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# Bio-effect-monitoring of long-term thermal wastes on the oyster, *Crassostrea gigas*, using heat shock proteins

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ABSTRACT

We bio-monitored the stress of oyster, *Crassostrea gigas*, for possible long term effects of thermal waste from a power plant. The expression level of its heat shock proteins (HSPs) was measured by real time-reverse transcript PCR along with their density and growth in the field. Oyster size varied in a distance dependent pattern. Physics modeling for evaluation of spreading of the thermal effluent revealed that station A is affected by the thermal effluents abundance, and the size of *C. gigas* showed a negative relationship with distance to the power plant. The abundance and size of *C. gigas* were smallest at station A, which was closest to the thermal effluent outlet. The kinetics of changes in the hsp70 and hsp90 mRNA levels in the mantle of *C. gigas* were also investigated. Regardless of the higher expression level of hsp70 mRNA than hsp90, both hsp70 and hsp90 mRNA levels were significantly higher at station A. The expression levels decreased inversely with distance from the thermal effluent outlet, with expression of hsp70 mRNA at station A being approximately 7-fold higher than at station B and 15-fold higher than at station C. Similarly, expression of hsp90 mRNA at station A was approximately 14-fold higher than at station B and 22-fold higher than at station C. The present findings provide new insights on biological correlation among the growth of individuals and population size and the molecular index in *C. gigas* following thermal effects.

## 1. Introduction

Fossil fuel and nuclear power plants release heated effluent into local marine environments (Hamrick and Mills, 2000) that could lead to increases in water temperature of as much as 8 °C (Laws, 1993). Such increases could have particularly serious effects on benthic communities, because many of these organisms are sessile or sedentary (Bamber and Spencer, 1984). Accordingly, several studies have investigated the problems associated with such heated effluents impacting intertidal benthic communities (Bamber and Spencer, 1984; Chou et al., 2004; Crema and Pagliai, 1980; Lardicci et al., 1999; Suresh et al., 1993).

The oyster, *Crassostrea gigas*, is one of the most prevailing benthic invertebrates in a rocky intertidal zone, and has been intensively investigated with respect to its molecular and biological characteristics, such as immune responses (Cheng et al., 2016) and transcriptional changes following exposure to a pesticide (Rondon et al., 2016).

It is well known that organisms in general and also oysters respond to acute heat shock by altering the gene transcription and translation of heat shock proteins (Snyder et al., 2001). Heat shock proteins (HSPs) are highly conserved proteins that are upregulated in response to acute physical and chemical stressors, such as increased temperature, tissue trauma, heavy metals toxicity, radiation, infection, and normal changes associated with cellular development or differentiation (Lindquist and Craig, 1988; Schlesinger, 1990). In the presence of such stressors, HSPs act as molecular chaperones that protect the structure and function of proteins from denaturation and promote the refolding of unfolded proteins that are partially denatured (Nover and Scharf, 1997; Parsell and Lindquist, 1993; Sanders, 1993). Stress can also lead to the upregulation of constitutive heat shock proteins and/or the synthesis of inducible isoforms (Morimoto et al., 1994). HSPs are classified into several groups based on their molecular masses; namely, HSP90 (85–90 kDa), HSP70 (68–72 kDa), HSP20–30 (20–30 kDa), and others (Feder and Hofmann, 1999).

The HSP70 family is the most highly conserved and largest of all stress protein families. HSP70 has been shown to be the primary family

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of HSP that is responsive to thermal stress. The role and expression of HSP70 have been intensively investigated in marine benthos such as molluscs (Clark and Peck, 2009; Clegg et al., 1998; Snyder et al., 2001), echinoderms (Dong and Dong, 2008; Holm et al., 2008), and cnidarians (Rossi and Snyder, 2001; Snyder and Rossi, 2004). Additionally, the basic features of HSP70 with induced thermo-tolerance in the oyster (*C. gigas*) has been well characterized (Clegg et al., 1998; Farcy et al., 2009). Three isoforms of the HSP70 family have been reported in *C. gigas* (Hamdoun et al., 2003). Two proteins (77 kDa and 72 kDa) are constitutively expressed, and their level of expression increases after acute thermal stress. In contrast, the third isoform protein (69 kDa) shows induced expression and is typically only detectable after acute thermal stress.

Although the HSP90 family has been characterized in a wide range of organisms (Morimoto et al., 1994; Parsell and Lindquist, 1993), it has been much less investigated than HSP70 in molluscs, especially in *C. gigas* (Choi et al., 2008; Jo et al., 2008). The HSP90 family, which has been reported in all organisms, is the least understood of the major HSPs in terms of their cellular function (Farcy et al., 2007; Parsell and Lindquist, 1993). Isoforms of the HSP70 and HSP90 families are involved in the stabilization of different types of intracellular receptors. Both HSP70 and HSP90 function as stabilizers of steroid hormone receptor complexes (Hutchison et al., 1994). In addition, HSP90 functions as a dimer binding to and stabilizing aryl hydrocarbon receptor proteins such as steroid receptors and protein kinases (Csermely et al., 1998).

Although the functions of HSPs, including HSP70 and HSP90, have been widely studied in diverse organisms, only few field studies have tested the *in situ* responses to such temperature stresses. Therefore, as part of a broader study on damage caused to fisheries in response to discharged thermal effluents, we examined the constitutive 72kD isoform (hsp70) and the 90kD isoform (hsp90) of heat shock proteins of *C. gigas* inhabiting nearby thermal effluent outlets. We also examined the abundance and size distribution of *C. gigas* according to the distance from thermal effluent outlets.

## 2. Materials and methods

### 2.1. Study sites

To know the relationship between distance from the thermal effluent point and biological effects to oyster inhabiting, the studied sites were selected to enable comparisons among zones within a transect gradient near thermal effluent outlets of a power plant (Fig. 1). Each site was separated by 2217 m (St. A), 3713 m (St. B), and 4718 m (St. C) from the thermal effluent outlet. The geographic information pertaining to the study site is provided in Table 1. Sampling was performed during low tide, and ocean temperature was measured (YSI Model 55) for each study site (Table 2).

### 2.2. Sampling design

The abundance of *C. gigas* on the upper intertidal zone was determined by randomly placing a frame of 50 × 50 cm (0.25 $m^2$) within the area covered by *C. gigas*, after which the oysters inside the frame were counted. Since the sampling areas were limited to the areas dominated by *C. gigas*, the number of replicates varied between 10 and 20 per site. The length distribution of *C. gigas* was investigated by measuring the shell length (longest diameter of the shell) of 10 oysters that were randomly selected on an area dominated by *C. gigas*. Shell length was measured with vernier calipers to the nearest millimeter (Fig. 2).

### 2.3. Modeling

The EFDC (Environmental Fluid Dynamics Code) model developed at the Virginia Institute of Marine Science (Hamrick, 1992) was used for a precursory study. This approach was used for a wide range of environmental and biological studies, such as simulations of pollutant and thermal waste diffusion (Hamrick and Mills, 2000) and simulation of organismal transport in Ireland (Bedri et al., 2011) and Korea (Lee et al., 2015).

To estimate the impacted range, we used the temperature data describing thermal wastes from the power plant and the ocean temperature data measured at the Dae-San observatory station provided from the Korea hydrographic and oceanographic agency (Fig. 1; Table 2). Specifically, thermal wastes were 9.4 °C warmer than intake seawater in winter and 5.7 °C higher than seawater in summer. Modeling of the spread of the thermal effluent revealed that it impacted station (St) A (Fig. 1).

### 2.4. Quantification of heat shock protein

All chemicals, reagents and kits used in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), Qiagen (Valencia, CA, USA), Invitrogen (Carlsbad, CA, USA) or Promega (Madison, WI, USA). Oligonucleotide synthesis and DNA nucleotide sequencing were performed at Co. Bionics (Seoul, South Korea).

The whole bodies of sampled oysters (n = 10) were immediately pooled in a tube with RNA later reagent (Sigma-Aldrich Co., St. Louis, MO, USA), then frozen under liquid nitrogen to stabilize the RNA in their organs. After transfer to the laboratory, total RNA from the mantle of *C. gigas* was isolated by homogenizing the tissue with RNAiso Plus (TaKaRa Bio, Shiga, Japan). The total RNA was then purified and quantified according to the manufacturer's instructions. The amount and quality of RNA was quantified using a spectrophotometer. The first strand cDNA was made as a template for real time reverse transcriptase-polymerase chain reaction (RT-PCR) using an RNA PCR kit (AMV TaKaRa Bio, Shiga, Japan) Ver.3.0 (TaKaRa Bio, Shiga, Japan) following the manufacturers' protocols.

Real-time RT-PCR was conducted to study the expression pattern of hsp70 and hsp90. Primers for RT-PCR were designed using previously reported full-length cDNA sequences of *C. gigas* (hsp70: AF144646; hsp90: AF144646). All sequences information of primers used in this study are provided in Table 3. SYBR® Premix Ex Taq™ (TaKaRa Bio, Shiga, Japan) was used to detect specific PCR products according to the manufacturers' instructions. RT-PCR was conducted as follows: initial denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 20 s, and extension at 72 °C for 10 s and then 7 min at 72 °C for final extension. Amplification and detection of SYBR® Green were performed using a TaKaRa Thermal Cycler Dice® TP815 system (TaKaRa Bio, Shiga, Japan).

All data were expressed relative to the actin mRNA of *C. gigas*, which was used as a house keeping reference to normalize the expression levels between the samples after verification of stability during the experiment. The specificity of the real time RT-PCR products was determined by melting curve analysis. All experiments were analyzed in triplicate. Fold changes in the gene expression relative to the control were determined using the standard $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

### 2.5. Statistical analyses

The abundance and size survey results were expressed as means ± standard deviations (SD). Additionally, the expression levels of mRNA were expressed as the means ± the standard errors of the mean (Abdallah et al., 2007). One-way ANOVA followed by Duncan's multiple range comparison tests was used to check the differences in abundance, size and mRNA expression of *C. gigas* at each station investigated. Statistical analyses were conducted using the SPSS statistical package (Version 10.0; SPSS Inc., Chicago, IL, USA). The normality of data was checked by Shapiro-Wilk test. Data differences