J. Aquaculture
Vol. 20(4), 205-211, 2007

한국양식학회지
Journal of Aquaculture
©Korean Aquaculture Society

Osmoregulation and mRNA expression of a heat shock protein 68 and glucose-regulated protein 78 in the Pacific oyster *Crassostrea gigas* in response to salinity changes

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Stress-inducible proteins may function in part as molecular chaperones, protecting cells from damage due to various stresses and helping to maintain homeostasis. We examined the mRNA expression patterns of a 68-kDa heat shock protein (HSP68) and 78-kDa glucose-regulated protein (GRP78) in relation to physiological changes in Pacific oysters (*Crassostrea gigas*) under osmotic stress. Expression of HSP68 and GRP78 mRNA in the gill significantly increased until 48 h in a hypersaline environment (HRE) and 72 h in a hyposaline environment (HOE), and then decreased. Osmolality and the concentrations of Na^+, Cl^-, and Ca^{2+} in the hemolymph of HRE oysters significantly increased until 72 h (the highest value) and then gradually decreased; in HOE oysters, these values significantly decreased until 72 h (the lowest value), and then increased. These results suggest that osmolality and Na^+, Cl^-, and Ca^{2+} concentrations were stabilized by HSP68 and GRP78, and indicate that these two stress-induced proteins play an important role in regulating the metabolism and protecting the cells of the Pacific oysters exposed to salinity changes.

Keywords: Pacific oyster, *Crassostrea gigas*, HSP68, GRP78, Salinity change, Osmolality

Introduction

The coastal habitats of Pacific oysters (*Crassostrea gigas*) in Korea are subject to frequent and rapid changes in salinity. For example, high water temperature and prolonged drought in the summer may lead to a hypersaline environment (HRE), while typhoons and local runoff during the rainy season often results in a hyposaline environment (HOE). Salinity is highly relevant to growth, maturation, distribution, food intake, energy activation, and metabolic activities of many types of bivalve, including the Pacific oyster (Mills, 2000; Navarro et al., 2000). Fluctuations in salinity induce several osmotic responses in bivalves aimed at preserving cellular volume, such as controlling the accumulation of organic compounds called osmolytes (Somero and Bowlus, 1983). In teleosts, osmotic regulation has also been studied at the cytological level to clarify the molecular mechanisms involved in responses to salinity changes (Lee et al., 2006; Chang et al., 2007; Choi et al., 2007). However, few studies have addressed the osmotic responses of bivalves.

Bivalve defense mechanisms involve the circulation of blood cells called hemocytes (Cheng, 1981). In *C. gigas*, two types of hemocytes exist, which are differentiated by their morphology: hyalinocytes and granulocytes (Cheng, 1981). Recent studies on the response of bivalves to salinity changes have described hemocyte activation (Gagnaire et al., 2006) and the release of amino acids from the hemolymph (Hosoi et al., 2003). However, it remains unknown how various ions, including Na^+, Cl^-, and Ca^{2+}, in the hemolymph of bivalves, and osmotic pressure, respond to salinity fluctuations.

Similarly, little is known about the salinity-induced physiological stress responses (and the expression of corresponding stress-related genes) of the Pacific oyster. Many organisms synthesize heat shock proteins (HSPs) in response to stressors such as changes in salinity, water temperature, and heavy-metal content (Beckmann et al., 1990), and simultaneously exhibit numerous physiological responses (Schlesinger et al., 1992). HSPs play an important role in maintaining homeostasis by protecting cellular structures and functions (Sanders, 1993; Forsyth et al., 1997; Iwama et al., 1998, 1999; Ackerman and Iwama, 2001) and preventing cellular degeneration. HSPs may also limit damage to cells by inducing proteolysis or restoring cellular structure (Hartl, 1996; Bukau and Horwich, 1998; Morimoto, 1998). The Asian clam (*Potamocorbula amurensis*) shows time-dependent changes in HSP70 in response

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to salinity changes; as salinity decreases, so does HSP70, and vice versa (Werner, 2004; Werner and Hinton, 1999, 2000).

Glucose-regulated protein (GRP) is a member of the HSP family and plays an important role in many cellular reactions (Lee, 1987; Hightower, 1991). GRP is synthesized in response to glucose shortage, glycosylation suppression, and Ca^{2+} insufficiency in the endoplasmic reticulum (Lee, 1987; Little et al., 1994), but only a few preliminary studies have addressed the regulation of GRP genes. GRP78 is a calcium-binding protein that has significant sequence homology with HSP70 (Mazzarella and Green, 1987) and is involved in cellular protection (Hendershot et al., 1988; Craig et al., 1994). However, in contrast to HSP, GRP is found mainly in the endoplasmic reticulum, the major site of Ca^{2+} storage within the cell (Little et al., 1996). GRP temporarily interferes with the secretion and synthesis of membrane proteins, and may permanently bind to mutant proteins (Craven et al., 1997). While Yokoyama et al. (2006) reported the cDNA cloning of GRP78 expressed in the gills and adductor muscles of Pacific oysters exposed to a high water temperature, the expression of GRP78 in response to changes in salinity has not been extensively studied.

We investigated the osmoregulation and stress responses of Pacific oysters by comparing the mRNA expressions of HSP68 and GRP78 in the gills of oysters under different salinities. We also measured osmolality and related physiological responses, such as changes in the concentrations of Na^+, Cl^-, and Ca^{2+} ions.

Materials and Methods

Oysters

One-year-old Pacific oysters (average shell length: 112 ± 10.7 mm; shell height: 31.1 ± 5.4 mm; weight: 20.3 ± 3.9 g) obtained from an oyster hatchery on Dae-bu Island, Goseong, Gyeongnam, Korea, were housed in 50-L circulation filter tanks at a density of 50 oysters per tank. During experiments, the water temperature was kept at 17 ± 1°C and the photoperiod (L:D) was 12:12 h. Each experimental and control group consisted of five oysters.

Salinity treatment

After the oysters were acclimated to seawater of normal salinity (35 psu) for 48 h, the experimental group was subjected to a salinity of either 52.5 psu (HRE; 150% seawater salinity) or 17.5 psu (HOE; 50% seawater salinity). Samples were collected from five oysters 0, 12, 24, 48, 72, 96, and 192 h later. The oysters were not fed during the experiments.

Hemolymph analysis

Hemolymph was drawn and the gills were extracted from oysters in the two experimental groups. Hemolymph was drawn from the pericardial cavity with a 3-mL syringe, then separated by centrifugation (4°C, 10,000 rpm, 5 min) and stored at –80°C until analysis. Hemolymph osmolality was measured with the Vapor Pressure Osmometer (Vapro 5520; Wescor, Logan, UT, USA), Na^+ and Cl^- concentrations were determined with the Biochemistry Autoanalyzer (model 7180; Hitach Corp., Tokyo, Japan), and Ca^{2+} concentrations were assessed with the AVL 9180 (AVL, Roswell, GA, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For RT-PCR amplifications, degenerate primers were designed using highly conserved regions of Pacific oyster HSP68 (GenBank accession no. AB122062), GRP78 (AB122065), and 28S rRNA (AF137051) (Table 1). Total RNA was extracted from the gill using a Trizol kit (Gibco/BRL, Grand Island, NY, USA). Reverse transcription was conducted using M-MLV reverse transcriptase (Bioneer, Seoul, Korea) and cDNA was synthesized using 2.5 µg total RNA, according to the PCR protocol. PCR was performed using AccuPower RT/PCR PreMix (Bioneer). BS Taq Master Mix (2×) (Biosesang, Sungnam, Korea) was used, and the PCR cycle was as follows: initial denaturation for 5 min at 94°C, denaturation for 30 s at 94°C, annealing for 30 s at 54°C, 35 extensions for 1 min at 72°C, and a final extension for 7 min at 72°C. The PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 µg/µL), with amplified 28s rRNA as the control.

Statistical analysis

Significant differences among the data from each experiment were obtained using a one-way analysis of variance (ANOVA Tukey's test or LSD test) and the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). The significance level was $P < 0.05$.

Table 1. Oligo primers used for the RT-PCR of *Crassostrea gigas*

Primers	DNA sequence
HSP68-F	5'-TCAACTGCTGGTGACACACA-3'
HSP68-R	5'-TGGTCATGGCAAACTCACCT-3'
GRP78-F	5'-CTTCGGGATATCAAGCTACG-3'
GRP78-R	5'-TCCGGATCAATCCGATCCTGA-3'
28s rRNA-F	5'-TGCTCTGGACTGTCCTAGGA-3'
28s rRNA-R	5'-ACCGATTCGCCACTGACCAT-3'

Results

Expression of HSP68 mRNA

HSP68 mRNA expression of HRE oysters (52.5 psu salinity) gradually increased with time, and peaked 48 h after the start of the experiment. Then it rapidly decreased (Fig. 1A). Similarly, HSP68 mRNA expression in HOE oysters (17.5 psu salinity) also gradually increased with time, albeit more slowly, and peaked at 72 h (Fig. 1C). At 35 psu, the salinity of normal seawater, HSP68 mRNA expression did not change significantly throughout the experiment (Fig. 1B).

Expression of GRP78 mRNA

GRP78 mRNA expression in HRE oysters gradually increased with time and peaked between 48 and 72 h, after which it slowly decreased (Fig. 2A). GRP78 mRNA expression in HOE oysters also gradually increased with time until 72 h (Fig. 2C). Thus, the changes in GRP78 mRNA expression

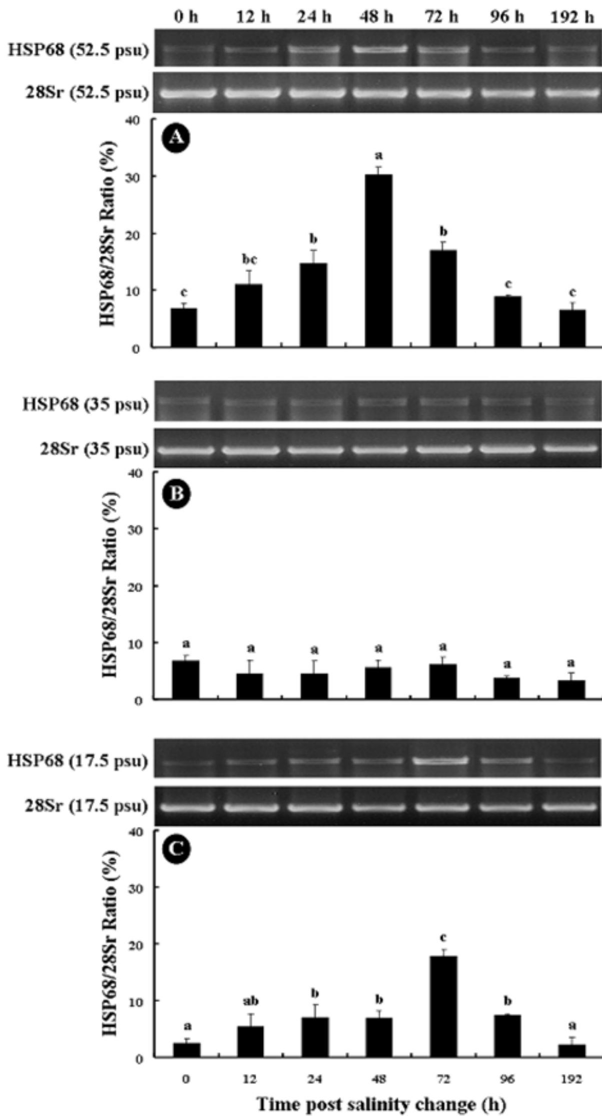

Fig. 1. The mRNA expression levels of a 68-kDa heat shock protein (HSP68) of *Crassostrea gigas* in a hypersaline environment of 52.5 psu (A), normal salinity of 35 psu (B), and hyposaline condition of 17.5 psu (C). Total RNA was extracted 0, 12, 24, 48, 72, 96, and 192 h after treatment, and 2.5 μg was used for PCR. The values are percent increases relative to control values. The expression level of each sample was normalized with respect to the 28S rRNA signal and represents a relative expression. Values with different letters are significantly different ($P < 0.05$) from each other. Values are means ± SD (n = 5).

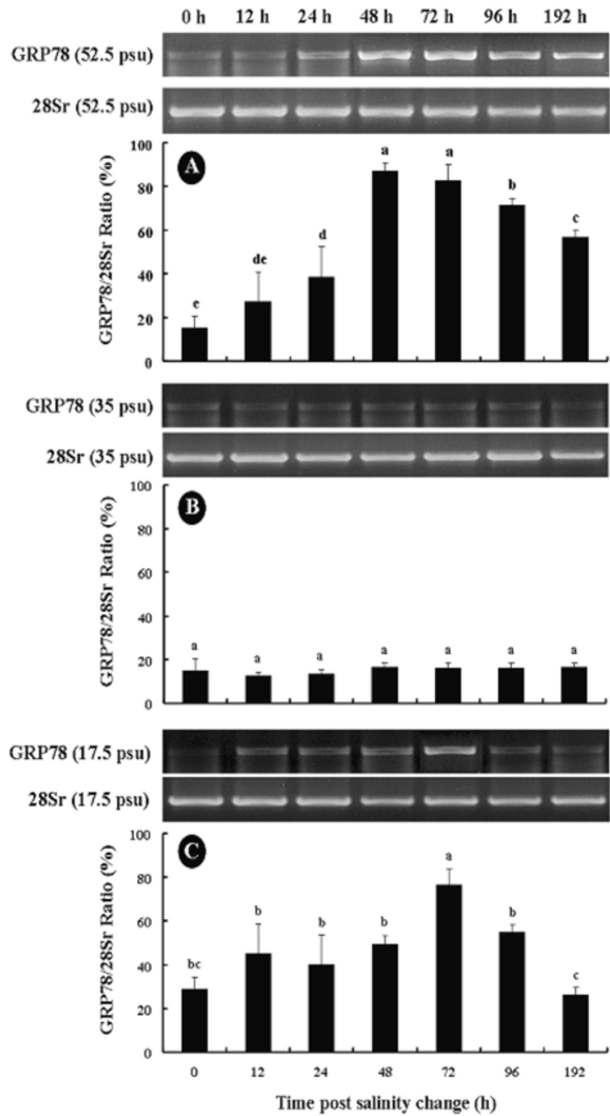

Fig. 2. The mRNA expression levels of a 78-kDa glucose-regulated protein (GRP78) of *Crassostrea gigas* in a hypersaline environment of 52.5 psu (A), normal salinity of 35 psu (B), and hyposaline condition of 17.5 psu (C). Total RNA was extracted 0, 12, 24, 48, 72, 96, and 192 h after treatment, and 2.5 μg was used for PCR. The values are percent increases relative to control values. The expression level of each sample was normalized with respect to the 28S rRNA signal and represents a relative expression. Values with different letters are significantly different ($P < 0.05$) from each other. Values are means ± SD (n = 5).

resembled those of HSP68 mRNA. In the control group exposed to 35 psu salinity, GRP78 expression did not change significantly with time (Fig. 2B).

Osmolality

The highest hemolymph osmolality of HRE oysters was 1429.7 ± 5.9 mOsm/kg, which occurred 48–72 h after exposure. Thereafter, osmolality significantly decreased and eventually stabilized at 96–192 h (Fig. 3A). The hemolymph osmolality of HOE oysters rapidly decreased beginning 12 h after exposure, and was maintained in the range of 443.7 ± 4.1 to 481 ± 23 mOsm/kg until 192 h, with a gradual increase beginning at 92 h (Fig. 3B).

Na^+, Cl^-, Ca^{2+} concentrations

The concentrations of Na^+, Cl^-, and Ca^{2+} ions in the hemolymph of HRE oysters began to significantly increase 12 h after exposure; they peaked at 72 h (at 657.7 ± 5.7, 715 ± 3.1, and 172.3 ± 2.1 mmol/L, respectively), and then gradually decreased (Fig. 4). In HOE oysters, the concentrations of these ions significantly decreased until reaching their lowest levels 72 h after exposure (153.3 ± 12.5, 142.3 ± 2.3, and 29.1 ± 6.8 mmol/L, respectively), and then slowly increased (Fig. 5).

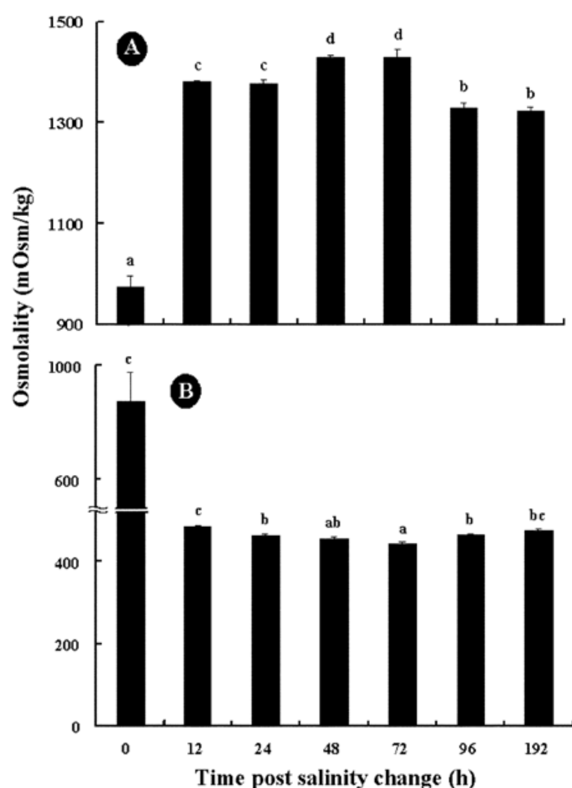

Fig. 3. Hemolymph osmolality during adaptation to hypersaline conditions of 52.5 psu (A) and hyposaline conditions of 17.5 psu (B). Hemolymph was extracted from the oyster and used to measure osmolality. Values with different letters are significantly different (*P* < 0.05) from each other. Values are means ± SD (n = 5).

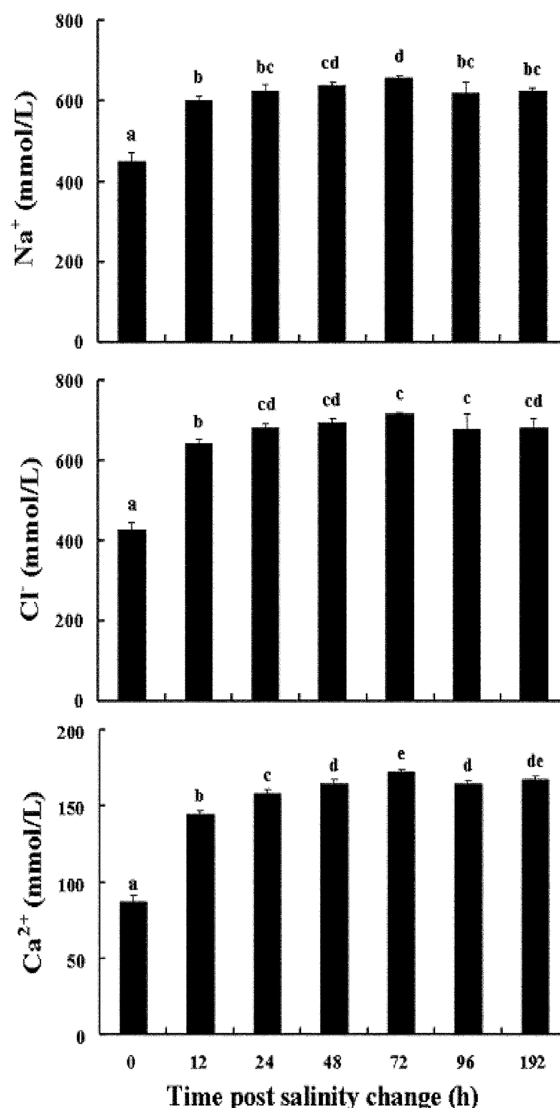

Fig. 4. Changes in hemolymph Na^+, Cl^-, and Ca^{2+} concentrations in response to hypersaline conditions (52.5 psu) for 192 h. Hemolymph was extracted from the oysters and used to measure Na^+, Cl^-, and Ca^{2+} concentrations. Values with different letters are significantly different (*P* < 0.05) from each other. Values are means ± SD (n = 5).

Discussion

The Pacific oyster is an euryhaline bivalve that is relatively resistant to environmental stresses, such as changes in salinity (Gagnaire et al., 2006). However, localized torrential downpours, for example, may rapidly change salinity, resulting in acute changes in osmotic pressure that disrupt homeostasis. This would result in abnormal moisture content, intracellular concentrations of nutrients and salts, and cellu-

lar osmotic pressure (Morgan and Iwama, 1991). Eastern oysters (*Crassostrea virginica*) in extreme hyposaline (0–5 psu) water show inhibited growth and abnormal shell movement and seawater transport (Loosanoff, 1953). In addition, changes to the osmotic balance of heart tissues have adverse effects on heart function and inhibit acetylcholinesterase activity (Dimock, 1967). While these reports described the effects of salinity changes on overall homeostasis and osmoregulation in oysters, the cellular and molecular events behind these effects were not determined.

Gagnaire et al. (2006) reported that oysters perished starting from the third day of exposure to salinities of either 5 psu or 60 psu, and that mortality was very high after the sixth day. However, in our study, no individuals perished when exposed to salinities of 52.5 and 17.5 psu. This suggests that euryhaline Pacific oysters can survive within a salinity range of 17.5 to 52.5 psu.

We found that hemolymph osmolality changed immediately after a salinity shift, indicating that the Pacific oyster can respond quickly to sudden changes in its environment. Moreover, following exposure to a salinity of 52.5 psu, hemolymph osmolality was higher than the osmotic pressure of the hypersaline seawater. This was most likely attributable to the removal of moisture from the oysters' bodies, and thus to an increase in the salt and nutrient concentrations in response to the high external osmotic pressure. The osmotic pressure of hemolymph in oysters exposed to 17.5 psu was lower than the osmotic pressure of the hyposaline seawater, and resulted in a pattern of osmosis opposite to that observed under hypersaline conditions.

The expression of HSP68 and GRP78 mRNA with respect to salinity changes was highest at 48 h under hypersaline conditions and at 72 h under hyposaline conditions. These increases were most likely cellular responses aimed at preventing secondary damage induced by the external stress (Teh et al., 1999; Werner and Hinton, 1999, 2000). Such physiological reactions are representative of the normal metabolic responses of living organisms to avoid death due to rapid environmental changes (Ciavarra and Simeone, 1990; Hightower, 1991). Our results regarding the patterns of HSP68 mRNA expression are similar to those reported for HSP70 protein in the estuarine Asian clam *P. amurensis* (Werner and Hinton, 2000; Werner, 2004). The expression of GRP78 mRNA followed the same pattern observed for HSP68 mRNA. This was not unexpected since GRP78 is a member of the HSP family and performs similar functions, i.e., maintaining cell shape and protecting cells against stress-induced damage (Hendershot et al., 1988; Craig et al., 1994).

Previous studies on fugu (*Takifugu rubripes*) (Lee et al., 2006), black porgy (*Acanthopagrus schlegeli*) (Chang et al., 2007), and flounder (*Platichthys flesus*) (Bond et al., 2002) demonstrated that these species, under salinity stress, possess osmoregulatory abilities that allow them to recover the salinity level of normal seawater (35 psu). However, in contrast to fishes, Pacific oysters can achieve a balance between internal osmolality and the osmolality of their environment, and perhaps modify the osmolality of their environment. In this respect, our results are similar to those of Hosoi et al. (2003), who found that stabilization at 800 mOsm/kg·H_20 was reached 8 h after Pacific oysters were exposed to a rapid increase in osmotic pressure from 15 to 30 psu, and conversely, back to 380 mOsm/kg·H_20 after 8 h following a rapid decrease in the osmotic pressure from 30 to 15 psu. Furthermore, as in fishes, the Na^+ and Cl^- concentrations accounted for approximately 70% of the osmolality and were representative ions influenced by osmoregulation in the Pacific oyster (Lee et al., 2006; Chang et al., 2007).

HSP68 and GRP78 mRNA expression in Pacific oysters in response to variations in salinity did not change immediately, but rather when the internal osmotic pressure and the concentrations of Na^+, Cl^-, and Ca^{2+} were at their highest or lowest for a fixed length of time. This result is consistent with Piano et al. (2005), who reported that HSP70 mRNA expression in the flat oyster (*Ostrea edulis*) peaked 3 h after the bivalve was exposed for 1 h to a water temperature of 35°C followed by exposure to 18°C.

We found that Na^+, Cl^-, and Ca^{2+} levels and hemolymph osmolality changed immediately when Pacific oysters were shifted to the experimental environments. The same tendency was seen with HSP68 and GRP78 mRNA expression. In addition, stabilization of osmotic pressure and Na^+, Cl^-, and Ca^{2+} levels occurred after HSP68 and GRP78 mRNA expression had stabilized. Further studies on salinity changes and osmolality- and stress-related genes are needed to determine the relationships between these cellular events.

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Manuscript Received: May 23, 2007
Revision Accepted: October 4, 2007