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Transcriptomic analysis of immunity in rainbow trout (*Oncorhynchus mykiss*) gills infected by *Ichthyophthirius multifiliis*

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ABSTRACT

The parasite *Ichthyophthirius multifiliis* infecting skin, fins and gills of a wide range of freshwater fish species, including rainbow trout, is known to induce a protective immune response in the host. Although a number of studies have reported activation of several immune genes in infected fish host, the immune response picture is still considered incomplete. In order to address this issue, a comparative transcriptomic analysis was performed on infected versus uninfected rainbow trout gills and it showed that a total of 3352 (7.2%) out of 46,585 identified gene sequences were significantly regulated after parasite infection. Of differentially expressed gene sequences, 1796 genes were up-regulated and 1556 genes were down-regulated. These were classified into 61 Gene Ontology (GO) terms and mapped to 282 reference canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Infection of *I. multifiliis* induced a clear differential expression of immune genes, related to both innate and adaptive immunity. A total of 268 (6.86%) regulated gene sequences were known to take part in 16 immune-related pathways. These involved pathways related to the innate immunity such as the Chemokine signaling pathway, Platelet activation, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, and Leukocyte transendothelial migration. Elevated transcription of genes encoding the TLR 8 gene and chemokines (CCL4, CCL19, CCL28, CXCL8, CXCL11, CXCL13, CXCL14) was recorded indicating their roles in recognition of *I. multifiliis* and subsequent induction of the inflammatory response, respectively. A number of upregulated genes in infected gills were associated with antigen processing/presentation and T and B cell receptor signaling (including B cell marker CD22 involved in B cell development). Overall the analysis supports the notion that *I. multifiliis* induces a massive and varied innate response upon which a range of adaptive immune responses are established which may contribute to the long lasting protection of immunized rainbow trout.

1. Introduction

The parasitic ciliate *Ichthyophthirius multifiliis* is the causative agent in one of the most problematic fish diseases among freshwater fishes, white spot disease, which is associated with high morbidity, mortality and significant economic losses in the aquaculture industry worldwide [1–3]. This parasite can infect fins, skin and gills of almost all species of freshwater fish, including rainbow trout [1,4,5]. Fish surviving the infection may develop protective immunity [3,6] which suggests that immunoprophylactic control of the disease through vaccination is feasible. A limited number of genes related to the immune system of rainbow trout has been confirmed involved in the host response to infection [7–12], but recent progress within transcriptome profiling allows a far higher resolution of the immune response picture of rainbow trout exposed to *I. multifiliis*. RNA-Seq analysis is a powerful tool to investigate the functional complexity of transcriptomes in many organisms [13] and the technology allows an in-depth understanding of physiological processes [14] including immune-related genes in the fish [15]. Transcriptomic approaches have been successfully used in analyzing host immune reactions to parasite infection in large yellow croaker [16,17], orange-spotted grouper [18] and Tibetan highland fish [19]. Extensive analysis at the transcriptome level with regard to *I. multifiliis* infection in the latter fish species showed an innate immunity profile as response to the infection with focus on toll-like receptors (TLRs), cytokines and complement components in the host defense [19]. We have applied a transcriptomic analysis for an in-depth analysis

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of the immune response mechanism in rainbow trout gills following exposure to the parasite. Key immune genes with roles in the mucosal immune response were identified and could be assigned to 16 immune-related pathways associated with both adaptive and innate factors. The basic and detailed information on the immune mechanism of rainbow trout provided by the present study may support future applied research on immunoprophylactic approaches against *I. multifiliis* infection.

2. Materials and methods

2.1. Ethics statement

All experiments were conducted at Laboratory of Aquatic Pathobiology Facilities at the University of Copenhagen and were performed in accordance with and approved by the Experimental Animal Inspectorate under the Ministry of Food, Agriculture and Fisheries (license: 2013-15-0201-00764).

2.2. Experimental fish and housing conditions

Rainbow trout fry were hatched from certified and disinfected eyed eggs (Fousing strain, Jutland, Denmark) at 7 °C and reared (12–13 °C) at the Bornholm Salmon hatchery (Nexø, Denmark) (pathogen-free recirculated closed system). Fish were transported (3 h duration in oxygenated containers) from the hatchery to our experimental fish facility (water temperature of 12–14 °C) where they were kept (30 d) in 200 L fish tanks until initiation of the experiment. The body weight of the fish varied from 5.5 g to 8.5 g and the total body length ranged from 8.0 cm to 9.5 cm. Two weeks prior to the experiment, rainbow trout were transferred to a thermostat controlled room (pre-set at 15 °C) and acclimated in 60 L tanks containing freshwater (municipal tap water, Frederiksberg) equipped with internal biofilters (Eheim, Germany), plastic plants (enrichment), and continuous aeration using air stones. Water (50%) in tanks was changed daily during the experiment. Fish were fed every second day (1% of biomass) using a commercial pelleted feed (Biomar, Denmark). The experiment was performed in duplicate. A total of 80 fish were randomly distributed in 4 tanks whereby each aquarium contained 20 fish. Throughout the experimental period, the water temperature was kept at 15–16 °C (all tanks measured by hand-held thermometers), temperature variation was kept at a minimum by using acclimatized water for replenishment and water quality (NH_3 < 0.5, NO_2 < 0.1, pH 7.6) was monitored with standard test kits (Merck, Germany).

2.3. Infection procedure

Fish in two fish tanks (each containing 20 fish) were exposed (day 0) to infective theronts by adding a solution of parasites (2400 theronts/fish). Corresponding tanks were sham exposed by pouring a similar volume of pure water into the tanks. Theronts were produced from tomocysts developed from tomonts released from infected fish using a procedure as described by Sigh et al. [7,8]. Food was withheld 24 h prior to infection and during the infection procedure (24 h duration). Internal biofilters were shut off and fish-rearing water was not changed only during the infection procedure whereafter feeding was reinstalled but kept at a minimum (0.5 of the biomass daily).

2.4. Sampling

Samples for transcriptomic analysis were collected from fish in each tank (control and infected group) at day 8 after exposure. At this time point the parasites in the epidermis (the trophont stage) of the infected fish is near their maximal development but not yet ready to burst out of the fish host. Fish were collected by a hand-net, rapidly euthanized by immersion into an overdose MS-222 (300 mg/L) followed by dissection and tissue sampling. Gill samples (infected by trophonts) were dissected under the dissection microscope and subsequently placed into 2 mL tubes containing RNA*later*® (Cat. No. R0901, Sigma-Aldrich). Samples were pre-stored at 4 °C for 24 h and then stored at −80 °C until RNA purification.

2.5. RNA extraction, library construction and sequencing

Tissue samples placed in 1.5 mL tubes containing Lysis Solution and 2-mercaptoethanol were homogenized by TissueLyser II (Qiagen, USA). Total RNA was extracted using a RNA purification kit (Cat. No. RTN350-KT, Sigma-Aldrich), according to the manufacturer's instruction and subsequently treated with DNase I (Cat. No. EN0521, Thermo Scientific, USA). The purified RNA was stored at −80 °C until further analysis. QPCR analysis was conducted to elucidate expression of selected immune genes and to confirm the presence of parasites in infected gills samples (see *2.7*). In addition, total RNA was sent out for sequencing provided by ZF-Genomics, Leiden-Netherlands. Quality and integrity of the total RNA were for that purpose checked on an Agilent Bioanalyzer 2100 total RNA Nano series II chip (Agilent, Amstelveen, Netherlands). Illumina RNAseq libraries were prepared from 500 ng total RNA using the Illumina TruSeq™ Stranded mRNA LT Sample Prep Kit according to the manufacturer's instructions (Cat. No. RS-122-2101, Illumina Inc. San Diego, CA, USA). All RNAseq libraries (150–750 bp inserts) were sequenced on an Illumina HiSeq2500 sequencer as 1 × 50 nucleotides single-end reads according to the manufacturer's protocol. Image analysis and base calling were done using the Illumina pipeline.

2.6. RNA-seq validation using qPCR

In order to confirm the RNA-seq results, eight immune-related genes which were chosen based on our previous confirmed role in rainbow trout immune responses to Ich, were analyzed. The qPCR analysis was conducted using the same total RNA used in RNA-seq analysis. RNA concentration was determined using NanoDrop 2000 (Thermo Scientific, USA) and the quality of RNA was assessed using 2% agarose gel electrophoresis with ethidium bromide (EtBr) staining.

cDNA synthesis was performed on 1000 ng total RNA in a 20 µl setup using MultiScribe Reverse Transcription reagent following the manufacturer's instructions (Thermo Fisher Scientific, Lithuania). Total RNA was reversely transcribed using random hexamers (Roche, Switzerland). Reactions were performed in a T100™ Thermal Cycler (BioRad, USA) under the following conditions: 25 °C for 10 min, 37 °C for 60 min, and 95 °C for 5 min. The synthesized cDNA was diluted ten times using RNase free water (Life Technologies, UK) and subsequently stored −20 °C until further use.

Quantitative PCR assays were performed in an AriaMx Real-Time PCR system (Agilent Technologies, USA) using the Brilliant® II QPCR master mix (Stratagene, USA). The synthesized cDNA was used as a template for qPCR analysis with specific primer and corresponding probe designed for the selected genes (Supplementary Table S1). The qPCR reactions were carried out in a 12.5 µl total reaction volume consisting of 6.25 µl of Brilliant® II QPCR master mix (Agilent Technologies, USA), forward and reverse primer (0.8 µM each), TaqMan® probe (0.4 µM), 2.75 µl DNase/RNase free water, and 2.5 µl cDNA template (5 ng/µl). The PCR conditions were 95 °C for 15 min followed by 45 cycles of 95 °C for 10 s and 60 °C for 45 °C. In order to detect contamination during assays, negative controls without template and reverse transcriptase were run in each assay. The elongation factor 1-α gene (EF1-α) was used as an internal control (reference gene).

2.7. Data analysis

Reads were aligned to the rainbow trout reference genome (http://www.genoscope.cns.fr/trout/data/) using TopHat (version 2.0.5) [20] and on average 53.4% of the RNAseq reads could be mapped. The

resulting files were filtered using SAMtools (version 0.1.18) [21] to exclude secondary alignment of reads. For statistical comparison of gene expression levels between groups, aligned fragments per predicted gene were counted from SAM alignment files using the Python package HTSeq (version 0.5.3p9) [22]. To make comparisons across samples possible, these fragment counts were corrected for the total amount of sequencing performed for each sample. As a correction scalling factor, we employed library size estimates determined using the R/Bioconductor (release 2.11) package DESeq [23]. Read counts were normalized by dividing the raw counts obtained from HTSeq by its scale factor. Correction for false positives is included in the statistical analysis of gene expression through DESeq. The cut-off for significance was set to adjusted $p < 0.05$ and at least 2-fold change. A sequence homology search was performed using BLAST program in the National Center for Biotechnology Information (NCBI) database. Gene Ontology (GO) annotation and KEGG analysis were performed by DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov). The annotation result was categorized with respect to Biological Process, Molecular Function, and Cellular Component. Manual literature reviews were also performed in order to gain an overview of gene pathway networks, especially in immune-related pathway.

The relative expression levels of the selected genes analyzed by qPCR were determined using the $2^{-\Delta\Delta Ct}$ method [24] and presented as the fold increase or decrease of the infected fish compared to control fish. A Student's *t*-test was used to evaluate statistically any difference between infected and non-infected control fish with a probability level of 0.05. The minimum acceptable fold change was set at least 2-fold change.

3. Results

3.1. Sequencing and functional annotation

cDNA libraries from gill tissue of ten infected and ten control specimens were analyzed via Illumina sequencing in a single-read 50-nt run which generated, on average, 16,405,706 raw reads per sample. On average, 53% of the raw reads (8,738,493 reads) mapped to the rainbow trout (*Oncorhynchus mykiss*) reference genome. The reads have been deposited in the NCBI GEO repository under accession number GSE115675. A total of 44,007 distinct sequences (94.47%) of unigenes was annotated by BLAST-based gene identification. A similarity distribution analysis showed that 33% of unigenes had a strong similarity to reference sequences of *Homo sapiens, followed by Mus musculus* (20.2%), *Danio rerio* (12%), *Rattus norvegicus* (7.1%), and *Bos taurus* (4.8%) (Fig. 1).

3.2. Differentially expressed genes (DEGs)

In total, 3355 of the 46,585 (7.2%) unigenes showed significant differential expression when applying the criteria for the adjusted p-value < 0.05 and the absolute value of fold changes greater than 2. Transcriptome comparison of the gill tissue revealed that a total of 1799 (53.6%) unigenes were up-regulated while 1556 (46.4%) unigenes were down-regulated following infection (Fig. 2). In addition, a total of 155 (4.6%) DEGs were mapped to unknown genes, 86 of which were up-regulated and 69 of which were down-regulated. Using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, unigenes related to the immune system and diseases process were identified.

3.3. Gene ontology (GO)

The potential functions of DEGs were determined in terms of biological process, molecular function and cellular component according to the GO database. Distribution of the unigenes in different GO categories at level 2 is shown in Fig. 3, which was further classified into 61 sub-categories. The DEGs in terms of biological process were clustered into 25 sub-categories, the most enriched components of which were 'cell process' (2553 unigenes), 'single-organism process' (2254 unigenes), 'biological regulation' and (1857 unigenes). In addition, 965 and 410 unigenes were involved in 'signaling' and 'immune system process' category, respectively. For the term Molecular Function, DEGs were distributed into 14 sub-categories, of which the major part was related to the terms 'binding' (2260 unigenes) and 'catalytic activity' (983 unigenes). With regard to the term Cellular Component, many DEGs were assigned to 'cell' (2596 unigenes), 'cell part' (2593 unigenes), and 'organelle' (2111 unigenes) among 22 sub-categories.

Within the biological process category, it was noteworthy that many immune-related unigenes listed with 'response to stimulus' (57.8%) and 'immune system process' (12.8%) (Fig. 3). Analysis of level 3 GO term distribution showed that most of the significant unigenes within the response to stimulus category were involved in 'cellular response to stimulus' (1154 unigenes), followed by 'response to stress' (659 unigenes), and 'response to chemical' (649 unigenes). In the immune system process category, the highest number of unigenes were expressed in the categories 'immune response' (211 unigenes), 'immune system development' (166 unigenes), 'leukocyte activation' (112 unigenes), and 'immune effector process' (109 unigenes) (Fig. 4).

3.4. KEGG pathway

A total 1184 of the 3200 (37%) DEGs (mapped to 952 genes) was assigned to 282 KEGG pathways. All the annotated pathways were grouped into six major categories: Metabolism (11 pathways), Genetic Information and Processing (4 pathways), Environmental Information and Processing (3 pathways), Cellular Processes (4 pathways), Organismal System (9 pathways), and Human Diseases (10 pathways). The highest number of unigenes related to Organismal System which contained 1128 unigenes, followed by Human Diseases (1097 unigenes), Environmental Information Processing (784 unigenes), Metabolism (641 unigenes), Cellular Processes (394 Unigenes), and Genetic Information Processing (326 unigenes). Furthermore, some pathways which had the most significant number of unigenes were associated with immune system and disease process such as 'immune system' in Organismal system, 'cancers: overview' and 'infectious diseases: viral' in Human Diseases, 'signal transduction' and 'signaling molecules interaction' in Environmental Information processing (Fig. 5).

According to the KEGG pathway assignment, a total of 268 immune-related of unigenes were identified in 16 immune pathways (Table 1). Chemokine signaling pathway and Platelet activation had most of the unigenes accounting for 37 unigenes (mapped to 27 genes) and 34 unigenes (mapped to 24 genes), respectively. Other immune pathways with highest number of unigenes included Toll-like receptor signaling pathway (22 unigenes mapped to 17 genes), Hematopoietic cell lineage (20 unigenes mapped to 13 genes), Leukocyte transendothelial migration (20 unigenes mapped to 15 genes), NOD-like receptor signaling pathway (20 unigenes mapped to 13 genes), Natural killer cell mediated cytotoxicity (18 unigenes mapped to 12 genes), RIG-I-like receptor signaling pathway (17 unigenes mapped to 12 genes), Cytosolic DNA-sensing pathway (15 unigenes mapped to 12 genes), and Fc gamma R-mediated phagocytosis (15 unigenes mapped to 12 genes). DEGs involved in 16 immune pathways during parasite *I. multifiliis* infection are listed in Table S2.

Of immune related DEGs, 76 genes were up-regulated, such as chemokine (C−C motif) ligand 4 (CCL4), CD22 molecule (CD22), heat shock protein 4 (Hspa4), interleukin 8-like 2 (IL8L2), toll like receptor 8 (TLR8), etc. While 47 genes were down-regulated, such as C−C motif chemokine ligand 20 (CCL20), G protein subunit gamma 2 (GNG2), interleukin 12B (IL12B), phospholipase C beta 4 (PLCB4), PTK2 protein tyrosine kinase 2 (Ptk2), etc. Parts of these immune-related DEGs divided into 6 KEGG pathways are shown in Table 2 and discussed later.

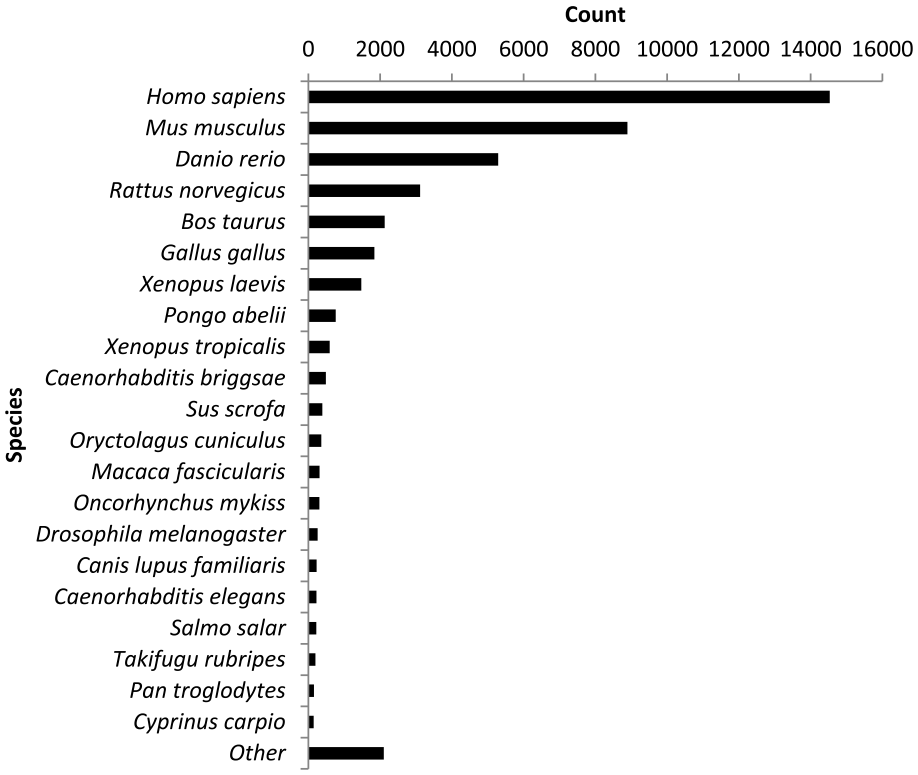

Fig. 1. BLAST analysis of unigenes that were represented in the rainbow trout gill transcriptome.

The *I. multifiliis* infection also significantly affected genes related to signaling pathway associated with immune response. Thus, a total of 553 unigenes identified within 23 signal transduction pathways. PI3K-Akt signaling pathway (81 unigenes), MAPK signaling pathway (51 unigenes), and FoxO signaling pathway (45 unigenes) were three sub-categories of signal transduction which had most unigenes. (Table S3). Among them, a number key immune-associated DEGs were shown in Table 2 including MAPK 9, 11, 13, STAT1, Pik3r5 etc.

3.5. qPCR validation of differentially expressed genes

Quantitative RT-PCR analysis of expression of selected immune-related genes in rainbow trout gills corresponded to RNA-seq results. As shown in Fig. 6, of the selected genes, two genes encoding IL-8 and SAA were significantly up-regulated whereas the gene encoding IL-17C1 was significantly down-regulated. A non-significant trend for down-regulation of genes encoding CD4 and TNFα as found in the qPCR analysis

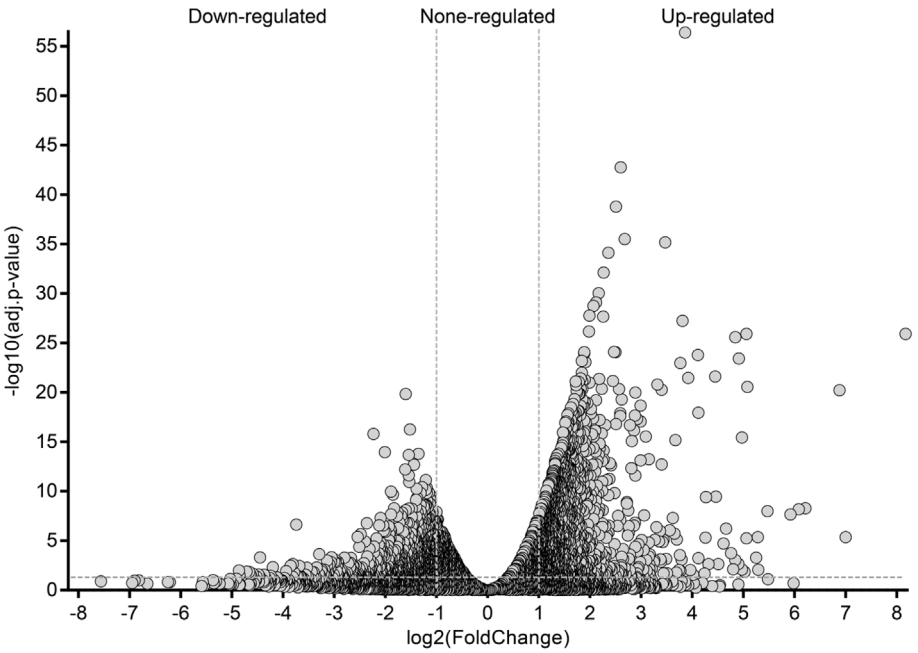

Fig. 2. Volcano plot of DEGs from rainbow trout gill infected by Ich. The horizontal and vertical dotted lines show the adjusted p-value equal to 0.05 and the minimum acceptable fold change, respectively. The value of log2FoldChange for all genes was analyzed as log2 (infected/control).

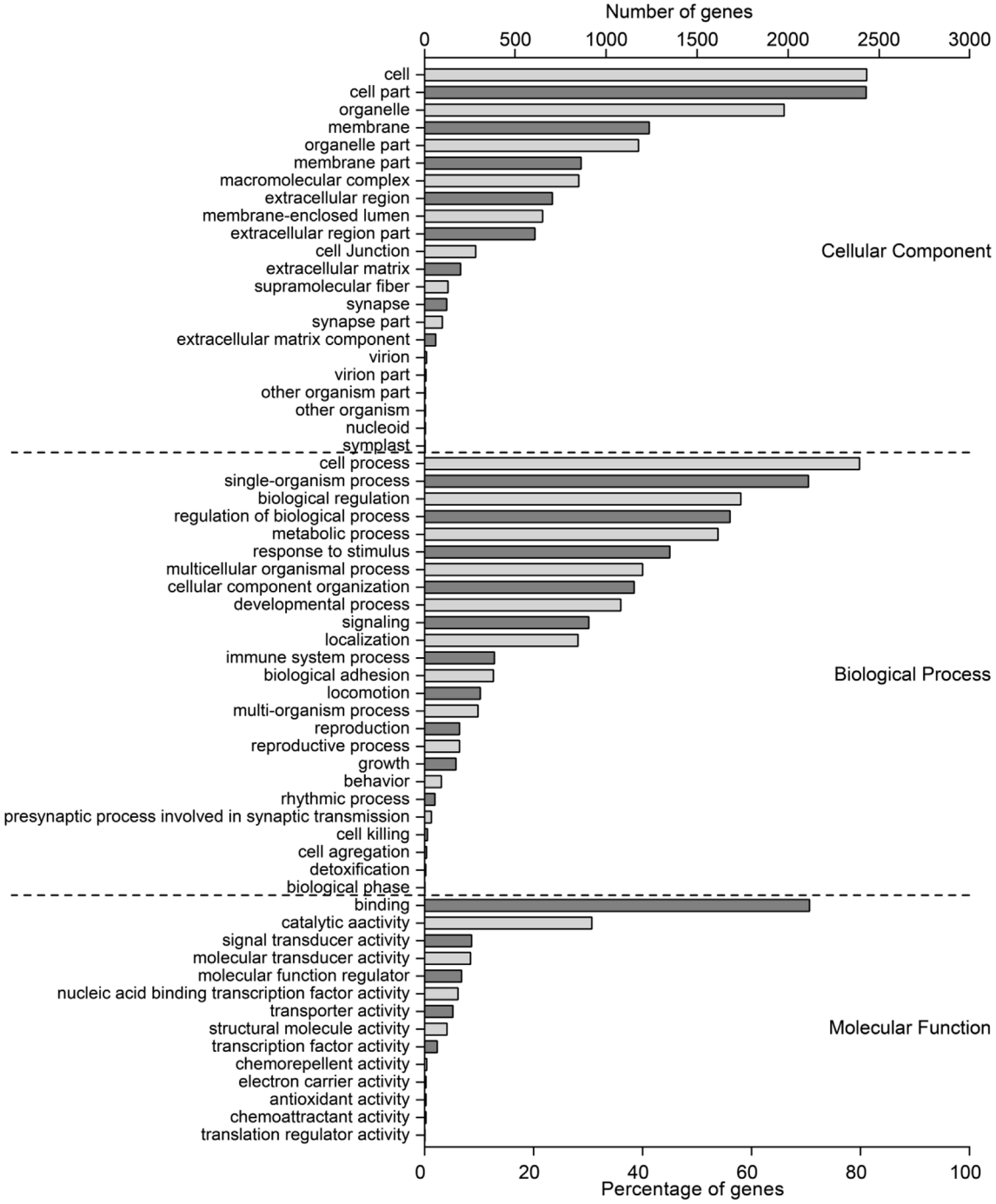

Fig. 3. GO annotation of DEGs from the gill rainbow trout infected by Ich. DEGs allocated to three major categories subdivided into sub-categories.

was the only inconsistency noted between the two approaches.

4. Discussion

Mucosal immune responses in vertebrates, including mammals, play a central role in health and disease. Basic studies performed in fish have demonstrated that teleosts represent an important evolutionary threshold between innate immunity, as performed in invertebrates, and adaptive response mechanisms in vertebrates [25]. The present study was performed to extend our understanding of the mucosal immune response of rainbow trout. We investigated gills infected by the parasitic ciliate *I. multifiliis* as rainbow trout gills have been demonstrated to respond more vividly and dynamically to external stimuli when compared to skin [26,27]. Real-time quantitative PCR has up until now described expression of selected immune genes and clearly demonstrated their importance when fish respond to pathogen exposure [10] but the genes investigated in those studies are likely to account for merely a minor part of the immune processes occurring in mucosal surfaces. The transcriptomic analysis presented here aims at filling in gaps in our understanding of these pivotal processes.

A total of 46,585 unigenes were assembled from the gill transcriptome and 44,007 unigenes (94.47%) provided a strong similarity with reference sequences in NCBI databases. Sequencing errors or potentially novel genes may explain that 2578 (0.55%) unigenes failed to match the database [28]. The functional annotation of the transcriptome in the present study was higher than other recent

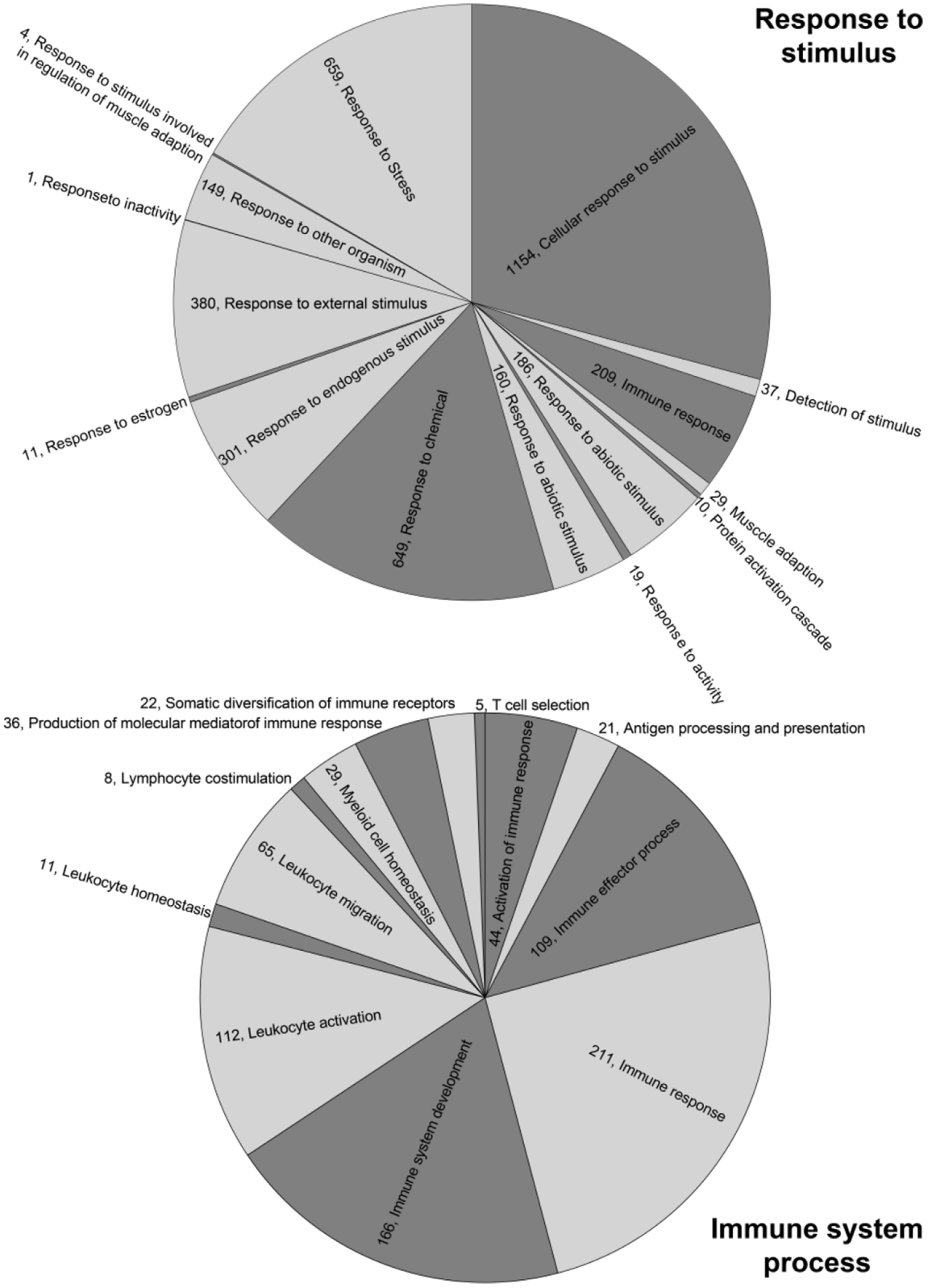

Fig. 4. Distribution of DEGs in response to stimulus and immune system process categories.

transcriptomic studies in rainbow trout [29,30] and other fish species [28,31,32]. According to our similarity analysis, we found that a total of 14,534 (33%) unigenes had a significant similarity to reference sequence of *Homo sapiens*, and only 5289 (12%) unigenes had a strong similarity to zebrafish reference sequences (*Danio rerio*). Moreover, only 306 (0.7%) unigenes showed similarity to reference sequences of *Oncorhynchus mykiss* but this should be explained by the incomplete annotation of sequences in the rainbow trout database. The percentage similarity was lower than found in a previous rainbow trout study reporting spleen transcript homology to reach 983 BLAST hits (3.73%) in the rainbow trout database [29].

With regard to gene ontology (GO) annotation, the annotated DEGs

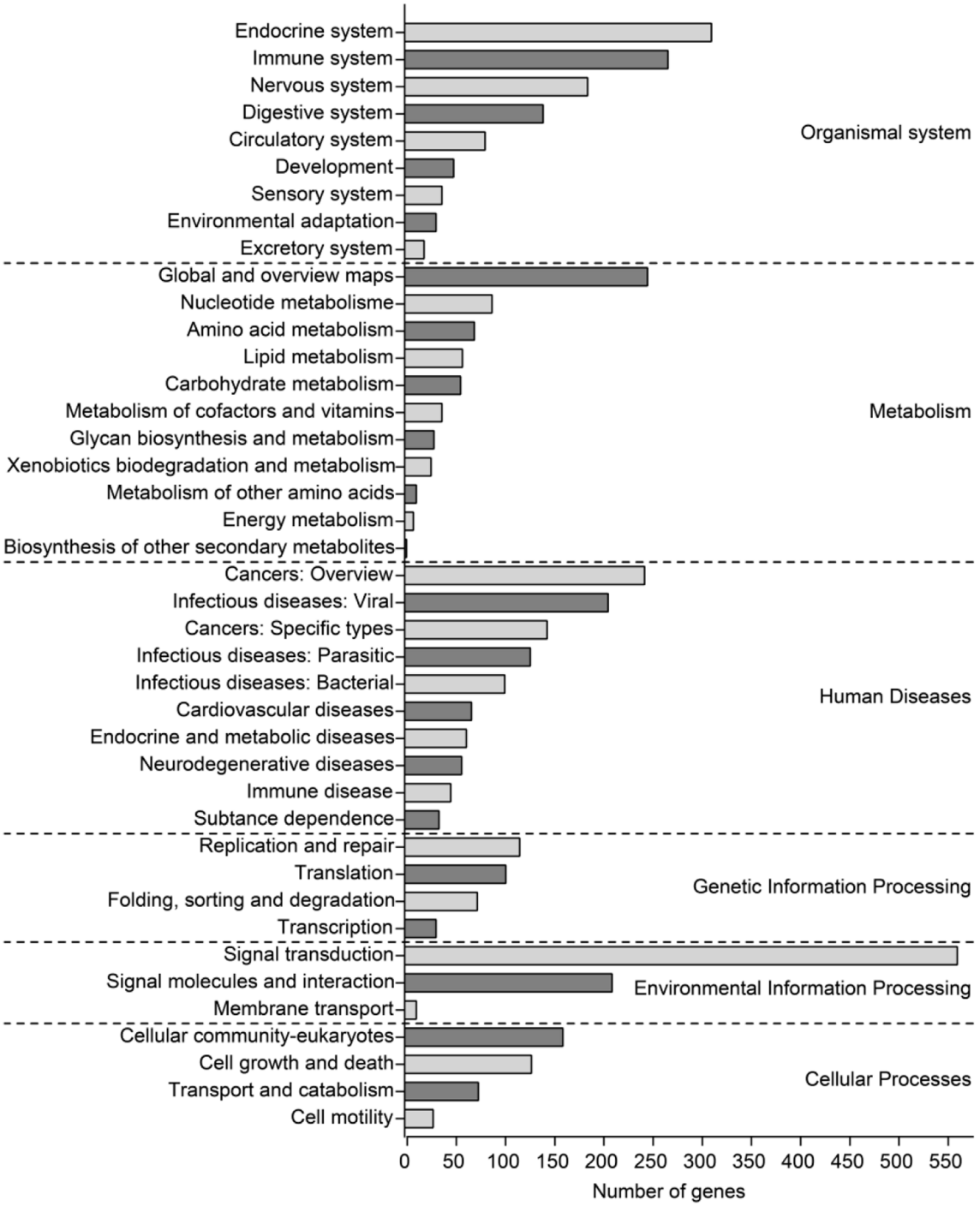

Fig. 5. KEGG classification of DEGs from the gill rainbow trout infected by Ich.

were mainly assigned to various terms of the biological process category corresponding to recent transcriptome analyses of rainbow trout [29,33], Japanese puffer [15], blunt snout bream [34], Tilapia [35,36], Ya-fish [31], grass carp [14], largemouth bass [32], and sea trout [37]. In line with those studies, the highly represented GO terms were cellular process and single-organism process for biological process, binding and catalytic activity for molecular function, cell and cell part for cellular component, indicating that these sub-categories to some extent, directly or indirectly, are connected to immune responses in rainbow trout. It has previously been suggested that immune-related proteins could be included within the groups cellular process, response to stimulus, signaling, immune system process categories and in binding and catalytic activity categories [29,38]. In agreement with the study on Japanese puffer (*Takifugu rubripes*), hundreds of gill immune-related genes were grouped within the category immune system process which again could be listed within the subgroups 'immune effector process', 'immune response', and 'leukocyte activation' [15]. In addition, the numbers of significantly expressed unigenes in those categories were higher than those of untreated rainbow trout [33] and brown trout [37], which further supports activation of rainbow trout immune defense mechanisms following infection [36].

Infection of rainbow trout with *I. multifiliis* induced differential expression of genes related to both the innate and the adaptive immune system but it was noteworthy that most genes were associated with

Table 1
The detailed distribution of DEGs in 16 immune-related pathways categorized from KEGG pathway assignment.

No.	Pathway ID	Pathway	Gene number
1	ko04062	Chemokine signaling pathway	37
2	ko04611	Platelet activation	34
3	ko04620	Toll-like receptor signaling pathway	22
4	ko04640	Hematopoietic cell lineage	20
5	ko04670	Leukocyte transendothelial migration	20
6	ko04621	NOD-like receptor signaling pathway	20
7	ko04650	Natural killer cell mediated cytotoxicity	18
8	ko04622	RIG-I-like receptor signaling pathway	17
9	ko04623	Cytosolic DNA-sensing pathway	15
10	ko04666	Fc gamma R-mediated phagocytosis	15
11	ko04660	T cell receptor signaling pathway	11
12	ko04610	Complement and coagulation cascades	10
13	ko04612	Antigen processing and presentation	9
14	ko04664	Fc epsilon RI signaling pathway	9
15	ko04662	B cell receptor signaling pathway	8
16	ko04672	Intestinal immune network for IgA production	4

pathways playing pivotal roles in innate immunity. These included 1) Chemokine signaling pathway [39], 2) Platelet activation [40,41], 3) Toll-like receptor signaling pathway [31], 4) NOD-like receptor signaling pathway [42], and 5) Leukocyte transendothelial migration [43]. Similar expression profiles were noted in lymphoid tissues of large yellow croaker [17] and orange-spotted grouper skin [18] infected by *C. irritans*, another parasitic ciliate. The DEGs associated with adaptive immunity comprised 1) T cell receptor signaling pathway, 2) Antigen processing and presentation, and 3) B cell receptor signaling pathway [29].

The chemokine signaling pathway has previously been reported activated in response to parasite infection including *I. multifiliis* [19] and *C. irritans* [16–18], and bacterial infection [14,28,34,44,45]. The pathway involves among other elements a series of chemokines [46] inducing inflammatory responses following infection [47]. Chemokines control migration and positioning of immune cells in exposed tissues by binding to the corresponding receptor on the surface of cell target and are critical elements in the innate immune system and cross-talk between involve in innate and adaptive players [39]. Thus, CCL4 interacts with macrophage and NK-cell migrations, and T cell-dendritic cell interaction whereas CCL19 interact with T cell and dendritic cell migration, and CCL28 play a role in T cell and B cell homing to the mucosa [16,39]. Chemokine and chemokine receptors found significantly up-regulated in rainbow trout gill exposed to *I. multifiliis* infection were more diverse than found in Tibetan highland fish (with only chemokine CXCL12 and chemokine receptor CCRL1) exposed to the parasite. However, chemokine CCL4, CCL19, CXCL8, CXCL11, and chemokine receptor CCR3 and CXCR1 were correspondingly up-regulated in the skin of orange-spotted grouper infected by *C. irritans* [18] framing the importance of this chemokine group in mucosal responses towards skin parasites. CXCL8 (IL-8) was previously noted to take part in the inflammatory response in rainbow trout gill [10] and skin [12,48] infected by Ich. It has a chemo-attractive effect on neutrophils [49,50] by interacting with chemokine receptor CXCR1 on this cell type [39]. A strong expression of chemokine CK11 was previously found in rainbow trout mucosal tissues skin and gill as a consequence of viral infection [51,52]. We found that chemokines CCL4L1, Ccl20 and chemokines receptors Ccr6, Ccr7, CCR9 were suppressed in response to *I. multifiliis* infection which suggests a complex interaction of chemokine signaling during these responses. Similar dynamic expressions were found in Amur sturgeon infected with *Y.ruckeri* where CCL19 was activated while CCL13 and CXC4 were suppressed [53].

Platelets have the capacity to interact with almost all immune cells, and are not only involved in innate immunity. Platelets are in mammals activated by binding their receptors to agonists such as collagen,

Table 2
Representative immune-related genes differentially expressed after an *I. multifiliis* infection.

Gene name	Description	Fold	Change
Chemokine signaling pathway			
ADCY2	adenylate cyclase 2	0.45	Down
CCL28	C−C motif chemokine ligand 28	2.69	Up
CCR3	C−C motif chemokine receptor 3	2.62	Up
CCR9	C−C motif chemokine receptor 9	0.37	Down
Ccl19	chemokine (C−C motif) ligand 19	10.17	Up
Ccl20	chemokine (C−C motif) ligand 20	0.18	Down
CCL4	chemokine (C−C motif) ligand 4	7.63	Up
CCL4L1	chemokine (C−C motif) ligand 4-like 1	0.42	Down
Ccr6	chemokine (C−C motif) receptor 6	0.35	Down
Ccr7	chemokine (C−C motif) receptor 7	0.46	Down
Ccr9	chemokine (C−C motif) receptor 9	2.39	Up
CXCL11	C-X-C motif chemokine ligand 11	3.07	Up
CXCL13	C-X-C motif chemokine ligand 13	27.94	Up
CXCL14	C-X-C motif chemokine ligand 14	2.31	Up
CXCL8	C-X-C motif chemokine ligand 8	4.73	Up
CXCR1	C-X-C motif chemokine receptor 1	17.28	Up
GNG12	G protein subunit gamma 12	2.11	Up
GNG2	G protein subunit gamma 2	0.50	Down
Gng5	G protein subunit gamma 5	0.40	Down
Jak2	Janus kinase 2	3.45	Up
NCF1	neutrophil cytosolic factor 1	2.52	Up
NFKBIA	NFKB inhibitor alpha	0.42	Down
PARD3	par-3 family cell polarity regulator	0.48	Down
Pik3r5	phosphoinositide-3-kinase, regulatory subunit 5, p101	2.74	Up
PLCB4	phospholipase C beta 4	0.49	Down
PPBP	pro-platelet basic protein	4.44	Up
Ptk2	PTK2 protein tyrosine kinase 2	0.48	Down
STAT1	signal transducer and activator of transcription 1, 91 kDa	3.59	Up
Platelet activation			
ADCY2	adenylate cyclase 2	0.45	Down
COL1A2	collagen type I alpha 2 chain	0.26	Down
COL3A1	collagen type III alpha 1 chain	0.40	Down
COL24A1	collagen type XXIV alpha 1 chain	0.48	Down
Col2a1	collagen, type II, alpha 1	0.42	Down
Col11a1	collagen, type XI, alpha 1	0.49	Down
FCER1G	Fc fragment of IgE receptor Ig	2.65	Down
GP1BA	glycoprotein Ib platelet alpha subunit	0.41	Down
Gucy1a3	guanylate cyclase 1 soluble subunit alpha 3	0.27	Down
Itpr3	inositol 1,4,5-triphosphate receptor 3	2.68	Up
ITPR1	inositol 1,4,5-trisphosphate receptor type 1	0.48	Down
ITGB1	integrin subunit beta 1	0.45	Down
Lcp2	lymphocyte cytosolic protein 2	4.70	Up
MAPK11	mitogen-activated protein kinase 11	3.25	Up
MAPK13	mitogen-activated protein kinase 13	2.01	Up
MYLK4	myosin light chain kinase family member 4	0.29	Down
Pik3r5	phosphoinositide-3-kinase, regulatory subunit 5, p101	2.74	Up
Pla2g4f	phospholipase A2, group IVF	5.02	Up
PLCB4	phospholipase C beta 4	0.49	Down
PTGS1	prostaglandin-endoperoxide synthase 1	2.23	Up
PRKG2	protein kinase, cGMP-dependent, type II	0.42	Down
Arhgef12	Rho guanine nucleotide exchange factor (GEF) 12	0.49	Down
TLN2	talin 2	0.44	Down
Toll-like receptor signaling pathway			
Casp8	caspase 8	3.20	Up
CCL4	chemokine (C−C motif) ligand 4	8.87	Up
CCL4L1	chemokine (C−C motif) ligand 4-like 1	0.42	Down
CXCL11	C-X-C motif chemokine ligand 11	3.07	Up
CXCL8	C-X-C motif chemokine ligand 8	4.73	Up
IRF3	interferon regulatory factor 3	3.66	Up
Irf7	interferon regulatory factor 7	4.32	Up
IL12B	interleukin 12B	0.47	Down
IL8L2	interleukin 8-like 2	5.47	Up
MAPK11	mitogen-activated protein kinase 11	3.25	Up
MAPK13	mitogen-activated protein kinase 13	2.01	Up
Mapk9	mitogen-activated protein kinase 9	3.05	Up
NFKBIA	NFKB inhibitor alpha	0.42	Down
Pik3r5	phosphoinositide-3-kinase, regulatory subunit 5, p101	2.74	Up

(*continued on next page*)

Table 2 (*continued*)

Gene name	Description	Fold	Change
LOC101078177	proto-oncogene c-Fos-like	2.75	Up
STAT1	signal transducer and activator of transcription 1, 91 kDa	3.59	Up
TLR8	toll like receptor 8	4.11	Up
Antigen processing and presentation			
CALR	calreticulin	3.53	Up
HSPA4	heat shock protein family A (Hsp70) member 4	2.77	Up
LGMN	legumain	3.63	Up
TAP2	transporter 2, ATP binding cassette subfamily B member	2.34	Up
T cell receptor signaling pathway			
Pdpk1	3-phosphoinositide dependent protein kinase-1	0.39	Down
Lcp2	lymphocyte cytosolic protein 2	4.70	Up
MAPK11	mitogen-activated protein kinase 11	3.25	Up
MAPK13	mitogen-activated protein kinase 13	2.01	Up
NFKBIA	NFKB inhibitor alpha	0.42	Down
NFATC1	nuclear factor of activated T-cells 1	0.47	Down
PAK5	p21 (RAC1) activated kinase 5	0.30	Down
PAK6	p21 (RAC1) activated kinase 6	2.36	Up
Pik3r5	phosphoinositide-3-kinase, regulatory subunit 5, p101	2.74	Up
B cell receptor signaling pathway			
CD22	CD22 antigen	2.81	Up
Inppl1	inositol polyphosphate phosphatase-like 1	0.49	Down
NFATC1	nuclear factor of activated T-cells 1	0.47	Down
NFKBIA	NFKB inhibitor alpha	0.42	Down
Pik3r5	phosphoinositide-3-kinase, regulatory subunit 5, p101	2.74	Up

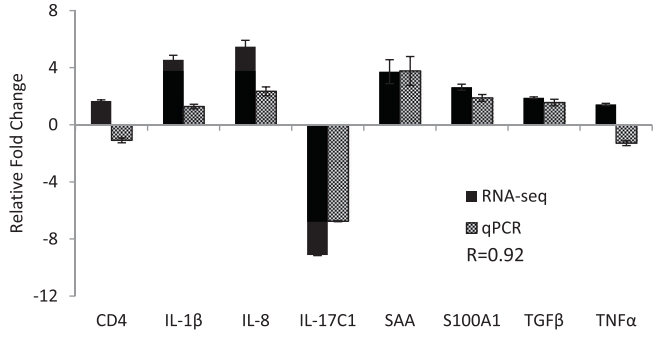

Fig. 6. Validation of RNA-seq analysis by comparing with qPCR analysis on selected immune genes in rainbow trout. CD4, T-cell surface glycoprotein CD4; IL-1β, Interleukin-1 beta; IL-8, Interleukin-8; IL-17C1, Interleukin-17C1; SAA, Serum amyloid A protein; S100A1, Protein S100-A1; TGFβ, Transforming growth factor beta, TNFα, Tumor necrosis factor alpha. Gene expression values are presented as relative fold change (mean ± SEM), which were calculated by comparing infected group to control group (N = 10 samples).

thrombin, ADP, TxA_2, and epinephrine whereafter they secrete a number of inflammatory mediators including chemokines/cytokines and express various toll-like receptors (TLRs). Mammalian platelets trigger release of cytokines when binding neutrophils, monocytes, and lymphocytes [40,41,54] and the present study indicates that this cell type also in fish play a central role. A number of genes associated with platelet activation including FCER1G, ITPR3, Lcp2, MAPK11, MAPK13, Pik3r5, PLA2G4F, and PTGS1 were activated in the gill tissue after parasite infection and it is noteworthy that adaptor protein FCER1G, Prostaglandin-endoperoxide synthase 1 PTGS1, protein kinase MAPK14, and intracellular signaling molecule ITPR1 are associated with platelet activation in mammals [55].

Toll-like receptors (TLRs) are key molecules of the innate immune system alerting the presence of microbial pathogens through recognition of pathogen-associated molecular patterns (PAMPs) [31,56]. A total of 20 different TLRs have been reported in teleost fish and more than 10 TLRs genes have been identified in rainbow trout including TLR1, 2, 3, 5, 5b, 5s, 7, 8, 8a1, 8a2, 9, 20 and 22 [29,56–58]. In the present study, only TLR8 was significantly up-regulated suggesting that this gene was involved in recognizing *I. multifiliis*. However, Tibetan highland fish regulated not only TLR8 but also TLR4 and TLR5M as response to this infection [19] and channel catfish activated TLR1, 2, and 9 but not TLR8 after infection [59]. Orange-spotted grouper [18,60–62] and large yellow croaker [16] expressed TLR1, 2, 5, 5S, 9, and 21 following *C. irritans* infection. Due to its role in anti-viral immunity [58] TLR8 activation was unexpected in this parasite-host system. It could be speculated that viruses infecting the parasite *I. multifiliis* might be involved, a possibility that should be further investigated. We have at present no reports on virus infections of *I. multifiliis* although numerous protozoan parasites serve as hosts and vectors of viruses [64]. TLR8 ligands include single-stranded RNA [61] and unmethylated CpG DNA [59] which may be present in parasites [63]. In this study, up-regulation of genes involved in MAPK signaling pathway such as MAPK9, 11, 13 suggested that TLR8 could have transmitted the signals to AP-1 by activating the downstream transcription factors p38, JNK, and IRF7 through a MyD88-dependent signaling pathway inducing the expression of pro-inflammatory cytokines CCL4, CXCL11, CXCL8, and IL8L2 [18,34,56,65]. Further, parasite infection could affect to suppression of pro-inflammatory cytokines in rainbow trout since down-regulation of IL12B, a key pro-inflammatory cytokine that bridges both innate and adaptive immunity [66], was seen after infection.

Antigen processing and presentation are central elements of adaptive immunity in teleost. Antigens are processed and presented to specific lymphocytes when bound to major histocompatibility complex (MHC) I and II [67–69]. CALR, Hspa4, and TAP2 genes are important in MHC class I antigen processing and presentation. Thus, the CALR gene is involved in the formation of the MHC class I and TAP2 (a subunit of transporter associated with antigen processing (TAP) complex located in the endoplasm reticulum (ER) membrane) transports the peptide antigen across the ER membrane for subsequent loading into the peptide-binding groove of MHC I [69,70]. Further, the cytoplasmic protein, Hspa4, is involved in regulation of the antigen processing and presentation [71]. LGMN contributes to antigen processing for presentation on MHC class II complex [72,73], and it is noteworthy that MHC II in rainbow trout gills has been demonstrated as a prominent molecule during *I. multifiliis* infection [10]. T-cells become activated when the T-cell receptor recognizes the antigen presented by MHC molecules. In a recent study, the T-cell receptor signaling pathway was found involved in the immune response of grouper infected by *C. irritans* suggesting a role of teleost T-cells in adaptive immunity as described in mammals [74]. Some genes involved in T-cell receptor signaling pathway were activated in the present study including Lcp2, MAPK11, MAPK13, Nfkbia, PAK6, and Pik3r5. These genes encode proteins which activate and trigger downstream signaling in this pathway. Lcp2 encodes the adaptor protein SLP-76 which is important in the development of T cells and propagating signal downstream of T-cell receptor in mammals [75,76]. B cells are involved in the antigen-specific defense through binding of antigen to the B cell receptor [29]. *I. multifiliis* exposure induced expression of a gene encoding B-cell receptor CD22 taking an important role in the B-cell receptor signaling pathway in rainbow trout as well as in orange-spotted grouper [18]. B-cell receptor CD22 plays a pivotal role in B cell development and antibody production [77] and these results indicate the specific B-cell response of rainbow trout may occur locally at the infected site against the parasite infection [18].

Playing a critical role in the immune system, a number of signaling pathways related to signal transduction is normally involved in response to parasite infection. Some of the representative genes in signal transduction were in the present study found up-regulated and as these may further induce other pathways in the immune system their role is central. MAPK9, 11 and 12 are the member of the MAPK signaling pathway involving both innate and adaptive immunity [78–80]. The

gene encoding the signal transduction of activator STAT1 is a member of the JAK-STAT signaling pathway playing a critical role in the immune system [81,82]. Our results suggested that this signaling pathway was likely involved in chemokine and TLR signaling pathway in rainbow trout, similar to mammals. The gene encoding Pik3r5 is a member of the PI3K-Akt signaling pathway which can activate other pathways related to innate and adaptive immunity such as Toll-like receptor signaling pathway, B cell and T cell receptor signaling pathway [83–85].

Almost half of the differentially expressed genes (46.4%) in rainbow trout gills were down-regulated after infection and may function as a regulatory mechanism of rainbow trout responses. It was assumed that down-regulation of ADCY2, GNG2, Gng5, PARD3, PLCB4, Ptk2 might be involved in the negative regulation of the inflammatory response. Also Wang et al. studying large yellow croaker with *C. irritans* infection noted a corresponding negative regulation mechanism related to the inflammatory response [16].

In conclusion, a transcriptomic profile of rainbow trout gills exposed to the parasitic ciliate *I. multifiliis* was reported for the first time. A total of 3355 differentially expressed unigenes were identified. Of these were 1184 unigenes (mapped to 952 genes) annotated to 282 KEGG pathways and 268 unigenes were associated with 16 immune pathways. Most unigenes were related to innate immune system pathways (Chemokine signaling pathway, Platelet activation, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, and Leukocyte transendothelial migration) although a number of unigenes was related to adaptive responses (antigen processing and presentation, T and B cell receptor signaling pathway). The present study gave a far better resolution of the immune response of rainbow trout exposed to a parasitic protozoan than has ever been presented previously. The identification of a series of immune genes involved in several but important pathways was useful for understanding of immune mechanism of the rainbow trout responding to the parasite *I. multifiliis*. Our results provide tools to link innate and adaptive immune elements in the process and present basic information which will be useful in the future studies related to immunoprophylaxis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.075.

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