Transcriptional Profiles of Genes Related to Stress and Immune Response in Rainbow Trout (Oncorhynchus mykiss) Symptomatically or Asymptomatically Infected With Vibrio anguillarum

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Rainbow trout (Oncorhynchus mykiss) is one of the most common aquaculture fish species worldwide. Vibriosis disease outbreaks cause significant setbacks to aquaculture. The stress and immune responses are bidirectionally modulated in response to the health challenges. Therefore, an investigation into the regulatory mechanisms of the stress and immune responses in trout is invaluable for identifying potential vibriosis treatments. We investigated the transcriptional profiles of genes associated with stress and trout immune functions after Vibrio anguillarum infection. We compared the control trout (CT, 0.9% saline injection), asymptomatic trout (AT, surviving trout with minor or no symptoms after bacteria injection), and symptomatic trout (ST, moribund trout with severe symptoms after bacteria injection). Our results showed activated immunomodulatory genes in the cytokine network and downregulated glucocorticoid and mineralocorticoid receptors in both AT and ST, indicating activation of the proinflammatory cytokine cascade as a common response in AT and ST. Moreover, the AT specifically activated the complement- and TNF-associated immune defenses in response to V. anguillarum infection. However, the complement and coagulation cascades, as well as steroid hormone homeostasis in ST, were disturbed by V. anguillarum. Our studies provide new insights toward understanding regulatory mechanisms in stress and immune functions in response to diseases.

Keywords: rainbow trout, vibriosis, stress responses, immune functions, RNA-Seq
HIGHLIGHTS

- Asymptomatic and symptomatic trout mounted different immune responses
- *V. anguillarum* infection activated the proinflammatory cytokine cascade
- The complement- and TNF-related immune defenses were specifically activated in asymptomatic trout
- Diverse functions were identified among three novel c3-1 subtypes

INTRODUCTION

Teleosts have to cope with various challenges, including the diversity of the potential environmental stimuli and pathogen load (1, 2). Although teleosts respond differently to stressors and the immune responses also remain species-specific, environmental and aquaculture insults can trigger defensive reactions of fish, including the activation of the stress response (3, 4). Based on energy balance, the stress response results in energy redistribution with the ultimate purpose to restore homeostasis, thus saving the energy that is not necessary to survive and enabling fishes to prepare for “fight” or “flight” (5–7). For example, a slightly activated stress response could enhance immune competence (fight), while a prolonged stress response suppresses immune function (flight) (8).

Cortisol and its receptors [glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (9)] play an important role in regulating crosstalk between the stress response and immune networks. Activation of the GR (or MR) may serve as an early danger alarm and enable the immune system to prepare for the fight against health challenges (10, 11). Moreover, GR (or MR) activation modulates the leukocyte-regulated immune responses and negotiates the initiation and efficacy of immune functions (1). Inflammation serves as the first step of immunomodulation in response to infection or irritation (12). Proinflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα) (5), act as an important defense mechanism against pathogens. The stress response typically regulates the immune response by suppressing the synthesis and release of proinflammatory cytokines in both mammals and teleosts (13–15).

In the mid-1980s, a series of papers published in *Science* showed that proinflammatory cytokines act as stress-response regulators [reviewed in (16)]. Another previous study showed that cytokines regulate stress responses in mammals by decreasing GR expression, blocking GR translocation, and...
disrupting GR-DNA binding in the nucleus (17). In response to pathogen infection, the homeostatic interaction between the stress response and cytokine-induced inflammation in teleosts is more complicated, showing no negative or positive correlation among various teleosts. For example, the stress response (mimicked by cortisol) does not affect cytokine gene expression in rainbow trout (*Oncorhynchus mykiss*); however, the stress response did reduce the stimulated gene expression of all cytokines in gilthead sea bream (*Sparus aurata*) (11). In the European sea bass (*Dicentrarchus labrax*), genes associated with glucocorticoid synthesis and inflammatory responses are simultaneously upregulated after *Vibrio anguillarum* infection (5). These studies indicate that the interplay between stress and immune responses is differentially regulated in various teleost species.

In addition to the cytokines, the complement cascade is also involved in immunomodulation in response to pathogen invasion. The complement system, which was identified a century ago, is the most ancient and essential immune system component [reviewed in (18–20)]. The complement system is the first immune response against invading pathogens and orchestrates the subsequent immunological and inflammatory processes associated with detection, destruction, and elimination of the microbial intruders [Reviewed in (18–20)]. The mammalian complement repertoire includes ~35 plasma (hydrophilic)- and membrane (hydrophobic)-bound complement proteins (21). Although the mammalian complement system can be activated by the classical, lectin, or alternative pathways, all three pathways share the common step of activating the component C3 (18). The physiological functions and signaling cascades of the complement system are mostly conserved between mammals and teleosts (22, 23). An activated complement system will release complement protein fragments that typically kill the microbial intruders and orchestrate immunological and inflammatory homeostasis (22). Early studies in rainbow trout showed that the complement system accounts for resistance to furunculosis or vibriosis (24, 25). These two highly contagious diseases cause excessive trout mortality, which leads to significant aquacultural economic loss.

Infectious diseases are constant threats to aquaculture and larviculture, causing significant financial losses due to high infectivity and mortality (11). *V. anguillarum*, the causative agent of vibriosis, is a gram-negative bacteria that causes severe, frequently deadly hemorrhagic septicemia in teleosts (26, 27). The previous studies showed that fish exhibit higher individual variations in response to pathogen infection (28–30). Genetic factors that favor the survival of asymptomatic individuals could be used as targets for selecting disease-resistant fish, thus reducing economic loss from infectious disease (31). Although accumulating studies have been focused on generating disease (or stress)-resistant fish strains, the mechanisms remain largely unknown (31, 32). Investigation of the target genes and pathways associated with disease-resistant could potentially provide molecular markers for genetic breeding.

Rainbow trout (*Oncorhynchus mykiss*) is one of the most common aquaculture fish species worldwide (Food and Agriculture Organization of the United Nations); however, the trout industry is severely affected by vibriosis (27). In this study, the RNA-Seq datasets were retrieved from our previous studies (33, 34), and we analyzed a total of 27 RNA-Seq libraries. Briefly, we investigated control trout (0.9% saline-injection), asymptomatic trout (AT; surviving trout with minor or no symptoms after *V. anguillarum* injection), and symptomatic trout (ST; moribund trout with severe symptoms after *V. anguillarum* injection). The brain, kidney, and spleen were collected for RNA-Seq. Previous studies in trout revealed important genes involved in regulating stress responses and immune functions (35–40); therefore, we targeted these candidate genes (Figure 1). Our studies showed that complement- and TNF-associated immune defenses were specifically activated in AT. Our studies provide new insights into the stress-immune network in response to pathogen infection in trout and provide potential molecular markers for genetic breeding of disease-resistant trout populations.

**MATERIALS AND METHODS**

**Ethics Statement**

Experiments in this study were conducted in accordance with Guidelines of Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201), U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and use of laboratory animals (NIH Publications No. 8023, revised 1978) National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 8023, revised 1978). No endangered or protected animal species were used. The effects of sex were not considered because trout juveniles are sexually immature.

**Animals**

Rainbow trout juveniles were obtained from Linqu Salmon and Trout Aquatic Breeding LLC (Weifang, Shandong, China). These juveniles were from the same full-sibling family batch and spawned on the same day with synchronized development. Trout were acclimatized for 14 days in indoor cuboidal tanks equipped with a water pump, chiller system, sand filter, and biofilter at the Experimental Fish Facility in Key Laboratory of Mariculture, Ocean University of China. According to the Standards of Linxia Salmon and National Trout Elite Breeding and Protection Farm (Linxia, Gansu, China, Approved by Department of Agriculture, China, 2009), trout were cultured at ~16°C and ~7 mg/L of dissolved oxygen. Trout were fed a commercial pellet twice a day at 7% of total body weight.

**V. anguillarum**

The *V. anguillarum* strain was obtained from the Laboratory of Pathology and Immunology of Aquatic Animals, Ocean University of China (41, 42). The bacteria were grown overnight at 28°C in 2216E medium. The bacterial suspension was then centrifuged and resuspended with 0.01 M phosphate-buffered saline (PBS, pH = 7.2). *V. anguillarum* suspension density was adjusted to serial dilutions for preliminary testing: 10^{6}, 10^{7}, or 10^{8} colony forming units (CFU/ml) (33).
Experimental Design

This manuscript used the same RNA-Seq samples previously described in two papers evaluating the growth hormone and insulin-like growth factor axes, as well as the caspase gene family in rainbow trout (33, 34). Previous studies showed 10^7 to 10^9 CFU/ml of *V. anguillarum* could cause vibriosis in rainbow trout and other teleosts (41–43). Our published paper further showed that *V. anguillarum* of 10^7 CFU/ml at 20°C exhibited mild to moderate symptoms of vibriosis disease with a relatively lower mortality (33). Therefore, trout were challenged by 10^7 CFU/ml. In the control group, 90 trout were randomly distributed into three tanks, with 30 trout in each tank. The control trout (CT) were intraperitoneally injected with 200 μl physiological saline (saline-challenged, 0.9% NaCl). In the challenged group, 90 trout were equally and randomly distributed into three tanks. Trout of the challenged group were challenged by intraperitoneal injection of 200 μl *V. anguillarum* (10^7 CFU/ml). In challenged groups, the first three erratically swimming moribund trout showing severe symptoms, such as hemorrhage in fins, in tank #1 were pooled as sample #1 of the symptomatic trout (ST). After 120 h post-challenge, the three surviving trout with minor or no symptoms were pooled as sample #1 of the asymptomatic trout (AT). Likewise, sample #2 of ST and AT, as well as sample #3 of ST and AT, were collected from tank #2 and tank #3, respectively (Figure 1). The control trout were injected with 0.9% NaCl and then sampled with the same protocol. (A) was partly adapted from Figure 1 in our previous paper (33). (B) Based on previous studies (35–40), genes in the brain, kidney, and spleen associated with stress and immune functions were investigated in CT, ST, and AT.

RNA-Seq Analysis

A total of 27 libraries [3 tissues (brain, kidney, spleen) × 3 replicated samples (each sample contained three pooled individuals × 3 treatment groups)] was constructed via the TruSeq™ RNA Sample Prep Kit (Illumina, CA, USA). This study used the same RNA-Seq data with our previously published paper (33, 34), but we focused on different functional genes and used various analyses. The sequence reads are available from the NCBI sequence read archive (SRA) with the accession number of PRJNA667799.

Novel Gene(s) Identification

The amino acid sequences of trout novel C3-1 proteins, and zebrafish (*Danio rerio*), southern catfish (*Silurus meridionalis*), rat (*Rattus norvegicus*), and human (*Homo sapiens*) C3 proteins were used for the phylogenetic analysis and sequence alignment. Phylogenetic analyses were plotted using the Neighbor-joining (N-J) method via MEGA 7, with 1000 bootstrap replications for phylogeny. The SWISS-MODEL between trout and mammalian C3 proteins was generated using the SWISS-MODEL (https://swissmodel.expasy.org/) (44, 45). The mammalian C3 with an intact thioester at 3Å resolution [PDB ID: 2B39 (46)] was used as the template. Comparison of the domains between trout and mammalian C3 and the cartoon, stick, and sphere structures of the proteins were generated with the PyMOL software package (47, 48).
**RESULTS**

**Differentially Expressed Genes Between ST and CT**

The heatmap displayed the transcriptional profile of genes associated with the stress response, cytokines and cellular functions, and the complement system between ST and CT (Figures 2A–C). The overall transcriptional profiles of target genes in ST and CT in response to *V. anguillarum* infection were summarized by PCA (Figure 2D). Red dots show the vector containing overall gene expression in ST, and green dots showed the vector containing overall gene expression in CT. Separated PCA vectors were present, indicating that the *V. anguillarum* infection resulted in different profiles of genes associated with the stress response, cytokines and cellular functions, and the complement system between ST and CT (Figure 2D). The loading plot of PCA shows the genes exerting stronger influences on PCA analysis (Figure 2E), points far away from the zero point, Table S1).

The volcano plots showed that, compared to CT, the ST showed significantly downregulated kidney mrrα, mrrβ, c7-2, and cd93, and spleen grα, grβ, c7-2, and c1qa, and brain c7-2 and c3-4 (Figures 2F, G). Compared to CT, the kidney il1l, mbl-h2, and c3-1b1, and spleen il1l1, il1l2, il8, tnfα2, c3-1a, c3-1b1, and c3-3 were significantly upregulated in ST (Figures 2F, G). The genes showed in volcano plots were labeled in the loading plot (Figure 2E).

The correlation analysis of all target genes is depicted using a heatmap (Figure 2H and Figure S2). The Pearson correlation coefficients showed that the kidney mrrα or mrrβ exhibited strong negative relationships with the cytokines of il1lβ3, il4, il8, and tnfα3 (Figures 2H–J). The spleen grβ showed negative relationships with il1l1, il1l2, il8, and tnfα2 (Figures 2H, K).

**Differentially Expressed Genes Between AT and CT**

The transcriptional profiles of genes involved in the stress response, cytokines, cellular functions, and the complement system between AT and CT were shown by heatmap (Figures 3A–C). Separated PCA plots indicate that genes related to cytokines, the stress response, cellular functions, and the complement system were differently expressed in AT and CT (Figure 3D). The loading plot showed the genes significantly involved in the separated PCA plots (Figure 3E, points far away from the zero point, Table S2).

The volcano plots showed that, compared to CT, the AT showed downregulated kidney c7-2, cd93, and spleen mrrα, mrrβ, grβ, hsdl1lβ2, c7-2, c6, and c8g, and brain c7-2 (Figures 3F, G). The AT exhibited significantly upregulated kidney il1l2, il1l4, c4, and mbl-h2, and spleen il1l1, il1l2, il1l3, il6, tnfα3, cfb, cpf1, c3-lb2, c3-3, and bcf2-b (Figures 3F, G). These genes were highlighted in the loading plot (Figure 3E).

Heatmap showing the Pearson correlation coefficients of genes (Figure 3H and Figure S3). Pearson correlation coefficients showed that the spleen mrrα or mrrβ exhibited strong negative relationships with the cytokines of il1lβ1, il1lβ2, il1lβ3 and tnfα3 (Figures 3H–J). The spleen grβ exhibited negative Pearson correlation coefficients with il1lβ3, and tnfα3 (Figures 3H, L), while the kidney grα showed negative relationships with il1lβ1, il1lβ3, tnfα1 and tnfα3 (Figures 3H, K).

**Identification of Novel c3 Gene Subtypes**

We identified three novel c3 gene subtypes in RNA-Seq data. Based on the alignment of the amino acid sequences, these three C3 proteins showed the conserved functional domains, including the ANATO domain, thioester domain, and C3-convertase cleavage site (Figure 5A and the whole sequences alignment are shown in Figure S7). Based on mammalian C3 (PDB ID: 2B39), the SWISS-MODEL illustrated conserved motifs between trout and mammalian C3 with blue cartoons (Figure 5B and Figures S7, S8, S9A, S9B, S9C). Red and green boxes mark the ANATO and thioester domains, respectively (Figures 5B, C). The comparison of the thioester (green) and ANATO (red) domains between trout and mammalian C3 are shown in
FIGURE 2 | Transcriptional profiles of genes in stress and immune functions between ST and CT. (A–C) The heatmap of genes related to the stress response (A), cytokines and cellular functions (B), and the complement system (C). The heatmap is generated by the values of log10 (normalized count+1). The red shows high expression, and green shows low expression. More details are shown in Table 1. Basal gene expression is shown in Figure S5. (D, E) PCA (D) and loading plots (E) of genes related to the stress response, cytokines, cellular functions, and the complement system. The red dots show the vector of overall gene expression in CT, and the green dots show the vector of overall gene expression in ST. Details of the loading plot are shown in Table S1. (F, G) Volcano plots of genes of the stress response and cytokine (F), and the complement system (G). Negative and positive Log2FoldChange show down-regulation and upregulation, respectively (ST vs. CT). More details are shown in Table 1. (H–K) Correlations of genes related to the stress response, cytokines, cellular functions, and the complement system. The detailed view of (H) is shown in Figure S2. Gene abbreviations are shown in Table 1.
FIGURE 3 | Transcriptional profiles of genes in stress and immune functions between AT and CT. (A–C) The heatmap of genes of the stress response (A), cytokines and cellular functions (B), and the complement system (C). The heatmap is generated by the values of log10 (normalized count+1). The red shows high expression, and green shows low expression. More details are shown in Table 1. Basal gene expression is shown in Figure S5. (D, E) PCA (D) and loading plots (E) of genes related to the stress response, cytokines, cellular functions, and the complement system. The red dots show the vector of overall gene expression in CT, and the blue dots show the vector of overall gene expression in AT. Details of the loading plot are shown in Table S2. (F, G) Volcano plots of genes of the stress response and cytokines (F), and the complement system (G). Negative and positive Log2FoldChange show down-regulation and upregulation, respectively (AT vs. CT). More details are shown in Table 1. (H–L) Correlations of genes related to the stress response, cytokines, cellular functions, and the complement system. The detailed view of (H) is shown in Figure S3. Gene abbreviations are shown in Table 1.
Figure 4 | Transcriptional profiles of genes in stress and immune functions between ST and AT. (A–C) The heatmap of genes of the stress response (A), cytokines and cellular functions (B), and the complement system (C). The heatmap is generated by the values of log10 (normalized count+1). The red shows high expression, and the blue shows low expression. More details are shown in Table 1. Basal gene expression is shown in Figure S5. (D, E) PCA (D) and loading plots (E) of genes related to the stress response, cytokines, cellular functions, and the complement system. The blue dots show the vector of overall gene expression in AT, and the green dots show the vector of overall gene expression in ST. Details of the loading plot are shown in Table S3. (F, G) Volcano plots of genes related to the stress response and cytokines (F), and the complement system (G). Negative and positive Log2FoldChange show down-regulation and upregulation, respectively (ST vs. AT). More details are shown in Table 1. (H–K) Correlations of genes related to the stress response, cytokines, cellular functions, and the complement system. The detailed view of (H) is shown in Figure S4. Gene abbreviations are shown in Table 1.
Compared to CT, the ST showed significantly upregulated c3-1a and c3-1b1 expression in the kidney and spleen (Figures 5D, E).

**Functional Enrichment Analysis of DEGs**

Compared to CT, the AT showed upregulated Ko04610 (complement and coagulation cascades) in the kidney and spleen (Figure 5D). In contrast, the ST showed a downregulated Ko04610 pathway in the brain, kidney, and spleen (Figure 5D). No significant changes in the Ko04610 pathway were observed in the kidney and spleen between AT and ST. The overlapping genes in the Ko04610 pathway are shown in Venn diagrams (Figures S9G, S9H), and their expression levels among CT, AT, or ST were shown by heatmap (Figures S9I–S9L).

We showed gα and gβ were shared in the list of DEGs between groups of CT and ST or CT and AT. In the gene ontologies (GO) terms involved in gα and gβ, 8 GO terms were shared between the comparisons of CT and ST or CT and AT (Figure 6A, details in Table 2). Three GO terms were specifically enriched in the comparison of CT and ST (Figure 6B, details in Table 2), and five GO terms were specifically enriched in the comparison of CT and AT (Figure 6B, details in Table 2). Likewise, ifnα subtypes were shared in the DEGs list between CT and ST or CT and AT (Figure 6C, details in Table 2). Three GO terms were specifically enriched in the comparison of CT and ST (Figure 6D, details in Table 2), and 14 GO terms were specifically enriched in the comparison of CT and AT (Figure 6D, details in Table 2).

Genes of mrxα and mrfβ were identified in the list of DEGs between the comparison of ST and AT. Based on the KEGG database, four pathways that are associated with functions of steroid hormones were enriched (Figure 7A), including ko04960 (aldosterone-regulated sodium reabsorption, Figure 7B), ko04978 (mineral absorption), ko00140 (steroid hormone biosynthesis, Figure 7C) and ko04913 (ovarian steroidogenesis, Figure 7D).

**DISCUSSION**

Several studies have already focused on reactions of stress- and immune-related functions to *V. anguillarum* infection in teleosts, showing the teleosts exhibit species-specific modulations (1, 5, 11). Therefore, we evaluated stress response and immune network changes in trout after *V. anguillarum* infection. Previous studies evaluated the immunomodulation of cartoons (Figure 5C and Figures S9B, S9C). The conserved amino acid sequences of GCGEQ in thioester domain were labeled (Figure 5C, top figure). The locations were adjacent, and the identities were identical for both GCGEQ sequences of mammalian and trout C3 (Figure 5C, top figure; Figure S9C).

Likewise, the ANATO domains of both mammalian and trout C3 are similarly organized, and their amino acid sequences were highly identical (Figure 5C, bottom figure; Figure S9B). The gene expression levels of three c3 were shown (Figures S9D–F). Compared to CT, the ST showed significantly upregulated c3-1a and c3-1b1 expression in the kidney and spleen (Figures S9D, E).
FIGURE 6 | Enriched GO terms associated with gr subtypes (A, B) or associated with tnfα subtypes (C, D). (A) The enriched GO terms shared in comparisons of CT vs. ST and CT vs. AT. (B) The enriched GO terms specifically identified in CT vs. ST or CT vs. AT. (C) The enriched GO terms shared in comparisons of CT vs. ST and CT vs. AT. (D) The enriched GO terms that are specifically identified in CT vs. ST or CT vs. AT. Details for GO terms annotation are shown in Table 2. (E) M1 macrophage polarization potentially activates proinflammatory cytokine cascade response. (F) The phosphorylated STAT dimer enhances TNFα-regulated immunomodulation, thus enabling the trout in AT to fight off the pathogen infection.

FIGURE 7 | Enriched KEGG pathways (A) and transcriptional levels of DEGs from enriched pathways (B–D). (A) The enriched KEGG pathways in comparisons of AT vs. ST. (B) Transcriptional levels of DEGs from enriched KEGG pathway of ko04960 (aldosterone-regulated hydromineral balance). (C) Transcriptional levels of DEGs from enriched KEGG pathway of ko04913 (steroidogenesis). (D) Transcriptional levels of DEGs from enriched KEGG pathway of ko00140 (steroid hormone biosynthesis). (E) The enriched KEGG pathways showed endocrine dyshomeostasis resulting from V. anguillarum infection might serve as a lethal factor in trout of ST.
European sea bass and flounder (*Paralichthys olivaceus*) with *V. anguillarum* concentration of $10^7$ CFU/ml (5, 54). Consistently, our preliminary trial showed trout challenged by $10^7$ CFU/ml of *V. anguillarum* exerted mild to moderate symptoms compared to trout infected by $10^8$ or $10^9$ CFU/ml of *V. anguillarum* (33). In brief, trout challenged by $10^7$ CFU/ml of *V. anguillarum* began to die within 24 h after challenge and the mortality is around 20% within 120 h after challenge (33). Moreover, the RNA-seq and qPCR data consistently showed the ST and AT exerted different expressions of genes in caspase family (34). For example, ST showed higher up-regulated *casp8*, which is involved in apoptosis regulation, pathogen detection and immunomodulation (34). In this study, based on multivariate analysis of PCA, significant differences in the transcriptional profiles of stress and immune-related genes were observed in trout between the pairwise comparisons of CT, AT, and ST (Figures 2D–4D and Figure S1). The analysis of gene expression and pathway enrichment showed that the proinflammatory cytokine cascade response, which is potentially caused by M1 macrophage polarization, is activated in both AT and ST (Figures 6 and 8). However, the complement system showed phenotype-specific responses between AT and ST (Figures 6 and 8).

**Complement System**

The C3 serves as a major acute-phase protein (55). The expression of c3 gene subtypes is significantly upregulated in response to bacterial or LPS stimulation in multiple teleosts, including the dojo loach (*Misturnus anguillicaudatus*), rainbow trout, southern catfish (*Silurus meridionalis*), and grass carp (*Ctenopharyngodon idella*) (56–59). Consistently, our study found that the trout c3 gene subtypes showed upregulation in responses to *V. anguillarum* infection. Salmonidae species, such as trout and salmon, experienced four rounds of genome duplication. Consequently, the genetic expansions are characterized by duplicated functional gene copies (paralogs) in Salmonidae fishes (60, 61). Previous studies identified multiple trout c3 subtypes (*c3-1, c3-3, and c3-4*) with functional diversity (62, 63). Our study identified three novel subtypes within c3-1 (*c3-1a, c3-1b1, and c3-1b2*) (Figure 5 and Figures S7–S9). These genes exhibited conserved sequence identity but specific expression patterns in responses to *V. anguillarum* infection (Figure 5 and Figures S7–S9), indicating that these genes can encode bioactive proteins with diversity in functions.

The complement system served as a major governor of inflammatory responses (64). The homeostasis of inflammatory reactions plays a vital role in modulating health balance. Either inefficient or overactive activation of the complement system could disturb the homeostasis, which is detrimental for health balance (64, 65). Compared to CT, the kidney and spleen of ST exhibited downregulated complement cascades (Ko04610). Previous studies in mice indicated that the inefficient activation of complement cascades might be associated with increased susceptibility to infectious diseases (64, 66). Therefore, the ST showed severe symptoms in response to *V. anguillarum* infection.

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**FIGURE 8** | Putative pathways involved in defense mechanism, hemostasis, and inflammatory responses based on RNA-Seq signatures.
The complement and coagulation cascades belong to a complex inflammation regulatory network (67, 68). In most of the pathophysiological processes, both the complement and coagulation cascades are activated simultaneously (69). Consistent with the downregulated complement cascades, key genes in coagulation cascades were downregulated in the kidney and spleen of ST, including vwf subtypes (von Willebrand factor), α2m (α-2-macroglobulin), and f3α (Coagulation factor XIII A chain). The ST also showed downregulated platelet activation (ko04611, Figure S10). The downregulated coagulation cascades and platelet activation probably caused severe hemorrhages in the fins, kidneys, and other visceral masses in ST, all of which were lethal to the trout. Studies in biomedical and fishery sciences showed healthy individuals could efficiently regulate the complement system, thus not only preventing the complement(s) exhaustion but also enabling the complement(s) to restore (21, 70). However, the moribund trout might fail to efficiently regulate the complement system. The complement exhaustion further reduced the defense to pathogen infection and eventually caused the worse outcomes (death) (71, 72).

The complement system can activate the innate immune system and thus play an essential role in linking the innate and adaptive systems in mammals and teleosts (18, 35, 55, 73). The AT showed upregulated complement and coagulation cascades, enabling the AT to fight the inflammatory pathogenesis and prevents life-threatening bleeding (69). Consistently, AT had higher fga and fgb expression (fga and fgb: fibrinogen αβ chain, which has a significant function in hemostasis, Figure S10). Based on these pieces of evidence, we propose that the different responses of complement and coagulation cascades might linked to varying phenotypes of trout in response to V. anguillarum infection. A recent study showed that complement cascades serve as a bridge between immunomodulation in trout in response to bacterial infection (74), consistent with what we found in our study.

Cytokine Networks

The cytokine networks govern the normal development and physiology in animals, and dysregulations of cytokine networks are involved in pathophysiological alternations (75). In humans, the IL1 serves as the most potent endogenous pyrogens in organisms affected by infectious diseases (76, 77). Likewise, IL1 plays an apical role in initiating inflammatory responses in teleosts (78), and V. anguillarum infection results in significantly upregulated il1β in teleosts, including Atlantic cod (Gadus morhua), sea bream, and European sea bream (79–82). Our study showed that ST and AT exhibited significantly upregulated il1β subtypes (Figures 2 and 3, Table 1). ST and AT also showed upregulated infxα subtypes (Figures 2 and 3, Table 1), which was consistent with a previous study showing that the functions of IL1 and TNF largely overlap in teleosts (83). Indeed, the IL1 and TNF work synergistically, and the TNF usually acts as the first cytokine to follow an IL1 surge in an inflammatory response (83). Like IL1β, IL11 could regulate a series of important immunomodulatory effects by affecting proliferation and differentiation of hematopoietic progenitors, thus serving as a multifunctional modulator (84, 85). Studies showed kidney il11 was significantly upregulated in response to bacterial pathogens in golden pompano (Trachinotus ovatus) (86), which is in line with our results (Figures 2 and 3, Table 1).

In addition to upregulated cytokine genes (il1β subtypes, tnfxα subtypes, and il111), ST and AT showed specifically upregulated il8 and il6, respectively (Figures 2 and 3). IL6 and IL8 are two important proinflammatory cytokines that play an important role in regulating local or systemic inflammation (87). Studies showed both IL1α and IL1β subtypes could initiate the signal transduction and trigger the expression of IL6 and IL8 (12, 88). Consistently, this study revealed strong positive relationships between the expression of il6/il8 and il1β subtypes (Figures 2 and 3, Figures S2, S3, S6). For example, the il1β3 and il6 were both upregulated in AT rather than ST (Figures 3). During evolution, the IL1α is evolving faster than IL1β, thus resulting in decreased sequence and functional homology between trout and mammalian IL1α orthologs (89, 90). Our further studies will investigate whether the evolutionally conserved IL1β exhibits subtype-specific IL6/IL8 expression regulation.

Compared to trout in ST, trout in AT exhibited more upregulated GO terms associated with immune defenses and the resulting intracellular signaling (Figure 6 and Table 2), including GO:0051607, defense response to virus; GO:0035631, CD40 receptor complex; GO:0002768, immune response-regulating cell surface receptor signaling pathway; GO:2000353, positive regulation of endothelial cell apoptotic process; GO:0043123, positive regulation of I-kB kinase/NF-kB signaling; GO:0051092, positive regulation of NF-kB transcription factor activity; and GO:0042531, positive regulation of tyrosine phosphorylation of STAT protein. Despite limited studies on TNF-regulated intercellular and intracellular signaling transduction in teleosts, the in vivo studies on humans and rodents provide a potential model that could describe the immune mechanisms specifically activated in AT. Relevant to the GO terms of GO:2000353, GO:0043123, GO:0051092, and GO:0042531, previous biomedical studies showed TNFxα activates the intracellular NF-kB signaling, while the cytoplasmatic STAT serves as a negative regulator of TNFxα-triggered NF-kB activation (91). The activation of NF-kB signaling and NF-kB transcriptional factors maintains an evolutionarily conserved and important role in initiating and coordinating the innate and adaptive immune responses (92). The phosphorylated STAT dimer will translate and localize to the nucleus, where it cannot interact with the TNFxα-receptor complex. STAT localization to the nucleus allows a more robust NF-kB activation (91), enabling the trout to activate the immune defenses in response to V. anguillarum infection (Figure 6E).

Glucocorticoid Receptor and Mineralocorticoid Receptor

In addition to the GR, the teleost MR also serves as a receptor for stress perception. Our results showed the asymptomatic trout showed upregulated kidney mrα and mrβ expression. Consistently, previous studies showed MR and/or GR are expressed in immune tissues and regulate the immunomodulation (93–95). Moreover, increased stress hormone levels are observed in trout and zebrafish treated with the V. anguillarum vaccine (1, 96).
## Table 1: Gene list of Figures 2-4

| Gene | Full Name | Function Description | Gene ID | ST vs. CT | AT vs. CT | ST vs. AT |
|------|-----------|----------------------|---------|-----------|-----------|-----------|
| pamcβ | pro-opiomelanocortin β | stimulate the adrenal glands to release cortisol. | The stress response | NM_001124719.1 | down | down |
| mra (K) | mineralocorticoid receptor α | mineralocorticoids/glucocorticoid receptor | | NM_001124730.1 | down | down |
| mrc (K) | mineralocorticoid receptor β | mineralocorticoids/glucocorticoid receptor | | NM_001124740.1 | down | down |
| mra (S) | mineralocorticoid receptor α | mineralocorticoids/glucocorticoid receptor | | NM_001124730.1 | down | down |
| mrc (S) | mineralocorticoid receptor β | mineralocorticoids/glucocorticoid receptor | | NM_001124740.1 | down | down |
| grα (S) | glucocorticoid receptor α | regulate inflammation, cellular proliferation, and differentiation | | NM_001124482.1 | down | down |
| grδ (S) | glucocorticoid receptor δ | | | | | |
| hsd11b2 (S) | corticosteroid 11β dehydrogenase isozyme 2 | catalyzes the conversion of cortisol to the inactive metabolite cortisone | | NM_001124218.1 | down | down |
| hsd11b2 (K) | corticosteroid 11β dehydrogenase isozyme 2 | catalyzes the conversion of cortisol to the inactive metabolite cortisone | | NM_001124218.1 | down | down |
| il1b1 (S) | interleukin 1 β1 | endogenous pyrogen | | NM_001124347.2 | up | up |
| il1b2 (S) | interleukin 1 β2 | endogenous pyrogen | | XM_021622166.1 | up | up |
| il1b3 (S) | interleukin 1 β3 | endogenous pyrogen | | XM_021590496.1/ AJ557021.2 | up | up |
| tnfα2 (S) | tumor necrosis factor α2 | potent pyrogen by stimulation of interleukin-1 | | NM_001124374.1 | up | |
| tnfα3 (S) | tumor necrosis factor α3 | potent pyrogen by stimulation of interleukin-1 | | XM_021559781.1 | up | |
| il8 (S) | interleukin 8 | stimulate lymphocyte and monocyte differentiation | | NM_001124657.1 | up | |
| il11 (K) | interleukin 11 | response to an inflammatory stimulus | | NM_001124362.1 | up | |
| sodc (K) | extracellular superoxide dismutase (Cu-Zn) | convert superoxide radicals into hydrogen peroxide and oxygen | | XM_021619043.1 | down | |
| cat (B) | catalase | protect cells from the toxic effects of hydrogen peroxide | | XM_021564294.1 | down | |
| c3-1a (S) | Complement C3-1A | activation of the complement system | | XM_021561545.2 | up | |
| c3-1a (K) | Complement C3-1B | activation of the complement system | | XM_021561577.2 | up | |
| c3-1b (S) | Complement C3-1B | activation of the complement system | | XM_021561577.2 | up | |
| c3-1b (K) | Complement C3-1B | activation of the complement system | | XM_021561577.2 | up | |
| c3-1b2 (S) | Complement C3-1B2 | activation of the complement system | | XM_021596453.2 | up | |
| c3-3 (S) | Complement C3-3 | activation of the complement system | | XM_021568201.2 | up | |
| c3-4 (B) | Complement C3-4 | activation of the complement system | | XM_021557344.2 | down | down |
| c4 (K) | Complement C4 | classical complement pathway | | NM_001124385.1 | up | up |
| c6 (S) | Complement C6 | play a key role in the innate and adaptive immune response | | NM_001124621.1 | down | |
| c7-1 (S) | Complement C7-1 | play a key role in the innate and adaptive immune response | | NM_001124618.1 | up | up |
| c7-1b (S) | Complement C7-2 | play a key role in the innate and adaptive immune response | | NM_001124407.1 | down | down |
| c7-2 (K) | Complement C7-2 | play a key role in the innate and adaptive immune response | | NM_001124407.1 | down | down |
| c7-2a (S) | Complement C7-2 | play a key role in the innate and adaptive immune response | | NM_001124407.1 | down | down |
| c7-3 (S) | Complement C7-2 | play a key role in the innate and adaptive immune response | | NM_001124407.1 | down | down |
| c8g (S) | Complement component C8 gamma chain | regulate complement binding | | NP_001117880.1 | down | |
| c8g (B) | Complement component C8 gamma chain | regulate complement binding | | NP_001117880.1 | up | |

(Continued)
## TABLE 2 | The enriched GO term lists.

| GO Term | Function Description | Gene ID | Expression patterns between the pairwise comparisons |
|---------|----------------------|---------|-----------------------------------------------------|
| GO:0004883 | glucocorticoid receptor activity | XM_036933232.1 | ST vs. CT up, AT vs. CT up |
| GO:0038050 | glucocorticoid-activated sequence-specific DNA binding | NM_001124201 | ST vs. CT up, AT vs. CT up |
| GO:0031963 | cortisol receptor activity | XM_021566443.2 | ST vs. CT up, AT vs. CT up |
| GO:1990794 | basolateral part of the cell | XM_036968033.1 | ST vs. CT down, AT vs. CT down |
| GO:0005496 | steroid-binding | XM_021574853.2 | ST vs. CT down, AT vs. CT down |
| GO:0045944 | positive regulation of transcription by RNA polymerase II | XM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0046688 | response to copper ion | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0038500 | core promoter sequence-specific DNA binding | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0039183 | cellular response to steroid hormone stimulus | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0006955 | immune response-regulating cell surface receptor signaling pathway | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:006325 | chromatin organization | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0005631 | defense response to viruses | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0048504 | positive regulation of cytosolic calcium concentration | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0002768 | immune response-regulating cell surface receptor signaling pathway | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0003053 | negative regulation of endothelial cell apoptotic process | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0001023 | regulation of immunoglobulin secretion | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0051092 | positive regulation of NF-κB transcription factor activity | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0043569 | positive regulation of blood vessel endothelial cell migration | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0042531 | positive regulation of tyrosine phosphorylation of STAT protein | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0042113 | B cell activation | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0000897 | external side of plasma membrane | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
| GO:0043547 | positive regulation of GTPase activity | BM_001160480.1 | ST vs. CT up, AT vs. CT up |
Indeed, bidirectional communication exists between stress and immune responses, and low levels of stress (eustress) may result in enhanced immune competence (97). The slightly upregulated mrca and mrfβ could act as an alarm and stimulate the immune system to fight against V. anguillarum infection, consistent with previous studies (10, 11).

Studies on humans, rodents, and other mammals showed that cytokines could affect the genes associated with the stress response through cytokine-specific mechanisms. For example, IL1 and IL6 exhibit positive effects, while the TNFα exhibits the opposite manners (16, 98, 99). These cytokines have also been reported to dysregulate and/or block the functions of GR subtypes (17). In teleosts, the immune responses regulated by the interactions between the genes in the stress response and cytokine networks are not homogeneous. Previous studies showed it is greatly affected by specific characteristics of challenges (environmental stressors or disease pathogens), target tissues (in vitro or in vivo; peripheral tissues or mucosal surfaces), and the adaptive life story of each species (bream, bass, or trout) (1, 5, 13, 100). In this study, the results showed that downregulated mr and gr subtypes exhibited strong negative relationships with cytokine genes of il1β and tnfα subtypes in AT and ST (Figures 2 and 3), which were partially consistent with the results in mammalian studies. Previous studies in sea bream showed that the stress response can suppress the gene expression of cytokines (13). These results indicated that the stress response and cytokine networks are intimately and bidirectionally linked, enabling teleosts to cope with challengers from environmental stimuli and/or pathogen invasion (8, 10, 83, 97).

After V. anguillarum infection, ST and AT both exhibited significantly downregulated GO terms associated with functions of grα and grβ (GO:0004883, glucocorticoid receptor activity; GO:0031963, cortisol receptor activity; GO:0005496, steroid binding), and significantly upregulated GO terms that are involved in tnfα-regulated immune responses (GO:0042832, defense response to protozoan; GO:0030890, positive regulation of B cell proliferation) (Figure 6 and Table 2). The upregulated il1β, il6, il8, and tnfα genes are markers of M1 macrophage polarization, which activates the proinflammatory cytokine cascade against the pathogen invasion (101). The M1 macrophage-triggered proinflammatory cytokine cascade is suppressed by glucocorticoids and GR in basal conditions, but is activated by downregulated glucocorticoids and GR in an active infection (102). In this study, both ST and AT exhibited downregulated GO terms associated with cortisol and cortisot receptor functions and upregulated M1 macrophage polarization markers. These results suggest that activation of the proinflammatory cytokine cascade by M1 macrophage polarization is a general response for trout to fight pathogen invasion.

Compared to trout of AT, four KEGG pathways involved in steroid hormone biosynthesis and functions were downregulated in ST (Figure 7). Steroid hormones, which include corticosteroids and sex steroids, play an important role in regulating homeostasis via modulating metabolism, immunomodulation, salt balance, water balance, and reproduction. The KEGG analysis revealed that the genes associated with the biosynthesis of corticosteroids and sex steroids (ko04913 and ko00140) were significantly downregulated, suggesting that V. anguillarum infection severely dysregulated the homeostasis of the steroid hormone network in trout of ST (Figure 7E). The dyshomeostasis of steroid hormone might disturb the bidirectional link between stress and immune responses. Thus, steroid hormone receptors (such as kidney mr subtypes) might fail to transduce the “alarm” of pathogen infection to immune systems in symptomatic trout. Based on previous studies, the well orchestrated stress response can be divided into three phases: alarm, resistance, and exhaustion (103–105). Downregulated steroid hormone biosynthesis might indicate that the ST was in an exhaustion phase, which is consistent with the human study showing death may be associated with an exhausted adrenal cortex (106–108).

Previously published chapters in the book of Fish Physiology (Biology of Stress in Fish, Volume 35) showed that, with the perception of health challenges, the induction of neuroendocrine cascades serves as the primary responses. The secondary response to stressors includes the physiological adjustments of hydromineral balance and immune function (3, 7, 109). Hydromineral dysfunction is a typical stress response because the altered adrenaline, which is induced by stressors, can change the blood flow and permeability and dysregulate the cardiovascular and respiratory functions (7, 109, 110). Consistently, our studies showed significantly downregulated KEGG pathways associated with aldosterone-regulated salt and water balance (ko04960 and ko04978) (Figure 7E), indicating that ST trout show hydromineral dyshomeostasis. Previous studies in Chinook salmon (Oncorhynchus tshawytscha) showed the hydromineral balance is changed during euthanasia (111), which is consistent with our KEGG results. Based on this data, we propose positive feedback between severe infection and imminent death: (1) infection and its resulting stress response disturb the hydromineral homeostasis, thus resulting in a moribund condition. (2) The moribund condition further exacerbates the dyshomeostasis of hydromineral functions, leading to death.

**CONCLUSIONS**

Based on pairwise comparisons of CT, AT, and ST, we found the CT, AT, and ST show distinct transcriptional profiles of genes in stress and immune networks (Figure 8). The AT exhibited the eustress response, and eustress can stimulate the immune system to fight against bacterial infection. The ST exhibited a strong stress response, and the distress resulted in a secondary stress response, thus exacerbating immune dysfunctions and hydromineral dyshomeostasis. Regarding the immunomodulation, analysis of gene expression and pathway enrichments showed activation of the proinflammatory cytokine is a general response of AT and ST in responses to V. anguillarum infection. Additionally, the specifically upregulated complement and coagulation cascades and TNF-associated immune defenses probably enable the AT to fight the inflammatory pathogenesis and the resulting bleeding.
DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committees of Ocean University of China (permit number: 20141201).

AUTHOR CONTRIBUTIONS

Conceptualization: H-SW, J-FL, M-ZZ, and Z-SH. Project administration: Y-RX, CZ, and H-KZ. Supervision: H-SW, J-FL, and Z-SH. Methodology: Y-RX, CZ, H-KZ, X-DY, and M-QL. Writing—original draft: H-SW, Z-SH, and JD. Writing—review and editing: H-SW, Z-SH, and JD. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.639489/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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