The Israeli Journal of Aquaculture – Bamidgeh • ISSN 0792-156X • IJA.73.2021.1546886, 12 pages
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Expression analysis of three immune genes *IL-8, IL-6 and IL-1β* in the Japanese flounder (*Paralichthys olivaceus*)

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Key words: *Paralichthys olivaceus*, IL-8; IL-6; IL-1β, LPS, tissue expression

Abstract

Interleukin-8 (IL-8), Interleukin-6 (IL-6), and Interleukin-1β (IL-1β) are major immune-related genes that play important roles in the innate immune system of vertebrates. In this study, the expression levels of the three immune-related genes in eleven tissues of normal adult Japanese flounder (*Paralichthys olivaceus*) were examined using semi-quantitative RT-PCR at 13time points after challenge with Lipopolysaccharide (LPS). The results showed that the highest expression levels of the Japanese flounder *IL-8* were detected at 12h in the spleen, 12h in the head kidney, and 24h in the liver after the challenge with LPS. Interestingly, the expression levels of *IL-6* in the spleen and head kidney were highest at 48h. The liver had the highest expression level of *IL-6* at 36h.The highest expression levels of *IL-1β* were detected at 3h in the spleen and 9h in the head kidney and liver. These results indicated that *IL-8, IL-6*, and *IL-1β* also played important roles in the immune response of the Japanese flounder at lower temperatures. The study provided a basis for further research on the immune mechanisms of *IL-8, IL-6, IL-1β,* and the production of recombinant *IL-8, IL-6, or IL-1β* used in Japanese flounder cultivation.

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Introduction

The immune system is commonly divided into innate (non-specific) and adaptive (specific); innate response generally precedes adaptive response and activates and determines the nature of the adaptive response. Fish possess both the innate and adaptive immune systems, placing them in an intermediate phylogenetic position. The fish immune system is very different from that of mammals; the innate immune system of fish is the primary defense barrier since the adaptive immune system is inefficient due to its evolutionary status (Magnadóttir, 2006).

The immune system plays an important role in identifying and eliminating pathogens and participating in processes that maintain homeostasis following inflammatory reactions. The innate immune system is critical to the function of acquired immunity and determines the nature of the acquired responses (Carroll et al., 1998). The integration of innate and adaptive immunity dynamically regulates inflammation *in vivo* (Lo et al., 1999). Cytokines are important components of the immune system of fish. These are small glycoproteins that act as signaling molecules in a variety of immunological and inflammatory reactions (Thomson, 1994). Cytokines are conventionally divided into different families, including interleukins (IL), chemokines, interferons (IFN), tumor necrosis factors (TNF), and colony stimulating factors (CSF).

Interlerkin-8 (IL-8) is a monomeric polypeptide and was the first known chemokine. It belongs to the CXC subgroup of chemokines and plays a crucial role in recruiting neutrophils into the tissues (Mackay, 2001). Lipopolysaccharide (LPS) is a strong stimulus that can induce the production of IL-8 from monocytes, and many other factors may also induce IL-8 production, including IL-1 and TNF-α (Mackay, 2001).

IL-6 is a pleiotropic cytokine involved in the immune response, hematopoiesis, and inflammation. The molecular cloning and sequencing of the flounder IL-6 gene have beenreported as well as the up regulation of the expression of this gene after bacterial challenge (Nam et al., 2007). In addition, IL-6, a cytokine released during the early urinary infection (UTI), serves a dual role by initiating the inflammatory response while also repairing urothelial defenses (Wood et al., 2010).

Interleukin-1β(IL-1β) is a member of the IL-1 cytokine family; the structure includes a beta trefoil composed of 12 beta sheets. IL-1β plays a pivotal role in the inflammatory response and maturation and proliferation of many immune cell types. Induced *in vivo* with LPS, expression of the IL-1β transcript was detected by RT-PCR in the spleen and testes of *Scyliorhinus canicula*. The discovery of the *Scyliorhinus canicula IL-1β* gene demonstrated the importance of IL-1β in the cartilaginous fish immune system (Bird et al., 2002). It is demonstrated that the immune response in Japanese flounder is significantly influenced by IL-1β (Emmadi, 2005).

The Japanese flounder is an economically important marine fish species known for its rapid growth, good taste, and high nutritive value. With the massive increase in aquaculture in recent decades, the scale of Japanese flounder cultivation has been expanding continuously in China. In northern China, the seawater temperature is comparatively lower than in southern China, especially in winter. The lower temperatures may affect the growth of the Japanese flounder and its immune response.

In this study, the temporal expression patterns of the three immune genes *(IL-8, IL-6*, and *IL-1β*) of the Japanese flounder indifferent tissues were examined after infection

with Lipopolysaccharide (LPS) at lower temperatures. That LPS can stimulate the innate immune system of fish has previously been demonstrated (Engelsma et al., 2001). As many as 13 time points were selected to analyze the expression of *IL-8*, *IL-6*, and *IL-1β*. This study will be helpful for further research on the antiviral mechanisms of these three immune genes and the production and application of relevant recombinant *IL-8, IL-6, and IL-1β*.

Materials and Methods

Ethics and methods

This study was approved by the Animal Care and Use Committee of the Key Laboratory of Mariculture in North China (Dalian, Liaoning). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Japanese flounder

Normal adult individuals weighing 700g on average were raised at Key Laboratory of Mariculture, Ministry of Agriculture, Dalian Ocean University. They were kept in two tanks ($15m^3$) separately for one week before experimentation, and the water temperature was 8°C to 10°C.

Challenge and sampling

Lipopolysaccharide (LPS) (Sigma, L2880, 055:B5), the major constituent of the outer layer of Gram-negative bacteria, was dissolved with physiological saline (PS) to a concentration of 1mg/ml. Thirty-nine Japanese flounders were injected intraperitoneally with LPS (400μg/kg) (Lee et al., 2001) as the experimental group, and thirteen Japanese flounders were injected with an equal dose of PS as the control group. Then, three LPS-infected fish and one PS-injected fish were sacrificed at each sampling time point (3h, 6h, 9h, 12h, 24h, 36h, 48h, 60h, 72h, 84h, 96h, 108h, and 120h) after infection. Tissues of interest (spleen, head kidney, and liver) were dissected, quickly frozen in liquid nitrogen, and stored at −80°C until RNA extraction. Meanwhile, two normal adult fish were sacrificed, and tissues of the head kidney, spleen, liver, kidney, brain, skin, gill, muscle, heart, intestine, and gonad were removed and kept at −80°C until use.

Primer design

According to *IL-8* (GenBank accession number AF216646), *IL-6* (DQ267937), and *IL-1β* (AB070835) genes of Japanese flounder in the GenBank database, three pairs of primers (IL-8-F/R, IL-6-F/R, IL-1β-F/R) were designed separately (**Table 1**). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AB029337) gene of Japanese flounder was selected as the internal control, and a pair of primers (GAPDH-F/R) was designed (**Table 1**).

Table 1 Primers used in the present study

Primer ID	Primer sequence	Gene	Accession number	Fragment length	Tm [℃]	Location*
IL-8-F	5'-TGGTCTGTCTGTCCCTGTG-3'	IL-8 gene	AF216646	304bp	55	18-321
IL-8-R	5'-CTTGAAGCGATTTGTCCTC-3'					
IL-6-F	5'-AAGAGGAAAAGGCATAGTG-3'	IL-6 gene	DQ267937	385bp	52	84-468
IL-6-R	5'-CCTCTGTGGTTGCTATGTC-3'					
IL-1B-F	5'-AGCAAAGGTCACAAAGCAG-3'	IL-1B gene	AB070835	350bp	53	45-334
IL-1B-R	5'-ACAATGTCGTCGGAGTAGC-3'					
GAPDH-F	5'-ATGCTGGTGCCCACTATGT-3'	GAPDH gene	AB029337	577bp	56	260-836
GAPDH-R	5'-ACCTGGTGCTCGGTGTATG-3'					

*Location represents the position of the Japanese flounder IL-8, IL-6, IL-1B and GAPDH gene sequence, respectively

Isolation of RNA

Total RNA was extracted separately from selected tissues of Japanese flounder using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 50mg samples were homogenized with 1ml TRIzol reagent, and 0.2ml of chloroform was added after incubation at 20°C for 5 min. Tubes were shaken vigorously by hand for 15 seconds and incubated at 20°C for 3min. The mixture was centrifuged at 12000rpm, 4°C for 15 min. Subsequently, the supernatant was collected, and 0.5ml of isopropyl alcohol was added. After incubation at 20°C for 10min, the sample was centrifuged at 12000rpm, 4°C for 10 min. Finally, RNA was collected and washed with 1ml 75% ethanol once and then briefly dried, and the RNA pellet was dissolved in RNase-free water.

Semi-quantitative RT-PCR analysis of IL-8, IL-6, and IL-1β gene expression

The cDNA synthesis was carried out using M-MLV Transcriptase (TaKaRa) according to the manufacturer's instructions. Total RNAs from the same time points or tissues were equally and separately mixed for reverse transcription.

Primers (IL-8-F/R, IL-6-F/R, IL-1β-F/R, and GAPDH-F/R) were separately used for amplifyinganIL-8 fragment of 304bp, anIL-6 fragment of 385bp, anIL-1β fragment of 350bp, and a GAPDH fragment of 577bp. Polymerase chain reaction (PCR) was used for gene sequence amplification with the conditions as follows: one cycle of 5 min at 94°C; 30–32 cycles of 30s at 94°C, 30s at 52°C–60°C (details are listed in **Table 1**), 45s at 72°C, and one cycle of 7 min at 72°C.

PCR products were analyzed using1.0% agarose gels stained with ethidium bromide,

and the amplified DNAs were recorded with the Kodak Image Station 440CF. The integrated optical density (IOD) ratios of IL-8, IL-6, and IL-1β versus GAPDH were determined with a Gel-Pro analyzer to measure the relative mRNA expression. Each value represents the average of triplicate experiments. The data were analyzed using statistical product and service solutions (SPSS) 21.0 software.

Results

IL-8, IL-6, and IL-1β expression in the tissues of normal adult Japanese flounder

RT-PCR was conducted to analyze tissue expression levels of *IL-8, IL-6,* and *IL-1β*genes in normal Japanese flounders. The expression level of *IL-8* was the highest in the kidney and relatively high in the spleen, head kidney, skin, and gill. The expression level was very low in the liver, brain, muscle, heart, intestine, and gonad (**Figure 1 A**). The expression level of *IL-6*in blood and head kidney was the highest in the head kidney and relatively high in the spleen and gill. The expression of *IL-6* was very low in the liver, kidney, brain, skin, muscle, heart, intestine, and gonad (**Figure 1 B**). The *IL-1β* gene was strongly expressed in the head kidney and skin (**Figure 1C**). The expression level was very low in the spleen, liver, kidney, brain, gill, muscle, heart, intestine, and gonad.

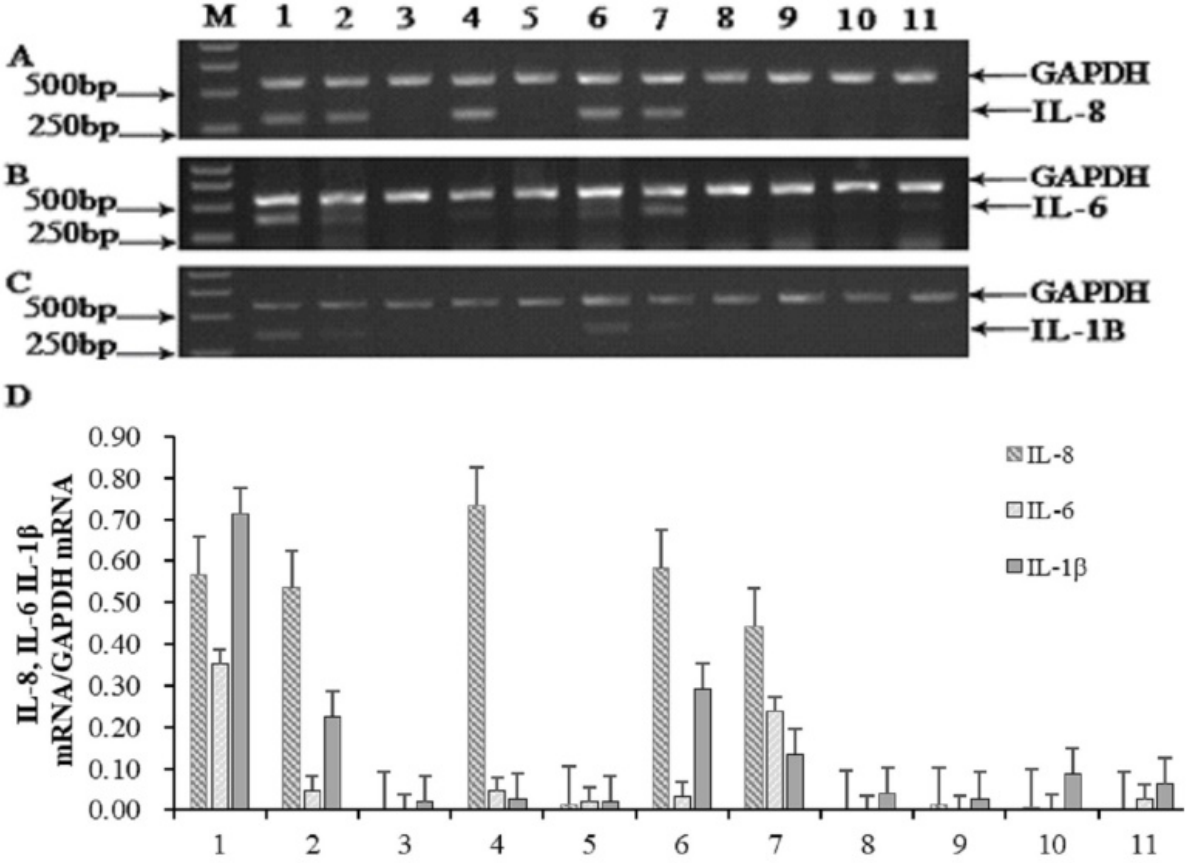

Figure 1 Semi-quantitative RT-PCR analysis of IL-8, IL-6 and IL-1β mRNA expression in various tissues of Japanese flounder. **A, B, C**: Expression of Japanese flounder *IL-8, IL-6* and *IL-1β* in various tissues, respectively; D: Densitometric analysis of semi-quantitative RT-PCR was used to obtain the relative IOD of IL-8, IL-6 and IL-1β versus GAPDH; M: molecular weight standard; 1-11: head kidney, spleen, liver, kidney, brain, skin, gill, muscle, heart, intestine, gonad. The Japanese flounder *IL-8* gene-specific RT-PCR products (304 bp), *IL-6* gene-specific RT-PCR products (385 bp) and *IL-1β* gene-specific RT-PCR products (350 bp) are indicated on the right margin, along with GAPDH RT-

PCR products (577 bp) as an internal control.

IL-8 expression in infected Japanese flounder tissues

The expression of *IL-8* in the Japanese flounder was analyzed in the spleen, head kidney, and liver after a challenge with LPS. In the three tissues, the expression of *IL-8* was dramatically up regulated after infection. The highest expression levels of *IL-8* were detected at 12h in the spleen and head kidney and 24h in the liver. The expression in the spleen began at 3h, decreased gradually at 24h, and returned to the background level after 36h (**Figure 2 A**). In the head kidney, the expression levels remained high from 9h to24h and then decreased gradually to the background levels (**Figure 2 C**). The expression in the liver began at 3h and reached the highest point at 24h, then declined to the background level at 36h (**Figure 2 E**). We also injected the Japanese flounder with physiological saline as a control, and the results showed that the *IL-8* expression in the spleen, head kidney, and liver was consistent with the levels in uninfected adult tissues, indicating that the injection of physiological saline was not harmful to the Japanese flounder (**Figure 2 B, D, F**).

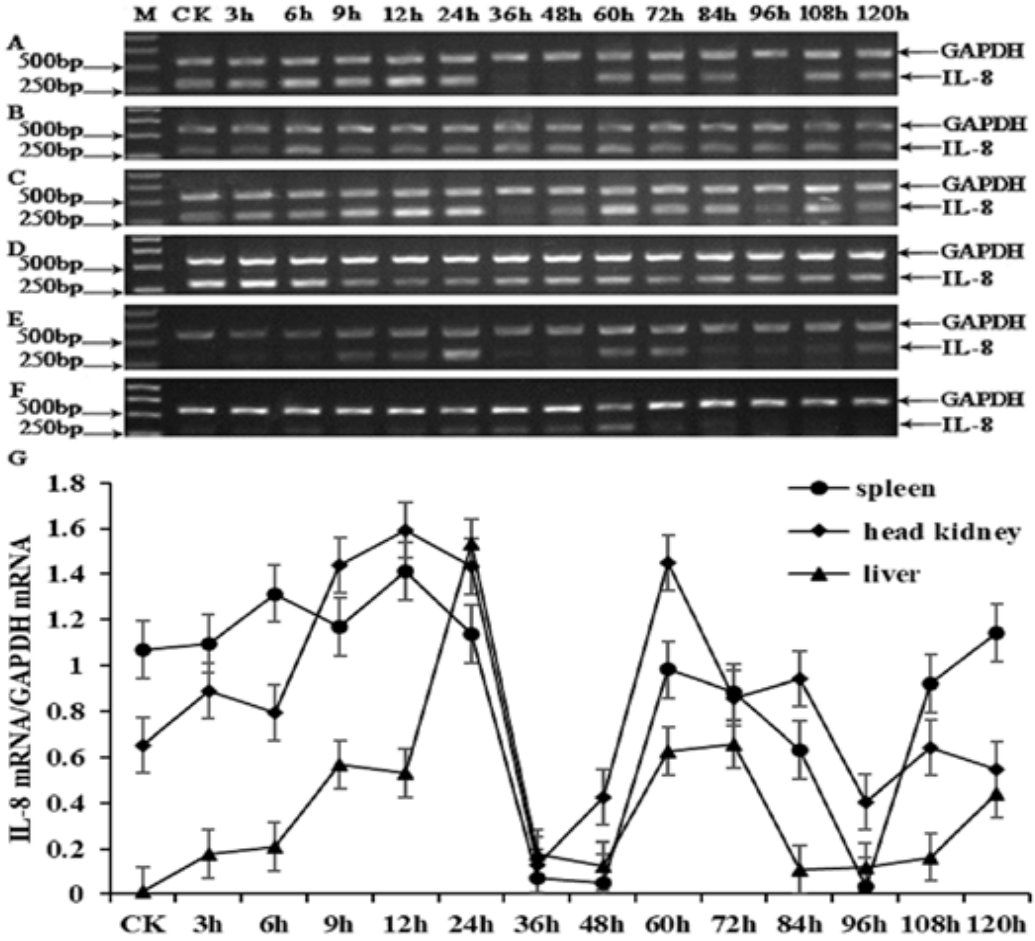

Figure 2 Semi-quantitative RT-PCR analysis of Japanese flounder IL-8 in infected tissues (spleen, head kidney, liver). A, C, E: spleen, head kidney, liver of Japanese flounder injected with LPS, respectively; B, D, F: spleen, head kidney, liver of Japanese flounder injected with PS, respectively; G: the relative IOD of IL-8 versus GAPDH in infected spleen, head kidney and liver; M: molecular weight standard; CK: uninfected tissues as controls; Different time points after challenge with LPS

are indicated on the above margin. The Japanese flounder IL-8 gene-specific RT-PCR products (304 bp) and GAPDH RT-PCR products (577 bp) are indicated on the right margin.

IL-6 expression in infected Japanese flounder tissues

The expression of *IL-6* in the spleen, head kidney, and liver was dramatically up regulated after the challenge. The expression in the spleen began at 6h and decreased to the background level at 60h (**Figure 3 A**). The highest expression level in the head kidney was detected at 9h and peaked at 48h, then returned to the background level at 84h (**Figure 3 C**). The expression level in the liver was high at 12h, peaked at 36h, and decreased at 48h (**Figure 3 E**). The expression of *IL-6* was very low at other time points. We also injected the Japanese flounder with physiological saline as a control, and the results showed that the injection of physiological saline did not influence the expression of *IL-6*in the three tissues (**Figure 3 B, D, F**).

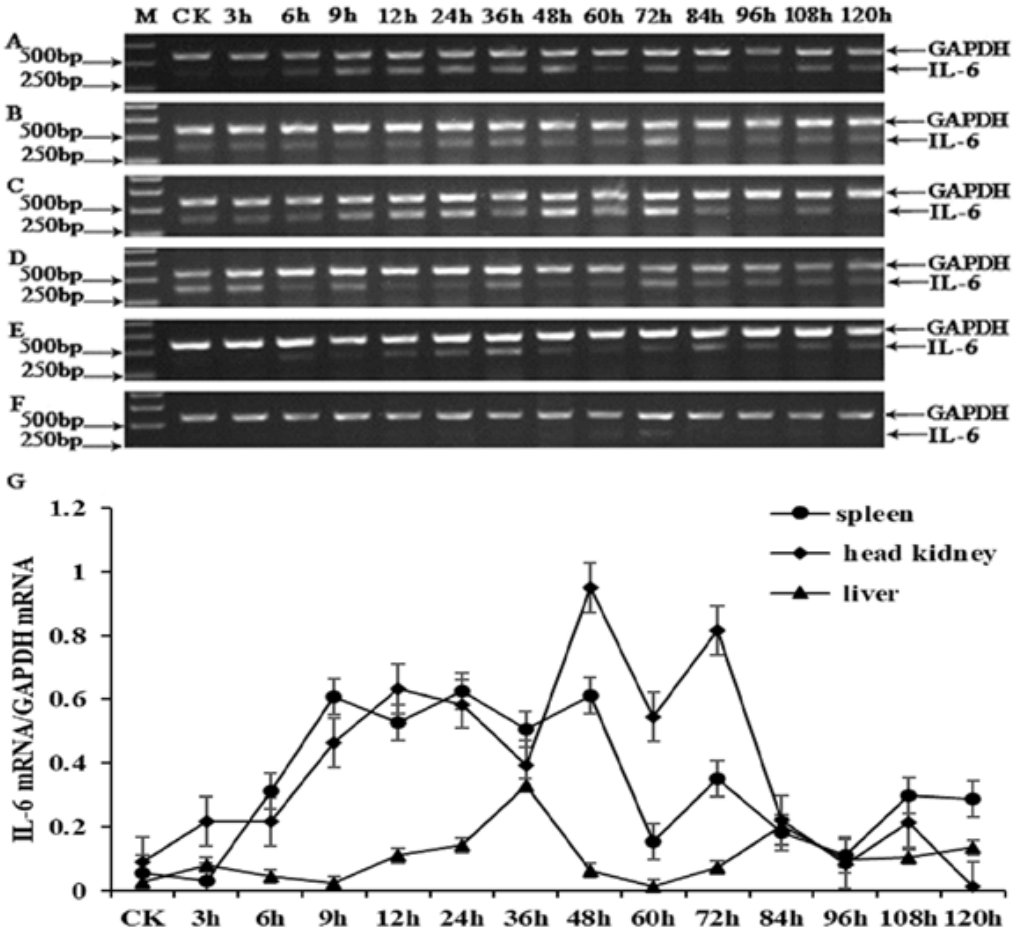

Figure 3 Semi-quantitative RT-PCR analysis of Japanese flounder IL-6 in infected tissues (spleen, head kidney, liver). A, C, E: spleen, head kidney, liver from the Japanese flounder injected with LPS, respectively; B, D, F: spleen, head kidney, liver from the Japanese flounder injected with PS, respectively; G: the relative IOD of IL-6 versus GAPDH in infected spleen, head kidney and liver; M: molecular weight standard; CK: uninfected tissues as controls; Different time points after the challenge with LPS are indicated on the above margin. The Japanese flounder IL-6 gene-specific RT-PCR products (385 bp) and GAPDH RT-PCR products (577 bp) are indicated on the right margin.

IL-1β expression in infected Japanese flounder tissues

The highest expression levels of the Japanese flounder *IL-1β* were detected at 3h in

the spleen and 9h in the head kidney and liver after infection. The expression in the spleen decreased gradually to the background level after 24h, and the expression in the head kidney, and the liver had similar trends (**Figure 4 A, C**). The expression level of *IL-1β* was very low at most time points, except at 36h. The expression of *IL-1β* in the spleen, head kidney, and liver of Japanese flounder injected with physiological saline was consistent with the level in uninfected adult tissues (**Figure 4 B, D, F**). The results indicated that physiological saline was not harmful to the Japanese flounder.

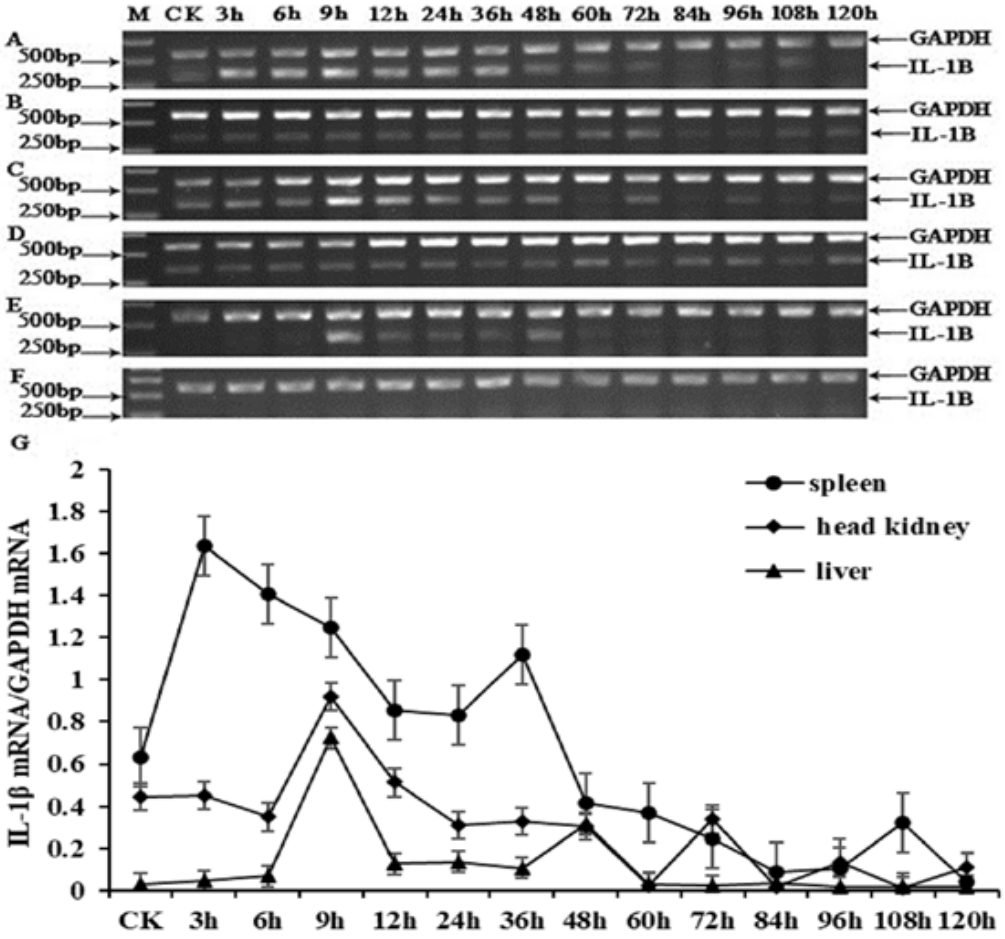

Figure 4 Semi-quantitative RT-PCR analysis of Japanese flounder IL-1β in infected tissues (spleen, head kidney, liver). A, C, E: spleen, head kidney, liver from the Japanese flounder injected with LPS, respectively; B, D, F: spleen, head kidney, liver from the Japanese flounder injected with PS, respectively; G: the relative IOD of *IL-1β* versus GAPDH in infected spleen, head kidney and liver; M: molecular weight standard; CK: uninfected tissues as controls; Different time points after the challenge with LPS are indicated on the above margin. The Japanese flounder *IL-1β* gene-specific RT-PCR products (330 bp) and GAPDH RT-PCR products (577 bp) are indicated on the right margin.

Discussion

Environmental factors such as temperature and seasonal changes have significant effects on the immune system of poikilothermic animals, including fish (Nikoskelainen et al., 2004). Fish reared in open air tanks or sea cages are subject to environmental variation. It is commonly assumed that innate immunity is more active at low temperatures, while the acquired immune system is more effective at high temperatures (Alcorn et al., 2002). In cod, the innate system parameters such as spontaneous hemolytic activity decreased

with increasing temperature, whereas the IgM serum concentration and naturalanti body activity increased (Magnadóttir et al., 1999). However, studies have also shown that there are examples of low temperature having adverse effects on innate immunity parameters. For example, in rainbow trout acclimatized at temperatures from 5°C to 20°C for 57 d, phagocytic activity, as well as the serum complement activity, increased with the temperature, while antibody activity was unaffected (Nikoskelainen et al., 2004). The seawater temperature in northern China is comparatively lower than in southern China, especially in winter. The lower temperature might affect the growth of the Japanese flounder and its immune response. In this study, the Japanese flounders were kept for one week at a lower water temperature of8°C-10°C before the experiment was carried out. The results showed that the expression levels of *IL-8, IL-6,* and *IL-1β* in the spleen, head kidney, and liver of Japanese flounders injected with LPS were different at 13 time points, indicating that the innate immune system of Japanese flounder could resist external infection at lower temperatures.

Our results showed that the three immune-related genes were differentially expressed in Japanese flounder tissues, indicating the sites of function in the body. There were high expression levels of *IL-8, IL-6,* and *IL-1β* in classical fish immune organs such as the head kidney, and spleen (**Figure 1**). The innate immune system is composed of three compartments: the epithelial and mucosal barrier, the humoral parameters, and the cellular components. The epithelial and mucosal barrier of the skin, gills, and the alimentary tract is an important disease barrier in fish. In addition to providing physical and mechanical protection, the fish mucus contains several immune defense parameters, including antimicrobial peptides, complement factors, and immunoglobulins (Aranishi and Mano, 2000; Magnadottir, 2006; Whyte, 2007; Subramanian et al., 2007). An experiment using the semi-quantitative RT-PCR technique (Lindensrom et al., 2003) examined the expression of the pro-inflammatory cytokine IL-1β and the type II IL-1β receptor in the skin of rainbow trout. The study found that a less obvious induction of IL-1β expression was seen in the initial phases of primary *G. derjavini* infection than in the secondary infection imposed just after recovery from the priming infection. In addition, significant up-regulation of *IL-8* and *IL-1β* was found in the gills of striped trumpeter in response to infection by the ectoparasite *Chondracanthus goldsmidi* (Covello et al., 2009). In our study, the expression of three genes was rather high in the skin and gill, suggesting that these cytokines (IL-8, IL-6, IL-1β) in the skin and gill and were important in the epithelial and mucosal barrier. A lower expression of *IL-1β* was also detected in the muscle of the intestine of Japanese flounder, but *IL-8* and *IL-6* were not detected. This suggested that some immune cytokines exist not only in immune organs but also in the organs that do not take part in the immune response.

IL-8 in the Japanese sea perch (*Lateolabrax japonicus*) was found to be a constitutive and inducible acute-phase protein that may be involved in the immune defense (Qiuet al., 2009). Expression analysis of Japanese flounder *IL-8* in infected tissues carried out in our study revealed that*IL-8* was highly expressed in the spleen and head kidney at 12h and the liver at 24h after challenge with LPS (**Figure 2**). There was also a high level of *L.japonicusIL-8* in the spleen and head kidney. The temporal expression of LjIL-8 mRNA in the spleen was up-regulated by lipopolyssacharide (LPS) stimulation and reached the maximum level at 6h post-stimulation (Qiu et al., 2009). Our result was similar to the

results of Qiu et al., indicating that LjIL-8 was a constitutive and inducible acute-phase protein that is involved in the immune defense of *L. japonicus*.

Importantly, *IL-6* is a multifunctional cytokine that can be induced by a plethora of chemical and physiological compounds, including the inflammatory cytokines TNF and IL-1(Berghe et al., 2000). Our study examined the expression of IL-6 in the main immune organs of infected the Japanese flounder. As shown in **Figures 3** and **4**, the expression of IL-6 after challenge in the spleen, head kidney, and liver was both abundant and persistent. IL-6 expression followed the expression of *IL-1β* in the spleen, liver, and head kidney after challenge with LPS. IL-6 showed high expression from 9h to 48h in the spleen, with the highest point at 48h and relatively high expression in the liver from 24h to 48h, with the highest point at 36h, and from 12h to 72h in the head kidney, with the highest level at 48h.*IL-1β* was highly expressed in the spleen at 3h and in the liver and head kidney at 9h after challenge with LPS. This suggests that the IL-6 promoter is multifunctional and responds to a wide variety of environmental stimuli, including cytokines such as IL-1β (**Figure 3**).

In fish, IL-1β was found to play a key role in the inflammatory response of the innate immune system that functions as the first line of inducible host defense against bacterial, fungal, and viral pathogens (Hoebe et al., 2004).Expression analysis of Japanese flounder *IL-1β* in infected tissues carried out in the present study revealed thatIL-1β was highly expressed in the spleen at 3h and the liver and head kidney at 9h after challenge with LPS (**Figure 4**).This result was very similar to the IL-1β expression in the spleen of the flounder *Paralichthys olivaceus* infected with outer membrane vesicle (OMV), where *IL-1β* was markedly induced at 3h post-injection, and strong induction was observed at 6h post-injection (Hong et al., 2009).

Acknowledgements

This study was supported by the National Marine Public Benefit Research Foundation, China (No. 201405003-2) and the Science and Technology Innovation Fund Project of Dalian city (2018J12SN070).

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