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Short communication

Expression of immune genes in Indian major carp, *Catla catla* challenged with *Flavobacterium columnare*

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ABSTRACT

Columnaris disease, caused by *Flavobacterium columnare*, is one of the important bacterial diseases responsible for large-scale mortalities in numerous freshwater fishes globally. This disease can cause up to 100% mortality within 24 h of infection and is considered to be a cause of concern for aquaculture industry. Despite being a serious disease, scarce information is available regarding host-pathogen interaction, particularly the modulation of different immune genes in response to *F. columnare* infection. Therefore, in the present study, an attempt has been made to study expression of important immune regulatory genes, namely *IL-1β*, *iNOS*, *INF-γ*, *IL-10*, *TGF-β*, *C3*, *MHC-I* and *MHC-II* in gills and kidney of *Catla catla* following experimental infection with *F. columnare*. The expression analysis of immune genes revealed that transcript levels of *IL-1β*, *iNOS*, *IL-10*, *TGF-β*, C3 and *MHC-I* were significantly up-regulated ($p < 0.05$) in both the organs of the infected catla. *IFN-γ* and *MHC-II* were up-regulated in gills of infected catla whereas, both the genes showed down-regulation in kidney. The results indicate that important immune genes of *C. catla* are modulated following infection with *F. columnare*. The knowledge thus generated will strengthen the understanding of molecular pathogenesis of *F. columnare* in Indian major carp *C. catla*.

1. Introduction

Immune mechanisms of teleosts encompass both non-specific and specific immune responses, including humoral and cellular components to protect against infectious diseases [1]. Innate immunity is considered as the first line of defence against invading pathogens, which depends on robust and coordinated gene-regulatory cascades [2–4]. These cascades get activated upon recognition of microbes or microbial-associated molecular pattern by pattern-recognition receptors of host [5], which leads to activation of key transcription factors such as cytokines and other key regulatory factors that orchestrate the inflammatory and regulatory responses. These immune regulatory cascades help in eliminating pathogens and subsequently development of adaptive immunity against the some specific pathogen [6]. Hence, understanding the mechanisms involved in immune response against invading pathogens is essential for developing effective prophylactic measures.

Columnaris disease, caused by *Flavobacterium columnare*, is one of the most frequently encountered and devastating bacterial disease in freshwater fish, affecting numerous wild and cultured fish species [7,8]. To date, the disease has been reported from more than 60 fish species from Asia, Europe, North and South America, Africa and Australia, and the host range of *F. columnare* is expanding [7,9,10]. The disease can result in acute mortalities up to 100% within a short span of time and is considered to be one of the major limiting factors in aquaculture [7]. Importantly, *Catla catla*, the fastest growing Indian major carp cultured in Indian subcontinent [11], is highly susceptible to this disease [12].

F. columnare mainly affects gill and skin tissues [10] and also has the capability to enter into the bloodstream and internal organs. To date, most of the studies on this pathogen have been carried out on the mode and dynamics of adhesion [13], virulence of different genomovars [14,15] and adaptive immunity of the host [16,17]. However, information regarding the mechanisms through which *F. columnare* modulates the immune response of the host to its advantage, is scarce. Considering the same, the present study has been carried out to study the expression pattern of important immune regulatory genes, namely interleukin-1β (*IL-1β*), inducible nitric oxide synthase (*iNOS*), interferon γ (*INF-γ*), interleukin-10 (*IL-10*), transforming growth factor β (*TGF-β*), complement component 3 (*C3*), major histocompatibility

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complex I and II (MHC-I and MHC-II) genes in *C. catla* following infection with *F. columnare*. The studies on spatio-temporal expression of immune genes against *F. columnare* will reveal mechanisms of host-pathogen interaction which would be useful for developing future control strategies against this important pathogen.

2. Materials and methods

2.1. Experimental animal

Healthy *C. catla* (53.4 ± 2.41 g and 15.6 ± 1.1 cm) juveniles were procured from Live Fish Germplasm Resource Centre of ICAR-National Bureau of Fish Genetic Resources, Lucknow, and held in fibre reinforced plastic (FRP) tanks (1000 L) in wet laboratory with static water system and constant aeration. Fish were fed twice daily with commercial diet (ABIS Exports India Pvt. Ltd., Chhattisgarh, India) @ 2% body weight and about one-third of water in the tanks was exchanged daily. The fish were acclimatized in laboratory for 2 weeks before conducting the experiment. During the experiment, temperature, dissolved oxygen, pH, ammonia and nitrite concentration were 24.5 ± 1.4 °C, 6.8 ± 0.78 mg/L, 8.2 ± 0.42, < 0.5 mg/L and < 0.2 mg/L, respectively. Prior to the experiment, five fish were sampled randomly for examination to ensure that they were free from parasitic infestation and *F. columnare* infection.

2.2. Bacteria and experimental infection

F. columnare isolate (BE-1), used in the present study, was previously isolated and the experimental infection was carried out as described earlier [18]. Briefly, thirty fish in the infected group were challenged by bath immersion for 60 min in 20 L of water containing lethal dose 50 (LD_{50}) of *F. columnare* (2.4×10^7 cfu/mL), calculated previously following Reed and Muench [19]. Further, 30 fish in the control group were challenged similarly by immersion in 20 L of water containing sterile cytophaga broth equal to the amount of bacterial culture used above. For studying immune gene expression, three fish from each group were randomly sampled at 3, 6, 12, 24, 48, 72 and 96 h post infection (hpi). Prior to collection of tissues, fish were euthanised with 200 mg/L of MS222 (Sigma-Aldrich, St. Louis, MO, USA), and gill and kidney tissues were taken aseptically in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) and stored at 80 °C. For histopathological examination, gills from the control and infected groups were fixed in 10% neutral buffered formalin at each sampling interval. The animal care and experimental challenge were approved by the Institutional Animal Ethics Committee of ICAR-NBFGR, Lucknow.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted separately from gill and kidney of three individual biological replicates from infected as well as control group at each time point. Total RNA was extracted using Trizol® Reagent (Invitrogen, USA) as per the manufacturer's protocol. The extracted RNA was treated with RNase-free DNase I (Thermo Scientific, USA) to remove residual genomic DNA. The quantity and purity of RNA was measured with Denovix DS-11 spectrophotometer (Denovix Inc., USA) and the integrity and purity of RNA were further evaluated by electrophoresis on agarose gel. One microgram of DNase-treated RNA was reverse-transcribed using RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, USA), primed with random hexamer primers following the manufacturer's instructions. The cDNA was diluted ten times in RNase-free water and stored at −20 °C for further use in quantitative RT-PCR.

2.4. Quantitative reverse transcription PCR

qRT-PCR was carried out using StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA). Briefly, 20 µL reaction mixture consisted of 10 µL 2x SYBR® Green PCR Master mix (Applied Biosystems, USA), 0.5 µL of each gene-specific primer (10 µM) and 5 µL of diluted cDNA as template. The primers for qRT-PCR were either self-designed using Primer3 software from sequence available in NCBI or sourced from published literature (Table 1). The qRT-PCR cycling conditions consisted of an initial denaturation at 95 °C (for 10 min), followed by 40 cycles of denaturation at 95 °C (30 s), annealing at 55 °C (50s) and extension at 72 °C (40s). To check qRT-PCR specificity, melt-curve analysis was performed following qRT-PCR amplification. Elongation factor-1α (EF-1α) was used as the reference gene for data normalization. For each sample, qRT-PCR was run in duplicate (technical replicates) along with a non template control (NTC). To analyse the fold-change in expression of selected immune genes, the $2^{-\Delta\Delta Ct}$ method was followed [20]. The data thus generated from three biological replicates were analysed with one-way analysis of variance (ANOVA) and Duncan test using SPSS program (SPSS, version 16, USA). Data were considered statistically significant at $p \leq 0.05$.

Table 1
Oligonucleotide primers used for amplification of catla cDNA target genes for qRT-PCR studies.

Primer	Primer sequence	Size (bp)	Accession number
EF-1F	CAATTTCTGGATGGCACGGTGAC	128	JX480501
EF-1R	GGCATCCAGGGCATCAAGAAGAG		
IL-1β F1	ACCCGCTTAACACACCAGCGACTT	100	AM932525
IL-1β R1	AACATCGGCCAACCTGTGTGCCT		
iNOS-F1	TGGCAGAATTCAGTGGGCTA	207	AY904363
iNOS-R1	TAGCCAGCGTATCGTACCAG		
INFγ-F1	CACGAGCTACACAATGCACA	248	KF590042
INFγ-R1	GCATACGTCTTGAGCTCTGC		
IL10-1F1	CAGGATCTCAAGCGGGATATG	133	HQ221996
IL10-1R1	CAAGCTCTCCCATGGCTTTA		
TGF-β F1	GTACACTACGGCGGAGGATTG	75	EU086521
TGF-β R1	CGCTTCGATTCGCTTTCTCT		
C3-HF	TTGGCTGGACTGTGAAACCA	130	XM026236332
C3-HR	AGGTGTATGATCCCACTTCCC		
MHC-I βF2	GCAGTATGGAAAGAGCAGCC	171	KJ703112
MHC-I βR2	TCACCAACCTCCACATCCTC		
MHC-II F2	TCTGACGTTGGCCCTTGAG	103	Self designed
MHC-II R2	GATGGCACCTTCAACATCTTCTC		

3. Results and discussion

The gills are recognised as one of the key immune-competent organs and act as the physical and immunological barrier against the invading pathogens. Therefore, understanding the immune response exhibited by gills following infection is important to unravel the mechanisms involved in host-pathogen interaction [21,22]. Furthermore, the kidney also plays an important role in innate as well as adaptive immunity, specifically in antigen processing and phagocytosis see Ref. [23]. Hence, in the current study, gene expression pattern of immune regulatory genes in gills and kidney of *C. catla* following *F. columnare* infection was studied to understand the host-pathogen interactions.

3.1. Clinical signs, gross and histopathological alterations

The experimentally infected fish started exhibiting clinical signs, namely lethargy and mild respiratory distress by 12 hpi. As the infection progressed, the severity of clinical signs increased, gills had large areas of white-yellowish discoloration and the fish were observed to be swimming near the water surface with accelerated opercular movements by 24 hpi (Fig. 1A and B). The severity of lesions was found to decrease from 48 hpi and by 96 hpi, the lesions appeared to have healed. During the experimental period, in the infected group, a total of 5 fish died. Out of these, two fish died by 24 hpi, two fish died during 24–48 hpi, and one fish died during 48–72 hpi. However, no clinical

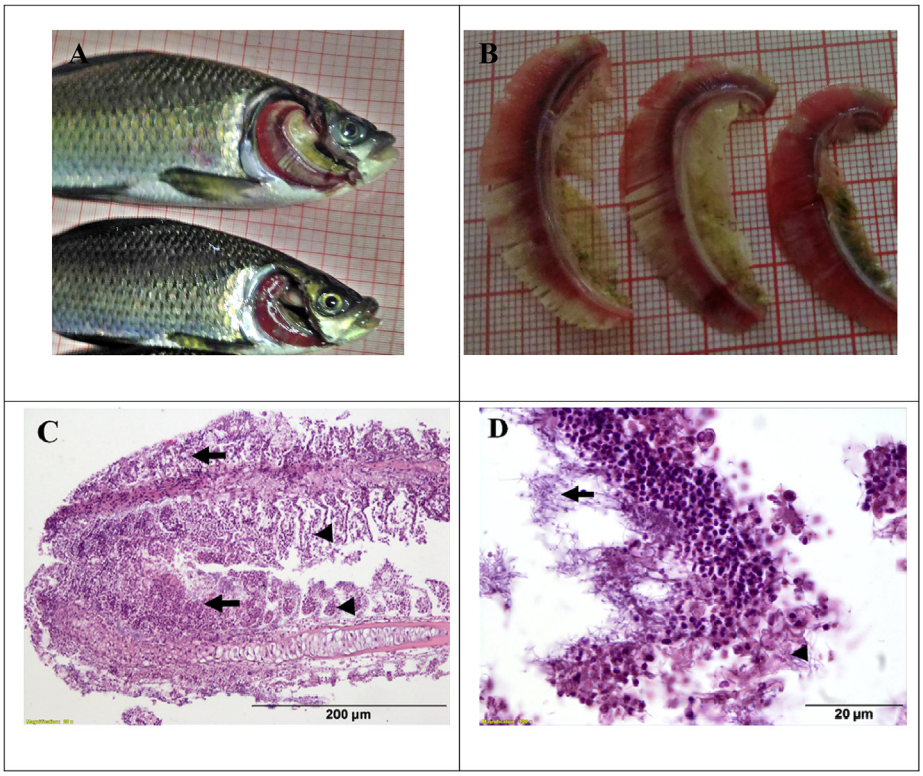

Fig. 1. Gross lesions and histopathology of gills of *Catla catla* infected with *Flavobacterium columnare*; large areas of white-yellowish discoloration in affected gills at 24 hpi (A–B). Gills showing fusion of secondary filaments, loss of architecture and inflammatory exudates (C), large clumps of bacterial cells (arrow) embedded in the desquamated mass of epithelial cells (arrowhead) at 24 hpi (D).

signs and mortality were observed in fish from control group. The histopathological alterations were found to be most severe from 24 to 48 hpi and included bacterial masses in gills, fusion of secondary filaments and sloughing of epithelial cells with a consequential disruption of gill architecture (Fig. 1C and D). The clinical signs, gross and histopathological lesions in gills of catla are in conformity with previous reports of *F. columnare* infection in *C. catla* [12,18], *Cyprinus carpio* [24], *Ictalurus punctatus* and *Danio rerio* [13].

3.2. Expression pattern of immune genes

In the current study, *IL-1β* mRNA expression level was found to be highly up-regulated ($p < 0.05$) in both the gills and kidney as early as 3 hpi by 7-fold and 139-fold (peak up-regulation), respectively. The expression level in kidney declined at subsequent time points and reached the normal level by 24 hpi. However, in the gills, the highest expression was recorded at 6 hpi (32-fold), which decreased to normal at the next time point i.e. 12 hpi (Fig. 2A and B). These results are in conformity with the previous reports of *Siniperca chuatsi* infected with *F. columnare* [25], *D. rerio* challenged with *Francisella* sp. [26] and *Monopterus albus* challenged with *Aeromonas hydrophila* [27]. *IL-1β* belongs to the pro-inflammatory group of cytokines, which induces a cascade of reactions leading to inflammation enabling the host to respond promptly to the infection [28]. Higher expression of *IL-1β* in the early stages of infection is crucial in inducing inflammatory response, whereas at later stages, its rapid restoration to the basal level might be due to regulatory and inhibitory mechanism of anti-inflammatory cytokines including *IL-10* and *TGF-β*, as excessive expression of *IL-1β* is directly associated with the damage to the host tissue [29,30].

The inducible nitric oxide synthase (iNOS) synthesizes nitric oxide (NO) from L-arginine, which plays an essential role in both innate and acquired immunity. NO acts as a potent antimicrobial and cytotoxic agent [31,32]. In the present study, the expression of *iNOS* was significantly up-regulated ($p < 0.05$) from 3 to 12 hpi in gills with maximum expression of 38-fold at 12 hpi in infected group. Thereafter, the expression level of the *iNOS* was found to decrease, and was comparable to the control group from 48 hpi onwards (Fig. 2C). In kidney, expression of *iNOS* gene was significantly up-regulated ($p < 0.05$) only at 24 hpi in infected group. At rest of the time points, no significant difference was observed between infected and control group (Fig. 2D). The results in the present study are corroborated with the previous reports in *I. punctatus* infected with *F. columnare* [33,34] and *Oncorhynchus mykiss* infected with *Renibacterium salmoninarum* [35] and *Neoparamoeba* sp. [36]. Up-regulation of *iNOS* in gills at early stages of infection might be due to the acute inflammatory response against invading pathogens [35]. As observed in the present study, higher expression of *iNOS* in kidney tissue has been reported in *Labeo rohita* and *I. punctatus* infected with *Edwardsiella tarda* [33,37]. Higher expression of *IL-1β* as observed in the present study might have been responsible for increased *iNOS* expression through activation of transcription factors such as NF-kB, and increased NO production has been linked with tissue damage [38]. The extensive necrosis in gills with loss of architecture as observed in the present study could be partly due to increased expression of *iNOS*.

The INF-γ is a pleiotropic and pro-inflammatory cytokine, which plays an important role in both innate and adaptive immune responses against microbial pathogens [39]. In this study, the mRNA expression level of *INF-γ* in gills of *F. columnare* challenged *C. catla* was altered significantly only at 96 hpi, with up-regulation ($p < 0.05$) of 5-fold (Fig. 2E). IFN-γ has previously been reported to be induced by bacteria as well as bacterial products and plays a protective role in the host response to several bacterial infections [40–42]. In addition, INF-γ is reported to enhance phagocytosis and stimulate the production of inflammatory cytokines, chemokines and other microbicidal molecules [43]. Hence, from the observations of this study and those reported earlier, it can be inferred that expression of *INF-γ* in gills might be playing a major role in defense against columnaris disease in *C. catla*. However, in kidney, *INF-γ* transcript level was found to be significantly down-regulated ($p < 0.05$) throughout the experiment, except at 3 hpi (Fig. 2F). Previously, down-regulated expression of *INF-γ* has been reported in kidney and spleen of *O. mykiss* following challenge with *Lactococcus garvieae* [44]. Similar observations have been reported by

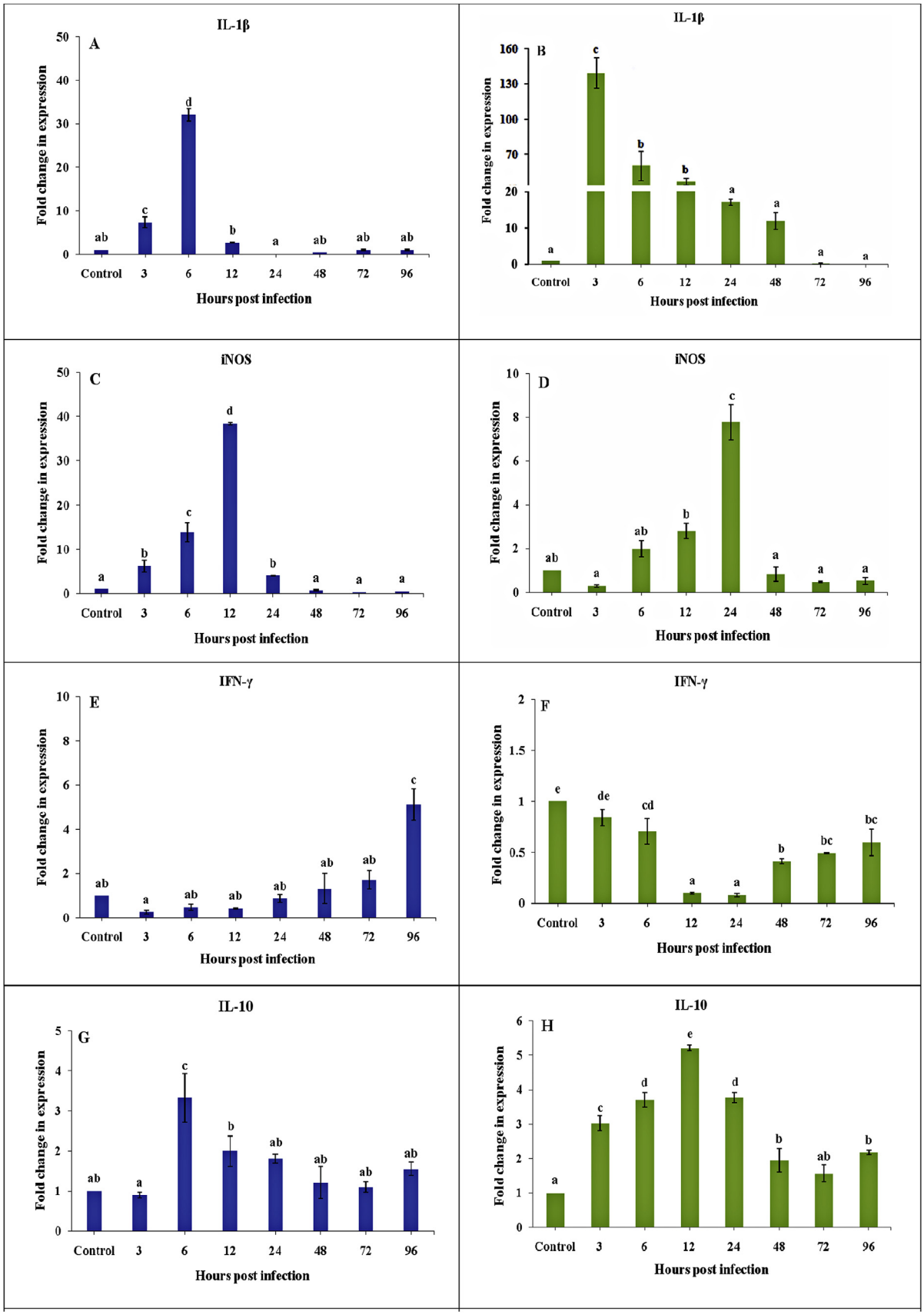

Fig. 2. Expression of immune genes in the gills and kidney of *Catla catla* at different time-points following challenge with *Flavobacterium columnare* (A–B-*IL-1β*, C–D-*iNOS*, E–F-*IFNγ*, G–H-*IL-10*, I–J-*TGF-β*, K–L-*C3*, M–N-*MHC-I* and O–P-*MHC-II*). Left side bar graphs indicates gills and right side bar graphs indicate kidney. Significant differences ($p < 0.05$) between control and challenge group are denoted by different letters. The bar depicts mean expression level obtained from three biological replicates at the specific time-point.

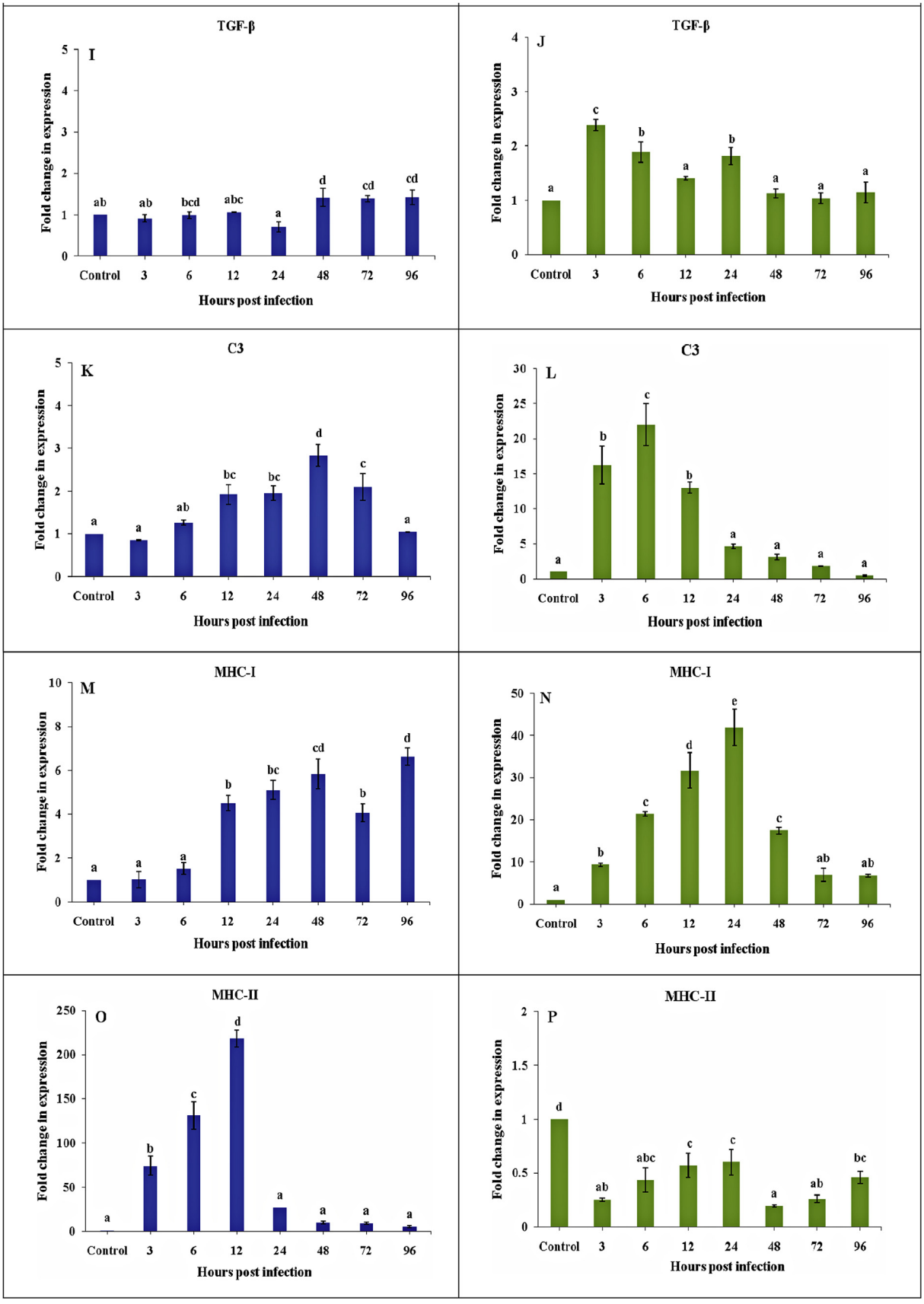

Fig. 2. (*continued*)

Chen et al. (2015) in *Larimichthys crocea* infected with *Cryptocaryon irritans* [45].

IL-10 is an anti-inflammatory as well as regulatory cytokine which limits excessive immune responses, thereby, preventing the tissue damage [46]. In the gills of *F. columnare* challenged *C. catla*, the mRNA expression level of *IL-10* was significantly up-regulated ($p < 0.05$) (3.32-fold) only at 6 hpi (Fig. 2G). In kidney, *IL-10* expression was significantly up-regulated ($p < 0.05$) from 3 to 48 hpi and 96 hpi with the maximum expression of 5-fold at 12 hpi (Fig. 2H). Expression level of *IL-10*, in the present study, is in accordance with earlier reports in gills and kidney of *C. catla* following *A. hydrophila* infection and LPS stimulation [46], kidney of *Cyprinus carpio* after injection with LPS [47] and *Gadus morhua* following infection with *Vibrio anguillarum* [48]. Up-regulation of *IL-10* in response to increased expression of *IL-1β* could be a host strategy to prevent excessive inflammatory response and thereby, limiting the tissue damage.

TGF-β is a pleiotropic cytokine that controls the initiation and resolution of inflammatory responses and also plays an important role in the immune system by regulating the proliferation, differentiation, and survival of lymphocyte population [49]. In the present study, the mRNA expression level of *TGF-β* in gills was significantly up-regulated ($p < 0.05$) following infection with *F. columnare* at later stages i.e. from 48 hpi onwards (Fig. 2I). However, in kidney, *TGF-β* expression was found to be significantly up-regulated from 3 to 24 hpi (except 12 hpi), with the maximum expression of 2.4-fold at 3 hpi (Fig. 2J). These results are in close agreement with the previous report in gills and spleen of *Pylodictis olivaris* following challenge with *F. columnare* and *E. ictaluri* [50]. Up-regulated expression of *TGF-β* in kidney, as observed in the present study, is in accordance with increased expression levels of *TGF-β* in *Ctenopharyngodon idella* challenged with *A. hydrophila* [30]. Higher expression of *TGF-β* has been also reported in *O. mykiss* following infection with *Aeromonas salmonicida* [51]. *TGF-β* is reported to suppress immune responses by the inhibition of inflammatory response and promoting the function of Treg cells [52]. Therefore, taking into account the results obtained in the present study and those reported earlier, it can be inferred that induced expression of *TGF-β* would have helped in resolution of inflammation [53].

Complement system, a key component of immune system (innate and adaptive immunity), has the ability to opsonise the pathogens for engulfment by phagocytes and plays a central role in inflammatory process ,and damages bacteria by creating pores in their membrane [54]. From the observation of C3 gene expression in gills of *C. catla*, significant difference was not observed in *F. columnare*-challenged animals up to 6 hpi. However, a significant up-regulation ($p < 0.05$) was recorded at 12 to 72 hpi with maximum expression level of 2.83-fold at 48 hpi (Fig. 2K). In kidney, from 3 to 12 hpi, the *C3* transcript level was found to be significantly up-regulated with the maximum expression of 22-fold at 6 hpi in challenged animals. Afterwards, the expression level reduced to the level of control and remained at similar level till 96 hpi (Fig. 2L). Similar up-regulated pattern of *C3* mRNA expression has been reported in the gills of *Misgurnus anguillicaudatus* and *O. mykiss* following infection with *A. hydrophila* and *Ichthyophthirius multifiliis*, respectively [55,56]. In case of kidney, the results of the present study are in accordance with *E. tarda* challenged *L. rohita* [37] and *Streptococcus iniae* challenged *Oreochromis niloticus* [57]. Higher expression of *C3* has been also reported in head kidney of *Miichthys miiuy* and *Epinephelus lanceolatus* after challenge with *Vibrio anguillarum* and *V. alginolyticus*, respectively [58,59]. Based on the observations from the present study, it can be inferred that up-regulated expression of *C3* following *F. columnare* infection might have played an important role in containing the infection [60]. In accordance with the present findings, an increase in C3 activity following *F. columnare* infection has been reported previously [18].

The MHC genes (MHC-I and MHC-II) are recognised as signature molecules for disease resistance in animals by modulating innate as well as adaptive immunity through antigen presentation and interactions with T-cell subtypes and NK cells. In the gills of catla challenged with *F. columnare*, the mRNA expression level of *MHC-I* was unaltered till 6 hpi. Further, by 12 hpi, expression of *MHC-I* was up-regulated and reached the maximum value of 6-fold at 96 hpi (Fig. 2M). In kidney, transcript level of *MHC-I* was significantly up-regulated from 3 hpi up to 48 hpi with maximum expression of 41-fold at 24 hpi. Thereafter, from 72 to 96 hpi, the expression level of *MHC-I* was similar to that of control (Fig. 2N). The results of the present study were corroborated with the study in *I. punctatus* and *M. anguillicaudatus* infected with *F. columnare* [61,62], *M. miiuy* challenged with *V. anguillarum* [63] and *C. idella* challenged with *A. hydrophila* [64]. Modulation of *MHC-I* is an evident phenomenon observed upon various stimuli i.e. LPS, bacteria, virus infection and tumor invasion [65]. Up-regulated expression of *MHC-I* in *C. catla* challenged with *F. columnare*, might be correlated with the active antigen presentation and pathogen clearance of this bacterium as a part of a cell-mediated immune response of host.

In the present study, expression level of *MHC-II* was significantly up-regulated at early stages of infection up to 12 hpi in the gills of challenged fish compared to the control, with maximum expression of 218-fold at 12 hpi (Fig. 2O). However, in kidney, expression level of *MHC-II* was significantly down-regulated throughout the experiment and recorded lowest fold change in expression of 0.19 fold at 48 hpi in challenged fish compared to the control (Fig. 2P). The results recorded in the present study are in close agreement with the *MHC-II* expression level reported from gills of *I. punctatus* challenged with *F. columnare* [61]. In addition, *E. lanceolatus* larvae challenged with *V. alginolyticus* also exhibited higher expression of *MHC-II* mRNA [59]. From these observations, it can be inferred that enhanced expression of MHC-II might help in either activating various antigen presenting proteins or modulate expression of several molecules such as proteasome, ubiquitin and TAP responsible for antigen presentation [66]. The down-regulated expression profile of *MHC-II* in kidney of *F. columnare*-infected *C. catla* is similar to the down-regulated expression of *MHC-II* recorded by Matsuyama et al. (2007) in *Paralichthys olivaceus* infected with *E. tarda* [67]. Similarly, down-regulated expression of *MHC-II* was reported in the kidney, liver and spleen of *Scophthalmus maximus* [68] and spleen of *Cynoglossus semilaevis* [69] when challenged with *V. anguillarum*. Based on the observations from the present study and those reported earlier [68,69], it can be inferred that disruption of cellular function occurs in immune organs following exposure to pathogenic bacteria resulting in changes in expression pattern of immune genes.

In conclusion, the modulation of the studied immune genes was observed in gills and kidney of *C. catla* at different time intervals following infection with *F. columnare*. The up- and down-regulation of these genes would be playing an important role during response of the host against *F. columnare* infection. The results add to the improved understanding of immune responses of catla against this important bacterial pathogen.

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References

[1] I. Hirono, T. Aoki, Immune-related genes of Japanese flounder, *Paralichthys olivaceus*, in: N. Shimizu, T. Aoki, I. Hirono, F. Takashima (Eds.), Aquatic Genomics, Springer-Verlag, Tokyo, Japan, 2003, pp. 286–300.
[2] Q. Huang, D. Liu, P. Majewski, L.C. Schulte, J.M. Korn, R.A. Young, E.S. Lander, N. Hacohen, The plasticity of dendritic cell responses to pathogens and their components, Science 294 (2001) 870–875.
[3] S.T. Smale, Selective transcription in response to an inflammatory stimulus, Cell

140 (2010) 833–844.
[4] R. Medzhitov, C.A. Janeway Jr., Innate immune recognition and control of adaptive immune responses, Seminars in Immunology, vol. 10, Academic Press, 1998, pp. 351–353.
[5] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol. 11 (2010) 373–384.
[6] K. Murphy, P. Travers, M. Walport, Janeway's Immunobiology, Garland Science, New York, 2008.
[7] A.M. Declercq, F. Haesebrouck, d.B.W. Van, P. Bossier, A. Decostere, Columnaris disease in fish: a review with emphasis on bacterium-host interactions, Vet. Res. 44 (2013) 27–43.
[8] H.T. Dong, S. Senapin, B. LaFrentz, C. Rodkhum, Virulence assay of rhizoid and non-rhizoid morphotypes of *Flavobacterium columnare* in red tilapia, *Oreochromis sp.*, fry, J. Fish Dis. 39 (2016) 649–655.
[9] T.P. Loch, M. Faisal, Emerging flavobacterial infections in fish: a review, J. Adv. Res. 6 (2015) 283–300.
[10] J. Plumb, L. Hanson, Health Maintenance and Principal Microbial Diseases of Cultured Fishes, fourth ed., Wiley-Blackwell, Oxford, UK, 2011.
[11] FAO, Cultured aquatic species information programme *Catla catla* (hamilton, 1822), http://www.fao.org/fishery/culturedspecies/Catla_catla/en, (2017) , Accessed date: 14 April 2018.
[12] D.K. Verma, G. Rathore, Molecular characterization of *Flavobacterium columnare* isolated from a natural outbreak of columnaris disease in farmed fish, *Catla catla* from India, J. Gen. Appl. Microbiol. 59 (2013) 417–424.
[13] O. Olivares-Fuster, S.A. Bullard, A. McElwain, M.J. Llosa, C.A. Arias, Adhesion dynamics of *Flavobacterium columnare* to channel catfish *Ictalurus punctatus* and zebrafish *Danio rerio* after immersion challenge, Dis. Aquat. Org. 96 (2011) 221–227.
[14] S. Thomas-Jinu, A. Goodwin, Morphological and genetic characteristics of *Flavobacterium columnare* isolates: correlations with virulence in fish, J. Fish Dis. 27 (2004) 29–35.
[15] A.M. Darwish, A.A. Ismaiel, Genetic diversity of *Flavobacterium columnare* examined by restriction fragment length polymorphism and sequencing of the 16S ribosomal RNA gene and the 16S–23S rDNA spacer, Mol. Cell. Probes 19 (2005) 267–274.
[16] L.D. Grabowski, S.E. LaPatra, K.D. Cain, Systemic and mucosal antibody response in tilapia, *Oreochromis niloticus* (L.), following immunization with *Flavobacterium columnare*, J. Fish Dis. 27 (2004) 573–581.
[17] C.A. Shoemaker, P.H. Klesius, J.D. Drennan, J. Evans, Efficacy of a modified live *Flavobacterium columnare* vaccine in fish, Fish Shellfish Immunol. 30 (2011) 304–308.
[18] Ravindra, P.K. Pradhan, V. Pande, M.K. Yadav, D.K. Verma, N. Sood, Modulation of the innate immune responses in Indian major carp, *Catla catla* following experimental infection with *Flavobacterium columnare*, Aquaculture 510 (2019) 22–31.
[19] L.J. Reed, H. Muench, A simple method of estimating fifty percent endpoints, Am. J. Epidemiol. 27 (1938) 493–497.
[20] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.
[21] E.O. Koppang, A. Kvellestad, U. Fischer, Fish Mucosal Immunity: Gill, Mucosal Health in Aquaculture, 2015, pp. 93–133.
[22] J. Xu, L. Feng, W.D. Jiang, P. Wu, Y. Liu, J. Jiang, S.Y. Kuang, L. Tang, W.N. Tang, Y.A. Zhang, X.Q. Zhou, Effects of dietary protein levels on the disease resistance, immune function and physical barrier function in the gill of grass carp (*Ctenopharyngodon idella*) after challenged with *Flavobacterium columnare*, Fish Shellfish Immunol. 57 (2016) 1–16.
[23] C. Kum, S. Sekkin, The Immune System Drugs in Fish: Immune Function, Immunoassay, Drugs, Intech Open Access Publisher, 2011.
[24] A.M. Declercq, K. Chiers, F. Haesebrouck, W. Van Den Broeck, J. Dewulf, M. Cornelissen, A. Decostere, Gill infection model for columnaris disease in common carp and rainbow trout, J. Aquat. Anim. Health 27 (2015) 1–11.
[25] W. Zhou, Y. Zhang, Y. Wen, W. Ji, Y. Zhou, Y. Ji, X. Liu, W. Wang, M. Asim, X. Liang, T. Ai, Analysis of the transcriptomic profilings of Mandarin fish (*Siniperca chuatsi*) infected with *Flavobacterium columnare* with an emphasis on immune responses, Fish Shellfish Immunol. 43 (2015) 111–119.
[26] L.N. Vojtech, G.E. Sanders, C. Conway, V. Ostland, J.D. Hansen, Host immune response and acute disease in a zebrafish model of *Francisella* pathogenesis, Infect. Immun. 77 (2009) 914–925.
[27] Q.Q. Xu, P. Xu, J.W. Zhou, T.S. Pan, R. Tuo, K. Ai, D.Q. Yang, Cloning and expression analysis of two pro-inflammatory cytokines, IL-1β and its receptor, IL-1R2, in the Asian swamp eel *Monopterus albus*, Mol. Biol. 50 (2016) 671–683.
[28] S. Bird, J. Zou, T. Wang, B. Munday, C. Cunningham, C.J. Secombes, Evolution of interleukin-1beta, Cytokine Growth Factor Rev. 13 (2002) 483–502.
[29] J.E. Sims, D.E. Smith, The IL-1 family: regulators of immunity, Nat. Rev. Immunol. 10 (2010) 89–102.
[30] X. Wang, X. Yang, C. Wen, Y. Gao, L. Qin, S. Zhang, A. Zhang, K. Yang, H. Zhou, Grass carp TGF-β1 impairs IL-1β signaling in the inflammatory responses: evidence for the potential of TGF-β1 to antagonize inflammation in fish, Dev. Comp. Immunol. 59 (2016) 121–127.
[31] C. Bogdan, Nitric oxide and the immune response, Nat. Immunol. 2 (2001) 907–916.
[32] F. Aktan, iNOS-mediated nitric oxide production and its regulation, Life Sci. 75 (2004) 639–653.
[33] J. Yao, C. Li, J. Zhang, S. Liu, J. Feng, R. Wang, Y. Li, C. Jiang, L. Song, A. Chen, Z. Liu, Expression of nitric oxide synthase (NOS) genes in channel catfish is highly regulated and time dependent after bacterial challenges, Dev. Comp. Immunol. 45 (2014) 74–86.
[34] Y. Ren, H. Zhao, B. Su, E. Peatman, C. Li, Expression profiling analysis of immune-related genes in channel catfish (*Ictalurus punctatus*) skin mucus following *Flavobacterium columnare* challenge, Fish Shellfish Immunol. 2 (2015) 537–542.
[35] J.J. Campos-Perez, A.E. Ellis, C.J. Secombes, Toxicity of nitric oxide and per-oxynitrite to bacterial pathogens of fish, Dis. Aquat. Org. 43 (2000) 109–115.
[36] A.R. Bridle, R.N. Morrison, B.F. Nowak, The expression of immune-regulatory genes in rainbow trout, *Oncorhynchus mykiss*, during amoebic gill disease (AGD), Fish Shellfish Immunol. 20 (2006) 346–364.
[37] B.R. Mohanty, P.K. Sahoo, Immune responses and expression profiles of some immune-related genes in Indian major carp, *Labeo rohita* to *Edwardsiella tarda* infection, Fish Shellfish Immunol. 28 (2010) 613–621.
[38] B. Bao, E. Peatman, P. Xu, P. Li, H. Zeng, C. He, Z. Liu, The catfish liver-expressed antimicrobial peptide 2 (LEAP-2) gene is expressed in a wide range of tissues and developmentally regulated, Mol. Immunol. 43 (2006) 367–377.
[39] R. Shtrichman, C.E. Samuel, The role of gamma interferon in antimicrobial immunity, Curr. Opin. Microbiol. 4 (2001) 251–259.
[40] C.W. Czarniecki, G. Sonnenfeld, Interferon-gamma and resistance to bacterial infections, APMIS 101 (1993) 1–17.
[41] L. Grayfer, M. Belosevic, Molecular characterization, expression and functional analysis of goldfish (*Carassius aurutus* L.) interferon gamma, Dev. Comp. Immunol. 33 (2009) 235–246.
[42] H. Beekhuizen, J.S. van de Gevel, Gamma interferon confers resistance to infection with *Staphylococcus aureus* in human vascular endothelial cells by cooperative proinflammatory and enhanced intrinsic antibacterial activities, Infect. Immun. 75 (2007) 5615–5626.
[43] F. Sun, E. Peatman, C. Li, S. Liu, Y. Jiang, Z. Zhou, Z. Liu, Transcriptomic signatures of attachment, NF-kB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection, Dev. Comp. Immunol. 38 (2012) 169–180.
[44] R. Castro, J. Coll, M. del Mar Blanco, A. Rodriguez-Bertos, L. Jouneau, J.F. Fernández-Garayzábal, A. Gibello, Spleen and head kidney differential gene expression patterns in trout infected with *Lactococcus garvieae* correlate with spleen granulomas, Vet. Res. 50 (2019) 32–45.
[45] R.N. Chen, Y.Q. Su, J. Wang, M. Liu, Y. Qiao, Y. Mao, Q.Z. Ke, K.H. Han, W.Q. Zheng, J.S. Zhang, C.W. Wu, Molecular characterization and expression analysis of interferon-gamma in the large yellow croaker *Larimichthys crocea*, Fish Shellfish Immunol. 46 (2015) 596–602.
[46] B. Swain, M. Samanta, M. Basu, P. Panda, B.R. Sahoo, N.K. Maiti, Molecular characterization, inductive expression and mechanism of interleukin-10 gene induction in the Indian major carp (*Catla catla*), Aquacult. Res. 43 (2012) 897–907.
[47] R. Savan, D. Igawa, M. Sakai, Cloning, characterization and expression analysis of interleukin-10 from the common carp, *Cyprinus carpio* L, Eur. J. Biochem. 270 (2003) 4647–4654.
[48] M. Seppola, A.N. Larsen, K. Steiro, B. Robertsen, I. Jensen, Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.), Mol. Immunol. 45 (2008) 887–897.
[49] G.C. Blobe, W.P. Schiemann, H.F. Lodish, Role of transforming growth factor beta in human disease, N. Engl. J. Med. 342 (18) (2000) 1350–1358.
[50] Y. Wang, S. Wang, H. Luo, Y. Lv, Transforming growth factor β1 gene play a novel role in innate immune response in *Pelteobagrus fulvidraco*, Turk, J. Fish. Aquat. Sci. 18 (2018) 539–546.
[51] I.E. Mulder, S. Wadsworth, C.J. Secombes, Cytokine expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) during infection with *Aeromonas salmonicida*, Fish Shellfish Immunol. 23 (2007) 747–759.
[52] T. Maehr, M.M. Costa, J.L.G. Vecino, S. Wadsworth, S.A. Martin, T. Wang, C.J. Secombes, Transforming growth factor-β1b: a second TGF-β1 paralogue in the rainbow trout (*Oncorhynchus mykiss*) that has a lower constitutive expression but is more responsive to immune stimulation, Fish Shellfish Immunol. 34 (2013) 420–432.
[53] H.C. Ingerslev, C.G. Ossum, T. Lindenstrøm, M.E. Nielsen, Fibroblasts express immune relevant genes and are important sentinel cells during tissue damage in rainbow trout (*Oncorhynchus mykiss*), PLoS One 5 (2010) 9304–9313.
[54] Jr, C.A. Janeway, P. Travers, M. Walport, M.J. Shlomchik, The complement system and innate immunity, Immunobiology: the Immune System in Health and Disease, fifth ed., Garland Science, New York, 2001.
[55] Y. Xu, Y. Yu, X. Zhang, Z. Huang, H. Li, S. Dong, Y. Liu, F. Dong, Z. Xu, Molecular characterization and expression analysis of complement component 3 in dojo loach (*Misgurnus anguillicaudatus*), Fish Shellfish Immunol. 72 (2018) 484–493.
[56] M.M. Olsen, P.W. Kania, R.D. Heinecke, K. Skjoedt, K.J. Rasmussen, K. Buchmann, Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: molecular and immunohistochemical studies, Fish Shellfish Immunol. 30 (2011) 859–869.
[57] J. Qiang, F. Tao, J. He, L. Sun, P. Xu, W. Bao, Effects of exposure to *Streptococcus iniae* on microRNA expression in the head kidney of genetically improved farmed tilapia (*Oreochromis niloticus*), BMC Genomics 18 (2017) 190–200.
[58] Y. Sun, R. Wang, T. Xu, Conserved structural complement component C3 in miiuy croaker *Miichthys miiuy* and their involvement in pathogenic bacteria induced immunity, Fish Shellfish Immunol. 35 (2013) 184–187.
[59] Y.D. Wang, S.J. Huang, H.N. Chou, W.L. Liao, H.Y. Gong, J.Y. Chen, Transcriptome analysis of the effect of *Vibrio alginolyticus* infection on the innate immunity-related TLR5-mediated induction of cytokines in *Epinephelus lanceolatus*, Fish Shellfish Immunol. 52 (2016) 31–43.
[60] A. Sahu, J.D. Lambris, Structure and biology of complement protein C3, a connecting link between innate and acquired immunity, Immunol. Rev. 180 (2001) 35–48.
[61] E. Peatman, C. Li, B.C. Peterson, D.L. Straus, B.D. Farmer, B.H. Beck, Basal polarization of the mucosal compartment in *Flavobacterium columnare* susceptible and resistant channel catfish (*Ictalurus punctatus*), Mol. Immunol. 56 (2013) 317–327.

[62] Z. Huang, Y. Yu, S. Dong, Y. Yin, Q. Wang, W. Yu, Y. Luo, J. Xu, L. Zhang, Y. Yu, T. Ai, Major histocompatibility complex class IIA and IIB genes of loach (*Misgurnus anguillicaudatus*): molecular cloning and expression analysis in response to bacterial and parasitic challenge, Aquaculture 500 (2019) 359–369.

[63] T.J. Xu, Y.N. Sun, Y.Z. Cheng, G. Shi, R.X. Wang, Genomic sequences comparison and differential expression of miiuy croaker MHC class I gene, after infection by *Vibrio anguillarum*, Dev. Comp. Immunol. 35 (2011) 483–489.

[64] Y. Dang, X. Xu, Y. Shen, M. Hu, M. Zhang, L. Li, L. Lv, J. Li, Transcriptome analysis of the innate immunity-related complement system in spleen tissue of *Ctenopharyngodon idella* infected with *Aeromonas hydrophila*, PLoS One 11 (2016) 0157413.

[65] A. Cuesta, J. Meseguer, M. Ángeles Esteban, Cloning and regulation of the major histocompatibility class I alpha gene in the teleost fish gilthead seabream, Fish Shellfish Immunol. 22 (2007) 718–726.

[66] S.A.M. Martin, S.C. Blaney, D.F. Houlihan, C.J. Secombes, Transcriptome response following administration of a live bacterial vaccine in Atlantic salmon (*Salmo salar*), Mol. Immunol. 43 (2006) 1900–1911.

[67] T. Matsuyama, A. Fujiwara, C. Nakayasu, T. Kamaishi, N. Oseko, I. Hirono, Gene expression of leucocytes in vaccinated Japanese flounder (*Paralichthys olivaceus*) during the course of experimental infection with *Edwardsiella tarda*, Fish Shellfish Immunol. 22 (2007) 598–607.

[68] Y.X. Zhang, S.L. Chen, Molecular identification, polymorphism, and expression analysis of major histocompatibility complex class IIA and B genes of turbot (*Scophthalmus maximus*), Mar. Biotechnol. 8 (2006) 611–623.

[69] T.J. Xu, S.L. Chen, X.S. Ji, Z.X. Sha, Molecular cloning, genomic structure, polymorphism and expression analysis of major histocompatibility complex class IIA and IIB genes of half-smooth tongue sole (*Cynoglossus semilaevis*), Fish Shellfish Immunol. 27 (2009) 192–201.