Fish and Shellfish Immunology 84 (2019) 865–875

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular characterization and expression analysis of complement component C3 in southern catfish (*Silurus meridionalis*) and a whole mount *in situ* hybridization study on its ontogeny

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ARTICLE INFO

Keywords:
Silurus meridionalis
Complement component C3
Expression analysis
Whole mount *in situ* hybridization
Ontogeny
Aeromonas hydrophila

ABSTRACT

The complement system plays an important role in protecting fish against attack by pathogens early in life. Complement component C3 is a central component in the complement system. The present work aimed to clone the full length C3 cDNA sequence of southern catfish (*Silurus meridionalis*), detect the tissue expression patterns of C3, investigate the ontogeny of C3 in embryo and larva, and assess the expression of C3 in response to pathogen infection. The full length C3 cDNA sequence of 5157 bp with an open reading frame (ORF) of 4938 bp was cloned from southern catfish. The deduced amino acid sequence showed similarity with other teleost fish. The mRNA expression of C3 was detected in liver, spleen, stomach, intestine, and head kidney with RT-PCR and *in situ* hybridization. Whole mount *in situ* hybridization results revealed that C3 was first expressed in the yolk syncytial layer at 34 h post fertilization (hpf), followed by the liver at 36 h post hatching (hph). When challenged with *Aeromonas hydrophila*, the transcripts of C3 showed a significant up-regulation in liver and spleen at 24 h. The results suggested that complement C3 played a key role in defense against invading pathogens in the early development stages of southern catfish. Therefore, these results provide important information to understand the functions of C3 during fish early development in Siluriformes.

1. Introduction

The vertebrate immune system is composed of two major subsystems: the adaptive system and the innate system [1,2]. The complement system is an important link between the innate and adaptive systems, and involves more than 35 soluble and membrane-bound proteins [3,4]. The complement systems can be activated through three pathways: the classical complement activation pathway, the alternative complement pathway, and the lectin complement pathway [5]. The classical pathway is triggered by antigen antibody complexes, whereas the alternative and lectin pathways are activated by direct binding of complement components to microbial surfaces [6]. Among the three activation pathways, C3 is the most important and central complement component present in the plasma that participates in all three pathways [7,8]. C3 is cleaved into C3a and C3b with the formation of the C3 convertase [3]. C3a is a small bioactive fragment and mediates many biological activities; C3b is a large fragment and undergoes an extensive conformational change to expose and cleave the thioester bond [9]. The binding of C3b to the active convertase changes the substratum specificity of the enzyme complex which starts to cleave C5 to C5a and C5b [10]. C5b initiates the assembly of the membrane attack complex (C5b-C9) [3].

Fish embryos and larvae are exposed to natural water environments and have to face a high density of pathogens [11,12]. Thus, the possession of an effective immune system in fish embryos and larvae is important for the survival of early fish stages in such environments [13]. Maternal innate immune factors including the complement component C3, lectins, protease inhibitors, and lysozymes can be transferred from mother to offspring [14]. Those maternal factors play an important role in the protection of early fish life against microbial attack [11]. However, recent studies show that complement components are synthesized by the embryo starting from an early developmental stage and can also fight invading pathogens at an early stage [12,15]. For example, the mRNA levels of C3 and Bf increased significantly when

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https://doi.org/10.1016/j.fsi.2018.10.083
Received 31 July 2018; Received in revised form 25 October 2018; Accepted 29 October 2018

the zebrafish embryo was challenged with lipopolysaccharide, suggesting that C3 and Bf expressed by the embryo are ready to react against bacterial infection [13].

C3 mRNA has been shown to be expressed in developing embryos or larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.) [16,17], Atlantic cod (*Gadus morhua* L.) [18,19], spotted wolffish (*Anarhichas minor* Olafsen) [20], rainbow trout (*Oncorhynchus mykiss*) [15], Atlantic salmon (*Salmo salar*) [21], carp (*Cyprinus carpio*) [22], zebrafish (*Danio rerio*) [13], Indian major carp (*Labeo rohita*) [23], and olive flounder (*Paralichthys olivaceus*) [24]. However, there is no study available on C3 in southern catfish (*Silurus meridionalis*) and the ontogeny of C3 in the early life of southern catfish. It has been suggested that the ontogenetic development of the immune system varies between different fish species [16]. Thus, studying the complement components in embryos and larvae of other fish is needed for better understanding of the innate immune mechanism during early development.

Southern catfish, belonging to the family Siluridae, are widely distributed south of the Yangtze River Basin [25]. Southern catfish is also an important economic fish in China because of its nutritional value, large size, fast growth, and high fecundity [26]. However, the outbreak of disease has risen with the increased stocking density, which limits the development of a sustainable culture of southern catfish [27,28]. *Aeromonas hydrophila* is a common pathogen in southern catfish and results in high mortality of the fish at the larva and juvenile stages [29]. Complement factors have been suggested to be involved in the innate response against *A. hydrophila* [30]. However, there is no available knowledge regarding the complement factors in southern catfish.

In the present study, the full-length cDNA sequence of complement C3 of southern catfish was cloned, and the mRNA expression of C3 in different tissues was measured. Furthermore, the ontogeny of C3 was determined using whole mount *in situ* hybridization and RT-PCR, and the expression level of C3 in southern catfish was assessed after infection with *A. hydrophila*.

2. Materials and methods

2.1. Experimental animals and sample preparation

Southern catfish with body lengths of 18.5 ± 2.3 cm and body weights of 65.5 ± 4.8 g were obtained from the Key Laboratory of Fisheries Science of Chongqing. The fish were maintained in several 100 L opaque tanks, and water temperature was adjusted to 25 ± 2 °C in the laboratory with an air conditioner. For the tissue-specific expression study, tissues from healthy fish including blood, stomach, liver, kidney, head kidney, intestine, muscle, brain, skin, gill, heart, and spleen were collected, immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA extraction and first strand cDNA preparation

Total RNA was extracted from southern catfish liver using TRIzol Reagent (Invitrogen, USA) following manufacturer's protocol. The RNA was quantified using a Nanodrop 2000 (Thermo Scientific, USA), and cDNA synthesis was performed with a M-Mulv Reverse Transcriptase cDNA Synthesis Kit (TaKaRa, Japan) according to the manufacturer's instructions.

2.3. Cloning of the full length cDNA of C3

Primers were designed from the conserved regions of complement C3 sequences of *O. mykis*, *D. rerio*, *Homo sapiens*, *Gallus gallus*, and *C. carpio*. The PCR was conducted with Takara PCR Amplification Kit (Takara, Japan). The PCR program was as follows: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min, with final extension at 72 °C for 10 min. The target products were purified using TIANgel Midi Purification Kit (TIANGEN, China). The purified products were cloned into the pMD-19T vector (Takara, Japan) for sequence determination. 5′-RACE and 3′-RACE were performed using a BD SMART RACE cDNA Amplification Kit (Clontech, USA) following manufacturer's protocol. The PCR products were subsequently cloned into the pMD-19T vector and sequenced. All primers used in the study were listed in Table 1.

Table 1
Primer sequences of complement C3 used in the present study. The primers of C3−1F and C3-1R, C3−2F and C3-2R, as well as C3−3F and C3-3R yielded three cDNA sequences of 1468 bp, 1266 bp, and 1578 bp, respectively. 3′ RACE and 5’ RACE yielded two cDNA sequences of 997 bp and 1194 bp, respectively.

Primers	Sequence (5‘→3′)	Use
C3−1F	CATTTGTCTTGATTGCCATGCAAG	Cloning
C3-1R	TCACTCGTAGGCCAGTATTC	
C3−2F	TGAAAGACTCTATCACTACCTGG	Cloning
C3-2R	CAGCAACCTGCCAGTAAGTA	
C3−3F	AGTGGCAGTGAAATGGTGGAAG	Cloning
C3-3R	TTCCACACGCACCTTCTGTAGTT	
C3−4F	AAGACCCGAACGGAGATGCCA	3′ RACE
C3-4R	ATGACCACATCCACATCTTCAGCAGG	5′ RACE
C3-EAF	TGTGCCAAAACAGTCGGAAGTC	Expression analysis
C3-EAR	GCTGAACCTTGTCTGAGCGTGT	
18S−F	CCTGAGAAACGGCTACCACATCC	Expression analysis
18S-R	AGCAACTTTAATATACGCTATTGGAG	
C3−5F	TAATGTAGACCCCAAATCCAGC	For *in situ* hybridization probe
C3-5R	TAGCCTCATTACGCCGACTC	For *in situ* hybridization probe

2.4. Sequence analysis

The southern catfish complement C3 sequence was analyzed at both nucleotide and amino acid levels using the Nucleic Translation of the EMBOSS explorer (http://emboss.bioinformatics.nl/). The amino acid sequence of southern catfish complement C3 was predicted and analyzed using the SMART (http://smart.embl-heidelberg.de/) [31]. Amino acid sequences of complement C3 of related species were obtained from NCBI GenBank. Multiple sequence alignments were conducted with BioEdit [32]. The phylogenetic tree was constructed by MEGA 4.0 software, using the Neighbor-Joining method and 1000 bootstrap replications [33].

2.5. Semi-quantitative RT-PCR analysis of C3 tissue expression pattern

The southern catfish C3 tissue expression pattern was analyzed by semi-quantitative RT-PCR. The PCR program was as follows: 94 °C for 3 min, followed by 33 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s, with final extension at 72 °C for 10 min. The expression of southern catfish 18S rRNA was used as an internal control. The negative control PCR with no cDNA template and the positive control PCR with southern catfish C3 template were included in the analysis. PCR products were electrophoresed on a 1.2% agarose gel, and then examined with a Gel DocTM XR documentation system (BioRad, USA).

2.6. In situ hybridization

Digoxigenin (DIG)-labeled RNA probe and control probe for C3 were prepared with DIG RNA Labeling Kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions.

Liver and intestine samples were washed two times with phosphate buffered saline (PBS, pH 7.4, RNase-free) and then fixed with 4% paraformaldehyde (PFA) for at least 24 h at 4 °C. Tissues were immersed with a graded ethanol series and stored in 100% ethanol for 24 h at −20 °C. The tissues were then dehydrated sequentially in ethanol and xylene, and embedded in paraffin. Ultrathin sections (8 μm in thickness) were prepared and mounted onto air plasma spraying (APS)-coated slides. Tissue sections were deparaffinized and rehydrated with xylene and ethanol. The slides were washed twice in PBS buffer. Sections were permeabilized by incubating in 20 μg/mL proteinase K at 37 °C for 30 min. Prehybridization was done by fixing the sections in prehybridization solution for 2 h at 60 °C. Hybridization was performed using DIG-labeled oligonucleotide anti-sense and sense probes in hybridization solution (50 μg/mL heparin, 50% formamide, 0.5 mg/mL torula yeast tRNA, 0.1% Tween-20, 5 × SSC (NaCl + Na_3citrate)) for 18 h at 60 °C in a humidified chamber. The cover slips were carefully removed by washing in 2 × SSC, followed by washing twice in 50% deionized formamide and 50% 2 × SSC for 15 min at 60 °C, twice in 2 × SSC for 15 min, and twice in 1 × SSC for 15 min, respectively. The RNA in the sections was removed with 20 μg/mL RNAase at 37 °C in 1 × SSC for 30 min, followed by washing three times in 0.1 × SSC for15 min at 37 °C, and then washed twice in washing buffer (0.1 M Tris + 0.15 M NaCl, pH 7.4) for 10 min. The sections were then incubated with blocking solution for 1 h. Anti-DIG Fab fragments at a dilution (in blocking solution) of 1:1000 were applied and allowed to react for 4 h at room temperature. The color reaction was developed over 3 h at 37 °C using Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. The reaction was visualized with a microscope (Nikon 80i, Japan).

2.7. Ontogeny of C3 in southern catfish

2.7.1. Preparation of parent fish, embryos, and larvae

Parent southern catfish were obtained from the Hechuan reach to the Beibei reach of Jialing River. The body length of the female was 92.6 cm with a body weight of 9.5 kg, and the body length of the male was 79.0 cm with a body weight of 5.8 kg. The parents were cultured for 1 week at room temperature. Then, chorionic gonadotropin (HCG) at a dose of 1000 U/kg and luteinizing hormone-releasing hormone analogues (LRH-A) at a dose of 25 μg/kg were injected into female fish by intraperitoneal injection, and HCG at a dose of 500 U/kg and LRH-A at a dose of 12.5 μg/kg were injected into male fish. Artificial insemination was conducted at 24 h after the injection of HCG and LRH-A.

Eggs, embryos, and larvae were collected at 0, 1, 2, 5, 7, 11, 13.5, 15, 17, 20.5, 22.5, 25.5, 30, 32, 34, 36, 39, 42, 47.5, 50, 52, 54, 58, and 63 h post fertilization (hpf), as well as 8, 16, 24, 32, 44, 56, 80, 104, 136, 172, and 212 h post hatching (hph). All samples were washed with 1 × PBS. Then the samples were frozen in liquid nitrogen and stored at −80 °C.

2.7.2. Expression of C3 in egg, embryo, and larva

The ontogeny expression of C3 in egg, embryo, and larva was analyzed by semi-quantitative RT-PCR. All samples were performed in triplicates, and the conditions used for RT-PCR were described in section 2.5. PCR products were electrophoresed on a 1.2% agarose gel, and then examined with a Gel DocTM XR documentation system (BioRad, USA).

2.7.3. Whole mount in situ hybridization

DIG-labeled RNA probe and control probe for C3 were prepared as section 2.6 described. Embryos were fixed in 4% PFA in phosphate saline solution (pH 7.4) for 24 h, followed by washing with 1 × PBT (10 × PBS 100 mL, DEPC water 890 mL, 10% Tween 20 10 mL) three times (5 min each). The embryos were dechorionated with 100% methanol for five times (5 min each) and then stored at −20 °C at least 24 h.

Embryos/eggs/larvae (EEL) were rehydrated with successive dilutions of methanol in PBT (75% methanol, 50% methanol, and 25% methanol; vol/vol), each for 5 min. Then, the EEL were washed four times with 1 × PBT for 5 min. The rehydrated embryos were digested with proteinase K (5 μg/mL) at room temperature for 30 min. The digested EEL were washed three times with PBT: two quick washes and one 5-min wash. The EEL were subsequently incubated in 4% PFA for 20 min and washed five times with 1 × PBT (for 5 min each). Prehybridization was conducted by incubating EEL with 200 μL prehybridization solution for 2–5 h in a 67 °C water bath. Next, the prehybridization solution was removed, and 200 μL hybridization solution containing 3% of probe stock solution (vol/vol) was added to incubate with the EEL overnight in a 67 °C water bath.

The next day, the hybridization reaction mixture containing the probe was removed, and 1 mL of prewarmed hybridization solution was added to wash the EEL for 20 min. Then, the EEL were washed with prewarmed solutions of 2 × SSCT (20 × SSC 100 mL, DEPC water 890 mL, 10% Tween 20 10 mL) in formamide (50% 2 × SSCT three times, 75% 2 × SSCT one time, 100% 2 × SSCT two times) for 20 min at 67 °C. The EEL were subsequently washed with prewarmed 1 × PBT for 5 min at 67 °C, followed by washing with 1 × PBT for 5 min at 37 °C. The embryos were then incubated with 1 mL blocking solution for 5 h at 37 °C. Lastly, the EEL were incubated with a solution of anti-DIG-AP antibody (1:2000) in blocking solution overnight at 4 °C.

On the third day, the EEL were washed three times with 1 × PBT for 5 min each, followed by washing six times with 1 × PBT for 30 min each. Then, the EEL were washed three times with NTMT (2 mL 100 mM pH 9.5 Tris HCl, 1 mL 50 mM $MgCl_2$, 2 mL 100 mM NaCl, 500 μL 0.1% Tween-20, and 14.5 mL water) solution for 5 min each. The EEL were subsequently transferred to a 24-well plate. The color reaction was developed at 37 °C using Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. The staining reaction was monitored every 15 min using a microscope. When the desired staining intensity was reached, the EEL were immediately washed three times with 1 × PBT. The reaction was subsequently stopped by the stop solution. Finaly, the EEL were stored in 0.5 mL 80% glycerol stock solution (vol/vol) at 4 °C. The reaction was visualized with a microscope (Nikon 80i, Japan).

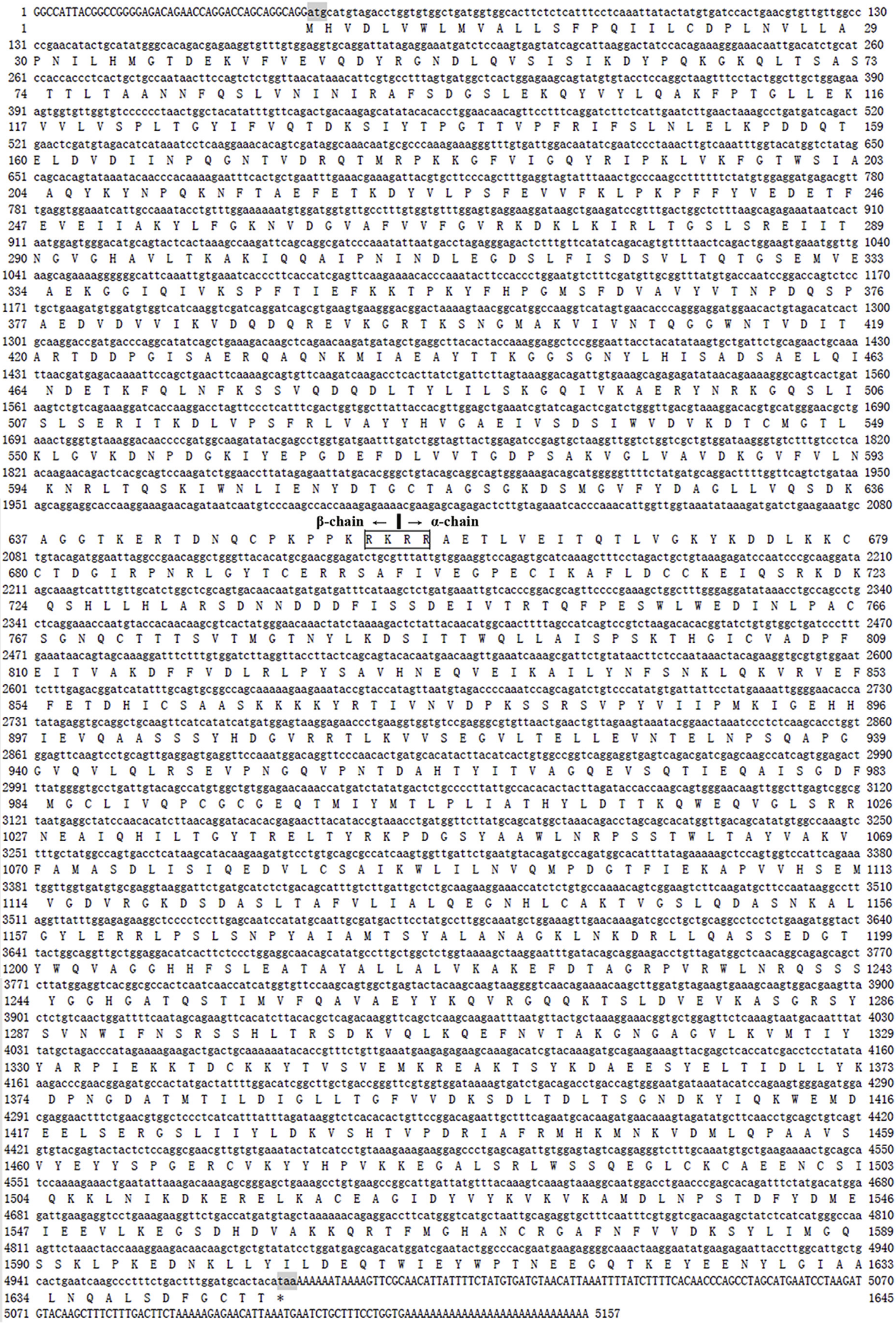
2.8. *A. hydrophila* infection and C3 expression analysis

Eighty southern catfish with body lengths of 18.5 ± 2.3 cm and body weights of 65.5 ± 4.8 g were randomly placed into two 100 L tanks (40 fish per group) containing 50 L water. Two hundred μL *A. hydrophila* solution at a concentration of 1.0×10^6 CFU/mL were injected into the fish in trial group by intraperitoneal injection. Two hundred μL 0.65% sodium chloride solution was injected into the fish in control group. Three fish were randomly sampled on days 1, 3, 7, 14, 21, and 35. The liver, intestine, and spleen were collected after fish were anesthetized with 150 mg/L tricaine methanesulfonate (MS-222, Sigma). All samples were immediately frozen in liquid nitrogen and stored at −80 °C for total RNA extraction.

C3 expression in liver, intestine, and spleen on different days after *A. hydrophila* infection was analyzed by semi-quantitative RT-PCR. All samples were performed in triplicate, and the conditions used for RT-PCR were described in section 2.5. PCR products were electrophoresed on a 1.2% agarose gel and then examined with a Gel DocTM XR documentation system (BioRad, USA).

2.9. Statistics

All experimental data are expressed as the mean ± SD (standard deviation). Assumptions of normality and homogeneity of variances were confirmed with the Shapiro-Wilk test and Levene's test, respectively using a statistical analysis system software (SPSS 19.0). Significance differences between the control and infected groups were determined using independent samples *t*-test. Results were considered statistically significant when $p < 0.05$ and $p < 0.01$.



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1                                            M  H  V  D  L  V  W  L  M  V  A  L  L  S  F  P  Q  I  I  L  C  D  P  L  N  V  L  L  A  29
131 ccgaacatactgcatatgggcacagacgagaaggtgtttgtggaggtgcaggattatagaggaaatgatctccaagtgagtatcagcattaaggactatccacagaaagggaaacaattgacatctgcat 260
30 P  N  I  L  H  M  G  T  D  E  K  V  F  V  E  V  Q  D  Y  R  G  N  D  L  Q  V  S  I  S  I  K  D  Y  P  Q  K  G  K  Q  L  T  S  A  S 73
261 ccaccaccctcactgctgccaataacttccagtctctggttaacataaacattcgtgcctttagtgatggctcactggagaagcagtatgtgtacctccaggctaagtttcctactggcttgctggagaa 390
74  T  T  L  T  A  A  N  N  F  Q  S  L  V  N  I  N  I  R  A  F  S  D  G  S  L  E  K  Q  Y  V  Y  L  Q  A  K  F  P  T  G  L  L  E  K 116
391 agtggtgttggtgtccccccctaactggctacatatttgttcagactgacaagagcatatacacacctggaacaacagttcctttcaggatcttctcattgaatcttgaactaaagcctgatgatcagact 520
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160 E  L  D  V  D  I  I  N  P  Q  G  N  T  V  D  R  Q  T  M  R  P  K  K  G  F  V  I  G  Q  Y  R  I  P  K  L  V  K  F  G  T  W  S  I  A 203
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                                              β-chain ← ▌→ α-chain
637  A  G  G  T  K  E  R  T  D  N  Q  C  P  K  P  P  K [R  K  R  R] A  E  T  L  V  E  I  T  Q  T  L  V  G  K  Y  K  D  D  L  K  K  C  679
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940 G  V  Q  V  L  Q  L  R  S  E  V  P  N  G  Q  V  P  N  T  D  A  H  T  Y  I  T  V  A  G  Q  E  V  S  Q  T  I  E  Q  A  I  S  G  D  F 983
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1634  L  N  Q  A  L  S  D  F  G  C  T  T  *                                                                                           1645
5071 GTACAAGCTTTCTTTGACTTCTAAAAAGAGAACATTAAATGAATCTGCTTTCCTGGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 5157
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Fig. 1. Nucleotide sequence of the southern catfish C3 cDNA and deduced amino acid sequence. Initiation codon and stop codon (gray shadow); the potential processing site for the cleavage of the alpha and beta subunits (box).

3. Results

3.1. C3 molecular characterization from *S. meridionalis*

Both the complete nucleotide and deduced amino acid sequences of southern catfish C3 are shown in Fig. 1. The full-length C3 sequence of 5157 bp consisted of a 5′ UTR of 43 bp, an open reading frame (ORF) of 4938 bp, and a 3′ UTR of 176 bp (Fig. 1). The 3′ UTR of C3 had the characteristic polyadenylation signals (AATAAA) at position of 4986 bp. The ORF of C3 sequence encoded a predicted protein of 1645 amino acids with a molecular formula $C_{8200}H_{12940}N_{2184}O_{2505}S_{58}$, a molecular mass of 184 kDa, and a predicted isoelectric point (pI) of 6.06.

3.2. Sequence analysis and multiple alignment

The deduced amino acid sequence showed a signal peptide at position 1–22 amino acids with a sequence of MHVDLVWLMVALLSFPQIILCD (Fig. 1). The domain search analysis revealed that C3 had a putative *N*-glycosylation site at position N^{1312} and three O-glycosylation sites at positions T^{137}, T^{750}, and T^{958} (Fig. 1). The anaphylatoxin domain (ANATO) was at position 679–714 amino acids, the α2-macroglobulin family domain (A2M) was at position 755–853 amino acids, the thioester site (GCGEQ) was at position 993–997 amino acids, and the C3-convertase cleavage site (Arg-Ser) was at position 731–733 amino acids (Figs. 1 and 2).

3.3. Phylogenetic analysis

Teleosts clustered into a group with southern catfish C3 being closest to Cyprinidae fish. Amphibians, reptiles, birds, and mammals clustered together. *Strongylocentrotus putpuratus* and *Lethenteron japonicum* clustered into a different group (Fig. 3).

3.4. Tissue distribution of C3

The expression levels of C3 were detected in blood, liver, spleen, kidney, head-kidney, stomach, intestine, skin, gill, brain, and heart using semi-quantitative RT-PCR with 18S rRNA as the internal reference gene. It was observed that C3 was highly expressed in the liver, followed by the spleen, stomach, and intestine, and weakly expressed in head kidney. There was no expression of C3 in blood, skin, kidney, muscle, gill, brain, and heart (Fig. 4).

3.5. *In situ* hybridization

In situ hybridization revealed the presence of C3 transcripts in liver cells and intestine epithelial cells. There was no expression of C3 in the endothelial cells of liver blood vessels (Fig. 5).

3.6. Ontogeny of C3 in southern catfish

RT-PCR results showed that expression of the C3 gene was not detected in eggs, fertilized eggs (0 hpf), multicellular stage (5 hpf), late blastula stage (13.5 hpf), late gastrula stage (20.5 hpf), neurula stage (22.5 hpf), or the blastopore closure stage (25.5 hpf) (Fig. 6). A weak expression of C3 was first observed at the eye primordia formation stage (30 hpf) (Fig. 6). With embryo development, the expression of C3 increased at the tail bud stage of 34–36 hpf and significantly increased at the tail bud stage of 39 hpf (Fig. 6). High C3 expression was also detected at the cardiac formation stage (42 hpf), anal primordia appearance stage (47.5 hpf), otolith appearance stage (50 hpf), blood circulation stage (52 hpf), prehatching stage (58 hpf), and hatching stage (63 hpf) (Fig. 6). The expression of C3 was decreased at 8 and 24 hph, and then it was up-regulated to a high level from 32 to 212 hph (Fig. 6).

In situ hybridization results revealed that C3 expression was not visible at 20.5, 25.5, and 30 hpf (Fig. 7 A-F). A low signal of C3 was observed at 34 hpf and distributed in the yolk syncytial layer (Fig. 7 G). With embryo development, C3 transcription was clearly observed at 36 hpf, and located all over the yolk sac at 52 hpf (Fig. 7 I–S). At 58 and 63 hpf, the transcription signal was strong in the abdomen of the yolk sac and decreased in the dorsum of the embryo (Figs. 7T and . 8 A-D). At 12 and 24 hph, the transcription signal was strong in the abdomen and post-abdomen of the yolk sac (Fig. 8E–L). At 36 and 48 hph, C3 mRNA was expressed in the liver and the yolk sac (Fig. 8M − U). The yolk sac was gradually absorbed with the growth of the larva. At 104 hph, the yolk sac was completely absorbed, and the transcription signal was presented only in the liver (Fig. 8V–Y).

3.7. Expression of C3 in response to *A. hydrophila* infection

C3 mRNA expression was up-regulated significantly in the liver and spleen on day 1 after challenging the fish with *A. hydrophila* ($p < 0.05$) (Fig. 9A and B). In the liver of the infection group, C3 expression reached its maximum on day 3, and decreased significantly on day 7 (Fig. 9 A). There was no significant difference between the infection group and control group from day 7 to day 35 (Fig. 9 A). In the spleen, C3 expression reached its peak on day 7, and decreased significantly from day 14 to day 35 (Fig. 9 B). In the intestine, C3 expression showed no significant change at all time points post infection (Fig. 9 C).

4. Discussion

In this study, the full-length 5157-bp cDNA sequence encoding C3, consisting of a 5′ UTR of 43 bp, an open reading frame (ORF) of 4938

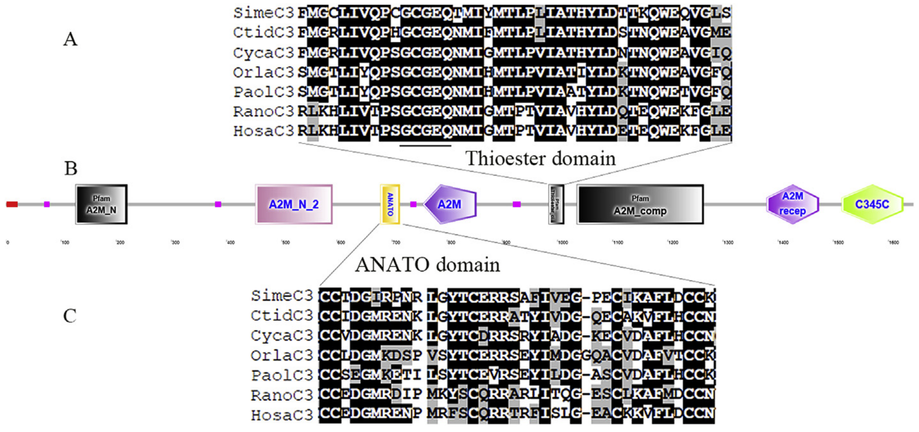

Fig. 2. The predicted protein domain characteristics of complement component C3 (B). A2M − N and A2M-N-2, the alpha2-macroglobulin *N*-terminus regions 1 and 2; ANATO: the anaphylatoxin domain; A2M, the alpha2-macroglobulin C-terminus region; A2M-COMP, the alpha2-macroglobulin complement component domain; A2M-RECEP, the alpha2-macroglobulin receptor domain. C345C, the complement C3/4/5 C-terminal region. Multiple alignments of the southern catfish C3 thioester domain (A), ANATO domain (C), and with other C3 proteins. The GenBank accession numbers of the sequences used in the alignment are shown as follows: CtidC3 (*Ctenopharyngodon idella*), AAQ74974.1; CycaC3 (*Cyprinus carpio*), BAA36619; OrlaC3 (*Oryzias latipes*), NP_001098552; PaolC3 (*Paralichthys olivaceus*), BAA88901; RanoC3 (*Rattus norvegicus*) NP_058690; HosaC3 (*Homo sapiens*), AAA85332; Sime (*Silurus meridionalis*) C3, MH700481.

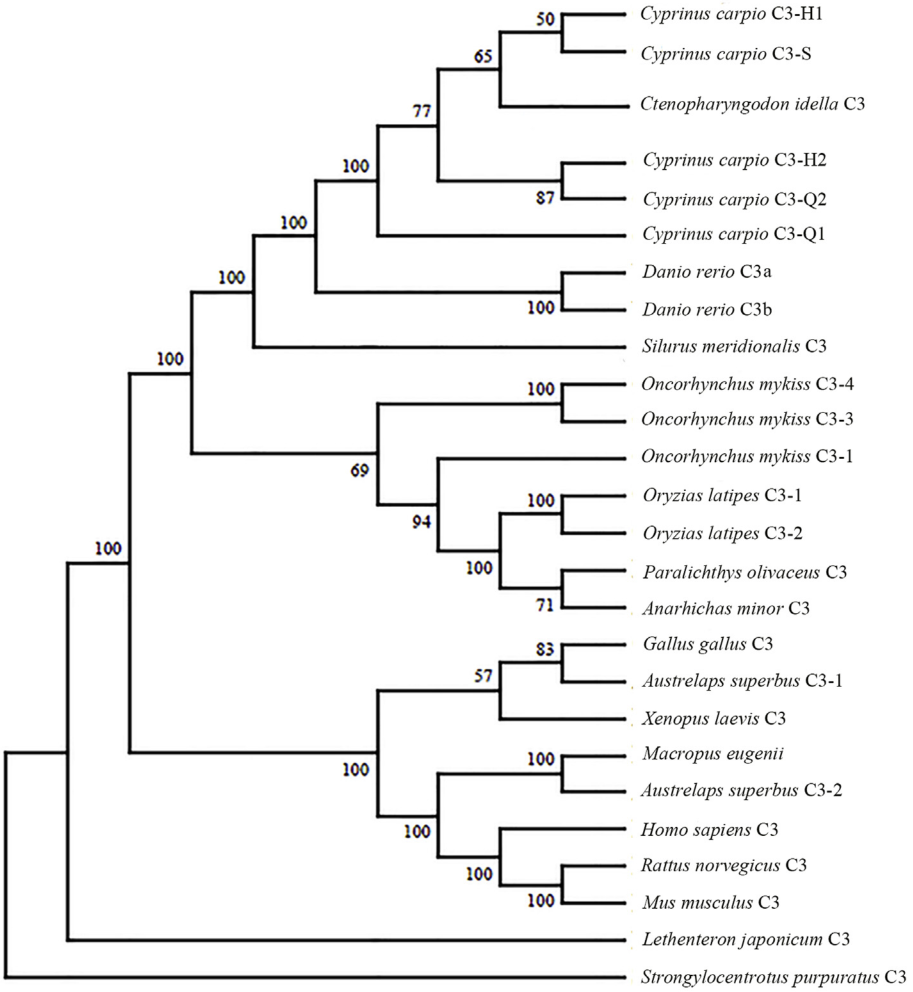

Fig. 3. Phylogenetic tree of southern catfish and other species were constructed using MEGA 4.0. The GenBank accession numbers for the sequences are as follows: *Anarhichas minor* C3, CAC29154; *Paralichthys olivaceus* C3, BAA88901; *Oncorhynchus mykiss* C3-1, P98093; *Oncorhynchus mykiss* C3-3, AAC60015; *Cyprinus carpio* C3−S, BAA36621; *Cyprinus carpio* C3−H1, BAA36619; *Cyprinus carpio* C3−H2, BAA36620; *Cyprinus carpio* C3-Q1, BAA36622; *Cyprinus carpio* C3-Q2, BAA36623; *Ctenopharyngodon idella* C3, AAQ74974.1; *Oryzias latipes* C3-1, NP_001098552; *Oryzias latipes* C3-2, NP_001098553; *Danio rerio* C3b, CAX13555; *Danio rerio* C3a, NP_001032313; *Macropus eugenii* C3, AAW69835; *Xenopus laevis* C3, AAB60608; *Mus musculus* C3, NP_033908; *Rattus norvegicus* C3, NP_058690; *Austrelaps superbus* C3-1, ABA28746; *Austrelaps superbus* C3-2, ABA28747; *Strongylocentrotus purpuratus* C3, AAC14396; *Homo sapiens* C3, AAA85332; *Lethenteron japonicum* C3, BAA00983; *Silurus meridionalis* C3, MH700481.

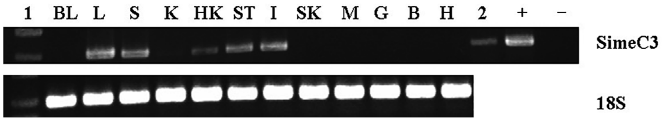

Fig. 4. Expression analysis of the C3 gene in various tissues of healthy southern catfish. 18S rRNA was used as an internal control. BL, blood; L, liver; S, spleen; K, kidney; HK, head kidney; ST, stomach; I, intestine; SK, skin; M, muscle; G, gills; B, Brain; H, heart; 1 and 2, DL 2000 maker; +, positive control; -, negative control.

bp, and a 3′ UTR of 176 bp, was cloned from the liver of southern catfish, the tissue distribution of C3 was detected, and C3 expression post-bacterial infection was evaluated. The ontogeny of C3 in southern catfish was investigated with whole mount *in situ* hybridization and RT-PCR. This is the first report of a complete sequence of C3 in southern catfish.

The deduced amino acid sequence analysis revealed that C3 of southern catfish was similar to other fish in having two chains (α and β), with a thioester bond in the α-chain. The thioester domain (GCGEQ) is a highly conserved region in all species and is responsible for its ability to attach covalently to target molecules [7,34]. A previous study suggested that Pro^{1007} and Pro^{1020} were necessary for stable thioester formation [35]. In this study, Pro^{991} and Pro^{1004} are found in the C3 sequence of southern catfish. The processing site of RKRR (in the β-α domain) and the C3-convertase cleavage site (Arg-Ser) is conserved in southern catfish C3. The His^{1126} and Glu^{1128}, which are suggested to be involved in binding specificity of C3 in humans [36], are also conserved in C3 at positions 1110 and 1112. These sequence data indicate that southern catfish C3 functions are similar to the C3 molecules of other animals.

Most of the teleost fish were found to possess multiple isoforms of C3. For example, eight isoforms were reported in *D. rerio* [37]. Five C3 variants were characterized in carp [38], five isoforms were reported in *Sparus aurata* [39], three subtypes were characterized in *O. mykiss* [40], and three isoforms were discovered in dojo loach (*Misgurnus anguillicaudatus*) [30]. These multiple isoforms of C3 have shown differences in their binding efficiencies to various complement-activating surfaces [41]. The coexistence of multiple forms of C3 in the same animal might increase the number of possible pathogens recognized by these proteins, and therefore, enhance the protective effect of the organism's complement system [40]. In the present study, only one type of C3 was isolated using primers designed from conserved regions. Similar to southern catfish, a single isotype of C3 is reported in *Epinephelus coioides* [42], *L. rohita* [7], and *Chionodraco hamatus* [9].

Liver has been recognized as a main production site for the majority of complement components in mammals [43]. Fish is in accordance with the phenomenon; the highest expression of C3 is in the liver [44]. The liver is an important organ for innate immunity, playing a key role in host defenses against pathogens [24]. In the present study, the *in situ* hybridization and RT-PCR results demonstrated that C3 is highly expressed in the liver of southern catfish. This indicated that liver was indeed the main supplier of C3 in southern catfish.

Extrahepatic expression of complement components play an important role in regulation of local immune responses including crosstalk with the adaptive immune system, as well as damage and repair of

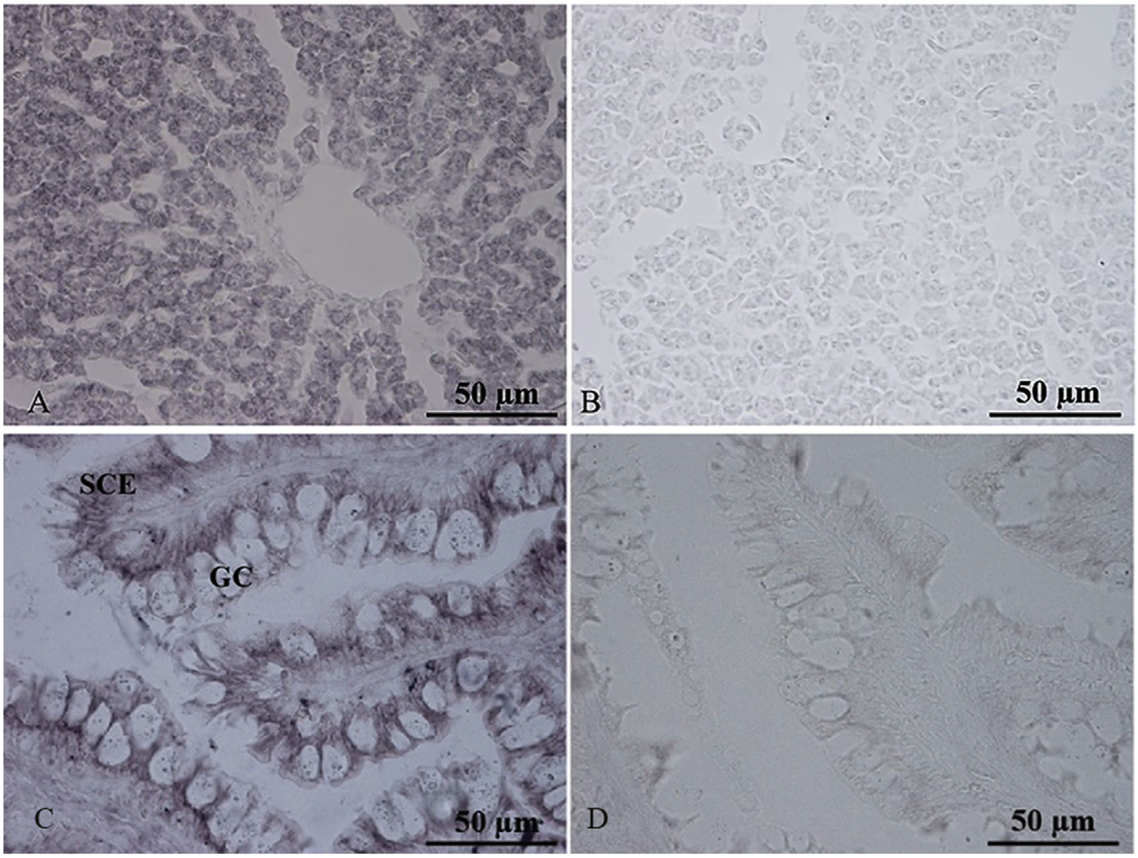

Fig. 5. Expression of complement C3 in liver (A and B) and intestine (C and D) of southern catfish determined by *in situ* hybridization. SCE, simple columnar epithelium; GC, goblet cell. B and D, negative control by sense probe. Scale Bar = 50 µm.

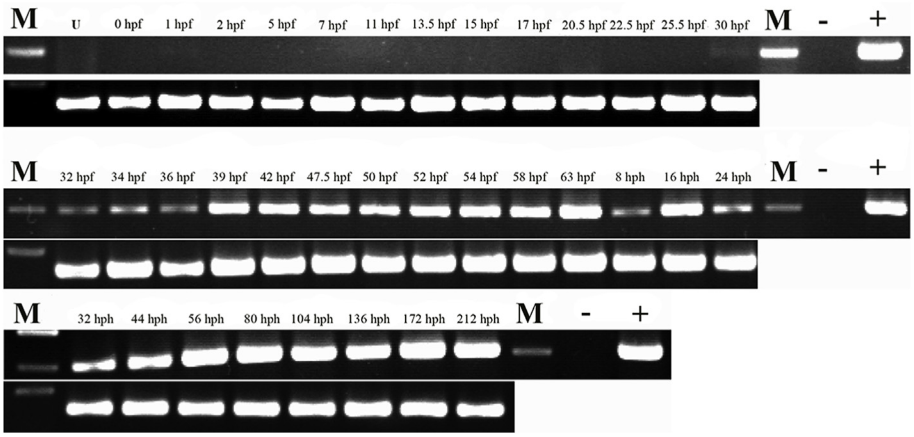

Fig. 6. Expression of C3 mRNA during ontogeny. Eggs, embryos, and hatchlings were sampled at different time points. The RNA was extracted and analyzed by RT-PCR. M, Marker DL2000; U, unfertilization; hpf, hour post fertilization; hph, hour post hatching; +, positive control; -: negative control; 18S rRNA was used as a positive control to normalize the samples.

tissues [12]. Although C3 of southern catfish was detected in spleen, stomach, intestine, and head kidney, it was not expressed in blood, skin, kidney, muscle, gill, brain, and heart. The previous study showed that extrahepatic expression of C3 changed with different fish [21,23,45]. In Atlantic salmon (*Salmo salar*), C3 was synthesized in heart, gonads, muscle, intestine, skin, gill, pylorus, and spleen [21]. While in olive flounder, the C3 gene was not expressed in extrahepatic sites [24]. These distribution differences at extrahepatic sites may be due to the multiple isoforms of C3 isolated in different fish. For example, there are two different expression patterns of C3 in zebrafish as follows: C3.1, C3.2/3, C3.6, and C3.7/8 were primarily expressed in the spleen and liver, but C3.4 and C3.5 were mainly expressed in the kidney and the intestine [37].

In situ hybridization revealed the presence of C3 transcripts in intestine epithelial cells. A previous study suggested that complement C3 might play an important role in the mucosal tissues of teleost fish [30]. Mucosal immunity is a common functional entity and is located in all mucosal tissues of teleost fish including the intestine [46]. C3, mainly found in squamous epithelial cells and fibroblasts of the intestine, are most likely acting in defense against invading pathogens ingested through the mouth [16]. The presence of C3 in intestine epithelial cells indicates that C3 might participate in the mucosal immune system of the intestine.

Whole mount *in situ* hybridization revealed that C3 transcripts were not detected in the unfertilized eggs of southern catfish. In Atlantic salmon, C3 protein was presented in the unfertilized eggs using the method of immunoblotting, but C3 transcript was only expressed post fertilization [21]. C3 transcripts were also not presented in the eggs of

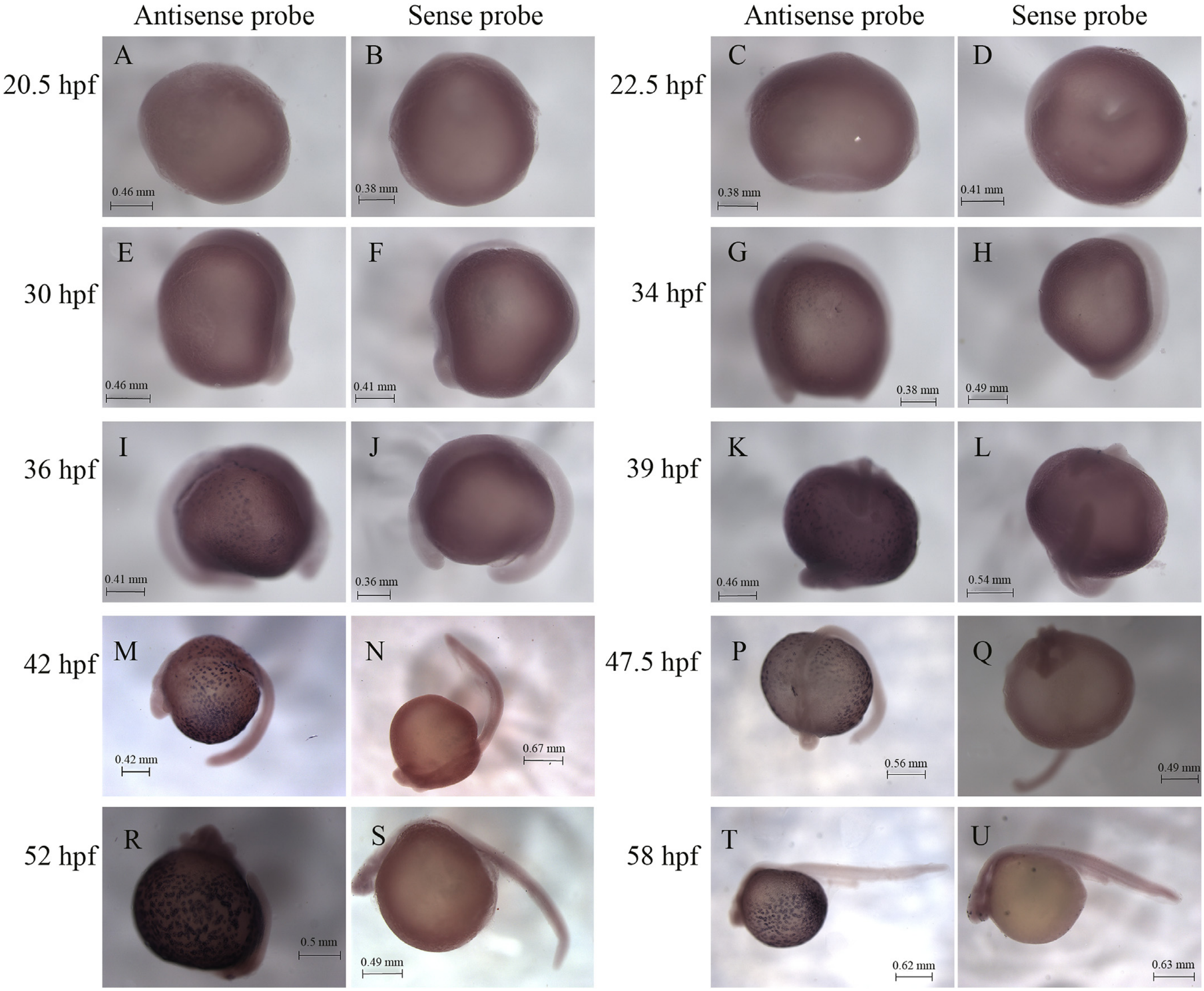

Fig. 7. Whole mount *in situ* hybridization analysis of C3 mRNA in embryos post-fertilization. C3 was not expressed at 20.5 (A, B), 25.5 (C, D), and 30 hpf (E, F). The expression of C3 was presented on the yolk sac at 34, 36, 39, 42, 47.5, 52, and 58 hpf using the antisense probe. The sense probe served as a negative control. hpf, hour post fertilization. Scale bars of A to U were 0.46, 0.38, 0.38, 0.41, 0.46, 0.41, 0.38, 0.49, 0.41, 0.36, 0.46, 0.54, 0.42, 0.67, 0.56, 0.49, 0.50, 0.49, 0.62, and 0.63 mm, respectively.

Indian major carp [23], rainbow trout [15], zebrafish [13], and spotted wolffish [20]. These results indicated that the maternal C3 mRNA was not transferred into the eggs in those fish.

The expression of C3 mRNA has been studied in several teleost fish. Results have shown that the ontogenetic development of C3 varies between different fish species [15,20,22,24]. In large yellow croaker, the expression of C3 starts at the two-cell embryo stage [45]. In zebrafish, the mRNA of C3 was expressed at 2 hpf [13]. In Indian major carp [23], rainbow trout [15], Atlantic salmon [13], Atlantic cod [11], and Atlantic halibut [16], the C3 transcripts were detected at 6 hpf, 7 dpf, 14 dpf, 1 dph, and 15dph, respectively. However, in carp, the expression of C3 was detected as early as 0 hpf [22]. In the present study, C3 transcript of southern catfish was first detected at 32 hpf with RT-PCR, and it was observed with a low signal at 34 hpf by whole mount *in situ* hybridization. Therefore, the ontogeny of southern catfish C3 was different from those of the other reported fish.

Even though liver is the main production site for majority of the complement components, it is not able to produce significant amounts of C3 at the earliest time points in the embryo [20]. The yolk syncytial layer, a unique structure for fish, is the primary site for the expression of C3 [20,22]. C3 transcripts around the yolk syncytial layer were detected with whole mount *in situ* hybridization. With embryo development, C3 expression was presented and located all over the yolk sac at 52 hpf. Liver is the second site for the expression of C3 and plays a role in the resorption of yolk. Increased expression of C3 was detected in hepatic cells during liver formation of southern catfish. In contrast, the expression of C3 in the yolk syncytial layer decreased significantly. The same phenomenon was also reported in the carp, Atlantic cod, and spotted wolffish [11,20,22]. C3 expression in embryo might play a role in defense against invading pathogens.

In most cases, C3 behaved as a major acute-phase protein in response to bacterial stimulation [12]. In a previous study, expression levels of three isoforms of C3 in the liver, spleen, skin, and gills of dojo loach were significantly upregulated after infection of *A. hydrophila* [30]. Similar results for C3 were also discovered in the liver of rainbow trout stimulated with LPS [47]. In this study, the expression of C3 was up-regulated significantly in the liver and spleen at 24 h post-*A. hydrophila* infection. Therefore, C3 of southern catfish also participated in defense against bacterial infection.

In conclusion, the full-length cDNA of C3 from southern catfish was

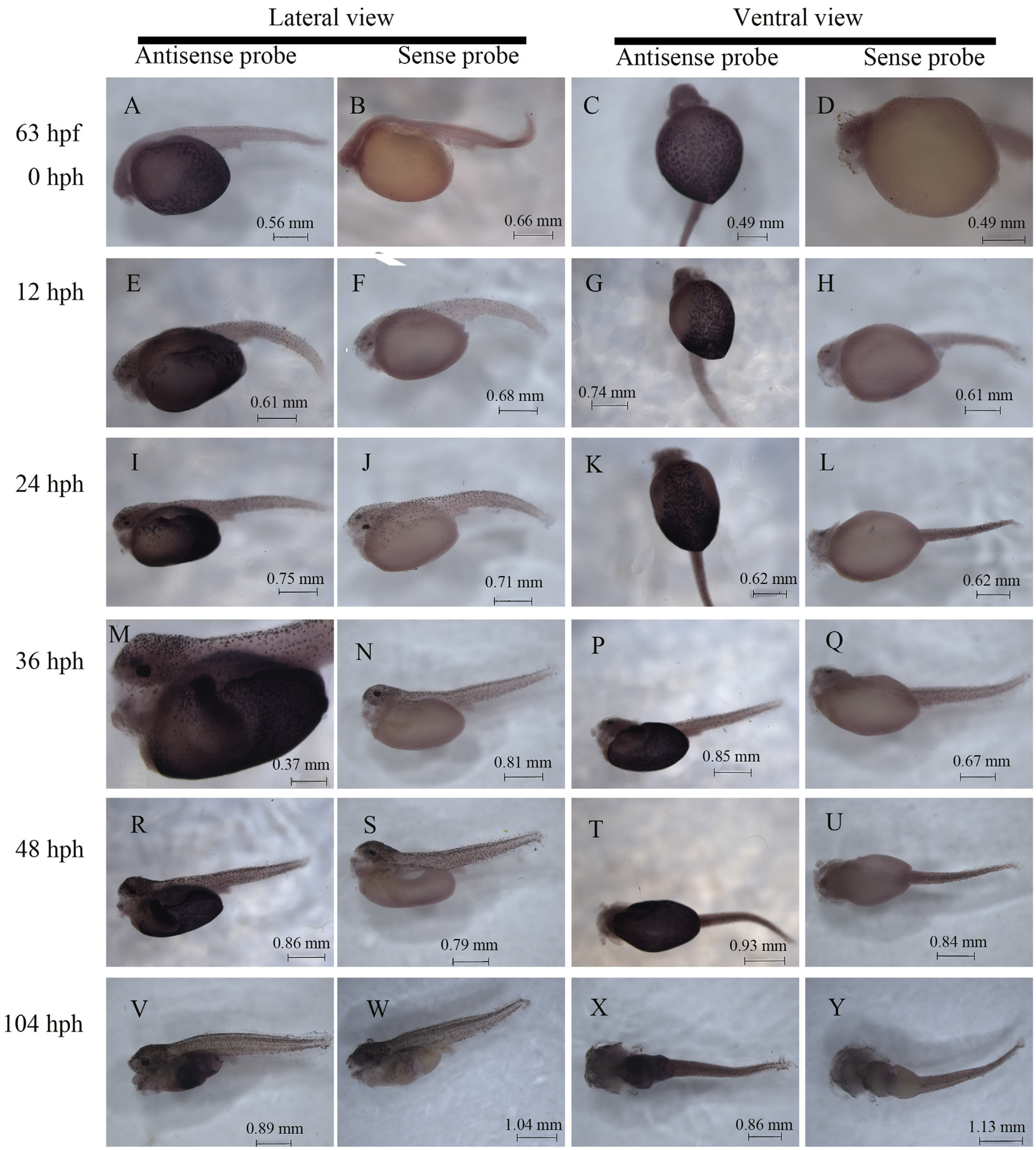

Fig. 8. Whole mount *in situ* hybridization analysis of C3 mRNA in larvae post-hatching. The expression of C3 in liver was detected at 36 hph using the antisense probe. All yolk was absorbed at 104 hph and the expression of C3 was presented only in the liver of southern catfish. The sense probe served as a negative control. hph, hour post hatching. Scale bars of A to U were 0.56, 0.66, 0.49, 0.49, 0.61, 0.68, 0.74, 0.61, 0.75, 0.71, 0.62, 0.62, 0.37, 0.81, 0.85, 0.67, 0.86, 0.79, 0.93, 0.84, 0.89, 1.04, 0.86, 1.13 mm, respectively.

identified and characterized, and the amino acid sequence of C3 was deduced. C3 was expressed in liver, spleen, stomach, intestine, and head-kidney. The primary site of embryonic C3 expression was the yolk syncytial layer, followed by the liver. Challenge experiments with *A. hydrophila* showed a significant upregulation of C3 in liver and spleen. Taken together, these results suggested that complement C3 played an important role in improving the defense ability of southern catfish against pathogens infection. The results provided an important information to understand the ontogeny of C3 in catfish belonging to Siluriformes.

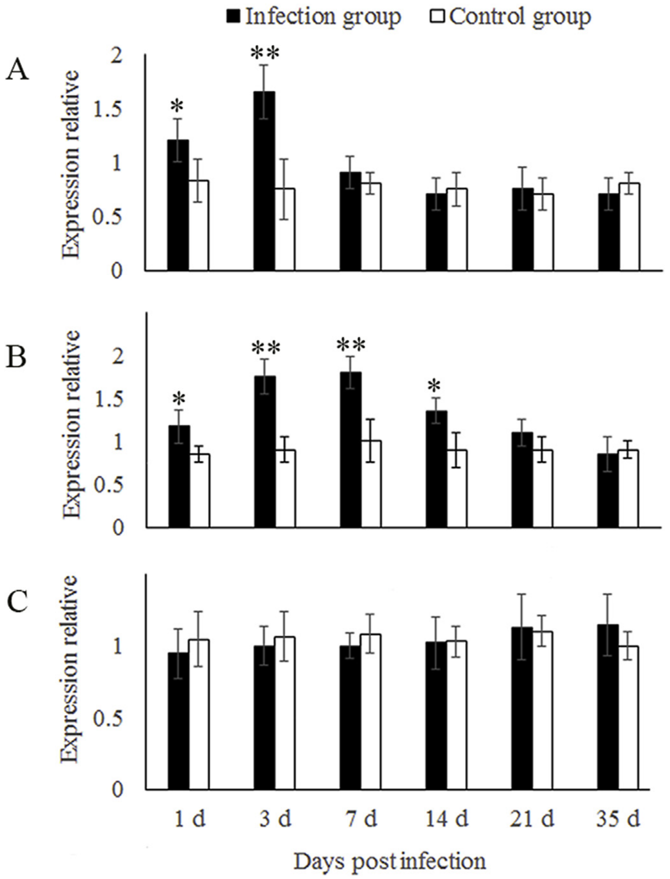

Fig. 9. The expression levels of C3 on 1, 3, 7, 14, 21, and 35 days in liver (A), spleen (B), and intestine (C) after infection with *Aeromonas hydrophila* determined by real-time PCR. Expression levels are normalized against 18S rRNA. Bars represented the mean ± SD (n = 3) for each time. *$p < 0.05$, **$p < 0.01$.

Conflicts of interest

The authors have no competing interests to declare.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 41476100, 31702382), the Science and Technology Project of Guangdong Province (2016B090918112, 2016B090918029), the Guangzhou Science and Technology Project (201604020040), the Marine and Fishery Special Project of Fish Diseases Treatment and Prevention in Guangdong Province (YueCaiNong [2016] No.11), as well as the China Postdoctoral Science Foundation (2017M612847).

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