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Author: Kathryn M. McRae Barbara Good James P. Hanrahan Matthew S. McCabe Paul Cormican Torres Sweeney Mary J. O'Connell Orla M. Keane

PII: S0304-4017(16)30179-0
DOI: http://dx.doi.org/doi:10.1016/j.vetpar.2016.05.014
Reference: VETPAR 8014

To appear in: *Veterinary Parasitology*

Received date: 10-12-2015
Revised date: 3-5-2016
Accepted date: 12-5-2016

Please cite this article as: McRae, Kathryn M., Good, Barbara, Hanrahan, James P., McCabe, Matthew S., Cormican, Paul, Sweeney, Torres, O'Connell, Mary J., Keane, Orla M., Transcriptional profiling of the ovine abomasal lymph node reveals a role for timing of the immune response in gastrointestinal nematode resistance.Veterinary Parasitology http://dx.doi.org/10.1016/j.vetpar.2016.05.014

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

Kathryn M. McRae[1,2†], Barbara Good [3], James P. Hanrahan[3], Matthew S. McCabe[1], Paul Cormican[1], Torres Sweeney[4], Mary J. O'Connell[2] & Orla M. Keane[1§]

[1]Animal & Bioscience Department, Teagasc, Grange, Dunsany, Co. Meath, Ireland.

[2]Bioinformatics & Molecular Evolution Group, School of Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland.

[3]Animal & Bioscience Department, Teagasc, Athenry, Co. Galway, Ireland.

[4]School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Republic of Ireland

Current address: [†]AgResearch, Invermay Agricultural Centre, Mosgiel, New Zealand

[§]Corresponding author

Email addresses:

KMM: kathryn.mcrae@agresearch.co.nz

BG: barbara.good@teagasc.ie

JPH: sjohanrahan@gmail.com

MMC: matthew.mccabe@teagasc.ie

PC: paul.cormican@teagasc.ie

TS: torres.sweeney@ucd.ie

MJO'C: mary.oconnell@dcu.ie

OMK: orla.keane@teagasc.ie

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

Abstract

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

Scottish Blackface lambs, with divergent phenotypes for resistance, were challenged with 30,000 *Teladorsagia circumcincta* larvae (L3), and abomasal lymph nodes recovered at 7 and 14 days post-infection (dpi). High-throughput sequencing of cDNA from the abomasal lymph node was used to quantitatively sample the transcriptome with an average of 32 million reads per sample. A total of 194 and 144 genes were differentially expressed between resistant and susceptible lambs at 7 and 14 dpi respectively. Differentially expressed networks and biological processes were identified using Ingenuity Pathway Analysis. 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 animals respond to infection earlier than susceptible animals. Twenty-four Single Nucleotide Polymorphisms (SNP) within 11 differentially expressed genes, were tested for association with gastrointestinal nematode resistance in the

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

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

Keywords

Scottish Blackface; Sheep; *Teladorsagia circumcincta*; Host-parasite interaction; Transcriptome.

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1 Ethical approval

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

2.2 Animals

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

Flock FEC (eggs per gram (epg)) was monitored weekly, from when lambs were approximately 8 weeks of age, using the FECPAK method (Fecpak). Eggs were distinguished as *Nematodirus* spp. (FEC_{NEM}) and 'other trichostrongyles' spp (FEC_{OT}). When FEC_{OT} reached approximately 600 epg the lambs were individually sampled twice (FEC1A and FEC1B), 1 week apart, and FEC was determined for each sample using the modified McMaster method (Anon, 1986). $FEC1A_{OT}$ and $FEC1B_{OT}$ were averaged to give $FEC1_{OT}$, the first phenotypic measurement of resistance. Following FEC1B the lambs were treated with a non-persistent macrocyclic lactone (ML, Oramec, Merial Animal Health Ltd) in accordance with manufacturer's recommendations. Flock FEC was again monitored weekly until FEC_{OT} reached approximately 600 epg when 2 more FEC (1 week apart) per individual were

153 completed (FEC2A and FEC2B) and the average computed to generate $FEC2_{OT}$, 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 $\ln(FEC_{OT} + 25)$
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 ($FEC1_{OT}$ and $FEC2_{OT}$) 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 –

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

2.4 Phenotypic measurements and analysis

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

2.5 Tissue collection and RNA extraction

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

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

2.6 Library preparation and sequencing

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

2.7 Bioinformatics analysis

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

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

2.8 cDNA synthesis and RT-qPCR

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

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

2.9 SNP genotyping and analysis

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

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

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

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

SNP effects for FEC traits (FEC1 and FEC2 for *Nematodirus* and for 'other trichostrongyles') were estimated in ASReml (Gilmour et al., 2009) by fitting the SNP, one at a time, as fixed effects. The model also included sex and grazing group as fixed effects. 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.

3. Results

3.1 Phenotypic measurements

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

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

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

3.2 RNA Sequencing

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

3.3 Differential gene expression

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

3.4 Genes DE between HighFEC and LowFEC animals

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

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

3.5 Immune response over time

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

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

3.6 Visualisation across multiple analyses using Ingenuity Pathway Analysis

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

3.7 Validation of expression

In order to validate the results of the RNA sequencing, a panel of 8 DE genes were chosen for validation by RT-qPCR. These were *ALB*, *ASZ1*, *CXCL11*, *GABBR2*, *GSDMA*, *LYVE1*, *MFI2* and *STPG1*. The gene expression pattern, in terms of direction and magnitude of 7 of the 8 genes was reproducible by qPCR

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

3.8 SNP analysis

The results of an association analysis between SNP in DE genes and FEC are shown in Table 5. The results yielded a range of significance for each individual SNP; however, no single SNP reached significance after Bonferroni correction for multiple comparisons (Abdi, 2007). Four SNP were, however, suggestively associated with $FEC2_{OT}$: 2 coding SNP within *SLC30A2*, and 2 non-coding SNP within *ALB*. Both SNP within *SLC30A2* code for missense variants; however, SNP OAR2_239929582 is classified as 'tolerated' by the SIFT algorithm (score = 1) (Kumar et al., 2009), whereas SNP OAR2_239931409 is classified as 'deleterious' (score = 0). The latter SNP has a minor allele (A) frequency of 0.03 within the set of individuals studied, with only 14 out of 237 animals heterozygous and no animal homozygous for this allele. However, the allele was not out of Hardy-Weinberg equilibrium (P = 0.90).

4. Discussion

4.1 Phenotypic differences between resistant and susceptible lambs

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

4.2 Response to GIN infection in phenotypes divergent for resistance

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

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

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

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

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

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

Like their HighFEC counterparts, LowFEC animals expressed higher levels of *MX2* at 7 dpi than at 14 dpi. While all of the DE genes that were increased in LowFEC animals at 14 dpi are currently annotated as “novel protein coding” genes in Ensembl (Ensembl Release 78), several are orthologous to *Bos taurus* and *Homo sapiens* MHC genes. ENSOARG00000016098 is a 1-to-1 ortholog of *BOLA-DRB3*. ENSOARG00000002985 and ENSOARG00000015866 are one-to-many orthologs of *BOLA-DQA1* and *BOLA-DQB*, respectively. ENSOARG00000010572 is a many-to-many ortholog of multiple human MHC class I genes. The MHC is involved in the induction and regulation of the immune response, and associations between the MHC genes and both resistance and susceptibility to GIN have been found in multiple studies reviewed by Venturina et al. (2013) (Venturina et al., 2013).

4.4 Pathway analysis

Biological processes involving 'inflammatory response', 'attraction of T lymphocytes' and 'binding of leucocytes' were more highly expressed in resistant animals at 7 dpi. Therefore, these animals appear to be generating an earlier immune response to infection than susceptible animals, through an increase in migration of cells involved in the response to pathogens. Increased cellular recruitment to the lymph node in resistant animals was also indicated by the increased weight of the abomasal lymph node tissue. Upstream regulators of the genes involved in these processes included TNF, IFN-α, IFN-β and IFN-λ. The pro-inflammatory cytokine TNF is produced in the gastric mucosa during inflammation and can promote gastrointestinal homeostasis, although excess TNF production can contribute to gastric mucosal inflammation and injury (Wallace and Ma, 2001), and has been implicated in the intestinal pathology of nematode infections (Lawrence et al., 1998). Following deliberate infection with *T. circumcincta*, elevated *TNF* expression has been observed in both the abomasal lymph node of challenged compared to unchallenged lambs at 5 days post infection (Craig et al., 2007), and in the abomasal mucosa of *DRB1*1101* carrier lambs compared to their non-carrier counterparts at 3 days post infection (Hassan et al., 2011). The type I IFNs (α and β) can be produced by almost every cell type, including leukocytes. While IFNs are best known for their role in the cellular response to viral infections, they also possess immunomodulatory activities (González-Navajas et al., 2012). The connection between type I IFNs and several human autoimmune and inflammatory disorders is well known, with several inflammatory syndromes shown to benefit from the administration of type I IFNs (González-Navajas et al., 2012). While the antiviral capability of type III IFNs, including IFN-λ, 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

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

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

4.6 SNP analysis

We hypothesised that markers in DE genes may be associated with resistance. The goal was not to identify causative mutations *per se* but to test markers which may be in linkage disequilibrium with causative mutations. As DE genes detected by tagwise dispersion were more consistent between animals within a group, markers in these genes were tested for association with FEC. While a number of SNP in *ALB* and *SLC30A2* were suggestively associated with $FEC2_{OT}$ they were not significant after correction for multiple testing. The present study is limited by the number of animals (237) available for association analysis. Previous studies have involved between 752 and 1275 individuals (Riggio et al., 2013; Sall et al., 2012) for GWAS. Future work on validating the SNP of interest from this study would require a larger number of Scottish Blackface animals. As noted in reports on multiple studies, host resistance to GIN appears to be mediated by many genes, each with a relatively small effect (Kemper et al., 2011; Riggio et al., 2014). The candidate gene approach is therefore unlikely to capture all of the variation underlying known phenotypic differences; future work on identifying SNP to be used in selection programmes will likely focus on utilising a panel of SNP in addition to searching for individual causative mutations. Despite these limitations, suggestive associations between $FEC2_{OT}$ and multiple SNP within the same gene indicate that genotyping SNP within DE genes may be a valid way of discovering polymorphisms associated with GIN resistance.

5. Conclusions

Transcriptional profiling of the abomasal lymph node during a controlled challenge with *T. circumcincta* indicated that in resistant (LowFEC) Scottish Blackface lambs, 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

Conflict of Interest Statement

The authors declare that they have no conflict of interests.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Assumpta Glynn, Margaret Murray, Joe Larkin, Kevin Fitzsimmons, Simone Sebastiano, Luke O'Malley P.J. Hastings and Henry Walsh. Special thanks to Tom McNeilly from the Moredun Research Institute for supplying the *T. circumcincta* L3 antigen, and Rudiger Brauning and John McEwan from AgResearch for supplying the flanking sequences for the Ovine Infinium® HD SNP BeadChip SNP. Kathryn McRae was supported by a Teagasc Walsh fellowship (grant number 6001) and the Allan and Grace Kay Overseas Scholarship.

Figures

Figure 1 – Experimental design.

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

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

Mean (±s.e) faecal egg count of HighFEC (n = 10) and LowFEC (n = 10) individuals during natural challenge (A). Mean (±s.e) circulating basophil numbers (B), Mean (±s.e) serum anti-*Teladorsagia circumcincta* IgA (C) and Mean (±s.e) mucosa anti-*T. circumcincta* IgA (D) in HighFEC and LowFEC animals during a controlled challenge with 3 x 10^4 *T. circumcincta* L3 larvae.

Figure 3 - Top 20 DE diseases and biological functions.

Heat map of the top 20 IPA-derived diseases and biological functions from genes DE between HighFEC and LowFEC animals at 7 or 14 dpi, sorted by P value.

Figure 4 - Top 10 IPA-derived upstream regulators.

Heat map of the top 10 IPA-derived upstream regulators from genes DE between HighFEC and LowFEC animals at 7 or 14 dpi, sorted by P value.

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

Comparison		**Common dispersion** *FDR <0.05*	**Tagwise dispersion** *FDR <0.1*
HighFEC vs LowFEC	7 dpi	194	7
	14 dpi	144	6
7 vs 14 dpi	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[1]	CPM[2]	FDR[3]
7 dpi	HighFEC	ENSOARG00000013782	*ALB*	Albumin	166.3	1.7	<0.01
		ENSOARG00000020224	*COL9A2*	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	*OSBPL5*	Oxysterol binding protein-like 5	0.4	2.1	0.02
		ENSOARG00000005490	*SLC30A2*	Solute carrier family 30 (zinc transporter), member 2	0.5	46.1	0.03
14 dpi	HighFEC	ENSOARG00000020373	*MFI2*	Antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	3.7	19.3	<0.01
		ENSOARG00000011529	*ZFR2*	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	*ECT2L*	Epithelial cell transforming 2 like	0.2	166.9	0.04
HighFEC	7 dpi	ENSOARG00000013782	*ALB*	Albumin	0.2	202.5	<0.01
		ENSOARG00000010231	*MX2*	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	*LYVE1*	Lymphatic vessel endothelial hyaluronan receptor 1	0.3	28.1	0.07
		ENSOARG00000019641	*LSAMP*	Limbic system-associated membrane protein	0.7	151.9	0.08
		ENSOARG00000019517	*CHI3L2*	Chitinase 3-like 2	0.5	38.4	0.08
LowFEC	7 dpi	ENSOARG00000010231	*MX2*	Myxovirus (influenza virus) resistance 2 (mouse)	0.5	8.2	<0.01
		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	*MAB21L3*	Mab-21-like 3 (*C. elegans*)	0.4	5.4	0.04
	14 dpi	ENSOARG00000016098	-	Uncharacterized protein	0.6	12.8	<0.01
		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
ENSOARG00000005126	-	Uncharacterized protein	5.2	3.6	0.10

[1]FC = fold-change. [2]CPM = counts-per-million. [3]FDR = false discovery rate.

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

Comparison	Network	IPA Score[1]	Genes
7 dpi HighFEC vs LowFEC	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
14 dpi HighFEC vs LowFEC	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
HighFEC 7 vs 14 dpi	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
LowFEC 7 vs 14 dpi	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

[1]**IPA** network **score is expressed as the –log(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[1]	Ratio[2]	Genes
7 dpi	Pathogenesis of Multiple Sclerosis	0.00002	0.44	***CXCL10***, ***CXCL11***, ***CCR5***, ***CXCL9***
HighFEC vs LowFEC[3]	Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.01	0.04	***CCR5***, *COL13A1*, *LAMA1*, ***LBP***, ***CXCL9***, *COL9A2*, *MMP9*
LowFEC	Pathogenesis of Multiple Sclerosis	0.000007	0.44	***CXCL10***, ***CXCL11***, *CCL5*, ***CXCL9***
7 vs 14 dpi [4]	IL-17A Signaling in Gastric Cells	0.01	0.12	***CXCL10***, ***CXCL11***, ***CCL5***
	Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde	0.02	0.25	*KMO*, ***IDO1***
	Granulocyte Adhesion and Diapedesis	0.04	0.03	***CXCL10***, ***CXCL11***, ***CCL22***, *CCL5*, ***CXCL9***
	Agranulocyte Adhesion and Diapedesis	0.04	0.03	***CXCL10***, ***CXCL11***, ***CCL22***, *CCL5*, ***CXCL9***
	NAD biosynthesis II (from tryptophan)	0.04	0.13	*KMO*, ***IDO1***

[1]BH P value = Benjamini-Hochberg corrected P value. [2]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. [3]Genes more highly expressed in LowFEC animals are in bold, while genes more highly expressed in HighFEC animals are normal typeface. [4]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[1]	Position	SNP ID[2]	Consequence[3]	SIFT[4]	MAF[5]	Alleles[6]	n[7]	P value[8]			
										FEC1 $_{OT}$	*FEC1* $_{NEM}$	*FEC2* $_{O}$ $_{T}$	*FEC2* $_{N}$ $_{EM}$
ENSOARG00000020224	*COL9A2*	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	*MFI2*	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	*SLC30A2*	2	239929582	rs408045395	Missense	1	0.27	G/T	237	0.97	0.93	**0.03**	0.35
		2	239931409	rs414806237	Missense	0	0.03	T/A	237	0.64	0.48	**0.03**	0.47
		2	239932238	rs426472558	Synonymous	-	0.47	T/C	237	0.97	0.38	0.25	0.84
ENSOARG00000011529	*ZFR2*	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	*ALB*	6	88140673	rs399322137	N/A	-	0.39	G/A	237	0.23	0.58	**0.03**	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	**0.03**	0.59
ENSOARG00000000928	*ECT2L*	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	*OSBPL5*	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

[1]Chromosome, [2]SNP = dbSNP (release 140) [3]Consequence = consequence of variant on the protein sequence, N/A indicates variant is intronic. [4]SIFT = effect of substitution on protein function: green indicates change is predicted to be tolerated, red indicates change is predicted to be deleterious. [5]MAF = minor allele frequency. [6]Alleles = first allele/second allele (second allele is the minor allele). [7]n = number of animals with genotypes. [8]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

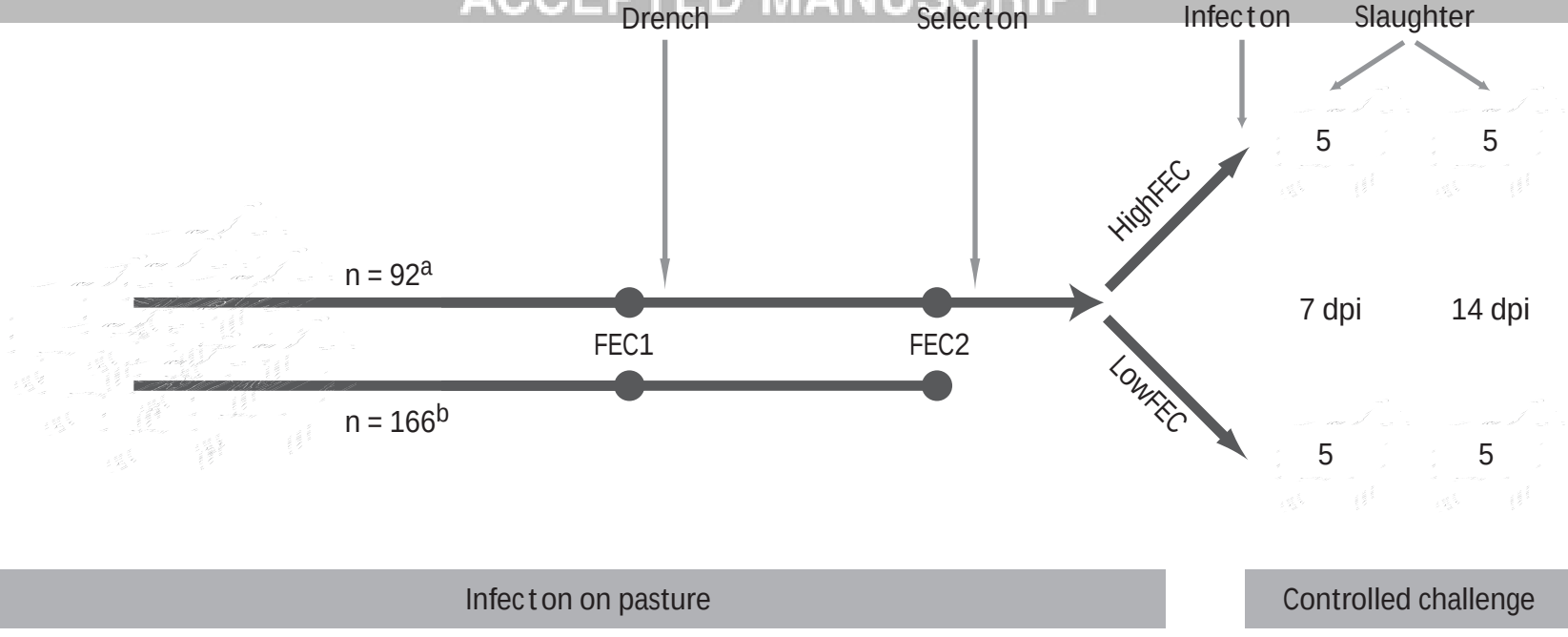

Figure 2

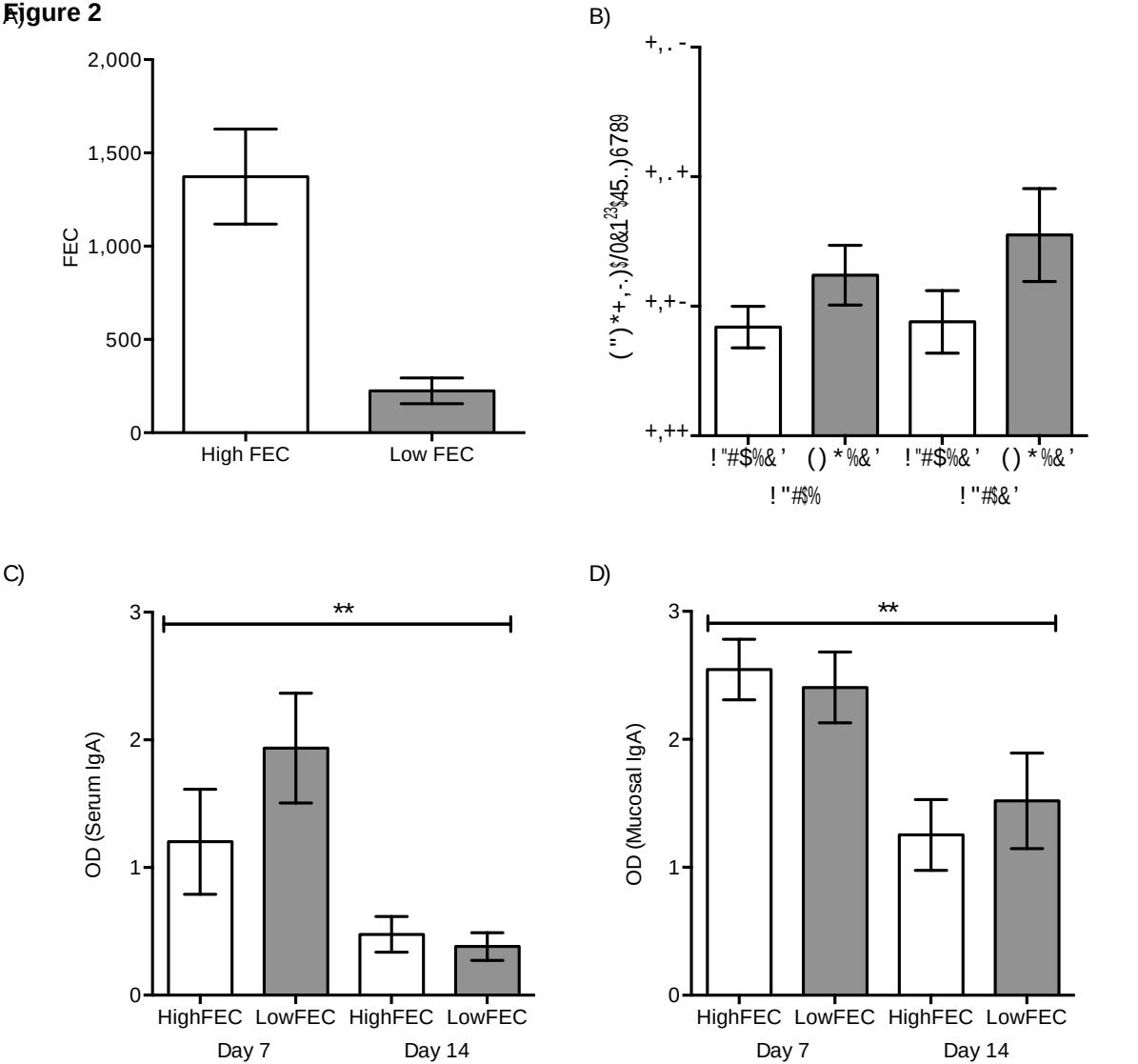
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Figure 3

Figure 4