

Innate and early immune responses to *Yersinia ruckeri* in the spleen of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Background: *Yersinia ruckeri* is a pathogen that can cause enteric redmouth disease in salmonid species, damaging global production of economically important fish including rainbow trout (*Oncorhynchus mykiss*). Herein, we conducted the transcriptomic profiling of spleen samples from rainbow trout at 24 h post-*Y. ruckeri* infection via RNA-seq in an effort to more fully understand their immunological responses.

Results: We identified 2498 differentially expressed genes (DEGs), of which 2083 and 415 were up- and down-regulated, respectively. We then conducted a more in-depth assessment of 78 DEGs associated with the immune system including CCR9, CXCL11, IL-1 β , CARD9, IFN, CASP8, NF- κ B, NOD1, TLR8a2, HSP90, and MAPK11, revealing these genes to be associated with 20 different immunological KEGG pathways including the Cytokine-cytokine receptor interaction, Toll-like receptor signaling, RIG-I-like receptor signaling, NOD-like receptor signaling, and MAPK signaling pathways. Additionally, the differential expression of 8 of these DEGs was validated by a qPCR approach and their immunological importance was then discussed.

Conclusions: Our findings provide preliminary insight regarding the innate and early immune responses of rainbow trout following *Y. ruckeri* infection and the base for future studies of host-pathogen interactions in rainbow trout.

Background

Yersinia ruckeri is a pathogen that can cause enteric redmouth disease (ERM) or yersiniosis, resulting in significant mortality and economic losses associated with the global production of rainbow trout (*Oncorhynchus mykiss*). Rainbow trout are highly susceptible to ERM, although other species of fish can also be affected by this disease [1, 2]. Multiple studies have sought to clarify the immunological responses of fish species to *Y. ruckeri* infection. In one study, Raida et al. determined that very susceptible trout species exhibited a robust and rapid-onset septicemic response to infection associated with the production of high levels of pro-inflammatory cytokines [3]. Similarly, these pro-inflammatory cytokines were also upregulated in the spleen of the vaccinated rainbow trout following *Y. ruckeri* challenge, albeit to a lesser extent than in naïve fish [4]. The spleen is a key secondary lymphoid organ that is thus closely associated with rainbow trout responses to *Y. ruckeri* infection, and significant changes in the expression of splenic immune-related genes have been detected following *Y. ruckeri* challenge [5, 6]. However, no systematic analyses of patterns of rainbow trout splenic gene expression after *Y. ruckeri* infection have been conducted to date.

RNA sequencing (RNA-seq) is a high-throughput approach to analyzing transcriptomes that has frequently been employed in studies of fish species [7]. Several recent studies based on RNA-Seq analysis have explored rainbow trout responses to a range of pathogen types, such as splenic responses to *Aeromonas salmonicida* [8, 9], infectious hematopoietic necrosis virus (IHNV) [10], and *Ichthyophthirius multifiliis* [11]. Such transcriptomic analyses have offered new insights into the etiology of these

diseases, and similar studies of *Y. ruckeri* infections may highlight viable approaches for treating or preventing yersiniosis in rainbow trout farming.

As such, we herein conducted a transcriptomic study assessing rainbow trout splenic immune responses to *Y. ruckeri* infection. After identifying infection-related differentially expressed genes (DEGs), we validated a subset of these genes via qPCR and conducted the functional annotation of immune-associated DEGs. Together, our data offer a preliminary insight for future research regarding the immunological mechanisms involved in rainbow trout defensive response against *Y. ruckeri*.

Results

RNA-sequencing and data processing

Genes associated with rainbow trout immune response to *Y. ruckeri* infection were identified by assessing spleen samples from YR-infected and control uninfected fish via RNA-seq. In total, six cDNA libraries were prepared (from 3 per group), and raw data were generated (Table S1) and deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA675742.

Following the completion of filtering, 44.07 G bp of clean data were extracted, with over 93.15–93.55% of the bases reads having a phred quality value ≥ 30 in the non-infected group compared to 92.87–93.43% in the YR-infected group. These quality scores were consistent with excellent quality data. Reads from these two groups exhibited GC contents of 49.14–49.64% and 49.00–49.18%, respectively (Table 1).

Table 1
Characteristics of RNA-seq data

| Samples | Clean reads (M) | Clean bases (Gb) | GC Content (%) | Q30 (%) |
|------------------------------|-----------------|------------------|----------------|---------|
| non-infected rainbow trout 1 | 26.9191 | 8.0181 | 49.14 | 93.18 |
| non-infected rainbow trout 2 | 23.4555 | 6.9991 | 49.64 | 93.55 |
| non-infected rainbow trout 3 | 25.5238 | 7.6123 | 49.33 | 93.15 |
| YR-infected rainbow trout 1 | 22.5961 | 6.7523 | 49.18 | 92.87 |
| YR-infected rainbow trout 2 | 23.5341 | 7.0248 | 49.00 | 93.43 |
| YR-infected rainbow trout 3 | 25.7487 | 7.6694 | 49.18 | 93.32 |
| Non-infected group | 75.8984 | 22.6295 | 49.32 | 93.29 |
| YR-infected group | 72.0286 | 21.4465 | 49.12 | 93.21 |
| Total | 147.927 | 44.076 | 49.25 | 93.25 |

The total number of expressed genes detected in samples from uninfected rainbow trout was slightly higher than that detected in YR-infected rainbow trout (Fig. 1).

Read Mapping To The Reference Genome

Cleaned reads were mapped to the *O. mykiss* reference genome, with 84.81–85.99% of these reads ultimately matching perfectly. Over 70% of reads aligned to exonic regions in each library, of which 78.05–78.24% in the uninfected group and 78.53–79.11% in the YR-infected groups mapping to unique reads whereas 6.76–7.38% in the uninfected group and 6.81–7.17% in the YR-infected groups mapping to multiple reads. A total of 123.7985 (41.90%) and 125.0329 (42.32%) M reads in the uninfected and YR-infected groups mapped to reference genome sense and antisense strands, respectively (Table 2). Besides, some new genes were detected and classified with the NR, Swiss-Prot, GO, COG, KOG, Pfam, and KEGG databases (Table S2).

Table 2
RNA-seq alignment details and mapping ratios

| Samples | Total reads (M) | Mapped reads (M) | Uniq mapped reads (M) | Multiple map reads (M) | Reads map to '+' | Reads map to '-' |
|------------------------------|-----------------|-------------------|-----------------------|------------------------|-------------------|-------------------|
| Non-infected rainbow trout 1 | 53.8382 | 45.6616 (84.81%) | 42.0247 (78.06%) | 3.6369 (6.76%) | 22.4170 (41.64%) | 22.6348 (42.04%) |
| Non-infected rainbow trout 2 | 46.9109 | 40.1607 (85.61%) | 36.7008 (78.24%) | 3.4599 (7.38%) | 19.7468 (42.09%) | 19.9220 (42.47%) |
| Non-infected rainbow trout 3 | 51.0477 | 43.3188 (84.86%) | 39.8427 (78.05%) | 3.4761 (6.81%) | 21.3261 (41.78%) | 21.4872 (42.09%) |
| YR-infected rainbow trout 1 | 45.1922 | 38.7941 (85.84%) | 35.5522 (78.67%) | 3.2418 (7.17%) | 19.0209 (42.09%) | 19.2198 (42.53%) |
| YR-infected rainbow trout 2 | 47.0682 | 40.4739 (85.99%) | 37.2354 (79.11%) | 3.2386 (6.88%) | 19.7989 (42.06%) | 20.0358 (42.57%) |
| YR-infected rainbow trout 3 | 51.4973 | 43.9318 (85.31%) | 40.4383 (78.53%) | 3.4934 (6.78%) | 21.4897 (41.73%) | 21.7333 (42.20%) |
| Non-infected group | 50.5989 | 129.1411 (85.09%) | 118.5682 (78.12%) | 10.5729 (6.98%) | 63.4899 (41.84%) | 64.0440 (42.20%) |
| YR-infected group | 47.9192 | 123.1998 (85.71%) | 113.2259 (78.77%) | 9.9738 (6.94%) | 60.3095 (41.96%) | 60.9889 (42.43%) |
| Total | 295.5624 | 252.3409 (85.40%) | 231.7941 (78.44%) | 20.5467 (6.96%) | 123.7985 (41.90%) | 125.0329 (42.32%) |

DEG Identification And Analysis

The Pearson's correlation coefficient values were used to assess relative gene expression in the uninfected and YR-infected groups (Fig. S1). A total of 2498 DEGs were identified by comparing these groups, of which 2083 (83.39%) were up-regulated and 415 (16.61%) were down-regulated, in YR-infected fish compared to uninfected fish (Table S3). Volcano and MA plots were also used to represent these gene expression trends (Fig. S2).

Of these DEGs, 2431 were classified successfully using the NR, Swiss-Prot, GO, COG, KOG, Pfam, and KEGG databases (Table 3). With respect to new genes, many DEGs were annotated using the NR and

eggNOG databases, but few were annotated in the COG database.

Table 3
Summary statistics regarding DEG functional annotation

| Annotated databases | NR | Swiss-Prot | GO | COG | KOG | Pfam | KEGG | eggNOG | All |
|--|-------|------------|-------|-------|-------|-------|-------|--------|------|
| DEGs number | 2421 | 1679 | 1766 | 584 | 1539 | 2116 | 1533 | 2295 | 2431 |
| Ratio (%) | 99.59 | 69.07 | 72.65 | 24.02 | 63.31 | 87.04 | 63.06 | 94.41 | |
| To better understand the functional roles of detected DEGs, GO annotation was next performed by categorizing these DEGs into 23 biological processes (BPs), 19 cellular components (CCs), and 16 molecular functions (MFs). Cellular (42.07%), single-organism (36.51%), metabolic (30.75%), and biological (29.64%) processes were the most dominant categories of BPs, while membrane (27.94%), cell (26.34%), cell part (25.66%), and membrane part (24.70%) were the most enriched CCs and binding (40.07%) and catalytic activity (19.26%) were the most dominant MFs (Fig. 2). | | | | | | | | | |

In addition, KEGG pathway enrichment analyses were performed to assess the functional roles of these DEGs during *Y. ruckeri* infection in rainbow trout. Assembled DEGs were analyzed with the KEGG database, leading to their classification into 6 categories (Fig. S3). KEGG enrichment results, including the top 9 pathways enriched for > 50 genes ($p < 0.05$), are shown in Fig. 3. Four highly enriched pathways were detected through this KEGG analysis, including the NOD-like receptor signaling, cytokine-cytokine receptor interaction, Toll-like receptor signaling, and RIG-I-like receptor signaling pathways. The preferential enrichment of these pathways suggests that many of the genes differentially expressed between uninfected and YR-infected rainbow trout were related to the immune system.

Identification Of Immune-related DEGs

To better understand the intracellular signaling pathways during *Y. ruckeri* infection in rainbow trout, we therefore focused on 78 immune response-related DEGs identified in this study, including two new genes (Table S4). A heatmap was constructed based upon the fold-change expression values for these DEGs (Fig. 4), clearly demonstrating that almost all of these genes (74) were upregulated in spleen samples from YR-infected fish compared to spleen samples from uninfected fish, whereas only 4 genes were down-regulated after infection.

Further analysis of these immune-related DEGs revealed them to be primarily associated with 20 immunological KEGG pathways, including the MAPK signaling, Cytokine-cytokine receptor interaction, Toll-like receptor signaling, RIG-I-like receptor signaling, NOD-like receptor signaling, FoxO signaling, mTOR signaling, apoptosis, TGF-beta signaling, regulation of autophagy, ErbB signaling, cell adhesion molecule (CAM), intestinal immune network for IgA production, cytosolic DNA-sensing, phosphatidylinositol signaling system, and p53 signaling pathways (Table 4). The top 3 pathways enriched in these genes included the NOD-like receptor signaling (31 genes), RIG-I-like signaling (35 genes), and Toll-like receptor signaling (51 genes) pathways (Fig. 5).

Table 4
Immune-related DEGs

| Gene ID | Type | Log ₂ Fold | Putative homolog protein | KEGG pathway |
|------------|------|-----------------------|--|---|
| Gene 10807 | up | 12.0338 | Interleukin-1 beta | ko04620: Toll-like receptor signaling pathway |
| Gene 24642 | up | 10.6682 | Interleukin-8 | ko04060: Cytokine-cytokine receptor interaction |
| Gene 22618 | up | 9.3812 | interleukin-8 like | ko04621: NOD-like receptor signaling pathway |
| Gene 4948 | up | 8.4892 | Tumour Necrosis Factor | ko04150: mTOR signaling pathway |
| Gene 28337 | down | -2.5818 | Mitogen-activated protein kinase 11 | ko04010: MAPK signaling pathway |
| Gene 25622 | up | 10.1562 | Interleukin-6 | ko04060: Cytokine-cytokine receptor interaction |
| Gene 34157 | up | 9.0084 | Interleukin-6 | ko04060: Cytokine-cytokine receptor interaction |
| Gene 34403 | up | 6.5996 | Tumor necrosis factor | ko04060: Cytokine-cytokine receptor interaction |
| Gene 25550 | up | 6.1699 | Tumor necrosis factor | ko04060: Cytokine-cytokine receptor interaction |
| Gene 2178 | down | -1.1786 | NOD1 | ko04621: NOD-like receptor signaling pathway |
| Gene 20638 | up | 4.6720 | Small cytokines (intecrine/chemokine) | ko04060: Cytokine-cytokine receptor interaction |
| Gene 26188 | up | 3.7094 | Mab-21 protein | ko04623: Cytosolic DNA-sensing pathway |
| Gene 44284 | up | 3.1644 | Immunoglobulin V-set domain | ko04514: Cell adhesion molecules (CAMs) |
| Gene 23752 | up | 2.9294 | Phosphoinositide 3-kinase regulatory subunit | ko04012: ErbB signaling pathway |
| Gene 5944 | up | 2.4306 | Interferon alpha/beta receptor | ko04060: Cytokine-cytokine receptor interaction |

Validation Of Selected Degs By Qrt-pcr

As expected, all the eight immune-related DEGs exhibited similar expression trends when measured via both qPCR and RNA-Seq analysis, confirming the reliability of our analytical techniques (Fig. 6).

Discussion

ERM is a serious disease that impacts global salmonid populations [12]. While some studies have begun to characterize rainbow trout immune responses to *Y. ruckeri* infection [6, 13], no systematic transcriptomic analyses of these responses have been conducted to date. The spleen plays central roles in orchestrating innate and adaptive immune responses in fish. Herein, we sequenced the spleen transcriptomes of rainbow trout infected with YR in comparison with those of control uninfected rainbow trout and we identified 2498 DEGs between these populations, of which 2083 were up-regulated whereas 415 were down-regulated in infected rainbow trout. Immune response-related DEGs were then assessed in additional detail in an effort to explore the basis of innate immune responses against *Y. ruckeri* infection in rainbow trout.

Cytokines are secreted by a range of cell types, and they act as immune response regulators that can be classified as interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs), and chemokines [14]. Of the 78 immune-associated DEGs in the present study, 31 were classified into the cytokine-cytokine receptor interaction pathway, including chemokine (C-X-C motif) ligand (*CXCL11*), C-C motif chemokine receptor 9 (*CCR9*), caspase recruitment domain-containing protein (*CARD9*), *IL-12*, *IL-1 β* , and *IFN*. Chemokines control the migration of particular immune cell subsets and coordinate both adaptive and innate immune responses to stressors [15]. The transcription of *CXCL11* in rainbow trout has previously been shown to be induced in response to *Y. ruckeri* infection [16]. Herein, we observed the upregulation of both *CXCL11* and *CCR9* in the spleens of rainbow trout infected with this bacterium, consistent with the pathogen-induced chemokine regulation. *CARD9*, which is normally activated by CLRs [17], was 1.39-fold downregulated in response to *Y. ruckeri*.

Apoptosis is an important determinant of cellular survival in both physiological and pathological contexts, and can be triggered by factors such as hypoxia, chemical exposure, temperature stress, or immune responses to particular stimuli. Upon bacterial infection, a host's cells may undergo apoptotic death to mitigate the spread of the pathogen within host tissues [18]. Herein, we observed the upregulation of caspase 8 (*CASP8*), receptor-interacting serine/threonine-protein kinase 1-like (*RIPK1*), transcription factor p65-like (*TF65*), inhibitor of apoptosis protein-like protein (*IAP*), and NF-kappa-B inhibitor alpha-like (*I κ B α*) following YR infection in rainbow trout. Caspases are proteases that serve as essential regulators of apoptotic cell death, with *CASP8* having showed to be an upstream regulator of apoptotic cascades in fish [19]. Marked *CASP8* upregulation has also previously been detected in head-kidney and spleen leukocytes of *Totoaba macdonaldi* at 24 h post-infection with *Vibrio parahaemolyticus* and *Aeromonas veronii* [20]. *NF- κ B* can control innate and adaptive immune-related gene expression, inducing apoptosis in response to numerous stimuli [21]. At the same time, *NF- κ B* activation induces *I κ B α* expression in rainbow trout, in turn resulting in the feedback inhibition of *NF- κ B* [22]. Upregulation of *I κ B α* , *IAPs* and *RIPK1* detected in this study can suggest the compensatory activation of some inhibitors of apoptotic cell death, underscoring the complexities of cellular responses to *Y. ruckeri* in rainbow trout. Additional work must be done in order to understand in depth how the apoptotic processes.

Pattern recognition receptors (PRRs) serve as innate sensors that can rapidly detect and respond to conserved damage- and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively), resulting in the induction of immune-related gene expression and anti-pathogen responses. PRRs detected in aquatic species to date include TLRs, NLRs, RLRs, and CLRs [23]. In the present study, we identified over 10 members of the TLR, NLR, and RLR gene families that were differentially expressed in the spleens of rainbow trout at 24 h post-*Y. ruckeri* infection, including heat shock protein 90 (*HSP90*), tumor necrosis factor alpha-induced protein 3-like (*TNFAP3*), nucleotide-binding oligomerization domain-containing protein 1-like (*NOD1*), *IL-1*, *IL-12*, toll-like receptor 8a2 (*TLR8a2*), and interferon α (*IFN α*). HSPs are important regulators of fish immune responses [24, 25], and *HSP90* upregulation detected in the present research may be linked to the rainbow trout innate immune defenses to *Y. ruckeri* infection. *NOD1* modulates the innate immune response of fish to bacterial peptidoglycan. Loss- and gain-of-function experiments have suggested that *NOD1* can control rainbow trout pro-inflammatory cytokines in rainbow trout [26]. Palti et al. first reported the presence of the *TLR8a2* gene in rainbow trout, which they found to be somewhat downregulated in response to treatment with the human agonist of TLR7/8 known as R848 [27]. We found both *NOD1* and *TLR8a2* to have been downregulated in rainbow trout during the early stages of *Y. ruckeri* infection. Overall, these findings suggest that the PRRs were differentially expressed in rainbow trout and may be important mediators of the initial induction of immunological responses to bacterial infection.

The MAPK signaling pathway is responsive to diverse extracellular stimuli and can modulate transcription factor expression and activation, controlling a range of biological processes including proliferation, apoptosis, and gene transcription. Recent evidence indicates that fish MAPKs can be induced by a range of stimuli. For example, flagellin treatment is associated with *MAPK11* upregulation in rock bream (*Oplegnathus fasciatus*) [28]. In contrast, in the present study we observed a 2.58-fold decrease of *MAPK11* expression in the spleen of rainbow trout following *Y. ruckeri* infection, although additional validation of these results is warranted. We also observed a significant *MAPK8* upregulation upon *Y. ruckeri* infection in rainbow trout. Moreover, a total of 22 DEGs involved in the MAPK signaling pathway seems to play key roles in the rainbow trout response to infection with this bacterium.

Conclusions

In summary, we conducted a transcriptomic analysis of spleen samples from rainbow trout infected with *Y. ruckeri* in an effort to better understand the immunological basis for responses to this pathogen, leading to the identification of several key immune-related DEGs. Overall, our results will provide a preliminary insight regarding the innate and early immune responses of rainbow trout following *Y. ruckeri* infection and the base for future studies of host-pathogen interactions in rainbow trout.

Methods

Experimental fish and bacteria

Healthy rainbow trout (~ 10 g) were obtained from Benxi Agrimarine Industries Inc. and housed in a 540 L fiberglass circulating water tank at a constant temperature of $14 \pm 0.2^\circ\text{C}$ with a 12 h light/dark cycle and an 8.0 mg/L oxygen saturation. Fish were maintained under these conditions for 2 weeks and were fed commercial rainbow trout feed.

Y. ruckeri strain BH1206 was isolated from infected rainbow trout, confirmed to be pathogenic, and used for challenge experiments as previously published [29]. Bacteria were grown for 24 h in TSB medium (BD Difco, USA) and collected by spinning for 5 min at 6,000 xg prior to resuspension in sterile PBS (pH 7.2) at $6 \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$.

Bacterial Challenge And Sampling

Prior to challenge test, healthy rainbow trout were kept under laboratory conditions in flow-through tanks at approximately 14°C with continuous aeration and fed twice a day at 1.2% of body weight with commercial fish feed. A subset of experimental fish was microscopically and bacteriologically examined to verify freedom of *Y. ruckeri* infection. Tricaine methanesulfonate (MS222) was used to anesthetize fish prior to the challenge or tissue sample collection. Experimental infection was induced by intraperitoneally (i.p.) injecting fish with 100 μL of BH1206 bacteria at $6 \times 10^5 \text{ CFU}$ per gram of fish body weight. An equivalent volume of PBS was injected into uninfected control fish. At 24 h post-infection, three fish per group were sacrificed by an overdose of anesthetic, and spleens were collected, washed to remove blood and fat, snap-frozen with liquid nitrogen, finally stored in liquid nitrogen tank. To confirm the presence of *Y. ruckeri* in experimental fish, the kidney was sampled to perform bacteriological examination.

RNA Isolation

Splenic RNA was isolated using Trizol (Invitrogen, USA), after which RNA integrity and purity were evaluated via 1% agarose gel electrophoresis and using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), while a Qubit RNA Assay Kit and a Qubit 2.0 Fluorometer (Life Technologies, CA, USA) were utilized to measure RNA concentration. After RNA preparation, all downstream library preparation and sequencing were performed by Biomarker technologies CO., LTD (Beijing, China).

Library Construction And Sequencing

A total of 3 μg RNA per spleen sample was utilized for library construction using a NEBNext Ultra RNA Library Prep kit for Illumina (NEB, USA), with samples being affixed with appropriate barcodes. Following DNase I treatment, the remaining mRNA was purified and sheared into 200–250 bp fragments as discussed previously [30]. Library quality was assessed with an Agilent Bioanalyzer 2100 instrument, and a cBot Cluster Generation System with TruSeq PE Cluster Kit v4-cBot-HS (Illumina) was used to cluster barcoded samples. An Illumina HiSeq 2500 platform was then used for the paired-end sequencing of these prepared library samples.

Data Processing

Raw data were initially cleaned by removing reads that contained adapter sequences, poly-N sequences, and low-quality reads with the FastQC program (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), after which clean data Q30, GC-content, and sequence duplication levels were calculated. The Trinity software [31] was then used to assemble reads into EST clusters, followed by de novo assembly and alignment to the rainbow trout reference genome (<http://www.genoscope.cns.fr/trout/data/>) with TopHat (v.2.0.5). Functional annotation was performed by comparing unigenes to the following databases: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins) [32]; Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database) [33]; GO (Gene Ontology) [34].

DEGs Identification

The RSEM software was used to assess unigene expression based upon reads per kilobase of exon per million mapped reads (RPKM) [35]. The DESeq R package (1.10.1) was used to identify DEGs between infected and non-infected fish using a negative binomial distribution-based model, with P values being adjusted as indicated by the Benjamini and Hochberg approach to reduce the false discovery rate. DEGs were considered as those genes with an adjusted P-value < 0.05, and were represented with volcano and MA plots. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for functional enrichment analysis of DEGs, with pathways that had a Q-value ≤ 0.05 after correcting for multiple testing being considered significantly enriched.

Validation Of Immune-related DEGs By QRT-PCR

To confirm the results of RNA-sequencing, eight immune-related DEGs (*IL-1 β* , *IL-8*, *TNF*, *NOD1*, *CARD9*, *TLR8a2*, *CCR9*, and *HSP90*) were chosen for qRT-PCR-based validation using the same RNA samples prepared for RNA-seq using primers designed with the Premier primer 5 software (Table 5). *EF-1 α* was used as a normalization control for these analyses. SYBR Green dye (Takara, China) and an ABI PRISM 7500 Fast Real-time PCR instrument were used for qRT-PCR based on provided protocols. All reactions were conducted in triplicate with the following thermocycler settings: 60 s at 95°C; 40 cycles of 15 s at 95°C, 45 s at 60°C. Melt curve analyses were conducted to confirm the specificity of amplification products. Relative gene expression was assessed via the $2^{-\Delta\Delta CT}$ approach [36].

Table 5
qRT-PCR Primers Lists

| Gene_ID | Gene name | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|-------------------|--------------------------------|---------------------------------|---------------------------------|
| Gene10807 | <i>IL-1β</i> | CAACTAAGATGGCCGCAA | TCGGTACATACTCTAAACCTC |
| Gene24642 | <i>IL-8</i> | ATTTATAAGCTTGATAGGCTG | GTTGTATATAAGAAACCGACT |
| Gene25550 | <i>TNF</i> | CAGGAGCATCACTACCTTC | TTACTAGAACTTTCTGCGGAT |
| Gene2178 | <i>NOD1</i> | ATACAACTGCTACCCCGACCA | AGGCACATTCACCAGGTCCA |
| Gene14361 | <i>HSP90</i> | GATCCTTCACCGTCAAAGTCG | TCACTTCCTTGTCACGCTCC |
| Gene45413 | <i>CCR9</i> | ATCTTGAATTTAAGCGCCTGT | ACATCATCCTCACCAACCGTA |
| Gene19781 | <i>CARD9</i> | TGACAACACTGACACGGAT | ATGCACATGAAGAGATACAAGC |
| newGene59965 | <i>TLR8a2</i> | CTCTGCCATTTTGATTGGGA | CCCCTAAGAAATCCACGAGA |
| Housekeeping gene | <i>EF-1α</i> | GATCCAGAAGGAGGTCACCA | TTACGTTGACCTTCCATCC |

Abbreviations

YR: *Yersinia ruckeri*; DEGs: Differentially expressed genes; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; ILs: Interleukins; IFNs: Interferons; TNFs: Tumor necrosis factors; CCR9: C-C motif chemokine receptor 9; CARD9: Caspase recruitment domain-containing protein; CASP8: Caspase 8; RIPK1: Receptor-interacting serine/threonine-protein kinase 1-like; TF65: Transcription factor p65-like; IAP: Inhibitor of apoptosis protein-like protein; *I κ B α* : NF-kappa-B inhibitor alpha-like; PPRs: Pattern recognition receptors; HSP90: Heat shock protein 90; NOD1: Nucleotide-binding oligomerization domain-containing protein 1-like; TLRs: Toll-like receptors.

Declarations

Ethics approval and consent to participate

The study was carried out in compliance with the ARRIVE guidelines. The Committee of the Ethics on Animal Care and Experiments at Heilongjiang River Fisheries Research Institute of Chinese Academy of Fishery Sciences also approved this study, which was consistent with the Guidelines of European Union Council Directive 2010/63/EU for the protection of research animals.

Consent for publication

Not applicable.

Availability of data and materials

The dataset(s) supporting the conclusions of this article is(are) included within the article (and its additional file(s)).

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

DW and SMS participated in the design of this study, and carried out the experiments. DW drafted the manuscript. SWL provided assistance for data analysis, background information collection and manuscript editing. DFS and TYL acquired the funding, carried out the conception of this study and reviewed the manuscript. All authors have read and approved the content of the manuscript.

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Figures

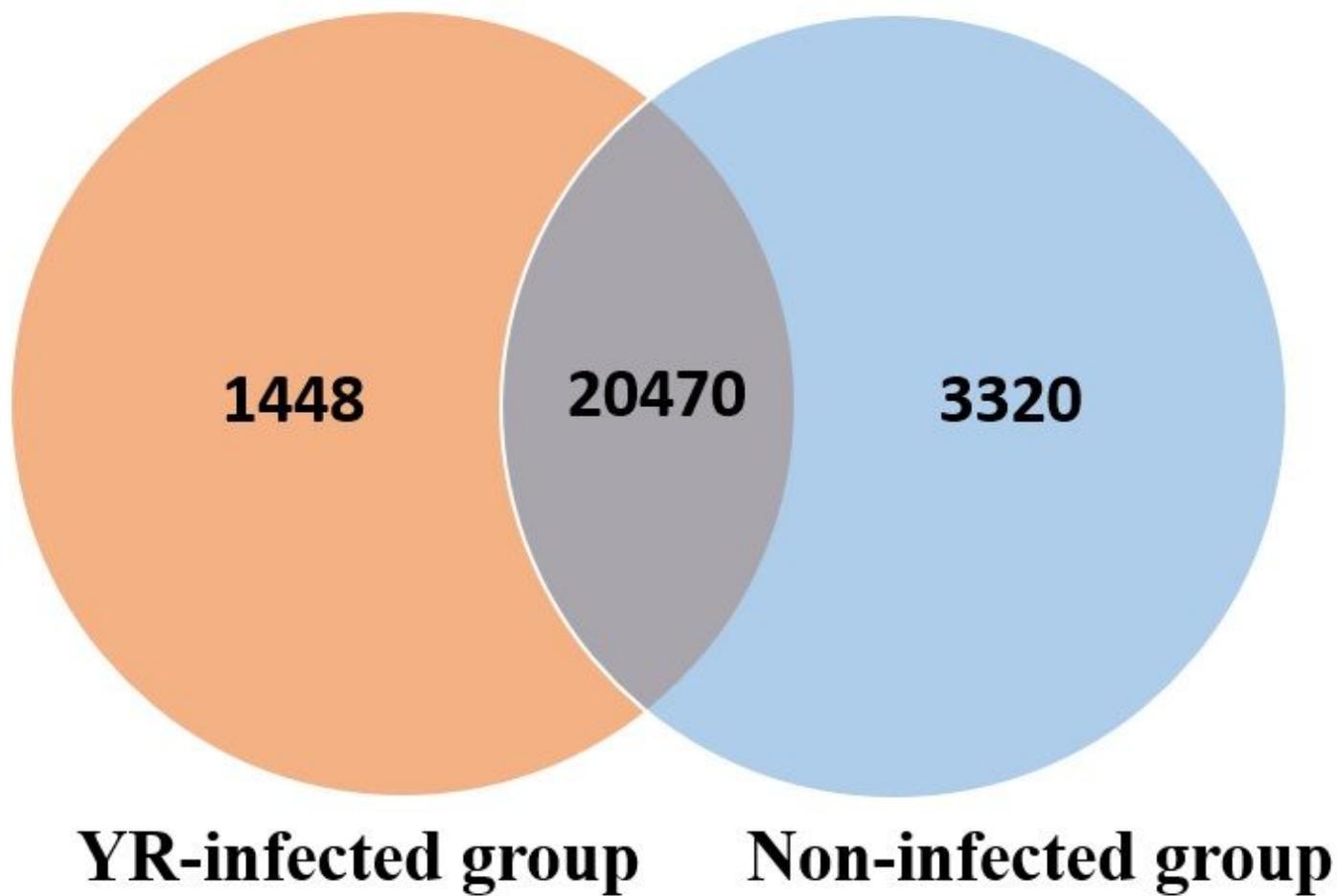


Figure 1

A Venn diagram indicating the numbers of genes detected in YR-infected and uninfected rainbow trout spleen samples

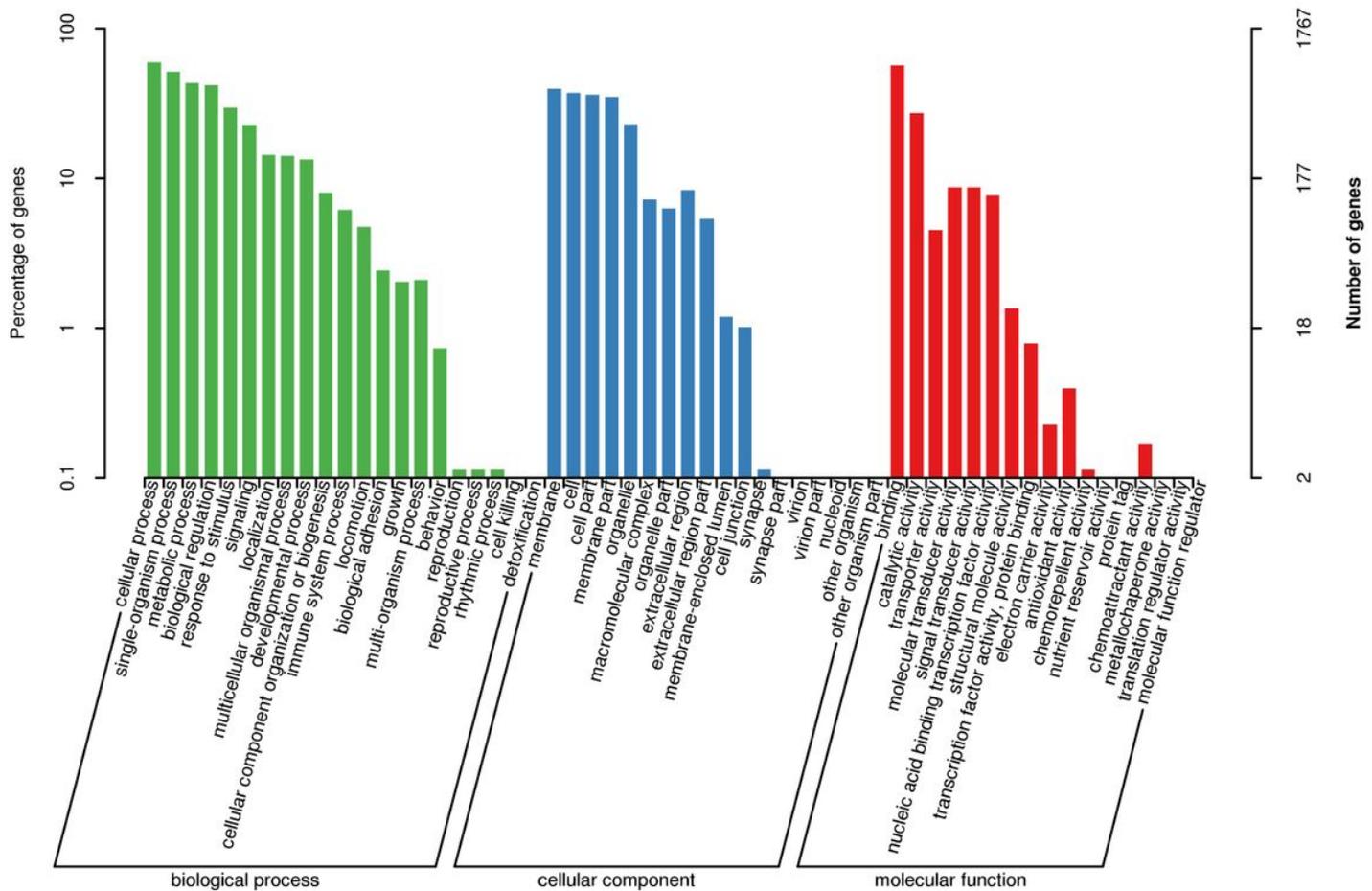


Figure 2

GO annotation of DEGs. DEGs were classified based on their enrichment in specific biological processes, cellular components, and molecular functions

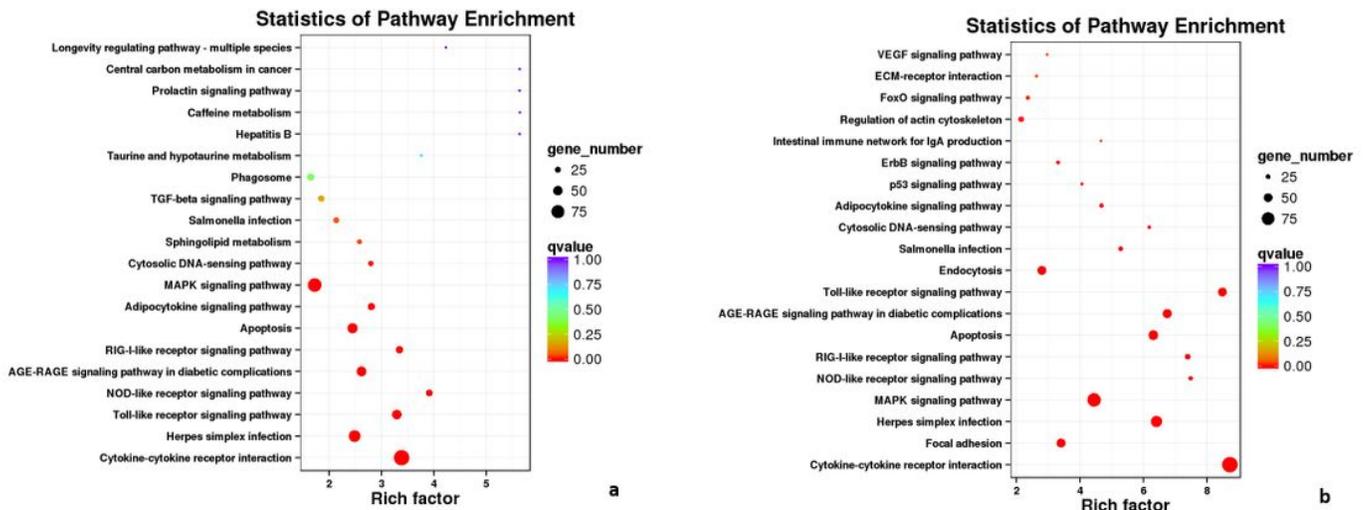


Figure 3

KEGG pathway enrichment results. Rich factor corresponds to the ratio of the total DEGs relative to total genes in the indicated pathways. (a) KEGG pathway enrichment results for all DEGs. (b) KEGG pathway enrichment results for the top 9 pathways

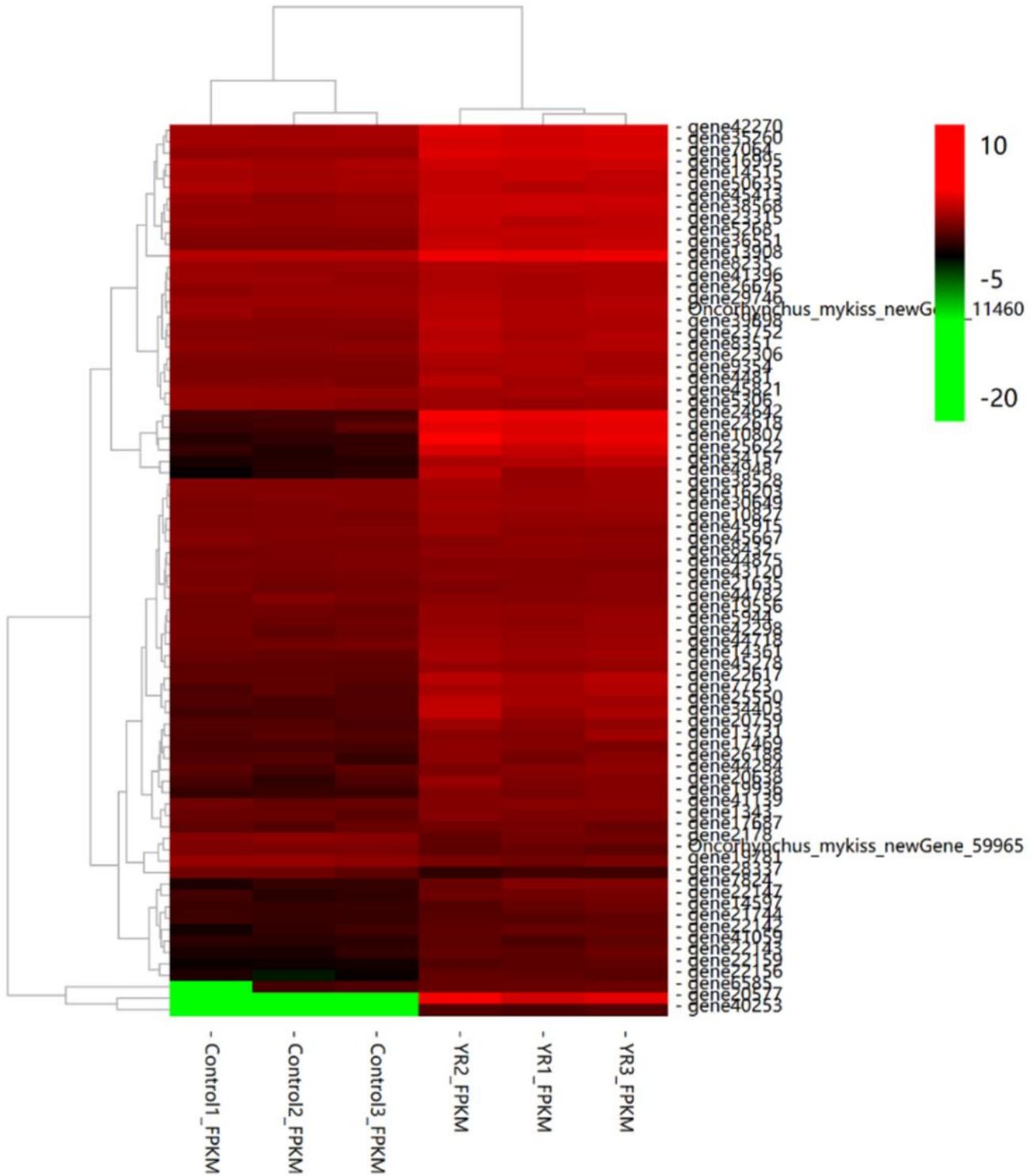


Figure 4

Statistics of Pathway Enrichment

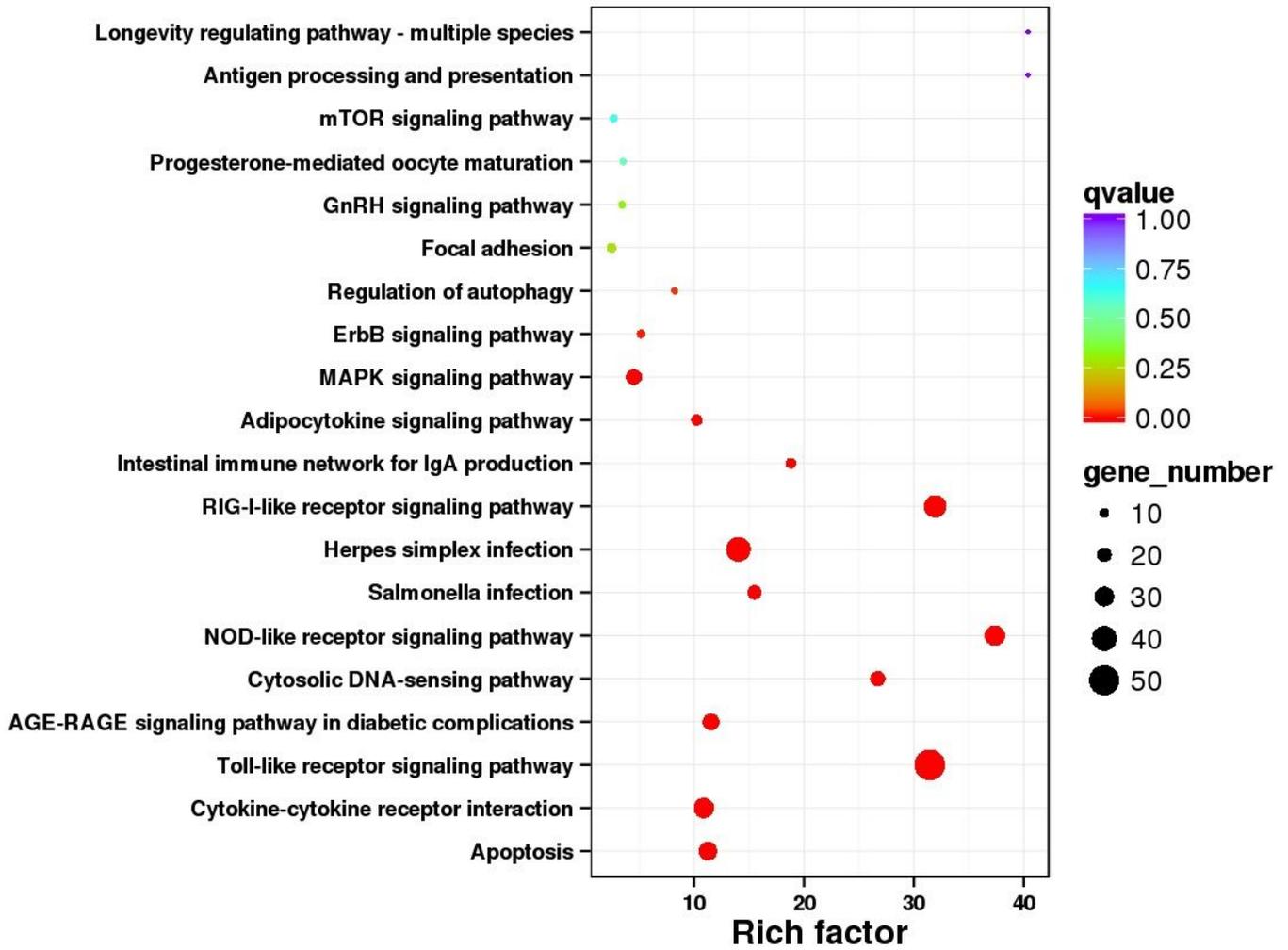


Figure 5

KEGG pathways enriched in genes differentially expressed between uninfected and YR-infected rainbow trout

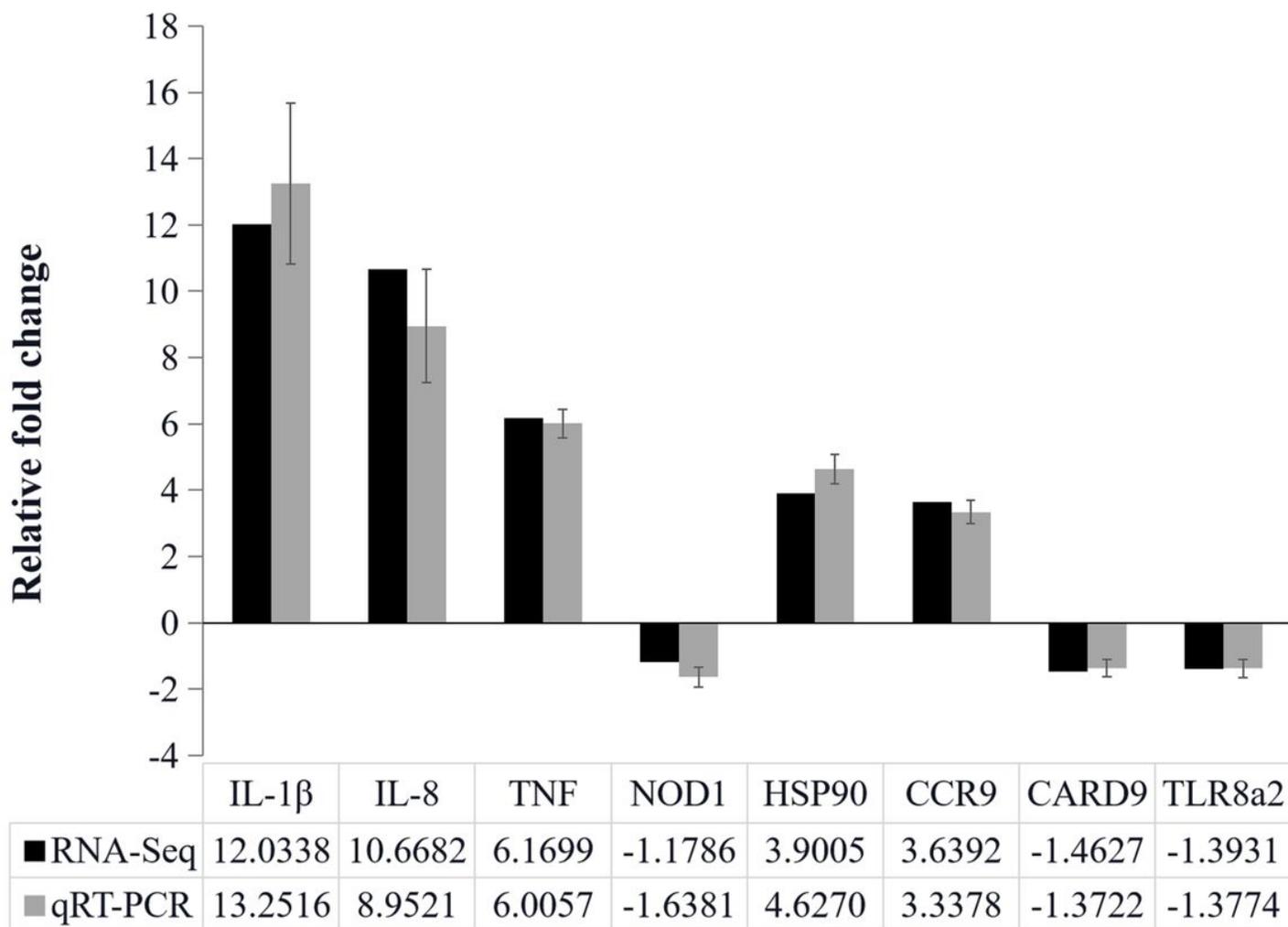


Figure 6

Comparison of DEG expression in qPCR and RNA-seq analyses. Relative gene expression levels were normalized to EF-1 α

Supplementary Files

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