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Combined effects of acute thermal and hypo-osmotic stresses on osmolality and *hsp*70, *hsp*90 and *sod* expression in the sea cucumber *Apostichopus japonicus* Selenka

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Abstract The combined effects of acute temperature and salinity on osmolality, expressions of heat shock proteins mRNA (*hsp*70, *hsp*90a and *hsp*90b) and superoxide dismutase mRNA (*sod*) were investigated in the sea cucumber *Apostichopus japonicus* Selenka. There were 12 treatments (combinations of temperature at 16, 20, 24 and 28 °C and salinity at 22, 27 and 32 ppt). In low salinity environments, the osmolality of the sea cucumber's coelomic fluid decreased immediately and reached osmotic balance within 6 h. The decline of osmolality after 2 h of hypo-osmotic stress was faster at high temperatures (28 °C) than that at low temperatures (16 and 20 °C). Cellular level stress was indicated by up-regulation of *hsp*70, *hsp*90s and *sod* mRNA, and the maximal expression of all genes occurred at 6 h after stresses. The up-regulation of *hsp*s and *sod* mRNA indicated the emergence of protein denaturation and oxidative damage and also suggested an increase in energy consumption at high temperature and low salinity. These results indicated that high temperature and low salinity could change biochemical pathways and energy budgets and then potentially impair the osmoregulation of the sea cucumber. Therefore, effective ways should be taken (e.g., draining off the upper freshwater, exchanging water and adding man-made sea water) to prevent the damage to sea cucumber culture caused by low salinity induced by rainstorms, especially at high temperature.

Keywords Sea cucumber · *Apostichopus japonicus* · Temperature · Salinity · Osmolality · Heat shock proteins · Superoxide dismutase

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Introduction

The sea cucumber *Apostichopus japonicus* Selenka, which is believed to have aphrodisiac and curative properties, has been a prevailing aquaculture species in China (Fu et al. 2005). The total area of sea cucumber farming reached 153,626 hectare in China, and the total output was 137,754 tons in 2011 (DOF 2012). Cultivation in field ponds is one of the main culture modes in northern China. Because these ponds are usually shallow (2–3 m in depth), environmental factors are relatively sensitive to changing weather and tidal cycles. Based on our observations, the water temperature in these ponds can rapidly increase from 20 to 30 °C, and frequently exceeds 30 °C during the low tidal period in summer (Dong et al. 2008a). Furthermore, the salinity can also decrease to ~20 ppt after a summer rainstorm within several hours, which can cause large-scale motality of sea cucumber (Meng et al. 2011).

Temperature and salinity are critical factors affecting the behavior, metabolism, growth, life cycle and intra- and interspecific relationships of aquatic organisms (Kinne 1971). As previous studies have described, temperature (Dong et al. 2010; Wang et al. 2011, 2012) and salinity (Yuan et al. 2006; Meng et al. 2011) can influence growth, survival and physiological performance of the sea cucumber *A. japonicus*. Because of the dramatic changes of temperature and salinity in the aquaculture ponds after rainstorms, it is crucial to elucidate the combined effects of temperature and salinity on the physiological performance of sea cucumbers.

The osmolality of coelomic fluid in the sea cucumber changes with changes in salinity of the ambient water (Binyon 1972; Diehl 1986). Vidolin et al. (2002) reported that the gray sea cucumber (*Holothuria grisea*) could temporally regulate the osmolality of its coelomic fluids by possibly reducing the permeability of its body wall. In *A. japonicus*, the osmolality of the coelomic fluid changed rapidly and stabilized at 6 h after the osmotic shock when salinity changed from 32 ppt to different salinities (20, 25, 30 and 40 ppt) (Dong et al. 2008b). The osmoregulatory capacity of aquatic animals can be affected by temperature (Williams 1960; Charmantier 1998). For example, the osmoregulation of the shrimp *Penaeus stylirostris* was diminished at low temperature (Lemaire et al. 2002). In *A. japonicus*, an earlier heat shock can affect the activities of Na^+/K^+ ATPase and this then affects osmolality of coelomic fluids of this species (Dong et al. 2008b).

When animals suffer either heat stress (Tomanek and Somero 1999; Dutton and Hofmann 2009) or osmotic stress (Pan et al. 2000; Niu et al. 2008), heat shock proteins (HSPs) are induced. Acting as molecular chaperones, HSPs assist in the refolding of stress-denatured proteins and prevent those proteins from aggregating in the cell, thus helping the cell to cope with potential damage (Feige et al. 1996; Hartl 1996; Frydman and Höhfeld 1997; Morimoto 1998). Some studies have shown that HSPs play an important role in the sea cucumber *A. japonicus*, resisting both thermal stress (Dong and Dong 2008; Ji et al. 2008; Meng et al. 2009; Wang et al. 2011, 2012) and osmotic stress (Dong et al. 2008b; Meng et al. 2011). Superoxide dismutase (SOD), a primary antioxidase that is directly involved in eliminating reactive oxygen species (ROS), has been used as a main index of antioxidant defense (Wilhelm-Filho et al. 1993, 2001; Leiniö and Lehtonen 2005). In *A. japonicus*, the activity of SOD increased significantly during large fluctuations of temperature (Dong et al. 2008b) and in the early stage of estivation (Ji et al. 2008). However, there is a lack of knowledge about the combined effects of temperature and salinity on the expression of *hsp*s mRNA in the sea cucumber.

In the present study, the osmolality of the coelomic fluid and the expression of *hsp*s and *sod* mRNA levels in juveniles of the sea cucumber *A. japonicus* were investigated at

different temperatures (16, 20, 24 and 28 °C) and salinities (22, 27 and 32 ppt) to elucidate the physiological response of sea cucumbers to acute changes of temperature and salinity. These results should be valuable in improving the management of sea cucumber aquaculture during rainstorms in summer.

Materials and methods

Collection and acclimation of animals

About 700 juvenile sea cucumbers, with a mean body wet weight of 0.92 ± 0.20 g (mean ± SE), were collected from Jimo Aquatic Seeding Breeding Farm, Qingdao, P. R. China. The animals were acclimated in several aquariums (1,700 × 750 × 350 mm) at 16 °C for 4 weeks. During acclimation, sea cucumbers were fed ad libitum with a laboratory-made formulated diet (9.94 ± 0.17 % moisture, 18.58 ± 0.23 % crude protein, 2.67 ± 0.06 % fat, 42.66 ± 0.54 % ash and 8.16 ± 0.00 kJ g^{-1} energy), containing powders of *Sargassum* spp., fishmeal, sea mud, wheat, vitamin and mineral premixes.

Aeration was provided continuously, and lighting was provided by incandescent lamps at a photoperiod of 12 h light: 12 h dark. Seawater was filtered using a sand filter, and the salinity was ~32 ppt. One-half or two-thirds of the rearing water was exchanged with fresh seawater daily. Water pH was about 7.8, and the concentration of ammonia was less than 0.24 mg l^{-1}. Water temperature, salinity, pH and ammonia concentration were determined with a mercury thermometer (accuracy ± 0.2 °C), a salinity refractometer (AIAGO, Japan), a pH meter (PH3150i, WTW, Germany), and a hypobromite oxidation method (Wu 2007), respectively.

Experimental protocol

Based on water temperature and salinity values in June–August along the coast of Shandong Province, 12 environmental regimes were designated as combination of temperatures of 16, 20, 24 and 28 °C (T_{16}, T_{20}, T_{24} and T_{28}) and salinities of 22, 27 and 32 (S_{22}, S_{27} and S_{32}). Each regime had three replications. After acclimation at 16 °C and 32 ppt (control) for 1 month, 360 sea cucumbers (ten individuals in each group) were allocated into 36 50-L plastic aquariums and reared in filtered aerated seawater maintained in the conditions as mentioned above. These aquariums were fully randomized and not blocked. Target temperatures above ambient were maintained by 300-W heaters connected to a temperature regulator provided with a thermocouple. The salinities of seawater were adjusted by adding aerated tap water. The rearing conditions were similar to those used in the acclimation period.

Osmolality, *hsp*s and *sod* mRNA expressions

After being transferred to the preprepared seawater at different temperatures and salinities, one specimen was sampled at 2, 6, 12, 24, 36 and 48 h from each glass aquarium for measuring the osmolality of the coelomic fluid ($n = 3$). Their body wall and intestine were quickly removed and frozen in liquid nitrogen (~−200 °C) for analysis of *hsp*s and *sod* mRNA expressions ($n = 3$). About 30 μl, coelomic fluid was extracted using 1.0 ml disposable syringes and No. 21 gauge 1.5″ needles. To make sure that all the fluid was

from the coelom, the body wall was pierced carefully at a small angle until the tip of the needle reached the coelomic cavity, without touching internal organs. The osmolality was measured using a Fiske 210 Micro-Sample Osmometer (Advanced Instruments, USA).

Total RNA was isolated from approximately 80 mg of body wall tissues by Trizol Reagent (Invitrogen, USA). A sample of 1 μg of total RNA was used as the template for synthesis of the first strand of cDNA. Partial β-actin gene (312 bp) was selected as reference housekeeping to normalize the level of expression between the samples amplified using the primers from Meng et al. (2009). Primers of the four genes *hsp*70, *hsp*90a, *hsp*90b and *sod* (Hsp70-F and Hsp70-R, Hsp90a-F and Hsp90a-R, Hsp90b-F and Hsp90b-R, Sod-F and Sod-R) were designed based on the sequences from GenBank (Ajhsp70, accession no. GH985449; Aj90a, accession no. JF907619; Aj90b, accession no. GH550976; Ajsod, accession no. X64060.1) as shown in Table 1.

Semi-quantitative PCR conditions and components for *hsp*s and β-actin were optimized, especially for the amplification cycles and annealing temperatures. The optimized PCR was carried out in 25 μl reactions containing 2.5 μl of 10 × PCR buffer, 1.6 μl of $MgCl_2$ (25 mmol l^{-1}), 2.0 μl of dNTP (2.5 mmol l^{-1}), 1 μl of each primer (10 pmol ml^{-1}), 15.875 μl of PCR-grade water, 0.125 μl (5 U μl^{-1}) of Taq polymerase (Promega, USA) and 1 μl of cDNA reaction mix. The PCR program was preceded by initial denaturation for 5 min at 94 °C, followed by 30 cycles (for *hsp*70, *hsp*90a, *hsp*90b and *sod*) or 27 cycles (for β-actin) of 94 °C for 45 s, 51 °C (for *hsp*70, *hsp*90a) or 49 °C (for *hsp*90b and *sod*) or 54 °C (for β-actin) for 45 s, 72 °C for 1 min and a final extension step at 72 °C for 10 min. The PCR products were electrophoresed in 1.2 % agarose gel stained with ethidium bromide (EB), after which the products were purified from the gel and sequenced to confirm the specificity of RT-PCR amplification. Electropheretic images and the optical densities of amplified bands were analyzed using GeneTools software (Syngene, USA). The abundance of *hsps* and *sod* mRNA was normalized to the corresponding β-actin abundance in all samples and expressed as the ratio of optical densities of genes and β-actin ($C_{hsp70}/C_{\beta\text{-actin}}$, $C_{hsp90a}/C_{\beta\text{-actin}}$, $C_{hsp90b}/C_{\beta\text{-actin}}$ or $C_{sod}/C_{\beta\text{-actin}}$).

Statistical analyses

The data were analyzed using SPSS for Windows (version 13.0; SPSS, Chicago, IL, USA). Data were tested for normal distribution using the Shapiro–Wilk test and for homogeneity of variances using the Levene test. Two-way ANOVA followed by post hoc Tukey multiple range test was applied to analyze the differences in osmolality of coelomic fluids in different temperature and salinity treatments. One-way ANOVA followed by post hoc Tukey multiple range test was applied to test the effect of salinity at each level of temperature and the effect of temperature at each level of salinity. The differences in gene expressions in different treatments were analyzed using one-way ANOVA followed by post hoc Tukey multiple range test or Kruskal–Wallis H test followed by Siegel–Tukey test depending on heterogeneity variances. Differences were considered significant at $P < 0.05$.

Results

Osmolality

The osmolality of the coelomic fluid changed immediately when the animals were transferred to low salinity treatments and reached osmotic balance within 6 h (Fig. 1, dotted

Table 1 Primer sets designed for semi-quantitative RT-PCR analysis of *hsp*s mRNA in the sea cucumber *Apostichopus japonicus*

Primers	Primer sequences
Hsp70-F	5′-ATGCCTAGAACCAGTAGAGAAAG-3′
Hsp70-R	5′-TGTCGTTCGTGATGGTGATT-3′
Hsp90a-F	5′-TTGTTGAAAGGGAGGAGG-3′
Hsp90a-R	5′-GGCATCAGAGGCGTTAGA-3′
Hsp90b-F	5′-TCTTTCTTAGGGAACTCATCTC-3′
Hsp90b-R	5′-CCTGTAGCATTCGTCATCG-3′
Sod-F	5′-CACGCAACTTACACCAATA-3′
Sod-R	5′-CAATACCAAACAAAGGCAC-3′
β-actin-F	5′-ACACGGTATCGTCACAAACTGG-3′
β-actin-R	5′-AGGATAGCGTGAGGAAGAGCAT-3′

lines represent the osmolality of ambient water in each experimental regime). Two-way ANOVA analysis showed that the interaction between temperature and salinity was significant (Table 2). The osmolality of coelomic fluid after 2 h of hypo-osmotic exposure (ΔOP_{2h}) differed statistically between different temperatures within the same salinity (Fig. 2). Values of ΔOP_{2h} at high temperature (28 °C) were significantly higher than those at low temperatures (16 and 20 °C) in low salinities (27 and 22 ppt).

Expression of *hsp*s and *sod* mRNA

The expressions of *hsp*70, *hsp*90 s and *sod* mRNA showed similar temporal patterns in different treatments (Fig. 3, 28 °C as examples). Levels of *hsp*s mRNA increased immediately after thermal and osmotic stresses, and the maximum values of *hsp*s and *sod* mRNA in all treatments occurred at 6 h after the stresses and then decreased to the initial levels.

The maximum values of mRNAs in the body wall at 6 h were variable among different salinity treatments (Fig. 4). For *hsp*70, the levels in S_{22} and S_{27} were significantly higher than that in S_{32}, and there was no significant difference between S_{27} and S_{22}. For *hsp*90a, there was no statistical difference among the three salinity treatments. For *hsp*90b, the expression pattern was similar to that of *hsp*70. Gene levels at S_{27} and S_{22} were significantly higher than that at S_{32}. For *sod*, there was no statistical difference among the three salinity treatments. The expressions of mRNAs in the intestine were identical to those in the body wall (Tables 3, 4; Fig. 5).

The maximum levels of mRNAs in the body wall were also distinct in different temperature treatments (Fig. 4). The levels of *hsp*s mRNA (*hsp*70, *hsp*90a and *hsp*90b) were up-regulated with temperature increase. The levels of *hsp*s mRNA in T_{24} and T_{28} were significantly higher than that of T_{16}, and there were significant differences between T_{28} and T_{20}. There was no significant difference in *sod* mRNA among different temperature treatments. In the intestine, the levels of *hsp*s mRNA in T_{24} and T_{28} were significantly higher than that of T_{16}. The levels of *sod* mRNA in T_{28} was significantly higher than that of T_{16} (Tables 3, 4; Fig. 5).

Discussion

Juveniles of sea cucumber *Apostichopus japonicas* Selenka prefer to live in the low intertidal zone (+0.4 m above Chart Datum) on rocky substrates (Yusuke et al. 2006). In

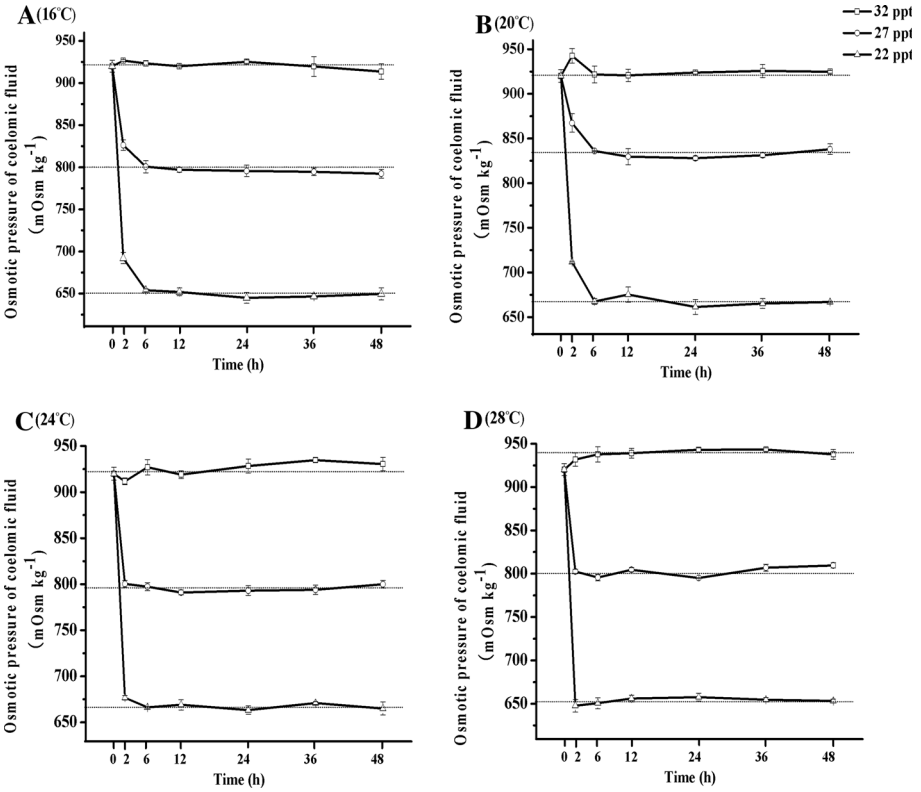

Fig. 1 The osmolality of the coelomic fluid in the sea cucumber *Apostichopus japonicus* at selected time points in 32, 27 and 22 ppt at **A** 16 °C, **B** 20 °C, **C** 24 °C and **D** 28 °C (mean ± S.E., $n = 3$). *Dotted lines* indicate the osmolarity of surrounding water in each treatment

Table 2 Effects of temperature and salinity on the differences in osmotic pressure of coelomic fluid of sea cucumbers *Apostichopus japonicas* between 0 and 2 h ($n = 3$)

Source	Type III sum of squares	*df*	Mean square	*F*	*P*
Temperature	12,565.11	3	4,188.37	40.81	< 0.001
Salinity	366,501.56	2	183,250.78	1,785.39	< 0.001
Temperature × Salinity	4,182.89	6	697.15	6.79	< 0.001

this area, sea cucumbers encounter extreme temperature and salinity variations during low spring tides, especially after a rainstorm in summer when the body temperature of the sea cucumber can increase rapidly from 20 °C to over 30 °C and the salinity can decrease from 32 to 20 ppt (Dong and Meng, unpublished data). So, we investigated combined effects of acute temperature and salinity stresses on related physiological responses of this species.

The osmolality of coelomic fluid in the sea cucumber changed rapidly with the change of ambient water salinity, and this change was temperature dependent. In the present study, osmolality of coelomic fluid reached osmotic balance with the environment within 6 h. The rapid change of osmolality in *A. japonicus* facing hypo-osmotic stress indicates that this species has poor ability for osmoregulation as previous studies described (Ruppert et al. 1994; Dong

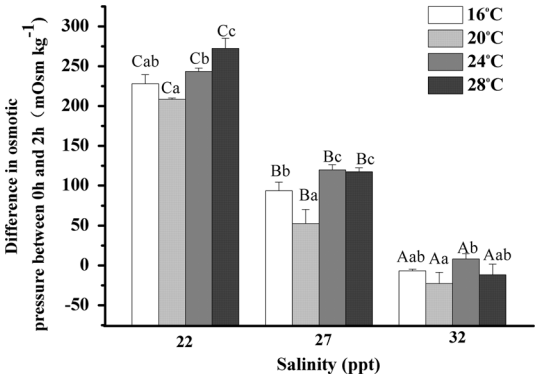

Fig. 2 The differences in osmolality of coelomic fluid of sea cucumbers *Apostichopus japonicus* from different treatments between 0 and 2 h. Differences between these groups were assessed by two-way ANOVA followed by post hoc Duncan multiple range test. Values with different *lower case letters* are significantly different ($P < 0.05$) among different temperature levels at the same salinity, and values with different *capital letters* are significantly different ($P < 0.05$) among different salinity levels at the same temperature (mean ± S.E., $n = 3$)

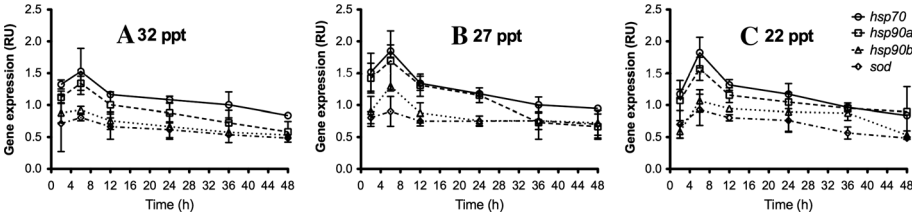

Fig. 3 Temporal patterns of heat shock proteins mRNA (*hsp*70, *hsp*90a and *hsp*90b) and superoxide dismutase mRNA (*sod*) in **A** 32 ppt, **B** 27 ppt and **C** 22 ppt in the body wall of sea cucumbers *Apostichopus japonicus* at 28 °C (mean ± S.E., $n = 3$)

et al. 2008b; Meng et al. 2011). Furthermore, the change of osmolality in the first 2 h was variable among different temperature treatments, and the osmolality of coelomic fluids of the sea cucumber is temperature dependent. High temperatures (24 and 28 °C), above the optimal temperature for growth (16–20 °C), can aggravate the decrease in osmolality of the coelomic fluid in low salinity.

The rapid decrease in osmolality of coelomic fluid in the sea cucumber is related to the limited energy supply against salinity stress. The aerobic metabolism decreased at high temperatures, as described in our previous studies (Dong et al. 2008a; Ji et al. 2008). When water temperature was over 24 °C, the oxygen consumption decreased continually with the elongation of thermal stress. Therefore, the limited energy supply is an important reason for the rapid decrease in osmolality of the sea cucumber at high temperatures.

Besides the decrease in aerobic metabolism, the increase in energy consumption is another reason for the low cellular energy status at high temperatures. In the present study, significant up-regulation of *hsp*70 and *hsp*90 s mRNA occurred at high temperatures. Large amounts of energy are required at several events in the heat shock responses, such as the activation of transcription of heat shock genes, the synthesis of HSPs, the repair and replacement of damaged proteins and the ATP-requiring chaperoning by HSPs (Somero 2002). Hence, less energy is allocated into growth, reproduction, defenses against salinity

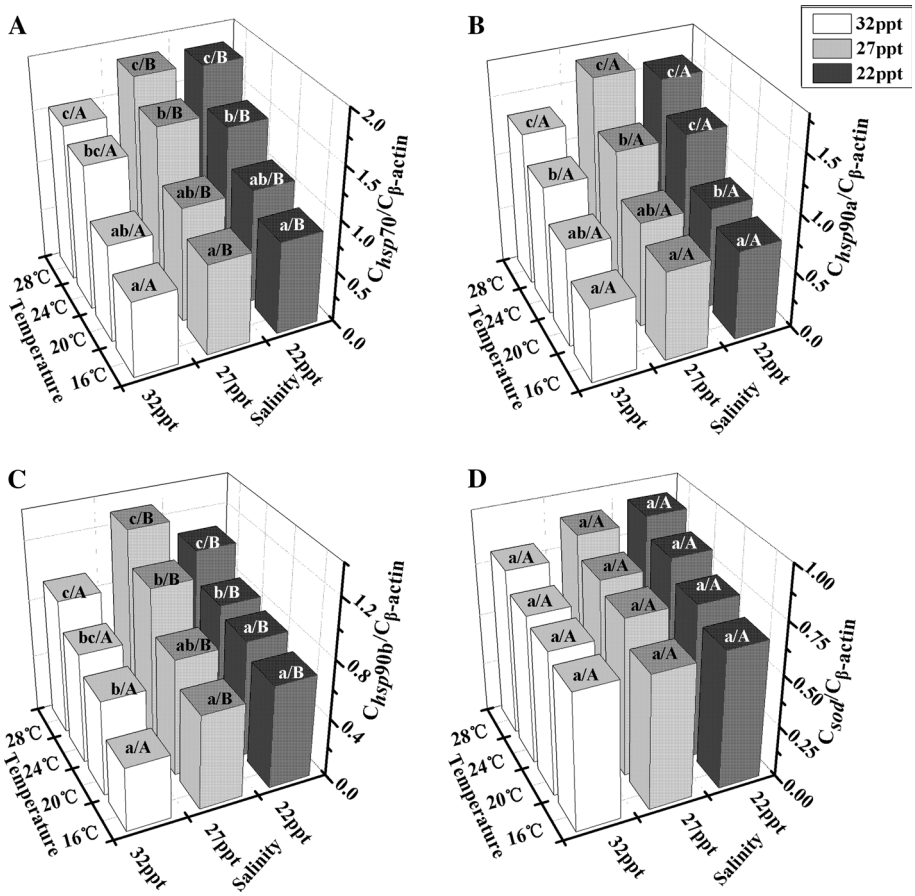

Fig. 4 Levels of **A** *hsp*70, **B** *hsp*90a, **C** *hsp*90b and **D** *sod* mRNA in the body wall of sea cucumbers *Apostichopus japonicus* at 6 h after the temperature and salinity stresses. Differences between these groups were assessed using one-way ANOVA followed by post hoc Duncan multiple range test or Kruskal–Wallis H test followed by post hoc S–N–K test. Data in the same column having different *lower case letters* are significantly different ($P < 0.05$) among different temperature levels, and data in the same row having different *capital letters* are significantly different ($P < 0.05$) among different salinity levels ($n = 3$)

stress (Na^+/K^+ ATPase synthesis, for example) and so on (Somero 2002; Tadashi et al. 2004).

Heat shock proteins (HSPs) and SOD are often regarded as defense mechanisms to environmental stresses (Feder and Hofmann 1999; Parihar et al. 1997). The expression of HSPs and SOD was tissue specific, and the internal organs were more sensitive than the body wall (Ji et al. 2008). This might be the reason for the lack of significant effects on *sod* expression in the body wall. The up-regulation of HSP at high temperature indicates an increase in protein damage. Previous studies showed that when suffered osmotic stress, the concentrations of intracellular ions of echinoderms was altered, so did enzymes of intermediary metabolism. The enzyme Na^+/K^+-ATPase (the 'sodium pump'), which is responsible for the regulation of Na^+ and K^+ by transferring ions, is fundamental to osmotic regulation in most eukaryotic cells (Balshaw et al. 2001; Jorgensen et al. 2003).

Table 3 One-way ANOVA table of gene expressions of *hsp*70, *hsp*90a, *hsp*90b and *sod* mRNA in the body wall and intestine of sea cucumbers *Apostichopus japonicas* at 6 h after the temperature and salinity stresses ($n = 3$)

Source	Sum of squares	*df*	Mean squares	*F* ratios	*P* value
Body wall					
*hsp*70					
T_{16}	0.050	2	0.025	5.992	0.037
T_{20}	0.058	2	0.029	12.797	0.007
T_{24}	0.066	2	0.033	5.210	0.045
T_{28}	0.059	2	0.034	5.431	0.042
S_{22}	1.482	3	0.494	15.463	0.001
S_{27}	1.775	3	0.592	11.554	0.003
S_{32}	1.277	3	0.426	4.755	0.035
*hsp*90a					
T_{16}	0.008	2	0.003	0.923	0.502
T_{24}	0.056	2	0.028	0.197	0.826
T_{28}	0.191	2	0.095	1.431	0.430
S_{32}	0.827	3	0.276	18.422	0.001
*hsp*90b					
T_{16}	0.118	2	0.059	34.341	0.001
T_{20}	0.146	2	0.023	5.905	0.029
T_{24}	0.164	2	0.082	5.143	0.045
S_{27}	0.767	3	0.256	29.012	0.000
S_{32}	0.378	3	0.126	10.531	0.004
sod					
T_{16}	0.080	2	0.040	1.880	0.232
T_{20}	0.008	2	0.004	0.813	0.487
T_{24}	0.023	2	0.012	0.155	0.860
T_{28}	0.025	2	0.012	0.215	0.737
S_{27}	0.101	3	0.034	0.918	0.475
S_{32}	1.498	3	0.466	3.460	0.056
Intestine					
hsp70					
T_{24}	0.164	2	0.090	5.351	0.047
T_{28}	0.048	2	0.030	6.069	0.037
S_{22}	1.683	3	0.561	6.440	0.016
S_{27}	1.269	3	0.490	4.620	0.035
S_{32}	1.348	3	0.449	5.110	0.029
hsp90a					
T_{16}	0.191	2	0.094	1.506	0.401
T_{28}	0.177	2	0.088	1.207	0.363
S_{32}	1.353	3	0.451	7.578	0.010
hsp90b					
T_{16}	0.074	2	0.037	11.000	0.010
T_{20}	0.049	2	0.024	5.604	0.042
T_{24}	0.048	2	0.024	5.634	0.047
T_{28}	0.050	2	0.026	5.198	0.044

Table 3 continued

Source	Sum of squares	*df*	Mean squares	*F* ratios	*P* value
S_{22}	1.042	3	0.347	18.268	0.001
S_{27}	1.312	3	0.404	4.463	0.037
S_{32}	0.972	3	0.324	56.382	0.000
sod					
T_{24}	0.010	2	0.005	3.570	0.095
T_{28}	0.085	2	0.043	0.720	0.524
S_{32}	0.143	3	0.048	29.793	0.000

Table 4 Kruskal–Wallis H test table of gene expressions of *hsp*70, *hsp*90a, *hsp*90b and *sod* mRNA in the body wall and intestine of sea cucumbers *Apostichopus japonicas* at 6 h after the temperature and salinity stresses ($n = 3$)

Source	Chi square	*df*	*P* value
Body wall			
*hsp*90a			
T_{20}	0.267	2	0.875
S_{27}	6.282	3	0.099
S_{22}	9.667	3	0.022
*hsp*90b			
T_{28}	5.689	2	0.061
S_{22}	8.897	3	0.031
sod			
S_{22}	1.769	3	0.622
Intestine			
*hsp*70			
T_{16}	5.600	2	0.061
T_{20}	2.039	2	0.361
*hsp*90a			
T_{20}	1.689	2	0.430
T_{24}	0.800	2	0.670
S_{22}	9.974	3	0.019
S_{27}	9.974	3	0.019
sod			
T_{16}	3.289	2	0.193
T_{20}	0.267	2	0.875
S_{22}	1.667	3	0.644
S_{27}	5.051	3	0.168

The activity of Na^+/K^+ ATPase of *A. japonicus* increased initially and reached a maximum value within 6 h after osmotic challenge (Dong et al. 2008b). As previous studies described, the activity of Na^+/K^+-ATPase in the estuarine crab, *Chasmagnathus granulate* Dana was the highest at 30 °C and a significant enzyme inhibition was observed at 40 °C (Castilho et al. 2001). The activity of Na^+/K^+-ATPase in the turbot *Scophthalmus maximus* was positively correlated with temperature from 10 to 22 °C, but when the temperature was increased further, the enzyme activity was inhibited or even lost (Imsland et al. 2003). Therefore, the stability of Na^+/K^+ ATPase is impaired at high temperatures.

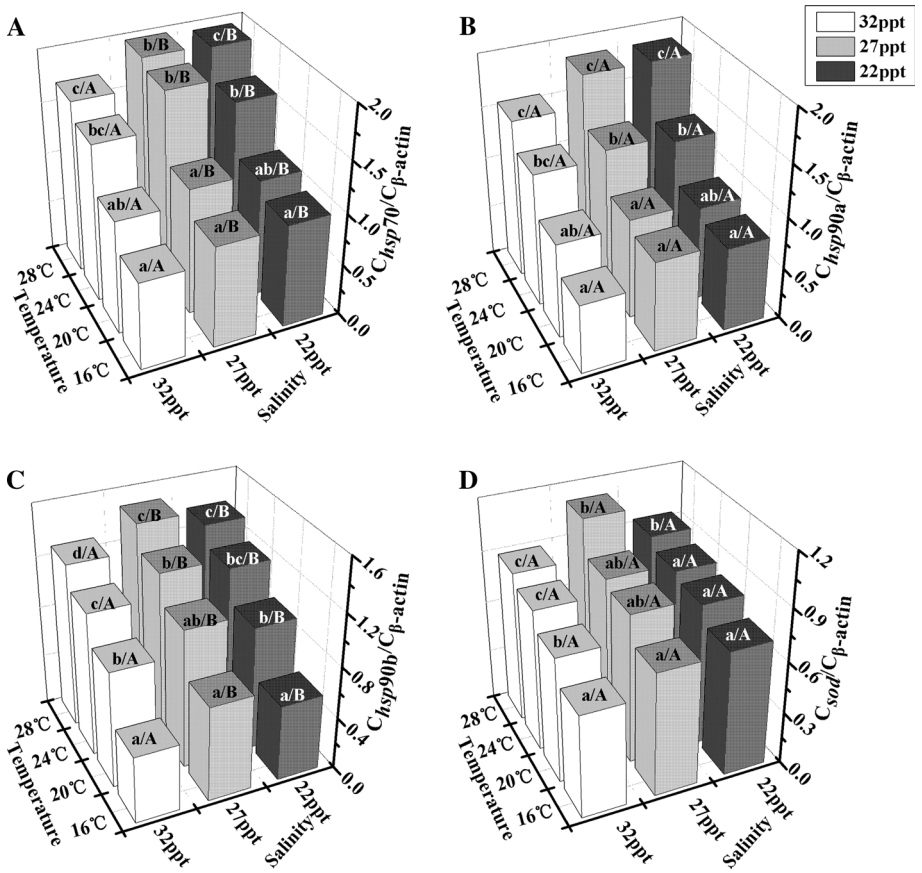

Fig. 5 Levels of **A** *hsp*70, **B** *hsp*90a, **C** *hsp*90b and **D** *sod* mRNA in the intestine of sea cucumbers *Apostichopus japonicus* at 6 h after the temperature and salinity stresses. Differences between these groups were assessed using one-way ANOVA followed by post hoc Duncan multiple range test or Kruskal–Wallis H test followed by post hoc S–N–K test. Data in the same column having different *lower case letters* are significantly different ($P < 0.05$) among different temperature levels, and data in the same row having different *capital letters* are significantly different ($P < 0.05$) among different salinity levels ($n = 3$)

The poor osmoregulatory capability at high temperature is also related to the change of biochemical pathways at high temperature. The enhancement of HSP during thermal stress blocks the synthesis of non-heat shock protein in some organisms because of the preferential translation of *hsps* mRNA (Storti et al. 1980; Morimoto et al. 1990). Therefore, the transcription and translation of proteins/enzymes involved in osmotic regulation are blocked at high temperatures, and then the osmoregulatory capability is decreased. Further, studies should be carried out to clarify the effect of thermal stress on osmotic regulation of the sea cucumber *A. japonicus*, especially the interaction between HSP and Na^+/K^+ ATPase.

In conclusion, the osmoregulation and *hsp*s mRNA expression of the sea cucumber were affected by temperature. The decrease in osmolality of sea cucumber's coelomic fluid in a hypo-osmotic environment is aggravated at high temperatures. Therefore, effective ways should be taken (e.g., draining off the upper freshwater, exchanging water and adding man-

made sea water) to prevent dramatic changes of salinity in sea cucumber culture ponds induced by rainstorms in summer.

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