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ISSN 0792 - 156X

PUBLISHER:

The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB)

The Israeli Journal of Aquaculture - Bamidgeh, IJA_71.2019.1556, 11 pages

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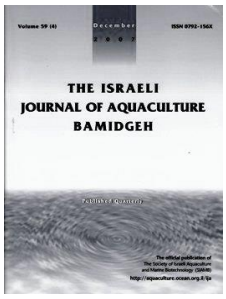

Pathogenicity and Immune Response of Starry Flounder, *Platichthys stellatus*, Infected with *Vibrio anguillarum*

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Keywords: Starry flounder; *vibrio anguillarum* infection; pathogenicity; immune response; serum analysis

Abstract

Vibrio anguillarum is the aetiological agent of vibriosis, a disease affecting many marine fish species. The occurrence of vibriosis in starry flounder, *Platichthys stellatus*, grown in an aquaculture farm has demonstrated the urgent need for information on pathogenic infection and immune response for efficient disease management. This is the first study to report *Vibrio anguillarum* isolation and infection in starry flounder. We evaluated immune responses, serum biochemical parameters, and cumulative mortality of the fish by experimentally challenging healthy fish. The expression levels of five immune genes (TNF, TNFR, IL-6, MHC-II, and CXC) were measured by real-time quantitative PCR. The transcriptional levels of the genes encoding tumor necrosis factor (TNF), TNF receptor (TNFR), interleukin-6 (IL-6), the major histocompatibility complex (MHC-II), and a chemokine (CXC) in the head-kidney of *V. anguillarum* infected fish were significantly upregulated compared with control fish and biochemical indices including the alanine aminotransferase, total serum protein, and glucose levels of infected fish differed significantly from those of control. Additionally, Starry flounder infected with *V. anguillarum* at 1.67×10^6 and 1.67×10^8CFU/mL showed 53%, and 100% mortality, respectively. This study furthers our understanding of the immune and serum biochemical alterations, and mortality induced by bacterial infections, depending on pathogen concentration. This may advance strategies for control of *V. anguillarum* in cultured starry flounder.

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Introduction

Starry flounder *Platichthys stellatus,* is a cold-water, benthic euryhaline fish that can adapt to wide range of environmental salinities ranging from complete freshwater to seawater. The fish is found across the North Pacific Ocean spanning the waters of Korea, Japan, the Sea of Okhotsk, the Bering Sea, and Alaska to California (Orcutt, 1950; Kramer et al., 1995). This important sport and food fish, acclimates well to indoor culture, remaining hardy at high densities and exhibiting rapid growth (Ding et al., 2010). From 2006, commercial culture increased steadily. In 2015, 1,841 tons of fish were sold (KOSTAT, 2016). However, as culture has developed, the frequency of pathogenic infections has also risen together with high mortality rates. Bacteria are often the principal causes (Austin & Austin, 1999).

Streptococcus parauberis (Cho et al., 2008), *S. iniaei*, *Edwardsiella tarda* (Park et al., 2016) and *E. ictaluri* (Tong et al., 2015), cause streptococcal disease and Edwardsiellosis, respectively, resulting in severe economic losses (Cho et al., 2008). Recently, vibriosis was detected in a starry flounder fish farm located on Jeju Island (personal communication). Vibriosis is one of the most prevalent bacterial fish diseases. *Vibrio anguillarum* in particular, which caused major disease outbreaks (Zorrilla et al., 2003; Afonso et al., 2005) was isolated from farmed populations. This study is the first to report *V*. *anguillarum* infection in starry flounder grown in an aquaculture farm.

Several studies have published detection (Avesever, 2015) and control measures; these include antibiotics, enriched diets, and vaccination (Teuber, 2001; Li et al., 2015), but the best way of preventing and/or controlling diseases in aquaculture farms is by strengthening fish defense mechanisms (Lee et al., 2011). In the present study, we collected the serum biochemical and immune gene expression data in relation to cumulative mortality of experimentally infected starry flounder and identified fish immune-related defense mechanisms against the invading bacterial pathogen. The data obtained can be effectively utilized in the disease management of starry flounder aquaculture.

Materials and methods

Screening for and identification of the pathogen.

Infected fish were collected from a fish farm on Jeju Island. Conventional PCR was performed to amplify the *empA* gene (439 bp) of *V. anguillarum* (Xiao et al., 2009) using the primer pair *empAF* (5′-CAGGCTCGCAGTATTGTGC-3′) and *empAR* (5′-CGTCACCAGAATTCGCATC-3′).The isolate was then cultured in Brain-heart infusion (BHI) broth for 24 h at 28°C and DNA was then extracted using a QIAmp DNA kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions; this DNA served as a template. Positive and negative controls (the latter without any template DNA) were included. PCR products were sequenced on an ABI 3730XL DNA analyzer (Applied Biosystems, Carlsbad, CA, USA) and the results were subjected to multiple sequence alignment using ClustalW (http://www.clustal.org) running MEGA v. 5.1 software.

Fish.

Starry flounder weighing 57 ± 3.6 g (n = 450) obtained from the Jeju fish farm were acclimated in the laboratory for at least 1 week before being experimentally infected with *V. anguillarum*. The seawater temperature and salinity were maintained at 16 ± 0.5°C and 30.0 ± 0.3% during both the acclimation and experimental periods; the water was continuously aerated.

Challenge, sampling, and cumulative mortality.

Healthy fish were selected based on overall appearance and vitality (Patel et al., 2009; Pridgeon et al., 2012) and randomly divided into one control and three infected

fish groups (15 fish/group in triplicate). Freshly isolated *V. anguillarum* was cultured in BHI medium at 28°C overnight, washed, and resuspended in sterile saline to obtained three different concentrations namely 1.67×10^3, 1.67×10^6 and 1.67×10^8 CFU/ml to assess the LD_{50} concentration. All the infected fish received 0.1 mL of bacterial suspension intraperitoneally; control fish received an equal volume of sterile saline. Cumulative mortality was recorded in all the challenged fish groups over 15 days and the LD_{50} concentration was determined.

For the challenge experiment, fish (n=270) were divided into two groups namely control and *Vibrio anguillarum* (Va) challenged (1.67×10^6 CFU/mL) groups (45 fish/group, in triplicate) and sampled at 0, 1, 3, 6, and 12 h, and at 1, 3, 5 and 7 days, post-infection (n= 5 fish). Blood and head-kidney were dissected from each sampled fish and stored at appropriate temperature until used (blood at room temperature for serum collection was stored at -20°C and head-kidney at -80°C for RNA extraction).

Serum biochemical analysis.

Blood from each control and *V. anguillarum* (LD_{50} concentration) infected starry flounder fish was collected via caudal puncture; the serum was prepared and stored at -20°C. The levels of serum biochemicals such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), amylase (AMY), bilirubin, total protein (TP), cholesterol, calcium, potassium, sodium, phosphorus, glucose, and galactose were measured using a VetScan comprehensive diagnostic portfolio (Calxis, Uiwang, South Korea).

Expression of genes of the immune system.

Total RNAs were extracted from the head-kidney of control and *V. anguillarum* infected fish using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. We evaluated the expression levels of five genes of the immune system namely, tumor necrosis factor receptor (TNFR), tumor necrosis factor (TNF), interleukin (IL-6), Major Histocompatability-II (MHCII), and Chemokine (CXC). The β-actin gene served as an internal control (Table 1).

Table 1. Primers used to amplify five immune genes in qPCR

Gene	*Primer sequences 5′ to 3′*	*Product size (bp)*	*References*
TNF – F	TGAGGGATGACCGAACCAC	148	(Cho et al., 2008)
TNF – R	GGACTGGCAGCAGAAAGAAGA		
TNFR – F	ACCCTGGATGGGCATATCA	213	(Cho et al., 2008)
TNFR – R	GCTGTCTGTTTGTGGCTTGG		
MHC-II – F	AGCAAAGTCCGCAGCAAAG	187	(Cho et al., 2008)
MHC-II – R	AGAAGCAGAGGAAACCCAGAGA		
IL-6 – F	ACAGACACAGCAGATTGCCATAGA	197	(Cho et al., 2008)
IL-6 – R	GCTCCCATCCATCCCTCTTAC		
CXC – F	GATGGGTTTGCTCTCTGTCTT	123	(Cho et al., 2008)
CXC – R	TCGTTGCTGTAATGGTGTTCCT		
- F β-actin	GATGCTGTTGTAGGTGGT	106	(Cho et al., 2008)
- R β-actin	AAAGCCAACAGGGAGAAG		

qPCR was performed using an Mx3000P Real-Time PCR System (Stratagene, San Diego, CA, USA) and the SYBR Green technique. Amplification was performed in a 96-well plate; each 25-μL reaction volume containing 12.5 μL of 2× Brilliant III Ultra-Fast SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA, USA), 2.5 μL of each of the forward and reverse primers (10 μM), 1 μL of template solution (1 μg cDNA), and 6.5 μL of Diethylpyrocarbonate-treated and sterile filtered water (DEPC-treated) water. The thermal profile for qPCR was 95°C for 10 min; followed by 40 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s. Following amplification, a melting curve analysis was performed with a thermal profile from 95°C to 65°C at a rate of 0.1°C per second with continuous acquisition of fluorescence data. Data analysis was performed using the

inbuilt program of the Mx3000P Real-Time PCR System. A standard curve was prepared for each gene using the vector containing a specific *Platichthys stellatus* cDNA fragment as template; the relative expression ratio (R) of each mRNA was calculated using the formula $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\ [test]-\Delta Ct\ [\beta\text{-actin}])}$ (Livak and Schmittgen, 2001). Real-time PCR efficiencies were measured by amplifying a dilution series of cDNA and applying the $10^{(-1/slope)}$ method, the results were consistent between the β-actin and the target genes. All data are presented as means with standard deviations.

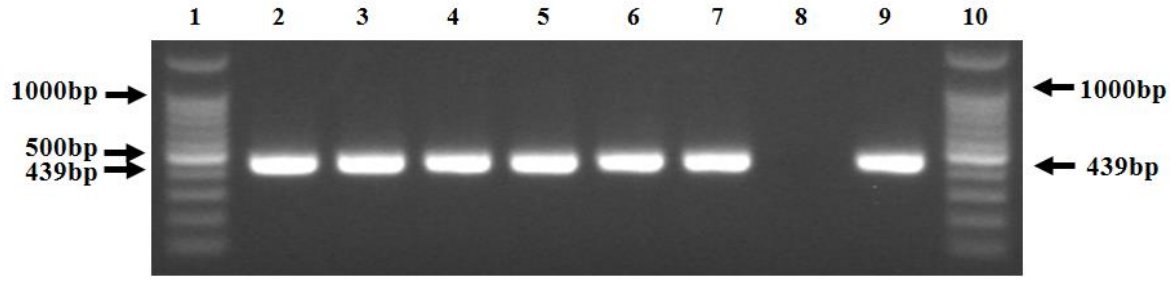
Statistical analysis.

All tests were performed in triplicate. Data were analyzed using SPSS software version 18 (SPSS Inc., Chicago, IL, USA); we employed one-way analysis of variance followed by Tukey's test to compare means between individual treatments. A *P*-value <0.05 was considered significant.

Results

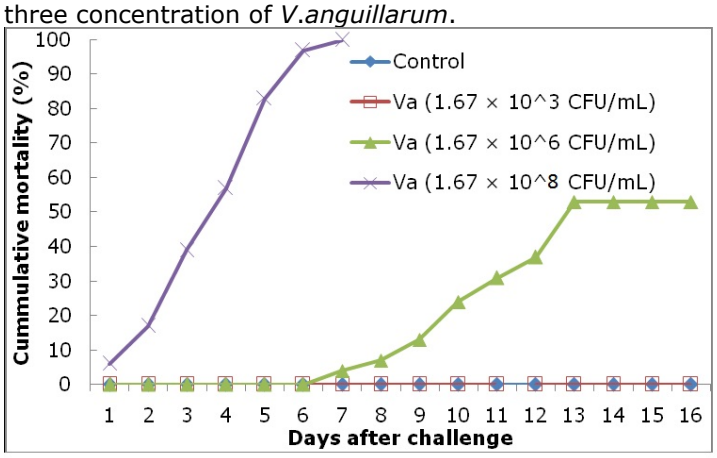
PCR analysis of the *empA* gene of the pathogenic bacterial strain amplified a 439-bp fragment (Fig. 1).

Figure 1. PCR amplification of an *empA* gene fragment from *Vibrio anguillarum* isolated from infected fish (in triplicate). Columns: 1 & 10; SiZer™-100 bp DNA Marker (iNtRON, Korea), 2–7; *empA* gene, 8; negative control, 9; positive control, 10; SiZer™-100 bp DNA Marker.



We recorded cumulative mortality rates over 15 consecutive days (Fig. 2) and the lethal concentration (LD_{50}) of *V. anguillarum* was identified as 1.67×10^6 CFU/m with the initial mortality of 4% at 6 days post-infection (dpi) which rose to 53% by 13 dpi, and then ceased. However, 100% mortality was observed in the 1.67×10^8 CFU/mL fish group while no fish died in the control and 1.67×10^3 CFU/mL fish group.

Figure 2. Cumulative mortality of starry flounder, *Platichthys stellatus*, intraperitoneally challenged three concentration of *V.anguillarum*.



Serum biochemical levels

The TP, ALT, and glucose levels differed significantly (all $P < 0.05$) between infected and control fish. The TP levels in infected fish decreased significantly (compared

with control fish) from 1 to 12 h post infection (hpi); increased significantly from 1 dpi to 3 dpi; and then decreased gradually (Fig. 3A). The glucose levels in infected fish decreased significantly ($P < 0.05$) to 3 dpi and increased at 5 and 7 dpi (Fig. 3C).

Figure 3. Serum biochemical levels in *V.anguillarum*-infected (1.67×10^6 CFU/mL) and control starry flounder, *P.stellatus.* 3A – Total serum protein; 3B – Serum ALT; 3C – Serum glucose.

Fig. 3A.

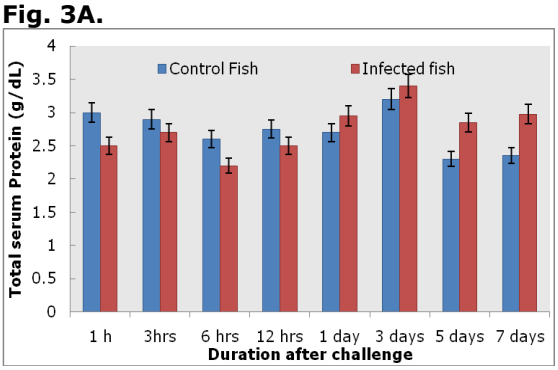


Fig. 3B.

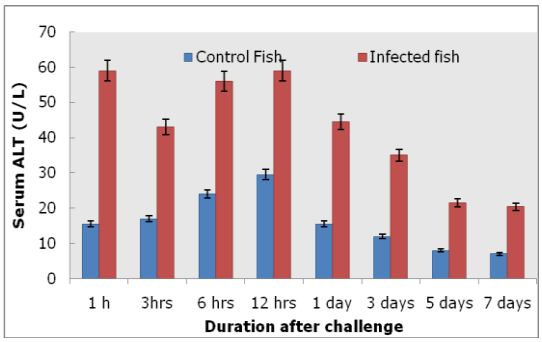
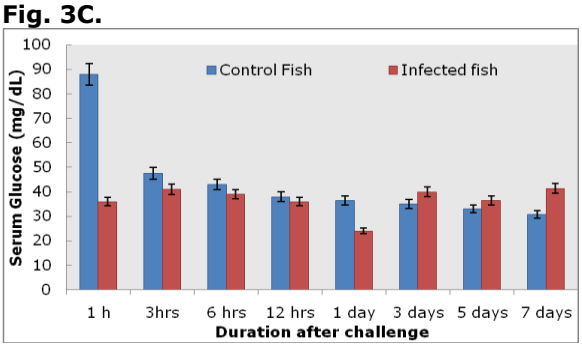


Fig. 3C.



No other biochemical parameter differed significantly between control and infected fish (see Table 2 in appendix at end of article).

Immune genes expression

The real-time qPCR data on five genes of the immune system expressed in the head-kidney of control and infected fish are shown in Figures 4A–4E. TNF gene expression was significantly elevated ($P < 0.05$) in *V. anguillarum* infected starry flounder fish when compared with control fish.

Figure 4. Relative mRNA expression levels from the TNF (4A), TNFR (4B), IL-6 (4C), MHC-II (4D) and CXC (4E) genes in the head-kidney of control and *V. anguillarum*-infected starry flounder, *P. stellatus*, as assessed using SYBR Green qPCR. All samples were normalized to the β-actin expression level (internal control). The relative levels of target gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (the Ct value of the target gene minus the Ct value of the β-actin gene). Data are means ± S.D. (n = 5).

Fig. 4A

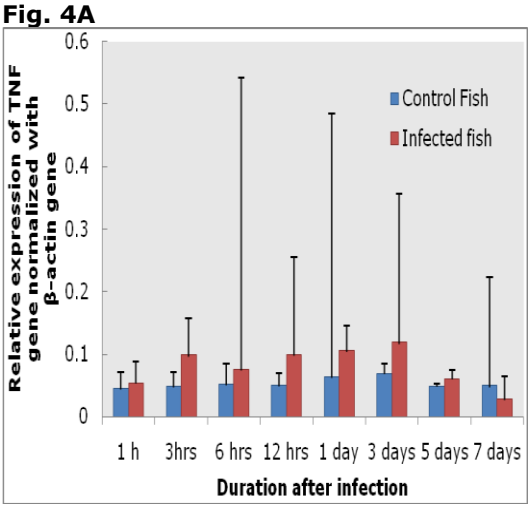


Fig. 4B

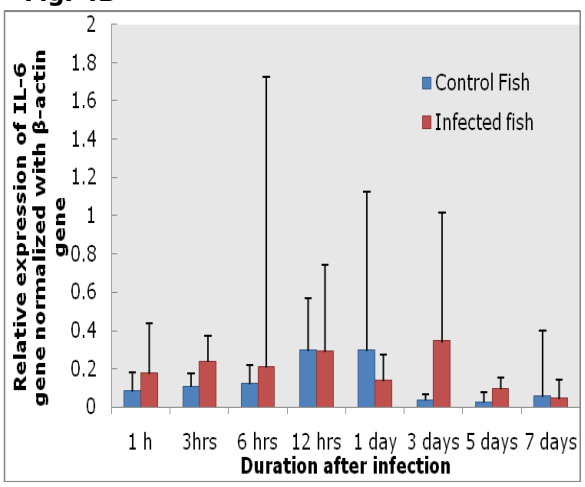


Fig. 4C

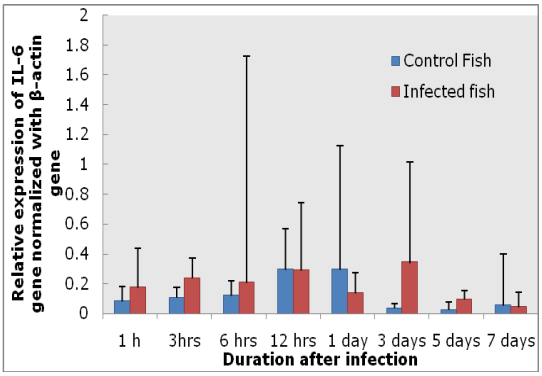


Fig. 4D

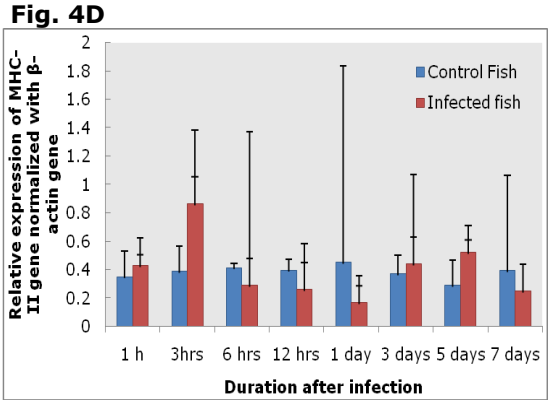
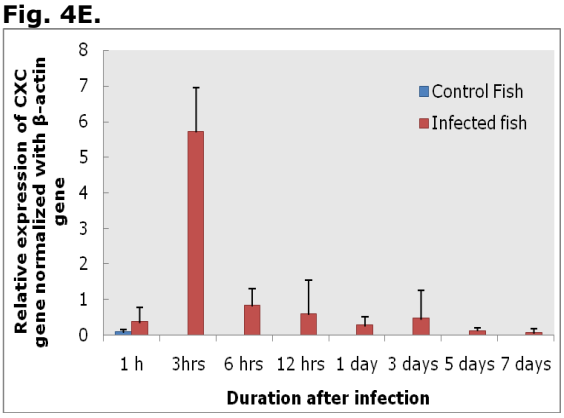


Fig. 4E.



Discussion

The amplified 439-bp *empA* gene (Fig. 1) sequence was 100% similar to that of *empA* of *V.anguillarum* (Xiao et al., 2009). *EmpA* encodes a zinc metalloproteinase, a notable virulence factor (Norqvist et al., 1990; Milton et al., 1992; Mo et al., 2002; Denkin and Nelson, 2004); this sequence serves to both identify *V. anguillarum* and to estimate its virulence level (Xiao et al., 2009).

About 61% of turbots (*Scophthalmus maximus*) died when challenged with 8.3×10^6 *V. anguillarum* per fish (Chair et al. 1994). A previous report has shown that the exotoxin produced by *V. anguillarum* plays a vital role in infecting flounders (Mo et al., 2002). Moreover, Chair et al., (1994) reported increased mortality after 3 dpi, due to the cumulative effect of bacterial action in turbot fed with artemia encapsulated with *Vibrio anguillarum.* These studies assessed the sensitivity or pathogenicity of the bacteria in fish.

The biochemical parameters differ significantly (*P<0.05)* between the control and infected fish groups. These are attributable to impairment of protein synthesis and/or liver damage caused by the pathogen (Stoskoph, 1993; Buhler et al., 2000). Similar patterns have been reported in tilapia infected with *Streptococcus agalactiae* (Evans et al., 2006; Alsaid et al., 2014) and catfish (*Silurus asotus*) infected with *E. tarda* (Yu et al., 2010).

The elevations in serum ALT levels in infected fish evident at all time points suggest that the viscera (including the liver and kidney) suffered severe damage (Fig. 3B), in agreement with previous data on tilapia and *Anguilla anguilla* infected with *S. agalactiae* (Alsaid et al., 2014) and *V. anguillarum* (Khalil et al., 2011) respectively. As noted in the cited works, we found that the increased serum ALT levels were associated with damage to the hematopoietic organs of the starry flounder. Similar ALT elevations following bacterial infection have been reported in the Atlantic salmon *Salmo solar*

(Waagbø et al., 1988), Nile tilapia *Oreochromis niloticus* (Chen et al., 2004), and brook trout *Salvelinus fontinalis* (Řehulka and Minařík, 2007).

The expression of five immune genes like TNFR, TNF, IL-6, MHC-II, and CXC, differ significantly in infected fish. Particularly elevated expression of TNF gene in *V. anguillarum* infected starry flounder. A similar result in Chinook salmon infected with *V. anguillarum* was reported (Ching et al. 2010). TNF, a pro-inflammatory cytokine, is expressed soon after infection and plays a key role in regulating inflammation (Zhang et al., 2012). Many fish TNFs have been produced in bacteria as monomers, dimers, and trimers; the recombinant TNFs activate fish macrophages/phagocytes, enhancing their ability to kill microbes (Zou et al., 2003). *In vitro* TNF treatment of trout head-kidney leucocytes and monocytes/macrophages triggered the expression of several immune system genes associated with inflammation and the antimicrobial response (Hong et al., 2013; Zou et al., 2003a). In the present study, TNF also induced expression of TNFR, IL-6, and MHC-II in infected fish; the levels were higher than those of control fish. Moreover, in zebrafish, TNF regulated cell survival and mediated resistance to infectious disease (Wiens and Glenney, 2011).

CXC gene expression was significantly upregulated in infected fish by 3 hpi, and fell by 6 hpi, but remained elevated (compared with control fish) at all time points. Chemokines are a large family of small (8–12 kDa) proteins orchestrating lymphocyte migration and adhesion. Chemokines organize the immune system, coordinating the actions of primary and secondary lymphoid organs under both physiological and pathological conditions (Stein and Nombela-Arrieta, 2005). Fish cytokines also play roles in development and hematopoiesis, attracting leucocytes to sites of infection and activating the antimicrobial mechanisms of such cells to counter invaders (Zou and Secombes, 2016).

Thus, activation of five genes of the immune system and the notable changes in the levels of various serum markers of starry flounder infected with *V. anguillarum* indicate that the fish mount a significant immune response to the pathogenic challenge. It is important to enhance host immune systems to prevent pathogenic invasions. However, prevailing statistical variation could be improved through further challenge experiments with various pathogens. More studies on this highly profitable aquaculture species must further be conducted. Hopefully, the results of this study could lead to further revelations on the mechanisms related to the immune responses in *P. stellatus* and hence, their diseases management strategies.

Acknowledgment

The authors are grateful to acknowledge to National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2016HID5A1911152, 2017R1A2B4005688) and the 2017 scientific promotion program funded by Jeju National University. This study was also supported by the Ocean and fisheries Research Institute, Jeju Special Self-Governing Province.

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Appendix:

Table 2. Serum biochemical indices of *Vibrio anguillarum* infected and control starry flounder, *Platichthys stellatus* at different time points.

Parameters	1hpi		3hpi		6hpi		12hpi		1dpi		3dpi		5dpi		7dpi	
	Control	infected	Control	infected	Control	infected	Control	infected	Control	infected	Control	infected	Control	infected	Control	infected
Albumin (g/dL)	1.65±0.10	1.4±0.15	1.35±0.11	1.45±0.17	1.5±0.1	1.85±0.16	1.85±0.2	1.42±0.02	1.25±0.03	1.4±0.12	1.2±0.10	1.35±0.09	1.1±0.10	1.47±0.11	1.15±0.2	1.34±0.14
ALKP (U/L)	24±0.13	22±0.10	20.5±0.12	21±0.02	21.5±0.10	23±0.03	25.5±0.15	22±0.12	19±0.11	20.5±0.17	22±0.15	27±0.12	13±0.17	20±0.16	11±0.1	19±0.15
Amylase (U/L)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Bilirubin (mg/dL)	0.25±0.01	0.2±0.15	0.3±0.1	0.25±0.08	0.32±0.05	0.24±0.03	0.3±0.07	0.2±0.01	0.2±0.11	0.3±0.15	0.2±0.12	0.2±0.02	0.2±0.05	0.2±0.08	0.2±0.04	0.2±0.06
Bun (mg/dL)	6.5±0.04	6±0.07	6.5±0.17	10±0.15	6.4±0.12	7±0.15	4.5±0.11	6±0.17	6.5±0.03	6.5±0.15	8±0.05	4±0.04	6±0.12	5±0.16	5±0.11	5.5±0.15
Calcium (mg/dL)	10.9±0.15	9.8±0.1	9.8±0.02	9.35±0.12	9.4±0.01	9.2±0.13	9.6±0.14	9.8±0.04	9.6±0.15	9.4±0.17	10.2±0.4	9.9±0.11	8.8±0.05	9.35±0.02	8.2±0.03	9.15±0.1
Phosphorus(mg/dL)	10.4±0.01	10.4±0.3	9.15±0.15	7.45±0.07	8.56±0.16	6.45±0.18	7.75±0.08	10.4±0.11	7.95±0.15	6.65±0.05	6.9±0.13	8.55±0.15	6.3±0.14	7.43±0.07	6.1±0.1	7.3±0.02
Creatinine(mg/dL)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2
Na+ (mmol/L)	157.5±0.2	148±0.1	151.5±0.1	145.5±0.2	143±0.05	141±0.15	147±0.15	148±0.21	147.5±0.04	150.5±0.17	150±0.11	146±0.12	144±0.19	145±0.15	142±0.04	143±0.15
K+ (mmol/L)	4.25±0.06	4.2±0.15	4.1±0.04	3.55±0.17	4.02±0.11	3.3±0.24	3.8±0.3	4.2±0.21	4.55±0.15	4.45±0.02	3.9±0.15	4.1±0.07	3.8±0.08	3.95±0.03	3.73±0.1	3.82±0.06
Globulin (g/dL)	1.35±0.19	1.1±0.12	1.55±0.02	1.25±0.07	1.43±0.04	1.06±0.08	0.75±0.1	1.1±0.15	1.45±0.14	0.95±0.21	1.7±0.05	1.55±0.04	1.2±0.15	1.37±0.16	1.1±0.09	1.25±0.11

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