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Individual and combined effects of salinity and lipopolysaccharides on the immune response of juvenile *Takifugu fasciatus*

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Abstract Lipopolysaccharides (LPS) and salinity are important variables in aquatic environments. High concentration of LPS and large changes in salinity seriously threat the survival of a variety of organisms, including fish. To reveal the effects of salinity and LPS on a fish immune response, we measured the immune-related parameters (total leukocyte count, total serum protein, albumin and globulin concentrations, complement C3 concentration, and lysozyme activity) and genes (the expressions of *TNF-α*, *IL-1β*, and *SOCS1–3* at the mRNA and protein levels) of juvenile *Takifugu fasciatus* exposed to phosphate buffered saline (PBS) or LPS (25 μg mL^{-1}) under different salinities (0, 15, and 30 ppt) for 24 h. Changes in key immunological indicators suggested that the LPS challenge induced considerable damage to *T. fasciatus*, whereas an increase in salinity mitigated the harmful effects. Moreover, although the immune responses in blood and other selected tissues (gill and kidney) were suppressed with an increase in salinity, the increased response in liver in saltwater enabled *T. fasciatus* to conquer large salinity variation during migration. The appropriate addition of salts appeared to be a sensible strategy to mitigate LPS-induced toxicity in the aquaculture of *T. fasciatus*.

Keywords Salinity · Lipopolysaccharides · Immune response · *Takifugu fasciatus*

Abbreviations

LPS	Lipopolysaccharides
T. fasciatus	*Takifugu fasciatus*
IL-1β	Interleukin-1β
TNF-α	Tumor necrosis factor-α
SOCS	Suppressors of cytokine signaling
ROS	Reactive oxygen species
GCs	Glucocorticoids
qRT-PCR	Quantitative real-time PCR
AKI	Acute kidney injury

Introduction

As a component of the Gram-negative bacterial outer membrane, lipopolysaccharides (LPS) is an important environmental variable in fresh, brackish, and marine waters due to fluctuation of microbial products and outbreaks of infectious diseases (Maeda et al. 1983). Although administration of LPS has been verified to enhance resistance against bacterial pathogens

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(Selvaraj et al. 2009), high levels of LPS are toxic to a variety of organisms, including fish. Salinity is also an important variable in aquatic environments. Large salinity variation seriously affects the growth and development and may cause even death of aquatic organisms (Yang et al. 2010). Currently, more and more studies have focused on the function of salinity in mitigating toxicity caused by environmental pollutants, such as nitrite and cadmium (Wang et al. 2017; Wang et al. 2016a). However, the effects of salinity and its function in mediating LPS-induced toxicity remain largely unexplored from the point of fish innate immune response.

In fish, the innate immune system is the first line of defense against pathogenic infections (Magnadóttir 2006). Many parameters and genes have been used as immunological indicators. The changes in leukocyte count and total serum protein, albumin, and globulin concentrations have been widely used to demonstrate tissue damage and diagnose fish diseases (Elasely et al. 2014; Yang et al. 2015). Complement C3 is an important component of fish innate immunity, playing an indispensable role in various immune effector functions, such as elimination of invading pathogens, promotion of inflammatory responses, and clearance of homeostatic cells (Ichiki et al. 2012). In fish, complement C3 is primarily synthesized in liver and expressed in a wide assortment of organs, such as gill, intestine, and skin (Boshra et al. 2006). Lysozyme, referred to as N–acetylmuramide glycanohydrolase or muramidase, is a bacteriolytic enzyme which acts as an opsonin of the complement system (Magnadóttir 2006). Magnadóttir et al. have confirmed that the lysozyme from the mother helps prevent the *Aeromonas salmonicida* infection when the fish has not reached the complete maturity of the immune system (Magnadottir et al. 2005). Inflammatory cytokines also play important roles in immunity of fish. Interleukin-1β (IL-1β) is a prototypic pro-inflammatory cytokine that can affect nearly every cell type, and it is often in concern with tumor necrosis factor (TNF) (Huising et al. 2004). In addition, suppressors of cytokine signaling (SOCS) are thought to play central roles in an innate immune response through negative feedback on cytokine signaling (Jin et al. 2008). Regulation of innate immune response is critical because excessive inflammatory reactions can be deleterious (Skjesol et al. 2014).

As a delicious and commercially farmed fish, *Takifugu fasciatus* is widely distributed in the South China Sea, the East China Sea, and inland waters in China and Korean Peninsula (Kato et al. 2005). In recent years, the expansion of farming land and limitations in disease prevention have caused serious bacterial diseases in the aquaculture of *T. fasciatus*. Moreover, with the establishment of dams in the middle and upper reaches of estuaries and overexploitation of groundwater for agricultural irrigation, aquaculture practices are now vulnerable to seawater intrusion (Kalbus et al. 2016). *T. fasciatus* is an anadromous species, which frequently encounters large salinity changes in its lifetime due to the migratory habit (Yang and Chen 2008). Additionally, studies have shown that estuarine animals in their early stage are more sensitive to numerous environmental factors than adults (Wang et al. 2017). These special characteristics suggest that the juvenile *T. fasciatus* is an ideal model to assess the individual and combined effects of salinity and LPS on the fish innate immune response.

In the present study, we first assessed the immune parameters (total leukocyte count, total serum protein, albumin and globulin concentrations, complement C3 concentration, and lysozyme activity) in blood and immune-related tissues (gill, kidney, and liver) under salinity and LPS challenge. Next, the expressions of immune-related genes (*TNF-α*, *IL-1β*, and *SOCS1–3*) at the mRNA and protein levels were analyzed. Our findings revealed the effects of salinity and LPS on immune response and provided valuable evidence to further clarify the significance of the application of salts to counteract LPS-induced damage in the aquaculture of *T. fasciatus*.

Materials and methods

Experimental fish

T. fasciatus (9 ± 1.25 cm in length, 20 ± 2.05 g in weight) were obtained from Zhongyang Group Co., Ltd. of *T. fasciatus* (Jiangsu Province, China). A total of 108 individuals were randomly transferred to 18 aquariums equipped with a bio-filtered water recirculation system (cooling and heating functions, volume 200 L; flow rate 5 L min^{-1}; 25 ± 1 °C; pH 7.5 ± 0.4; 0 ppt) and reared with the artificial compound feed twice a day at 25 ± 1 °C. After acclimation under laboratory conditions for 1 week, the fish were used for the experiments.

Experimental design

The experiment protocol was approved by the Ethics Committee of Experimental Animals at Nanjing Normal University (SYXK2015-0028). Firstly, juveniles were randomly and evenly divided into different salinity groups: 0 ppt, 15 ppt, and 30 ppt. To acclimate to the designated salinity, refined industrial salt was added to increase salinity by 3 ppt every 6 h until it reached the preset treatment concentrations. Afterwards, the juvenile fish, adapted to a specific salinity treatment for 1 week, were injected intraperitoneally with either 0.1 mL PBS (0.14 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) or LPS (25 μg mL^{-1}, Sigma, USA). The injection volume was proportionally adjusted according to the body weight of the individuals. Hematoxylin and eosin (H&E) staining was conducted to check tissue damages. All treatments were conducted in triplicate.

After 24 h of exposure, the juveniles ($n = 3$) were randomly taken from each aquarium and euthanized with MS-222 solution (0.05%, Sigma, USA). Then, blood samples were collected from the heart by a sterilized syringe containing heparin solution (Jiancheng Bioengineering, Nanjing, China). Serum was separated by centrifugation (3500 rpm, 4 °C, 10 min) for analysis of serum parameters. After blood collection, samples of gill, kidney, and liver were rapidly excised, frozen in liquid nitrogen, and stored at − 80 °C prior to further analysis.

Total leukocyte count and total serum protein, albumin, and globulin concentrations

Leukocytes were counted using an automatic hematology analyzer (BC-2800vet, Shenzhen, China). Total serum protein, albumin, and globulin concentrations were assayed by an automatic biochemical analyzer (Chemray 240, Shenzhen, China).

Complement C3 assay

Samples were weighed (wet mass) and homogenized with 10 volumes of 0.86% saline. The homogenates were centrifuged at 2500 rpm for 10 min at 4 °C, and the recovered supernatants were used to determine complement C3 concentration with a C3 ELISA kit (Jiancheng, Nanjing, China) as described by Abdollahi et al. (2016). The optical density (OD) was measured with a Synergy™ H1 Hybrid multimode microplate reader (BioTek Instruments, Winooski, VT, USA) at 450 nm. Protein concentration in the crude extract was determined following the Bradford method (Bradford 1976). The C3 values were assayed in triplicate and presented as μg mg^{-1} protein.

Lysozyme activity

According to a previously described method by Chen et al. (2017), the prepared samples were employed to determine the lysozyme activity in triplicate with a commercial kit (Jiangcheng, Nanjing, China). A unit of lysozyme activity was defined as the amount of enzyme causing a reduction in absorbance of 0.001 per min at 530 nm. The OD value of each sample was measured using a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). The lysozyme values were expressed as U mg^{-1} protein.

Gene expression at the mRNA level

Total RNA was isolated from tissues using High Purity RNA Fast Extract Reagent (Bioteke, Beijing, China), and the first-strand cDNA was synthesized using HiScript™ QRT SuperMix through a qPCR + gDNA wiper (Vazyme, Nanjing, China). Then, the expression of *TNF-α*, *IL-1β*, and *SOCS1–3* at the mRNA level was determined by quantitative real-time PCR (qRT-PCR), and *β-actin* was employed as an internal control (Wang et al. 2016b). The primers of *TNF-α*, *IL-1β*, and *SOCS1–3* were designed based on our previous transcriptome data of *T. fasciatus*. Table 1 lists the primers used in this study. The experiments were conducted in triplicate on an ABI StepOne™ Plus System (Applied Biosystems, USA) in a 20-μL reaction system consisting of 10 μL of SYBR Green master mix (Vazyme, Nanjing, China), 4 μL of cDNA (500 ng), and 3 μL of forward and reverse primers (2 mM). Briefly, after an initial denaturation step at 95 °C for 30 s, the amplifications were carried out with 40 cycles at a melting temperature of 95 °C for 10 s and an annealing temperature of 60 °C for 30 s. The relative expression levels were then calculated using the $2^{-\Delta\Delta Ct}$ method and were subjected to statistical analysis.

Western blotting analysis

The frozen samples were homogenized and prepared using a total protein extraction kit (KeyGen BioTech,

Table 1 Primers used for quantitative real-time PCR

Name	Primer sequence (5′-3′)	Melting temperature (°C)	Amplicon size (bp)
SOCS1-F	CTCTCCTCGCAGAGACCAAC	60	245
SOCS1-R	GAGGAGCGTCCCTCAACATA		
SOCS2-F	TGAGAGCATCCAAACGACTG	60	158
SOCS2-R	GCAGAATCTCTTTGGCTTCG		
SOCS3-F	CCAGAACCCTCTGACCCTCT	60	122
SOCS3-R	ATGGCGTTGCTAAACTTGCT		
TNF-α-F	TGGTGGTTCAGCACACACAG	60	133
TNF-α-R	TGCCTGAGAACACAGCATGA		
IL-1β-F	TGAAGAGCGTGGTCAACCTG	60	128
IL-1β-R	CTCTTCCACGATGGTGTCCA		
β-actin-F	AAGCGTGCGTGACATCAA	60	143
β-actin-R	TGGGCTAACGGAACCTCT		

Nanjing, China), and then the extracted proteins were stored at − 80 °C until Western blotting analysis. Proteins were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and then electro-transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were incubated with TBS containing 0.05% Tween-20 and 5% albumin bovine V (Solarbio, Beijing, China) at room temperature for 2 h to block non-specific bindings. Subsequently, the PVDF membranes were incubated with the primary antibodies, including three rabbit antibodies against SOCS1 (1:1000; D160748; Sangon Biotech, Shanghai, China), SOCS2 (1:1000; BS2914; Bioworld Technology, Minnesota, USA), and SOCS3 (1:500; D221242; Sangon Biotech, Shanghai, China), and one mouse antibody against β-actin (1:2600; A5441; Sigma, St. Louis, MO, USA), at 4 °C overnight, and then the membranes were washed and incubated with goat anti-rabbit IgG secondary antibody (L3012; SAB, Baltimore Ave, MD, USA) or goat anti-mouse IgG secondary antibody (L3032; SAB, Baltimore Ave, MD, USA) at room temperature for 2 h. Immunoreactive bands were visualized with a chemiluminescence reagent (Perkin-Elmer Life Science, USA). The densitometry analysis was performed using ImageJ software.

Statistical analysis

Statistical analysis was performed using SPSS 22.0. All data were presented as means ± SD (standard deviation). Two-way ANOVA was employed to assess interaction between salinity and LPS on the immune response. As interaction occurred, one-way ANOVA followed by Tukey's multiple range comparison test was used to determine the treatment response. $P < 0.05$ was considered statistically significant.

Results

Immune parameters

Total leukocyte count and total serum protein, albumin, and globulin concentrations

Total leukocyte count was significantly decreased under LPS challenge (Table 2). Salinity itself also downregulated the count, while there was an increase at 15 ppt and a decrease at 30 ppt under LPS challenge. The serum protein, albumin, and globulin concentrations were not altered under LPS challenge, while their concentrations were suppressed with the increase of salinity (Table 2). LPS challenge had no significant effect on serum protein, albumin, and globulin concentrations, and there was no significant interaction between salinity and LPS. Two-way ANOVA showed that significant interaction was detected between salinity and LPS on total leukocyte count ($P < 0.001$).

Complement C3 assay

The C3 concentration was negatively correlated with LPS at 0 ppt, except for that in kidney (Fig. 1). Although salinity itself downregulated the C3 concentration in gill

Table 2 Total leukocyte count and total serum protein, albumin, and globulin concentrations observed in juvenile *T. fasciatus* exposed to PBS or LPS (25 μg mL^{-1}) under different salinity levels (0, 15, and 30 ppt) for 24 h

Parameters	Groups	0 ppt	15 ppt	30 ppt
Total leukocyte count (10^9 L^{-1})	PBS	$64.60 \pm 5.23^{A*}$	$49.00 \pm 5.18^{B*}$	$17.20 \pm 1.36^{C*}$
	LPS	3.30 ± 0.19^{a}	5.10 ± 0.22^{b}	2.50 ± 0.14^{c}
Total protein (g L^{-1})	PBS	51.46 ± 4.02^{A}	38.70 ± 3.24^{B}	32.86 ± 3.29^{B}
	LPS	47.20 ± 4.31^{a}	38.68 ± 2.20^{b}	30.56 ± 2.68^{c}
Albumin (g L^{-1})	PBS	24.30 ± 1.69^{A}	18.47 ± 1.23^{B}	16.71 ± 1.26^{B}
	LPS	22.27 ± 0.73^{a}	17.98 ± 1.45^{b}	15.88 ± 1.33^{c}
Globulin (g L^{-1})	PBS	27.17 ± 1.06^{A}	20.22 ± 1.32^{B}	16.15 ± 1.01^{C}
	LPS	24.93 ± 1.97^{a}	20.70 ± 1.37^{b}	14.68 ± 0.92^{c}

All values represent means ± SD ($n = 3$ independent fish). Capital letters denote significant differences at different salinities (0, 15, and 30 ppt) with PBS injection. Lowercase letters indicate significant differences at different salinities with LPS challenge. The asterisk indicates significant differences between PBS and LPS injection at the same salinity

(Fig. 1a), its concentration in liver reached the highest at 15 ppt and was maintained at a high level even at 30 ppt (Fig. 1b, c). Moreover, when the juveniles were exposed to LPS, the decrease of C3 concentration at 0 ppt was inhibited at 15 ppt and 30 ppt (Fig. 1a, c). There was significant interaction between salinity and LPS on C3 concentration ($P < 0.05$).

Lysozyme activity

After LPS challenge, the lysozyme activity was significantly decreased at 0 ppt and 15 ppt, except for that in kidney (Fig. 2). Salinity itself downregulated lysozyme activity in gill and kidney (Fig. 2a, b), while its activity in liver reached the highest at 15 ppt and returned to the control level at 30 ppt (Fig. 2c). Additionally, under LPS challenge, the lysozyme activity at 30 ppt was significantly higher compared with that at 0 ppt and 15 ppt (Fig. 2a, c). There was significant interaction between salinity and LPS on lysozyme activity ($P < 0.05$).

Transcription patterns after challenged with salinity and LPS

Cytokine expression

When the juveniles were challenged with LPS at 0 ppt, the expressions of *TNF-α* and *IL-1β* at the mRNA level were significantly increased in each tissue (Figs. 3 and 4). Conversely, a significant decrease occurred at 15 ppt compared with the corresponding PBS-treated group (Figs. 3 and 4). Salinity alone upregulated the expressions of *TNF-α* and *IL-1β* at the mRNA level in gill and kidney

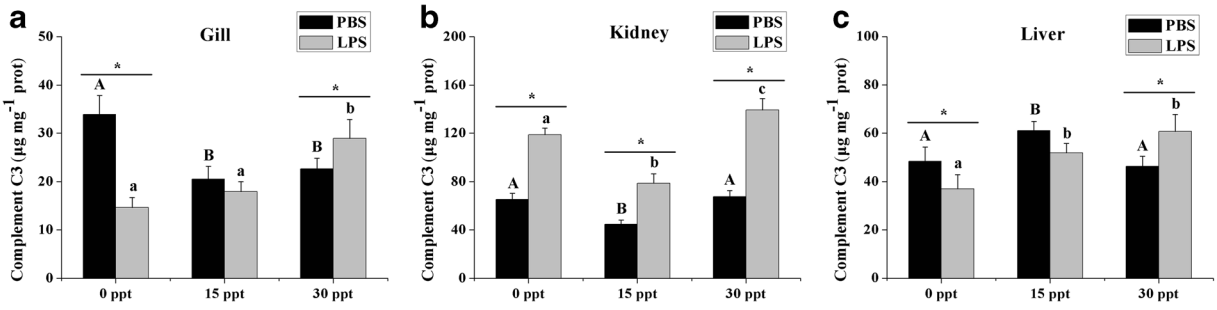

Fig. 1 Effects of salinity and LPS challenge on complement C3 concentration in gill (**a**), kidney (**b**), and liver (**c**) of juvenile *T. fasciatus*. All values represent means ± SD ($n = 3$ independent fish). Capital letters denote significant differences at different salinities (0, 15, and 30 ppt) with PBS injection. Lowercase letters indicate significant differences at different salinities with LPS challenge. The asterisk indicates significant differences between PBS and LPS injection at the same salinity

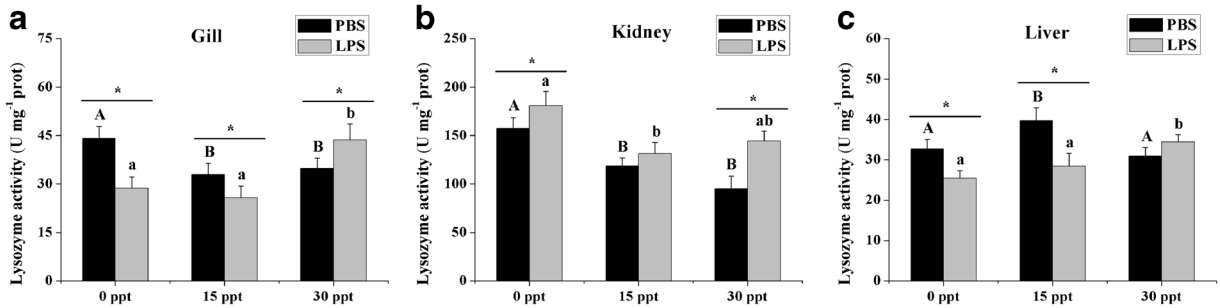

Fig. 2 Effects of salinity and LPS challenge on lysozyme activity in gill (**a**), kidney (**b**), and liver (**c**) of juvenile *T. fasciatus*. All values represent means ± SD ($n = 3$ independent fish). Capital letters denote significant differences at different salinities (0, 15, and 30 ppt) with PBS injection. Lowercase letters indicate significant differences at different salinities with LPS challenge. The asterisk indicates significant differences between PBS and LPS injection at the same salinity

(Figs. 3a, b and 4a, b), while their mRNA expressions in liver exhibited an opposite trend (Figs. 3c and 4c). At 15 ppt, the expressions of *TNF-α* and *IL-1β* at the mRNA level in gill and kidney dropped to the lowest under LPS challenge compared with that at 0 ppt and 30 ppt (Fig. 3a, b and 4c). Overall, there was significant interaction between salinity and LPS on the expressions of *TNF-α* and *IL-1β* at the mRNA level ($P < 0.05$).

SOCS expression

LPS challenge decreased the *SOCS1* expression at the mRNA level in gill and liver at 0 ppt compared with the corresponding PBS-treated group (Fig. 5a, g). However, in kidney, the *SOCS1* expression at the mRNA level was positively correlated with LPS challenge at 0 ppt (Fig. 5d). The *SOCS1* expression at the mRNA level was significantly decreased with the increase of salinity in PBS-treated groups in gill and kidney (Fig. 5a, d), while its mRNA expression in liver exhibited a contrasting pattern (Fig. 5g). Moreover, the *SOCS1* expression at the mRNA level in liver was significantly increased at 15 ppt and 30 ppt under LPS challenge (Fig. 5g). The *SOCS2* expression at the mRNA level in gill was significantly decreased under LPS challenge at 0 ppt, while its mRNA expression in liver remained unchanged (Fig. 5b, h). Salinity itself downregulated the *SOCS2* expression at the mRNA level in gill (Fig. 5b), while its mRNA expression in liver reached the highest at 15 ppt and was maintained at a high level even at 30 ppt (Fig. 5h). Similarly, the *SOCS2* expression at the mRNA level in liver was significantly increased at 30 ppt under LPS challenge (Fig. 5h). For *SOCS3*, its mRNA expression was decreased at 0 ppt in gill and kidney (Fig. 5c, f). Irrespective of PBS/LPS exposure, the *SOCS3* expression at the mRNA level in liver was significantly increased at 15 ppt and decreased to the control level at 30 ppt (Fig. 5i). There was significant interaction between salinity and LPS on the expressions of three *SOCS* isoforms at the mRNA level ($P < 0.05$), except for *SOCS3* in liver.

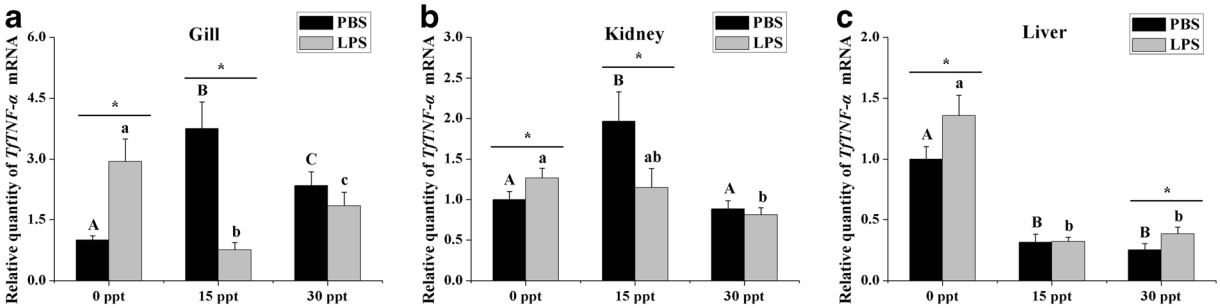

Fig. 3 Effects of salinity and LPS challenge on mRNA abundance of *TNF-α* in gill (**a**), kidney (**b**), and liver (**c**). All values represent means ± SD ($n = 3$ independent fish). Capital letters denote significant differences at different salinities (0, 15, and 30 ppt) with PBS injection. Lowercase letters indicate significant differences at different salinities with LPS challenge. The asterisk indicates significant differences between PBS and LPS injection at the same salinity

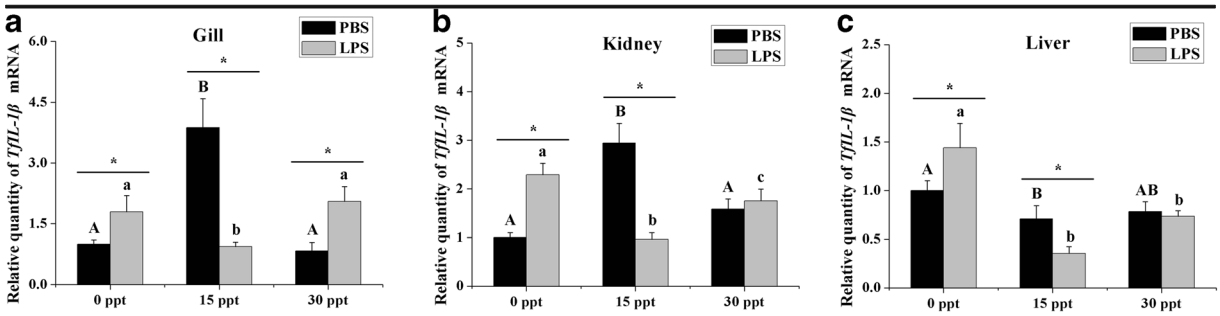

Fig. 4 Effects of salinity and LPS challenge on mRNA abundance of *IL-1β* in gill (**a**), kidney (**b**), and liver (**c**). All values represent means ± SD ($n = 3$ independent fish). Capital letters denote significant differences at different salinities (0, 15, and 30 ppt) with PBS injection. Lowercase letters indicate significant differences at different salinities with LPS challenge. The asterisk indicates significant differences between PBS and LPS injection at the same salinity

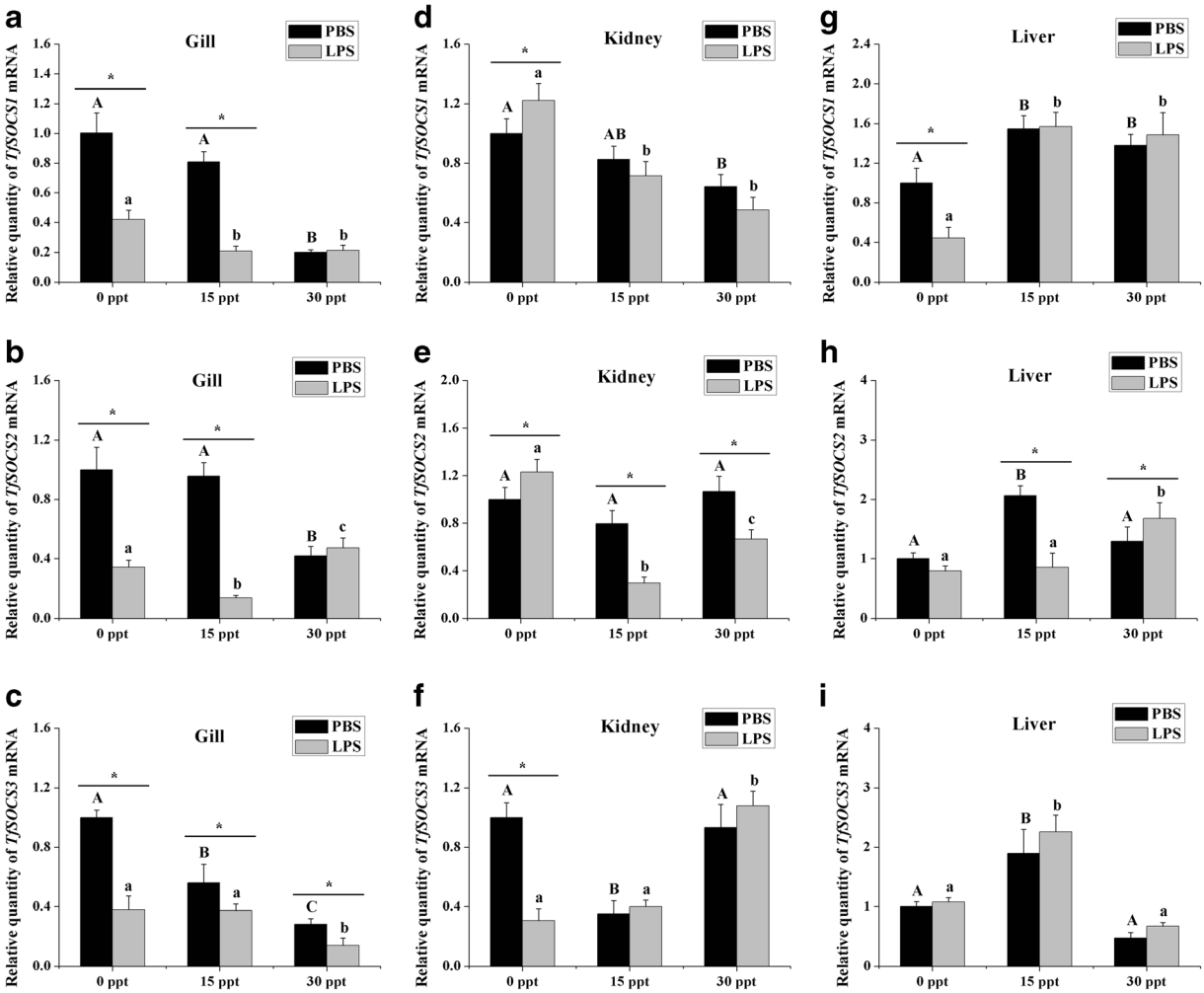

Fig. 5 Effects of salinity and LPS challenge on mRNA abundance of *SOCS1–3* in gill (**a**, **b**, **c**), kidney (**d**, **e**, **f**), and liver (**g**, **h**, **i**). All values represent means ± SD ($n = 3$ independent fish). Capital letters denote significant differences at different salinities (0, 15, and 30 ppt) with PBS injection. Lowercase letters indicate significant differences at different salinities with LPS challenge. The asterisk indicates significant differences between PBS and LPS injection at the same salinity

Western blotting analysis after challenged with salinity and LPS

The Western blotting analysis showed that anti-SOCS1, 2, and 3 could successfully detect the antigens, and the Mw of SOCS1, SOCS2, SOCS3 and β-actin was approximately 24, 22, 27, and 42 kDa, respectively. The expressions of the three SOCS isoforms at the protein level were significantly decreased at 0 ppt under LPS challenge, except for SOCS1 and 2 in kidney (Fig. 6). Salinity, irrespective of PBS/LPS exposure, downregulated the SOCS1 expression at the protein level in gill and kidney (Fig. 6a, d), while its protein expression in liver was significantly increased at 15 ppt, and maintained at a high level even at 30 ppt (Fig. 6g). Additionally, the SOCS2 expression at the protein level in liver was significantly increased at 30 ppt under LPS challenge (Fig. 6h). For SOCS3, irrespective of PBS/LPS exposure, its protein expression in liver was also significantly increased at 15 ppt and decreased to the control

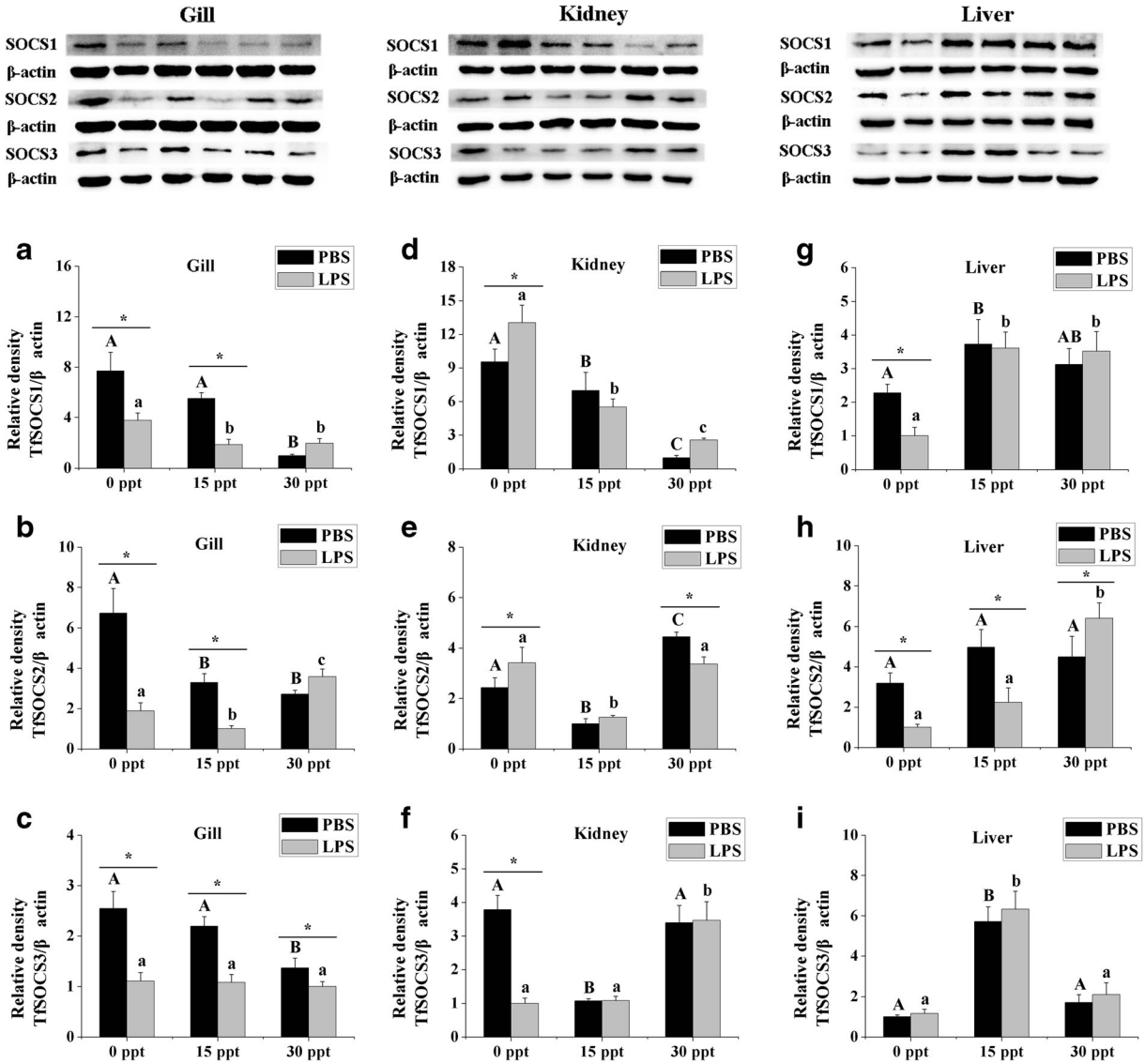

Fig. 6 Effects of salinity and LPS challenge on protein abundance of SOCS1–3 in gill (**a**, **b**, **c**), kidney (**d**, **e**, **f**), and liver (**g**, **h**, **i**). All values represent means ± SD ($n = 3$ independent fish). Capital letters denote significant differences at different salinities (0, 15, and 30 ppt) with PBS injection. Lowercase letters indicate significant differences at different salinities with LPS challenge. The asterisk indicates significant differences between PBS and LPS injection at the same salinity

level at 30 ppt (Fig. 6i). There was significant interaction between salinity and LPS on the expressions of three SOCS isoforms at the protein level ($P < 0.05$), except for SOCS3 in liver.

Discussion

It has been well documented that *T. fasciatus* can tolerate a wide salinity variation and live well in river (0 ppt), estuary water (15 ppt), and sea (30 ppt) (Yang and Chen 2008). LPS, derived from the cellular wall of Gram-negative bacteria, is a key factor that causes sepsis (Ye et al. 2017). Currently, a substantial body of literature has indicated that salinity can act as a strong protective factor. In *Fundulus heteroclitus*, the highest level of reactive oxygen species (ROS) and greatest damage occurred at 0 ppt, and the least was detected at 35 ppt (Loro et al. 2012). Moreover, Wang et al. have confirmed that salinity itself decreased the toxicity of cadmium and nitrite to juvenile *T. fasciatus* (Wang et al. 2017; Wang et al. 2016a). Here, we investigated the individual and combined effects of salinity and LPS on the immune response of juvenile *T. fasciatus*. Our results supported that salinity mitigated the LPS-induced damage, and the maintenance of immune competence in saltwater enabled *T. fasciatus* to tolerate salinity variation during migration.

Blood parameters

There was a clear interaction between salinity and LPS on total leukocyte count. A key finding from this study was that the leukocyte count was significantly decreased under LPS challenge at 0 ppt, while an increase of the count occurred at 15 ppt. However, fresh water to seawater transfer in rainbow trout showed sustained elevation in total leukocyte count (Taylor et al. 2007). Leukocytosis in some cases may be due to protective reaction, in which leukocytes protect the body when foreign substances invade the body (Afaq and Rana 2009). Our results indicated that salinity might reduce the impact caused by LPS. Increases in serum protein, albumin, and globulin concentrations are thought to be associated with a stronger innate immune response in fish (Wiegertjes et al. 1996). However, in the present study, LPS challenge exerted no significant effect on the total serum protein, albumin, and globulin concentrations, and their concentrations were negatively correlated with the increase of salinity. Therefore, we speculated that the mitigating effect of salinity on LPS toxicity did not directly reflect in the total serum protein, albumin, and globulin concentrations.

Complement C3 and lysozyme

The complement C3 and lysozyme presented similar response patterns in our study. Under LPS challenge, the complement C3 concentration and lysozyme activity were significantly decreased at 0 ppt in gill and liver. However, Paulsen et al. (2003) reported that LPS stimulated a 5- to 6-fold increase in the lysozyme production in salmon macrophages. Our results suggested that LPS challenge in freshwater could compromise the response of complement and lysozyme in these tissues. We also observed that the complement C3 concentration and lysozyme activity were negatively correlated with salinity in gill and kidney. However, irrespective of PBS/LPS exposure, the complement C3 concentration and lysozyme activity in liver were significantly elevated at 15 ppt, and maintained at a high level even at 30 ppt. Consistent with our results, the lysozyme concentrations in *Salmo trutta* were significantly increased after transferred from freshwater to seawater (Marc et al. 2010). It is clear that upregulation of complement C3 in fish is a pervasive innate immune response of the body to injury, trauma, or injection (Bayne and Gerwick 2001). Moreover, lysozyme activity is closely related to innate immune response, and it sensitively responses to infections and invasion by foreign materials (Paulsen et al. 2003; Sieroslawska et al. 2012). The rise of lysozyme suggests elevation of various humoral factors that can protect the host during pathogen invasion (Harikrishnan et al. 2010). Collectively, we proposed that the activated complement C3 and lysozyme in liver in saltwater enabled *T. fasciatus* to tolerate LPS challenge in their migratory life.

Cytokines

In the present study, the expressions of *TNF-α* and *IL-1β* at the mRNA level were significantly increased under LPS challenge at 0 ppt. Consistent with our results, the increased levels of *TNF-α* and *IL-1β* are characterized in serum and kidney tissues during the course of LPS-induced acute kidney injury (AKI) (Ye et al. 2017). Similar result was also found with LPS-stimulated expression of *TNF-α* in head kidney of carp

(Savan and Sakai 2004). All these results supported that *TNF-α* and *IL-1β* are cytokines that induce or facilitate inflammatory response under LPS challenge. However, overproduction of LPS-induced pro-inflammatory molecules, such as *TNF-α* and *IL-1β*, can lead to endotoxin shock, which is a severe systemic inflammatory response triggered by the interaction of LPS with host cells (Novoa et al. 2009). *ILs* and *TNF-α* were involved in the osmoregulatory signaling network in gill cells of *Triakis semifasciata* and *Oreochromis mossambicus* (Kültz 2012; Dowd et al. 2010). There was further interaction between salinity and LPS on the expressions of *TNF-α* and *IL-1β* at the mRNA level. We observed that LPS challenge decreased the expressions of *TNF-α* and *IL-1β* at the mRNA level with the increase of salinity, especially at 15 ppt. An increasing body of evidence has demonstrated that suppression of cytokine production can attenuate LPS-induced acute kidney injury (AKI) (Xu et al. 2014). Moreover, in mammals, glucocorticoids (GCs) and catecholamines, the major stress hormones, decrease the expressions of multiple inflammatory genes that are activated during the inflammatory process, limiting the inflammation itself to avoid toxicity and tissue damage (Calcagni and Elenkov 2010). Therefore, we demonstrated that the decreased expressions of *TNF-α* and *IL-1β* at the mRNA level in saltwater were associated with alleviating tissue damage caused by LPS.

SOCS

While cytokines play indispensable roles in mediating immune and inflammatory responses in complex organisms, excessive cytokine signaling can lead to chronic inflammation and diseases (Shepherd et al. 2012). The *SOCS* genes act as key negative regulators of cytokine signaling (Kile and Alexander 2001). In the present study, we, for the first time, investigated the effects of salinity and LPS on the expression profiles of *SOCS* genes at both the mRNA and protein levels. Our results showed that LPS challenge significantly decreased the expressions of the three *SOCS* genes at the mRNA and protein levels at 0 ppt in a tissue- and isoform-specific manner. The decreased expressions of *SOCS* genes corresponded with the increased levels of cytokines, and this might be related to LPS-induced tissue damage. We also noticed that although salinity itself decreased the expressions of the three *SOCS* genes at the mRNA and protein levels in gill, their expressions in liver were significantly increased at 15 ppt and maintained at the control level even at 30 ppt. This finding corresponded with the lower levels of cytokine expression that we observed in saltwater under LPS challenge. As *SOCS* genes are involved in attenuating the inflammatory response to cytokines in animals, including fish, we proposed that the elevated expressions of *SOCS* genes at the mRNA and protein levels in liver were vital for *T. fasciatus* to conquer salinity variation during migration. Strikingly, under LPS challenge, the expressions of the three *SOCS* genes at the mRNA and protein levels were positively correlated with salinity, specifically in liver. Consistent with our results, emaciation per se increases the expressions of liver *SOCS2* and *SOCS3* in Arctic charr (Philip et al. 2014). Therefore, we demonstrated that the elevated expressions of the three *SOCS* isoforms contributed to the reduction of LPS-induced tissue damage. There was a significant interaction between salinity and LPS on the expressions of *SOCS1* and *SOCS2* at the mRNA and protein levels, while no significant interaction effect was observed on liver *SOCS3* expression no matter at the mRNA level or the protein level. As observed in our study, *SOCS* genes responded to challenges in a tissue- and isoform-specific manner in fish (Philip et al. 2012; Shepherd et al. 2012). This might be related to their special roles in mediating response when *T. fasciatus* encountered with salinity and LPS challenge. Although the expressions of the three *SOCS* isoforms at the protein level were consistent with their mRNA expressions in most cases, a slight difference still existed between their mRNA expressions and protein expressions. For example, the mRNA level of *SOCS2* in kidney was not altered with the increase of salinity in PBS-treated groups, while its protein level was downregulated remarkably at 15 ppt. Additionally, the *SOCS2* expression at the mRNA level in liver was not altered at 0 ppt under LPS challenge, while its protein expression was significantly decreased. These results indicated that the relation between mRNA and protein was not strictly linear, and the amounts of the two molecules were mainly determined by translation and protein degradation (De et al. 2009).

Conclusions

In the present study, we investigated the individual and combined effects of salinity and LPS on the immune response of juvenile *T. fasciatus*. Based on our observation of several immune-related parameters and genes in

blood and tissues, we speculated that LPS challenge induced considerable damage to *T. fasciatus*, whereas an increase in salinity mitigated such harmful effects. Moreover, the increased immune response in liver in saltwater was vital for *T. fasciatus* to live well in a wide salinity variation during migration. Collectively, we strongly supported that when encountering with LPS-induced damage, the addition of salts (or salt water) was a sensible strategy to improve the fitness of *T. fasciatus*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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