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Cloning, Characterization and Functional Analysis of *Caspase 8-like* Gene in Apoptosis of *Crassostrea hongkongensis* Response to Hyper-Salinity Stress

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Abstract: *Caspase-8*, a member of the caspase family, is an initiating caspase and plays a crucial role in apoptosis. In this study, the full-length cDNA of *caspase8-like* (*CASP8-like*) was isolated from *Crassostrea hongkongensis* (*C. hongkongensis*) by RACE-PCR. *ChCASP8-like* contained a 1599-bp open reading frame (ORF) encoding 533 amino acids with two conserved death effector domains (DEDs) and a cysteine aspartase cysteine structural domain (CASc). Amino acid sequence comparison showed that *ChCASP8-like* shared the highest identity (85.4%) with *CASP8-like* of *C. angulata*. The tissue expression profile showed that *ChCASP8-like* was constitutively expressed in gills, hepatopancreas, mantle, adductor muscle, hemocytes and gonads, and was significantly upregulated in hemocytes, hepatopancreas and gills under hyper-salinity stress. The apoptosis-related genes, including *ATR*, *CHK1*, *BCL-XL*, *CASP8-like*, *CASP9* and *CASP3*, were significantly activated by hyper-salinity stress, but were remarkably inhibited by *ChCASP8-like* silencing. The caspase 8 activity was increased by 1.7-fold after hyper-salinity stress, and was inhibited by 9.4% by *ChCASP8-like* silencing. Moreover, *ChCASP8-like* silencing clearly alleviated the apoptosis resulting from hyper-salinity stress. These results collectively demonstrated that *ChCASP8-like* played a crucial role in inducing apoptosis against hyper-salinity stress.

Keywords: *Crassostrea hongkongensis*; *caspase8-like*; apoptosis; hyper-salinity stress

Key Contribution: In this study; a novel *CASP8-like* gene was characterized from *C. hongkongensis*. The tissue expression profile showed that *ChCASP8-like* had constitutive expression in all tissues, and was significantly upregulated in gills, hepatopancreas and hemocytes after hyper-salinity stress. Apoptosis-related gene transcripts and caspase 8 activity were significantly increased under hyper-salinity stress, and significantly decreased after *ChCASP8-like* interference. Moreover, exposure to hyper-salinity stress caused severe apoptosis, which could be alleviated by *ChCASP8-like* silencing.

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1. Introduction

Apoptosis, or programmed cell death, plays an important role in the homeostasis and function of the immune system [1–3]. Apoptosis is divided into intrinsic and extrinsic apoptosis pathways based on the nature of initiating signals [4]. The intrinsic apoptosis pathway is activated to release cytochrome c from the mitochondria to promote cell apoptosis when the organism suffers from DNA damage, growth arrest, or virus infection [5].

The extrinsic apoptosis pathway is activated through interactions between extracellular ligands and death receptors, and initiates downstream caspase proteins [4].

Caspases (cysteine-dependent aspartyl-specific protease) are essential for the initiation and execution of apoptosis and inflammatory response [6]. As a member of the caspase family, *caspase 8* (*Casp8*) has been well characterized as an initiator caspase involved in extrinsic apoptosis in vertebrates [7]. *Caspase 8* associated with Fas-associated protein with the death domain (FADD) to form the death-inducing signaling complex (DISC) [6] undergoes self-cleavage and activates downstream caspase proteins to trigger apoptosis. The *Caspase-8-like* gene, which is the most similar to *caspase 8*, is considered to function as an initiator caspase and plays crucial roles in extrinsic apoptotic pathways in some mollusk species [4,8]. However, some researchers found that *caspase-8-like* worked as a caspase suppressor to inhibit apoptosis and immune signaling in silkworm (*Bombyx mori*) [9]. The contradiction in this research implies that functional differences in *caspase-8-like* exist in different invertebrates.

Crassostrea hongkongensis (*C. hongkongensis*), a commercially valuable marine bivalve in South China, lives in estuaries and exhibits remarkable euryhaline traits with an optimal salinity range of 10–25 ppt [10]. However, large salinity fluctuations in estuary areas often pose a significant threat to oysters, especially hyper-salinity stress due to drought and high temperature. Salinity fluctuation is a crucial environmental factor that affects the reproduction, growth, development and survival of aquatic animals. Changes in salinity create osmotic gradients between intra- and extracellular environments of aquatic species. If not compensated for, these changes might disrupt cell volume, impair protein function and ultimately lead to death [11]. Therefore, shellfish living in estuaries must adjust their physiology to maintain homeostasis in the intracellular environment of their organisms during periods of fluctuating salinity [12,13]. However, prolonged exposure to high salinity can decrease the immunity of bivalves, resulting in outbreaks of various diseases and mass mortality [14,15]. Apoptosis is a crucial process in oyster cells that effectively eliminates damaged, senescent and infected cells without triggering inflammation. The *CASP8* gene of *C. hongkongensis* has been identified and confirmed to activate the NF-κB and p53 signaling pathways in the immune response against bacterial challenge in a previous report. However, there was no elaborate description on the function of the *CASP8-like* gene in the apoptosis of *C. hongkongensis*.

In this study, a novel *CASP8-like* gene was cloned from *C. hongkongensis* and named *ChCASP8-like*. The expression profiles in different tissues were analyzed before and after hyper-salinity stress. The function of *ChCASP8-like* in apoptosis was identified by RNAi, TUNEL (Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling) and caspase activity assays.

2. Materials and Methods

2.1. Experimental Animals

First-aged healthy *C. hongkongensis* (mean weight of 142.2 ± 10.0 g, mean shell length of 6.1 ± 1.0 cm, mean shell width of 4.0 ± 0.5 cm, mean shell height of 9.9 ± 1.0 cm) were collected from an aquaculture farm in Zhanjiang, Guangdong Province, China. The oysters were temporarily raised in 20 ± 0.5‰ seawater at 25.0 ± 0.5 °C for 3 days and fed with *Isochrysis zhanjiangensis* once a day.

2.2. RNA Isolation and cDNA Synthesis

Total RNA was respectively isolated from 2 g of the gonads, adductor, mantle, gills, hepatopancreas, and hemocytes using TRIzol Reagent (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. The integrity of the RNA was verified through 1.0% agarose gel electrophoresis. RNA quality was checked by observing the 260/280 and 260/230 absorbance ratios using a NanoDrop 2000 Spectrophotometer (Thermo Fisher, Waltham, MA, USA). The cDNA was synthesized using 0.1 μg of RNA as a template and 1 μL of 0.1 μg/μL random primer. The first-strand cDNA template was synthesized using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions.

2.3. cDNA Cloning and Sequence Analysis

Following previous reports [16], the full-length cDNA of *CASP8-like* was cloned using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA, USA), the 5′ RACE system for rapid amplification of cDNA ends (Thermo Fisher, Waltham, MA, USA) and the SMART RACE cDNA amplification kit (Clontech, CA, USA). Based on the *ChCASP8-like* partial sequences in the transcriptome data, specific primers were designed using Primer Premier 5.0 (https://www.premierbiosoft.com, accessed on 8 April 2023) (Table 1). The *ChCASP8-like*-outer-F and UMP primers were used to amply the 3′ sequence of 667 bp, and *ChCASP8-like*-outer-R and UMP primers were used to amply the 5′ sequence of 606 bp. The PCR products were detected using a gel imaging system (Bio-Rad, Hercules, CA, USA), and the target fragments were collected. Nested-PCR was applied using *ChCASP8-like*-inner-F and *ChCASP8-like*-inner-R to enrich the specific DNA band. The full-length cDNA sequence of *ChCASP8-like* was validated by conducting a test-PCR using primers *ChCASP8-like*-test-F and *ChCASP8-like*-test-R. The primer sequences are shown in Table 1.

Table 1. Primers used in the study.

Primer	Forward Primer/Reverse Primer (5′–3′)	Application	Product (bp)
ChCASP8-outer-F	CCAGTGGTCCTATGGCGGAAGTGATG	3′RACE	667
ChCASP8-inner-F	TGACAATGGGTCTTTCTTCGTTCAATCC	nest-3′RACE	543
ChCASP8-outer-R	CAGCAACGGAAACAGT	5′RACE	606
ChCASP8-inner-R	GTCCAGACAGTCCCACACG	nest-5′RACE	561
UPM	TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE	
NUP	AAGCAGTGGTATCAACGCAGAGT	RACE universal primer	
ChCASP8-test-F	TCGTGTGGGACTGTCTGGA	cDNA test	1689
ChCASP8-test-R	TGCCTAGACCTCGCTTTCAA	cDNA test	
ChCASP8-siRNA-F	GCGTAATACGACTCACTATAGGGGATTCTGCGTCATCTTCA	RNA interference	469
ChCASP8-siRNA-R	GCGTAATACGACTCACTATAGGGACTTCCGTCATCACTTCC	RNA interference	
GFP-siRNA-F	GATCACTAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA	RNA interference	717
GFP-siRNA-R	GATCACTAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA	RNA interference	
β-actin-F	GTGCTACGTTGCCCTGGACTT	qRT-PCR	110
β-actin-R	TCGCTCGTTGCCAATGGTGAT	qRT-PCR	
ChCASP8-F	AACTGTTTCCGTTGCTGA	qRT-PCR	89
ChCASP8-R	TACTCGCCGACTTCTTGT	qRT-PCR	
ChCASP3-F	AGGCTGGCTGATTATGGG	qRT-PCR	120
ChCASP3-R	TCGTTTGTGACGGTTTGC	qRT-PCR	
ChATR-F	CCTTCCCAACAGACCCAA	qRT-PCR	130
ChATR-R	TCGCTGCCGTTCATCGTG	qRT-PCR	
CASP9-F	CGAGGTGGAAAGGAGAAC	qRT-PCR	146
CASP9-R	CTGGGTCAGACTGGAAAGA	qRT-PCR	
ChCHK1-F	CACACGAAAGGAGTTACCCACAGAG	qRT-PCR	105
ChCHK1-R	TCGAAACACAGTAGCCAGTCCAAAG	qRT-PCR	
ChBCL-XL-F	ACTCGTGGACTCTATCGTGGACTG	qRT-PCR	99
ChBCL-XL-R	GCAATTCTAAGCGACTCCCATCCC	qRT-PCR	

Using the blast program (http://www.ncbi.nlm.nih.gov/, accessed on 10 June 2023), the full-length cDNA of *CASP8* was analyzed. The open reading frame (ORF) was identified with the ORF Finder program (https://www.ncbi.nlm.nih.gov/orffinder/, accessed on 16 September 2023). The molecular weight and theoretical isoelectric point (pI) were analyzed using a program online (http://web.expasy.org/cgibin/protparam/protparam, accessed on 23 December 2023). The TMHMM procedure (https://web.expasy.org/protparam/, accessed on 25 December 2023) was used to predict the transmembrane domain, and the SignalP program (http://www.cbs.dtu.dk/services/SignalP/, accessed on 25 December 2023) was used to predict signal peptides. Multiple sequence alignments were created by the ClustalX program. The phylogenetic tree was constructed through the neighbor-joining method using MEGA7.0 software.

2.4. Salt Stress Experiment and Sampling

Healthy oysters were randomly divided into two groups and cultured in two tanks with hyper-salinity seawater (40‰) and natural seawater (20‰), respectively. In total, 25 experimental individuals were placed in each tank. The water used for aquaculture was created by mixing seawater with sea crystals to produce 40‰ seawater (Yantong, Jiangxi, China) [17–19]. After 0 and 48 h of salt stress, the adductor muscle, mantle, gills, hepatopancreas and gonads of 5 individuals were collected for subsequent gene expression analysis. The hemolymph was withdrawn from the pericardial cavity of experimental oysters using a 1 mL sterile syringe. Each hemolymph sample was a mixture of three individuals, and 5 parallel samples were collected at each time point. The hemolymph was centrifuged at 3000 rpm at 4.0 °C for 5 min to separate the hemolymph supernatant and hemocytes. The hemocytes were suspended in Trizol reagent (TransGen Biotech, Beijing, China) for RNA extraction.

2.5. RNA Interference (RNAi) Experiment

RNAi was performed to test the function of *ChCASP8-like*. The specific small interfering RNA (*ChCASP8-like*-siRNA) was synthesized by *ChCASP8-like*-siRNA-F and *ChCASP8-like*-siRNA-R (Table 1). The green fluorescent protein (GFP) was cloned from the pEGFP-N3 plasmid, and GFP-siRNA was generated by GFP-siRNA-F and GFP-siRNA-R primers as a negative control (NC). The siRNA was synthesized with the T7 High Efficiency Transcription Kit (TransGen, Beijing, China) and purified with the EasyPure RNA Purification Kit (TransGen, Beijing, China).

In the RNAi experiment, each oyster was intramuscularly injected with 50.0 µL of 1.0 µg/µL siRNA and reinjected with the same dose on the fourth day to enhance the silencing effect. After the injection, the oysters were subjected to salt stress for 48 h. The hemocytes from 3 individuals were mixed as a sample, and 5 parallel samples were collected for TUNEL assay. The gills, hepatopancreas, mantle, adductor muscle and gonads were collected from each experimental individual for gene expression analysis and caspase activity analysis. Five parallel samples were collected.

2.6. Quantitative Real-Time PCR (qRT-PCR) Analysis

The qRT-PCR was performed using the LightCycler 96 instrument (Roche, Basel, Switzerland) following the manufacturer's protocol for PerfecterStart Green qPCR SuperMix (TransGen Biotech, Beijing, China). The reaction was run in a 10 µL volume containing 20 ng of cDNA, 0.3 µM of each primer and 5 µL of Green qPCR SuperMix. The optimal PCR conditions were established as follows: 95 °C for 2 min followed by 35 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 20 s. Each sample was run in triplicate, along with the internal control gene β-actin. The specific primers are listed in Table 1. The relative expression level of each target gene was calculated by the $2^{-\Delta\Delta Ct}$ method [20].

2.7. Caspase 8 and Caspase 3 Activity Analysis in Gills of *C. hongkongensis*

The caspase 8 and 3 activities of *C. hongkongensis* were determined using the Caspase 8 and Caspase 3 Assay Kit (Nanjing Jiancheng, Jiangsu, China) according to the manufacturer's instructions. Briefly, 50 mg of gills was incubated with 50 µL of pre-cooled lysate and homogenized on ice for 15 min. Then, the mixture was centrifuged at 12,000 rpm for 10 min at 4 °C to separate the supernatant. A small amount of supernatant was used to determine the protein concentration by the Bradford method. For caspase 3 activity analysis, 50 µL of supernatant containing 200 µg of protein was mixed with 5 µL of Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroaniline) substrate and 50 µL of 2× buffer in the dark at 37 °C for 4 h. For caspase 8 activity analysis, 50 µL of supernatant containing 200 µg of protein was mixed with 5 µL of Ac-IETD-pNA (acetyl-Ile-Glu-Thr-Asp p-nitroaniline) substrate and 50 µL of 2× buffer. The concentrations of *p*NA released from the substrate by caspase 8 and caspase 3 were calculated according to the absorbance values at 405 nm [21].

The activities of caspase 8 and caspase 3 were assessed by measuring the OD405 values of the treated tissues in comparison to the control tissues.

2.8. TUNEL Assay

TUNEL assay was designed to detect apoptotic cells that undergo extensive DNA degradation in early and late stages of apoptosis [22].The TUNEL assay was performed according to the manufacturer's instruction of the TUNEL Apoptosis Detection Kit (Alexa Fluor 488). The hemocytes of experimental oysters were collected by centrifugation at 2000 rpm for 5 min. The hemocytes were resuspended in PBS (2×10^7/mL) and gently spread on polylysine-coated slides. Then, the hemocytes on the slide were fixed with 4% paraformaldehyde for 25 min at 4 °C and washed with PBS twice. The slide was incubated with 100 μL of 20 μg/mL protease K solution for 5 min at room temperature and washed with PBS three times. Samples were incubated in 5× Equilibration Buffer (Yesen, Shanghai, China) for 20 min and stained with 2 μg/mL of DAPI in the dark for 5 min. The hemocytes were examined under a fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA).

2.9. Statistical Analysis

Data were subjected to statistical analyses using SPSS 22.0 software (IBM, Armonk, NY, USA). A one-way analysis of variance (ANOVA) was performed to determine the significant difference in different samples by SPSS (24.0 version, IBM, USA). The significant differences among samples (N = 5) were presented as * $p < 0.05$, and highly significant differences were shown as ** $p < 0.01$.

3. Results

3.1. Cloning and Sequence Analysis of Caspase8-like from C. hongkongensis

The full-length cDNA sequence of the *CASP8-like* gene was cloned from *C. hongkongensis* by RACE-PCR (GenBank accession number: OR066208) and named *Chcaspase8-like* (*ChCASP8-like*). As shown in Figure 1A, the full cDNA sequence of *ChCASP8-like* was 2015 bp in length, containing a 1599-bp open reading frame (ORF), an 84-bp 5′ untranslated region (UTR) and a 332-bp 3′ UTR with a polyadenylation signal sequence (aataaa) located upstream of the poly (A) tail. The ORF encoded 533 amino acids. The bioinformatics analysis of cDNA sequences did not reveal signal peptides or transmembrane domains. The predicted polypeptide sequence contained a conserved cysteine aspartase cysteine structural domain (CASc) in the C-terminal and two death effector domains (DEDs) in the N-terminal (Figures 1B and 2A). The deduced molecular mass of *ChCASP8-like* protein was 59.17 kDa with a theoretical isoelectric point (pI) of 5.37.

3.2. Multiple Sequence Alignment and Phylogenetic Analysis

The amino acid sequence of ChCASP8-like was compared among different species. The ChCASP8-like shared the highest identity (85.4%) with CASP8-like of *Crassostrea angulata* (*C. angulate)*, 56.7% with CASP8-like of *Crassostrea virginica* (*C. virginica*) and 49.2% with CASP8-like of *Ostrea edulis* (*O. edulis*). However, the *ChCASP8-like* shared low identity with the *CASP8* gene of *C. hongkongensis*, which was only 30.0%. Lower identity was reported among *CASP8* of *C. hongkongensis* and vertebrates, ranging from 23.1% to 27.7% (Figure 2A).

The phylogenetic trees were constructed using MEGA7 to indicate the evolutionary relationship of *caspase 8* from different species (Figure 2B). The results showed that all *caspase 8-like* genes from the referred mollusks were clustered into one branch, including *C. hongkongnsis*, *C. angulata*, *C. virginica and O. edulis.* All *caspase 8* genes from referred species were grouped into a big cluster, in which caspase 8 from vertebrates were classified to a close cluster, including *Danio rerio*, *Mauremys reevesii*, *Gallus gallus* and *Homo sapiens*. The *caspase 8-like* and *caspase 8* gene of *C. hongkongnsis* exhibited farther distance.

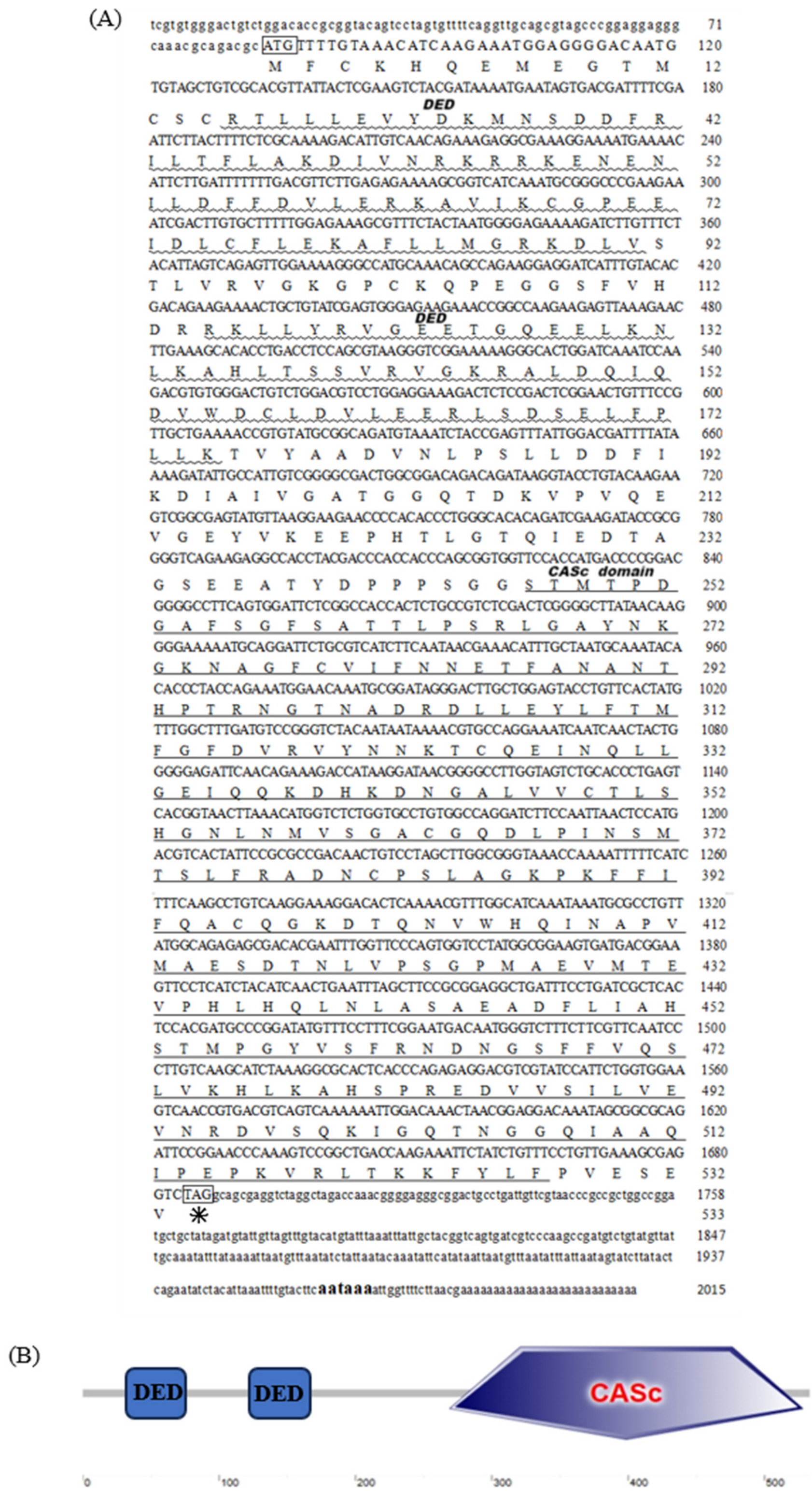

Figure 1. (**A**) Nucleotide and deduced amino acid sequences of *ChCASP8-like*. 5′UTR and 3′UTR sequences are shown in lowercase letters, and ORF sequences are shown in uppercase letters. The start codon (ATG) and the stop codon (TAA) are marked with boxes. The conserved CASc domain is underlined, and DEDs are shown with a wavy line. The putative polyadenylation signal (aataaa) is marked in bold. "✳" represents the termination codon. (**B**) The conserved DEDs and CASc domain of *ChCASP8-like* were predicted byConserved Domain Database (CDD).

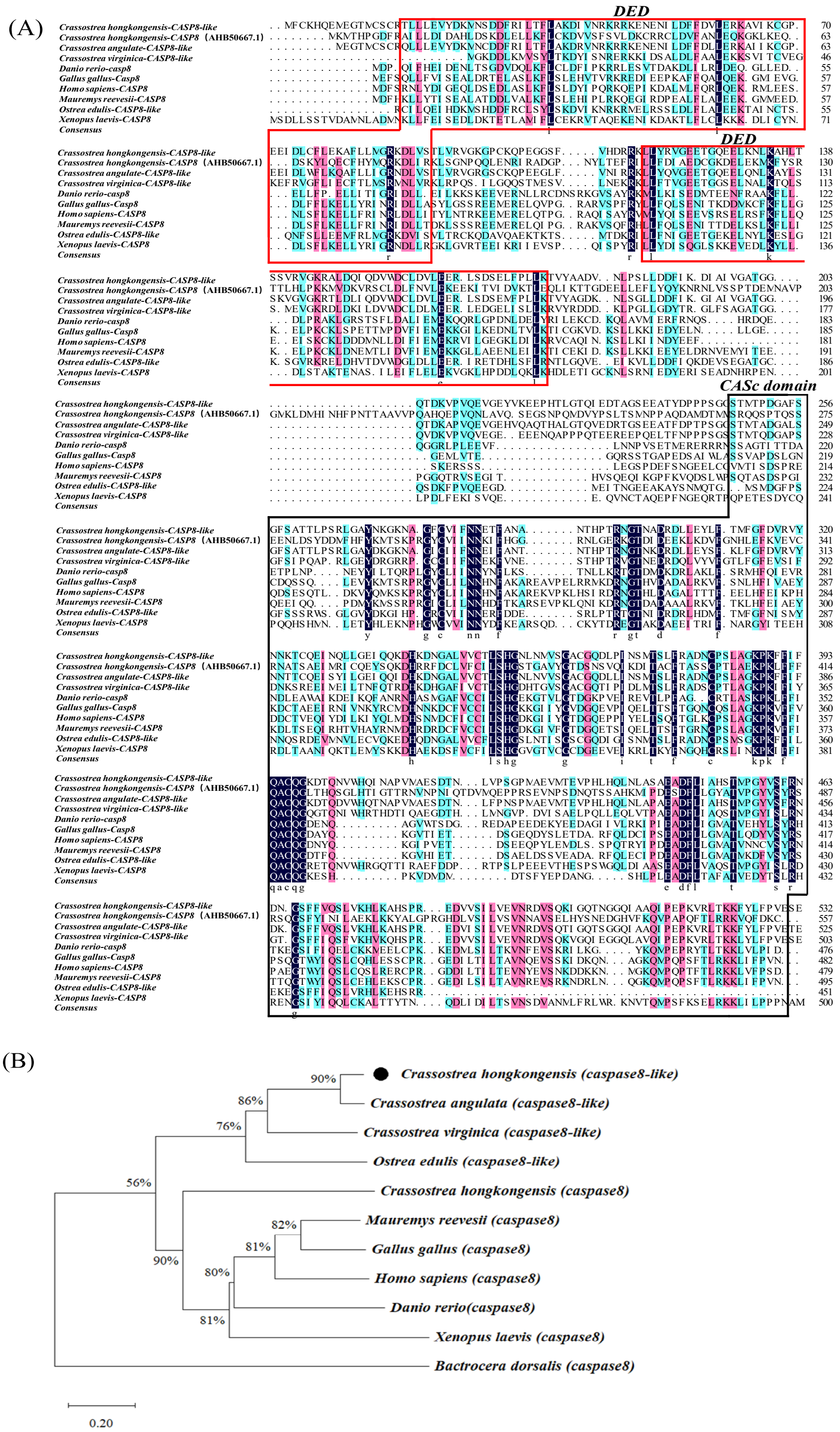

Figure 2. Amino acid sequence comparison and phylogenetic analysis. (**A**) Multiple sequence comparison of *caspase 8-like* and *caspase 8* genes from different species, including *Crassostrea hongkongnsis* (*CASP8-like*, OR066208), *Crassostrea hongkongnsis* (*CASP8*, AHB50667.1), *Crassostrea angulata* (*CASP8-like*, XP_052712040.1), *Crassostrea virginica* (*CASP8-like*, XP_022294614.1), *Danio rerio* (*casp8*, NP_571585.2), *Gallus gallus* (*Casp8*, NP_989923.3), *Homo sapiens* (*CASP8*, AAD24962.1), *Mauremys reevesii* (*CASP8*, XP_039350847.1), *Ostrea edulis* (*CASP8-like*, XP_048769780.1), *Xenopus laevis* (*CASP8*, NP_001079034.1) and *Bactrocera dorsalis* (*CASP8*, JAC47606.1). Identical residues were shown in black, highly conserved residues in red, and conserved residues in blue. The CASc and DED structural domains are shown in black and red boxes, respectively. (**B**) Phylogenetic tree analysis. The number on the node indicates the bootstrap value determined by bootstrap analysis for 1000 repetitions. • represents the *caspase 8-like* gene of *C. hongkongnsis* in this research.

3.3. *ChCASP8-like* Expression Profile in Different Tissues

The relative expression levels of *ChCASP8-like* in different tissues were detected by qRT-PCR before and after salt stress. As shown in Figure 3, *ChCASP8-like* was constitutively expressed in all analyzed tissues, including gills, hepatopancreas, mantle, adductor muscle, hemocytes and gonads. Before salt stress, *ChCASP8-like* had the highest expression level in the adductor muscle, a higher level in the gills and mantle, and lower level in the hepatopancreas, gonads and hemocytes. *ChCASP8-like* transcripts were significantly up-regulated in immune tissues such as gills, hepatopancreas and hemocytes after 48 h of hyper-salinity stress, including a 3.2-fold increase in the gills ($p < 0.01$), a 3.0-fold increase in the hepatopancreas ($p < 0.01$) and a 3.0-fold increase in hemocytes ($p < 0.01$), but it was significantly downregulated by 1.7-fold in the adductor muscle ($p < 0.05$). These data indicated that *ChCASP8-like* was involved in the immune response against hyper-salinity stress.

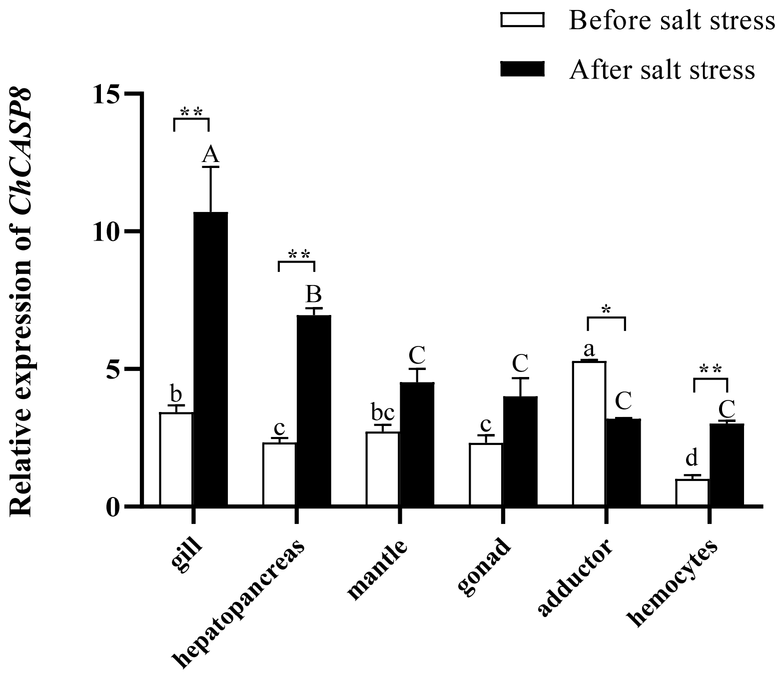

Figure 3. The relative expression of *ChCASP8-like* in different tissues before and after hyper-salinity stress. Different lowercase letters (abc) indicate significant differences among tissues before hyper-salinity stress ($p < 0.05$). Different capital letters (ABC) indicate significant differences among tissues after hyper-salinity stress ($p < 0.05$). Expression differences in the same tissue before and after hyper-salinity stress are indicated using * ($p < 0.05$) or ** ($p < 0.01$) ($N = 5$).

3.4. The Transcriptions of Apoptosis-Related Genes Were Stimulated by Hyper-Salinity Stress, but Were Inhibited by *ChCASP8-like* Silence

To analyze the function of *ChCASP8-like* in the analyzed tissues following hyper-salinity stress, *ATR*, the master regulator of the DNA replication stress response [23–25], was significantly increased by 5.9-fold ($p < 0.01$). *CHK1*, an important kinase involved in the S phase DNA damage checkpoint, was remarkably upregulated by 9.9-fold ($p < 0.01$). The *CASP8-like,* an initiator caspase [26], was significantly upregulated by 5.4-fold ($p < 0.01$). *CASP9*, an efficient executor of apoptosis [27], was significantly increased by 8.0-fold ($p < 0.01$). *CASP3* and *BCL-XL* transcripts showed no significant difference ($p > 0.05$). These results indicated that hyper-salinity stress induced the expression of most apoptosis-related genes.

However, under *ChCASP8-like* silence and hyper-salinity stress, the transcripts of *ATR, CHK1, BCL-XL, CASP8-like, CASP9* and *CASP3* were significantly inhibited compared to the hyper-salinity stress groups, and recovered close to the control level (NC group) (Figure 4). These data indicated that the stimulatory effects of hyper-salinity stress on apoptosis-related genes were blocked by *ChCASP8-like* silencing.

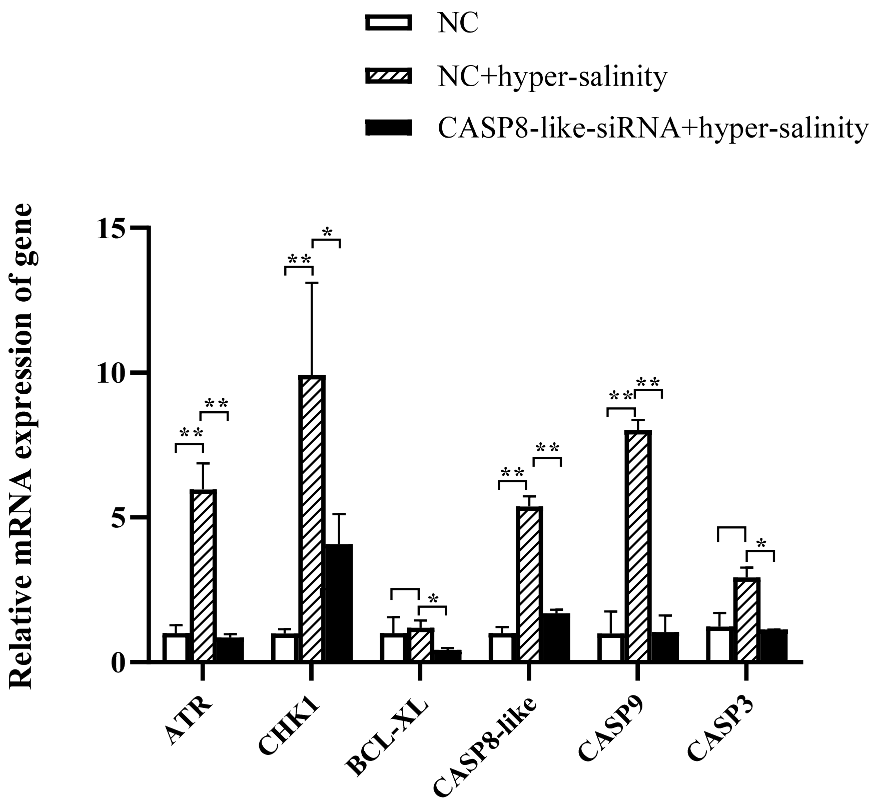

Figure 4. Transcriptional levels of *ATR*, *CASP8-like*, *BCL-XL*, *CASP9* and *CASP3* in *C. hongkongnsis* after *ChCASP8-like* interference and hyper-salinity stress in gill tissue. NC means the negative control group. β-actin was used as an internal reference. Data are presented as mean ± standard deviation (N = 5). * means $p < 0.05$ and ** means $p < 0.01$.

3.5. The Caspase 8 Activity Was Increased by Hyper-Salinity Stress, but Was Inhibited by ChCASP8-like Silence

Caspase 8 *activity* is an important indicator of the apoptosis degree. The caspase 8 activity was examined after hyper-salinity stress and *ChCASP8-like* silencing in gills. As shown in Figure 5A, compared with the control group, the caspase 8 activity was significantly increased by 1.7-fold ($p < 0.01$) after hyper-salinity stress. With *ChCASP8-like* knockdown and hyper-salinity stress, the caspase 8 activity was inhibited by 9.4% ($p < 0.05$) compared to the hyper-salinity stress group. The data showed that caspase 8 activity was significantly activated by hyper-salinity stress, but was slightly reduced by *ChCASP8-like* silencing, consistent with apoptotic gene expression analysis.

Caspase 3 was a key executor of apoptosis and played a crucial role in the final step of apoptosis. Therefore, the caspase 3 activity was analyzed here. The caspase 3 activity assay showed no significant difference after hyper-salinity stress and *ChCASP8-like* silencing (Figure 5B).

3.6. ChCASP8-like Silencing Alleviated the Apoptosis Resulted from Hyper-Salinity Stress

To further explore the effect of *ChCASP8-like* apoptosis resulted from hyper-salinity stress, the TUNEL assay was performed to detect the DNA damage of hemocytes in the *ChCASP8*-like-siRNA and GFP-siRNA groups. As shown in Figure 6, without the hyper-salinity stress, almost no damaged cells were found in the NC group. After hyper-salinity stress, approximately 87.5% of the hemocytes showed DNA breaks in the positive cells. After silencing *ChCASP8-like*, fewer DNA breaks were observed in hemocytes in approximately 36.4% of the positive cells. The data indicated that exposure to hyper-salinity stress caused severe apoptosis, which could be alleviated by *ChCASP8-like* silence. The

ChCASP8-like played a crucial role in activating apoptosis against hyper-salinity stress in oysters.

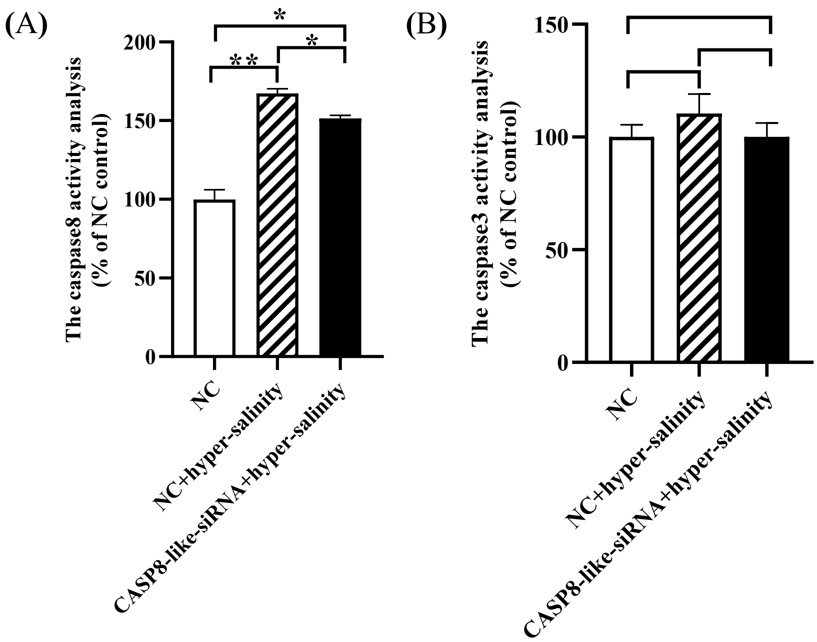

Figure 5. Caspase 8 and caspase 3 activity were analyzed after hyper-salinity stress and *ChCASP8-like* silencing in gills. (**A**) Caspase 8 analysis. (**B**) Caspase 3 analysis. Data are presented as mean ± standard deviation (N = 5). * means $p < 0.05$ and ** means $p < 0.01$.

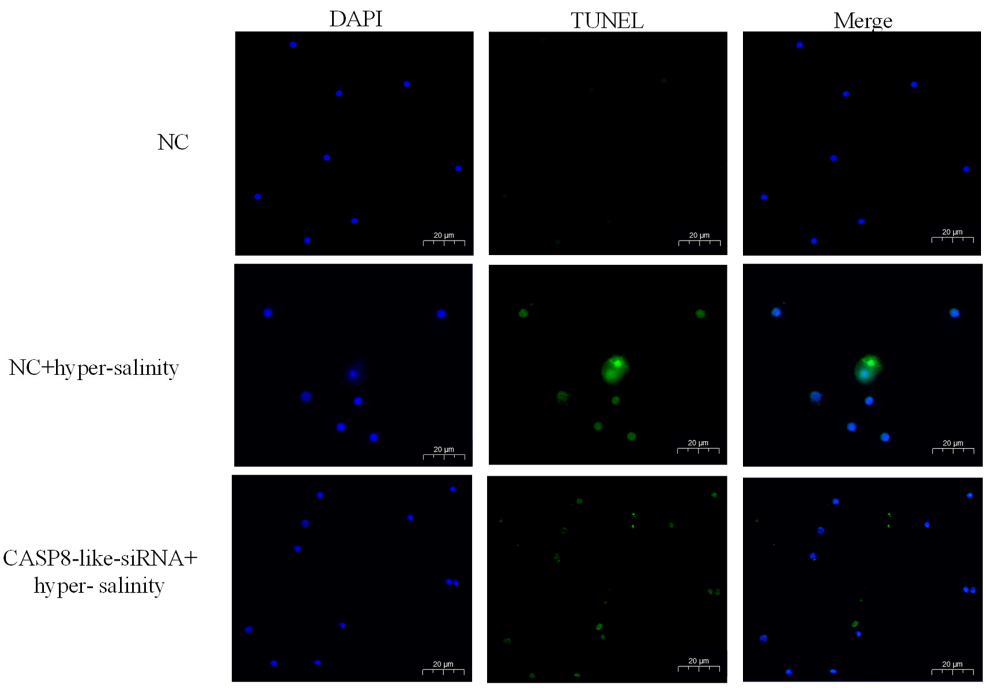

Figure 6. Fluorescence micrographs of apoptotic hemocytes with and without *ChCASP8-like* silencing under hyper-salinity stress. Green fluorescence indicated TUNEL-positive apoptotic nuclei and blue fluorescence indicated total nuclei.

4. Discussion

Oysters, a keystone bivalve living in estuarine and intertidal zones, are subject to frequent environmental disturbances, such as rapid salinity fluctuations [10,28]. *C. hongkongensis*, the major aquaculture species in South China, is prone to hypersalinity-related mass mortality. It is essential to investigate the strategy of *C. hongkongensis* against the threat of fluctuating salinity. Apoptosis is an important survival pathway of organism response to salinity stress by orderly caspase events.

The *caspase-8-like* gene has been characterized in several mollusk species, including *Haliotis discus* [29], *Mytilus galloprovincialis* [30], *C. hongkongensis* and *Crassostrea gigas* [4]. In vertebrates, *caspase 8* had two DED motifs, which were responsible for the self-activation of the inactive proenzyme [31]. In mammals, DEDs formed intracellular filaments and transmitted the external death signals to downstream effectors by cleaving *caspase-8* [32]. This was critical for *caspase 8* activation and the subsequent induction of apoptosis.

Amino acid sequence alignment showed that CASP8-like of *C. hongkongensis* had the highest identity (86.5%) with CASP8-like of *Crassostrea angulate*. The phylogenetic tree analysis also showed that the *ChCASP8-like* genes of *Crassostrea angulate*, *Crassostrea virginica* and *Ostrea edulis* were grouped into an evolutionary branch. Therefore, the novel caspase gene of *C. hongkongensis* was named as *ChASP8-like*. However, it is worth mentioning that the amino acid sequence of *ChCASP8-like* shared low identity (30.0%) with *CASP8* of *C. hongkongensis* (AHB50667.1) [8]. We hypothesized that *ChCASP8-like* and CASP8 were different isoforms. *Caspase-8* was found to be effective in activating the NF-kB pathway and p53/p21 pathway in oysters after bacterial infection [8]. In our study, we found that salt stress activated DNA damage repair-related genes (e.g., *ATR* and *CHK1*), apoptosis-related genes (e.g., *caspase 9* and *caspase 3*) and *BCL-XL* in the p53 signaling pathway. This implied that salinity stress and bacterial challenge might stimulate different immune pathways, which were mediated by *CASP8-like* and *CASP8* genes in *C. hongkongensis*, respectively

The expression profile showed that *ChCASP8-like* had constitutive expression in several tissues, with high expression levels in the gonads, gill, hepatopancreas and mantle. The expression levels were significantly upregulated in all immune tissue by hyper-salinity stress, especially in gills, hepatopancreas and hemocytes. Similarly, *caspase 8* from *C. virginica* was widely expressed in various tissues and developmental stages [4]. High levels of *CASP8* transcripts were also found in gills, hemocytes and digestive glands of mussels in response to high-temperature stress in *Mytilus coruscus* and *Mytilus galloprovincialis* [30], These data further indicated the hyper-salinity stress stimulated the immune response by activating of *ChCASP8-like* in *C. hongkongensis*.

Caspase 8, situated at the apex of the apoptotic cascade, is crucial for activating downstream executioner caspases by cleaving them and leading to cell death [33,34]. Therefore, the caspase 8 activity was examined after *ChCASP8-like* silencing and hyper-salinity stress in this study. The increased caspase 8 activity after hyper-salinity stress suggested that apoptosis had been activated. Apoptosis was mainly divided into two pathways, the intrinsic pathway and extrinsic pathway. The promoter that regulated the intrinsic pathway of apoptosis was *caspase 9*, which could bind to the adapter protein apoptosis protease activator 1 (APAF1) upon exposure of the caspase recruitment domain (CARD domain). When apoptosis was induced by positive or negative stimuli, the mitochondrial membrane was altered, allowing apoptotic proteins (such as cytochrome c, Smac/Diablo, and HtrA2/Omi) to move from the mitochondria and activate apoptosis [35]. The extrinsic pathway was mediated by the extracellular death ligands of the TNF family (TNFα, tumor necrosis factor α; FasL, the ligand for Fas and the TNF-associated apoptosis-inducing ligands) and was triggered by the recruitment of FADD and *caspase 8* to the death receptor [36,37]. *Caspase 8* was activated and cleaved its substrates *caspase 3* and *caspase 7*, ultimately leading to apoptosis. In our study, the decreased caspase 8 activity after RNA interference confirmed the key role of *ChCASP8* in apoptosis. *Caspase 3* was the key executor of apoptosis and was required to cleave the substrate of the apoptotic pathway during the final step [38]. However, there was no significant difference in caspase 3 activity in this research. The functions of *caspase 3* and *caspase 7* were reported to overlap in the regulation of apoptosis. A plausible explanation was that the increase in caspase 8 activity may also activate caspase 7 activity, not only caspase 3. This hypothesis needs to be further investigated.

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay is designed to detect apoptotic cells that undergo extensive DNA degradation in early and late stages of apoptosis [22]. The TUNEL assay is based on the ability of TdT to label blunt ends of double-stranded DNA breaks independent of a template, and has been widely used as a measure of apoptotic cell death [39]. In this study, the number of TUNEL-positive hemocytes were significantly increased after hyper-salinity stress, while significantly decreased after silencing *caspase8-like*. These data confirmed that the hemocytes suffered severe DNA damage from salt stress, and *caspase 8* played an important role in inducing apoptosis.

5. Conclusions

In conclusion, a novel *CASP8-like* gene was characterized from *C. hongkongensis*. The tissue expression profile showed that *ChCASP8-like* had constitutive expression in all tissues, and was significantly upregulated in hemocytes, hepatopancreas and gills by hyper-salinity stress. Apoptosis-related gene transcripts and caspase 8 activity were significantly increased after hyper-salinity stress, and significantly decreased after *ChCASP8-like* interference. Moreover, exposure to hyper-salinity stress caused severe apoptosis, which could be alleviated by *ChCASP8-like* silence. The results indicated that *ChCASP8-like* played a crucial role in activating apoptosis against hyper-salinity stress in oysters.

Author Contributions: J.L. performed experiments and wrote the manuscript. Z.Y. performed experiments. Y.L. (Yang Leng) analyzed the data and provided the experimental animals. J.Z. analyzed the data and verified the data. F.Y. designed the experiments and revised the manuscript. Y.L. (Yishan Lu) revised the manuscript. J.C. analyzed the data and proved the data. W.H. analyzed the data and verified the data. Y.Z. contributed to the graphing. Y.W. performed the experiments. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Our research was exempt from ethical evaluation because the Animal Ethics Committee's scope of approval is limited to studies involving vertebrates in China (Laboratory Animal Act, Chapter 5 Article 25).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data involved in this study have been deposited in the NCBI Bioproject under the accession number OR066208. The data that support the study findings are available upon request.

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