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ISSN 0792 - 156X

PUBLISHER:

The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB)

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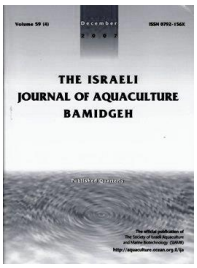

Heat Shock Protein 70 Gene of Ya-Fish (*Schizothorax Prenanti*) Responses to Thermal Stress and Bacterial Challenges

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Keywords: *Hsp70*; *Schizothorax prenanti*; gene expression; thermal stress; *Streptococcus agalactiae*; *Aeromonas hydrophila*

Abstract

The homeostasis of Ya-fish (*Schizothorax prenanti*) health may be affected by a wide variety of stressful stimuli from the complex aquatic environment. Heat shock proteins are extensively distributed and highly conserved in various animals, particularly Hsp70 which is a novel and potential biomarker for stressful environmental factors and disease conditions. The expression of *SpHsp70* was detected to evaluate the potential use of *SpHsp70* as a warning signal for some stimuli. In this study, freshwater *Schizothorax prenanti* were exposed to thermal stress and pathogenic bacteria in the aquatic environment. Results showed that *SpHsp70* were ubiquitously and differentially expressed in nine examined organs/tissues. In Ya-fish, stessor-specific reactions were observed after different bacterial challenges. At the acute stage, *SpHsp70* expression was significantly up-regulated in the kidney after *Streptococcus agalactiae* challenge, however *SpHsp70* expression significantly increased in the spleen after *Aeromonas hydrophila* challenge. These reactions suggest that the response of *SpHsp70* to bacterial stimuli depends on different stressors. Hence, thermal sensitivity and stressor-specificity precluded the simple use of *SpHsp70* as warning information in *S. prenanti*.

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Introduction

Schizothoracinae sp. are mainly distributed on the Qinghai-Tibetan Plateau and its peripheral areas and have successfully adapted, physiologically and in their cellular responses, to high-altitudes (Zhang et al., 2016). They are an endemic edible fish in the upper Yangtze River. Over the last decade the wild populations of *S. prenanti* have dramatically declined due to human impact. To protect this natural resource and provide safe animal protein, artificial culture of *S. prenanti* has developed in the southwest of China. However, it is susceptible to several bacterial pathogens, including *Aeromonas hydrophila* (Du et al., 2011), *Streptococcus agalactiae* (Geng et al., 2012), and *Edwardsiella tarda* (Zhou et al., 2016) in aquaculture. Therefore, monitoring the health status of *S. prenanti* is significant for long term aquaculture management. An interesting assumption is to establish sensitive and reliable warning information regarding the health status of *S. prenanti.*

Heat shock proteins (HSPs) are recognized as the most inducible indicators of stress. They were first described as stress proteins in relation to heat shock (Ritossa, 1962). The universality among divergent organisms of HSPs response to heat shock has been widely recognized. Heat shock protein 70 (Hsp70) has functions similar to other HSPs, maintaining cellular homeostasis of the cellular network, assisting in the folding processes of nascent proteins, and mediating the repair and degradation of denatured proteins (Mayer and Bukau, 2005). Much research has reported biomarker effects of environmental stressors on Hsp70 in turtles (Tedeschi et al., 2015), tilapia (Bilotta and Brazier, 2008), crucian carp (An et al., 2014), mandarin fish (Xu et al. 2015) and mud crabs *Scylla paramamosain* (Cheng et al. 2018). In addition to responding to environmental stressors, recent reports have revealed the response of Hsp70 to biological stressors. In humans, Hsp70 is regarded as a useful marker that reflects various health conditions such as inflammation, sepsis, asthma, and cancers (Qu et al., 2015). Commensal bacteria and pathogenic bacteria are an essential component of the aquatic microbiological flora. In fish, the infection of pathogenic bacteria triggers up-regulation of *Hsp70* mRNA (Han et al., 2017). Therefore, as a sensitive indicator, *Hsp70* may provide warning information of bacterial infection in *S. prenanti*.

The aim of this experiment was to evaluate the response/induction of *S. prenanti Hsp70* following thermal stress and pathogenic bacterial infection and to better understand the expression characteristics of *SpHsp70.*

Materials and Methods

Subjects.

Juvenile *S. prenanti, (*weight 45-62g, age 12-16 months) were collected from a local fish farm in Ya'an (Sichuan, China). The fish were acclimated in tanks (100cm×40cm×60cm) for 1 week where feeding and water exchange was conducted as described (Pu et al., 2016). Five fish were randomly selected to isolate bacteria on Trypticase Soy Broth and Brain Heart Infusion (BXB, Beijing, China) (Du et al., 2011; Geng et al., 2012). Prior to experimental treatment or organ extraction, fish were anesthetized in 0.02% MS-222. The animal experiments were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University.

Tissue specificity and thermal treatment.

Five healthy *S. prenanti* were used for analysis of the specific expression of *SpHsp70* in the liver, kidney, spleen, blood, brain, heart, skin, gills, and intestine. The fish were held at a temperature of 20°C before thermal treatment. Six fish were directly shifted to tanks wih a constant temperature of 25°C for 2h. The liver, spleen, kidney, and blood of fish were sampled after the fish had been held in a tank with water temperature of 20°C for 1h. A control group of six fish were transferred following the same procedure to a tank with filled with the same temperature (20°C) water.

Bacterial challenge.

Considering the different epidemiologic temperatures of bacteria, the bacterial challenge experiment was divided in two treatments, Gram-positive treatment and Gram-negative bacteria treatment. The Gram-positive bacterial challenge group (n=20) was intraperitoneally inoculated with 0.1ml *S. agalactiae* (1.5×10^7CFU/ml) and was maintained at 23±1°C. The Gram-negative bacteria group (n=20) was intraperitoneally inoculated with 0.1ml *A. hydrophila* (1.5×10^7 CFU/ml) and was maintained at 20°C. The respective control group (n=20) was given an intraperitoneal injection with the same volume of phosphate-buffered saline (PBS) at respective temperatures. Then, the liver, spleen, kidney, and blood of fish were sampled at 6, 24, 72, and 120 h post-challenge (hpc) from each group.

RNA isolation and quantitative polymerase chain reaction (qPCR) assay of SpHsp70.

RNA extraction and cDNAs synthesis were conducted as described previously (Pu et al., 2016). Approximately 0.1-0.2ml of blood per fish was extracted from the caudal vein and immediately transferred to Cell Lysis Buffer (BIKW, Beijing, China). The samples were transferred to RNA/DNA sample protector (TaKaRa, Dalian, China), and stored at -80°C until use. Total blood RNA was purified with the Blood Total Protein, total RNA, DNA, and microRNA-kit (BIKW) was purified using the RNAiso Plus kit (TaKaRa). The quantity and quality of RNA were assessed by OD260/280 test and 1.5% agarose electrophoresis. cDNA was synthesized by the PrimeScriptTM RT reagent kit with gDNA Eraser (TaKaRa).

The cDNA samples were used as template for qPCR with a pair of specific primers (*Hsp70*-F: 5′-CGGCCATGAACCCCAACAAC-3′, *Hsp70*-R: 5′- TGAACTTTCGGCTTCCCTCCA -3′) designed to amplify fragments of *SpHsp70* (Genbank accession: KC521443.1). *β-actin* and *18S-rRNA* served as endogenous reference genes to verify the quality and quantity of samples. The primers used were *β-actin* F(5′- CGAGCTGTCTTCCCATCCA -3′) and *β-actin* R(5′- TCACCAACGTAGCTGTCTTTCTG -3′), *18S-rRNA* F(5′- ACCACCCACAGAATCGAG AAA -3′) and *18S-rRNA* R(5′-GCCTGCGGCTTAATTTG ACT -3′). The qPCR amplification program was: 94°C for 5 min; 30 cycles of 94°C for 30 s, 58.5°C for 30 s, 72°C for 16 s; 72°C for 10 min, followed by a melting curve program (65–95°C with a heating rate of 0.5°C/s). The annealing temperatures of *β-actin* and *18S-rRNA* were 60°C and 58°C. Three replicates were taken for every sample.

Statistical analysis.

The expression data were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Quantitative data for *SpHsp70* mRNA levels were obtained by qPCR with *β-actin* and *18S rRNA* as endogenous reference genes to normalize the expression levels of samples. The statistical difference between the control group and treatments were tested by Student's t-test. All data are presented as the mean ± standard error (M ± SE). Differences between means were considered significant at the 95% confidence level ($P < 0.05$).

Results

Tissue specificity of SpHsp70 expression.

SpHsp70 transcripts were extensively and differentially expressed in the nine examined organs (Fig.1); these included hematic system (blood, heart), urinary system (kidney), respiratory system (gill), integumentary system (skin), immune system (spleen), nervous system (brain) and digestive system (liver, intestine). Among these organs, the highest expression of *SpHsp70* was in the blood (141.9 fold) and the lowest in the intestine (1.0 fold). A relatively high expression was observed in the kidney (28.3 fold), gill (17.3 fold), skin (15.0 fold) and heart (12.8 fold). The relative low expression of *SpHsp70* was detected in the spleen and in the liver (Fig.1).

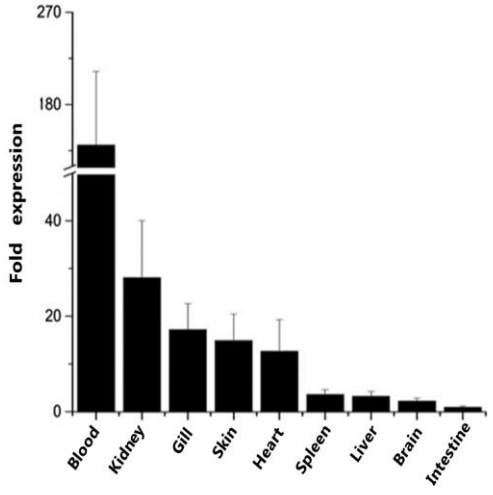

Fig. 1 The relative mRNA expression of *SpHsp70* in different organs. Quantitative data were obtained by qPCR. Results were normalized by *β-actin* and *18S rRNA*. Intestine was taken as control. Values are represented as mean ± standard error (n=5).

Expression pattern of SpHsp70 after thermal stress.

Compared with control group, significant up-regulated expressions of *SpHsp70* were observed in kidney (28.4 fold), spleen (9.5 fold), blood (5.4 fold), and liver (2.9 fold) after thermal stress (Fig.2). The results exhibited that *SpHsp70* was a sensitive factor for response to thermal stress. Among these organs, the kidney was the most sensitive organ to heat shock.

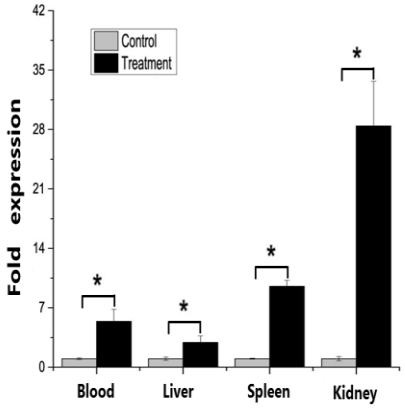

Fig. 2 The relative mRNA expression of *SpHsp70* after thermal stress. Quantitative data were obtained by qPCR. Results were normalized by *β-actin* and *18S rRNA*. Values were represented as mean ± standard error (n=6). Control groups were taken as the calibrator. Asterisk (*) indicated a significant difference ($P<0.05$).

Expression patterns of SpHsp70 after bacteria challenge.

mRNA expression of *SpHsp70* in the bood samples was induced after *S. agalactiae* challenge at 6hpc (2.1 fold) and 24hpc (7.9 fold), but it significantly decreased at 120hpc (0.7 fold) (Fig.3a). There was no *SpHsp70* expression after *A. hydrophila* challenge at 6hpc. A significant down-regulation was observed from 72hpc (0.4-0.6 fold) compared with control group (Fig.3b).

In the liver, the response of *SpHsp70,* after two different bacteria challenges was similar to changes in the blood. At 6bpc, *S. agalactiae* challenge stimulated up-regulated expression of *SpHsp70* (3.9 fold), and subsequently decreased at 72hpc and 120hpc after both bacteria challenges. There was significant down-regulated expression after *A. hydrophila* challenge at 72hpc (0.4 fold) (Fig.3d).

In the spleen, *SpHsp70* expression decreased with significant down-regulation at 72hpc (0.3 fold) and increased at 120hpc (2.5 fold) after *S. agalactiae* challenge (Fig. 3e). The opposite tendency was observed after *A. hydrophila* challenge. The expression increased significantly at 6hpc (4.1 fold) and then recovered to the control level (Fig. 3f).

In the kidney, *SpHsp70* expression was induced by *S. agalactiae* challenge at 6hpc, 24hpc and 120hpc, and was significantly up-regulated at 6hpc (6.8 fold) (Fig. 3g). However, *SpHsp70* expression was similar to the changes in the blood and liver after *A. hydrophila* challenge. Significant down-regulation appeared at 72hpc (0.4 fold) (Fig. 3h).

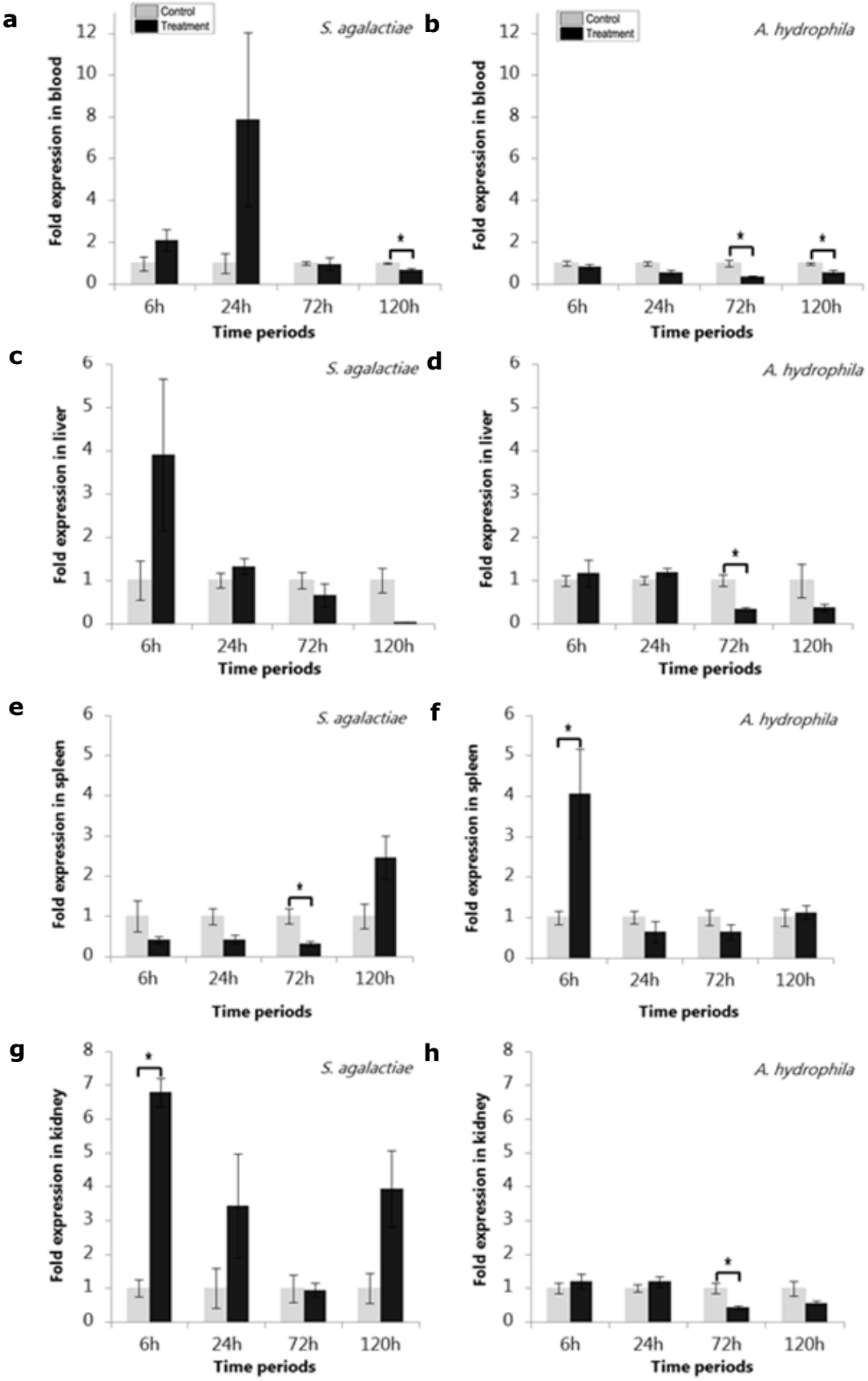

Fig. 3 The relative mRNA expression of *SpHsp70* after bacteria challenge. Quantitative data were obtained by qPCR. Results were normalized by *β-actin* and *18S rRNA*. Control groups were taken as the calibrator. Values were represented as mean ± standard error (n=5). Asterisk (*) indicated a significant difference (P<0.05).

The Schizothoracinae family is one of the diverse orders of cyprinids in the Qinghai-Tibetan Plateau and periphery, which are adapted to the diurnal temperature differences in the local environment. Heat shock protein 70 (Hsp70) can read the structure of nascent polypeptide chains, refold the denatured proteins, and degrade the damaged proteins. As the chaperone protein, it regulates and controls cell homeostasis, proliferation, differentiation, and cell death (Mayer and Bukau, 2005). *SpHsp70* was ubiquitously expressed in all examined organs in this study. This was consistent with the findings in *Siniperca chuatsi* (Wang et al., 2014), *Oreochromis niloticus* (Zhang et al., 2014) and *Huso dauricus* (Peng et al., 2016), but the findings herein exhibited tissue-specific differences of SpHsp70 expression in *S. prenanti.* Tissue-specific differentiation is related to organs and the rate of protein renovation. In this study, relative expression of *SpHsp70* in different tissues was similar to previous reported results in *S. prenanti* (Li et al., 2015). Results indicated that tissue differentiation of *Hsp70* was species-specific.

HSPs are sensitive to heat shock and have been widely discussed as biomarkers of environmental stress in aquatic animals. Temperature change can be a common environmental stressor either in wild or cultured fish. The expression of *Hsp70* plays a critical role in thermal stress in many fish (Giri et al., 2014; Liu et al., 2017). It is worth considering that some fish may not respond to heat shock. The classic example of this phenomenon is that the highly cold-adapted, stenothermal Antarctic fish *Trematomus bernacchi* could not induce *Hsp70* under high-temperature stress (Hofmann et al., 2000). *SpHsp70* cDNA of *S. prenanti* has a highly conserved sequence which shared 97.9% identity with *Cyprinus carpio* (Li et al., 2015). The function of the gene is the result of its adaptive evolution. In this study, the expression levels of *SpHsp70* were significantly elevated in kidney, spleen, liver, and blood after thermal stress. A similar *Hsp70* expression was observed in *Labeo rohita* (Giri et al., 2014) and *Paralichthys olivaceus* (Liu et al., 2017). Under thermal stress, the rapid response of *Hsp70* was helpful in maintaining cellular homeostasis and tissue function. In this study, *SpHsp70* expression was highest in the kidney, which was considered as the most sensitive organ to thermal stress. The main function of kidney is the maintenance of water and sodium metabolism. Research has explained that chronic or repeated episodes of heat stress accompanying dehydration or volume depletion can cause repeated subclinical kidney injury (Fabiana B.Nerbass et al., 2017).

Pathogens are biological stressors in aquaculture (Iwama et al., 2004). Studies have suggested that bacterial challenge triggers *Hsp70* mRNA expression (Han et al., 2017). *S. agalactiae*, a gram-positive coccus, is a kind of harmless commensal strain in ordinary situations. But pathogenic *S. agalactiae* causes serious fish infection and zoonotic infection associated with freshwater fish when faced with harsh biological environments (Kalimuddin et al., 2017). In this study, the *S. agalactiae* challenge differed from the serious damage caused by *S. prenanti* causing systemic sepsis as described by Geng (Geng et al., 2012). In other findings, researchers found that, as intracellular bacteria, *S. agalactiae* could persist inside macrophages after nonopsonic phagocytosis, and eventually trigger programmed cell death (PCD) in macrophages and monocytes (Ulett et al., 2003). In our study, significant up-regulation of *SpHsp70* in liver, blood, and kidney at the early stage (6hpc-24hpc) may be due to the fact that HSP70 over-expression protected the cell against PCD; this has been partially corroborated by Chang (Chang et al., 2014). At 120hpc, a down-regulated expression was observed in the blood ($P<0.05$). Down-regulation of *Hsp70* was also reported in channel catfish (Shtratnikova et al., 2016) and sea bream (Deane and Woo, 2005) after bacterial infection. Research provided an evidence that down-regulation of *Hsp70* can eventually aggravate hypoxia-induced apoptosis by increasing the expression of NF-κB and caspase-3 (Xie et al., 2017).

In the spleen, *SpHsp70* expression significantly decreased at 72hpc ($P<0.05$) but recovered at 120phc. The spleen is a phagocytic filter that removes bacteria from the

bloodstream and plays a role in sequestering bacteria. It was the main site where splenic necrosis was observed in *S. prenanti* after *S. agalactiae* infection after histopathology research (Geng et al., 2012). Nucleus changes caused by DNA degradation were characteristic of necrosis. DNA damage associated with cell death, induced by down-regulation of heat shock transcription factor (*Hsf1*), is a critical component for *Hsp70* induction (Kim et al., 2012). Therefore, we speculated that the *Hsp70* down-regulation was mainly due to the confrontation between bacterium and host organs.

A. hydrophila, a gram-negative motile rod, is an opportunistic pathogen of fish which typically causes hemorrhagic septicemia. In *S. prenanti*, skin ulceration is the usual symptom of *A. hydrophila* infection (Du et al., 2011). In the blood, liver and kidney, *A. hydrophila* did not up-regulate the expression of *SpHsp70* at the early stage (6hpc-24hpc) after infection. This suggests rather that *A. hydrophila* was not recognized as a danger to the host organism. The expression of *SpHsp70* in the spleen was significantly up-regulated at 6phc ($P<0.05$). However, our findings on its expression in the liver after *A. hydrophila* infection differed from another published report (Li et al., 2015). Different host stage, challenge temperature, and strain virulence may affect the infection status and expression level of the gene. When comparing the mRNA relative expression of *SpHsp70* during Gram-positive bacterial (*S. agalactiae*) challenge, with Gram-negative bacterial (*A. hydrophila*) challenge, distinct trends were observed in four examined organs. Our results showed that stressors-specific response of *SpHsp70* reacted differently in various organs and tissues when challenged with pathogenic bacteria.

In conclusion, *SpHsp70* was sensitive to thermal stress, and the most sensitive organ was the kidney. We suggest that bacteria stimulate the *SpHsp70* response and this depends on different bacterial stressors. Hence, thermal sensitivity and stressor-specificity precludes the simple use of *SpHsp70* as a warning signal in *S. prenanti.*

Acknowledgements

This work was supported by the double branch plan of Sichuan Agricultural University.

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