

Effects of heat stress on the mRNA expression of Hsp 70 and Hsp 90 in white shrimp, *Exopalaemon carinicauda*

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Exopalaemon carinicauda is an economic species which is frequently exposed to heat stress such as the thermal discharge from power stations. The mRNA expression of heat shock protein (HSP) families, HSP 70 and HSP 90, in *E. carinicauda* was investigated by real-time quantitative PCR following acute heat stress. HSP 70 mRNA expression levels increased at 2h, and then decreased till the end of the experiment when shrimps stressed by 19 °C, 23 °C and 27 °C. The HSP 90 mRNA expression levels decreased at 2h, and then increased till the end of the experiment when shrimps stressed by 19 °C, 23 °C and 27 °C. Differences between HSP 70 and HSP 90 mRNA expression of shrimp can present the different roles stressed by heat stress. Therefore, the mRNA expression of HSP 70 and HSP 90 could be developed as biomarkers to indicate heat stress in shrimp.

[Keywords: HSP 70; HSP 90; heat stress; *Exopalaemon carinicauda*; biomarker]

Introduction

Marine ecosystem will be threatened with thermal discharge from power stations (fossil fuel and nuclear power) located on the coastal regions of China. Temperature is a very important factor that affects marine organisms and ecosystems. The influence of coastal power station thermal discharge on the benthos¹⁻³, fish⁴⁻⁶, phytoplankton⁷⁻⁸ as well as culturable bacteria⁹ were previously investigated.

However, the impact of thermal discharge on the expression of HSPs in marine shrimps has not been investigated. HSPs are molecular chaperones that are involved in maintaining routine cellular functions, with a crucial role in protein folding, unfolding, aggregation, degradation, and transport¹⁰. HSPs are classified into major families according to their molecular weight (e.g., HSP 100, HSP 90, HSP 70, and HSP 27)¹¹. In addition, HSPs are

highly conserved proteins that are synthesised in most organisms in response to various environmental stresses and stimuli, such as heat shock, cold shock, heavy metals, bacteria, and osmotic changes¹². Studies of HSPs in fish including fish cell lines, primary cell culture and whole fish exposed to heat shock, most investigators reported that the increase in HSP 70 or HSP 90 in different levels of physiological organization was the most prominent response in most heat shock experiments¹². Their involvement in inducible stress tolerance (thermotolerance) raises some fundamental questions regarding the regulation of this protection and whether fish in nature can be conditioned by one stressor to better tolerate a subsequent insult¹³. Studies of HSPs in shrimps or mollusc such as HSP 60, HSP 70 and HSP 90 mRNA levels were sensitively induced by

heat shock¹⁴⁻¹⁷. Among the HSP families, HSP 70 and HSP 90 are the most prominent groups and are present in all cell types¹⁸.

The mRNA expression levels of HSP in marine organisms exposed to acute or chronic heat stress have been widely studied. For instance, the changes of HSP 70 in *Mytilus edulis*¹⁹⁻²⁰, *Goniopora djiboutiensis*²¹, *Gillichthys mirabilis*²², *Ostrea edulis*¹⁶, *Mytilus galloprovincialis*²³ and *Tigriopus japonicus*²⁴ stressed by heat shock have been evaluated. The changes of HSP 60 and HSP 70 in *Litopenaeus vannamei* following acute heat stress was studied¹⁵, and the changes of HSP 70 and HSP 90 in *Acropora millepora*²⁵, *Crassostrea gigas*¹⁷, *Sparus aurata*²⁶, and *Symbiodinium* sp.²⁷ during short or long-term exposure to increasing temperature have also been studied. Additionally, the three HSP genes (HSP 60, HSP 70 and HSP 90) were also analysed together in *Acipenser medirostris* after heat stress¹⁸. The results of most of these studies showed that the mRNA expression of HSP 70 and HSP 90 were induced within the first few hours after thermal stress^{16, 18, 23, 26}. However, the up-regulated levels of HSP 70 and down-regulated levels of HSP 90 in *Symbiodinium* sp at elevated temperatures suggested diverse roles of these molecular chaperones during heat stress responses²⁷.

Although many studies have investigated changes in the gene expression of HSPs in marine organisms after exposure to heat stress, little is known about the transcript changes of HSP 70 and HSP 90 in muscle of heat shocked *E. carinicauda*, which is a common palaemonid along the coasts of China and one of the most significant small shrimps of economic importance in China²⁸. The primary aim of the present study is to examine whether HSPs (HSP 70 and HSP 90) transcriptional responses can be used as biochemical indicators of thermal stress in coastal marine shrimps (*E. carinicauda*) in China. The second aim is to evaluate the impacts of discharging water from thermal power plants on the fishery resources and aquatic ecosystem.

Materials and Methods

E. carinicauda, averaging 6.21 (± 1.35) cm in body length and 2.18 (± 0.47) g in body weight, were obtained from a local market in Xiangshan city (Zhejiang province, China) on November 2010 and maintained in tanks (200 L) under 25‰ salinity and 15 (± 1) °C for one week before the beginning of the experiment.

In the experiment, 20 shrimps were kept in 20-L acrylic aquaria containing 18 L seawater at different temperatures of 15, 19, 23, 27, 31 and 35 °C, respectively. Each treatment group was replicated. The mortality was recorded and three live individuals were randomly sampled from each group (15, 19, 23 and 27 °C group only) after exposure for 2, 12, 24 and 48 h, and the muscle tissue was excised and stored in RNastore reagent (Tiangen).

Total RNA was purified from the samples using the TRIzol reagent (Invitrogen), genomic DNA was digested with Dnase I (Invitrogen), and the purified RNA was stored at -80°C until use. The first-strand cDNA synthesis was performed using M-MLV reverse transcriptase (Invitrogen) to transcribe the poly (A) mRNA with Oligo-dT and random 6-mer primers. The reaction conditions were those recommended by the manufacturer's instructions. The cDNA was maintained at -20 °C until use as the template for the quantitative real-time PCR (qRT-PCR).

The mRNA expression of the target genes in the muscle tissue was measured by qRT-PCR. Specific primers were designed based on published *E. carinicauda* mRNA sequences (GenBank accession nos. HQ185257, HQ162267, and FL589653) using Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA). Primer sequences are shown in Table 1.

The qRT-PCR amplifications were carried out with an ABI StepOnePlus Detection system (Applied Biosystems, Foster City, CA, USA). Amplifications were performed in a 96-well plate with a 25- μ L reaction volume containing 1.0 μ L of 25 $\times$ SYBR Green $\square$, 0.8 μ L of PCR Forward Primer (10 μ M), 0.8 μ L of PCR Reverse Primer (10

μM), 0.4 μL of ROX Reference Dye II (50×), 1.0 μL of cDNA template and 14.7 μL of diethylpyrocarbonate water (DEPC-water). Thermal profile for the SYBR Green qRT-PCR consisted of an initial step at 95 °C for 90 s, followed by 40 cycles of 95 °C for 5 s and 58 °C for 30 s. In this experiment, a preliminary trial showed that the β-actin gene had a steady expression in this experimental species. Therefore, the β-actin gene was used as the housekeeping gene in all of the qRT-PCR assays, and the primers were designed based on the β-actin gene sequence (GenBank accession: FL589653). The HSP 70, HSP 90 and the β-actin qRT-PCR primers are listed in Table 1. Standard curve and the gene expression

levels were analysed automatically by the system, as was the setting of the base line. A melting curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that only one product was amplified and detected. For the experimental results of the qRT-PCR, the observations at the various temperatures were calculated to derive the means and standard errors. Significance of the differences in expression was determined using one-way ANOVA followed by Duncan's multiple range tests; with a threshold significance level of $P < 0.05$. All of the statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA) (Zhou et al. 2010)¹⁵.

Table 1—HSP genes of *E. carinicauda* and their corresponding PCR primers used for qRT-PCR

Target gene	GenBank#	Primer sequence (5'→3')	Position	Product (bp)
HSP70Fr	HQ185257	CGATGGTGCTCATCAAAATA	445-647	203
HSP70Rr	HQ185257	CTTTTGTGTCGAGGCCGTAAG	445-647	203
HSP90Fr	HQ162267	CCTCCAAGCTCGAGAATGTC	176-409	234
HSP90Rr	HQ162267	CGACGAGGTAAGCGGAGTAG	176-409	234
β-actin Fr	FL589653	GCCGTGACCTCACAGACTACCT	153-337	185
β-actin Rr	FL589653	TTGATGTCTCGCACGATTCTC	153-337	185

Results

Table 2 showed the mortality of *E. carinicauda* exposed to the different temperatures. *E. carinicauda* could survive under the relative low temperature groups (15, 19, 23 and 27 °C). However, it began to die when the water temperature reached 31 °C, resulting in 10 %

mortality within 2 h, and the mortality increased rapidly within 4 h (70 % mortality). All of the individuals were dead at 35°C within 4 h. Results showed that *E. carinicauda* could not survive normally when heat shock more than 16 °C (from 15°C to 31°C) compared the control group in autumn.

Table 2—Effect of water temperature on the mortality of *E. carinicauda* (%)

Treatment time (h)	Temperature (°C)					
	15	19	23	27	31	35
0	0	0	0	0	0	0
2	0	0	0	0	10	50
4	0	0	0	0	70	100
6	0	0	0	0	80	100
24	0	0	0	0	90	100
48	0	0	0	0	100	100

The mRNA expression of HSP 70 was examined using qRT-PCR, and these changes of HSP 70 in muscle were investigated after the exposure to

various temperatures. Levels of HSP 70 at 15 °C (control group) did not change significantly within 24 h and decreased by 18.27 % when compared to

the values at 48 h ($P<0.05$) (Fig. 1A). The levels of HSP 70 at 19 °C increased rapidly and significantly within 2 h and 12 h ($P<0.05$) and decreased thereafter (Fig. 1B). The fluctuations of the mRNA expression of HSP 70 at the other temperatures (23 °C and 27 °C) were very similar with those of the 19 °C treatment, peaking at 2 h-12 h (1.17-2.06 fold) (Fig. 1B, C and D).

The mRNA expression of HSP 90 in shrimp muscle at 15 °C decreased to 57.36 % ($P<0.05$) in 12 h, 98.34 % ($P<0.05$) in 24 h and 73.49 %

($P<0.05$) in 48 h, respectively (Fig. 2A). The levels of HSP 90 gene expression in muscle at 19 °C decreased to 70.52 % ($P<0.05$) in 2 h, 85.05 % ($P<0.05$) in 12 h, 88.58 % ($P<0.05$) in 24 h and 86.94 % ($P<0.05$) in 48 h, respectively (Fig. 2B). The mRNA expression of HSP 90 at 23 °C was down-regulated at 24 h and then increased significantly (Fig. 2C). The levels of HSP 90 at 27 °C decreased rapidly in 24 h and increased rapidly at 48 h (Fig. 2D).

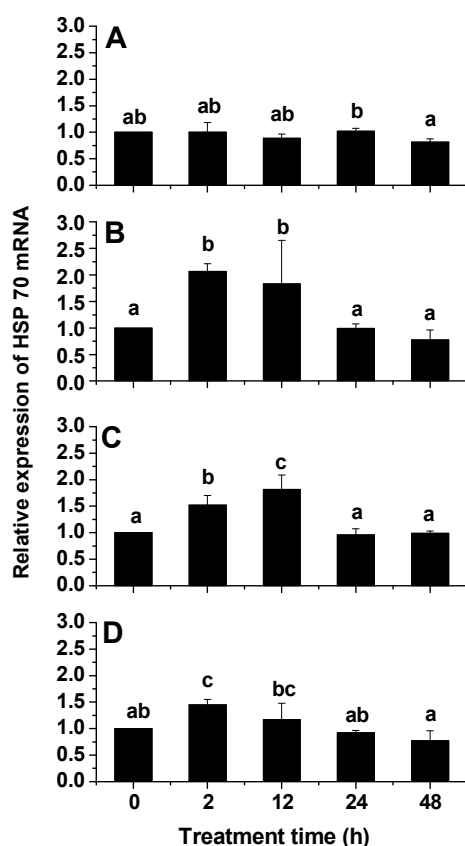


Fig. 1—HSP 70 mRNA levels were expressed as means $\pm$ SD, $n = 3$) in *E. carinicauda* exposed to different temperatures (15°C: A, 19°C:B, 23°C:C and 27°C:D) after 2h, 12h, 24h and 48h. Control group shrimp were maintained at 15°C. Within each HSP class and graph different letters (a, b, c) indicate significantly different groups ($P<0.05$).

Discussion

Rapid changes in coastal seawater temperature as a result of thermal discharge from power stations are bringing marine organisms close to their

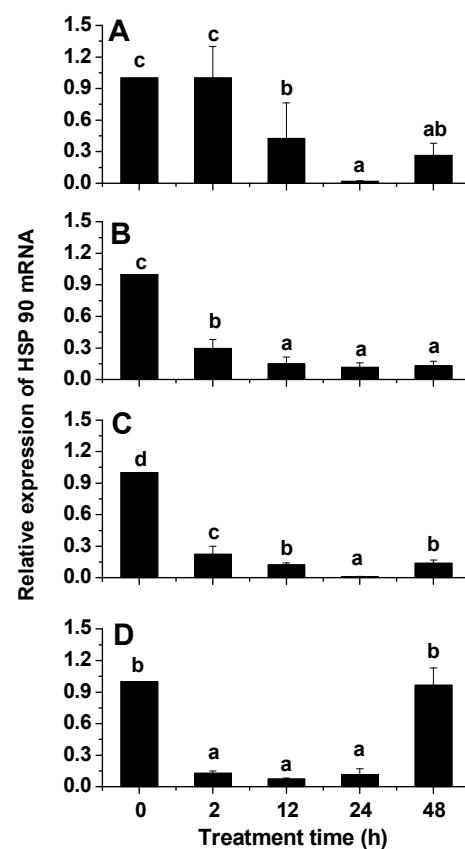


Fig. 2—HSP 90 mRNA levels were expressed as means $\pm$ SD, $n = 3$) in *E. carinicauda* stressed by different temperatures (15°C: A, 19°C:B, 23°C:C and 27°C:D) after 2h, 12h, 24h and 48h. Control group shrimp were maintained at 15°C. Within each HSP class and graph different letters (a, b, c, d) indicate significantly different groups ($P<0.05$).

thermal tolerance limits. Data obtained in this study demonstrated that *E. carinicauda* could not survive in seawater with temperatures over 27 °C long-term (Table 2). Mortality of the shrimps increased most

drastically during the exposure to 31 °C, and 90 % of the shrimps died within 24 h. This result was consistent with the previous reports which found the lethal temperatures of marine coastal invertebrate species were about 30 °C and the mean upper incipient lethal temperature of Decapoda was 32.9 °C⁶. It had been reported that mussels (*M. galloprovincialis*) and gilthead seabream (*S. aurata*) could not survive when the temperature of the seawater exceeded 26 °C (the ambient temperature was 18 °C) in the Mediterranean^{23, 26}. It was proposed that mussels relied on stored fuels to replenish the consumed ATP, the higher temperature might cause the depletion of stores and, thus, contribute to cell death²³. In this study, the shrimps could not survive when heat shock exceeded 16 °C, the mortality of *E. carinicauda* might be caused by the same reason.

HSP 70 is known to assist in the folding of nascent polypeptide chains and to mediate the repair and degradation of altered or denatured proteins¹³, furthermore, it is a family of stress-inducible proteins²⁹. The mRNA expression of HSP 70 represents a profound essentiality for the synthesis of corresponding protein; it is substantial to reveal the mRNA expression profile in various stimulations. In the present study, the mRNA expression of HSP 70 in *E. carinicauda* increased rapidly in 2 h when exposed to 19 °C and 27 °C and then decreased significantly ($P < 0.05$) (Fig. 1B and D), and the mRNA expression of HSP 70 in *E. carinicauda* increased rapidly in 12 h when exposed to 23 °C and then decreased significantly ($P < 0.05$) (Fig. 1C). As a control, the mRNA expression of HSP 70 in *E. carinicauda* exposed to 15 °C maintained relatively stable level (Fig. 1A). Up-regulation of HSP 70 expression under heat shock treatment has been observed in molluscs^{16-17, 19} and shrimps^{15, 30}. The mRNA expression of HSP 70 was very sensitive to heat shock. Some previous studies indicated that the HSP 70 mRNA was expressed in the first hours (3h or 6h) only after heat shock^{15-16, 25}. Rapid induction of HSP 70 mRNA to the heat stress represents the molecular adaptation in which a large number of denatured

proteins require the function of HSP in the cells to reduce the excess of unfolded proteins¹⁵.

HSP 90 is active in supporting various components of the cytoskeleton and steroid hormone receptors¹³. It was reported that HSP 90 was likely involved in heat shock transcription factor 1 (HSF 1) trimerization to activate a target gene and to protect the cell against death after heat shock³¹. In this study, the mRNA expression of HSP 90 of the shrimps was decreased significantly in 2 h when exposed to 19, 23 and 27 °C, as compared with 15 °C (Fig. 2). Prolonging exposure to higher temperature (23 and 27 °C) led to a significant increase in 48 h (Fig. 2C, D). The mRNA expression of HSP 90 in *Symbiodinium* was down-regulated by elevated temperatures at the onset of the experiments in both symbiotic and cultured environments²⁷. Heat shock treatment induced reduction in HSP 90 expression has also been shown in *Arabidopsis*³² and *Drosophila*³³. On the other hand, up-regulation of HSP 90 expression under heat shock treatment has been observed in fish¹², coral²⁵, oyster¹⁷ and shrimp¹⁴. It is a reasonable that the mRNA expression of HSP 90 in different species may be different under heat shock.

Comparable changes in mRNA expression of HSP 70 and HSP 90 genes were found for *E. carinicauda* exposed to elevated temperatures. In the present study, a differential regulation of two inducible HSPs under heat shock was revealed. The down-regulation of HSP 90 by elevated temperatures at the onset and the up-regulation of HSP 90 by the severe elevated temperatures at the later times suggest the diverse roles of these molecular chaperones (compared with HSP 70) during the heat stress response. In addition, some previous studies reported that marine organisms exhibited different responses in different seasons under heat stress. It was reported that the response to heat stress the HSP 70 mRNA levels in *C. gigas* was stronger and quicker in summer than in winter¹⁷ and the similar result was found in *M. edulis*²⁰. In the present study, heat stress resulted in an increase in HSP 70 and a decrease in HSP 90 during the first few hours in *E. carinicauda* in

autumn; further studies are required to explain the mechanisms driving these molecular regulations and their potential protective role for the prevention of cell damage in *E. carinicauda* exposed to heat stress in different season. Additional experiments should be examined on the relationship between the differential mRNA expression of HSPs and the regulation of a HSF that is involved in activation of heat-inducible genes and heat stress. Moreover, it should be focused on those other parameters as well as physiological and genetic factors such as oxidative stress (superoxide dismutases, catalases, glutathione peroxidases and so on), phosphorylation of stress-activated protein kinases (p38 mitogen-activated protein kinase, cJun-N-terminal kinases) and metabolic capacities (citrate synthase, malate dehydrogenase, pyruvate kinase and so on).

Conclusion

In summary, the mortality data showed that *E. carinicauda* cannot survive normally with heat shock more than 16 °C. The expression of mRNA expression of HSP 70 and HSP 90 in *E. carinicauda* changed sensitively in response to heat shock. Up and down regulated expression of HSP70 or down and up regulated expression of HSP 90 after heat shock indicated that these two HSP genes might play very important roles to allow shrimp to cope with heat stress.

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