

mRNA expression profiles of heat shock proteins of wild and salinity-tolerant swimming crabs, *Portunus trituberculatus*, subjected to low salinity stress

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ABSTRACT. Challenged by the low salinity, 4 parts per thousand (4 ppt), for 72 h, the survivals of swimming crabs (*Portunus trituberculatus*) were collected as the screened group (SG, tolerant to low salinity). Aiming at identifying the mechanism of low salinity tolerance, quantitative real-time PCR was employed to investigate the expression profiles of 4 HSP genes (*HSP60*, *HSP70*, *HSP90-1*, *HSP90-2*) in the hepatopancreas of wild (WG) and screened (SG) groups of *P. trituberculatus* exposed to low salinity (4 ppt). The results showed that 3 of the candidate genes (*HSP60*, *HSP70*, *HSP90-1*) exhibited similarly downregulated expression profiles in the first 3 h ($P < 0.05$), which became upregulated from 3 h to 72 h after being subjected to low salinity conditions. In contrast, the expression profile of the *HSP90-2* gene was upregulated during the first 6 h for the WG, and during the first 12 h for the SG, after which it became downregulated. *HSP90-1* and *HSP90-2* were highly expressed at 12 h after low salinity challenge in the SG, but not the WG. The response of these 2 genes to salinity stress indicates

their suitability as biomarkers to differentiate SG from WG crabs. The results indicate that HSP genes are involved in the adaptation of crabs to low salinity exposure, and that different HSPs have diverse functions in response to low salinity stress in *P. trituberculatus*. In addition, HSP expression in SG indicates that this group is more tolerant to low salinity conditions compared to WG.

Key words: *Portunus trituberculatus*; Low salinity stress; qRT-PCR; HSP

INTRODUCTION

Heat shock proteins (HSPs) are a family of highly conserved proteins involved in the stress response formed during the process of biological evolution (Feder and Hofmann, 1999). HSPs are classified into 5 families based on their molecular weights; including HSP100, HSP90, HSP70, HSP60, and small HSPs (Georgopoulos and Welch, 1993; Moseley, 1997). These proteins have been reported to be present in all organisms from bacteria to mammals, and play critical roles in protein folding, assembly, intracellular localization, and the regulation of gene expression (Morimoto, 1998; Terasawa et al., 2005). Abnormal environment factors (such as high temperature, heavy metals, high or low salinity) that could potentially damage cells stimulated the production of HSPs (Deane et al., 2002; Spees et al., 2002a). To date, several studies have reported the expression modes of HSPs under salinity stress. For example, when Atlantic salmon (*Salmo salar*) are transferred to high-salinity sea water, HSP90 levels significantly increased (Pan et al., 2000). In addition, the expression of HSP70 and HSP90 in the abdominal muscle of the lobster *Homarus americanus* exhibits major changes under both low and high salinities (Spees et al., 2002b).

The swimming crab, *P. trituberculatus*, is widely distributed in the coastal waters of Japan, Korea, and China (Dai et al., 1986), and is a commercially important fishery and aquaculture species in China (Sun, 1984). As a euryhaline crab species, *P. trituberculatus* tolerates a wide range of ambient salinities, from 10 ppt to 38 ppt, with optimal salinities of 20 ppt to 35 ppt (Ji, 2005). However, some individuals exhibit high flexibility to extremely low salinity. Such individuals have been screened for use in the field of genetics and breeding, due to their great practical value in production practices. In our previous research, a large number of crabs (2.97 ± 0.44 g) were placed in a salinity of 4 ppt for about 72 h, with the surviving individuals being collected for culture under normal conditions. Subsequently, the surviving individuals were designated as the screened group (SG). Then, SG and wild group (WG) individuals were separately bred under the same environmental conditions for the further experiments.

The entire life history of the swimming crab is influenced by external environmental salinity. Although the saline adaptability of swimming crabs involves a series of reactions at the individual, protein, and molecular level, the osmotic regulation mechanism at the molecular level might underlie this species' adaption to saline conditions. The molecular characterization of HSP60, HSP70, and HSP90 from *P. trituberculatus* have been previously reported (Zhang et al., 2009; Cui et al., 2010; Xu and Qin, 2012). In this paper, the expression levels of *HSP60*, *HSP70*, *HSP90-1*, and *HSP90-2* in the hepatopancreas of WG and SG swimming crabs, *P. trituberculatus*, during 72 h exposure to low salinity (4 ppt) were detected, with the purpose of identifying the mechanism of low salinity tolerance.

MATERIAL AND METHODS

Animals and sample treatment

WG and SG swimming crabs (*P. trituberculatus*) were collected from a commercial farm in Xiangshan, China. Individuals were fed with frozen fish once daily from 5pm to 6pm. For the salinity challenge, the normal salinity of seawater was 30 ppt, and a normal temperature was maintained at $(27.8 \pm 1^\circ\text{C})$. Then, crabs, averaging $(64.1 \pm 6.4 \text{ g})$ of WG and $(66.5 \pm 4.3 \text{ g})$ of SG in body weight, were exposed to 4 ppt salinity stress, and sampled at 0, 3, 6, 12, 24, 48, and 72 h after challenge. The hepatopancreas of each crab was sampled and stored at -80°C until analysis.

RNA purification and first-strand synthesis of cDNA

Total RNA was extracted by the RNAiso Plus (Takara, Tokyo, Japan) following manufacturer protocols. The integrity of RNA was detected on 1.0% agarose gel, and the RNA was stored at -80°C before use. The first-strand of cDNA was obtained by a RevertAid™ First Strand cDNA Synthesis Kit (Thermo, MD, USA) in a 20- μL volume containing total 5 μg RNA and 5 μM oligo (dT) primer. The mixture was incubated at 42°C for 1 h, terminated by heating at 70°C for 5 min, and subsequently stored at -80°C .

Quantification of mRNA expression levels

qRT-PCR was performed by SYBR Premix Ex Taq™ (Takara, Tokyo, Japan). Amplification was carried out on a 96-well plate in 20- μL reaction volume containing 10 μL 2xSYBR Premix Taq™ (Takara, Tokyo, Japan), 0.3 μL PCR forward primer (10 pM), 0.3 μL reverse primer (10 pM), 1.5 μL cDNA template (samples diluted 5 times), and 7.9 μL ddH₂O. The thermal profile for real-time PCR was 95°C for 2 min, followed by 40 cycles at 95°C for 5 s and 60°C for 15 s. Four pairs of specific primers (HSP60F/HSP60R, HSP70F/HSP70R, HSP90-1F/HSP90-1R, and HSP90-2F/HSP90-2R) were used to detect the transcripts of *HSP60*, *HSP70*, *HSP90-1*, and *HSP90-2* respectively. β -actin was used as the internal control. All primers used in this study are shown in Table 1. A melting curve analysis (60 to 95°C , with increments of 0.5°C per second) was added after the amplification to confirm that only one product was amplified and detected. ddH₂O was used instead of cDNA template as PCR negative control.

Table 1. Informations of primers used in this study.

Primers	Sequence (5' to 3')	Sequence information
HSP60F	AGGAGCGAATGGCAGCACT	Q-RT HSP60 primer
HSP60R	ATCCAGGGCAGGCAAGCAG	Q-RT HSP60 primer
HSP70F	CCGTATCCCTAAGACCCAGAAA	Q-RT HSP70 primer
HSP70R	CAGCCTCAGACTTATCACCAAAA	Q-RT HSP70 primer
HSP90-1F	CTATCCCATCAGGCTCCTTGTT	Q-RT HSP90-1 primer
HSP90-1R	CACCTACATCCTCAATCTTGGCTT	Q-RT HSP90-1 primer
HSP90-2F	TCTCTTCGCCTTCGCCTCTCA	Q-RT HSP90-2 primer
HSP90-2R	AAGCGTTCGTGGATGGTGCCT	Q-RT HSP90-2 primer
β -actin-F	TCACACACTGTCCCATCTACG	Q-RT actin primer
β -actin-R	ACCACGCTCGGTACAGATTTC	Q-RT actin primer

Data analysis

All experiments were performed in triplicate. Gene expression level was determined by the $2^{-\Delta\Delta C_t}$ method. All data are reported as means \pm SD (standard deviation) in terms of their relative mRNA expression. The results were analyzed by one-way analysis of variance (one-way ANOVA), followed by the Duncan multiple range tests using the SPSS software 19.0. P values less than 0.05 were considered to be statistically significant.

RESULTS

Expression profiles of HSP60 after low salinity challenge

The *HSP60* expression level in the 2 groups of swimming crabs followed a similar pattern (Figure 1). For the WG (Figure 1A), *HSP60* expression significantly decreased at 3 h ($P < 0.05$). From 3 to 48 h, HSP expression alternately increased and decreased, but with no significant difference ($P > 0.05$), then it sharply increased at 72 h ($P > 0.05$). For the SG (Figure 1B), *HSP60* expression was significantly reduced during the first 3 h ($P < 0.05$), but gradually rose from 3 to 48 h, and then dramatically rose to the highest level at 72 h ($P < 0.05$).

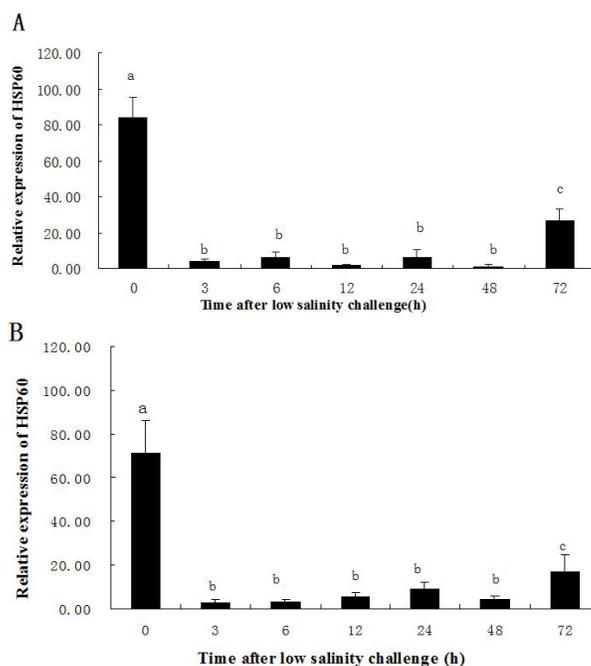


Figure 1. Relative expression level of *HSP60* in hepatopancreases after low salinity (4 ppt) stress of wild swimming crab. **A.** Screened swimming crab; **B.** measured by SYBR Green RT-PCR. The β -actin gene is used as an internal control to calibrate the cDNA template for all the samples in each determination. Each bar represents the mean value from four determinations with standard error. The hepatopancreases were collected from four individual crabs at 0, 3, 6, 12, 24, 48, and 72 h. Different lowercase letters indicate statistically significant differences ($P < 0.05$) in relative expression of each gene among different sampling time points.

Expression profiles of HSP70 after low salinity challenge

As shown in Figure 2, the *HSP70* transcripts from both groups under low salinity (4 ppt) stress had a curvilinear trend as time progressed. Compared to the control (untreated 0 h), the expression of *HSP70* in both groups initially declined in the first 3 h ($P < 0.05$) and then increased (Figure 2). In contrast, at 48 h post-stress, the *HSP70* transcripts of SG (Figure 2B) almost recovered to the original level, whereas that of WG (Figure 2A) was maintained at a low level until 72 h.

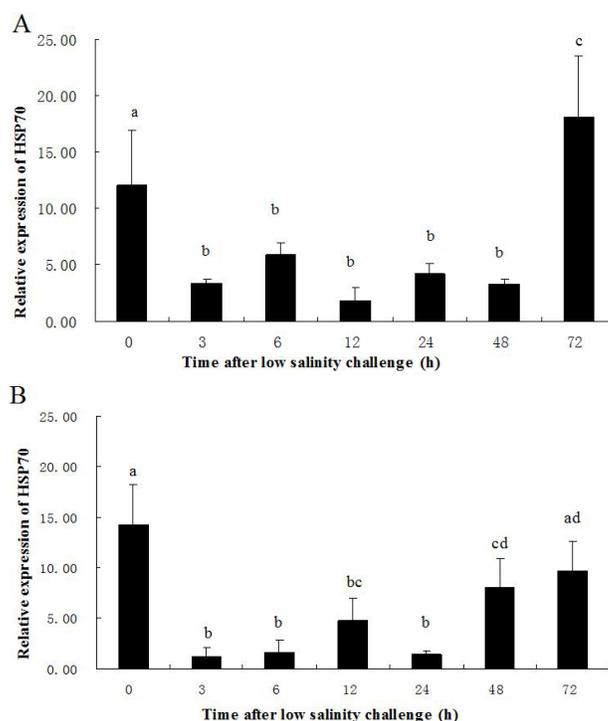


Figure 2. *HSP70* transcript temporal expression relative to β -actin in hepatopancrease of wild swimming crab. **A.** Screened swimming crab; **B.** after low salinity (4 ppt) stimulation was analyzed by RT-PCT. Each bar represents the mean value from four determinations with standard error. The hepatopancreases were collected from four individual crabs at 0, 3, 6, 12, 24, 48, and 72 h. Different lowercase letters indicate statistically significant differences ($P < 0.05$) in relative expression of each gene among different sampling time points.

Expression profiles of HSP90-1 after low salinity challenge

The expression pattern of *HSP90-1* in the 2 groups is shown in Figure 3. At 3 h after stress, the expression level in both groups rapidly declined ($P < 0.05$). However, the expression of WG (Figure 3A) was maintained at an extremely low level, without significant change ($P > 0.05$) from 6 to 48 h, but markedly increased at 72 h ($P < 0.05$). In comparison, that of SG (Figure 3B) rose to a higher level at 12 and 24 h ($P < 0.05$), then decreased ($P < 0.05$) at 48 h, and remained relatively stable from 48 to 72 h ($P > 0.05$).

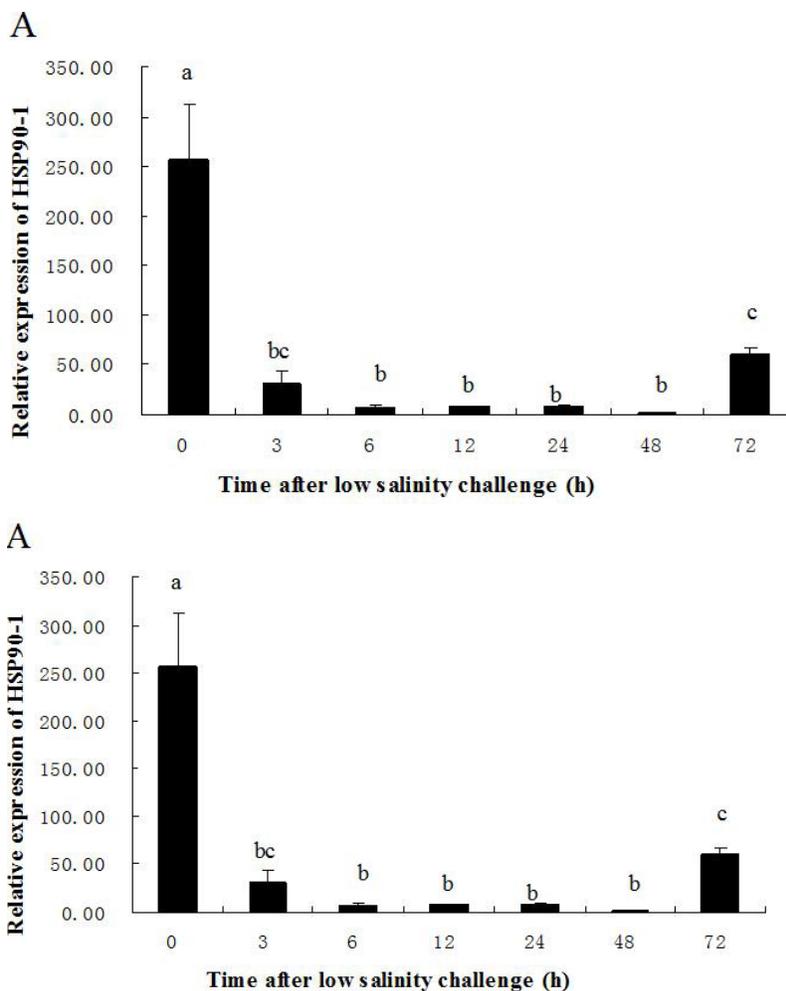


Figure 3. Temporal expression of *HSP90-1* relative to β -actin of wild swimming crab. **A.** Screened crab; **B.** analyzed by real-time PCR in *Portunus trituberculatus* hepatopancreases after low salinity challenge. Each bar represents the mean value from four determinations with standard error. The hepatopancreases were collected from four individual crab at 0, 3, 6, 12, 24, 48, and 72 h. Different lowercase letters indicate statistically significant differences ($P < 0.05$) in relative expression of each gene among different sampling time points.

Expression profiles of HSP90-2 after low salinity challenge

HSP90-2 exhibited the opposite trend to *HSP60*, *HSP70*, and *HSP90-1* (Figure 4). For the WG (Figure 4A), *HSP90-2* expression was significantly upregulated from 0 to 6 h ($P < 0.05$), and reached a maximum at 6 h. Afterwards, the expression level gradually decreased, with a further decline by 72 h, but remained higher compared the initial level. For the SG (Figure 4B), *HSP90-2* expression gradually rose from 0 h to 6 h ($P > 0.05$), and reached a dramatic maximum at 12 h ($P < 0.05$). Then, the expression level dropped significantly from 12 h to 24

h, and remained relatively stable from 24 to 72 h ($P > 0.05$); however, the expression level at 72 h was twice as high compared to the original level.

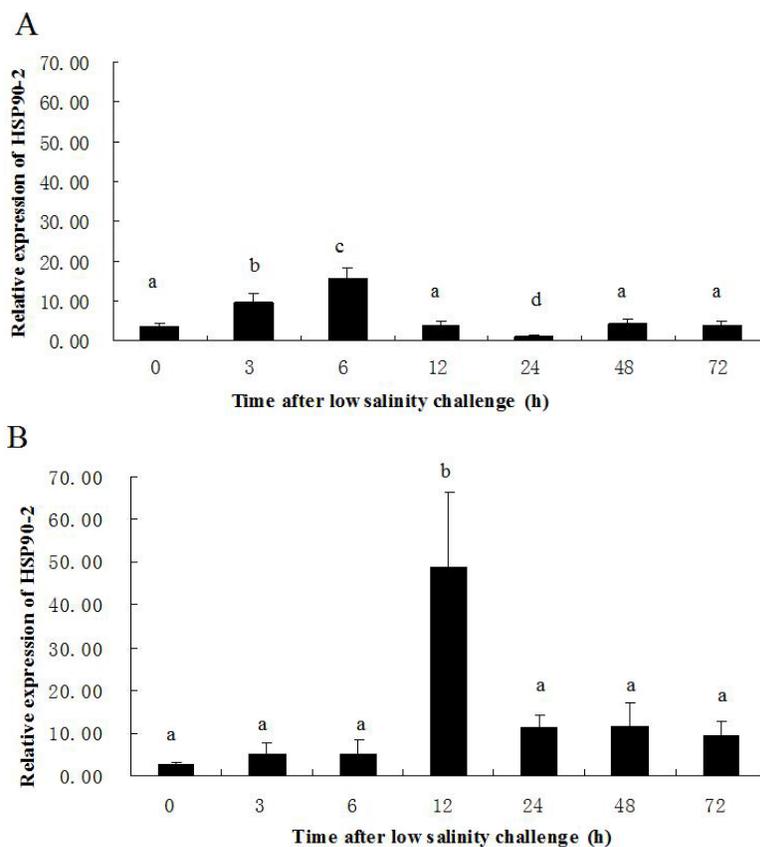


Figure 4. Relative mRNA levels of *HSP90-2* genes in hepatopancrease of wild swimming crab. **A.** Screened swimming crab; **B.** after exposure to low salinity (4 ppt) was evaluated by RT-PCR. Each bar represents the mean value from four determinations with standard error. The hepatopancreases were collected from four individual crabs at 0, 3, 6, 12, 24, 48, and 72 h. Different lowercase letters indicate statistically significant differences ($P < 0.05$) in relative expression of each gene among different sampling time points.

DISCUSSION

The *HSP* genes are considered to be critical elements during the salinity acclimation process (Xu and Liu, 2011). In crustaceans, the hepatopancreas has been identified as the vital tissue for immune defense (Söderhäll and Cerenius, 1998). The involvement of *HSP* genes in both high and low salinity responses has been demonstrated in many aquatic animals. It has been demonstrated that the expression level of *HSP70* gradually increases after low salinity exposure in the gill of *Mytilus galloprovincialis* (Hamer et al., 2004), but decreases in the gill of *Chamelea gallina* (Monari et al., 2011). The overexpression of *HSP70* has also been re-

ported in the hemocytes of *Scylla paramamosain* after high salinity stress (Yang et al., 2013). The expression of *HSP90* was induced when *Crassostrea hongkongensis* (Fu et al., 2011) and *Crassostrea gigas* (Jo et al., 2008) were subject to salinity stress. In the lobster, *Homarus americanus*, the expression of *HSP70* and *HSP90* in the abdominal muscle increased after salinity stress, whereas *HSP90* levels remained unchanged in the hepatopancreas (Spees et al., 2002b). These studies demonstrate that HSP genes play critical roles in the ability of animals to cope with abnormal salinity challenge.

The *HSP60* expression pattern in the 2 groups of swimming crabs differs to that in the gill of *P. trituberculatus* (Xu and Qin, 2012) and the pleopod of the shrimp, *Litopenaeus vannamei* (Huang et al., 2011) when subject to 10-ppt salinity challenges. This difference might arise because *HSP60* exhibits tissue-specific expression patterns or because the salinity in our study was much lower compared to that used in previous studies. For the WG (Figure 1A), HSP expression significantly decreased at 3 h. Then, from 6 to 48 h, HSP expression alternately increased and decreased with no significant difference ($P > 0.05$), and reached the lowest point at 48 h. However, at 72 h, HSP mRNA expression was significantly enhanced. For the SG (Figure 1B), the *HSP60* transcript level was sharply downregulated during the first 3 h ($P < 0.05$), but gradually increased from 3 to 72 h, which might strengthen the organisms immunity to protect it from further damage (Ranford and Henderson, 2002; Tsan and Gao, 2004). In addition, it contributes to the folding of nascent proteins, post-translation. As a molecular chaperone, an important activity of *HSP60* is the mediation of the native folding of proteins (Ellis and van der Vies, 1991). It seems that the expression level was more unstable for the WG than the SG.

HSP70 indicates the presence of physiological stress (Dahlhoff et al., 2001), and exerts its specific protective function in all organisms (de la Vega et al., 2006). The transcriptional expression of *HSP70* showed a time-dependent response in the hepatopancreas after crabs were challenged with low salinity. In general, the expression level in both the SG and WG first decreased and then increased. A similar tendency was obtained in a previous study on *Eriocheir sinensis* (Sun et al., 2012); however, our results differed to a study on the lobster *Homarus americanus* (Spees et al., 2002b). This difference might have been influenced by the energy depletion of osmoregulation and the very low salinity (4 ppt) in our study. HSPs require ATP to function. Impaired stress protein function associated with reduced ATP levels has been previously established (Feige et al., 1996; Roberts et al., 1997). It has been postulated that energy depletion caused by increased osmoregulation under low salinity conditions might lead to reduced levels of *HSP70* proteins in the swimming crab, and has also been observed in all tissues of *P. amurensis* (Werner and Hinton, 2000) and in the gill of *Chamelea gallina* (Monari et al., 2011). In the current study, as time progressed, *HSP70* expression increased to protect organisms from further damage (de la Vega et al., 2006). For the SG (Figure 2B), expression levels remained low during the first 24 h. Then, *HSP70* expression increased at 48 h, and continued to increase ($P > 0.05$) until 72 h, almost reaching the initial level. However, for the WG (Figure 2A), the expression level remained low until 48 h, and then sharply rose at 72 h ($P < 0.05$). This result indicates that the SG had adapted to the low salinity when the stress time reached 48 h. In addition, this result also indicated that SG required less time to adapt to the low salinity environment compared to WG.

As a molecular chaperone, *HSP90* plays an important role in folding new peptides, along with refolding and stabilizing denatured proteins, under stressful conditions (Sreedhar et al., 2004). In vertebrates, 2 subtypes of *HSP90* have been identified; namely, the stress-inducible *HSP90 α* and the constitutively expressed *HSP90 β* (Heikkila, 2010). In *P. trituber-*

culatus, 2 complete HSP90s (*HSP90-1*, *HSP90-2*) genes that exhibit tissue-specificity have been reported (Zhang et al., 2009). In the present study, the expression level of *HSP90-1* was downregulated and then upregulated, but remained lower compared to the initial level. It is possible that the 4 ppt was so low that it fell beyond the salinity range that induces the production of *HSP90-1*; thus, disrupting protein synthesis in organisms (Huhtala et al., 2005). A similar expression pattern has been reported in the gill of *Crassostrea hongkongensis* under low salinity stress (Fu et al., 2011). In the current study, the high expression of *HSP90-1* at 12 and 24 h was apparent in the SG (Figure 3B), but not the WG (Figure 3A). The increase in expression level from 3 h to 24 h might contribute to the refolding of denatured proteins to protect organisms from environmental stress (Sanders, 1993; Grzesiuk and Mikulski, 2006). In comparison, the expression level decreased between 24 and 72 h, which might be because long-time exposures to low salinity are harmful to organisms.

However, the opposite trend appeared in the expression profile of *HSP90-2* in swimming crabs, which was consistent with a previous report (Zhang et al., 2009). The expression level of *HSP90-2* in the 2 groups first increased and then progressively decreased, which was similar to that observed in the gill of *C. hongkongensis* (Fu et al., 2011) and the branchial lamellae of *S. salar* (Pan et al., 2000) under high salinity stress. For the WG (Figure 4A), there was a dramatic increase during the first 6 h ($P < 0.05$) after challenge, and reached a maximum at 6 h. Then, the expression level dropped to almost the initial level at 12 h, and even sharply decreased to the lowest level at 24 h ($P < 0.05$). Afterwards, the expression level increased again from 24 to 72 h, and recovered to the initial level at 72 h. For the SG (Figure 4B), there was no obvious change during the first 6 h after challenge; however, a significant increase was observed at 12 h, and then the expression decreased again. Although the expression level from 24 to 72 h remained steady ($P > 0.05$), and higher compared to the original, there was no major difference compared to the original level ($P > 0.05$). The high expression level of *HSP90-2* during the first 6 h in the WG and at 12 h in the SG helps to protect organisms against environmental stress. Subsequently, the expression level was downregulated; hence, it is possible that the overexpression was insufficient to counteract the damage to proteins following a long period of low salinity stress, and then inhibited the metabolic process (Meng et al., 2011). We found that between 6 and 12 h the SG was post-challenged to increase the expression level to protect the organism (Grzesiuk and Mikulski, 2006). The SG might have some advantageous mechanisms enabling them to adapt better to low salinity conditions compared to the WG. We also inferred that *HSP90-1* and *HSP90-2* played quite different roles in the resistance to low salinity.

In conclusion, we compared the expression profiles of *HSP60*, *HSP70*, *HSP90-1*, and *HSP90-2* in the hepatopancreas of wild and salinity-tolerant swimming crabs subject to low salinity challenge. The *HSP60* and *HSP70* expression level of SG was obviously more stable and required less time to adapt the low salinity conditions compared to the WG. The high expression of *HSP90-1* and *HSP90-2* at 12 h indicated that the SG had a strong ability to adapt to a low salinity environment. This study provides a new insight about the relationship between HSPs and salinity acclimation, which might be beneficial for developing breeds that are resistant to low salinity.

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