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A temporal shift in regulatory networks and pathways in the bovine small intestine during *Cooperia oncophora* infection

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ABSTRACT

Cooperia oncophora is an important parasitic nematode of cattle with a wide distribution in temperate areas. Twenty Holstein nematode-naïve bull calves were experimentally infected with approximately 100,000 infective L3s and infection was allowed to progress for 7, 14, 28, 42 days, respectively. This experiment was conducted to identify putative recognition and inflammatory pathways in the host-parasite relationship. Gene expression profiles of the small intestine were compared using a high-density bovine 60 mer oligo microarray. A total of 310 genes were differentially expressed during the course of infection. The pathways and regulatory networks significantly impacted by the infection were analysed. A total of 22 canonical pathways and nine regulatory networks were significantly affected during infection. During the early phase of the infection (7 days p.i.), parasites suppressed the acute phase response and the complement system of the host. At 14 days p.i., three out of the six pathways impacted were related with retinoid X receptor (RXR) functions. At 28 days p.i., the effects on RXR were less evident. The host response shifted to lipid metabolism and signalling, especially eicosanoid production and signalling, suggesting that eicosanoid-mediated inflammation might be a major host defence mechanism. By 42 days p.i., the pathways impacted involved glycosphingolipid biosynthesis and transforming growth factor β (TGFβ signalling. The expression of cadherin-like 26 (CDH26) was strongly up-regulated starting at 14 days p.i. and peaked at 28 days p.i. The extent of its expression is positively correlated with the infiltration of eosinophils ($R = 0.82$) and coincides with the number of adult parasites in the tissue. CDH26 demonstrated an expression profile similar to two other cell adhesion molecules involved in recognition of carbohydrates on foreign organisms, collectin and galectin, suggesting that it may serve as a pattern recognition molecule for *C. oncophora*. These results provide a potential molecular roadmap for future studies aimed at defining host immune responses and understanding protective immunity against gastrointestinal nematodes.

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1. Introduction

Cooperia oncophora is one of the most important gastrointestinal nematodes infecting cattle in the temperate regions of the world. Nematode parasites cost the American cattle industry in excess of $2 billion per year due to decreased animal growth as well as increased treatment costs. The effect of parasite infection on cattle growth and intestinal pathology has been well studied (Coop et al., 1979; Armour et al., 1987; Rodrigues et al., 2004). Although clinical symptoms are generally mild, *C. oncophora* infection has been shown to reduce weight gain by up to 13.5% of total cattle bodyweights (Coop et al., 1979). The manifestations of host immunity against *C. oncophora* infection have long been observed and include reduced female fecundity and arrested parasite development. Since their invention 25–35 years ago, anthelmintic drugs have played a major role in the control of parasitic nematodes including *C. oncophora*. However, resistance of this species to anthelmintic drugs is becoming a worldwide issue (Vermunt et al., 1995; Stafford and Coles, 1999; Anziani et al., 2004). Ironically, the initial high efficacy and broad spectrum of these drugs has also resulted in a stagnation of research to identify alternative methods of parasite control. Understanding the mechanisms that contribute to protective immunity, immunosuppression and host resistance will have an important impact on the development of alternative control strategies.

Cooperia oncophora infection elicits a Th2-like immune response, characterised by up-regulation of IL-4 and the involvement of both eosinophils and mucosal IgA (Gasbarre et al., 2001; Kanobana et al., 2002). Host serological response to *C. oncophora* experimental infection has been extensively studied (Nieuwland et al., 1995; Kanobana et al., 2001, 2002, 2003a). *Cooperia oncophora*-specific serum and mucus IgG1 and IgA was strongly induced upon experimental challenge in cattle (Kanobana et al., 2003b). Moreover, the

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levels of *Cooperia*-specific IgA were significantly higher in intermediate responders than in low responders in cattle (Kanobana et al., 2001) and expulsion of the adult *Cooperia* worm appeared to be associated with a significant increase in mucosal IgA and an influx of eosinophils (Kanobana et al., 2002). Polymeric immunoglobulin receptor (PIGR), a gene responsible for trans-epithelial transport of polymeric immunoglobulins, such as IgA dimers and IgM pentamers, into mucosal and glandular secretions was strongly up-regulated in the heifers resistant to parasitic nematodes after experimental parasite challenge (Li et al., 2007b). Kanobana et al. (2003a,b) also showed that the peak in antibody titres was preceded by a significant increase in B cells and major histocompatibility complex $(MHCII)^+$ cells in the draining lymph nodes, suggesting that B cells may play an important role in development of acquired immunity against the parasite. Although important in our overall understanding of host–parasite interaction, the genetic basis and cytokine profiles in host resistance as well as the immune mechanisms responsible for protective immunity have not been identified. In this study, we aim to characterise transcriptional profiles in the bovine small intestines during *C. oncophora* experimental infection, especially temporal shifts in regulatory networks and pathways induced by the infection.

2. Materials and methods

2.1. Animals and parasitology

Twenty Holstein bull calves used in this experiment were purchased shortly after birth. The animals were fed with rations and maintained on concrete for the duration of the experiment. After reaching ~3 months of age, these nematode-naïve animals were orally infected with *C. oncophora* infective L3s (10^5 L3s per animal). The L3s were obtained from cultures maintained at the United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Beltsville facilities. The animal maintenance and handling were based on the protocol approved by The USDA-ARS Animal Care and Use Committee following Institutional Animal Care and Use Committees (IACUC) guidelines. The experiment was conducted in two blocks (10 animals in each block). The animals were sacrificed at 7, 14, 28 and 42 days p.i., respectively. There were four bull calves at each time point. Four naïve uninfected animals were used as controls. Small intestine (SI) tissues were collected from the jejunum approximately 3 m from the pyloric sphincter and snap frozen in liquid nitrogen prior to storage at −80 °C until total RNA was extracted. Tissues were also stored in OCT compound (Sakura Inc., Torrance, CA, USA) for cryo-sectioning. Fecal egg counts per gram of faeces (EPGs) were determined by double centrifugation using zinc sulphate and parasite burdens from the entire small intestines were determined as previously described (Li et al., 2007a,b).

2.2. Histology and eosinophil counts

Tissues were preserved in OCT and kept at −80 °C until cryo-sectioning. Tissues were sectioned at ~7.0 μM and eosinophils were enumerated on H & E stained slides. A total of 10 image fields (400×) were counted for each sample. The eosinophil data were analysed using the unpaired *t*-test.

2.3. RNA extraction, quantitative PCR, and microarray analysis

Total RNA extraction, *quantitative PCR*, microarray fabrication and hybridization were as previously described (Li and Li, 2006; Li et al., 2006). Briefly, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and further purified using an RNeasy Mini kit (Qiagen, Valenica, CA). RNA integrity was verified using a Bioanalyzer 1000 (Agilent, Palo Alto, CA). For *quantitative PCR*, the cDNA synthesis was performed with an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). *quantitative PCR* analysis was carried out with iQ SYBR Green Supermix kit (Bio-Rad) using 200 nM of each amplification primer and the first-strand cDNA (100 ng of the input total RNA equivalents) in a 25 μl reaction volume as previously described (Li et al., 2006). Amplification was carried out on an iCycler iQ^{TM} Real Time PCR Detection System (Bio-Rad) with the following profile: 95 °C for 60 s, 40 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. A melting curve analysis was performed for each primer pair. Standards for each gene were prepared from PCR products purified using the QIAquick purification kit (Qiagen). The expression levels were determined from a standard curve of known target cDNA copy numbers (1.0×10^2–1.0×10^7 molecules), which was analysed simultaneously with the experimental samples.

The bovine microarray used was described previously (Li and Li, 2006; Li et al., 2006). The microarray consists of 86,191 unique 60 mer oligonucleotides, representing 45,383 bovine sequences or genes. Each unique oligonucleotide was repeated four times on the microarray. After hybridization, scanning and image acquisition, the data were extracted from the raw images using NimbleScan software (NimbleGen, Madison, WI). A total of 20 microarrays were used in the experiment (GEO Accession No. GSE11950).

Relative signal intensities (log2) for each feature were generated using the robust multi-array average algorithm (Irizarry et al., 2003). Data was processed based on the quantile normalisation method (Bolstad et al., 2003). The background-adjusted, normalised and log-transformed intensity values were then analysed using Partek Genomics Suite (Partek Inc., St. Louis, MO, USA). There existed considerable biological variations in term of both worm burden and fecal egg counts as well as underlying gene expression in this outbred population, which were also reflected in the variable microarray data obtained. The permutation-based microarray data analysis tools such as significance analysis of microarray (SAM) seemed too stringent to identify any candidate genes. In this study, unpaired (two-sample) *t*-tests were performed based on the comparisons of all log2 transformed intensity values of the same genes between four uninfected controls versus each of four infected groups (7, 14, 28 and 42 days p.i.). There were four animals in each of the four infected groups. Two-sample *t*-tests were carried out amongst the eight microarrays. A *P*-value of 0.05 was used as a cut-off and only genes that demonstrated statistically significant expression across all eight microarrays, regardless of the magnitudes of their intensity values, were subjected to further data analysis. The gene list resulting from this test was quite reliable because only those genes with consistent gene expression values in all biological replicates could meet the *P*-value cut-off. The genes from this list which were further analysed using *z*-score tests by calculating the 95% cut off intervals, were designated as significant and are listed in Supplementary Table S1. The *z*-score test is a statistical tool used in inference which determines whether the difference between a sample mean and the population mean is large enough to be statistically significant. Briefly, this approach involves calculating a distribution of *z*-scores for all genes in each microarray, and employing the difference of the *z*-scores (*z*-ratio) between the two groups (the naïve control and the infected animals) to identify genes for which expression is significantly altered in infected groups. Because the entire challenge experiment was completed within a relatively short time span, time-matched naïve controls for each of the infected groups were not used. Instead, in the first block, two naïve controls were sacrificed together with the 7 days p.i. animals; and in the second block, the remaining two naïve controls were sampled at the same time as 28 days p.i. animals. The age difference between the naïve controls and each of the four infected groups at the end of the experiment was not statistically different. The expression patterns between the two batches of naïve controls were indistinguishable.

2.4. Network identification and canonical pathway analysis

Genes significantly regulated during the infection were analysed using Ingenuity Pathways Analysis (IPA) software v6.0 (Ingenuity Systems, Redwood City, CA) as described previously (Li and Capuco, 2008). Briefly, the significant genes with known gene identifiers (gene symbols) and their corresponding expression values were uploaded into the software. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the database. Networks of these focus genes were then algorithmically generated based on their connectivity and assigned a score. The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset but may not be an indication of the quality or significance of the network. The score takes into account the number of focus genes in the network and the size of the network to approximate how relevant this network is to the original list of focus genes. IPA uses a right-tailed Fisher's test to calculate the *P* value for networks. A score of 10, for example, would mean that there is a $P = 10^{-10}$ chance that the genes in that network are associated solely by chance. The network identified is then presented as a graph indicating the molecular relationships between genes/gene products. Each network or pathway was arbitrarily set to have a maximum of 35 focus genes. Genes or gene products are represented as nodes and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one literature reference of direct physical, transcriptional and enzymatic interactions or from canonical information stored in the Ingenuity Pathways Knowledge Base. The intensity of the node colour indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product, and edges are displayed with various labels indicating the nature of the relationship between the nodes as described in figure legends. A functional analysis of a network then identified the biological functions and/or diseases that were most significant to the genes in the network. Canonical pathways were identified from the IPA library of canonical pathways based on two parameters: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway and (2) a *P* value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

3. Results

3.1. EPG, parasite burden, and eosinophil counts

Up to 29% of the L3s used for infections were recovered from the small intestines at 28 days p.i., which is consistent with previously published observations (Coop et al., 1979). As expected, only the L4s (~10% of the total larvae used for infections) were recovered at 7 days p.i. At 14 days p.i. a significant variation in worms recovered was observed. Of the four animals in the 14 days p.i. group, no worms including larvae were recovered from one animal, only 50 larvae were recovered from another, while the remaining two animals had 9800 and 27,000 adult worms recovered, respectively. Different immune response types amongst calves may indeed exist (Kanobana et al., 2001). Adult worm burden was drastically reduced at 42 days p.i., from an average of 25,000 worms per animal to just 1600 worms per animal.

Table 1 summarizes EPG, worm burden and eosinophil counts for all groups. The control group averaged 25 eosinophils per graticule field in the lamina propria. At 7 days p.i., the mean eosinophil number was slightly reduced to 18. After the initial reduction, eosinophils accumulated in the lamina propria and peaked at 28 days p.i. with ~54 eosinophils per field. The numbers of eosinophil infiltrates were significantly higher at both 28 days p.i. and 42 days p.i. ($P < 0.01$), compared with those at 7 days p.i. and the naïve controls.

3.2. Gene expression profiles and quantitative PCR

Global expression analysis in the small intestine was conducted using a bovine high-density oligo microarray. Using a combination of cutoffs of $P < 0.05$ based on ANOVA and 2-fold changes, a total of 310 unique sequences/genes were identified to be significantly altered during the infection,. At 7 days p.i., 45 unique sequences were differentially regulated by the infection, with the majority of the genes being down-regulated. Amongst these down-regulated genes were complement component 4B (C4B), ECSIT, an evolutionarily conserved intermediate in the Toll/IL-1 signalling pathway, and squalene epoxidase (SQLE). The number of genes differentially regulated during the infection peaked at 14 days p.i. (131 sequences), following by 28 days p.i. (111 sequences) and 42 days p.i. (71 sequences), as summarized in Supplementary Table S1. Several genes were constantly up-regulated at all four time points; a notable example is glucosaminyl (*N*-acetyl) transferase 3, mucin type (GCNT3), a key enzyme in mucin biosynthesis, which was up-regulated greater than 5-fold at all time points. Cadherin-like 26 (CDH26), represented by two non-overlapping sequences, was strongly up-regulated starting at 14 days p.i. and peaked at 28 days p. (~173-fold). A similar expression profile was observed for both galectin OVGAL11 (LGALS13) and collectin-46 (SFTPD). Calponin 1, basic, smooth muscle (CNN1), on the other hand, was up-regulated more than 2-fold at 7 days p.i.; its expression peaked at 14 days p.i. and returned to basal levels by 42 days p.i., suggesting that enhanced smooth muscle contractility may indeed contribute to active expulsion of adult worms. In contrast, placental growth factor (PGF) was one of the genes whose expression was constantly repressed by the adult parasite.

A nucleotide search using the BLAST algorithm was conducted against the Reference mRNA Sequences Database (RefSeq; http://www.ncbi.nlm.nih.gov/RefSeq/) using both 60 mer oligo sequences

Table 1
Eggs per gram of faeces (EPG), *Cooperia oncophora* parasite burden and eosinophils in the bovine small intestine ($n = 4$).

	Control	7 days p.i.	14 days p.i.	28 days p.i.	42 days p.i.
Adult worm (♂)	0 ± 0.0	0 ± 0.0	3600 ± 5826.4	11,550 ± 1514.9	450 ± 353.6
Adult worm(♀)	0 ± 0.0	0 ± 0.0	5563 ± 7003.7	13,500 ± 2402.4	1163 ± 894.8
Larvae	0 ± 0.0	10,675 ± 7823.3	13 ± 25.0	25 ± 28.9	0 ± 0.0
EPG[a]	0	0	0	619	15
Eosinophils[b]	25 ± 6.4	18 ± 2.0	34 ± 4.6	54 ± 8.4[c]	46 ± 4.0[c]

[a] EPG data were taken prior to necropsy (not weekly mean).
[b] Mean numbers of eosinophils per 10 graticule fields per sample in the lamina propria.
[c] $P < 0.01$ based on unpaired *t* test.

Table 2
The genes significantly regulated in the bovine small intestine during *Cooperia oncophora* infection.

Sequence ID	Gene	Reference sequence ID	7 days p.i.	14 days p.i.	28 days p.i.	42 days p.i.
S00045630	AADAC	NM_001075791	0.83	0.45[a]	0.58	0.65
S00042890	ABCB4	XM_001251122	0.98	0.47[a]	0.89	1.03
S00038488	ABCC4	XM_610144	0.13[c]	0.86	1.13	0.69
S00019365	ABO	NM_001077926	0.92	0.79	0.76	0.42[c]
S00031144	ACOX2	NM_001102015	0.93	0.43[a]	0.74	0.73
S00036604	ADRA1B	XM_001250068	0.96	0.45[a]	0.45[a]	0.82
S00024510	ALDH8A1	NM_001075626	1.42	1.82	2.57	2.55[a]
S00021309	ALOX15	NM_174501	0.77	1.53	2.32[a]	1.14
S00021426	ALOX5	XM_613515	0.77	1.24	2.01[a]	1.63
S00020615	AMN	NM_001077041	1.23	0.48	0.67	0.40[a]
S00018801	ASB1	NM_001040445	1.1	2.11[a]	1.37	1.04
S00019902	ASPN	NM_001034309	1.41	1.22	2	2.12[a]
S00040188	AVIL	XM_599021	1.05	1.77	2.07[c]	1.44
S00029304	BCAS1	XM_001252931	0.73	1.68	1.82	2.12[a]
S00007942	BMPR1B	NM_001105328	1.59	1.09	2.17[a]	2.18[a]
S00039531	BVES	NM_007073	1.14	1.37	2.10[a]	1.45
S00028698	BZW1	XM_876130	0.93	2.35[a]	1.25	1.1
S00045253	BZW2	XM_584557	0.47	3.03[a]	0.71	0.7
S00003455	C1orf160	NM_001035451	0.48[a]	0.94	0.55	0.74
S00041335	C1RL	XM_584280	0.88	0.44[a]	0.7	0.61
S00019321	C20orf59	NM_001100378	0.83	2.00[a]	1.3	1.02
S00023831	C20orf70	NM_174803	0.79	0.37	0.07[a]	0.87
S00011367	C21orf45	NM_018944	1.01	1.98[a]	1.4	1.14
S00002913	C4B	NM_001002029	0.40[a]	0.53	0.73	0.62
S00034190	C9orf103	NM_001001551	0.91	0.36	0.69	0.72
S00031996	C9orf123	NM_033428	1.08	2.09[b]	1.66	1.4
S00022184	C9orf52	XM_585202	1.11	2.12[a]	1.32	1.19
S00017682	CA9	XM_866456	0.85	1.31	2.23[b]	1.4
S00028098	CAMLG	NM_001745	1.99[b]	0.94	1.06	1.16
S00044753	CAPN13	NM_001035360	0.92	1.68	3.55[b]	1.38
S00039707	CDH26	XM_869285	1.24	46.16[b]	157.19[c]	74.10[a]
S00039458	CEACAM1	NM_205788	0.72	0.48[a]	0.75	0.75
S00012717	CENPE	XR_027308	1.1	1.97	1.56	1.90[*]
S00028535	CES3	XM_590749	0.9	0.38[a]	0.64	0.62
S00045347	CHGA	NM_001275	0.49[b]	0.78	0.47	0.74
S00013159	CIB2	XM_869807	1.39	1.77	3.33[b]	1.86
S00038125	CLCA3	NM_181018	1.1	1.74	2.27[b]	1.93
S00012875	CNN1	NM_001299	2.18	5.03	4.91[a]	1.1
S00002286	COL1A1	NM_000088	0.21[a]	1.1	0.34	0.66
S00033748	COL29A1	NR_022012	1.11	1.36	2.91[a]	2.26
S00033044	CPA5	NM_001077905	1.22	0.43[a]	0.53	0.87
S00006020	CSF3R	NM_000760	0.97	0.37[b]	0.56	0.47[b]
S00035576	CYP2C18	NM_001076051	0.46[a]	0.74	0.65	0.64
S00017467	CYP4A11	NM_001077908	0.74	0.47	0.33[a]	0.51
S00006231	DDO	NM_173908	1.12	0.41[a]	0.66	0.58
S00006190	DGAT2	NM_205793	1.23	0.5	0.37[a]	0.39[a]
S00021489	DHX58	NM_001015545	0.8	0.73	0.39[a]	0.42
S00039731	DIMT1L	NM_001046036	1.3	3.03[a]	1.85[a]	1.59
S00034383	DIO2	NM_001010992	1.29	6.01[a]	4.19[a]	2.26[a]
S00044565	DR1	NM_001938	0.15[a]	1.42	0.41	0.54
S00008071	DUOX2	NM_014080	0.33	2.39	13.26[a]	4.56
S00019172	DUOXA2	XM_871774	0.65	2.37	8.34[a]	4.87
S00045542	ECSIT	NM_001034286	0.50[a]	1.5	1.08	0.77
S00044965	EDEM1	NM_014674	0.27[a]	1.11	0.45	0.7
S00014186	EFHB	XM_609023	1.27	1.29	2.10[b]	2.12[a]
S00012938	ENPP7	NM_178543	0.79	0.14[a]	0.48	0.33
S00025925	EOMES	XM_001251929	0.78	0.47[a]	0.35[b]	0.83
S00045013	FER1L6	XM_605757	1.22	1.41	2.01[a]	1.7
S00028191	FLVCR2	NM_017791	0.96	0.62	0.72	0.45[a]
S00045721	FOXD2	NM_004474	0.31[b]	0.55	0.73	0.38
S00030041	GCNT3	NM_205809	8.39	9.17[b]	5.67	10.52[a]
S00025058	GPD2	NM_001100296	0.93	2.05	2.26[a]	1.32
S00035688	GPR120	XM_865266	1.03	2.16[a]	1.61	0.92
S00022223	GPR155	NM_152529	0.7	0.44[a]	0.77	0.74
S00024408	HDC	NM_001024551	1.16	1.43	2.03[a]	1.58
S00021333	HIF3A	NM_001105342	1.02	0.26[a]	0.43	0.54
S00020518	HIST2H2AC	XM_583411	1.29	1.67	1.85	2.09[a]
S00041151	HLA-B	NM_005514	0.18[a]	0.54	1.21	0.23
S00002022	HLA-C	NM_001040498	0.94	2.18[a]	1.25	1.64
S00016178	HS6ST3	NM_153456	3.62[a]	0.75	6.17[c]	3.68
S00023477	HTATIP2	NM_001040563	1.16	2.12[a]	1.36	1.04
S00040774	HTR5B	NR_003947	0.79	0.47[a]	0.76	0.87
S00013263	IDH3A	NM_174644	0.76	2.46[a]	1.45	1.09
S00040506	IFI44	XM_872122	0.96	0.56	0.23[a]	0.33
S00036562	IGFBPL1	XM_001249651	2.07[a]	1.18	1.19	0.99
S00002156	IGLL1	NM_020070	1.98	1.64	2.09[a]	3.35[b]

Table 2 (*continued*)

Sequence ID	Gene	Reference sequence ID	7 days p.i.	14 days p.i.	28 days p.i.	42 days p.i.
S00021029	IL17RB	NM_018725	1.38	1.7	2.52[b]	2.07
S00042942	IL22RA1	NM_001034311	0.78	0.79	0.83	0.42[a]
S00019918	IL8RA	NM_174360	0.49[a]	0.94	0.92	0.95
S00006308	ISG15	NM_174366	0.98	0.55	0.22[a]	0.3
S00026647	ITGA4	NM_000885	0.96	1.26	1.37	2.49[b]
S00029472	KRT18	NM_199187	0.75	2.17[a]	1.2	0.84
S00000215	KRT7	NM_001046411	1.91	2.89[a]	1.71	1.47
S00008522	LGALS13	XM_593263	1.05	42.84[a]	65.52[b]	19.42
S00016590	LGALS3	NM_002306	1.76	1.51	2.09[a]	1.31
S00043675	LOC129607	XM_001252650	0.92	0.72	0.46[a]	0.40[a]
S00031966	LOC388335	NM_001004313	1.12	0.45[a]	0.57	0.61
S00035519	LOC651536	XM_940712	0.96	1.09	1.24	2.12[a]
S00014308	LRP8	NM_001097565	1.01	2.21[a]	1.24	1.05
S00033476	LRRC17	NM_001078150	1.21	0.48[b]	0.75	0.82
S00046170	MAMDC4	XM_593390	0.73	0.58	0.28[a]	0.49
S00029982	MCPH1	NM_001046004	1.43	1.77	3.43[b]	2.91[b]
S00006879	MIAT	NR_003491	0.76	1.13	2.17[a]	1.52
S00029985	MLZE	NM_001046004	1.18	1.67	3.30[b]	2.41[a]
S00036911	MS4A10	XM_582634	1.21	0.61	0.69	0.39[a]
S00026804	MX2	NM_173941	0.83	0.47	0.16[a]	0.32
S00038813	MYADM	NM_138373	0.89	6.09[a]	7.78	8.02
S00034117	NAP5	NM_207363	0.98	0.37[a]	0.81	0.8
S00043160	NARG1L	NM_024561	0.25[c]	1.27	1.12	1.28
S00009514	NEB	XM_613028	0.75	2.79[b]	4.63[c]	4.49[a]
S00032999	NIPA1	NM_144599	0.86	0.46[a]	0.71	0.58
S00022414	NXPH2	NM_174406	0.84	0.31[b]	0.36[a]	0.62
S00021414	NXPH3	XM_614523	0.85	1.17	2.37[a]	1.33
S00014865	OAS1	NM_001029846	1.05	0.78	0.42[a]	0.45[a]
S00025243	P2RX1	NM_002558	0.95	2.99[b]	3.02[a]	1.98
S00031595	PGF	NM_002632	1.14	0.45	0.54	0.44[a]
S00023224	PLK4	NM_001083427	0.89	2.01[a]	1.51	0.98
S00041447	PNPLA3	XM_583061	1.45	3.2	2.18[a]	1.32
S00013150	POLQ	NM_199420	1.04	2.09[a]	1.28	1.17
S00027634	PPFIBP2	NM_003621	3.58[a]	1.49	3.45[a]	2.33
S00009921	PPP1R14C	NM_001078068	0.68	1.91[a]	1.19	0.96
S00045633	PPTC7	XM_001107564	0.55	3.35[a]	5.56[b]	1.2
S00001986	PRSS2	NM_174690	8.48	36.55[b]	0.29	21.56[a]
S00034132	PTPN5	NM_001102293	0.67	0.39	0.47	0.27*
S00000831	PTPRN2	NM_130842	1.04	2.15[a]	1.51	1.37
S00030297	PYGM	NM_175786	1.19	1.28	2.35[a]	1.48
S00029595	RARRES2	NM_001046020	0.88	0.51[a]	0.74	0.7
S00042017	RGS4	NM_001046600	0.67	0.50[a]	0.79	1.21
S00034109	RND2	XM_587874	0.71	0.41	0.58	0.44[a]
S00037417	RP11	NM_207299	1.22	1.62	2.79[a]	1.22
S00000760	RPL38	NM_001035258	0.51[a]	0.92	1.73	1.06
S00028083	SAA1	NM_001075260	0.30[a]	0.22[b]	0.64	0.67
S00030236	SAA2	NM_181016	0.25	0.15[a]	0.43	0.57
S00020002	SCD	NM_173959	1.09	2.18[a]	1.91	1.46
S00003006	SERPINA5	NM_176646	1.04	0.6	1.05	0.51[a]
S00028312	SERPINB5	NM_002639	1.89	4.95[a]	2.03	1.9
S00007318	SFTPD	NM_001001856	1.15	9.03	41.75[a]	18.51
S00009139	SH2D6	NM_198482	0.95	1.76	3.24[a]	1.73
S00038017	SI	NM_001114189	0.42[a]	0.87	0.64	0.9
S00033464	SLC14A1	NM_001008666	0.82	0.5	0.47[b]	0.87
S00041150	SLC27A6	NM_001101169	1.33	0.41[b]	0.85	0.98
S00019646	SLC35A2	NM_176640	1.09	1.98[a]	1.12	0.84
S00007213	SLC35C1	NM_001101210	1.22	2.04[a]	1.61	1.04
S00008646	SLC38A1	XR_027995	0.94	2.97[a]	1.49	1.83
S00032437	SLCO2A1	NM_005630	0.87	0.66	0.81	0.47[c]
S00044767	SQLE	NM_001098061	0.16[a]	2.17	1.25	0.56
S00023096	ST3GAL1	NM_003033	1	1.44	2.66[a]	2.21
S00020322	TCEA3	NM_001046360	0.97	0.52[a]	0.84	0.77
S00010616	TCN1	XM_868213	0.67	2.21	4.47[a]	2.89
S00040967	TEP1	XM_582150	0.75	0.83	0.71	0.46[a]
S00006291	TIPIN	NM_001105336	0.59	0.86	0.46[a]	0.63
S00027948	TLR10	NM_001076918	0.93	0.49[b]	0.75	1.09
S00023092	TMEM151A	NM_001083782	0.79	0.46	0.38	0.33[a]
S00014980	TMEM59L	NM_001075301	0.74	1.1	0.49[a]	0.86
S00003903	TMEM66	NM_001076856	0.6	2.55[a]	0.73	1.04
S00017494	TMEM86A	NM_001035388	0.67	0.77	0.62	0.42[b]
S00021360	TNFSF9	NM_003811	1.09	3.05[a]	1.29	1.27
S00026694	TNS4	NM_001038155	1.04	3.62[a]	2.20[a]	1.39
S00046461	TRIM40	NM_138700	17.66[a]	1.2	2.49	1.15
S00011455	TRPM6	XM_605991	0.72	0.78	0.34[a]	0.47
S00016533	UBD	NM_006398	0.67	0.67	1.04	0.48[a]
S00003157	USP18	NM_001017940	0.8	0.58	0.28[a]	0.36
S00019176	VNN3	NM_001024556	1.05	0.43[a]	0.73	0.72

(*continued on next page*)

Table 2 (*continued*)

Sequence ID	Gene	Reference sequence ID	7 days p.i.	14 days p.i.	28 days p.i.	42 days p.i.
S00021046	YPEL2	XM_001251606	0.87	0.50[a]	0.74	0.88
S00023796	ZNF71	NM_021216	2.83	3.39[a]	3.53	2.94[a]

Numbers represent fold with control = 1.0. The values smaller than 1.0 indicate down-regulation.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

on the microarray and the expressed sequence tag (EST) sequences from which the 60 mer oligos were designed for all 310 sequences. A separate search using *tblastx* was conducted for those sequences without any significant hits by the nucleotide search using the BLAST algorithm using *E value* $<10^{-10}$ as a cutoff. After removing redundancy in which a gene was represented by multiple sequences, a total of 155 genes with annotation and approved gene symbols were identified (Table 2). These genes were subjected to the pathway and network analysis discussed below.

The expression of 12 selected genes was verified using *quantitative PCR* (Supplementary Table S2). Consistent with the microarray results, expression of TNFα, IL-1β and IL-6 remained unchanged during the infection (data not shown). However, expression of IL-4, IL-5 and IL-13 was up-regulated starting at 14 days p.i. (Fig. 1).

Expression of selected cell adhesion molecules, such as CDH26, collectin-46 and galectin OVGAL11 (LGALS13), was strongly up-regulated starting at 14 days p.i. based on the quantitative PCR results, in a good agreement with the microarray results. The expression of CDH26 strongly correlated with eosinophil numbers (Fig. 2) and adult worms ($R = 0.822$ and 0.839, respectively).

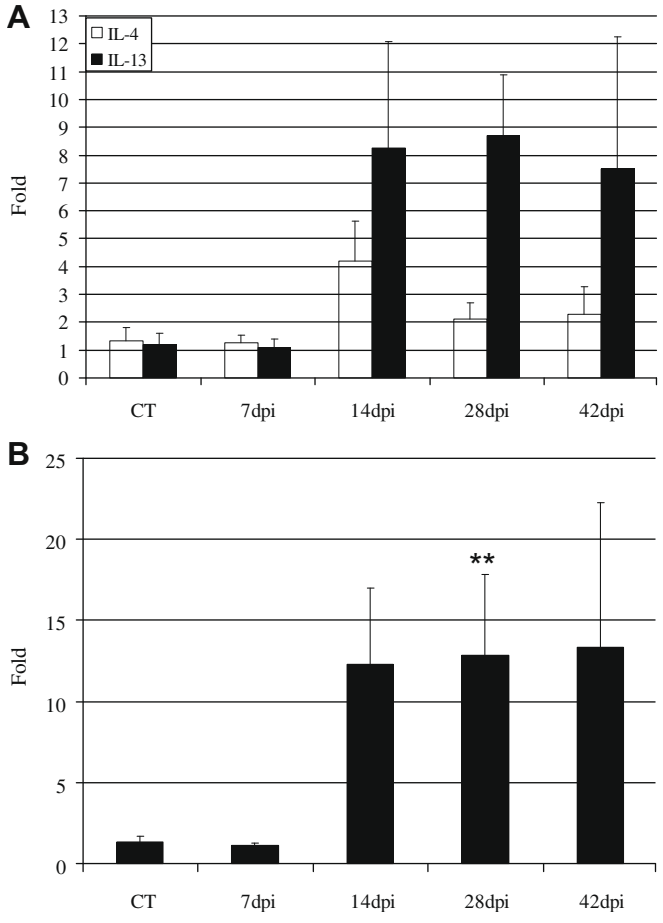

Fig. 1. The cytokine expression profiles in the bovine small intestine detected using quantitative PCR during *Cooperia oncophora* infection. Mean fold changes ($n = 4$) are presented with SEM as error bars. (A) IL-4 and IL-13. The expression of IL-13 was statistically significant ($P < 0.05$) at 14, 28 and 42 days p.i. (dpi). (B) IL-5. **Statistically significant at $P < 0.01$. CT, control.

3.3. Pathways and regulatory networks

A total of 22 canonical pathways were significantly impacted ($P < 0.05$) during the infection. Fig. 3 lists the top six canonical pathways regulated at each time point. At 7 days p.i., the infection dampened the acute phase response and the complement system as evidenced by down-regulation of the genes involved. At 14 days p.i., the top pathway affected based on the level of significance was sphingolipid metabolism. Three out of the six pathways affected were associated with RXR functions at this time point including activation of both PXR/RXR and TR/RXR. When the infection progressed to 28 days p.i., the majority of the pathways impacted were related to lipid metabolism and eicosanoid signalling. By 42 days p.i., the canonical pathways affected were glycosphingolipid biosynthesis and TGF-β signalling.

Nine regulatory networks with an IPA score greater than 10, indicating a less than 10^{-10} chance that the genes in the network are associated together solely due to random events, were identified during the course of infection (Fig. 4). These networks were generally associated with certain predominant molecular functions. For example, at 14 days p.i., molecular or cellular functions associated with all three networks included tissue morphology and inflammation. At 28 days p.i., inflammation, cell-to-cell signalling and interaction, and cellular growth and proliferation were some of the functions associated with the networks. By 42 days p.i., the network functions shifted to cellular movement and development.

4. Discussion

While host responses to *Ostertagia ostertagi*, a parasitic nematode causing considerable damage to the abomasum, have been well defined, the cytokine profiles and host response to *C. oncopho-*

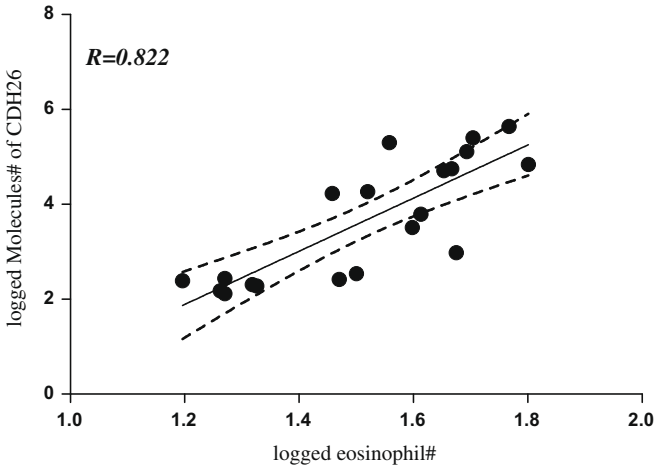

Fig. 2. Linear regression analysis between the number of cadherin-like 26 (CDH26) molecules and the number of eosinophils ($n = 20$). The dashed lines represent 95% Confidence Interval.

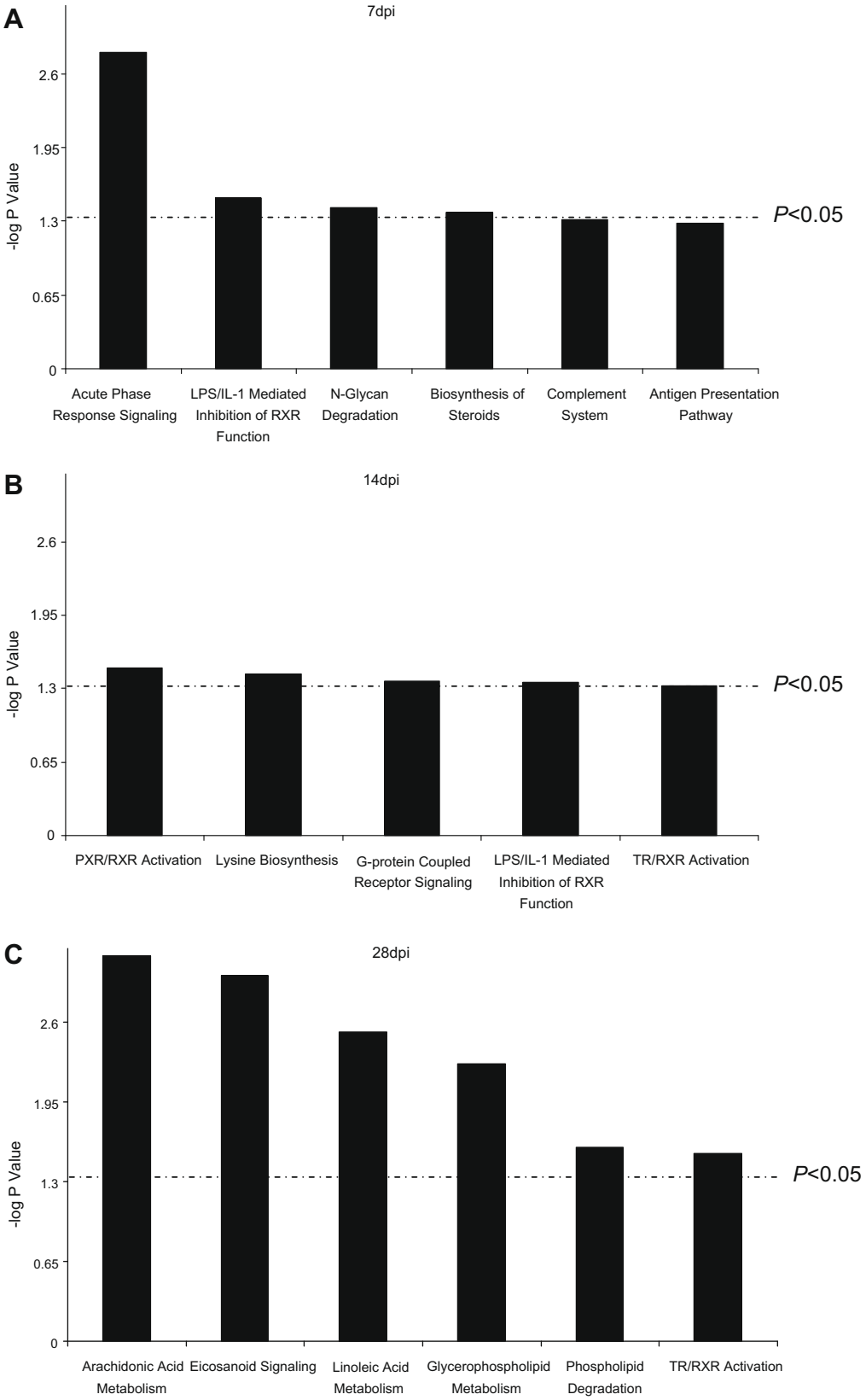

Fig. 3. Top six canonical pathways impacted during *Copperia oncophora* infection based upon the levels of significance. (A) 7 days p.i.; (B) 14 days p.i.; (C) 28 days p.i.; (D) 42 days p.i.

ra infection in cattle have not been extensively investigated. For example, in the abomasal lymph nodes, *Ostertagia* infection induced a decrease of $CD3^+$ cells and an increase in the percentage of IgM^+ cells (Canals et al., 1997). These changes in cell populations coincided with a strong up-regulation of Th2 cytokines (IL-4, IL-5, IL10 and IL-13) while Th1 cytokines, such as IL-2 and IFNγ, were

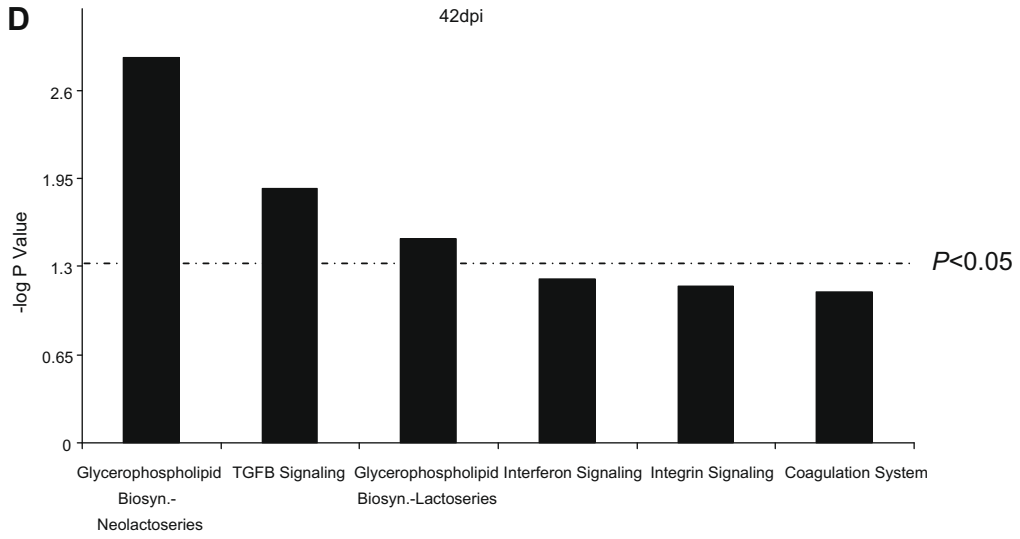

Fig. 3 (*continued*)

significantly decreased (Canals et al., 1997; Claerebout et al., 2005). In the abomasal mucosa, both IFNγ and cytokines were strongly up-regulated after infection (Almeria et al., 1997; Claerebout et al., 2005). In the cattle-*Cooperia* system, Kanobana et al. (2001) analysed differential responses of host responder types to experimental challenge and found that expulsion of adult worms was associated with significant increases in mucous IgA and IgG1 as well as an influx of eosinophils, independent of mast cells in the small intestine. In a typical Th2 response, as defined based mainly on rodent models, mucosal mast cells tended to be one of the major effector mechanisms. Our results indicate that while both Th2 cytokines IL-4 and IL-13 were strongly up-regulated during the course of infection starting at 7 days p.i., IFNγ remained largely unchanged, providing another piece of evidence that the distinct Th1/Th2 response types may not apply to gastrointestinal nematodes in cattle. Kanobana et al. (2003a,b) also characterized different T and B cell populations and their respective functions in the generation of protective immunity against *C. oncophora*. However, global expression profiles and pathways induced during *C. oncophora* infection have not been studied.

In the present study, we systematically analysed expression profiles, biological processes and pathways as well as regulatory networks impacted in the bovine small intestine during *C. oncophora* infection. We specifically compared the temporal shifts in expression patterns during the course of infection. Parasite infection impacted host immune responses significantly at all four time points tested (Fig. 5). However, underlying mechanisms were completely different. At 7 days p.i., the main processes related with immune response were rolling, homing, chemotaxis of leukocytes and complement activation, as indicated by the regulation of key genes in these processes. Parasite infection suppressed two canonical pathways: the acute phase response and the complement system, suggesting that the ability of the host to elicit and better maintain inflammatory responses at the sites of infection during the early phase of infection may render the host resistance or protective immunity (Li et al., 2007a,b). At 14 days p.i., the immune response shifted to priming and induction of lymphocytes, cell movement of leukocytes, neutrophil release and migration of dendritic cells as well as natural killer cells. At 42 days p.i., the major immune mechanism included proliferation of B-1 lymphocytes, rolling and accumulation of granulocytes, and rolling of eosinophils. In addition, migration of mast cells, eosinophil/granulocytes trafficking, eosinophil rolling and egression, neutrophil release and chemotaxis, and lymphocyte production became dominant at this point. Coinciding with these different mechanisms of immune responses, different sets of genes linked to inflammation, such as integrin, alpha 4 (ITGA4) and PGF, were regulated during the course of infection.

Our results also suggested that lipid metabolism was one of many biological processes that were significantly regulated at all four time points ($P < 0.05$). At 14 days p.i., the top canonical pathway impacted, based upon the level of significance related to lipid metabolism, was sphingolipid metabolism. At 28 days p.i., a majority of the pathways impacted were related to lipid metabolism, including phospholipid degradation, and eicosanoid signalling, and the metabolism of arachidonic acid, linoleic acid metabolism and glycerophospholipid (Fig. 3), as evidenced by the up-regulation of the key genes in the pathways, such as arachidonate 5-lipoxygenase (ALOX5) and arachidonate 15-lipoxygenase (ALOX15). At 7 days p.i., although no canonical pathway related with lipid metabolism was significantly affected, the genes regulating two major classes of molecules, cholesterol and retinoic acid, such as serum-amyloid A-like (SAA1) and endoplasmic reticulum (ER) degradation enhancer, mannosidase alpha-like 1 (EDEM1) as well as squalene epoxidase (SQLE), were significantly impacted. At 14 days p.i., SLC27A6, a gene involved in the uptake of polyunsaturated fatty acids (PUFA), linoleic acid and oleic acid, was down-regulated, as were genes involved in sphingolipid metabolism. By 28 days p.i., membrane-bound or lipid raft-associated phospholipids were beginning to be degraded to form arachidonic acid (a PUFA). Subsequently, eicosanoids, such as leukotrienes and prostaglandins, were generated. Lipoxin A4, leukotrienes and prostaglandins (PG) are known to induce inflammation. PG is also known to play an important role in promoting IgE and IgG production by synergizing with IL-4 while inhibiting IgM production and skewing Th1/Th2 balance (Roper et al., 1995, 2002). By 42 days p.i., the gene responsible for biosynthesis of triacylglycerol (DGAT2) was down-regulated. Because existent phospholipids are utilised to synthesize eicosanoids and enhance inflammatory response, it is possible that lipid balance in the gastrointestinal tract during *C. oncophora* infection is disrupted and that lipids, especially PUFA, are deficit. Indeed, PUFA, especially those in omega-3 ($n-3$) and omega-6 ($n-6$) families, such as docosahexaenoic acid (DHA), arachidonic acid and linoleic acid, have long been known to have strong immunomodulatory effects (Hwang, 2000) and dietary supplements of these molecules have been shown to alter lymphocyte functions in rats. Macrophages from fish oil-fed mice showed significantly

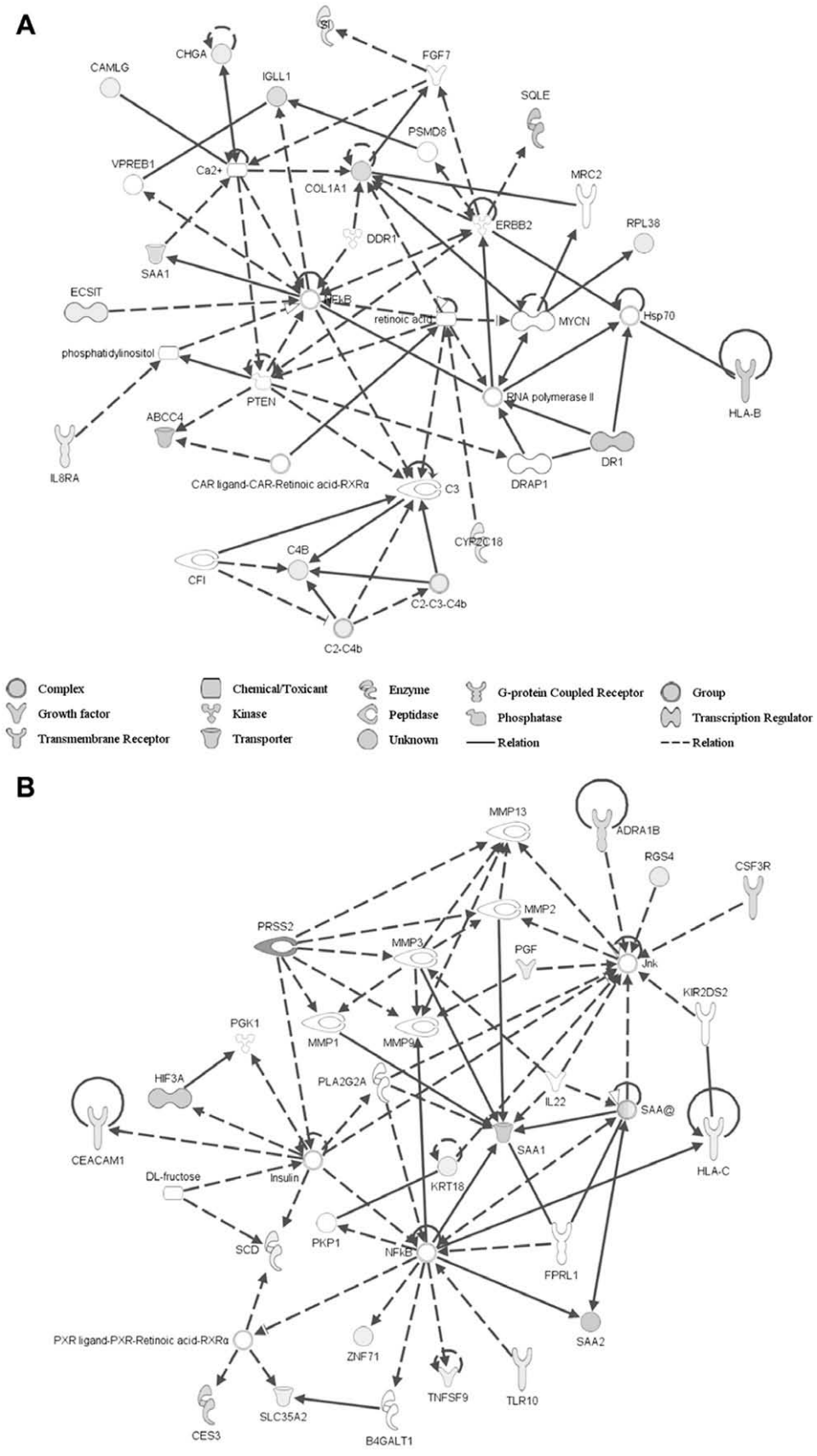

Fig. 4. A temporal shift in selected regulatory networks during *Cooperia oncophora* infection. (A) 7 days p.i. (score = 40, number of Focus Genes = 15); (B) 14 days p.i. (score = 36, number of focus genes = 17). One of the major cellular functions associated with this network was inflammatory disease; (C) 28 days p.i. (score = 25, number of focus genes = 12). Network functions included cellular growth and proliferation and cell-to-cell interaction; (D) 42 days p.i. (score = 28, number of focus genes = 12). The major cellular functions associated with this network included cellular development and tissue morphology.

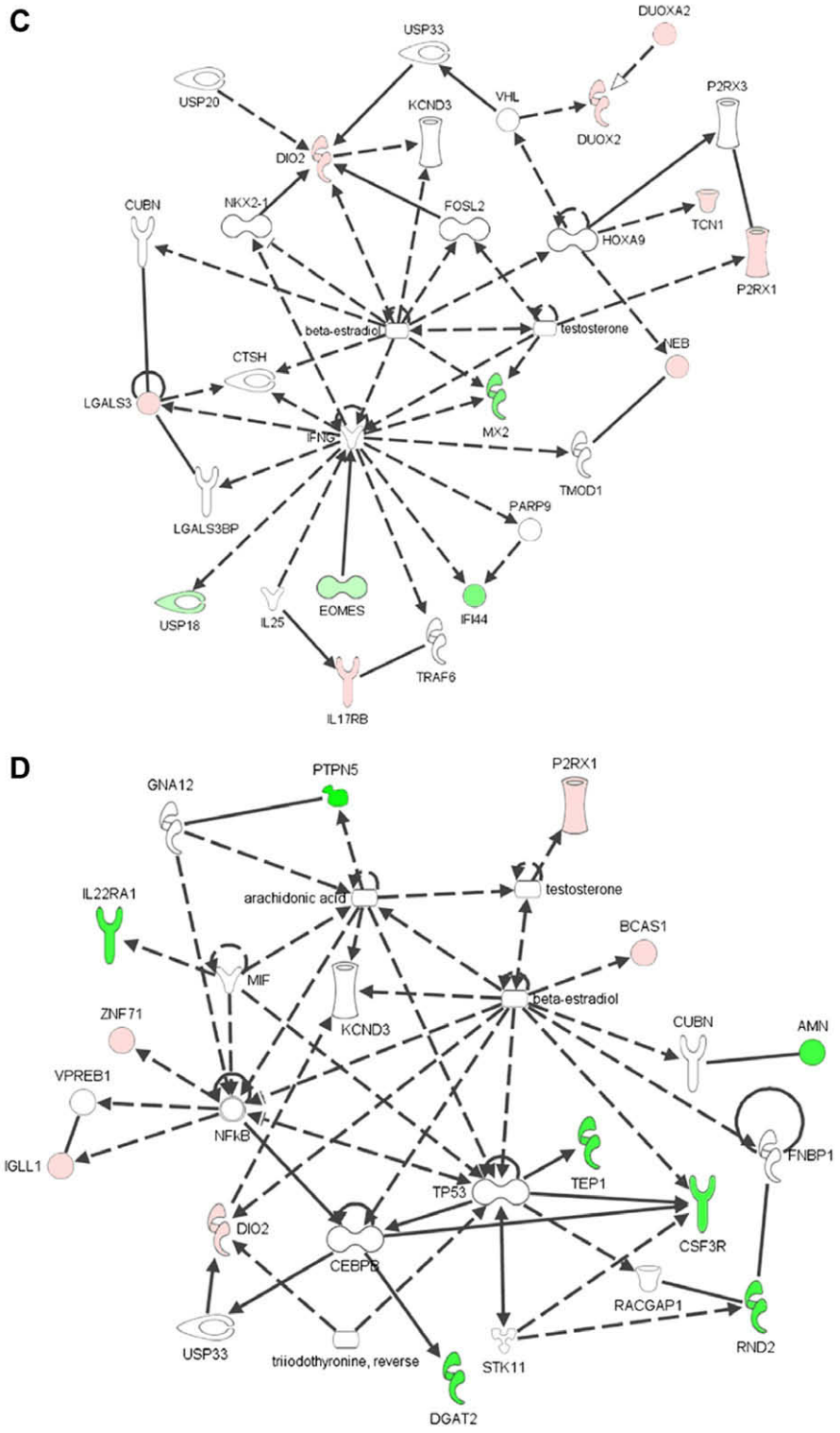

Fig. 4 (*continued*)

lower production of TNFα and higher production of nitrite than those from mice fed other diets (Wallace et al., 2000), suggesting that omega-3 PUFA may serve as a potent inhibitor for Th1 response. In ruminants, Muturi et al. (2005) investigated the effect of dietary PUFA on calves infected with both *O. ostertagi* and *C. oncophora*. Their results indicated that total fecal egg counts after the infection were decreased by 24% (not statistically significant) by fish oil (omega-3 PUFA) treatment. The numbers of mucosal mast cells and eosinophils were significantly lower in the fish oil group after infection compared with the groups that were infected and fed non-supplemented diets. More importantly, the number of intestinal immature parasites was significantly higher in the infected group fed with fish oil, suggesting that omega-3 fatty acids may indeed enhance protective immunity against *C. oncophora* via affecting worm development that resulted in an inhibition in the maturation of the L3s and a reduction in egg production. Our re-

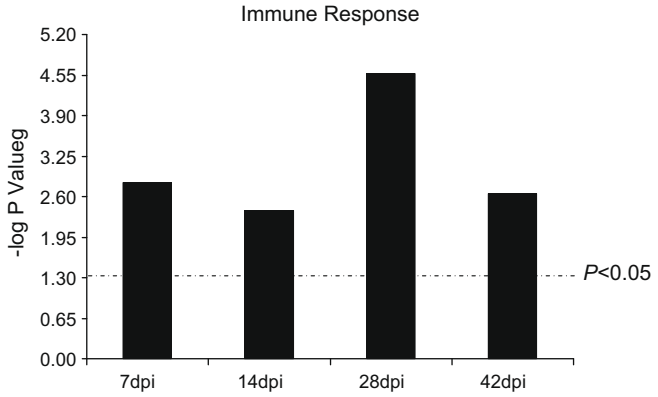

Fig. 5. Immune response affected during *Cooperia oncophora* infection based on the level of significance. dpi, days p.i.

sults provided possible molecular explanations of immunomodulatory effects of polyunsaturated fatty acids during the infection of parasitic nematodes in cattle.

Several canonical pathways associated with RXR were significantly impacted during *C. oncophora* infection. At both 7 and 14 days p.i., a pathway called LPS/IL-1β mediated inhibition of RXR functions was regulated. TR/RXR activation was significantly impacted at 14 and 28 days p.i., respectively. The relationship between these pathways and host immune response to gastrointestinal nematodes remains unclear at this point. However, it is known that retinoids inhibit lipopolysaccharide (LPS) stimulated production of IL-12 (a Th1 cytokine) in a dose-dependent manner via ligand-independent binding of nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) to RXR (Na et al., 1999). In addition, retinoic acid can inhibit cytokine expression in hepatic macrophage including reduction of mRNA levels of TNFα, nitric oxide synthase 2A (inducible, hepatocytes) (NOS2A, iNOS), IL-6, and IL-1β (Motomura et al., 2001). Recently, it has been observed that LPS-specific regulatory networks in which NF-κB played a focal role in the mouse submucosa overlapped with the LPS/IL-1β mediated inhibition of RXR functions (Saban et al., 2008). RXR is known to be capable of mediating retinoid signalling and may play critical roles in development, adult physiology and metabolic processes by partnering with a number of nuclear receptors to form functional heterodimers (Szanto et al., 2004). Also, 9-*cis* retinoic acid and docosahexaenoic acid (DHA) were suggested to be endogenous RXR ligands (de Urquiza et al., 2000). It is possible that regulation of RXR functions was an indirect effect resulting from the host lipid metabolism (including DHA) as discussed in the previous paragraph. In addition, the canonical pathway PXR/RXR activation was impacted at 14 days p.i. PXR is the so-called xenobiotic receptor. Recent evidence demonstrated mutual repression between PXR/RXR and NF-κB pathways, providing a possible link between xenobiotic response and inflammation (Zhou et al., 2006).

Cell adhesion molecules such as cadherins, integrins and lectins play essential roles in mediating cell–cell adhesion in virtually every aspect of a multicellular organism's life including pathogen recognition and host defence. For example, lectins, including those binding B-galactoside moieties (galectins), play an important role in activation of immune response and local inflammation via specific yet reversible binding of cell surface carbohydrates (Lasky, 1991; Drickamer and Taylor, 1993). At least five cell adhesion molecules were strongly up-regulated during *C. oncophora* infection in this study. These molecules include the α4 integrin (ITGA4), a molecule partially responsible for eosinophil homing (Rothenberg et al., 2001) and galectin OVGAL11, a gene recently cloned (Dunphy et al., 2000) from sheep abomasa infected with *Haemonchus contortus* (a common parasitic nematode having a similar enteric life cycle as *C. oncophora*). The difference in expression pattern of galectin between the sheep abomasum infected with *H. contortus* and the bovine small intestine infected with *C. oncophora* was obvious: the gene in the former case was up-regulated as early as 2 days p.i. while the gene in the latter was not up-regulated until 14 days p.i. In addition, adult worms did not induce expression of this gene in the sheep abomasums while in the bovine small intestine, the gene remained strongly up-regulated until 42 days p.i., which suggested that the expression of this gene was indeed directly induced by the parasite and not due to tissue damage or inflammatory response. As members of a Ca^{++}-dependent lectins (C-type lectins) family, collectins act as sentinels of innate immunity via their capability to recognise pathogen-associated molecular patterns (PAMPs) on foreign organisms (Gupta and Surolia, 2007). Upon recognition of the PAMPs, collectins inhibit infection by direct opsonization, neutralisation, agglutination, complement activation and phagocytosis, as well as acting as modulators of inflammatory and allergic responses and apoptosis. Collectin-46, which is expressed mainly in the bovine liver, thymus, mammary gland and gastrointestinal tract (Hansen et al., 2002), was strongly up-regulated during infection starting at 14 days p.i. and reaching a plateau at 28 days p.i. in this study. Another molecule that demonstrated a similar expression profile was CDH26, whose expression was up-regulated several 100-fold by quantitative PCR at 28 days p.i. The function of CDH26 remains unknown. However, CDH 26 was strongly up-regulated in patients with rheumatoid arthritis (Nakamura et al., 2006) and eosinophilic esophagitis, an inflammatory condition in which the wall of the oesophagus becomes filled with large numbers of eosinophils (Blanchard et al., 2007). Most recently, two functional oestrogen response elements (ERE) were identified in the 20 kb promoter region around the transcription start site (TSS) of CDH26, which was up-regulated by oestrogen and its regulation was under the control of ERα (Bourdeau et al., 2008). In the present study, the molecule numbers of CDH26 positively correlated with both the number of eosinophils and adult parasites in the tissue. Due to their similar overall expression profiles, it is possible that all three cell adhesion molecules, galectin OVGAL11, collectin-46 and CDH 26, were involved in recognition of carbohydrates on the cell surface of the nematodes in ruminants.

In conclusion, we presented evidence that there was a temporal shift in global expression profiles, regulatory networks and pathways during the course of *C. oncophora* infection in the bovine small intestine. Our results suggested different mechanisms of host immune responses at various time points p.i., coinciding with key life cycle stages of the parasites. Our data provided possible molecular explanations of immunomodulatory effects of polyunsaturated fatty acids during the infection of parasitic nematodes in cattle. While much direct evidence is still needed, it became clear that cell adhesion molecules such as galectins, collectins and CDH 26 may be involved in recognition of pathogen-associated molecular patterns in gastrointestinal nematodes in ruminants. Our results will undoubtedly provide a molecular roadmap for future studies to define host immune response and identify effector mechanisms of protective immunity against nematode infection in the gastrointestinal tract.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2008.11.007.

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