82	0.48
ENSOARG00000001778	<i>OSBPL5</i>	21	47759083	rs422189173	N/A	-	0.48	T/C	237	0.16	0.48	0.81	0.11
		21	47759570	rs411124861	N/A	-	0.48	T/C	237	0.13	0.29	0.97	0.06
		21	47792907	rs161640197	Synonymous	-	0.22	G/A	237	0.72	0.58	0.53	0.18

<sup>1</sup>Chromosome, <sup>2</sup>SNP = dbSNP (release 140) <sup>3</sup>Consequence = consequence of variant on the protein sequence, N/A indicates variant is intronic. <sup>4</sup>SIFT = effect of substitution on protein function: green indicates change is predicted to be tolerated, red indicates change is predicted to be deleterious. <sup>5</sup>MAF = minor allele frequency. <sup>6</sup>Alleles = first allele/second allele (second allele is the minor allele). <sup>7</sup>n = number of animals with genotypes. <sup>8</sup>P-value = uncorrected significance value for each variable. After Bonferroni correction the significance level thresholds were  $P < 2.08 \times 10^{-3}$  and  $P < 4.17 \times 10^{-2}$  for genome-wide significance ( $P < 0.05$ ) and suggestive significance (that is, one false positive per genome scan), respectively. Suggestive associations SNP are highlighted in bold.

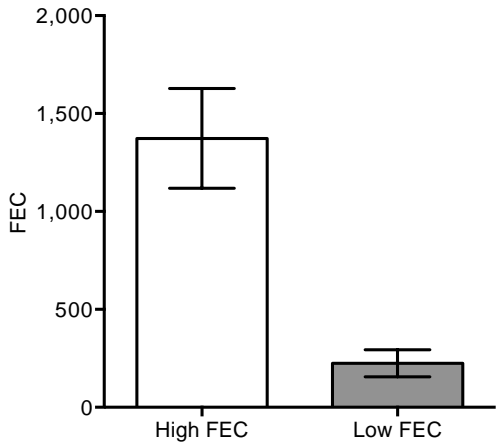
Figure 1

ACCEPTED MANUSCRIPT

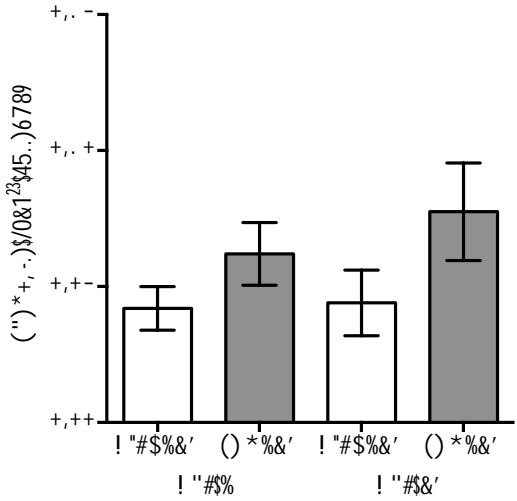




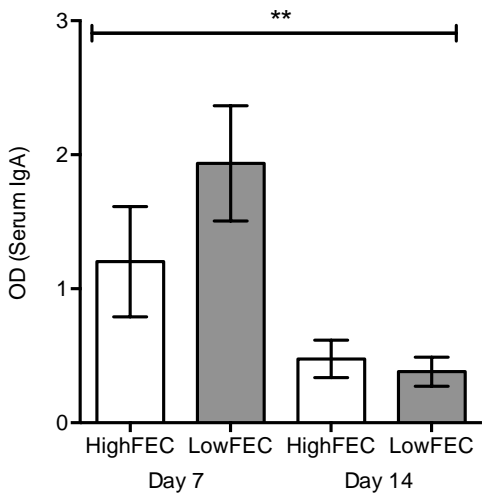
**Figure 2**



**B)**



**C)**



**D)**

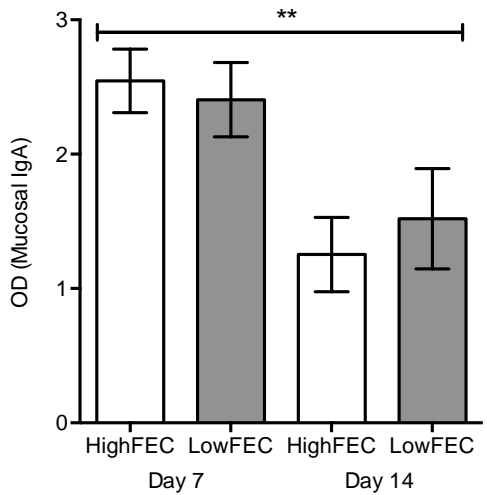


Figure 3

7 dpi

14 dpi

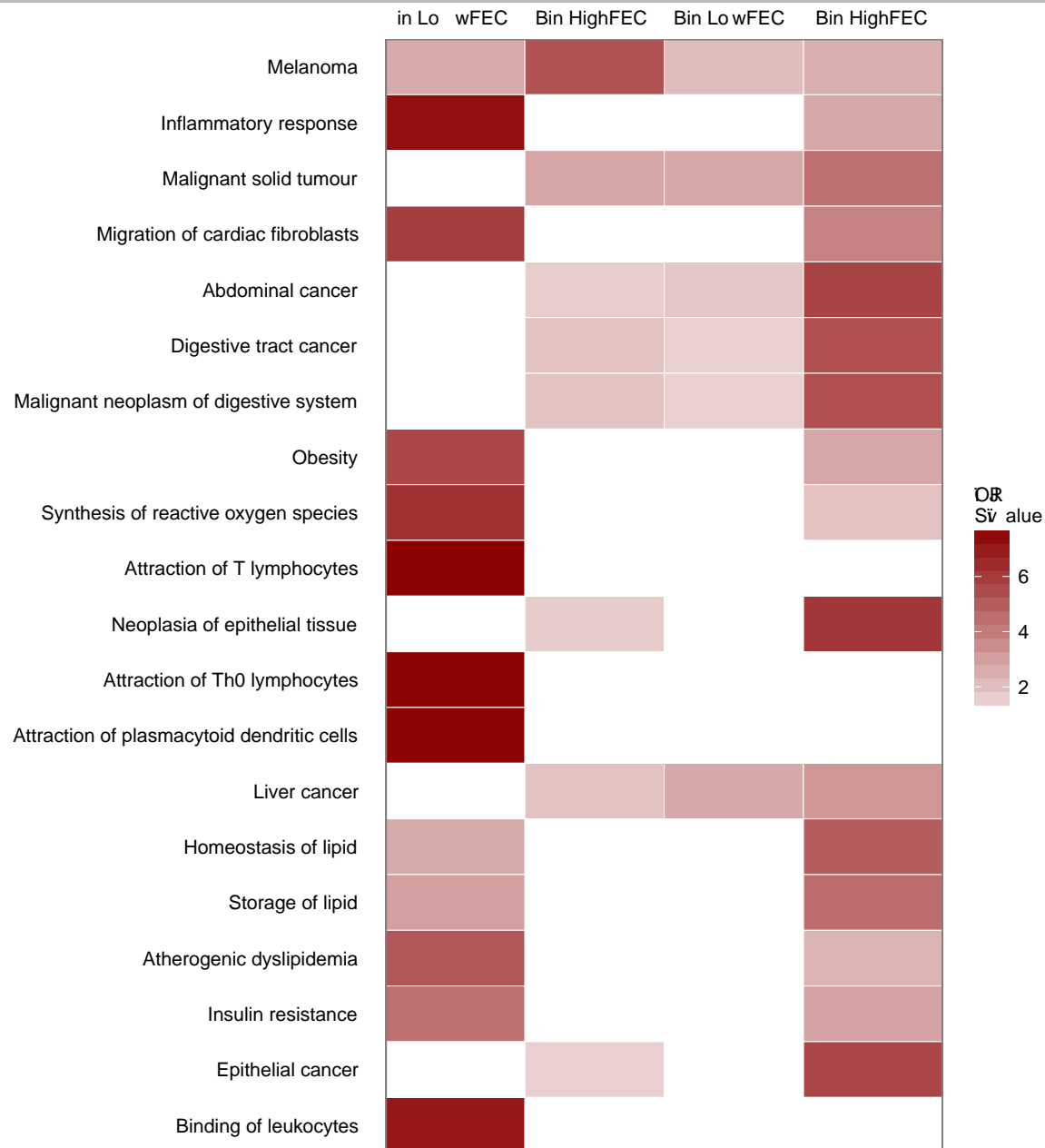


Figure 4

ACCEPTED MANUSCRIPT

7 dpi

14 dpi

Bin Lo wFEC

HighFEC

Bin Lo wFEC

HighFEC

/ $\beta$   
IFNL1  
TNF  
IFNB1  
IFNA1/IFNA13  
IFNA10  
IFNA7  
IFNA6  
IFNA14  
IFNA21

