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Short communication

Transcriptome analysis provides insights into molecular immune mechanisms of rabbitfish, *Siganus oramin* against *Cryptocaryon irritans* infection

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ABSTRACT

The rabbitfish *Siganus oramin* is resistant to the ciliate parasite *Cryptocaryon irritans*. L-amino acid oxidase (LAAO) protein from rabbitfish can kill *C. irritans in vitro*, however, other immune defence mechanisms against *C. irritans* remains unknown. Here, we generated transcriptomes of rabbitfish skin at 12 h post infection (PI) by *C. irritans*. The transcriptomes contained 238, 504, 124 clean reads were obtained and then assembled into 258,869 unigenes with an average length of 621 bp and an N50 of 833 bp. Among them, we obtained 418 differentially expressed genes (DEGs) in the skin of rabbitfish under *C. irritans* infection and control conditions, including 336 significantly up-regulated genes and 82 significantly down-regulated genes. Seven immune-related categories with 32 differentially expressed immune genes were obtained using Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. DEGs included innate immune molecules, such as LAAO, antimicrobial peptide, lysozyme g, as well as complement components, chemokines and chemokine receptors, NOD-like receptor/Toll-like receptor signaling pathway molecules, antigen processing and T/B cell activation and proliferation molecules. We further validated the expression results of nine immune-related DEGs using quantitative real-time PCR. This study provides new insights into the early immune response of a host that is resistant to *C. irritans*.

1. Introduction

Cryptocaryon irritans is a ciliated obligate parasite that causes the marine white spot disease in tropical and sub-tropical marine teleost [1,2]. The parasite targets the skin, fin, gill and cornea of its host, where it forms a large number of small white spots [3]. The infection severely impairs the physiological functions of the skin and gills, and it has a high mortality rate [3–5]. *C. irritans* is non-specific in its host selection and infects at least 100 species of teleost. However, not all species are susceptible to *C. irritans*, suggesting that some species are resistant against *C. irritans* [3,5–8].

The rabbitfish *Siganus oramin* is a marine teleost that is commonly cultured in Southeast China [9]. A previous field investigation during a *C. irritans* outbreak found that rabbitfish had significantly lower incidences of *C. irritans* infection, and even when infected with *C. irritans,* it did not show any symptoms of the marine white spot disease. Meanwhile, other fish species died from Cryptocaryoniasis during the outbreak season in the same sea area. Additionally, Wang et al. [10] examined the susceptibility of eight marine fish species from six different families that are cultured in Southern China, and found that the rabbitfish had significantly lower susceptibility to *C. irritans* than the other species of fish included in the study. Using artificial infection assays, we found that the number of parasites on the rabbitfish was decreased at 6 h post *C. irritans* infection (PI), which much shorter than other species where the parasitic stage of *C. irritans* lasts more than 2 days [2,11]. The tomonts were also smaller on rabbitfish compared to other fish species, implying that the rabbitfish evolved a resistance against *C. irritans* infection. Our previous study identified that the

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serum of rabbitfish could eliminate *C. irritans in vitro*, and a novel antiparasitic protein L-amino acid oxidase (LAAO) was isolated and purified from the serum [12]. However, other molecular immune mechanisms remain unknown.

Numerous studies have demonstrated that innate immunity and adaptive immunity play a role in combatting *C. irritans* infection [13–15]. However, these studies were conducted on hosts susceptible to *C. irritans*, such as *Epinephelus coioides* [16,17], *Trachinotus ovatus* [18] and *Larimichthys crocea* [19]. Studying the immune response of the rabbitfish, a species resistant to *C. irritans*, will enrich our understanding of the resistance mechanism in rabbitfish itself. To achieve this, we conducted a comparative transcriptome analysis of infected and uninfected rabbitfish skin, and identified genes involved in the immune response to *C. irritans*.

2. Materials and methods

2.1. Fish and parasite

Healthy rabbitfish (89.3 ± 8.7 g) were purchased from a commercial fish farm in Ningde City, Fujian Province, PR China. Ten fish were randomly screened for the presence of *C. irritans* on the gills and skin to ensure that the fish were not infected. The fish were cultured under laboratory conditions at 28 ± 1 °C with a flow-through water system (1000 L), and were acclimatized for at least two weeks before the experiment. Fish were fed twice daily with commercial pellet feed and their feces were siphoned off before feeding. All protocols involving fish were approved by the Institutional Animal Care and Use Committees at Sun Yat-Sen University.

A strain of *C. irritans* was obtained from a naturally infected large yellow croaker from a net cage in Ningde City. Parasite propagation and tomont collections were conducted in *L. crocea* (average 50 g) following the method of Jiang et al. [20].

2.2. C. irritans infection experiment and sample collection

In order to determine the time to collect samples for RNA-seq, 40 rabbitfish were artificially infected with a non-lethal dose of theronts (5×10^4/fish, 5 L seawater/fish) for 2 h. Fish were subsequently transferred to a new tank and raised under normal culture conditions. The gills of three fish were removed to count the number of trophonts at when the fish were transferred to the new tank (0 h), and 3 h, 6 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h and 120 h PI following protocols from Wang et al. [10]. Using the number of parasites found on the fish, the relative infection intensity (RII) was calculated as RII = Number of trophonts on second branchial piece/fish weight (g).

To collect RNA-seq samples, rabbitfish were divided into two groups containing 10 fishes each: the infected group where fish were treated with *C. irritans* following the protocol described above and the untreated control group. At 12 h PI, the skin of three fish was randomly sampled from the control (C, n = 3) and infected group (IN, n = 3). The samples were placed in RNAsafer Stabilizer Reagent (Takara, Dalian, China) immediately and placed at 4 °C overnight, then stored at −80 °C until RNA extraction.

2.3. RNA isolation, library preparation, and sequencing

Total RNA was extracted from samples using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The purity, quantity and integrity of total RNA were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc.) and 1% agrose gel. RNA with RNA integrity numbers (RIN) ≥7 were used for RNA-Seq library construction. Libraries were prepared using the TruSeq™ RNA Sample Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol, and libraries were sequenced on an Illumina/Hiseq-2500 platform (Illumina, San Diego, CA, USA) and 150 bp paired-end reads were generated at GENEWIZ, Inc. (Suzhou, China).

2.4. Data analysis of mRNA

Cutadapt [21] (version 1.9.1) was used to process raw reads to remove technical sequences, including adapters, polymerase chain reaction (PCR) primers, or fragments, as well as low-quality bases (Q < 20). Next, Trinity [22] was used with default parameters for de novo RNA assembly of clean data. Swiss-Prot, NCBI protein NR, Cluster of Orthologous Groups of proteins (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used for functional annotation of the transcriptome with highest sequence similarity and a typical cut-off *E*-value < $1.0\,E^{-5}$.

Gene and isoform expression levels were calculated using FPKM (fragments per kilobase of transcripts per million fragments mapped) values through RSEM (RNA Seq by Expectation Maximization) (v1.2.6) with default settings [23]. Differentially expressed genes (DEGs) analysis was performed on DESeq Bioconductor package with a negative binomial distribution model [24]. After adjusting for false discovery rate (FDR) using Benjamini and Hochberg's approach, genes with the absolute value of the log2 Ratio ≥ 1 and FDR ≤ 0.05 were defined as DEGs. All the DEGs were mapped to Gene Ontology (GO) and KEGG enrichment analysis, which were performed using the hypergeometric distribution test.

2.5. qPCR validation of DEGs

To verify the DEGs obtained from the transcriptome analysis, nine immune-related DEGs were selected for validation: complement component 4 (C4), complement component 7 (C7), E-selectin, interferon-gamma (IFN-γ), G-type lysozyme (LZM g), interferon regulatory factor 3 (IRF3), MHC class I alpha antigen (MHC I), signal transducer and activator of transcription 1 (STAT1) and Protein NLRC3 (NLRC3). cDNA was synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) following the manufacturer's protocol. Primers for the selected DEGs and *β*-actin were designed using Primer 5.0 (Premier Biosoft, Palo Alto, CA, USA) based on the assembled transcriptome sequence (Table S1). Real-time PCR was performed using a Roche LightCycler 480 Real-time PCR Detection System (Roche; www.roche.com) with SYBR Green Real-time PCR MasterMix (TaKaRa). PCR cycles were as follows: 95 °C for 3 min; followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s. The specificities of the PCR products were assessed by melting-curve analysis and sequencing. Each sample was amplified in triplicate. The mRNA expression level of the target gene was calculated relative to the reference gene using the $2^{-\Delta\Delta Ct}$ method [25] and statistical analyses were performed using SPSS (version 22.0; SPSS Inc., Chicago, IL, USA).

2.6. Statistical analysis

All data were analyzed using SPSS and one-way analysis of variance, followed by a Tukey test. The data are shown as mean ± s. e.m., where P < 0.05 was considered significant.

3. Results

3.1. Changes in the parasite numbers on fish

The relative infection intensity (RII) of rabbitfish was 3.25 ± 0.35 at 0 h PI. The RII began to decrease at 6 h PI, and was significantly lower at 12 h PI with 1.05 ± 0.47 RII (P < 0.05). RII continued to decline, until it reached about 0 at 96 h PI (Fig. 1).

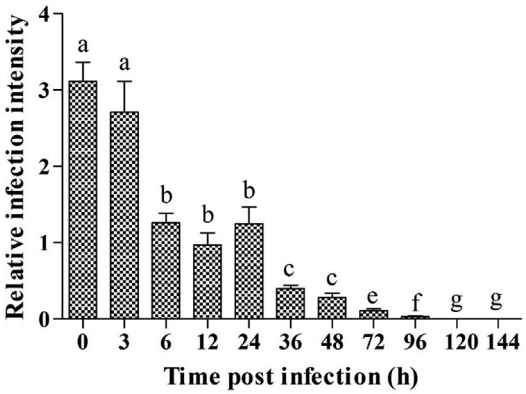

Fig. 1. Relative infection intensity in rabbitfish post *Cryptocaryon irritans* infection at different time points. Different superscripts indicate the significant difference ($P < 0.05$, Duncan's multiple comparison). Data shown are mean ± s. e.m, $N = 3$.

3.2. mRNA libraries

To identify genes that are involved in the rabbitfish's immune response against *C. irritans*, six cDNA libraries consisting of three infected samples (IN1, IN2, and IN3) and three control samples (C1, C2, and C3) were constructed and sequenced. A total of 241, 118, 224 raw reads were produced and deposited to NCBI (accession number SRP158990). The average of quality Q20 and Q30 were higher than 92.85% and 96.93% for each library, respectively, and the average GC content was 53.96% (Table 1). After filtering low quality reads, 238, 504, 124 clean reads were obtained and assembled into 258,869 unigenes with an average length of 621 bp and a N50 of 833 bp (Table 2).

Using BLASTp alignments with E-value $< 10^{-5}$ as cut-off, unigenes were aligned to NR, GO, COG, Swiss-Prot, and KEGG databases, where a total of 100,400 (38.78%) unigenes were annotated (Table 2). Among them, 66,877 (25.83%) unigenes were annotated by Swus-Prot and 95,775 (36.98%) unigenes were annotated by NCBI-NR. A total of 76,612 (29.59%) genes were annotated by GO and assigned into 59 subcategories that belonged to three major categories: biological process, cellular component and molecular function (Fig. S1). The COG database identified 47,283 (18.26%) annotated unigenes that clustered into 26 categories (Fig. S2). The KEGG database was used to annotate 40,065 (15.47%) unigenes that were classified into 43 KEGG pathways (Fig. S3).

3.3. DEGs, GO enrichment and KEGG pathway analysis

We compared the infected and control groups, and categorized genes as DEGs when the fold change of expression was > 2 ($P < 0.05$). The distribution trends of the DEGs were mapped in volcano plot (Fig. 2). We detected 418 DEGs, where 336 (80.4%) were upregulated and 82 (19.6%) were downregulated in the infected group.

DEGs were analyzed using GO and KEGG to evaluate the biological and functional roles of the DEGs. The GO enrichment analysis categorizes the role of genes into Molecular Function, Cellular Components and Biological Processes (Fig. S4). Most corresponding genes of DEGs in the Molecular Function category were involved in binding and catalytic activity, the Cellular Components category were involved in membrane and organelle function, and Biological Processes category were involved in metabolic processes.

We next aligned the transcriptome to the KEGG database to better predict the function of the DEGs (Fig. S5). The DEGs were mainly enriched infectious disease, signal transduction, and immunoregulation pathways. The most enriched pathways were herpes simplex infection and NOD-like receptor signaling pathway, where eight DEGs were enriched in these two pathways. Other enriched pathways were Toll-like receptor (TLR) signaling pathway, Measles, Hepatitis C, Amyotrophic

Table 2
Summary of de novo assembly of transcriptomic profiles of rabbitfish and annotation results.

	All_Unigene	Annoation results	Unigene	Precent (%)
sequences	258,869	Nr	95,775	36.98
bases	160699679	GO	76,612	29.59
Min	201	COG	47,283	18.26
Max	17,372	Swissprot	66,877	25.83
Average	621	KEGG	40,065	15.47
N50	833	Total	100,400	38.78

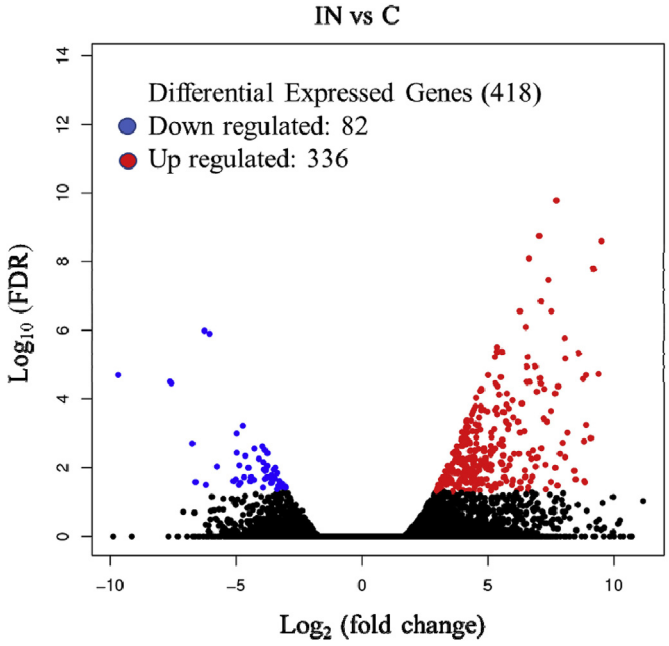

Fig. 2. Volcano plot of distribution trends for differentially expressed genes. The log2 fold (infected group/control group) indicates the mean expression level for each gene. Red dots represent significant up-regulated genes, green dots represent significant down-regulated genes and the black dots represent genes with no differential expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Summary statistics of the transcriptome sequencing for rabbitfish from control group and infected group.

Sample name	C1	C2	C3	IN1	IN2	IN3
Raw sequencing reads	43194852	37443562	41278526	37018696	37585254	44597334
Clean reads	42712376	37035128	40841728	36588564	37207442	44118886
Clean reads (%)	98.88	98.90	98.95	98.83	98.98	99.10
Q20 (%)	96.87	96.96	96.93	96.84	97.00	96.98
Q30 (%)	92.88	93.09	93.04	92.85	93.15	93.16
GC (%)	54.55	54.75	53.92	54.08	53.72	52.72

Table 3
Representative immune genes differentially expressed after *Cryptocaryon irritans* infection.

Gene name	Description	Fold change	FDR
Innate immune molecules			
AMP-NK	Antimicrobial peptide NK-lysin-like	28.9	2.99E-06
LAAO	Serum L-amino acid oxidase	30.1	3.18E-06
LZM g	lysozyme g protein	211.7	3.75E-15
Complement activation			
C7	Complement component 7	12.9	3.07E-06
C4	Complement component 4	7.8	0.0001
Chemokines and chemokine receptors			
IL8	Interleukin-8 like protein	26.8	5.13E-06
CXCL10	C-X-C motif chemokine 10-like	66.1	3.95E-06
CXCL20	C-C motif chemokine 20-like	75.5	3.97E-06
CXCR1	Chemokine XC receptor 1-like protein	39.4	2.31E-06
Cell adhesion and migration			
Gvin1	Interferon-induced very large GTPase 1	32.6	4.19E-09
SELE	E-selectin	15.3	1.82E-06
CD9	CD9 antigen 2	12.2	3.03E-06
Mef2d	myocyte-specific enhancer factor 2D isoform X1	47.5	9.70R-06
HSP90	Heat shock protein 90-alpha	21.7	2.90E-05
NOD-like receptor/Toll-like receptor signaling pathway			
STAT1	signal transducer and activator of transcription 1	15.8	5.58E-07
STAT2	signal transducer and activator of transcription 2	8.1	6.18E-05
IL10R2	interleukin-10 receptor subunit beta-like	19.5	1.48E-06
NLRC3	Protein NLRC3	24.2	1.70E-05
IRF7	Interferon regulatory factor 7	53.2	4.68E-05
NLRC5	Protein NLRC5	24.5	4.82E-05
IRF3	Interferon regulatory factor 3	63.6	4.92E-05
IRF4	Interferon regulatory factor 4	21.1	0.0001
INF-γ	Interferon-gamma	23.2	3.13E-08
IL1β	interleukin-1 beta	16.8	1.93E-04
Antigen processing and presentation			
CD83	CD83 antigen	20.6	1.27E-07
Ly9	T-lymphocyte surface antigen Ly-9	29.7	3.47E-06
TAP2	transporter-associated with antigen processing 2	21.3	9.53E-06
MHC I	MHC class I alpha antigen, partial	12.9	2.22E-05
MHC II	MHC class II antigen alpha chain	8.4	8.66E-05
TAP1	Antigen peptide transporter 1	9.3	5.75E-05
T/B cell activation and proliferation			
CD276	CD276 antigen	78.2	5.17E-05
CD200	OX-2 membrane glycoprotein	20.1	4.70E-05

lateral sclerosis (ALS), Glutamatergic synapse, Huntington's disease, Prolactin signaling pathway, Th17 cell differentiation, and Antigen processing and presentation.

3.4. Identification and clustering of immune-related DEGs

We next focused on DEGs that were annotated as immune-related in the GO and KEGG analyses to better understand the rabbitfish immune response against *C. irritans*. We identified 32 representative genes involved in innate and specific immunity (Table 3). These DEGs were further classified into seven subgroups: Innate immune molecules (6 DEGs), Complement activation (2 DEGs), Chemokines and chemokine receptors (4 DEGs), Cell adhesion and migration (5 DEGs), NOD-like receptor/TLR signaling pathway (9 DEGs), T/B cell activation and proliferation (6 DEGs) and Antigen processing and presentation (2 DEGs). In the present study, the expression for these immune related DEGs were all significantly up-regulate at 12 h PI.

3.5. Validation of DEGs from transcriptomes

We identified 418 DEGs by comparing the skin of rabbitfish infected with *C. irritans* and the untreated control group, demonstrating that the host experiences a dramatic change in gene expression when infected with *C. irritans*. To further validate the results from the RNA sequencing data, we selected nine immune DEGs for qRT-PCR analysis. The qRT-PCR expression patterns were consistent with the RNA-seq data (Fig. 3), and the statistical analysis showed correlation of r = 0.943 between the two types of analysis, confirming the reproducibility of the RNA-seq data.

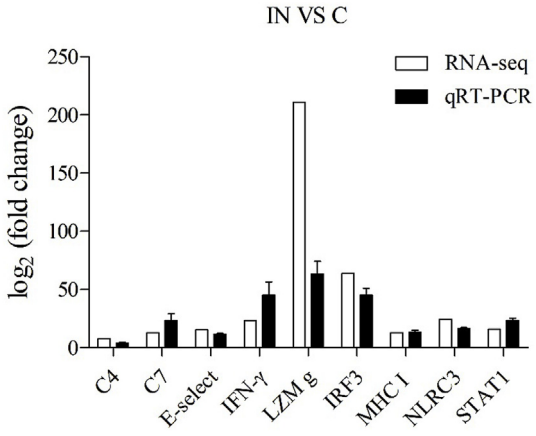

Fig. 3. Comparison of the expression profiles of nine immune function related genes as determined by RNA-seq (white) and qRT-PCR (black).

4. Discussion

A number of previous studies have shown that the rabbitfish is less susceptible to *C. irritans* than other teleost species [10,20]. *C. irritans* does not grow well on rabbitfish and most trophonts leave the host during its early developmental stages or grow into small tomonts, suggesting that the rabbitfish hinders the development of *C. irritans* [26]. We also found that the number of parasites on rabbitfish declined significantly at the early parasitic stage, therefore, it is likely that there are innate immune factors on the rabbitfish skin that prevent parasitic growth on the early stages of infection. The teleost skin is an important physiological barrier against pathogens, but is also considered to be the largest immunologically active mucosal organ of teleost species [27]. We generated transcriptomes of infected and uninfected rabbitfish skin and identified 418 DEGs in samples that were infected with *C. irritans*. Of these, 336 (80.4%) were up-regulated and 82 (19.6%) were down-regulated. It is worth noting that many of the DEGs fell into GO and KEGG pathways that were involved in the immune system (Table 3), suggesting that *C. irritans* infection triggered an immune response in the skin cells of rabbitfish. Similarly, the susceptible host *E. coioides* would be also triggered an immune response PI [28]. However, there were less DEGs in rabbitfish than those in *E. coioides* PI. Although the sampling time and species are different, it is evident that the number of up-regulated genes is significantly greater than down-regulated genes in the less susceptible host rabbitfish. In addition, the previously confirmed antiparasitic protein LAAO up-regulated 211.7 times in rabbitfish PI, which is not found in other transcriptomes of the susceptible host PI [19,28,29].

When the pathogens invade the skin of the host, the pathogen associated molecular patterns (PAMPs) will be first activated through the pattern recognition receptors (PRRs). Recognition of PAMPs leads to the activation of signaling pathways that work towards eradicating the pathogen. Mechanisms to attack pathogens include phagocytosis, activation of the complement cascade, production of inflammatory cytokines, secretion of interferons and antimicrobial peptides, and stimulation of the adaptive immune system [30]. We found that components of the NOD-like receptor and TLR signaling pathways, including IRF3, IRF7, STAT1, STAT2, NLRC3 and NLRC5, were significantly up-regulated in the transcriptome of the infected samples. IRF3 and IRF7

regulate type I interferon (IFN) response in mammals [31]. IFN-γ in fish induces antigen presentation, and enhances respiratory burst activity, phagocytic response and pro-inflammatory gene expression [32]; In this study, we found increased expression of IFN-γ in the skin of infected rabbitfish. In addition, the pro-inflammatory cytokine IL1β was also significantly up-regulated. Taken together, these results suggest that the above signaling pathways may be activated, leading to the secretion of cytokines against *C. irritans* in the infected samples.

In the elimination of pathogens, the innate immune molecules of the host play a central role in the resistance to pathogens in the first place [33,34]. DEGs in our dataset included innate immune genes such as LAAO, LZM g, and Antimicrobial peptide NK-lysin-like (AMP-NK), all of which were significantly up-regulated. LAAO is a classical flavoenzyme that can serve as an antibacterial protein on the epithelial surfaces of fish [35]. Previously, Wang et al. [12] found that purified LAAO from rabbitfish can eliminate *C. irritans in vitro* by lysing the *C. irritans* membranes. We found that LAAO was significant up-regulated 12 h PI in the skin, which is in lines with previous studies [20] that found up-regulated expression of LAAO from 6 to 24 h PI in gill and spleen of the rabbitfish. Lysozymes and antimicrobial peptides are important components of the innate immune system to defend against pathogens [36,37]. In species that are susceptible to *C. irritans*, lysozyme or antimicrobial peptides may be important for protecting against infections by parasites. Lysozyme activity is significantly higher in the blood of *E. coioides* and *L. crocea* after *C. irritans* infection [17,18]. Similarly, Zheng et al. [38] found five up-regulated antimicrobial peptide genes in the *L. crocea* liver after infection. Our results suggest that LZM g and AMP-NK may be involved in the rabbitfish's resistance against *C. irritans* infection.

The complement system, chemokines and chemokine receptors play an essential role in protecting hosts against *C. irritans* [16,19,27]. The complement system regulates innate and acquired immunity in fish [8,19]. C4 is an early component of complement activation, and C7 plays a role in the end effect of complement activation [39–42]. In the rabbitfish, we found that both C4 and C7 were up-regulated in infected samples. Chemokines recruit immune cells to migrate to the sites of injury or infection and regulate the inflammation response [43]. Chemokines and chemokine receptors are upregulated in other hosts that are infected by *C. irritans* [19,27,40], and we found that three chemokines (IL8, CXCL10 and CXCL20) and one chemokine receptors (CXCR1) were significantly up-regulated in the infected samples. IL-8 is an important chemokine that is involved in chemoattraction and activation of white blood cells. When fish are infected by parasites, neutrophils-like cells are often recruited to the local infection sites [43]. IL-8 serves as a chemoattractant and activates neutrophils by binding to receptors CXCR1 and CXCR2 [44]. We found that IL8 and CXCR1 were significantly up-regulated in infected samples, and these results suggest that neutrophils may participate in the rabbitfish's defense against *C. irritans* infection.

Although most *C. irritans* were eliminated by the rabbitfish at the early parasitic stage, DEGs associated with antigen presentation and T/B cell activation and proliferation were found in our dataset. Expression of MHC I, MHC II, TAP1, TAP2, Ly9, CD83, CD200 and CD276 were significantly up-regulated in infected samples. MHC molecules and CD83 are expressed on the surface of antigen presenting cells, and they are responsible for presenting antigens to T cells, as well as regulating specific B and T cell responses [45]. The results show that in addition to the innate immune response, infection by *C. irritans* leads to specific immune responses.

5. Conclusion

Here, we conducted a comparative transcriptomic analysis to identify DEGs involved in immune response against *C. irritans* in the rabbitfish. We found 418 DEGs in the skin of rabbitfish 12 h after *C. irritans* infection. By comparing DEGs related to immune response, we found that the rabbitfish activated innate immune molecules, complement and coagulation cascades pathway, chemokine signaling pathways, the NOD-like receptor, and TLR signaling pathways. The transcriptome also revealed that acquired immunity played a role in the rabbitfish's response to *C. irritans* infection, as we observed up-regulation of genes involved in antigen processing and presentation and T/B cell receptor signaling pathway were in the infected samples. In conclusion, rabbitfish relies primarily on innate immunity when infected by *C. irritans*, and acquired immunity further strengthens the resistance against the parasite.

Acknowledgments

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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.2019.02.039.

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