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# Stress levels over time in *Ruditapes philippinarum*: The effects of hypoxia and cold stress on hsp70 gene expression

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ABSTRACT

*Ruditapes philippinarum* is one of the most commercially important marine bivalves worldwide with a production over 4.0 million tons and 3.7 million USD in 2015. The Manila clam displays different shell color strains (white, zebra, and orange) in its natural habitat. Heat shock proteins (HSPs) play significant roles in response to adverse environmental stresses in aquatic animals. In this study, the expression levels of *HSP70* gene were analyzed by qPCR in gills of Manila clams under hypoxia and cold stresses. The expression pattern of *HSP70* was observed in different color strains of Manila clam under low temperature challenge. The expression of *HSP70* was up-regulated significantly up to 8 days in cultured Manila clam under hypoxia stress. *HSP70* expression in gill was inducible under hypoxia and low temperature stress. These findings contribute to our better understanding of the molecular response and biological function of HSPs in defending against low temperature and hypoxia challenges in *R. philippinarum*.

## 1. Introduction

Marine bivalve mollusks are benthic animals and often exposed to changes in temperature and oxygen concentration in their natural environment (Greenway and Storey, 2001). In aquatic environments, oxygen levels often fluctuate and marine bivalves experience hypoxic conditions on a regular basis (Greenway and Storey, 1999). In recent years, marine hypoxia has become one of the major ecological concerns, because of the increase of excessive anthropogenic input of organic matter and nutrients into coastal seawater (David et al., 2005). Water temperature is another important factor affecting the biological processes of aquatic animals, including their development, growth, reproduction, metabolism, behavior, geographic distributions and so on (Saucedo et al., 2004). For marine mollusks, especially those inhabiting in temperate and subtropical waters, where wide temperature variations usually occur over seasonal cycles and will have an influence on mollusks metabolic activities (Widdows, 1973a, b). Several studies have reported that the immune functions of mollusks are affected by variations in ambient temperature (Abele et al., 2002; Paillard et al., 2004; Renault et al., 2006). Aquatic animals are able to sense both transient fluctuations and seasonal variations in temperature and to respond to these changes by actively adjusting their physiological and biochemical activities to fit the ambient temperature regime. However, deleterious consequences will occur if the water temperature exceeds the species-specific thermal tolerance range (Thieringer et al., 1998).

Heat shock proteins (HSP) are a family of proteins which play an important role in normal growth and development, and it helps organisms to modulate stress response (Lindquist, 1986; Lindquist and Craig, 1988; Hartl, 1996). When environmental factors deviate from the optimal range of an organism, aerobic scope declines and a series of stress responses is elicited, including production of heat-shock proteins (HSP, molecular chaperones that assist in refolding of stress-damaged proteins). When organisms are challenged by different environmental stress such as temperature, salinity, hypoxia, heavy metals, bacteria and etc., the mRNA expression level of HSP would be increased to enable organisms to resist the adverse stressors so as to maintain the homeostasis and survival of cells (Kiang and Tsokos, 1998; Sørensen et al., 2003; Zhang et al., 2012). The oyster genome contains 88 heat shock protein 70 (HSP70) genes, which have crucial roles in protecting cells against heat and other stresses (Zhang et al., 2012). The HSP members are usually grouped according to their molecular weights, and it divided into three main families: HSP90s, HSP70s and small HSPs (Feder and Hofmann, 1999). Among these HSPs, the HSP70s are one of the most conserved and important protein families, which have been studied in many marine bivalve species, including oysters (Rathinam et al., 2000; Zhang and Zhang, 2012; Piano et al., 2004), mussels (Franzellitti and Fabbri, 2005), scallops (Wu et al., 2003; Song et al., 2006) and clams (Yue et al., 2011; Liu et al., 2015).

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Manila clam *Ruditapes philippinarum* is one of the most commercially important marine bivalves worldwide with a production over 4.0 million tons and 3.7 million USD in 2015 (FAO, 2017). In recent years, however, intensive aquaculture of this species has resulted in serious problems such as retarded growth and increased mortality. In particular, mass mortality of this species frequently occurs in summer and winter due to drastic environmental change such as hypoxia in summer and low temperature in winter (Zhang and Yan, 2006; Nie et al., 2016). This situation has not changed and large-scale death of farmed *R. philippinarum* has become a major constraint for the development of the clam culture industry. To genetically improve the clams, a selective breeding program was initiated in China in 2005 (Yan et al., 2005). Manila clam displays different shell color strains (white, zebra, and orange) in its natural habitats, which have been selected and cultivated for several generations through clams selective breeding programs (Zhang and Yan, 2010; Zhao et al., 2012; Yan et al., 2014). Previous studies have demonstrated that zebra strain and orange strain had stronger resistance to environmental stressors, such as temperature and salinity (Zhang and Yan, 2010; Xu et al., 2015; Nie et al., 2017). However, the molecular responses of *R. philippinarum* to low temperature and hypoxia stress remain largely unknown.

The expression levels and patterns of HSP are closely related to the environmental tolerance and resistance of an organism (Somero, 2012). Moreover, the level of HSP70 expression can be used as a biomarker to evaluate the temperature and oxygen level tolerance, and to assist molecular breeding for stress resistance *R. philippinarum*. Therefore, it is necessary to investigate the functions and expression characteristics of HSP70s under cold and hypoxia stresses due to the important roles of HSP70s in immune and stress resistance. In this study, we conduct the low temperature and hypoxia stress over a different period and quantified *HSP70* gene expression using quantitative real-time PCR (qRT-PCR). We hypothesized that *R. philippinarum* will feature a high plasticity in the production of stress proteins and will respond to sharp fluctuations in temperature and hypoxia by increased transcription of these proteins.

## 2. Materials and methods

### 2.1. Experimental animals

One wild population and three strains of color selected *R. philippinarum* were used in the low temperature experiment. Three strains of *R. philippinarum* (orange strain, white strain and zebra strain) were selected for shell color, fast growth and high survival rate for several generations, which originally collected from the natural population in Dalian and Fujian, China. The orange strain features orange shell color with high survival rate, and is the product of six generations of mass selection for faster growth. The white strain features white shell color with high growth rate, and has arisen from three generations of mass selection. The zebra strain is the product of seven generations of selection and features a striated shell with a high survival rate. Healthy *R. philippinarum* (shell length: 2.5 ± 0.5 cm) were collected from Zhangzidao (Dalian, Liaoning province, China) and maintained in 70 L tanks containing aerated sand-filtered seawater (salinity: 32 ppt, pH 8.1) at 22 ± 0.5 ℃ for one week prior to experimentation.

One cultured population of Manila clam (Zhuanghe, Dalian) with variegated color was used in the hypoxia experiment. Because the size of cultured clams (shell length: 3.5 ± 0.3 cm) collected from Zhuanghe are bigger than different shell color strains. Hence, only cultured Manila clam was used for hypoxia challenging in low oxygen experiment. One hundred of *R. philippinarum* were sacrificed for the low oxygen stress experiment. After being transported from the field to the laboratory, the clams were cleaned to remove any fouling and were acclimated in aerated 70 L plastic tanks, containing water at 12 ℃ ± 0.3 ℃ with salinity of 32. Other water quality were measured during the experiment (pH: 8.0 ± 0.2, dissolved oxygen: 7.0 ± 0.5 mg/L). All the clams were fed with Spirulina powder daily for one week before the hypoxia treatment, and water was exchanged once per day to discharge waste products from marine invertebrates.

### 2.2. Low temperature and hypoxia treatment

To investigate the temporal expression profile of HSP70 in Manila clams after cold shock treatment (−1℃), the healthy clams were randomly divided into two groups (cold stress experiment and control). The experimental clams were transferred from 20 ℃ to −1 ℃ for the acute cold stress, whereas the control clams were cultured in seawater with temperature at 20 ℃, and the salinity was kept stable at 32 ppt in the cold stress experiment. Clams exposed to the cold stress for up to 96 h and the samples were taken after the specific times (0, 3, 6, 12, 24, 48, 72 and 96 h). Gills were chosen for this study because of their high metabolic activity and essential physiological roles in response to stress. The gill samples were collected at 0, 3, 6, 12, 24, 48, 72 and 96 h post-exposure from the nine clams (3 pools of 3 individuals each) of the experimental group. Tissues were frozen in liquid nitrogen and stored at −80 ℃ for total RNA extraction.

To investigate the temporal expression profile of HSP70 in *R. philippinarum* after hypoxia stress, the healthy clams were randomly divided into two groups including hypoxia treatment group (2.0 mg/L) and normoxia control group (7.0 mg/L). Clams exposed to the hypoxia stress for up to 8 d and the samples were taken after the specific times. The gill samples were collected at 0, 2, 5 and 8 d post-exposure from nine clams (3 pools with 3 individuals each) of the experimental group. Tissues were snap frozen in liquid nitrogen and stored at –80 ℃ for total RNA extraction.

### 2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from 30 mg tissue samples using the RNA isolation kit (TianGene, Beijing, China) according to the manufacturer's protocol. RNA quantity, purity and integrity were verified spectrophotometrically (A260/A280) and by electrophoresis on 1% agarose gels. The concentration of total RNA used for cDNA synthesis was 200 ng/μL. The synthesized cDNA product was diluted appropriately (10×) and stored at −20 ℃ until further analysis. The expression of *HSP70* transcripts in the gill tissue of clams challenged with low temperature and hypoxia was determined by SYBR real-time fluorescence quantitative PCR method.

### 2.4. Expression analysis of HSP70

The *HSP70* gene was cloned and published in our previous study (Nie et al., 2017). Quantitative real-time PCR (qRT-PCR) was performed using SYBR® Premix Ex Taq™ II kit (TaKaRa). Stability of a reference gene was tested under the given experimental conditions. The β-actin gene from *R. philippinarum* was used as the reference gene due to the stability under given stresses. The quantitative real-time PCR was carried out in an Applied Biosystems 7500 Fast Real-time PCR System (Applied Biosystems, USA), and performed in a total volume of 20 μL (10 μL of The SYBR® Primix Ex Taq II (2×), 1 μL of cDNA template, 0.8 μL of each primer (10 μM), 0.4 μL of ROX reference Dye II (50×), and 7 μL of ddH2O). The amplification condition settings are as follows: 20 s at 95 ℃, 40 cycles of 3 s at 95 ℃ and 30 s at 60 ℃. To ensure data validity, the negative PCR control reactions were performed with $ddH_2O$ as templates, and each sample was run in triplicate. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The baseline was set automatically by the software to maintain consistency. The comparative Ct method was used to analyze the relative expression levels of *HSP70*. All data were given in terms of relative mRNA expression as means ± standard deviation (S.D, $n = 3$). Intergroup differences were analyzed by one-way analysis of variance