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Kinetics of pIgR and IgM immune responses in snakehead (*Channa argus*) to inactivated *Aeromonas hydrophila* via immersion and intraperitoneal injection

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This research was intended to investigate the kinetics of polymeric immunoglobulin receptor (pIgR) and its ligand immunoglobulins (Ig) in snakehead (*Channa argus*) through different vaccine delivery methods. First, we employed Oxford Nanopore Technologies (ONT) sequencing technology to acquire the snakehead fish's complete transcriptome, and the full coding sequence (CDS) of pIgR was identified and isolated through transcriptome sequencing named *Ch*pIgR. The CDS sequence spanned 1251 base pairs, translating into a protein that consists of 416 amino acids and has a molecular weight of around 45.80 kDa. The *Ch*pIgR had a pair of immunoglobulin-like domains in addition to a transmembrane region and an intracellular region. Furthermore, a polyclonal antibody targeting the snakehead recombinant *Ch*pIgR was constructed. *Ch*pIgR and IgM responses were analyzed after immunization with *Aeromonas hydrophila*. Although *ChpIgR* and *IgM* displayed a comparable transcription level, *ChpIgR* exhibited a more rapid increase and reached its peak earlier than *IgM*. Both vaccinated groups experienced an elevation in *ChpIgR* mRNA levels in the gill and spleen. On the other hand, the bath immersion group manifested that the skin, gills, and intestines had an elevated *IgM* mRNA expression, whereas the intraperitoneal vaccination group reported that the spleen and head kidney possessed a greater expression. The ELISA results indicated that the IgM and *Ch*pIgR levels reached their highest peak more rapidly in the skin and gill mucus in the bathing group, whereas they reached a greater peak in intestinal mucus in the injection group. The outcomes indicated that both bath and intraperitoneal vaccinations increased *Ch*pIgR and secretory Ig levels in mucus and bile, shedding light on the function of pIgR in immune response and laying the foundation for future studies on pIgR-linked immune defenses in teleosts.

Keywords Snakehead (*Channa argus*), Polymeric Immunoglobulin receptor (pIgR), Immune response, Immunoglobulin

Mucosal immunity system, serving as the initial defense mechanism in vertebrates, plays a crucial role in safeguarding organisms from harmful microorganisms in the environment[1]. The polymeric immunoglobulin receptor (pIgR) in this framework is important for transporting polymeric immunoglobulins (such as dimeric IgA and, in some animals, pentameric IgM) from the lower to the upper surface of epithelial cells[1–3]. This process results in secreting sIgA into mucosal secretions. Polymeric IgA is transported through pIgR to act as an immune barrier by preventing pathogens from attaching and entering cells, as well as aiding in the neutralization of harmful microorganisms within cells[2,4,5]. The cDNA of the pIgR gene from the chicken species (*Gallus gallus*) has been effectively obtained[6], while the pIgR gene of the African clawed frog species (*Xenopus laevis*) has been extensively examined[7]. Research has shown that the pIgR protein is crucial for protecting the immune system in mammals, birds, and amphibians.

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PIgR shows high expression levels in the mucosal tissues of teleosts, such as the liver, gills, skin, and intestines[8–15], suggesting its role in the defense of teleost mucosa, similar to mammals. The pIgR in fugu (*Takifugu rubripes*) and rainbow trout (*Oncorhynchus mykiss*) specifically interacts with IgT or IgM in the mucus of the intestines or skin, respectively. However, flounders (*Paralichthys olivaceus*) possess a secretory component (SC) in mucus, not in blood, indicating its role in polymeric immunoglobulins (pIg) transcytosis, similar to mammals. The pIgR expression levels is impacted by various types of bacteria[13,15,16] and viruses[12]. The expression levels of common carp (*Cyprinus carpio*) pIgR in the intestine rise at first following *Vibrio anguillarum* exposure, then decline[17], paralleling the expression pattern of pIgR in different organs of turbot such as the skin, gill, stomach, intestine, head-kidney, and spleen[16]. Furthermore, the zebrafish (*Danio rerio*) *pIgR* mRNA expression is increased following *Streptococcus iniae* infection, yet lowers post-SHRV (*Snakehead rhabdovirusm*) infection[12]. Significantly, the study conducted by Yang et al.[14] discovered that pIgR might potentially engage with four types of bacteria (*Escherichia coli*, *Aeromonas hydrophila*, *Staphylococcus aureus*, and *Bacillus subtilis*) in sea bass (*Lateolabrax japonicus*), implying that pIgR may play a part in identifying harmful bacteria. Few reports have specifically addressed the immunological reaction of pIgR in relation to Ig immunity[18,19]. Hence, further investigation is necessary to comprehend the connection between pIgR immune reactions and Ig immune reactions.

The Chinese people highly value the economic importance of the snakehead fish (*Channa argus*)[20]. However, raising snakehead fish has encountered challenges because of infectious illnesses, particularly from *A. hydrophila*, leading to significant financial losses[20]. Oxford Nanopore Technologies (ONT) is an advanced method that uses nanopores to sequence single molecules in real-time, allowing for accurate identification of intricate gene structures like variable splicing and fusion genes with the long-read segment technology[21]. Herein, we employed ONT sequencing technology to acquire the snakehead fish's complete transcriptome. The full coding sequence (CDS) of pIgR was identified and isolated through transcriptome sequencing named *Ch*pIgR. Subsequent to immunization with *A. hydrophila* via bathing and intraperitoneal injection, the changes in the expressions of pIgR and IgM genes were investigated. Additionally, polyclonal antibodies (Abs) targeting pIgR were generated to analyze the variations in *Ch*pIgR and IgM concentrations in mucous secretions. All results are helpful for understanding of the pIgR function in mucosal immunity and help to improve the immune defense of snakeheads against infections.

Results

Analysis of *Ch*pIgR CDS

Sequence cloning with primers pIgR-F and pIgR-R to give the complete CDS sequence. The CDS of *Ch*pIgR was 1251 bp (Fig. 1), which encodes a 416 amino acids protein. Predicted values for the *Ch*pIgR included a molecular weight that reaches 45.80 kDa and an isoelectric point which is at 5.79.

Analysis of alignment revealed the presence of numerous preserved patterns in vertebrate pIgRs within *Ch*pIgR, such as preserved KxxC and DxGxYxC patterns (x representing any amino acid) in ILD1 and preserved KxxC and DxGxYxC patterns in ILD2 (Fig. 2). The constructed phylogenetic tree showed that the homologous pIgR of amphibians, birds, and mammals were grouped, while the pIgR of teleosts formed a separate cluster (Fig. 3).

Production of polyclonal Abs targeting r*Ch*pIgR and rIgM

The SDS-PAGE analysis revealed successful expression of the *Ch*pIgR and IgM recombinant proteins (r*Ch*pIgR and rIgM) in snakehead using Arctic-Express™ as the host strain after induction with IPTG. The proteins showed up as separate bands, and their molecular weights were 47.2 and 66.7 kDa respectively (Fig. 4 A–B).

Rabbits were administered purified r*Ch*pIgR and rIgM by injection to induce the production of Abs. The specificity of the samples was confirmed using western blot experiments, employing total protein isolated from Arctic-Express™ cells that contained the recombinant plasmids pET-28a-*Ch*pIgR or pET-28a-IgM. The individual band identification was accomplished with Abs specific to r*Ch*pIgR or rIgM (Fig. 4C, lanes 1 and 3). Conversely, the serum obtained from animals that were not immunized did not show any reaction (Fig. 4C, lane 5). The Abs exhibited a comparable response to the protein identified by the His-specific mAb in the western blot analysis (Fig. 4C, lanes 2 and 4).

Variations of *ChpIgR* transcription levels in various tissues

Following injection and incubation, the expression level of the *ChpIgR* gene rose steadily within 72 h across all the evaluated tissues, with the maximum being achieved at 48 h (Fig. 5). In the case of the immersion group, the *ChpIgR* gene showed its highest relative expression in the skin and gills 12 h after the immunization process had been carried out. The levels reached the highest values, being 10.7-fold and 12.96-fold greater than the controls, respectively ($P < 0.05$). At 24 h after immunization, the liver, intestine, and spleen showed the highest levels of $P < 0.05$, significantly higher than controls by 2.86-, 9.25-, and 11.36-fold, respectively. *ChpIgR* expression in the head kidney achieved its greater point at 48 h, showing a significant increase of 2.53 times contrasted with the control ($P < 0.05$). The injected group showed a remarkable increase in the expression of the *ChpIgR* gene in the liver and spleen at 12 h. The levels in the liver and spleen were 4.87-fold and 14.66-fold higher, respectively, than those in the original control group ($P < 0.05$). After 24 h of immunization, *ChpIgR* attained its highest value ($P < 0.05$) within the skin, the gills, and the head kidney, and the levels were 9.93-, 10.25-, and 3.70-fold higher respectively in comparison with those in the original control group. The immersion group showed greater *ChpIgR* genes relative expression in the tissues of skin, gills and intestine. While the injected group showed more *ChpIgR* expression in the other organs included the liver, the spleen, and the head kidney. *ChpIgR* gene expression regulation was greater in the spleen and gills of the injection and immersion groups compared to other tissues.

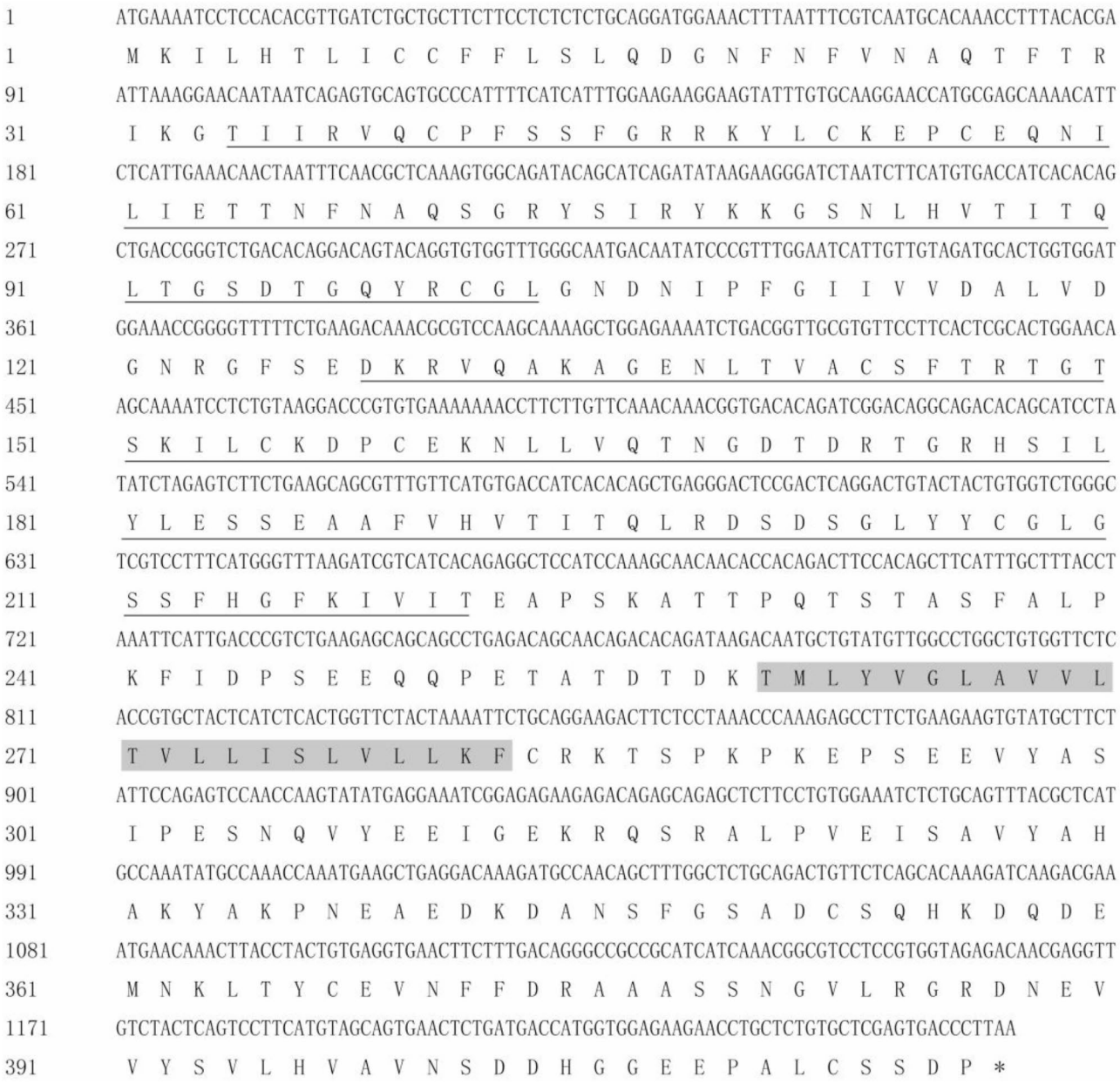

Fig. 1. Snakehead *Ch*pIgR's nucleotide and inferred amino acid sequences (GenBank accession number PQ001740). Underlined are the two Ig-like domains. The dark grey shading indicates the transmembrane region.

Variation in the expression of *IgM* in various tissues

The trend of *IgM* gene expression in each tissue was basically consistent with *ChpIgR*, showing a trend of rising first and then decreasing (Fig. 6). However, the level of the *IgM* gene increased slower than *ChpIgR*. The peak reached later than *ChpIgR*. Within the immersion cohort, *IgM* gene expression in the skin and gills reached their highest point four days after immunization, showing a 5.10- and 5.16-fold increase in comparison with the control group ($P < 0.05$), respectively. *IgM* gene expression reached their highest point at seven days post-immunization in the gut, head kidney, liver and spleen, showing a significant increase of 4.28-, 4.29-, 4.96-, and 3.93-fold contrasted with the control group ($P < 0.05$). *IgM* gene levels in various organs of the injection group reached their highest point seven days after immunization, with significant increases in comparison with the original control group ($P < 0.05$). The levels of spleen, head kidney, intestine, liver, gills and skin tissues exhibited peak values that were 9.09-, 8.95-, 6.10-, 6.00-, 3.95- and 3.48-fold higher respectively. The immersion group showed greater *IgM* genes relative expression in the tissues of skin and gills compared to the injected group. In contrast, in tissues included the intestine, liver, spleen, and head kidney, the injection group manifested an elevated *IgM* expression in contrast to the immersion group.

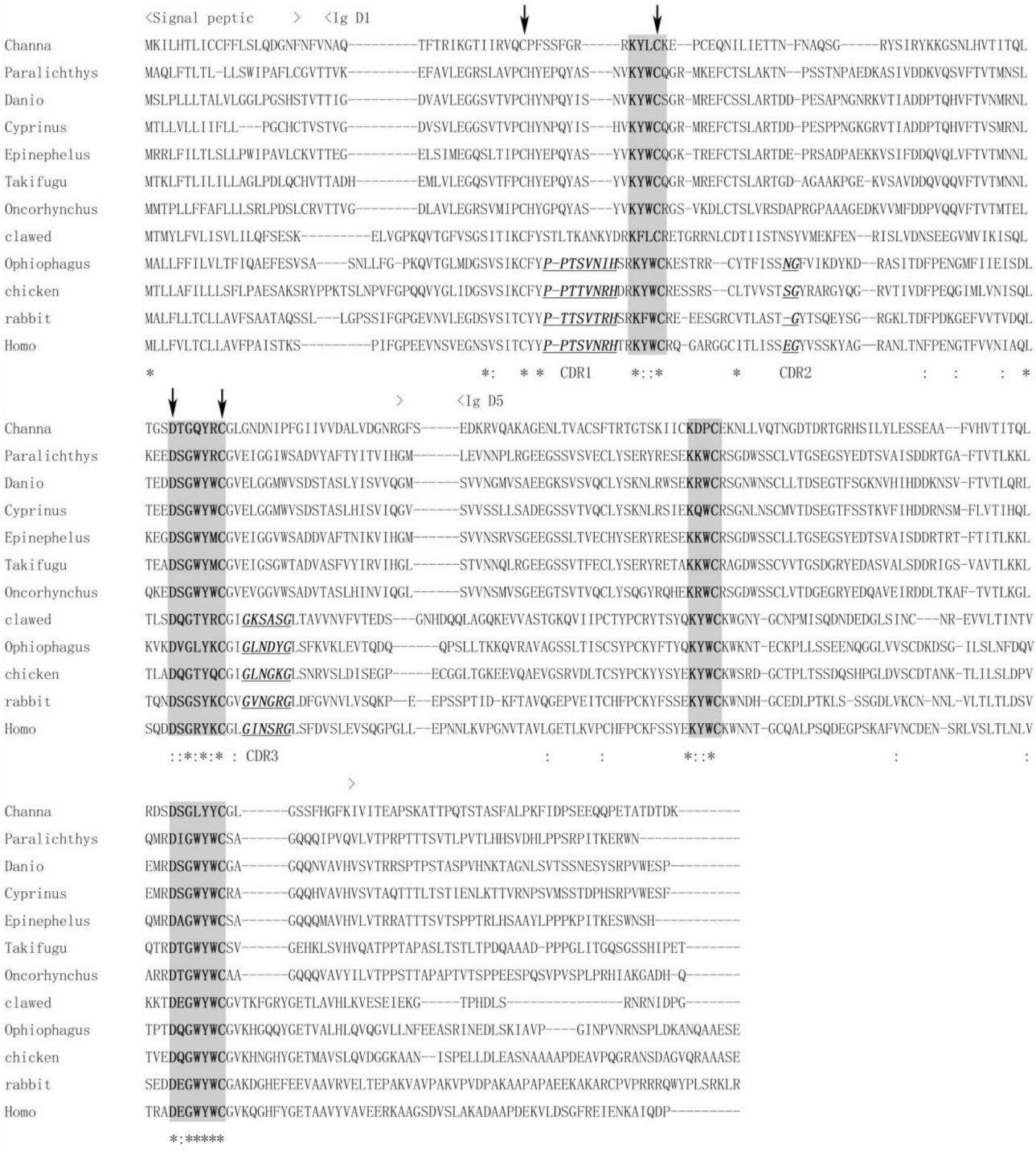

Fig. 2. Comparative alignment of snakehead *Ch*pIgR and pIgRs from several teleost and vertebrate species. The asterisk denotes residues that are the same in all the sequences, suggesting that the sequences are highly conserved. Bold preserved patterns in ILD1/5. Within the ILD1 of birds and mammals, there are CDR-like loops (CDR1/2/3) highlighted in bold and italics.

*Ch*pIgR protein kinetics in mucus and bile

Following vaccination with *A. hydrophila* via intraperitoneal injection and immersion, ELISA testing indicated that levels of *Ch*pIgR protein in exocrine secretions, initially increased within 28 days before returning to baseline levels (Fig. 7). Following immersion vaccination, the amounts of *Ch*pIgR protein peaked at 3 days after vaccination in the skin and gill mucus, at 5 days in the intestinal mucus, and at 7 days in the bile. *Ch*pIgR protein levels in intestinal mucus and bile achieved their greatest point 7 days following immunization in the injected group. In contrast, in skin and gill mucus, *Ch*pIgR peaked at 5 days after immunization. The levels of skin, gill,

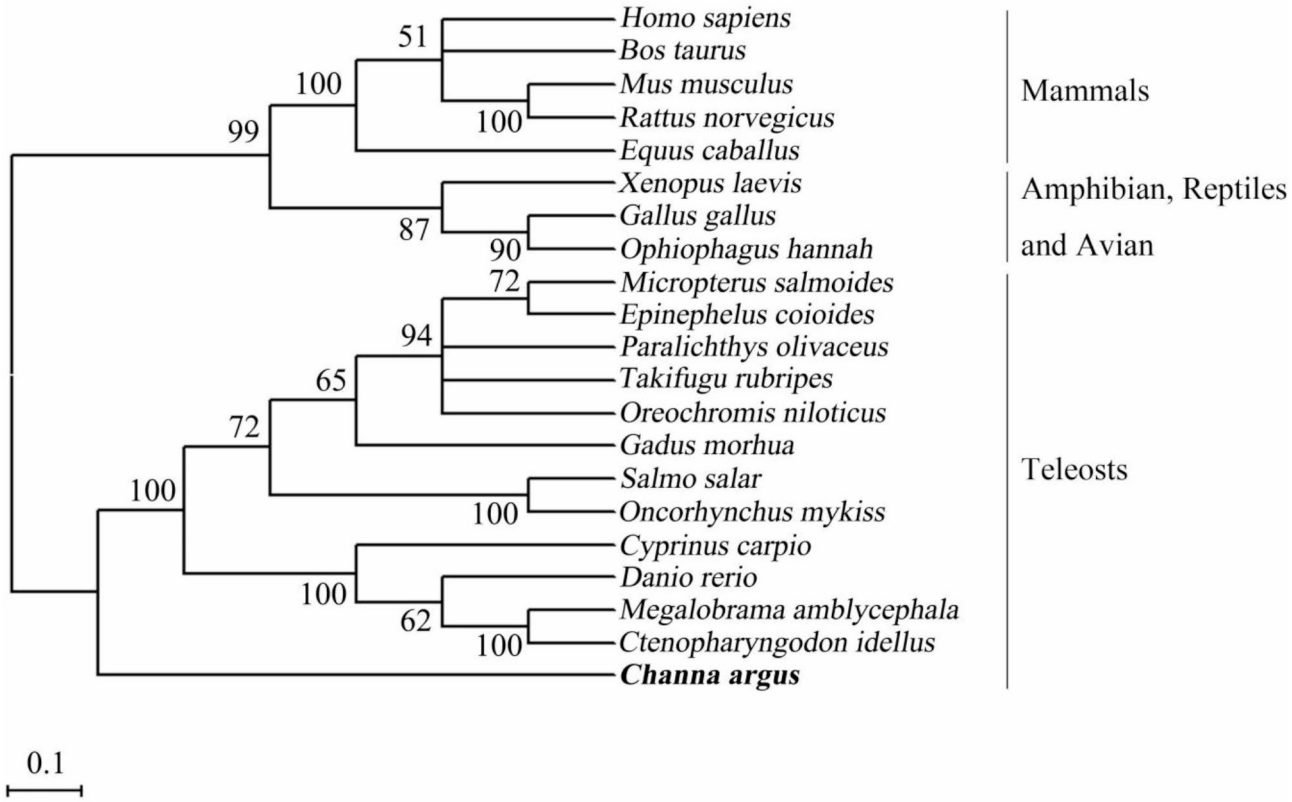

Fig. 3. A phylogenetic tree was created with the neighbor-joining technique, with the pIgR mature peptides from *Channa argus* and numerous vertebrates being involved. One thousand bootstrap replicates were performed on aligned sequences, with the branch numbers representing the bootstrap support. A scale bar is displayed in the bottom left corner.

and intestinal mucus were at their highest in the immersion group, whereas the level of bile was lower compared to the injection group.

IgM kinetics in mucus and bile

ELISA findings indicated that the levels of IgM protein in exocrine secretions, such as skin, gill, intestinal mucus, and bile, initially rose within 49 days and then returned to levels of the control group (Fig. 8). The trend of IgM protein levels and *Ch*pIgR protein levels were basically consistent in each immune group. However, the expression of IgM protein levels was increased later than that of *Ch*pIgR. In the immersion group, the expression level of skin mucus IgM peaked at 7 days after immunization, and the expression level of gill mucus IgM peaked at 14 days after immunization, in intestinal mucus and bile at 21 days after immunization. In the injection group, all exocrine IgM expression levels peaked at 21 days after immunization. The immersion group manifested the largest skin and gill mucus IgM levels contrasted with the injection group.

Discussion

In this investigation, according to the transcriptome data analysis, the CDS sequence of *Ch*pIgR was identified in snakehead. The primers were designed with the CDS sequence, and the *Ch*pIgR gene was cloned successfully. Using this information, we developed polyclonal Abs against the recombinant *Ch*pIgR and IgM proteins, analyzed the *ChpIgR* and *IgM* mRNA expression in snakehead, and investigated *Ch*pIgR and IgM protein levels in the bile, as well as mucus from the gills, skin, and intestines.

Mammals' pIgR homologs contain five potential Ig-like domains (ILDs), while birds and amphibians have four ILDs. Similar to other bony fish, the snakehead *Ch*pIgR had two ILDs that were similar to the ILD1 and ILD5 found in mammalian pIgR[8–12,15,16,22]. The results indicate that teleost pIgR containing two ILDs could transport IgM or IgT[8,22,23], while dimeric or trimeric IgA may need extra ILDs for attachment. A prior discovery indicating that IgM transcytosis remained almost unchanged even when ILD2/3 or ILD4/5 were removed provides support for this hypothesis[24]. Snakehead *Ch*pIgR in the current research exhibited numerous crucial amino acid residues in common with pIgRs found in other vertebrates. The pIgR ILD1 of vertebrates had similar critical motifs KxxC and DxGxYxC (where x exhibit any amino acid), while ILD5 of teleosts and different vertebrates contained the conserved KxxC and DxGxYxC. In line with the composition of pIgRs in other bony fish, the snakehead *Ch*pIgR was found to contain four of the five crucial amino acid residues of CWDC. This indicates that the teleost pIgR CWDC could help maintain the secondary structure. In contrast, teleosts lacked three CDR-like loops that were present in mammals and chickens, which were responsible for binding dimeric IgA and pentameric IgM[2,6,7,25].

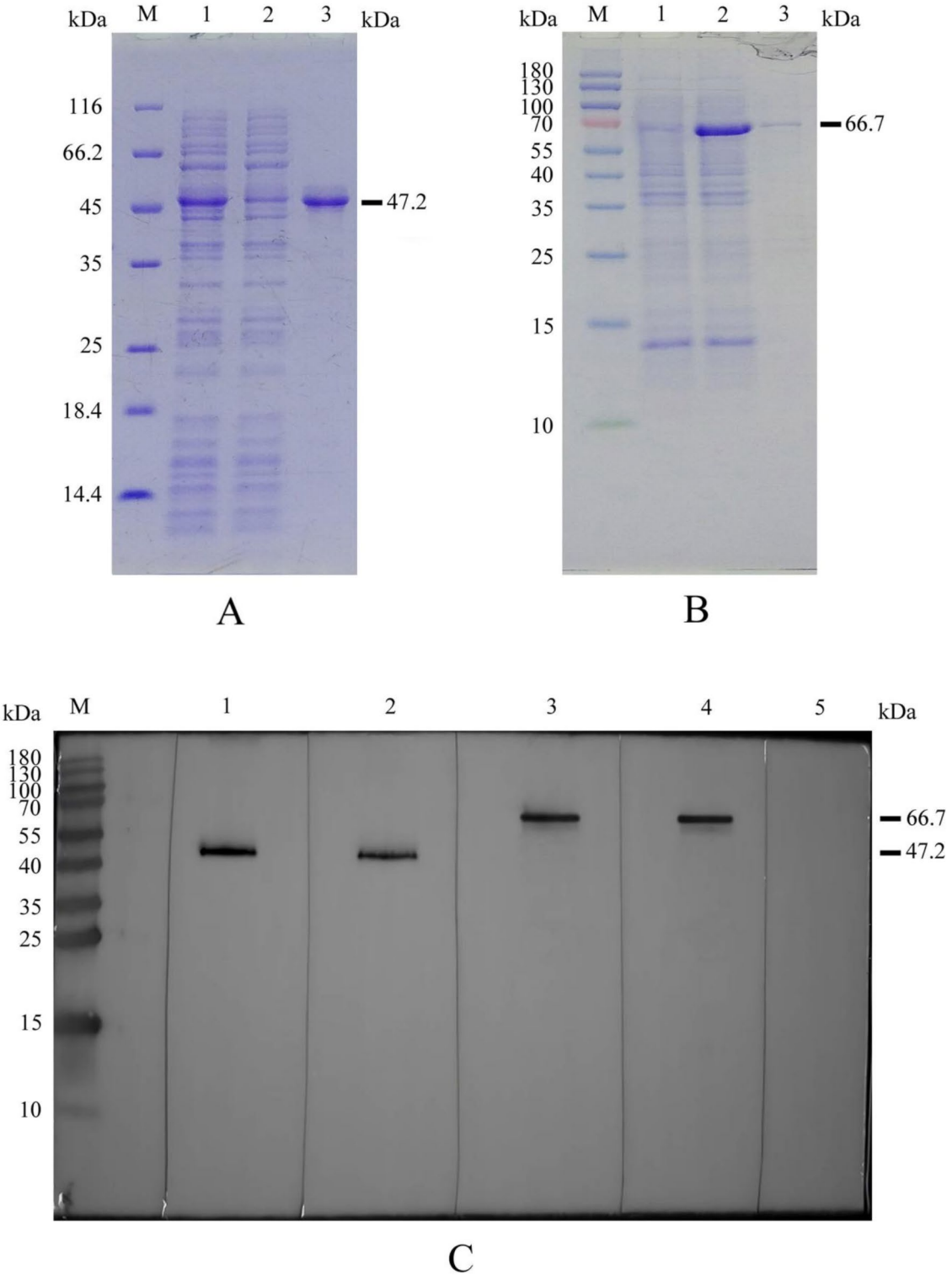

Fig. 4. Evaluation of protein separation using SDS-PAGE and western blotting. Lanes A1 and B2 display Arctic-Express™ cells that have been transfected with a recombinant plasmid and stimulated by IPTG. Conversely, lanes A2 and B1 depict Arctic-Express™ cells containing an empty plasmid that was stimulated by IPTG. Lane 3 displays purified recombinant snakehead *Ch*IgR or IgM. In the western blot analysis, lanes C1 and C3 exhibit Arctic-Express™ cells with recombinant plasmid immunostained with polyclonal Abs against *Ch*IgR and IgM, while lanes C2 and C4 show Arctic-Express™ cells with recombinant plasmid immunostained with anti-His mAb. Lane C5 serves as a negative control using non-immune rabbit serum.

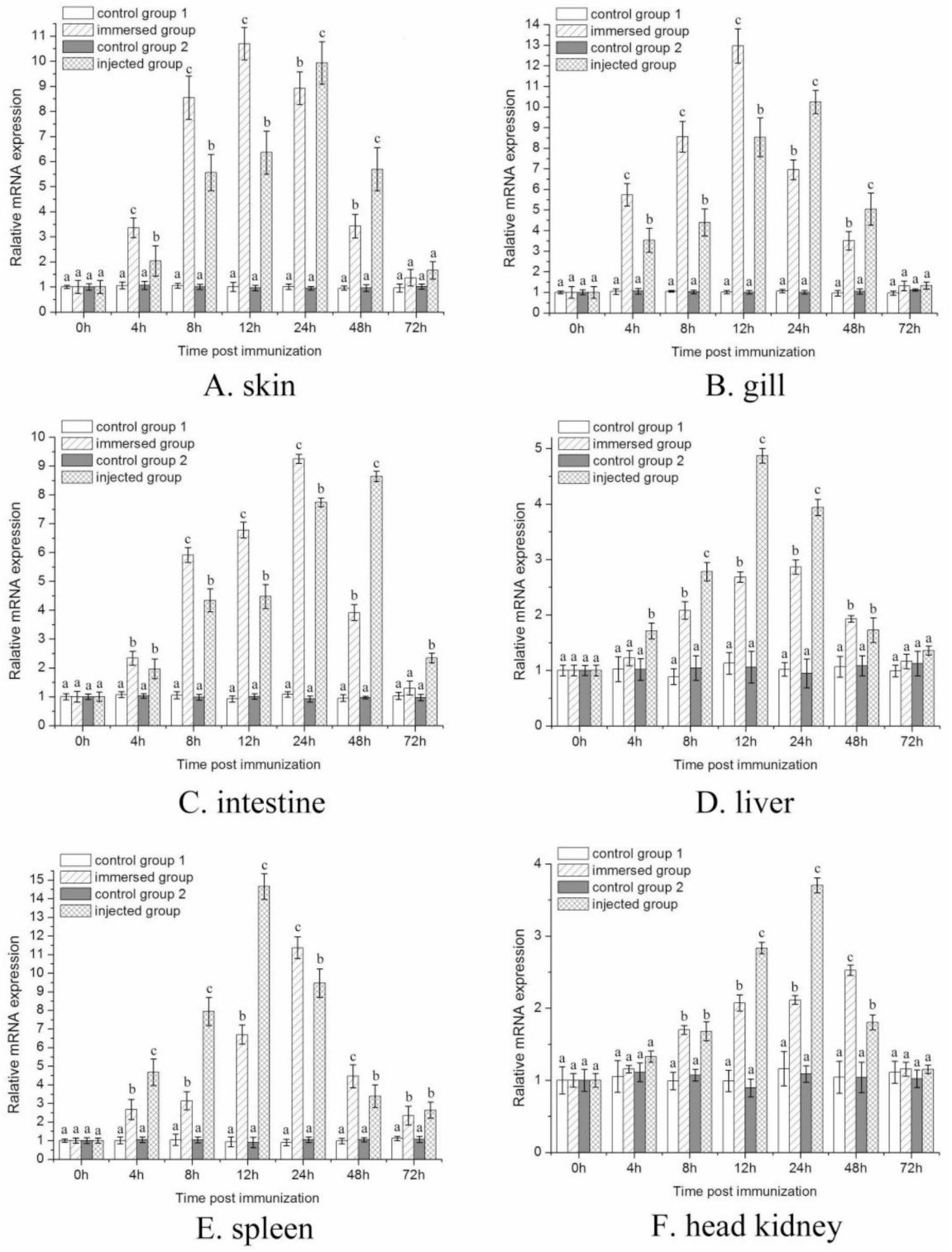

Fig. 5. Transcription levels of *ChpIgR* kinetics were studied in snakehead fish after being injected intraperitoneally and exposed to deactivated *A. hydrophila*. Letters "a", "b" and "c" on the bars represent the statistical significance ($P < 0.05$) compared to each other at same time point.

The lack of essential function patterns and CDR-like loops indicated that the way teleost pIgR binds to pIg may differ from that of mammals[8–12,15,16,22].

In this experiment, after injection and soaking immunization, the gene expression trend of *ChpIgR* and *IgM* in all tissues was generally consistent, with the trend of the first elevation and then hindrance. Both the injection and immersion groups exhibited a peak of *ChpIgR* within 48 h, with the immersion group reaching

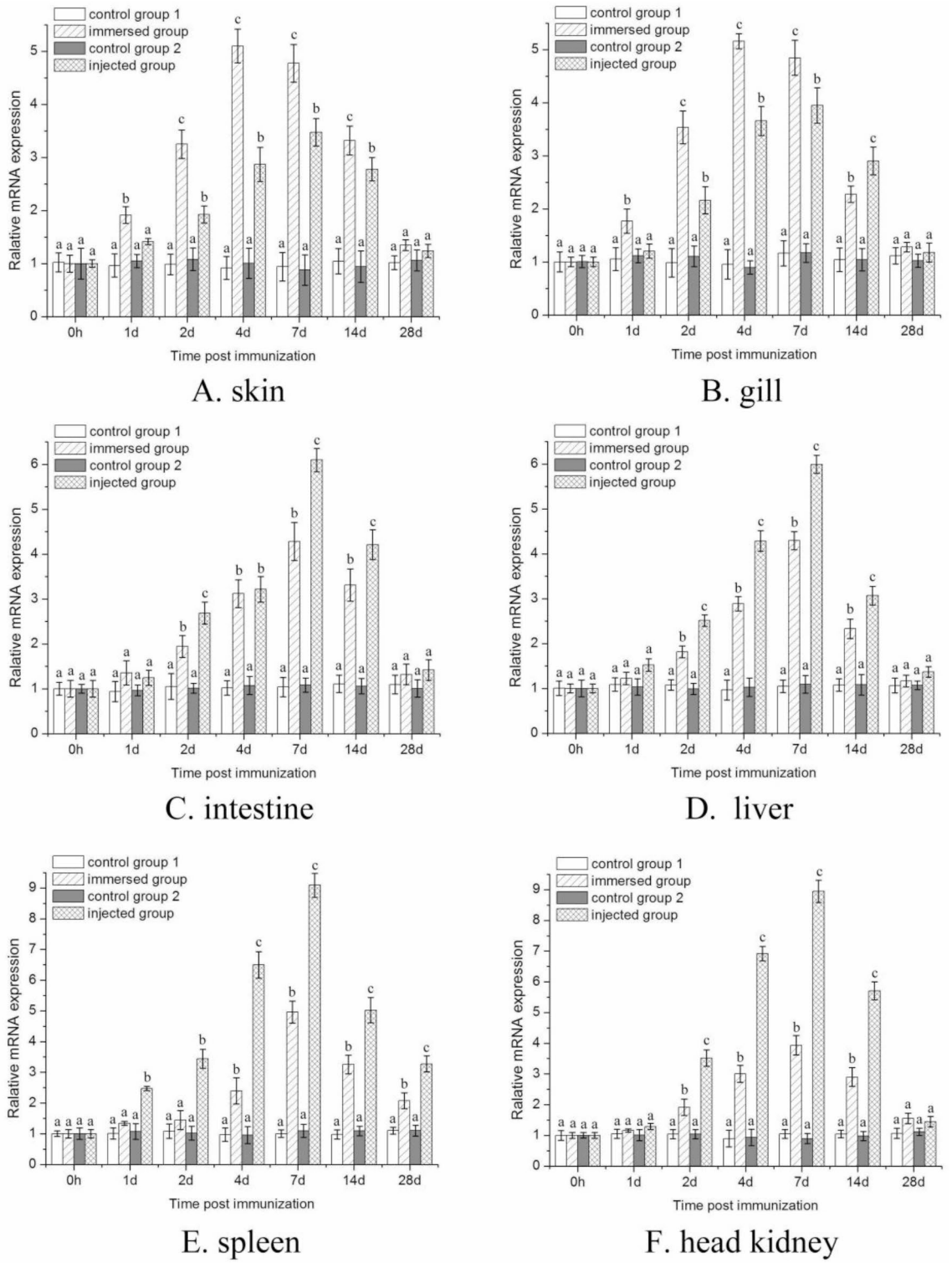

Fig. 6. Transcription kinetics of *IgM* levels were studied in snakehead fish after being injected intraperitoneally and exposed to deactivated *A. hydrophila*. Letters "a", "b" and "c" on the bars represent the statistical significance ($P < 0.05$) compared to each other at same time point.

its peak earlier in the gills and skin contrasted with the injection group. In the immersion group, the highest level of *IgM* gene expression was found at four days in the skin and gills, while in other tissues, it peaked at seven days. *IgM* expression peaked at seven days in all tissues of the immersion group. These results indicate that immune stimulation of *A. hydrophila* could induce upregulation of *ChpIgR* and *IgM* genes, and *ChpIgR* response was earlier than *IgM* while soaking immunization could induce *ChpIgR* and *IgM* expression response

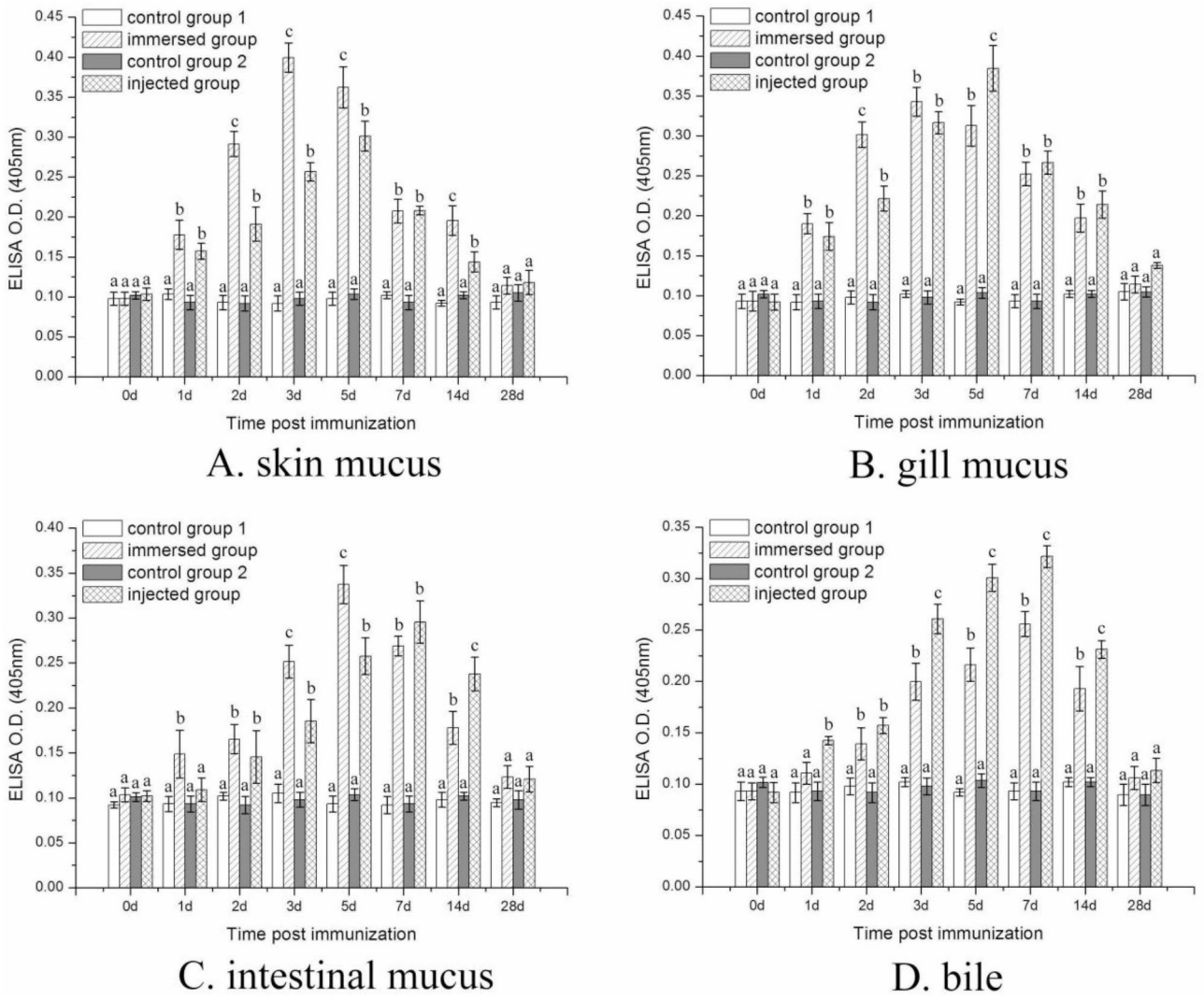

Fig. 7. The changes in *Ch*pIgR protein concentration after being injected intraperitoneally and exposed to deactivated *A. hydrophila*. A, B, C, D display the concentrations of *Ch*pIgR protein in the skin, gill, intestinal mucus, and bile of snakehead. Letters "a", "b" and "c" on the bars represent the statistical significance ($P < 0.05$) compared to each other at same time point.

earlier in mucosal immune tissues. Different results can be attributed to the various approaches used in experimentation[17,19]. Unlike intraperitoneal injection, bath vaccination allows for quicker responses from the gills and skin to *A. hydrophila*.

ChpIgR expression was quickly increased in tissues for a brief period, suggesting that pIgR contributes to the initial stages of an immune response. This reaction aligned with the results obtained in prior experiments conducted on Atlantic salmon, turbot, and grass carp[16,19,26]. Following intraperitoneal injection of *A. hydrophila*, snakehead showed a higher peak value of *ChpIgR* in the spleen compared to the gills and skin. Similarly, the spleen showed the maximum peak value of *ChpIgR* than the intestines in both turbot and flounder following antigen stimulation[16,17]. Thus, *ChpIgR* contributed to the immune system's response to pathogens in non-mucosal tissues. The choice of vaccination method would determine where the first immune response would occur, and the immune response of *ChpIgR* and *IgM* would demonstrate variability when several pathways delivered the same antigen.

The IgM expression level in skin mucus reached its highest point after 7 days of immersion immunization in snakehead, while in gill mucus, it peaked at 14 days, and in intestinal mucus and bile, it peaked at 21 days. At the same time, all exocrine IgM expression levels peaked at 21 days after injection immunization. The levels of skin mucus and gill mucus attained their maximum in the immersion group, although they were still lower than those in the injection group. This discovery was consistent with the IgM alterations observed in flounder after being exposed to *V. anguillarum*[27]. In summary, bath immunization effectively stimulated the synthesis of mucosal Abs. There is still much debate around the development and origins of fish mucus Ig[28–30]. Compared to intraperitoneal injection, the IgM protein level in the skin and gills increased faster with bath immunization, likely due to earlier contact with *A. hydrophila* during immersion immunization. According to our findings, the

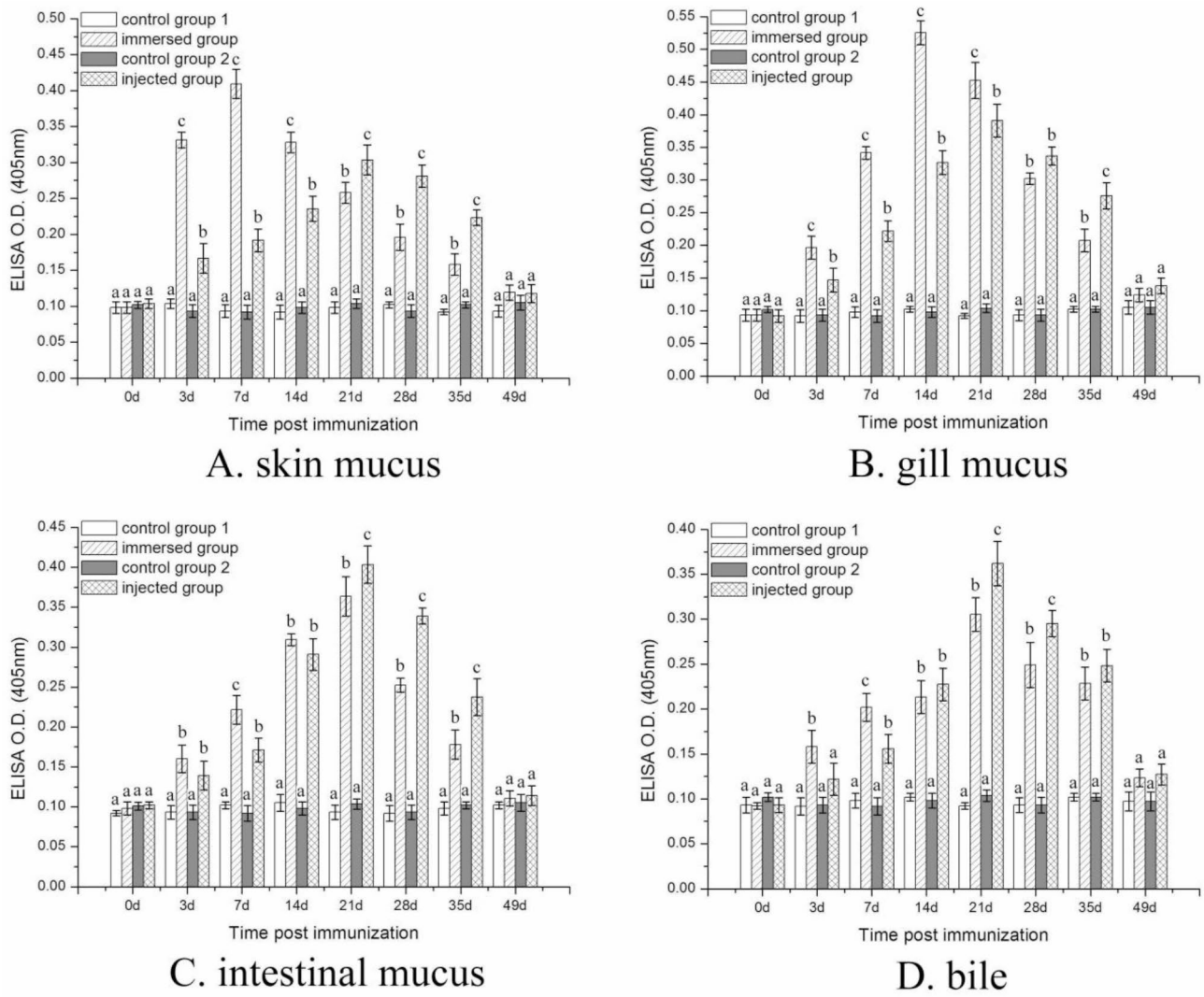

Fig. 8. The changes in IgM protein concentration after being injected intraperitoneally and exposed to deactivated *A. hydrophila*. A, B, C, D display the concentrations of IgM protein in the skin, gill, intestinal mucus, and bile of snakehead. Letters "a", "b" and "c" on the bars represent the statistical significance ($P < 0.05$) compared to each other at same time point.

Ig in snakehead skin mucus did not come from serum through passive transport. Additionally, we confirmed the presence of a separate mechanism for mucosal immune reactions, as suggested by Maki and Dickerson in their study on channel catfish[31]. In Antarctic teleost fish *Trematomus bernacchii*, IgM was observed in the liver and bile, accompanied by the aggregation of plasma cells in the hepatic portal tracts[32]. In addition, the presence of mucosal IgM was detected in the bile ducts of carp and flounder[18,29], suggesting that the liver could serve as a significant secretory organ for mucosal Ig, similar to mammals. Additionally, IgM was found in snakehead bile, and the IgM immune response in the bile was analogous to that of IgM in the intestinal mucus, indicating that pIgR facilitates the transcytosis of Ig from the liver to bile and then to the intestines[9,32].

In conclusion, we found the CDS sequence of *Ch*pIgR according to the transcriptome data analysis, and generated polyclonal Abs targeting the recombinant snakehead *Ch*pIgR and IgM proteins. Both bath immunization and vaccination through intraperitoneal injection were effective in eliciting *Ch*pIgR responses and enhancing the secretion of IgM in mucosal exocrine secretion, providing perspectives into fish mucosal immunity and laying the foundation for future studies on pIgR-linked immune defenses in teleosts.

Materials and methods

The establishment of a complementary DNA (cDNA) library

The snakehead (0.25 ± 0.05 kg) was obtained from Shandong Freshwater Fisheries Research Institute in good health. RNA was extracted from the snakehead liver with the RNA extraction kit (TIANGEN, Beijing, China). The creation of cDNA for sequencing followed the protocol provided by ONT (Oxford Nanopore Technologies). All procedures were carried out at Wuhan Benagen Technology Co., Ltd. The comprehensive cDNA libraries were created by using the cDNA-PCR Sequencing kit (ONT, Oxford, United Kingdom). Subsequently, the cDNA

Primer name	Primer sequence (5'-3') Application	
rpIgR F	**CATATG**CAGACCTTTACCCGCATTAA	gene cloning
rpIgR R	**CTCGAG**TTACGGATCGCTGCTGCAC	
rIgM F	**CATATG**CAGACCCTGACCGAAAGTG	gene cloning
rIgM R	**CTCGAG**TTACTGGGCAGTTTGGTTT	
qpIgR F	AGCTGAGGACAAAGATGC	qPCR primes
qpIgR R	GACTGAGTAGACAACCTCGT	
actin F	ATCGCCGCACTGGTTGTTGAC	internal control
actin R	CCTGTTGGCTTTGGGGTTC	
qIgM F	CTCAATGACCCCCCCTAA qPCR primes	qPCR primes
qIgM R	CACTGAAGGACGCTGAGT	

Table 1. Primers used in the present study. [a] M, A/C; Y, C/T; R, A/G; W, A/T. Underlined nucleotides indicated the restriction sites.

libraries were loaded onto the R9.4 sequencing chip and sequenced by means of the PromethION sequencer (ONT, Oxford, United Kingdom). The sequencing process lasted for a duration of 48–72 h. Following the sequencing process, NanoFilt (v2.8.0)[33] was implemented to filter raw fastq to exclude sequences that have quality scores less than 7 and sequences less than 50 bp. This resulted in the retention of only valid data for further analysis. The Pychopper program (v2.4.0) was used to ascertain complete sequences in precise sequencing data. Next, the filtered full sequences were compared to the snakehead reference genome with minimap2 (v2.17)[34] (https//ftp.ncbi.nlm.nih.gov/genomes/all/GCA/004/786/185/GCA_004786185.1_ASM478618v1/?spm=wolai.workspace.0.0.2f73767b7oxEuU).

Clone of the complete coding sequence for pIgR and sequence analysis

To determine the pIgR gene sequence found from the transcriptome sequencing results, ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) was implemented to ascertain the pIgR open reading frame (ORF) and the corresponding amino acid sequence. PCR primers covering the CDS were designed from this sequence: pIgR-F: ATGAAAATCCTCCACACGTTGAT, pIgR-R: TTAAGGGTCACTCGAGCACAGA. Liver RNA was isolated, and cDNA was synthesized following the protocol outlined by Xu et al.[19]. PCR amplification reactions with the CDS were performed using the first strand cDNA as a template and applying pIgR-F and pIgR-R primer pairs. The amplification product was sent to Chengdu Tsingke Biotech Co., Ltd. sequencing. The sequence obtained from sequencing was confirmed as the pIgR gene sequence by BLAST analysis. The sequence was named *Ch*pIgR, and the corresponding CDS was deposited in GenBank (Accession number PQ001740).

Protein analysis was conducted utilizing the ExPASY resources available at http://www.us.expasy.org. ClustalW programs were utilized to conduct multiple sequence alignments (http://www.ebi.ac.uk/clustalw). Employing the neighbor-joining method, we constructed a phylogenetic tree with MEGA relying upon the pIgR amino acid sequences from 21 vertebrates. Herein, the utilized accession numbers of pIgR-related proteins included *Micropterus salmoides* (MT919254), *Epinephelus coioides* (ACV91878), *Cyprinus carpio* (ADB97624), *Paralichthys olivaceus* (ADK91435), *Salmo salar* (ACX44838), *Takifugu rubripes* (BAF56575), *Oreochromis niloticus* (MK061423.1), *Gadus morhua* (AIR74929), *Oncorhynchus mykiss* (ADB81776), *Ophiophagus hannah* (ETE63349), *Danio rerio* (XP_694833), *Mus musculus* (AAA67440), *Megalobrama amblycephala* (OR677407.1), *Bos taurus* (NP_776568), *Ctenopharyngodon idellus* (KP768418), *Gallus gallus* (AAP69598), *Homo sapiens* (NP_002635), *Xenopus laevis* (ABK62772), *Oryctolagus cuniculus* (CAA25118), *Rattus norvegicus* (EDM09843), and *Equus caballus* (ACX69975.1).

Preparation of recombinant *Ch*pIgR and Ig

Specific primer design followed the CDS of *Ch*pIgR and gene sequences encoding IgM of snakehead (Genbank No. EU822510.1) (Table 1). Aiming at amplifying pIgR and IgM, primers were deployed to contain *EcoR* V and *Xho* I restriction sites, followed by cloning into pET28a. Afterward, Arctic-Express™ was administrated with the modified plasmids, and the desired clones were ascertained with PCR and confirmed by sequencing. Subsequently, the beneficial duplicates were added to an LB solution containing 50 μg/mL kanamycin and incubated at 37 °C in a rotating incubator until the OD_{600} value of the culture was between 0.6 and 0.8. Then the cell culture was subjected to a concentration of 1 mM/L IPTG in a stirring incubator at 37 °C for 6 h. The r*Ch*pIgR and rIgM proteins were subsequently isolated with denaturing conditions (8 M urea) on a column packed with Ni^{2+}-chelating Sepharose (GE Healthcare), following established techniques. Subsequently, the proteins were renatured in a TBS glycerol buffer using four cycles of dialysis. Each dialysis cycle lasted for a minimum of 12 h at 4 °C. Ultimately, proteins were isolated and identified with a 12% SDS-PAGE Gel. The BCA assay was employed to measure the quantity of the purified r*Ch*pIgR and rIgM[35].

The production of r*Ch*pIgR and rIgM polyclonal Abs

Typically, 200 μg of r*Ch*pIgR or rIgM was administered to New Zealand white rabbits by inoculation. Subsequent to two-week intervals, the rabbits were given their first booster dosage by the process of injection using a quantity of 200 μg of protein. Two additional doses of 100 μg of r*Ch*pIgR or rIgM were injected via the auricular vein every week. Serum samples from rabbits were obtained seven days after the final dose, and the levels of polyclonal Abs

against r*Ch*pIgR or rIgM were measured using ELISA. The Abs specificity was determined with western blot. Following the separation of the recombinant plasmid containing the Arctic-Express™ strain on SDS-PAGE Gel, the primary Abs were polyclonal Abs targeting r*Ch*pIgR or rIgM. A positive control was incorporated using anti-His mAb, while a negative control was incorporated using non-immune rabbit serum.

Preparing inactivated *A. hydrophila*

Our laboratory collected and stored the *A. hydrophila* specimen. *A. hydrophila* bacteria were cultured in LB medium for 48 h at 28 °C. Subsequently, we chose individual clones and introduced them into LB broth at 25 °C in a incubator with shaking. Subsequently, a solution of 0.5% formalin was used to deactivate the *A. hydrophila* for 72 h at a temperature of 4 °C. Afterward, the *A. hydrophila* were gathered through centrifugation, and the bacterial density was determined directly with an Accuri C6 flow cytometer (BD) before being stored at 4 °C for later usage.

Snakehead vaccinations and sampling

The snakehead (0.25 ± 0.05 kg) was obtained from Shandong Freshwater Fisheries Research Institute in good health. There were altogether 280 fish that were divided into four groups. The concentration of inactivated *A. hydrophila* was adjusted following the protocol outlined by Sheng et al.[18]. Briefly, healthy snakehead were injected at a dosage of 100 µL per fish, with the concentration of 1.0×10^8 CFU/mL, and throughout 20 days, no adverse reactions emerged. The first snakehead group was dipped into an inactivated bacteria solution with the concentration of 1.0×10^8 CFU/mL for 0.5 h, whereas the comparison snakehead group was submerged with a matching quantity of PBS for an identical period in water. The second set was vaccinated through intraperitoneal injection of the inactivated bacteria solution 100 µL with the concentration of 1.0×10^8 CFU/mL. Subsequently, three fish were randomly chosen in different time (0, 4, 8, 12, 24, 48, and 72 h, as well as 7, 14, and 28 days) from different group in order to evaluate gene expression. Prior to the dissection, the snakehead were killed with MS-222 at the concentration of 100 mg/L. RNA was isolated from immune organs contained the head kidney, skin, liver, gills, intestine, and spleen. Following that, the cDNA synthesis technique was conducted according to the approach presented by Xu et al.[19]. The samples were frozen at -20 °C, and the cDNA was preserved until it was ready for RT-PCR analysis.

For protein analysis, three fishes were randomly selected from each experiment group at various periods (0, 1, 2, 3, 5, 7, 14, 21, 28, 35, and 49 days) after immersing them. Subsequent to the dissection, the snakehead were killed with MS-222 at a dosage of 100 mg/L. Afterward, the skin was carefully scraped to gather skin mucus. The gathered mucus was then mixed with protease inhibitor solution (PBS 1X contained 1 mM PMSF and 0.5% BSA). Following three rinses with PBS 1X to eliminate any residual blood, the snakehead gills and the intestines were gathered and sliced. Subsequently, protease inhibitor buffer was added to the combination, which was then incubated at 4 °C for 2 h. The combination was periodically agitated throughout this incubation period. Gallbladder bile was collected and then rinsed with PBS. Following centrifugation of snakehead mucus and bile, the supernatant was kept at -70 °C until it was required.

Analysis of *Ch*pIgR and IgM

The *Ch*pIgR and IgM gene levels were ascertained using qRT-PCR. We utilized a Light Cycler 480 system (Roche) for the purpose of amplification using the SYBR Green PCR core Reagents (Applied Biosystems). The primer sequences are presented in Table 1. The amplification effectiveness of each primer was assessed by performing ten-fold serial dilutions on pooled cDNA, with the primer pairs showing efficiencies ranging from 1.95 to 2. The trial was performed three times, with actin serving as the control gene for every specimen. DEPC-treated water served as the negative control. The verification of a single PCR product was done by analyzing the amplification products post each PCR with the melting curve method. The primer pairs exhibited no primer-dimer artifacts and demonstrated a single peak in the dissociation curve when DEPC-treated H_2O was implemented as the template control throughout testing. To prevent gDNA contamination, cDNA was replaced with random RNA samples. For the analysis of relative expression levels, the $2^{-\triangle\triangle CT}$ technique outlined by Schmittgen and Livak[36] was employed.

ELISA analysis

The levels of *Ch*pIgR and IgM protein were analyzed following the ELISA protocol outlined by Sheng et al.[18]. Typically, 10 µg of mucus and bile was introduced to each well of ELISA plates overnight with 100 µL of carbonate-bicarbonate buffer with a pH of 9.6 (CB buffer, including 15 mM Na_2CO_3 and 35 mM $NaHCO_3$) at 4 °C. Afterward, They were rinsed three times by using 0.05% PBST. Subsequently, the protein's activity was inhibited over a period of 1 h at 37 °C by administering a 200 µL volume of a 4% BSA solution in PBS. Subsequently, the plates were cleaned and exposed to 100 µL of the polyclonal Abs in PBS with a dilution ratio of 1:1500 was used as the primary Abs. This treatment lasted for 1 h at 37 °C. Following an additional rinse, the plates were further treated with 100 µL of goat-anti-rabbit Ig-ALP conjugate in PBS (1: 4000) as a secondary Abs. This treatment lasted for 1 h at a temperature of 37 °C. After rinsing the plates once more, they were then treated with 100 µL of 50 mM carbonate-bicarbonate buffer (pH 9.8) containing 0.1% (w/v) pNPP (Sigma) and 0.5 mM $MgCl_2$. The sample was allowed to react for a duration of 30 min under dark conditions at ambient temperature. Following this, 50 µL of 2 M NaOH was put into each well, and the absorbance was quantified with an automated ELISA reader configured to detect light at a wavelength of 405 nm (Molecular Devices). The negative controls included wells that were coated with BSA and wells were incubated with non-immune rabbit serum in place of the primary Abs. The experiments were reproduced three times.

Data analysis

The data is displayed as averages. SPSS 16 was put into effect for the purpose of statistical analysis. The data was carefully examined with the use of one-way ANOVA, and Duncan's multiple comparisons of the averages were implemented accordingly. $P < 0.05$ demonstrated that the outcomes were statistically significant.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. The sequencing data were uploaded to the National Center for Biotechnology Information under Genbank number PQ001740.

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Author contributions
G.X. designed the entire experiment and wrote the manuscript. L.C. and X.C. participated in the data analysis. F.G., Y.J., X.L., H.X. and J.Z. participated in parts of the experiment. J.W. and Y.Q. edited and revised the paper. All authors approved the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical statement
All the procedures adhered to the Guide of the Institutional Animal Care and Use Committee at Shandong Freshwater Fisheries Research Institute.

Additional information
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