Article

**Schizothorax prenanti** Heat Shock Protein 27 Gene: Cloning, Expression, and Comparison with Other Heat Shock Protein Genes after Poly (I:C) Induction

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**Simple Summary:** *Schizothorax prenanti* is a valuable cold-water fish that is commercially farmed in southwest China. Numerous aquaculture farmers have recently adopted high-density farming to achieve greater economic benefits, but this has rendered *S. prenanti* more susceptible to microbial pathogens and resulted in economic losses. Hence, the immune mechanisms of *S. prenanti* against pathogens should be investigated. Heat shock proteins (Hsps) comprise a family of molecular chaperones that are involved in immune pathways. Here, we identified and cloned the cDNA encoding SpHsp27 gene and detected its tissue distribution and using polyinosinic-polycytidylic acid [Poly (I:C)] as a viral analog to challenge the fish. We also explored the expression of SpHsp27, SpHsp60, SpHsp70, and SpHsp90 in four immune organs from fish that were injected with Poly (I:C). We found that Poly (I:C) induced SpHsp27 expression in all of these tissues, and significantly upregulated most SpHsps genes compared with controls that were injected with phosphate-buffered saline. However, temporal expression and tissues were organ-specific. The present findings will help to further clarify the roles of Hsp genes in the mechanisms of antiviral immunity in fish.

**Abstract:** We identified and cloned cDNA encoding the heat shock protein (Hsp) 27 gene from *Schizothorax prenanti* (SpHsp27), and compared its expression with that of SpHsp60, SpHsp70, and SpHsp90 in the liver, head kidney, hindgut, and spleen of *S. prenanti* that were injected with polyinosinic-polycytidylic acid [Poly (I:C)]. The SpHsp27 partial cDNA (sequence length, 653 bp; estimated molecular mass, 5.31 kDa; theoretical isoelectric point, 5.09) contained an open reading frame of 636 bp and a gene encoding 211 amino acids. The SpHsp27 amino acid sequence shared 61.0–92.89% identity with Hsp27 sequences from other vertebrates and in seven *S. prenanti* tissues. Poly (I:C) significantly upregulated most SpHsps genes in the tissues at 12 or 24 h (p < 0.05) compared with control fish that were injected with phosphate-buffered saline. However, the intensity of responses of the four SpHsps was organ-specifically increased. The expression of SpHsp27 was increased 163-fold in the head kidney and 26.6-fold SpHsp27 in the liver at 24 h after Poly (I:C) injection. In contrast, SpHsp60 was increased 0.97–1.46-fold in four tissues and SpHsp90 was increased 1.21- and 1.16-fold in the liver and spleen at 12 h after Poly (I:C) injection. Our findings indicated that Poly (I:C) induced SpHsp27, SpHsp60, SpHsp70, and SpHsp90 expression and these organ-specific SpHsps are potentially involved in *S. prenanti* antiviral immunity or mediate pathological process.

**Keywords:** antiviral immunity; gene expression; heat shock protein 27; polyinosinic-polycytidylic acid; *Schizothorax prenanti*
1. Introduction

Heat shock proteins (Hsps) are a class of highly conserved proteins (molecular mass, 16–100 kDa) that are produced in prokaryote and euukaryote cells generally in response to stress [1]. Under normal conditions, Hsps help to ensure correct protein folding during synthesis and repair misassembled proteins [2]. The gene expression of Hsps is usually induced by elevated temperatures. However, many Hsps play pivotal roles in intracellular anti-stress and immune processes and are associated with autoimmune diseases [3,4]. They have been widely applied as biomarkers of biological, chemical, and physical stress [5,6]. Heat shock proteins are molecular chaperones that are categorized into Hsp100, Hsp90, Hsp70, Hsp60, and Hsp27 subfamilies of small heat shock proteins, (sHsps) according to their protein sequence homology and molecular mass. Small Hsps belong to a highly conserved class of Hsps consisting of 16–42 kDa proteins [7,8]. The vital functions of sHsps include the degradation of misfolded proteins [9] and the modulation of cell growth, apoptosis, as well as cytoskeleton elements [10].

Various organisms express evolutionarily conserved Hsps that protect against various environmental stressors [3,4]. Heat shock proteins have notable constitutive functions that are essential for protein metabolism in unstressed cells [11,12]. Therefore, heat shock protein genes (SpHsps) distribution in different tissues of Schizothorax prenanti should be investigated under normal conditions.

Fish Hsps have been assessed in coho salmon (Oncorhynchus kisutch) [13], gilthead sea bream (O. mykiss), rainbow trout (Sparus aurata) [14,15], common carp (Cyprinus carpio) [16], miiuy croaker (Mischthys miiuy) [17], and grass carp (Ctenopharyngodon idella) [18,19]. Li et al. (2015) cloned SpHsc70 and SpHsp70 and detected changes in their tissue-specific expression in response to challenge with Aeromonas hydrophila [20]. Pu et al. (2016) cloned and detected changes in SpHSP90 expression after Streptococcus agalactiae infection [21]. However, SpHSP27 has not yet been identified in fish.

Polyinosinic-polycytidylic acid [Poly (I:C)] is a viral analog that stimulates the immune system and induces a characteristic inflammatory response in organisms [22] and it has been used to study the antiviral mechanism of aquatic animals [23–36]. The role of Poly (I:C) in enhancing the natural immunity and reducing disease risk has attracted attention. Moreover, the immune responses to Poly (I:C) differs among fish species [23–26]. However, its effects on different immune tissues of S. prenanti have remained unknown. Heat shock protein 27 has been identified in zebrafish (Danio rerio), desert fish (Poeciliopsis lucida), and channel catfish (Ictalurus punctatus) [27–29]. However, the function of Hsp27 in these fish in response to viral challenge remains unclear.

Schizothorax prenanti belongs to the Schizothoracinae subfamily of the Cyprinidae family. It is mainly distributed in the upper reaches of the Yangtze River, including the Western Plateau of China. This economically important cold-water fish has been artificially cultivated and marketed for consumption at approximately USD6.7 per kilogram. Known as Ya-fish and Yang-fish in Sichuan and Shaanxi Provinces, respectively, this fish has been listed as a protected species in these regions due to the continual decline of wild populations [21,30,31].

In general, the liver, head kidney, and hindgut of the fish are regarded as immune organs that are central to immune responses [32,33] and Hsp gene expression has been assessed in fish spleens. Understanding how SpHsp expression varies after induction by Poly (I:C) might help to clarify the anti-viral and immune mechanisms of S. prenanti and a scientific basis for an in-depth study of SpHsps. Increasing numbers of aquaculture farmers have adopted high-density farming to achieve greater economic benefits, but this strategy renders S. prenanti more susceptible to microbial pathogens such as Streptococcus agalactiae [21] and Aeromonas hydrophila [20,31]. Although viral diseases of S. prenanti remain unknown, the immune response of this fish to viruses should be determined in advance. Therefore, we identified and cloned cDNA encoding SpHsp27 for the first time and analyzed SpHsp27, 60, 70, and 90 responses to Poly (I:C) by quantitative real-time RT-PCR (qRT-PCR).
Our findings will help to further clarify the roles of Hsp genes that are involved in the antiviral immunity mechanisms of fish.

2. Materials and Methods

2.1. Animal Treatment

We obtained healthy cultured *S. prenanti* (121.7 ± 28 g) in May 2020 from Qunfu Yangfish professional breeding cooperative, a commercial breeding aqua-farm in Hanzhong city, Shaanxi, China. The fish were maintained in glass tanks with a size of 60 × 30 × 40 cm³, and with aerated tap water at a temperature of 20 ± 1 °C. The tank filter was cleaned and about a quarter of the aerated tap water was replaced daily. The fish were fed with a commercial feed (floating compound feed with crude protein ≥32%, crude fat ≥3%) at a rate of 2% of their body weight twice daily. After 10 days acclimation, the fish were challenged with an intraperitoneal injection of Poly (I:C) (5 mg/kg body weight, P1530, sigma, St Louis, MO, USA), and the control group was injected with PBS at the same amount. To understand the expression of *SpHsps* in response to Poly (I:C) challenge, anatomical samples of Poly (I:C)-stimulated group and 5 mg/kg body weight PBS-stimulated group were taken at 12 h and 24 h after stimulation. The fish were anesthetized with eugenol at a concentration of 80 mg/L for 3 min before being dissected. The heart, liver, head kidney, hindgut, muscle, intraperitoneal fat, and spleen were collected from four healthy *S. prenanti* for examining the tissue distribution of *SpHsp27*. The liver, head kidney, hindgut, and spleen were collected from four *S. prenanti* of the PBS–injection and Poly (I:C) treatment at 12 and 24 h, respectively. The dissected tissues were preserved in liquid nitrogen for RNA extraction.

2.2. RNA Extraction and cDNA Synthesis

The tissue total RNA was extracted using the Trizol (Invitrogen, Carlsbad, USA) method. The total RNA concentration and purity were determined by RNA electrophoresis and the optical density absorption ratio (A260/280) in the Nanodrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA). First-stand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania), according to the method that was recommended by the manufacturer.

2.3. Partial cDNA Cloning of the *SpHsp27*

According to the transcriptome sequencing of *S. prenanti*, the specific Hsp27 primers were designed (Table 1, Hsp27-F and Hsp27-R) and synthetized by Tsingke Biotechnology Co., Ltd. (Xi’an, China). Liver cDNA was used as a template for *SpHsp27* amplification, with Primerstar® Max DNA polymerase (TaKaRa, Dalian, China). The PCR program was as follows: 35 cycles at 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 40 s. The PCR products were tailed A with 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 *Escherichia coli* DH5α cells. The positive bacteria clones were sequenced.

2.4. Sequence Analysis

The open reading frame (ORF) of *S. prenanti* Hsp27 (*SpHsp27*) was identified using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html (accessed on 10 May 2022)). The isoelectric point and molecular weight were predicted (https://web.expasy.org/compute_pi/, (accessed on 11 May 2022)). The secondary structure composition of *SpHsp27* protein was predicted (https://www.novopro.cn/tools/secondary-structure-prediction.html (accessed on 11 May 2022)). The three-dimensional structure of *SpHsp27* protein was predicted (https://swissmodel.expasy.org/ (accessed on 11 May 2022)). Multiple sequence alignments were performed using the Clustal X2 [34]. A phylogenetic tree of different vertebrates Hsp27 that was based on amino acid sequences was constructed by the neighbor-joining method using MEGA 4.0 software [35].
Table 1. The primers used for SpHsp27 clone and qRT-PCR.

| Primer                     | Sequence (5′-3′)                     | Annealing Temperature (°C) | Size (bp) |
|----------------------------|-------------------------------------|----------------------------|-----------|
| Primers for cloning        |                                     |                            |           |
| Hsp27-F                    | TTCAGCCATGGCGAGAGACGCATT             | 55                         | 653       |
| Hsp27-R                    | GTTGATGTCAGGGTTTTCTTTG              |                            |           |
| Primers for qRT-PCR        |                                     |                            |           |
| Hsp27-F                    | CTCGGGAATGTCTGAGATAAAG              | 62                         | 130       |
| Hsp27-R                    | CTCAATTTGCAAGCGGTTGAT               |                            |           |
| Hsp60-F                    | GGAGACCAAAACACGTGACTAC              | 62                         | 130       |
| Hsp60-R                    | GCACCGCTCCTCTTCTTCAT                |                            |           |
| Hsp70-F                    | CTCTGGAGAATTGACTGAAATAG             | 60                         | 106       |
| Hsp70-R                    | AGGTCACCGTATACCAAAAC               | 62                         | 182       |
| Hsp90-F                    | GACCACCTCCTTCTCCCTTTC              |                            |           |
| Hsp90-R                    | GACACCTCCTCCCTCCAT                 | 62                         | 126       |
| β-actin-F                  | GACCACCTCCTTCTGCATCCAT              |                            |           |
| β-actin-R                  | GTGATCTTCCCTTCTGATCCAT             |                            |           |

2.5. Tissue Distribution of SpHsp27 mRNA under Normal Conditions

The measured tissues in the control group included the heart, liver, spleen, head kidney, muscle, intraperitoneal fat, and hindgut. Total RNA was extracted and cDNA was synthesized as described previously (“RNA extraction and cDNA synthesis”). The qRT-PCR analysis was performed using the applied Biosystems Step One Plus (Life Technologies, Foster City, USA). The gene-specific primers are listed in Table 1. S. prenanti-specific β-actin primers were used to normalize the cDNA quantity for each tissue sample. Quantification of SpHsp27 and β-actin was performed in triplicate on all the samples using FastStart Essential DNA Green Master (Roche), according to the manufacturer’s instructions. The qRT-PCR data were calculated according to the $2^{-\Delta\Delta CT}$ method [36].

2.6. Detection of the Expression Patterns Induced by Poly (I:C)

For the Poly (I:C) challenge, 8 fish were injected intraperitoneally from behind the base of the pectoral fin with Poly (I:C) (5 mg/kg). Another 4 fish were injected intraperitoneally PBS at the same volume, as a control. A total of 4 fish samples were taken at 12 h and 24 h after Poly (I:C) injection, respectively. The head kidney, liver, hindgut, and spleen were collected from each fish and used to isolate the total RNA. To detect the expression of SpHsp27, SpHsp60, SpHsp70, and SpHsp90 changes after Poly (I:C) challenge, total RNA extraction, cDNA synthesis, and qRT-PCR were performed, as described previously (“Tissue distribution of SpHsp27 mRNA in unstressed conditions”). S. prenanti-specific β-actin primers were used to normalize the cDNA quantity for each tissue sample.

2.7. Statistical Analysis

SPSS 22.0 and Graphpad Prism 5.0 software were used for data analysis and histogram, respectively. The mRNA expression levels were analyzed by using the one-way ANOVA method. All the data are presented as the mean ± standard error (n = 4), and the statistically significant differences between PBS control and Poly (I:C) treatment groups at each time point are expressed with asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. corresponding control group at the time points.

3. Results

3.1. Identification, Structural, and Phylogenetic Analysis of SpHsp27

The 653-bp sequence of SpHsp27 cDNA included an ORF of 636 bp (Figure 1). The ORF encoded a predicted protein of 211 amino acids with a calculated molecular mass of 5.31 kDa and a theoretical isoelectric point of 5.09. Figure 2a shows the distribution of strands, helices, and coils, and Figure 2b shows the predicted 3D structure of SpHsp27 protein. The SpHsp27 amino acid sequence was the most similar to those of fish among animals and was the closest to goldfish (Carassius auratus) Hsp27 with 92.89% identity. The multiple sequence alignment in Figure 3 shows that SpHsp27 protein is moderately conserved and
contains a crystallin domain (dark green, with light blue region) and two putative actin interacting domains (light blue).

We analyzed the phylogeny of the SpHsp27 amino acid sequences to determine the evolutionary relationships between SpHsp27 and Hsp27 from other vertebrates, based on sequences in the GenBank database (Figure 4). The results revealed high amino acid sequence identity between SpHsp27 and the Hsp27 of Cyprinids, particularly goldfish.

Figure 1. Nucleotide and deduced amino acid sequences of SpHsp27. * Stop codon.

Figure 2. Predicted secondary structure and 3D-structural models of SpHsp27 protein. (a) Predicted secondary structure of SpHsp27 protein. Helix, ■; coil, ■; strand, □. (b) Three-dimensional model structures of predicted SpHsp27 protein.
Figure 2. Predicted secondary structure and 3D-structural models of SpHsp27 protein. (a) Predicted secondary structure of SpHsp27 protein. Helix, $\alpha$; coil, $\beta$; strand, $\beta$. (b) Three-dimensional model structures of predicted SpHsp27 protein.

Figure 3. Alignment of amino acid sequences of Prenant’s schizothoracin (Schizothorax prenanti) Hsp27 with Hsp27 proteins from goldfish (GenBank accession no. ABI26639), Sumatra Barb (GenBank accession no. NP_001008615), Mangrove killifish (GenBank accession no. AEM65174), Large yellow croaker (GenBank accession no. ADX98507), Nile tilapia (GenBank accession no. AAF13335), Rainbow trout (GenBank accession no. BAF80897), Domestic dog (GenBank accession no. AXQ88113), Wild boar (GenBank accession no. AAV54182), Human (GenBank accession no. BAB17232), Rat (GenBank accession no. AAA41353), and African clawed frog (GenBank accession no. ABF17872). Asterisk (*), identical; colon (:), conserved; and dot (.), semi-conserved residues. The conserved crystallin domain is shown in dark green (including light blue region). There are two putative actin interacting domains that are shown in light blue.

3.2. Tissue Distribution of SpHsp27 Expression in S. Prenanti

We quantified SpHsp27 mRNA expression in the heart, liver, spleen, head kidney, muscle, intraperitoneal fat, and hindgut tissues from four fish using qRT-PCR to determine SpHsp27 transcripts expression. The loading control for normalization was $\beta$-actin. Figure 5 shows the ubiquitous, but variable expression of SpHsp27 transcripts in all seven tissues. The expression of SpHsp27 was significantly higher in the heart and muscle than in all the other tissues, and in all other tissues except the heart, respectively (both $p < 0.05$). The expression SpHsp27 did not significantly differ among the liver, spleen, head kidney, intraperitoneal fat, and hindgut tissues.
Figure 4. Phylogenetic tree of relationships between S. prenanti Hsp27 and other vertebrates. The tree was constructed by the neighbor-joining method using MEGA 4.0 software. The numbers at the nodes indicate proportions of bootstrapping after 1,000 replications. • Schizothorax prenanti Hsp27.

Figure 5. Abundance of SpHsp27 transcripts in the heart, liver, spleen, head kidney, muscle, intraperitoneal fat, and intestine of S. prenanti as determined by qRT-PCR. The loading control for normalization was β-actin. (a, b, c) Means with different letters are significantly different from each other (p < 0.05). Values are shown as the means ± standard error (n = 4). Error bars, standard error of the means (n = 4 fish per group).
3.3. Expression of SpHsps after Challenge with Poly (I:C)

We quantified the transcripts in the liver, head kidney, hindgut, and spleen of S. prenanti by qRT-PCR at 12 and 24 h after injecting Poly (I:C) to determine changes in SpHsp27, SpHsp60, SpHsp70, and SpHsp90 expression.

3.3.1. Expression of SpHsp27 after Injection of Poly (I:C)

The levels of SpHsp27 transcripts were significantly higher in the liver and spleen tissues than those of the controls at 12 h after injecting S. prenanti with Poly (I:C) (\( p < 0.05 \), and \( p < 0.01 \), respectively (Figure 6) but did not significantly differ in the head kidney or the hindgut. However, SpHsp27 was overexpressed and significantly higher in the head kidney and liver that wereinjected after Poly (I:C) injection than in the controls at 24 and 12 h (\( p < 0.001 \)). Moreover, SpHsp27 transcripts were significantly more abundant in the hindgut and spleen at 24 h after Poly (I:C) injection compared with that at 12 h, and PBS (\( p < 0.01 \)).

![Figure 6. Levels of SpHsp27 transcripts that were determined by qRT-PCR in the liver, head kidney, hindgut, and spleen of S. prenanti at 12 and 24 h after Poly (I:C) injection. Values were normalized using \( \beta \)-actin. Statistically significant differences between the groups are indicated by asterisks (*** \( p < 0.001 \). PBS, phosphate-buffered saline (\( n = 4 \) fish per group).](image)

3.3.2. Expression of SpHsp60 after Challenge with Poly (I:C)

The expression of the SpHsp60 gene did not significantly change in the four tissues at 12 h after Poly (I:C) injection compared with the controls. However, the mRNA levels in the liver and head kidney were significantly higher (\( p < 0.001 \)) than that in the PBS controls and 12 h point after Poly (I:C) injection, whereas the those in the hindgut and spleen remained unchanged at 24 h after injection (Figure 7).
3.3.2. Expression of SpHsp60 after Challenge with Poly (I:C)

Figure 7 shows that transcripts of the SpHsp60 gene significantly increased in the liver and hindgut at 12 h after Poly (I:C) injection. The levels were significantly upregulated in the hindgut, liver, and head kidney at 12 and 24 h after Poly (I:C) injection (p < 0.001) compared to the PBS controls. The expression of β-actin was used as the normalization for qRT-PCR. Statistically significant differences between the groups are indicated by asterisks (** p < 0.01, *** p < 0.001). PBS, phosphate-buffered saline (n = 4 fish per group).

3.3.3. Expression Levels of SpHsp70 after Challenge with Poly (I:C)

Figure 8 shows that transcripts of the SpHsp70 gene were upregulated in all four tissues at 12 and 24 h after Poly (I:C) injection. The levels were significantly upregulated and downregulated in the head kidney at 12 and 24 h after Poly (I:C) injection (p < 0.001). The expression of the SpHsp70 gene significantly increased in the liver and hindgut (p < 0.001), as well as the spleen (p < 0.01) at 24 h after Poly (I:C) injection, and might not have reached a peak.
3.3.4. Expression of SpHsp90 after Challenge with Poly (I:C)

Figure 9 shows that SpHsp90 mRNA was significantly upregulated at 12 h after Poly (I:C) injection ($p < 0.001$) in the head kidney and hindgut, but not significantly altered in the liver and spleen. At 24 h after Poly (I:C) injection, SpHsp90 was significantly overexpressed in the liver ($p < 0.001$), and significantly higher in the head kidney than in the PBS control ($p < 0.001$) but did not differ from the level at 12 h after injection. These findings indicated that the increased expression of the SpHsp90 gene persisted in S. prenanti that were injected with Poly (I:C). Moreover, the expression in the hindgut significantly decreased to control levels at 24, compared with 12 h after Poly (I:C) injection ($p < 0.001$). Although the spleen is a major immune organ, SpHsp90 expression did not significantly change within 24 h of Poly (I:C) injection. We speculate that SpHsp90 gene expression in the spleen is involved later during the immune response. However, this speculation requires further investigation.

![Figure 9](image_url)

Figure 9. Levels of SpHsp27 transcripts in the liver, head kidney, hindgut, and spleen of S. prenanti at 12 and 24 h after Poly (I:C) injection that was determined by qRT-PCR. The values were normalized using β-actin. Statistically significant differences between the groups are indicated by asterisks (***, $p < 0.001$). PBS, phosphate-buffered saline (n = 4 fish per group).

4. Discussion

Small Hsps play important roles in cell homeostasis and in the immune responses of fish to a diverse range of environmental pathogens, including viruses [33,37–40]. Heat shock protein 27 is a vital member of the sHsp family and its expression is upregulated in cells that are exposed to viruses, bacteria, heat, and other stresses [41,42]. Moreover, Hsp27 participates in various signaling pathways and might be a significant therapeutic target [43]. Here, we identified and cloned SpHsp27 for the first time. Multiple sequence alignment showed that SpHsp27 protein is moderately conserved, and that its amino acid sequence has high identity with those of Cyprinids, particularly goldfish. The results of SpHsp27 amino acid sequencing and phylogenetic tree analysis revealed that S. prenanti is more closely related to goldfish. This finding was consistent with our previous findings of SpHsp60 [44], whereas SpHsp70 [20], Hsc70 [20], and SpHsp90 [21] were more closely related to common carp, allogynogenetic silver crucian carp (C. auratus gibelio), and zebrafish, respectively. Under non-stressed conditions, the expression of SpHsp27 was maximal in the heart, followed by muscle, consistent with the studies of zebrafish [45], rainbow trout [46], and sea perch (Lateolabrax japonicus) [47]. Le et al. (2017) speculated that the high expression level of Hsp27 in the muscle and heart of sea perch might be related to its important role in muscle maintenance [47]. Additionally, Robinson et al. (2010) have confirmed that Hsp27 plays a key role in stabilizing the actin cytoskeleton within the smooth muscle and
endothelial cells [48] To date, reports describing Hsp27 in fish are scant in the literature. We found that Poly (I:C) significantly upregulated SpHsp27 gene expression in various tissues, especially in the liver and head kidney. This differs from the findings of Stenberg et al. (2019), who found significantly downregulated Hsp27 gene expression in the head kidney leukocytes of Atlantic salmon (Salmo salar) that were incubated with Poly (I:C), compared with the controls [49].

Highly-conserved, multifunctional immunogenic Hsp60 is a mitochondrial matrix protein that is found in organisms ranging from primitive prokaryotes to higher eukaryotes [50,51]. The involvement of Hsp60 in regulating immune responses and modulating signaling pathways has been characterized [52,53]. The Hsp60 gene from pleopoda of the shrimp Litopenaeus vannamei, is significantly upregulated 24 h after infection with white spot syndrome virus [54]. The present findings of SpHsp60 expression in the liver and head kidney 24 h after Poly (I:C) injection is consistent with these results. We showed here that SpHsp60 gene expression notably remained unchanged in the hindgut and spleen within 24 h after Poly (I:C) injection. Huang et al. [54] suggested that Hsp60 plays an important role in shrimp health, especially in promoting inflammation, as well as specific and non-specific and immune responses against bacteria and viruses. Therefore, we inferred that Hsp60 plays a similar but organ-specific role in the anti-pathogen immunity of S. prenanti. However, Hsp60 and Hsp90 expression remains unchanged throughout progressive vibriosis, whereas the expression of Hsp70 and Hsc70 decreases in the kidney and liver tissues of sea bream (Sparus sarba) [55]. Eder et al. [56] showed that the response intensity of Chinook salmon (Oncorhynchus tschawytscha) Hsp60 depends on insecticide induction and is organ-specific. The present findings suggest that the intensity of the SpHsp60 gene response is also organ-specific, and that SpHsp60 expression in the liver and head kidney might be an early marker of viral infection in S. prenanti.

The Hsp70 family is encoded by non-inducible heat shock cognate protein 70 kDa (Hsc70) and inducible Hsp 70 kDa (Hsp70) genes [57]. Various types of stress induce Hsp70 expression, whereas Hsc70 expression remains unchanged or slightly upregulated [57,58]. Therefore, we investigated the SpHsp70 gene. Heat shock protein 70 suppresses apoptosis by directly associating with Apaf-1 [59]. The yellow drum (Nibea albiflora) Hsp70 gene (NaHsp70) is significantly upregulated in the liver at 12 h after Poly (I:C) infection, and peaks at 24 h [60]; our results were similar to these. However, NaHsp70 mRNA expression in the S. prenanti spleen was significantly upregulated at 12, 24, and 36 h, whereas SpHsp60 was not expressed at 12 h, but significantly upregulated at 24 h after Poly (I:C) injection. Moreover, NaHsp90 expression peaks at 12 h in the liver, whereas SpHsp90 expression in the liver peaked at 24 h after Poly (I:C) injection. Temporal expression also differed between NaHsp90 and SpHsp90 in spleen tissues. We speculate that this could be due to species-specific differences. The Hsp70 and Hsp90 genes in the two fish species were activated by Poly (I:C), although the timing of significant upregulation differed. Streptococcus agalactiae significantly induces SpHsp90 gene expression in the blood, liver, spleen, and trunk kidney, and peaks at 24 h in the liver, spleen, and kidney of S. prenanti [21]. Our results somewhat differed, suggesting that SpHsp90 gene expression is pathogen-specific. In summary, the SpHsp90 gene might be involved in resistance to bacterial and viral challenge and might be an early marker of infection. Although the spleen is a main immune organ, we found that SpHsp90 expression did not significantly change within 24 h of Poly (I:C) injection. We speculated that SpHsp90 expression in the spleen is involved in the immune response at a later time. However, this awaits confirmation by further study.

The expression of SpHsp27 at 24 h after Poly (I:C) injection was higher in the head kidney than in other tissues, suggesting that it is most suitable for detecting SpHsp27 24 h after viral infection. The expression profiles of the other three SpHsp genes were similar. For example, the hindgut is suitable for assessing SpHsp60 and SpHsp70 and the liver is suitable for assessing SpHsp60 and SpHsp90 at 24 h after viral injection. We also found that the liver and spleen were the most sensitive to Poly (I:C) at 12 h after injection and were thus suitable for evaluating SpHsp27 expression, whereas SpHsp70 could be evaluated in the head kidney,
and SpHsp90 could be evaluated in the head kidney and hindgut. Furthermore, regulation of the expression of the four SpHsp genes was quite complex. Many immune-related genes and signaling pathways are expressed at the transcriptional and translational levels.

Our findings showed that S. prenanti is very sensitive to Poly (I:C). The significantly increased expression of SpHsp27, SpHsp60, SpHsp70, and SpHsp90 after Poly (I:C) injection indicated that they potentially play important roles as molecular chaperones under virus-induced stress. In summary, Poly (I:C) induced the expression of four SpHsps with different tissue-specific and temporal profiles, indicating coordinated action against Poly (I:C). Further studies are needed to determine the role of SpHsps in the innate immune defense system against viral pathogens, or mediate pathological process, and in the involved signaling pathways.

5. Conclusions

In the present study, the partial cDNA encoding the full ORF of SpHsp27 genes of S. prenanti was successfully cloned and characterized. Phylogenetic analysis showed that the SpHsp27 protein is most closely related to Hsp27 from goldfish. Multiple sequence alignment showed that the SpHsp27 is moderately conserved. The SpHsp27 gene expressed in all tissues that were examined herein was cloned for the first time. Poly (I:C) organ-specifically-induced SpHsp27, SpHsp60, SpHsp70, and SpHsp90 that are potentially involved in antiviral immunity or mediate pathological process. Notably, SpHsp27 was the most sensitive to Poly (I:C) followed by SpHsp70.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Ethics Committee of the Shaanxi Institute of Zoology (protocol code: L22D003A51, date of approval: 4 July 2022) for studies involving animals.

Informed Consent Statement: Not applicable, as this research did not involve any humans.

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

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