

Effect of different thermal regimes on growth and physiological performance of the sea cucumber *Apostichopus japonicus* Selenka

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Abstract

This study deals with the effects of different thermal regimes on growth and physiological performance of the sea cucumber *Apostichopus japonicus* Selenka under controlled laboratory conditions. Eighty juvenile sea cucumbers, with a mean wet weight of 2.8 ± 0.8 g, were allocated to 20 experimental groups and allocated to constant temperature (18°C), and small ($18 \pm 2^\circ\text{C}$), medium ($18 \pm 4^\circ\text{C}$) and large ($18 \pm 6^\circ\text{C}$) daily temperature fluctuation regimes. Five groups were allocated to each regime. After the 35-day experimental period, the effects of different temperature regimes on growth, activities of glycolytic enzymes, enzymatic antioxidant defenses and expression of heat shock protein 70 (Hsp70) in juvenile sea cucumbers were measured. The small and medium temperature fluctuations could accelerate growth of sea cucumbers and the large temperature fluctuation retarded growth. Hexokinase activity at small and medium temperature treatments was significantly higher than that at the constant temperature. SOD activities at medium and large temperature fluctuations were significantly higher than that at the small temperature fluctuation treatment. These results indicated a possible higher utilization of carbohydrate through glycolytic cycle at the small and medium temperature fluctuations, and an enhancement of oxidative stress under the medium and the large temperature fluctuations. Hsp70 level at the large temperature fluctuation was significantly higher than at the other three treatments. The enhancement of Hsp70 at the large temperature fluctuation indicated a possible high level of protein damage which might result in an increased energetic cost that could retard growth of sea cucumbers. © 2008 Elsevier B.V. All rights reserved.

Keywords: Catalase; Hexokinase; Hsp70; Pyruvate kinase; Sea cucumber; Superoxide dimutase

1. Introduction

Water temperature is one of the most important factors affecting growth and other physiological performances in aquatic ectotherms. Many studies have demonstrated that temperature fluctuations have a significant effect on the growth of zooplankton (Van As et al., 1980), fish (Cox and Coutant, 1981; Diana, 1984; Konstantinov and Zdanovich, 1986; Konstantinov et al., 2003; Sierra et al., 1999; Zdanovich, 1999), shrimp (Miao and Tu, 1993, 1996) and sea star (Sanford, 2002). Further studies on fish and shrimp reveal that the effects of the amplitude of thermal fluctuations on aquatic animals are species-specific (Konstantinov et al., 1989; Tian et al., 2006). Though there have been many studies about the effects of temperature fluctuations on growth of

aquatic animals, few studies have been conducted to elucidate the inner physiological mechanism (Sastry and Ellington, 1978; Sastry, 1979).

A highly variable thermal environment, for example in intertidal zones, can induce thermal stresses to animals inhabiting these areas (Hofmann and Somero, 1995; Stillman and Somero, 1996; Roberts et al., 1997; Chapple et al., 1998; Brian et al., 2001). This thermal stress significantly affects the enzyme activity (Hand and Hardewig, 1996; Cordiner and Egginton, 1997; Hickey and Wells, 2003; Hardewig et al., 2004), antioxidant defenses (Buchner et al., 1996; Abele et al., 1998, 2002; Angel et al., 1999; Pörtner, 2002) and the expression of heat shock proteins (Feder and Hofmann, 1999; Helmuth and Hofmann, 2001; Halpin et al., 2004). The expression of heat shock protein affect energy metabolism (Dahlhoff et al., 2001), and may also affects growth, reproduction (Krebs and Feder, 1997a,b), and decrease an organism's fitness (Feder et al., 1992; Krebs and Loeschcke, 1994). A high intertidal species *Tegula funebris*

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grows slower than its low intertidal congeneric species *T. brunnea* due to the higher expression of heat shock protein (Tomanek and Sanford, 2003).

Apostichopus japonicus Selenka is an echinoderm, believed to have aphrodisiac and curative properties (Fu et al., 2005). In recent years, it has become an important aquaculture species in China. The area of sea cucumber farming has reached 10,000 ha in China, and the output of the dried *A. japonicus* (beche-de-mer) reached 5800 tons in 2000 (Chen, 2004). After incubation, most juvenile *A. japonicus* are reared indoors in China. Therefore it is important to design a favorable water temperature regime for growth of juvenile *A. japonicus*. In order to develop aquaculture techniques, the effect of temperature on growth of *A. japonicus* has been extensively studied. However, most studies have focused on the effect of constant temperatures (Liu et al., 1996; Li et al., 1996; Yu and Song, 1999; Dong et al., 2005; Yang et al., 2005) while research about the effects of temperature fluctuations on the sea cucumber is limited (Dong et al., 2006). The growth rate of juvenile *A. japonicus* increases with fluctuating temperatures when the mean temperature is below 18 °C (the constant temperature optimum for growth of the juvenile sea cucumber) and decreases at fluctuating temperatures when the mean temperature is above 18 °C (Dong et al., 2006).

The aim of the present study was to elucidate the effects of water temperature fluctuations on growth and physiological performance of the juvenile sea cucumber by measuring glycolytic enzyme activity, antioxidant defense and expression of heat shock protein 70.

2. Materials and methods

2.1. Collection and maintenance of animals

Juvenile sea cucumbers were collected at 15 °C from Penglai Aquaculture Farm, Yantai, P. R. China and acclimated at 18 °C for two weeks.

Seawater was filtered using a sand filter and the salinity was 28–30 ppt. One-half or two-thirds of the rearing water was exchanged by fresh equi-temperature seawater daily to ensure high water quality. Juveniles were fed *ad libitum* daily at

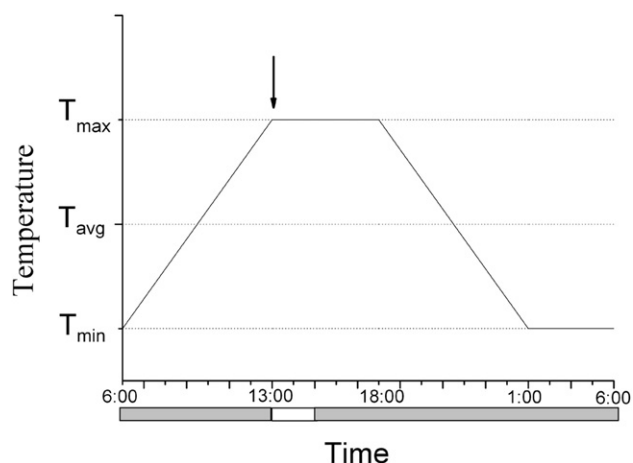


Fig. 1. Diagram of the diel temperature fluctuating mode. Photoperiod regime is depicted by horizontal white (light period) and black (dark period) bars. The arrow represents the feeding time.

Table 1

Effect of different times (Day 0, Day 10, Day 20, Day 30, and Day 35) and temperature treatments (CT, SF, MF, and LF) on group mean body weight in *Apostichopus japonicus* ($n=5$)¹

Source	Type III sum of squares	df	Mean square	F	p
Time	61.06	4	15.26	57.19	0.00
Treatment	7.72	3	2.57	1.35	0.29
Time × Treatment	17.91	12	1.49	5.59	0.00

¹Data were analyzed by repeated measure analysis of variance.

1300 h on a laboratory-made formulated feed ($22.9 \pm 0.2\%$ crude protein, $2.1 \pm 0.0\%$ fat, $34.7 \pm 0.6\%$ ash and $9.0 \pm 0.0\%$ moisture, 10.6 ± 0.0 kJ g⁻¹ energy), which mainly contained powdered *Sargassum* spp., fish meal, sea mud, wheat, vitamin and mineral premixes. Aeration was provided continuous during the whole experimental period. In the wild, *A. japonicus* lives under overhanging substrate with low light levels. Therefore, the photoperiod was designed as 2 h light: 22 h dark in this experiment.

2.2. Experiment design and procedure

After 24-h starvation, the initial individual wet weight measurements were taken by removing the animal from seawater for 1 min and removing excess external water by drying on sterile gauze. This method is a slight modification of that used by Battaglene et al. (1999). Juveniles, with a mean wet weight of 2.8 ± 0.8 g (S.D.), ranging 2–3 cm body length, were kept in glass aquaria (450 × 250 × 350 mm). A total of 20 experimental groups, each comprising four juvenile sea cucumbers, were acclimated to constant temperature treatment (CT, 18 °C), and small (SF, 18 ± 2 °C), medium (MF, 18 ± 4 °C) and large (LF, 18 ± 6 °C) temperature fluctuation treatments. Five groups were allocated to each treatment.

All individuals at each treatment were weighed every 10 days. At the end of the experiment, all specimens were sampled, weighed and immediately frozen in liquid nitrogen until further use.

Relative weight gain (RWG) was calculated by the following equation:

$$\text{RWG}(\%) = (\text{Wt} - \text{Wi}) \times 100 / \text{Wi}$$

where Wt and Wi are final and initial group mean body weight (g), respectively.

The fluctuating temperature treatments were programmed to follow a curve with different thermal amplitudes about 18 °C (Fig. 1). Temperatures of experimental aquaria were controlled by a laboratory-designed temperature control system described in Dong et al. (2006). This system was composed of programmed temperature-controller, heater, refrigerator, recirculation pump and cold water reservoir.

2.3. Sample preparation

One individual from each tank was randomly selected and 100–200 mg of body wall tissue was homogenized on ice by glass homogenizer for 2 min in 5 vol (w/v) of ice-cold 0.05 M PBS buffer (pH 7.5). The homogenate was centrifuged at 3000 ×g for 10 min at 4 °C. The supernatant fraction was collected and kept at –70 °C to measure enzymes' activities. Another individual from each tank was randomly selected and 50 mg of body wall tissue was homogenized by glass homogenizer for 2 min in 5 vol (w/v) homogenization buffer in cell lysis kit (BBI, Canada) containing protease inhibitor cocktail for heat shock protein assay. The homogenate was centrifuged at 10,000 ×g for 10 min. The supernatant fraction was collected and kept at –70 °C until used to measure the Hsp70 level.

2.4. Enzymes assay

Enzyme activity was analyzed according to the manual of analyses kits (Nanjing Jiancheng Bioengineering Institute, China). Activities of hexokinase (HK; EC 2.7.1.1) and pyruvate kinase (PK; EC 2.7.1.40) were analyzed according to Tanaka et al. (1962) and Valentine et al. (1967), respectively. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by a modification of the xanthine/xanthine oxidase method (McCord and Fridovich, 1969) and catalase (EC 1.11.1.6) activity was measured according to Goth (1991).

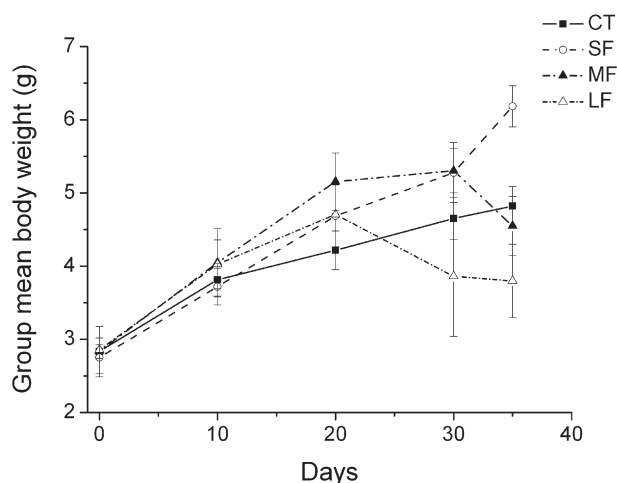


Fig. 2. Mean (± 1 S.E.) ($n=5$) of body weight for sea cucumbers (*A. japonicus*) at Day 0, Day 10, Day 20, Day 30 and Day 35 under 18 °C constant temperature (CT) (■), and 18 ± 2 °C (LF) (○), 18 ± 4 °C (MF) (▲), 18 ± 6 °C (LF) (△) fluctuating treatments.

2.5. Western blotting

Hsp70 was measured as previously described by Dong et al. (2007). Protein concentration was determined as described by Bradford (1976) with bovine serum albumin as standard. Gel electrophoresis of protein extracts was performed in 10% polyacrylamide gels (PAGE) according to Laemmli (1970). Equal quantity protein samples (20 μ g) in each well were subjected to gel electrophoresis in the presence of 2-mercaptoethanol. Semi-dry electro-transfer was performed according to Kyhse-Andersen (1984) onto PVDF-Immobilon membranes. Membranes were blocked and incubated with anti-Hsp70 Ab (H5147, Sigma, USA) (diluted 1:1000) for 2 h at 37 °C. Then the immune complexes were visualized by incubation with anti-mouse IgG (Horseradish peroxidase conjugated, HRP) followed by staining with diaminobezidin (DAB). Band intensity was quantified using GeneTools software (Syngene, USA).

An equal quantity of protein (20 μ g) of a sea cucumber which was subjected to an acute temperature flux (from 10 °C to 30 °C) for 1 h was also added to each gel. The Hsp70 level of this sample was designated as a standard for normalization. Hsp70 levels in this study were expressed as values relative to the level of the standard sample (Relative unit, RU) (%).

2.6. Statistics

The data were analyzed using SPSS for Windows (Version 11.0) statistical package. The assumption of homoscedasticity was tested with a Levene's test. A repeated measures analysis of variance was carried out on the group means of wet body weight ($n=5$), which were measured at different times, among different treatments (CT, SF, MF and LF). Mauchly's test of sphericity was used to verify the null hypothesis that the error covariance matrix of the orthonormalized transformed

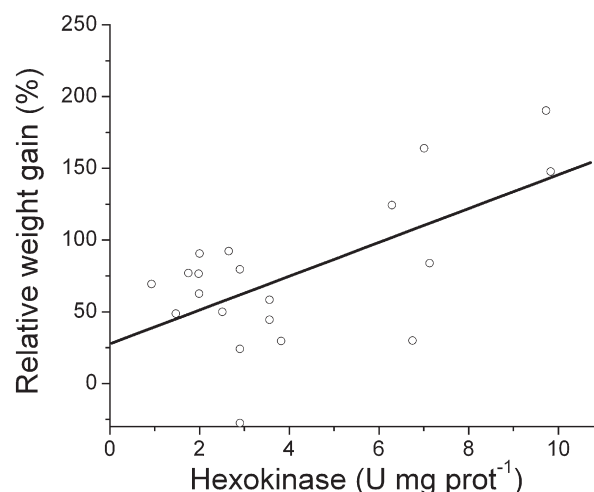


Fig. 3. Correlation of relative weight gain (RWG) with hexokinase. RWG was positively correlated with HK ($r=0.620$, $P=0.000$).

dependent variables was proportional to an identity matrix (approximately Chi-square=14.235; $df=9$; $p=0.116$). Univariate tests were used for this test.

Differences in survival rate among different treatments were analyzed with Chi-square test. Inter-treatment differences for hexokinase, pyruvate kinase, superoxide dimutase, catalase activities and Hsp70 level were analyzed with one-way ANOVA followed by post-hoc Duncan Multiple Range Tests. Pearson correlation analysis was used to detect possible associations of RGW with activities of HK, PK, SOD and Hsp70. Differences were considered significant if $p<0.05$.

3. Results

3.1. Growth and survival

The temporal change patterns of group means of wet body weight among different treatments were significantly different, and there was a significant interaction between different measurement times and temperature treatments (Table 1). The small and medium temperature fluctuations could enhance growth of sea cucumber. The body weight in CT sea cucumbers showed a gradual increase over the whole experimental period (Fig. 2). The mean body weight in SF sea cucumbers also kept growing during the 35-day experimental period. The mean body weight in MF sea cucumber increased to Day 30 and then decreased, and the mean body weight decreased 14.2% at Day 35 compared to that at Day 30. The mean body weight in LF sea cucumbers increased to Day 20, and then decreased thereafter.

At the end of 35-day experimental period, RWG was 70% \pm 16%, 132% \pm 22%, 61% \pm 16%, and 39% \pm 48% at 18 °C, 18 ± 2 °C, 18 ± 4 °C

Table 2

Activities of hexokinase, pyruvate kinase, SOD and catalase and Hsp70 levels in *Apostichopus japonicus* reared at different treatments for 35 days¹

	Treatment			
	CT (18 °C)	SF (18 ± 2 °C)	MF (18 ± 4 °C)	LF (18 ± 6 °C)
Hexokinase (U mg prot ⁻¹)	1.78 (0.28) ^a	7.14 (1.42) ^b	4.53 (0.84) ^c	2.90 (0.29) ^{ac}
Pyruvate kinase (U mg prot ⁻¹)	47.93 (4.07) ^{ab}	58.54 (6.24) ^b	43.49 (5.94) ^a	37.03 (2.26) ^a
SOD (U mg prot ⁻¹)	49.71 (5.96) ^{ab}	41.27 (8.82) ^a	62.89 (3.73) ^b	66.17 (4.57) ^b
Catalase (U mg prot ⁻¹)	42.59 (5.09) ^a	52.57 (4.29) ^a	48.74 (8.22) ^a	52.55 (4.92) ^a
Hsp70 (RU) ²	21.11 (10.66) ^a	7.69 (5.19) ^a	35.99 (17.37) ^a	97.01 (20.72) ^b

¹Values are means (S.E) ($n=5$). Means with different superscripts in same row are significantly different ($p<0.05$).

²An equal of quantity protein (20 μ g) of a sea cucumber that was subjected to an acute temperature flux (from 10 °C to 30 °C) for 1 h, was also added to each gel and the Hsp70 level was designed as a standard sample. Hsp70 levels in this study were shown as values relative to the level of the standard sample (Relative unit, RU) (%).

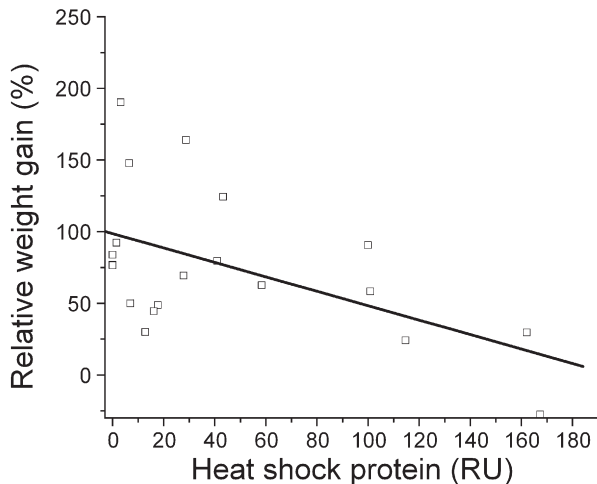


Fig. 4. Correlation of relative weight gain (RWG) with heat shock protein 70. RWG weight was negatively correlated with Hsp70 ($r=-0.534$, $p=0.021$).

and 18 ± 6 °C, respectively. The survival rate was $90\%\pm 14\%$, $100\%\pm 0\%$, $90\%\pm 14\%$ and $90\%\pm 22\%$ at 18 °C, 18 ± 2 °C, 18 ± 4 °C and 18 ± 6 °C, respectively. There were no significant differences among the survival rates (Chi-square test, $p=0.258$).

3.2. Activities of glycolytic enzymes

Among the four temperature treatments, the maximum activities of both HK and PK occurred in the SF sea cucumbers (Table 2). Compared to that at 18 °C, HK activity at SF and MF increased 401% and 255% , respectively ($F_{(3,16)}=7.524$, $p=0.002$); PK activity increased 122% at 18 ± 2 °C ($F_{(3,16)}=3.421$, $p=0.043$). HK activity was positively correlated with RGW ($r=0.620$, $p=0.000$; Fig. 3).

3.3. Superoxide dimutase and catalase activities

The effects of thermal amplitudes on superoxide dimutase activity were significant ($F_{(3,16)}=3.632$, $p=0.036$). Superoxide dimutase activity in the MF and LF sea cucumbers was significantly higher than that in SF sea cucumbers. There was no significant difference in catalase activity among the four treatments ($F_{(3,16)}=0.651$, $p=0.594$) (Table 2).

3.4. Expression of Hsp70

Tissue levels of Hsp70 were quantified as a direct index of the effects of thermal stress. The Hsp70 antibody we used for western analysis detected one band.

After the 35-day experiment, the effects of temperature fluctuations on Hsp70 expression compared to the control were significant ($F_{(3,16)}=7.121$, $p=0.003$). Hsp70 expression increased with the increase of thermal amplitudes. The minimum and maximum of Hsp70 levels occurred at 18 ± 2 °C and 18 ± 6 °C, respectively. The average Hsp70 level at 18 ± 6 °C was significantly higher than other treatments (Table 2).

Correlation analysis showed that the RGW was negatively correlated with the Hsp70 level ($r=-0.534$, $p=0.021$; Fig. 4).

4. Discussion

Temperature fluctuations can affect the growth of sea cucumbers (Dong et al., 2006). Results in the present study supported the previous conclusion and showed that the effects of thermal shifts on growth of sea cucumbers were significant.

The repeated measurements test showed that the effect of temperature fluctuation treatments on body weight was inconsistent among time. At most periods, small and medium temperature fluctuations could enhance growth. This result indicated that sea cucumbers could adapt to small and medium temperature fluctuations. However, too large a fluctuation (18 ± 6 °C) was too harsh a condition for growth.

The mechanisms by which temperature fluctuations influence growth of animals are still ambiguous. Previous studies attributed the increase of growth under fluctuating temperatures to enhanced food intake (Diana, 1984), decrease of metabolism (Pilditch and Grant, 1999; Tian et al., 2004) and to a change in the pattern of energy allocation (Cox and Coutant, 1981; Konstantinov et al., 1990; Pilditch and Grant, 1999; Tian et al., 2006). Our present study indicates that changes in physiological performance were important factors affecting growth of sea cucumbers under fluctuating temperatures. Compared to constant temperature, activities of HK and PK (HK activity at 18 ± 2 °C and 18 ± 4 °C and PK activity at 18 ± 2 °C) increased significantly under small and medium fluctuating temperatures. As two rate-limiting enzymes in the glycolytic cycle, HK and PK play important roles in the utilization of carbohydrate (Walzem et al., 1991; Hemre et al., 2002). Therefore, the higher activities of HK and PK indicated that a possible higher utilization efficiency of carbohydrate through the glycolytic cycle occurred at small and medium temperature fluctuations.

Superoxide dimutase activity at the medium and large temperature fluctuations was significantly higher than that at the small temperature fluctuation treatment. This result indicated an enhancement of oxidative stress under the medium and the large temperature fluctuations (Buchner et al., 1996; Pörtner, 2002). At the medium and large temperature fluctuation, the enhancement of superoxide dimutase activity was congruent with previous findings of higher oxygen consumption at high temperatures (oxygen consumption increase with the increase of temperatures within the scope of 10 to 25 °C) (Dong et al., 2005). Higher temperature fluctuation was therefore prone to enhance reactive oxygen species release, thereby increasing the risk of oxidative damage (Abele et al., 1998). Increased availability of superoxide dimutase alleviated oxidative stress and minimized hypoxia induced post-ischemic injury (Pörtner, 2002). In the present study, there were no significant differences in the catalase activity among the four temperature treatments. The difference of superoxide dimutase and catalase activities responding to temperature fluctuations indicated the differences concerning the inducibility of different antioxidant enzymes (Buchner et al., 1996; Abele et al., 1998). In Antarctic intertidal limpet *Nacella concinna*, the responses of superoxide dimutase and catalase to oxidative stress were different (Abele et al., 1998).

Heat shock proteins protect other proteins from unfolding, assist refolding denatured protein, or target them for degradation. These functions of heat shock proteins are viewed as critical for thermotolerance (Morimoto et al., 1994; Ellis, 1996; Feige et al., 1996; Frydman and Höhfeld, 1997). The expression of heat shock proteins is often regarded as a bioindicator of thermal stresses (Feder and Hofmann, 1999). In the present study, Hsp70 levels at 18 ± 2 °C and 18 ± 4 °C were similar to that at 18 °C.

This result indicated that the small and the medium temperature fluctuation did not increase the expression of inducible Hsp70 and sea cucumbers could adapt well to these conditions. However, Hsp70 levels increased significantly at the large temperature fluctuation (18 ± 6 °C). This high Hsp70 level indicated a high level of the protein damage at high temperature fluctuation (Lindquist, 1986; Feder and Hofmann, 1999). Correlation analysis showed that the elevated expression of Hsp70 might be one of the factors which retarded growth of sea cucumbers under large temperature fluctuations because increase of Hsp70 could result in an energetic cost (Feder and Hofmann, 1999). Furthermore, excess synthesis of heat shock proteins could be harmful if they are produced at the expense of other proteins (Tomanek and Somero, 2000).

In conclusion, large temperature fluctuations retarded growth of *A. japonicus* and induced heat shock response. However, small and medium temperature fluctuations accelerated growth of these sea cucumbers and should be useful in enhancing aquaculture performance of juveniles for farming and sea ranching.

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