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Molecular Cloning of Heat Shock Protein 60 (*Sp*HSP60) from *Schizothorax prenanti* and the Gene Expressions of Four *Sp*HSPs during Lipopolysaccharide (LPS) Infection

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Abstract: Heat shock proteins (HSPs) play a key role in anti-stress and immune processes and are associated with autoimmune diseases. In order to explore the immunological role of HSPs from *Schizothorax prenanti* (*S. prenanti*), *Sp*HSP60 was cloned for the first time in this study, and the gene expressions of *Sp*HSP27, *Sp*HSP60, *Sp*HSP70 and *Sp*HSP90 in the hepatopancreas, head kidney, hindgut and spleen were analyzed by quantitative real-time PCR (qPCR) after treatment with lipopolysaccharide (LPS). The open reading frame of the *Sp*HSP60 gene (GenBank accession number ON245159) is 1728 bp. It encodes a protein of 575 amino acids. Its C-terminus is a highly conserved and repeated glycine sequence, which is an important cofactor in ATP binding. Compared with the control group, most of the *Sp*HSPs were significantly upregulated in the tissues examined at 12 or 24 h after LPS challenge. The most abundant expression of *Sp*HSP70 was found in the head kidney at 24 h after LPS injection, followed by *Sp*HSP27 in the spleen at 24 h; both of these *Sp*HSPs displayed strong expression under the LPS stresses, about 20–70 fold more than that of *Sp*HSP60 and *Sp*HSP90. The temporal expression patterns of the four *Sp*HSP genes were different in the four tissues examined. Taken together, the results suggest that *Sp*HSP27, *Sp*HSP60, *Sp*HSP70 and *Sp*HSP90 participate in innate immunity stimulated by LPS, and the response intensity of the *Sp*HSPs was organ-specific, indicating they could provide early warning information against bacterial infection. The findings in our study will contribute to better understanding the biological processes and important roles of *Sp*HSPs involved in defending against pathogenic bacterial challenge.

Keywords: heat shock proteins; HSP60; *Schizothorax prenanti*; molecular cloning; tissue distribution; gene expression; lipopolysaccharides

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1. Introduction

According to homology and molecular mass, heat shock proteins (HSPs) are grouped into subfamilies such as HSP90, HSP70, HSP60 and small heat shock proteins (sHSPs) (16–30 kDa), such as HSP27. They are a group of molecular chaperones which are widely expressed in all organisms, from bacteria to plants to mammals, that show highly conservative evolution; they play protective roles against a variety of adverse stress stimuli, including bacterial infection [1].

To date, the molecular cloning and functional analysis of HSPs have been reported for fish such as *Oncorhynchus kisutch* [2], *Sparus aurata* and *O. mykiss* [3,4], *Cyprinus carpio* [5], *Miichthys miiuy* [6] and *Ctenopharyngodon idella* [7,8]; and the same of the HSPs of *Schizothorax prenanti* (*Sp*HSPs). Due to their important functions, HSPs have recently received considerable attention in aquatic animals. Pu et al. (2016) [9] cloned *Sp*HSP90 for the first time and detected changes in expression levels after bacterial *Streptococcus*

agalactiae infection. Meanwhile, Li et al. (2015) [10] cloned two types of heat shock proteins (*Sp*HSC70 and *Sp*HSP70) and detected changes of tissue-specific expression patterns in response to bacterial *Aeromonas hydrophila* challenge. However, at present, the remaining *Sp*HSP60 gene has not been cloned.

Fish are the perfect vertebrate model with which to research the physiology, function and regulation of HSPs, since they are exposed to pathogens and thermal and other stressors in their natural environment [11]. *S. prenanti*—belonging to the Schizothoracinae subfamily of the Cyprinidae family and commonly known locally as Ya-fish in Sichuan province and Yang-fish in Shaanxi province—is mainly distributed in the upper reaches of the Yangtze River, including the western plateau of the two Chinese provinces. As an economically important cold-water fish, it has been artificially cultivated for consumption and listed as a protected species in the two provinces, due to the continual decline of its wild populations [9,12,13].

Lipopolysaccharide (LPS) is a major component of the outer membranes of Gram-negative bacteria, and it can induce a cascade of immune stimulating and toxic pathophysiological activities in the body [14]. In order to explore the immune responses of *Sp*HSPs genes (*Sp*HSP27, *Sp*HSP60, *Sp*HSP70 and *Sp*HSP90) in various immune organs (hepatopancreas, head kidney, hindgut and spleen) after the bacterial mimic of LPS challenge, the *Sp*HSP60 was cloned for the first time in this study, and the patterns of tissue expression in response to LPS challenge of the four *Sp*HSPs were analyzed by quantitative real-time PCR (qPCR). Therefore, the results of our study will help us better understand the response of *Sp*HSP60. Moreover, the variations of *Sp*HSPs after LPS injection may provide a better explanation for the mechanisms of antibacterial and immune responses during LPS-associated infections, and provide a scientific basis for an in-depth study of *Sp*HSPs.

2. Materials and Methods

2.1. Animal Treatment

Healthy cultured *S. prenanti*, weighing 109.3 ± 27.1 g were obtained from the Qunfu Yang-fish professional breeding cooperative, a commercial breeding aquaculture farm in Hanzhong city (Shaanxi, China) in November 2020. The 12 fish were divided equally between two glass tanks which were equipped with a filter system. They were 60 × 30 × 40 cm, had aerated water and were kept at a temperature of 19 ± 1 °C. Fish were fed with commercial feed at a rate of 2% of their body weight twice daily at 08:00 a.m. and 16:00 p.m. for 10 d during acclimation. The photoperiod was 12 h (07:00–19:00) light and 12 h dark (19:00–07:00) during the acclimation and infection periods. The tank filter was cleaned once a day, and we replaced about a quarter of the aerated tap water. No mortality was observed during the trail.

After the acclimation, two fish were randomly selected from each tank, and a total of 4 fish were used to detect the tissue distribution of *Sp*HSP60 mRNA in unstressed conditions. The fish were anesthetized with eugenol at a concentration of 80 mg/L for 3 min, and then the tissues were collected, including the heart, hepatopancreas, head kidney, hindgut, muscle, adipose and spleen, and preserved in liquid nitrogen for RNA extraction.

The fish were challenged with an intraperitoneal injection of LPS (10 mg/kg, L2880, Sigma, St. Louis, MO, USA), and the control group was injected with the same volume of phosphate-buffered saline (PBS). Anatomical samples of the LPS-stimulated group and the PBS-stimulated group were taken at 12 and 24 h after injection, respectively. The tissues were dissected and preserved in liquid nitrogen for RNA extraction.

2.2. RNA Extraction and cDNA Synthesis

Tissue total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with some modifications [15]. Total RNA quality and concentration were determined by agarose gel electrophoresis and the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). First-stand cDNA was synthesized using the RevertAid

First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania), according to the method recommended by the manufacturer.

2.3. Full-Length cDNA Cloning of the SpHSP60 Gene

According to the transcriptome sequencing of *S. prenanti*, specific HSP60 full-length primers were designed (Table 1, HSP60-F and HSP60-R) with PrimerQuest™ Tool (https://sg.idtdna.com/pages/tools/primerquest (accessed on 9 January 2020)) and synthetized by Tsingke Biotechnology Co., Ltd. (Xi'an, China). Hepatopancreas cDNA was used as a template for *Sp*HSP60 amplification with Primerstar® Max DNA polymerase (TaKaRa, Dalian, China). The PCR program was as follows: 30 cycles at 98 °C for 10 s, 55 °C for 15 s and 72 °C for 1 min; and storage at 4 °C. PCR products were A-tailed with the DNA A-Tailing Kit (TaKaRa, Dalian, China). The obtained PCR products were ligated into a pMD19-T vector (TaKaRa, Dalian, China) and transformed into competent *E.coli* DH5α cells (TIANGEN BIOTECH, Beijing, China). Positive bacteria clones were sequenced.

Table 1. Primer sequences used in this study.

Primer	Sequence (5′-3′)	Annealing Temperature (°C)	Size (bp)
Primers for full-length PCR			
HSP60-F	CTGACGGACACGCGCATCCCTTCA	55	1826
HSP60-R	AATCATGTCCCCTGCCCACAACCCTTC		
Primers for quantitative real-time PCR analysis			
HSP27-F	CTCGGGAATGTCTGAGATAAAG	62	130
HSP27-R	CTCATGTTTGCCGGTGAT		
HSP60-F	GGAGAGCACAAACAGTGACTAC	62	130
HSP60-R	GACACGGTCCTTCTTCTCATTC		
HSP70-F	CTCTATGGTCCTGGTGAAGA	60	106
HSP70-R	CCTCTGGGAGTCATTGAAATAG		
HSP90-F	AGGTCACGGTCATCACTAAAC	62	182
HSP90-R	GACCACTTCCTTCACTCTCTTC		
β-actin-F	GACCACCTTCAACTCCATCAT	62	126
β-actin-R	GTGATCTCCTTCTGCATCCTATC		

2.4. Sequence Analysis

The open reading frame (ORF) of *S. prenanti* HSP60 (*Sp*HSP60) was identified using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html (accessed on 20 March 2022)). The isoelectric point and molecular weight were predicted (https://web.expasy.org/compute_pi/ (accessed on 21 March 2022)). The secondary structure composition of the *Sp*HSP60 protein was predicted (http://bioinf.cs.ucl.ac.uk/psipred (accessed on 21 March 2022)). The three-dimensional structure of the *Sp*HSP60 protein was predicted (https://swissmodel.expasy.org/ (accessed on 21 March 2022)). Multiple sequence alignments were performed using the Clustal X2 [16]. A phylogenetic tree of different species of HSP60s, based on full-length DNA sequences was constructed by the neighbor-joining method using MEGA 4.0 software [17].

2.5. Tissue Distribution of SpHSP60 mRNA in Unstressed Conditions

The mRNA levels in different tissues were measured, including the heart, hepatopancreas, spleen, head kidney, muscle, adipose and hindgut. Total RNA was extracted, and cDNA was synthesized as previously described. A real-time PCR analysis was performed using the Applied Biosystems Step One Plus system (Applied Biosystems, Foster City, CA, USA). The gene-specific primers are listed in Table 1. *S. prenanti*-specific β-actin primers served as internal controls to normalize cDNA quantity for each tissue sample. Quantification of *Sp*HSP60 and β-actin was performed in triplicate on all samples using the FastStart Essential DNA Green Master (Roche, Indianapolis, IN, USA), according to

the manufacturer's instructions. The qPCR data were calculated according to the $2^{-\Delta\Delta CT}$ method [18].

2.6. Detection of the Expression Patterns Induced by LPS

For the bacterial challenge, 8 fish were injected intraperitoneally from the base of the pectoral fin with LPS. The dose of injection was 10 mg/kg LPS per fish. Four fish were injected intraperitoneally with the same volume of PBS as controls. Four fish samples were taken at 12 and 24 h after LPS injection. The head kidney, hepatopancreas and hindgut of fish are generally regarded as immune organs which are central to immune responses [19]. These three tissues, plus the spleen—another vital immune organ—were collected from each fish and used to isolate total RNA. To detect the expression of *Sp*HSP27, *Sp*HSP60, *Sp*HSP70 and *Sp*HSP90 changes after the bacterial challenge, total RNA extraction, cDNA synthesis and qPCR were performed, as described in Section 2.5.

2.7. Statistical Analysis

GraphPad Prism 5 was used for the graphics and differences analysis, and a p value less than 0.05 was considered significant. All data are presented as mean ± standard error (n = 4). Differences between means were considered significant at the 95% confidence level. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were compared to the corresponding control group at the time points (fold-change as 1). Data were detected by one-way ANOVA followed by Tukey tests.

3. Results

3.1. Characterization of the SpHSP60 Gene cDNA

The full length of the *Sp*HSP60 gene (GenBank accession number ON245159) is 1826 bp, including an ORF of 1728 bp. The predicted ORF encodes a protein of 575 amino acids (Figure 1) that has a calculated molecular mass of 61.32 kDa and a theoretical isoelectric point of 5.54. The secondary structure composition of the *Sp*HSP60 protein is shown in Figure 2. According to the prediction result, the *Sp*HSP60 protein has strands, helices, coils, a transmembrane helix, etc. The predicted 3D structure of the *Sp*HSP60 protein (Figure 3a) shows that there is an adenosine-5′-diphosphate binding site, a magnesium ion binding site, a beryllium trifluoride ion binding site and a potassium ion binding site (Figure 3b). Multiple amino acid sequence alignment revealed a high degree of identity with HSP60.

3.2. Phylogenetic Analysis

A phylogenetic analysis of the amino acid sequences of *Sp*HSP60 was performed to study the evolutionary relationship with other vertebrates (Figure 4). The sequences were obtained from the GenBank database. The results showed that the amino acid sequence of *Sp*HSP60 has higher identity with Cyprinids and has the highest identity with the HSP60 of *Carassius auratus* (97.72%).

3.3. Tissue Distribution of SpHSP60 Expression in S. prenanti

The expression of *Sp*HSP60 mRNA was examined in different tissues from four unchallenged Yang fish using qPCR, with β-actin as the internal control. As shown in Figure 5, *Sp*HSP60 transcripts were ubiquitously expressed in all the tissues examined, including the heart, hepatopancreas, spleen, head kidney, muscle, adipose and hindgut; the levels obviously varied among different tissues. The relative expression of *Sp*HSP60 was significantly higher in the heart than in other tissues ($p < 0.05$), except for adipose. No significant difference in *Sp*HSP60 mRNA expression was observed in tissues except for the heart ($p > 0.05$). Indeed, the highest expression level of *Sp*HSP60 in the heart was nearly six times the lowest expression level in the hepatopancreas (Figure 5).

```
1       ctgacggacacgcgcatcccttcattcacctccagatcactctgcaaaATGCTGCGTTTACCCAGTGTAATGAAACAGATGAGGCCAGTG
1                                                       M  L  R  L  P  S  V  M  K  Q  M  R  P  V
91      TGCAGGGCGCTGGCCCCACACCTGACCCGTGCATATGCCAAGGAGGTCAAGTTTGGAGCAGATGCCCGTGCCCTCATGCTCCAGGGTGTT
15       C  R  A  L  A  P  H  L  T  R  A  Y  A  K  E  V  K  F  G  A  D  A  R  A  L  M  L  Q  G  V
181     GACCTGCTGGCTGATACTGTGGCCGTCACCATGGGACCAAAGGGTCGCACCGTTATCATTGAGCAGAGCTGGGGCAGCCCTAAAGTCACC
45       D  L  L  A  D  T  V  A  V  T  M  G  P  K  G  R  T  V  I  I  E  Q  S  W  G  S  P  K  V  T
271     AAAGATGGTGTCACAGTTGCCAAAAGTATCGACTTGAAGGATAAGTATAAGAACATCGGGGCCAGGCTGGTACAGGACGTGGCCAACAAC
75       K  D  G  V  T  V  A  K  S  I  D  L  K  D  K  Y  K  N  I  G  A  R  L  V  Q  D  V  A  N  N
361     ACAAATGAGGAGGCTGGAGATGGCACCACAACCGCCACAGTTTTGGCCCGTGCTATTGCCAAGGAGGGATTTGACACCATCAGCAAAGGC
105      T  N  E  E  A  G  D  G  T  T  T  A  T  V  L  A  R  A  I  A  K  E  G  F  D  T  I  S  K  G
451     GCCAACCCTGTGGAGATCCGTAGAGGAGTCATGTTGGCGGTGGAGGAAGTCATCAATGAACTCAAGAAACTCTCCAAGCCGGTCACAACA
135      A  N  P  V  E  I  R  R  G  V  M  L  A  V  E  E  V  I  N  E  L  K  K  L  S  K  P  V  T  T
541     CCAGAGGAAATTGCTCAGGTGGCCACTATTTCTGCCAATGGAGACACTGAAGTTGGTAACATCATCTCCAATGCTATGAAGAAAGTGGGC
165      P  E  E  I  A  Q  V  A  T  I  S  A  N  G  D  T  E  V  G  N  I  I  S  N  A  M  K  K  V  G
631     CGCAAGGGTGTTATTACAGTGAAGGATGGTAAAACCCTACATGATGAGCTTGAGATCATTGAGGGCATGAGGTTCGACCGTGGCTACATT
195      R  K  G  V  I  T  V  K  D  G  K  T  L  H  D  E  L  E  I  I  E  G  M  R  F  D  R  G  Y  I
721     TCTCCTTACTTCATCAACTCAGCTAAAGGCCAGAAGTGTGAGTTCCAGGATGCTTACCTACTTCTGAGTGAGAAGAAGATCTCCAGCGTA
225      S  P  Y  F  I  N  S  A  K  G  Q  K  C  E  F  Q  D  A  Y  L  L  L  S  E  K  K  I  S  S  V
811     CAGAGCATCGTGCCAGCACTGGAACTTGCCAACCAGCATCGCAAGCCTCTAGTGATCATCGCTGAAGATGTGGACGGAGAGGCACTCAGC
255      Q  S  I  V  P  A  L  E  L  A  N  Q  H  R  K  P  L  V  I  I  A  E  D  V  D  G  E  A  L  S
901     ACTCTGGTCCTCAACAGGTTGAAGGTTGGACTTCAGGTCGTTGCAGTCAAGGCTCCAGGATTCGGGGACAACAGGAAAAACCAGCTGCAG
285      T  L  V  L  N  R  L  K  V  G  L  Q  V  V  A  V  K  A  P  G  F  G  D  N  R  K  N  Q  L  Q
991     GATTTGGCAATTTCCACTGGAGGCACGGTGTTTGGTGATGATGCTGTGGGTTTGGCCATTGAGGATATTCAGGCACATGACTTTGGCAGA
315      D  L  A  I  S  T  G  G  T  V  F  G  D  D  A  V  G  L  A  I  E  D  I  Q  A  H  D  F  G  R
1081    GTCGGCGAGGTCATTGTGACAAAGGATGACACTATGCTCCTCAAAGGCCGTGGTGATCCAGCAACCATTGAGAAGCGTGTGAATGAGATC
345      V  G  E  V  I  V  T  K  D  D  T  M  L  L  K  G  R  G  D  P  A  T  I  E  K  R  V  N  E  I
1171    GCTGAACAGCTGGAGAGCACAAACAGTGACTACGAGAAGGAGAAACTCCACGAGCGTCTGGCCAAGCTCTCTGATGGAGTGGCTGTGATT
375      A  E  Q  L  E  S  T  N  S  D  Y  E  K  E  K  L  H  E  R  L  A  K  L  S  D  G  V  A  V  I
1261    AAGGTTGGAGGAACAAGTGACGTTGAAGTGAATGAGAAGAAGGACCGTGTCACTGATGCGCTGAATGCCACGCGAGCTGCTGTGGAGGAG
405      K  V  G  G  T  S  D  V  E  V  N  E  K  K  D  R  V  T  D  A  L  N  A  T  R  A  A  V  E  E
1351    GGAATCGTTCTTGGAGGAGGATGTGCCCTGCTGCGCTGCATCCCAGCCCTGGACAACATCAAGCCAGCCAATGATGATCTAAAGATTGGT
435      G  I  V  L  G  G  G  C  A  L  L  R  C  I  P  A  L  D  N  I  K  P  A  N  D  D  L  K  I  G
1441    ATCGACATTATTCGCAGAGCGCTTCGTATTCCAGCAATGACTATTGCCAAGAATGCAGGAGTTGAGGGCTCTCTGGTGGTGGAGAAGATC
465      I  D  I  I  R  R  A  L  R  I  P  A  M  T  I  A  K  N  A  G  V  E  G  S  L  V  V  E  K  I
1531    TTGCAGAGCGCTCCAGAGATTGGATACGATGCTATGAATGGAGAATATGTCAACATGGTCGAAAGAGGCATTATTGACCCCACAAAGGTT
495      L  Q  S  A  P  E  I  G  Y  D  A  M  N  G  E  Y  V  N  M  V  E  R  G  I  I  D  P  T  K  V
1621    GTGAGGACTGCACTGCTAGATGCTGCAGGTGTTGCATCTCTGCTGTCTACTGCTGAAGCCGTTGTCACCGAGATACCAAAGGAGGAGAAG
525      V  R  T  A  L  L  D  A  A  G  V  A  S  L  L  S  T  A  E  A  V  V  T  E  I  P  K  E  E  K
1711    GACATGCCGGCTGGAGGAATGGGTGGAATGGGAGGCATGGGTGGCATGGGAGGCATGGGATTCTAAaccgatctgcactgactttagtga
555      D  M  P  A  G  G  M  G  G  M  G  G  M  G  G  M  G  G  M  G  F  *
1801    aagggttgtgggcaggggacatgatt
```

Figure 1. The full-length cDNA nucleotide sequence and corresponding amino acid sequence of *Sp*HSP60; * indicates a stop codon.

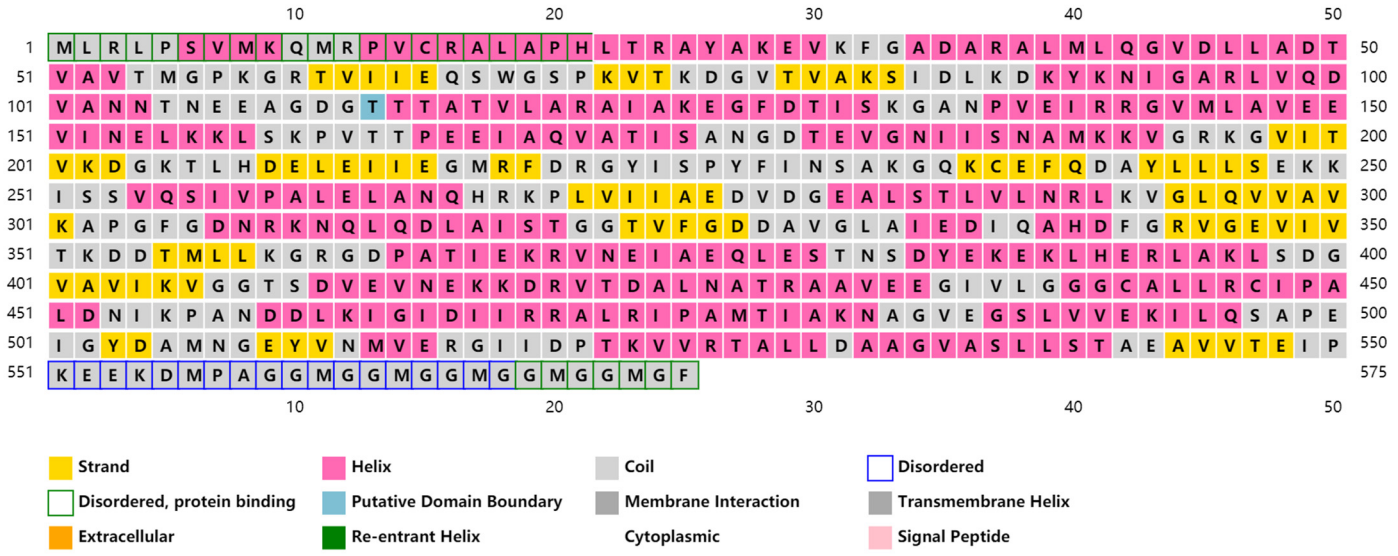

Figure 2. The secondary structure composition prediction of the *Sp*HSP60 protein.

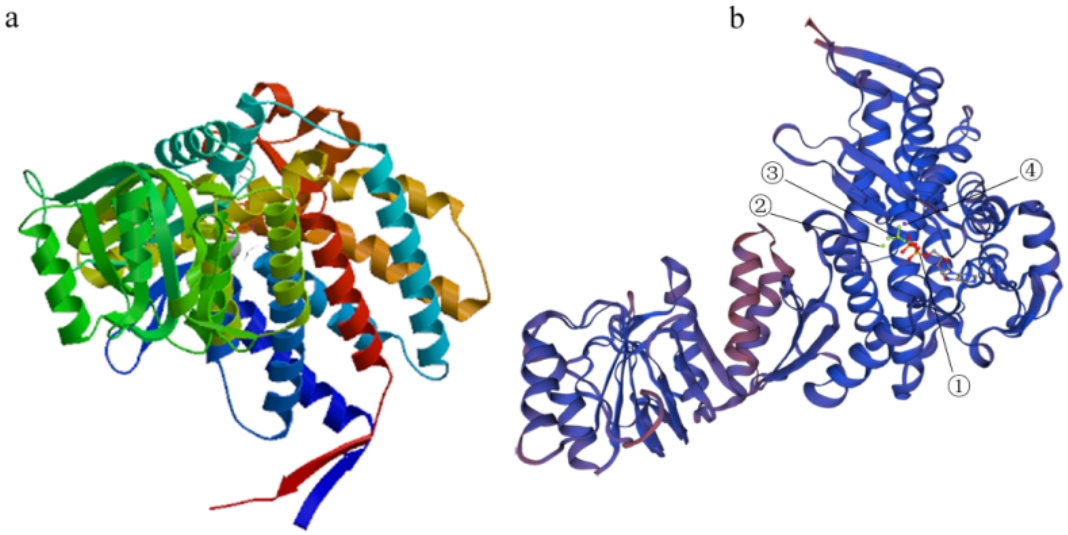

Figure 3. The 3D-structural models of *Sp*HSP60 and binding site prediction: (**a**) indicates the 3D-structural models of *Sp*HSP60; (**b**) indicates binding site prediction. ① Adenosine-5′-diphosphate binding site, ② magnesium ion binding site, ③ beryllium trifluoride ion binding site, ④ potassium ion binding site.

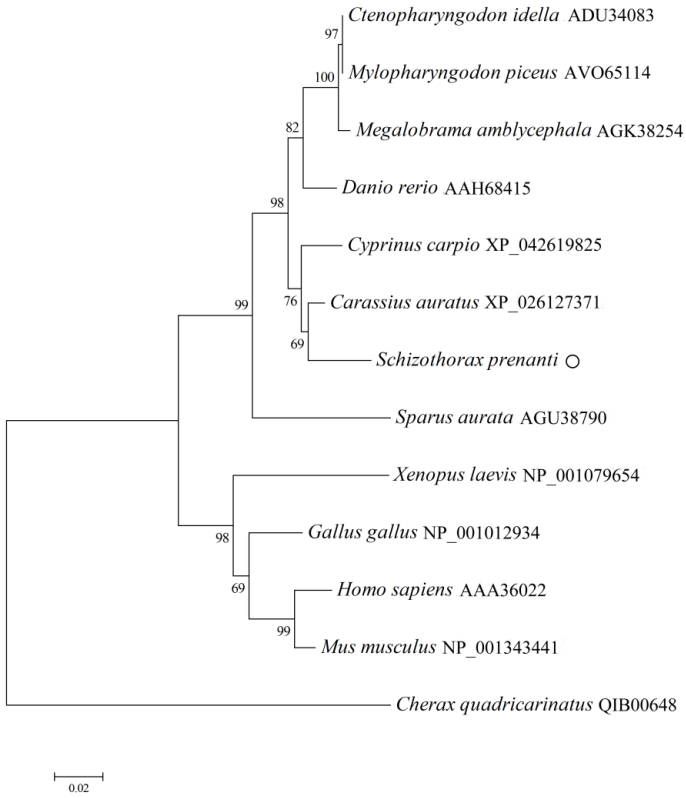

Figure 4. Phylogenetic tree of HSP60 amino acids from different vertebrates: ○ indicates *Sp*HSP60; the GenBank accession numbers of other vertebrates are marked.

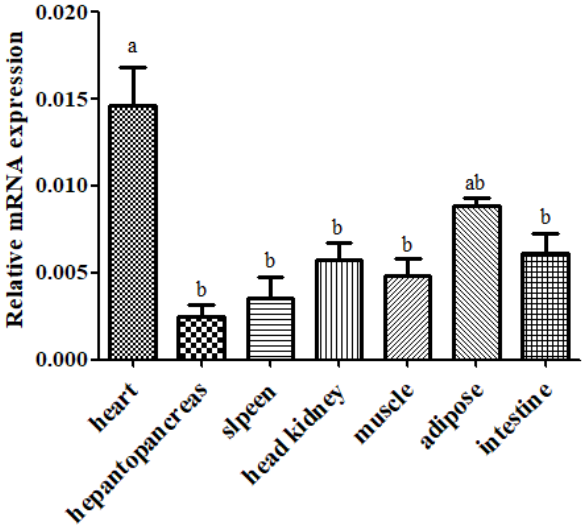

Figure 5. Transcript levels of *Sp*HSP60 in the various tissues of *S. prenanti*. Each value represents the mean ± SD (n = 4 fish per group); statistical significance was decided using one-way ANOVA followed by Tukey tests. Note: for a and b, different letters are significantly different ($p < 0.05$) from each other.

3.4. Expression Pattern Analysis of SpHSPs following LPS Challenge

To determine the changes in *Sp*HSP27, *Sp*HSP60, *Sp*HSP70 and *Sp*HSP90 after LPS injection at 12 and 24 h, the tissue-specific distributions of *Sp*HSP genes were determined in the hepatopancreas, head kidney, spleen and hindgut from *S. prenanti* using qPCR.

3.4.1. Expression of *Sp*HSP27 after Challenge with LPS

As shown in Figure 6, the mRNA relative expression levels of *Sp*HSP27 were upregulated with extreme significance in all the tissues examined at 24 h after LPS challenge ($p < 0.001$). The expression level peaked 12 h post-injection only in the spleen; this was also the highest expression level of the *Sp*HSP27 gene in this study, and was significantly higher than that of the PBS control group and the 24 h LPS injection group ($p < 0.001$). It was also overexpressed in the head kidney 24 h after LPS injection, second only to the level in the spleen 12 h after LPS injection. The transcript levels of *Sp*HSP27 in the hepatopancreas, head kidney and hindgut may have not reached their peak at 24 h after LPS challenge, whereas they were markedly higher than those in the PBS-injection group ($p < 0.001$). Indeed, the intestinal expression level of *Sp*HSP27 at 12 h was also higher with extreme significance than that of the PBS control group ($p < 0.01$).

3.4.2. Expression of *Sp*HSP60 after Challenge with LPS

The results of the temporal transcription level of *Sp*HSP60 after the fish were injected with LPS are shown in Figure 7. The gene in the four tissues examined showed a fairly consistent expression pattern within 24 h: significantly upregulated 12 h post-injection, and then downregulated 24 h post-injection. At 24 h of LPS stimulation, expression levels in the hepatopancreas, hindgut and spleen appeared to have no difference compared with those of the PBS control group, except that the level in the head kidney was higher with pronounced significance ($p < 0.05$). The results showed that the relative expression levels of *Sp*HSP60 in the hepatopancreas and spleen were extremely significantly increased at 12 h ($p < 0.001$), and then decreased with marked significance at 24 h ($p < 0.001$); there were no significant differences found in their 24 h relative expression levels compared with the PBS control group. The expression of *Sp*HSP60 in the head kidney had increased with extreme significance by 12 h ($p < 0.001$), and significantly decreased by 24 h ($p < 0.05$). The level at 24 h was still markedly higher than that of the control group ($p < 0.01$). The intestinal expression level had increased significantly by 12 h ($p < 0.05$), and no significance was found at 24 h.

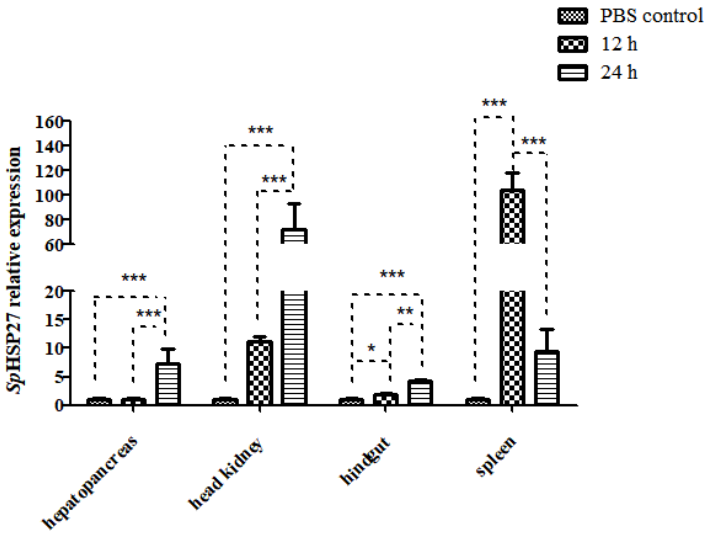

Figure 6. Transcript levels of *Sp*HSP27 at different times after LPS challenge. Each value represents a mean ± SD (n = 4 fish per group); statistical significance was decided using one-way ANOVA followed by Tukey tests. Note: an asterisk (*) over a column represents a statistically significant difference, one for $p < 0.05$, two for <0.01 and three for <0.001.

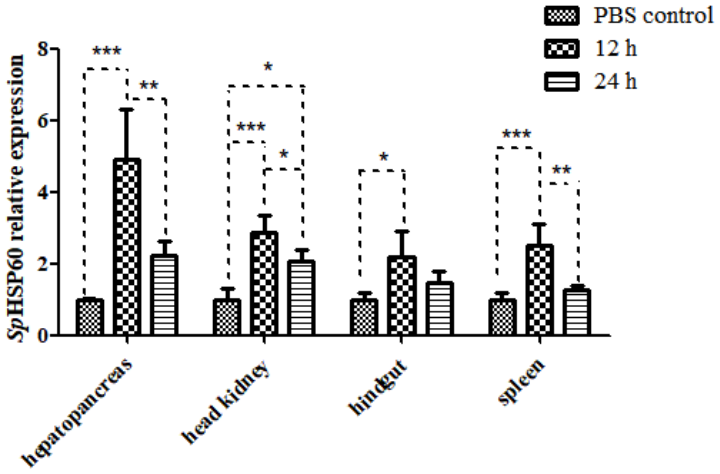

Figure 7. Transcript levels of *Sp*HSP60 in various tissues and at two time points after LPS challenge. Each value represents a mean ± SD (n = 4 fish per group); statistical significance was decided using one-way ANOVA followed by Tukey tests. Note: an asterisk (*) over a column represents a statistically significant difference, one for $p < 0.05$, two for <0.01 and three for <0.001. n = 4.

3.4.3. Expression of *Sp*HSP70 after Challenge with LPS

As shown in Figure 8, the transcription levels of *Sp*HSP70 in the four tissues examined were also evaluated after stimulating with LPS. In general, the expression level of the *Sp*HSP70 gene was the highest in the four induced *Sp*HSPs examined in this study. The level in the head kidney tissue was explosively upregulated 24 h after challenge with LPS. Compared to the PBS-injection group, the increased *Sp*HSP70 expression of the LPS-stimulated group at 12 h had no statistical significance generally, but the level in the hepatopancreas was higher with extreme significance at 12 h ($p < 0.001$). Moreover, the highest *Sp*HSP70 expression levels of the head kidney, hindgut and spleen appeared at 24 h, which were higher with pronounced significance than the PBS-injection groups' levels ($p < 0.001$). The hepatic *Sp*HSP70 at 24 h was decreased, but was still significantly higher than in the PBS-injection fish ($p < 0.001$).

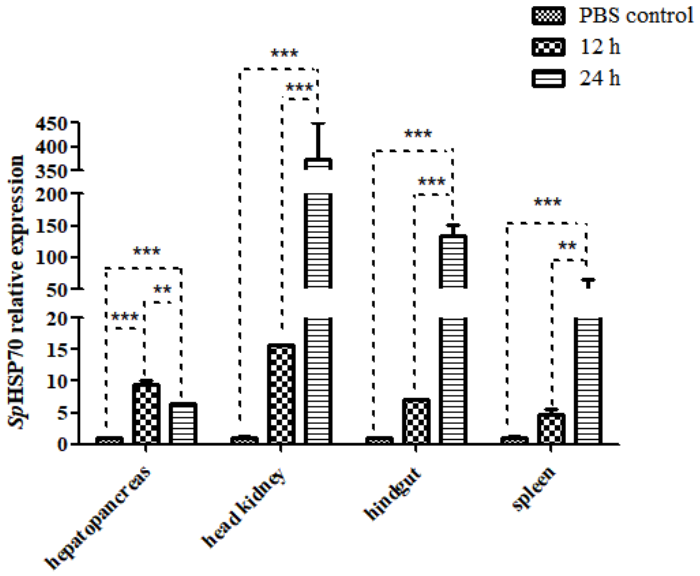

Figure 8. Transcript level of *Sp*HSP70 at different times after LPS challenge. Each value represents a mean ± SD (n = 4 fish per group); statistical significance was decided using one-way ANOVA followed by Tukey tests. Note: an asterisk (*) over a column represents a statistically significant difference, two for <0.01 and three for <0.001. n = 4.

3.4.4. Expression of *Sp*HSP90 after Challenge with LPS

The transcript levels of *Sp*HSP90 in the hepatopancreas and head kidney tissues showed no significant difference 12 h after LPS injection (Figure 9), whereas the levels in the two tissues strongly increased 24 h post LPS injection ($p < 0.01$). Notably, levels in the hindgut and spleen showed no significant changes either 12 or 24 h after LPS injection. The *Sp*HSP90 gene expression level was generally relatively low after LPS stimulation.

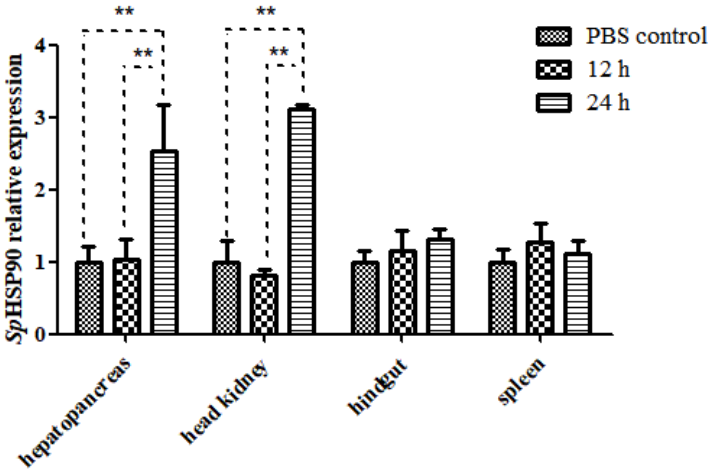

Figure 9. Transcript level of *Sp*HSP90 at different times after LPS challenge. Each value represents a mean ± SD (n = 4 fish per group); statistical significance was decided using one-way ANOVA followed by Tukey tests. Note: an asterisk (*) over a column represents a statistically significant difference, two for $p < 0.01$. n = 4.

4. Discussion

To date, due to there being few studies on HSPs of *S. prenanti*, we know little about the role of these genes in this cold-water fish. Only regulation under stress conditions of *Sp*HSP70, *Sp*HSC70 and *Sp*HSP90β has been reported [9,10]. This study cloned and analyzed the function of *Sp*HSP60 from *S. prenanti* for the first time. Its C-terminus is a

highly conserved and repeated glycine sequence, which is an important cofactor in ATP binding [20]. The amino acid sequence has high identity with those of *Cyprinids*—the highest with the HSP60 of *C. auratus*. The results of the phylogenetic trees for *Sp*HSP60 (this study) and *Sp*HSP70 [9] showed that *S. prenanti* is more closely related to *C. carpio*, whereas *Sp*HSP90 [10] was more closely related to *Danio rerio*.

It has been reported that HSPs have constitutive functions which are essential in the protein metabolism of the unstressed cell [21]. Among the different forms of HSPs, HSP60 is a mitochondrial matrix protein too, which is also a kind of highly conserved immunogenic molecule and a molecular chaperone. It is a well characterized protein mainly located in the mitochondria of eukaryotic cells. The chaperone influences the folding of other HSPs with constitutive functions, which are essential in the protein metabolism of the unstressed cell [22–25]. The results of this study showed that the expression level of *Sp*HSP60 under unstressed conditions was the highest in heart tissue, and significantly different. As is known to all, cardiomyocytes have the highest number of mitochondria. The significance of high HSP60 expression in the heart is consistent with the fact that HSP60 plays a key role in protecting the integrity of the heart [26]. HSP60 is abundant in mitochondria and plays a large role in the mitochondrial apoptosis pathway [27]. HSP60 can act as an early promoter of myocardial cell injury and heart failure [28]. The high expression of HSP60 can also explain why the heart will increase ATP supply under stress [29]. Our results support these findings and suggest that the HSP60 of fish may also be highly expressed in tissues which need more ATP for energy.

Understanding the expression patterns of genes in a variety of conditions, such as bacterial infections and different tissues, is a fundamental approach for researching the specific functions of genes [30]. Moreover, the analysis of gene expression can provide useful insights into the comprehension of genes. It is well known that HSPs are involved in the immune response in mammals [31–34]. In aquatic animals, HSPs are also observed to be highly expressed in various tissues under certain stress conditions [35]. However, whether HSP27, HSP60, HSP70 and HSP90 are involved in the immune processes of *S. prenanti* so far remains a question. In this study, the expression patterns of the four *Sp*HSPs under LPS stress were observed. From the analysis shown in Figure 6 to Figure 9, the four *Sp*HSPs mRNA transcripts were ubiquitously expressed in all the tissues examined. The differential temporal expression levels of mRNAs under LPS challenge were found. For example, *Sp*HSP70 is the most abundant in the head kidney, followed by *Sp*HSP27 in the spleen. *Sp*HSP60 and *Sp*HSP90 both displayed weak expression in all the tissues tested, about 20–70 fold less than *Sp*HSP70 and *Sp*HSP27 under the LPS stress. It was suggested that HSP70 is the main mediator of activation by bacterial LPS [36], and could activate the innate immune response by an LR4-mediated signal pathway [37]. Perhaps this explains why *Sp*HSP70 expression was the highest under LPS challenge in our study.

HSP27, as a member of the sHSPs family and the most widely distributed and well-studied HSPs in human beings [38,39], is less reported on for aquatic animals. HSP27 in zebrafish embryos has been studied, and the expression pattern partially overlaps with those reported for other heat shock proteins, but not completely [40]. It was reported that sHSPs, including HSP1, HSP2 and HSP20, regulated the physiological and biochemical processes of aquatic animals responding to pathogenic bacteria [41,42]. There are few reports about HSP27 in aquatic animals. The HSP27 gene was identified in *Carassius auratus*, and it inhibited protein aggregation and prevented cell apoptosis [43]. To our knowledge, this study reported the tissue expression pattern of *Sp*HSP27 after LPS challenge for the first time. The abundant expression level indicated that *Sp*HSP27 was significantly involved in the immune response and resistance to LPS infection, and the specific mechanism requires further study.

It has been reported that the hepatic HSP60 expression of *Megalobrama amblycephala* was upregulated with extreme significance at 24 h after *A. hydrophila* infection [44]. However, the results of this study showed that the *Sp*HSP60 expression level of the LPS-stimulated group at 12 h was extremely significantly higher than that of the PBS-injected group, and

24 h post-injection, the other three tissues showed a similar expression pattern of *Sp*HSP60. Although *Sp*HSP60 gene expression is low, as this study has shown, the transcript level was strongly upregulated under LPS challenge. We predict *Sp*HSP60 was also involved in the immune and antioxidant response of *S. prenanti*, along with *Sp*HSP90.

The HSP70 family consists of HSC70 and HSP70. HSC70 was found to be constitutively expressed in cells and usually remains unchanged or slightly upregulated during stress, whereas HSP70 is highly induced in response to stress [45,46]. In another study, the highest expression level of *Sp*HSP70 after bacterial stress was found in the kidney at 24 h after LPS infection, and a similar expression trend was observed within 24 h of bacterial infection. The difference was that no significance was found between the bacterial group and the PBS group at 12 or 24 h post-injection [10]. However, the levels were still significantly higher than those of the PBS-injected group at 12 and 24 h in this study. We speculate that this may be related to the different bacteria used in the two studies. *Mytilus galloprovincialis* was infected with three heat-killed bacteria, and it was found that mussel hemocytes responded differently to different heat-killed bacteria through the expression of HSP70. In other words, HSP70 expression failed to significantly regulate with the infection of *Vibrio splendidus* or *Micrococcus lysodeikticus*, at least during the 72 h post-injection, whereas *V. anguillarum* significantly elevated HSP70 gene expression from 12 to 48 h post-injection [47]. HSP70 was induced in juvenile *Oncorhynchus mykiss* injected with *V. anguillarum* [10] and *O. kisutch* infected with *Renibacterium salmoninarum* [2]. This is consistent with the results of this study. In contrast, HSP70 gene expression was downregulated in *Rhabdosargus sarba* during *V. alginolyticus* challenge [48].

HSP90 also plays a key role in protecting cells against stress damage, such as bacterial infection. It is a major chaperone in eukaryotes, and it can be coupled into complexes with over 400 different proteins [49–53]. There are two HSP90 groups (HSP90α and HSP90β) in vertebrates, which have evolved from common ancestral genes [53]. *Sp*HSP90 was firstly identified and characterized by Pu et al. (2016); their results showed that the *Sp*HSP90 gene was closely related to vertebrate β-isoforms and might have the same functions as the HSP90 genes from other organisms [10]. The expression patterns of *Sp*HSP90 reported in the kidney and hepatopancreas were similar to the results in this study at 24 h, whereas the pattern in the spleen was different. In summary, the results of this study support the conclusion of Pu et al. [10] that the *Sp*HSP90 gene could be induced by bacteria. In addition, *Sp*HSP90 may provide early warning information and be involved in the resistance to bacterial infection. Moreover, the head kidney is the most suitable for detecting *Sp*HSP90 during bacterial infection.

Notably, the four *Sp*HSPs' expression levels were distinct in different tissues, indicating that they may exert their protective effects by tissue- and time-specific mechanisms. It was documented that the upregulation of HSPs may be required for participating in the adaptive immune system, and may be transferred into the appropriate fish organs, which activate the fish body's immune ability to resist a pathogenic bacteria challenge [54].

5. Conclusions

In conclusion, we cloned HSP60 cDNA from *S. prenanti*. The *Sp*HSP60 gene is very similar to the HSP60 of teleosts. The amino acid sequence of *Sp*Hsp60 obtained in this study was highly conserved in evolution. *Sp*HSP60 mRNA was extensively expressed in all the tissues we examined. The significantly increased expression of *Sp*HSP27, *Sp*HSP60, *Sp*HSP70 and *Sp*HSP90 following exposure to LPS may indicate their important roles as molecular chaperones under bacterial stress. The response intensities of the *Sp*HSPs were organ-specific and may be involved in resistance to bacterial infection; therefore, the specific mechanism of *Sp*HSPs in *S. prenanti* needs further study.

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Institutional Review Board Statement: The experimental fish *S. prenanti* used in this study were obtained from a commercial aqua-farm and therefore did not require ethical approval.

Data Availability Statement: Publicly available datasets were analyzed in this study. The rest of the data presented in this study are available on request from the corresponding author.

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