Asian Fisheries Science **31** (2018):265–275

ISSN 0116-6514
E-ISSN 2073-3720
https://doi.org/10.33997/j.afs.2018.31.04.002

Asian Fisheries Science
The Journal of the Asian Fisheries Society

Impact of Short-term Hyposalinity Stress on Akoya Pearl Oyster, *Pinctada fucata* (Gould 1850)

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Abstract

The increase precipitation linked to climate change is well documented. In coastal waters, there is a frequent lowering of salinity during periods of rainfall and runoff from rivers and estuaries. This study assessed the effects of short-term hyposalinity stress on Akoya pearl oyster, *Pinctada fucata* (Gould 1850), focusing on the immunological parameters consisting of lysozyme and phenoloxidase (PO) activities. Akoya pearl oysters were exposed to short periods of 3 h daily for 30 days of hyposaline stress at 14 and 24 psu, followed by a recovery period that mimics the conditions typical for culture site at bays or estuaries experiencing heavy freshwater input, with a quick return to initial salinity of 34 psu. Lysozyme activity of oysters exposed to temporary salinity of 14 psu was 0.085 ± 0.028 mg mL^{-1}. This value was lower ($P < 0.5$) than lysozyme activity of oysters kept in temporary salinity of 24 psu (0.153 ± 0.030 mg mL^{-1}) and control (0.160 ± 0.031 mg mL^{-1}). Phenoloxidase activity of oysters exposed to temporary salinity of 14 and 24 psu was 0.013 ± 0.002 and 0.013 ± 0.001, respectively which was significantly lower than the control (0.016 ± 0.001). The results indicate that under short-term hyposalinity stress, Akoya pearl oysters might be susceptible to the threat of disease due to low lysozyme and PO activities.

Keywords: *Pinctada fucata*, salinity, hyposalinity stress, climate change, lysozyme activity, phenoloxidase activity

Introduction

Pearl farming is conducted to satisfy human attraction with pearls. The ability of pearl oyster to secrete pearl that was discovered since the late 1900s (Ikenoue 1992) had led to the production of cultured pearls with a current value of around US$500 million per year and provides a positive socio-economic impact especially for local communities (Southgate and Lucas 2008). However,

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pearl oyster culture habitat, as any other species in the coastal marine system, is being threatened by anthropogenic global climate change (IPCC 2014). Several drivers of climate change including warming of the water bodies, sea level rise, ocean acidification, change in weather pattern, and extreme weather events are directed to the coastal system (Barange and Perry 2009).

One of the threats of climate change on pearl farming is the increase in extremely heavy rain. It has been well documented that the increased precipitation is linked with climate change (Groisman et al. 2005; Min et al. 2011) and there is a potential lowering of salinity during periods of rainfall and runoff from rivers and estuaries in coastal waters (Raper and Braithwaite 2006; Southgate and Lucas 2008). Levinton et al. (2011) reported that short duration of low salinities disrupted the important thresholds for survival and metapopulational dynamics on the eastern oyster, *Crassostrea virginica* (Gmelin 1791). Tomanek et al. (2012) also reported that temporary hyposalinity caused the shift of the distribution range in blue mussel congeners, *Mytilus trossulus* Gould 1850, and *M. galloprovincialis* Lamarck 1819. A recent investigation of Manila clam, *Ruditapes philippinarum* (Adams and Reeve 1850) reported that the lysozyme activity decreased due to a short period of hyposalinity (Arisman et al. 2017).

Some species of pearl oyster can tolerate a considerable range of salinities. Adult Akoya pearl oyster, *Pinctada fucata* (Gould 1850), could survive in a salinity range of 18 to 35 psu (Katada 1959; Funakoshi et al. 1985). However, the immunological function of oysters significantly declined when Akoya, *Pinctada imbricata* (Gould 1850) and Sydney rock oyster, *Saccostrea glomerata* (Gould 1850) were exposed to low salinity (Butt et al. 2006; Kuchel et al. 2010). There is limited information on how short-term change of hyposalinity stress affects the oyster immune systems, especially in a framework of environmental alteration due to climate change.

A study was conducted to investigate the physiological responses to hyposalinity stress in Akoya pearl oyster to comprehend the potential impact of the increase of extreme precipitation on the oyster immune system. As suggested by Somero (2012) the intertidal species such as pearl oyster is an excellent model to study for evaluating climate change. In the present study, Akoya pearl oysters were exposed to temporary salinity changes to evaluate the impact on survival, lysozyme activity, and phenoloxidase (PO) activity. Lysozyme and PO have been known as the substantial immunological responses, that can be assessed in a short time and provide valuable information about the status of the oyster (Kuchel et al. 2010).

Materials and Methods

The study was conducted from May–June 2017 in the experimental water tank facility, at the Graduate School of Bioresources, Mie University, Japan. The adult stage of Akoya pearl oyster was obtained from Kashikojima Station of Wakasa Otsuki Pearl Cultivators Co., Ltd. in Shima City of Mie Prefecture, Japan. The adult oysters were measured according to Southgate and Lucas (2008),

with the average hinge length (HL) of 44.34 ± 4.33 mm, antero-posterior measurement (APM) of 49.13 ± 4.61 mm, and dorso-ventral measurement (DWM) of 53.02 ± 4.20 mm.

The oysters were reared in the closed recirculation system consisting of a 1.5 × 1 × 0.5 m indoor rearing glass-fibre tank. Artificial seawater was used by diluting sea salt (LIVESea® Salt, Delphis, Japan) with tap water with 24 h aeration before use. Ten oysters were placed into each of the nine 18 L (20 cm × 30 cm × 30 cm) transparent acrylic rearing vessels (RV) that were submerged into the glass-fibre tank to maintain the temperature of the system at 20 °C. Filtering system was partitioned inside the rearing tank, which consisted of polypropylene membrane as a mechanical filter and sintered glass substrate (Substrat pro, EHEIM GmbH, Germany) as a biological filter medium. The closed recirculation system was operated under laboratory lighting condition at 12L:12D. Water loss due to evaporation was replaced with fresh water, and the salinity was maintained at 34 psu by adding concentrated seawater solution to the original volume. Seawater in each RV was aerated to maintain constant oxygen supply and to keep food suspended and homogenous. Live feed, *Chaetoceros calcitrans* (Yanmar Co., Ltd., Japan) was given at 1 mL individual^{-1} day^{-1} (cell density 100 × 10^6 cell mL^{-1}). Seawater was changed as needed, by measuring phosphorus (PO_4^{3-}) and nitrogen (NH_3^-, NH_2^-, NH_3) content in seawater using handheld colourimeter for water quality measurement of aquaculture (DR890, Hach, USA). The closed recirculation system was acclimatised for 1 week at the salinity level of 34 psu before the start of the experiment.

The experimental design was established to mimic temporary hyposaline stress conditions that occurred in estuaries or bays during the heavy rain, followed by a quick return to normal condition with the incoming tides (Tomanek et al. 2012). Two experimental treatments were employed, at 14 psu and at 24 psu, and one control treatment at 34 psu, with three replicates. The oysters were exposed for 3 h salinity at 14 and 24 psu, by stopping inflow water into the RVs, and then an appropriate amount of seawater in the RVs was replaced with fresh water added gradually, approximately over a period of 5 min. After 3 h hyposaline exposure, concentrated seawater solution was added along with the resuming inflow of water into the RVs to maintain the initial salinity of 34 psu. Seawater salinity in each RV was checked using handheld refractometer (NEW S-100α, Tanaka Sanjiro Co., Ltd., Japan). The change of salinity was carried out daily, after 24 h recovery period, for 30 days.

To determine the growth rate, the sizes of the oysters were measured at the end of the experiment using the digital calliper according to Southgate and Lucas (2008). As for the survival, the occurrence of oyster mortality was recorded daily. The oysters were considered dead when their shells gaped and did not shut again after the external stimulus (Munari 2011).

Survival was calculated based on the following formula:

$$\text{Survival (\%)} = \frac{\text{number of oyster at the end of experiment}}{\text{number of initial oyster}} \times 100$$

Approximately 0.1 mL oyster hemolymph was withdrawn from the adductor muscle using a 1 mL sterile syringe (Terumo®, Terumo Co., Japan). After it was transferred into 5 mL sterile centrifuge tube, an equal amount of 0.1 M, pH 7.0 phosphate buffer was mixed using a vortex machine. The mixtures were then centrifuged at 5000 rpm for 30 min. The supernatant was collected into a sterile tube and stored at 10 °C for further analysis.

Lysozyme activity was measured according to Shugar's method of turbidity (Shugar, 1952). As much as 1 mg mL^{-1} cells of bacteria suspension, *Micrococcus lysodeikticus* (MP Biomedicals, LLC., France) was prepared using 0.1 M phosphate buffer pH 7.0, shortly before the assay. Six wells of a sterile microplate (96-wells microplate, AS ONE Co., Japan) were filled with 50 µL of the supernatants from each treatment. After which, 150 µL of *M. lysodeikticus* cells suspension was added quickly into all the wells which contain the supernatants. The absorbance of the sample was read at 0 and 1 min using Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories, Inc., Japan) at 20 °C and 450 nm of wavelength. As a standard, lysozyme from the chicken white egg (Wako Pure Chemical Industry Ltd., Japan) with the range concentration of 0.4, 0.2, 0.1 and 0.05 mg mL^{-1} was set using 0.1 M, pH 7.0 of phosphate buffer. A 50 µL of each concentration of standard lysozyme were added into microplate wells and was done in triplicate. Then 150 µL of *M. lysodeikticus* cells suspension was added quickly. Measurement of the absorbance at 450 nm wavelength was initiated continuously every 1 min interval for 4 min at 20 °C. Lysozyme activity was defined as lysozyme concentration in mg mL^{-1}, calculated using the calibration curve of standard lysozyme vs decrease in absorbance of the bacterial suspension after 1 min.

The PO activity was measured by preparing 1 mg mL^{-1} of L-DOPA (Tokyo Chemical Industry, Co., Ltd., Japan) using 0.1 M, pH 7.0 phosphate buffer as a substrate. Six wells of a sterile microplate (96-wells microplate, AS ONE Co., Japan) were filled with 50 µL of the supernatants from each treatment. After that, 150 µL of L-DOPA were added quickly. The absorbance of the sample was read at 0 min and 30 min using Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories, Inc., Japan) at 20 °C and 490 nm of wavelength. PO activity was calculated by subtracting the initial reading (at 0 min) from the reading taken at 30 min.

Data were checked for normal distribution (Shapiro-Wilk's test) and homogeneity of variance (Bartlett's test). Gehan-Breslow-Wilcoxon test was used for analysing the survival rate. Results were compared using one-way ANOVA followed by a posthoc test (Tukey test). All results are expressed as the mean ± standard deviation (SD). SPSS® Statistics version 23 (IBM®, New York, US) software was used for statistical analysis. The asterisks were used to mark the differences at the significant level of alpha 0.05 ($P < 0.05$).

Results

There was no visible effect on the growth of Akoya pearl oysters exposed to the temporary hyposaline condition. Size of HL, APM, and DVM did not increase over the course of the study ($P > 0.05$, Table 1). The survival rate of oysters exposed to temporary salinity at 14 psu and 24 psu was 97 % and 100 % respectively (Fig. 1). The control group at 34 psu with a survival rate of 97 % were not significantly different ($P > 0.05$) from the treatment groups. Water quality during the experimental period (Table 2), for rearing Akoya pearl oyster was under the acceptable range (Svobodova et al. 1993).

Table 1. Size of adult Akoya pearl oysters *Pinctada fucata*, before and after exposure to low salinity stress of 14 and 24 psu compared with control at 34 psu for 3 h day^{-1} for 30 days. Values are expressed as mean ± SD.

Salinity		Initial size (mm)	Final size (mm)
34 psu	*n*	30	29
	HL	43.18 ± 4.09	43.16 ± 3.97
	APM	48.13 ± 4.52	48.85 ± 4.13
	DVM	52.50 ± 3.82	52.10 ± 3.26
24 psu	*n*	30	30
	HL	41.89 ± 3.84	43.46 ± 3.99
	APM	46.99 ± 4.40	47.62 ± 3.73
	DVM	51.17 ± 3.92	51.71 ± 3.42
14 psu	*n*	30	29
	HL	46.37 ± 34.32	45.70 ± 4.57
	APM	51.64 ± 4.76	53.48 ± 5.06
	DVM	54.86 ± 4.72	56.39 ± 4.80

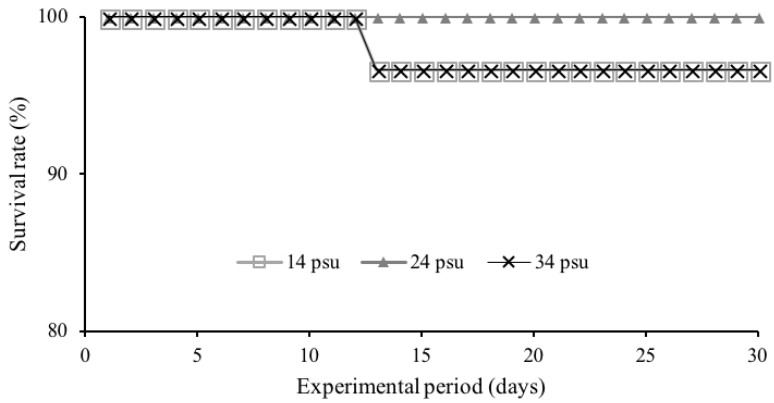


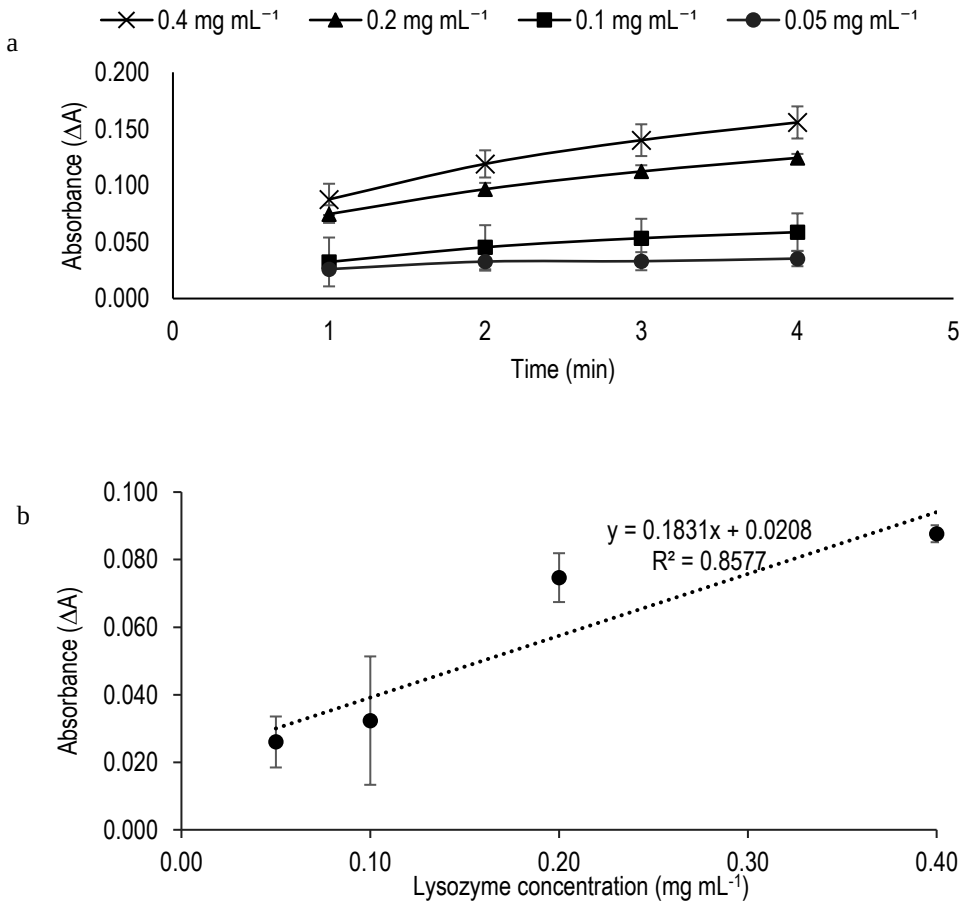
Fig. 1. Survival rate (%) of adult Akoya pearl oysters *Pinctada fucata*, exposed to low salinity stress of 14 and 24 psu compared with control (34 psu) for 3 h day^{-1} for 30 days. Each mark represents the mean of the samples (n=30).

Table 2. Water qualities of the recirculation system used for the 30 days experiment for exposure of short-term low salinity on adult pearl oyster *Pinctada fucata.*

Parameter	Concentration
Nitrate (NO_3^-)	2.20 ± 0.05 mg L^{-1}
Nitrite (NO_2^-)	0.13 ± 0.02 mg L^{-1}
Ammonia (NH_3)	0.04 ± 0.01 mg L^{-1}
Phosphate (PO_4^{3-})	0.46 ± 0.01 mg L^{-1}
pH	7.98 ± 0.03

Data were collected three times, in the beginning, middle and the end of the experiment. Values are mean ± SD.

Figure 2a shows 4 min reaction of bacteria suspensions with the standard lysozyme for four different enzyme concentrations. The calibration curve of lysozyme concentration vs the decrease of absorbance is shown in Fig. 2b. The linear regression equation and correlation coefficient of this standard curve were: y = 0.1831x + 0.0208 and R^2 = 0.8577, respectively. Under our experimental condition (pH 7.0, 20 °C), lysozyme activity of oyster exposed to the temporary salinity of 14 psu was 0.085 ± 0.028 mg mL^{-1} (Fig. 2c) which was significantly lower ($P < 0.05$) than oyster kept at 24 psu (0.153 ± 0.030 mg mL^{-1}) and the control treatment, at 34 psu (0.160 ± 0.031 mg mL^{-1}).



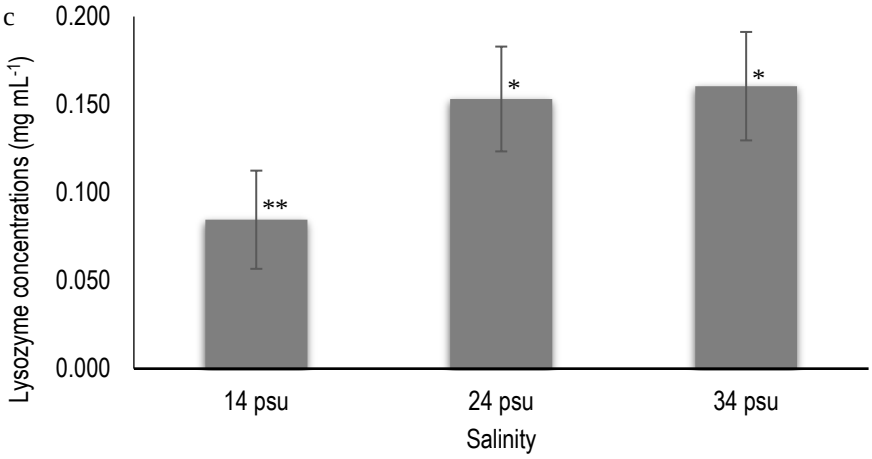


Fig. 2. Lysozyme activity of adult Akoya pearl oyster *Pinctada fucata*; (a) Decrease with the time of absorbance of bacteria suspensions of *Micrococcus lysodeikticus*, following the addition of chicken white egg lysozyme solutions at 20 °C and pH 7.0 in 0.1 M phosphate buffer, (b) Standard curve of lysozyme concentration vs decrease in absorbance of bacteria suspension (in 0.1 M phosphate buffer, pH 7.0 at 20 °C), produced during the first minute of reaction, (c) Lysozyme activity (mg mL^{-1}) when exposed to low salinity stress for 3 h day^{-1} for 30 days. Columns with a different number of stars are significantly different ($P < 0.05$). Bars denote mean ± SD.

The PO activity (Δ Abs) of adult pearl oyster exposed to the short-term salinity of 14 and 24 psu was 0.013 ± 0.002 and 0.013 ± 0.001, respectively (Fig. 3) which were significantly lower than the control oyster at 34 psu (PO activity = 0.016 ± 0.001). There was no significant difference between 14 and 24 psu treatments ($P > 0.05$).

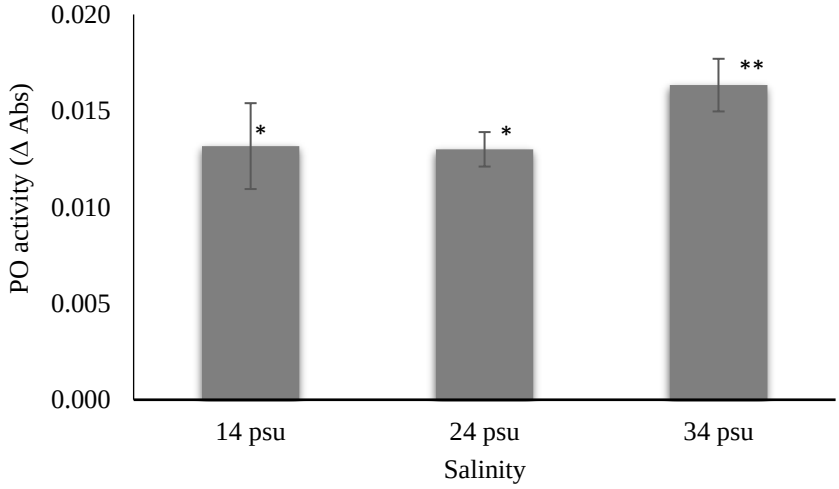


Fig. 3. Phenoloxidase activity (Δ Abs) of adult Akoya pearl oyster *Pinctada fucata* exposed to low salinity stress for 3 h day^{-1} for 30 days. Column with different number of stars are significant different ($P < 0.05$). Bars denote mean ± SD.

Discussion

The stress on oyster may increase the incidence of disease outbreaks which can result in mass mortalities (Le Bris et al. 2014). Cultured pearl oyster could experience various stressor of environmental alteration due to global climate change. Recent studies have provided information on the link between climate change and the increase of precipitation that potentially decrease salinity during periods of rainfall and runoff from rivers and estuaries in the coastal waters (Groisman et al. 2005; Raper and Braithwaite 2006; Southgate and Lucas 2008; Min et al. 2011). This experiment was conducted to mimic climate change caused by the increased incidences of severe rainfall by focusing on the short-term hyposalinity stress on adult Akoya pearl oyster, *P. fucata*. Investigation on growth, survival, and immunological responses of lysozyme and PO activity in controlled stress condition can provide important information about the health status of oyster, which is easily assessed in the short period (Kuchel et al. 2010). Levinton et al. (2011) conducted a similar study on eastern oysters, *C. virginica* at the culture site in the estuaries and they found that the increased precipitation reduced salinities and greatly increased oyster mortality, and there was a positive correlation between the shell growth with salinity. Another study in blue mussel congeners, *M. trossulus*, and *M. galloprovincialis* revealed that during 4 h treatments of hyposaline condition there was a change in the abundance of molecular chaperones of the endoplasmic reticulum indicating the protein unfolding, and a reduction in overall energy metabolism during recovery (Tomanek et al. 2012).

Salinity is one of the most important environmental factors associated with pearl oyster growth, survival, and disease prevalence (Southgate and Lucas 2008). It was reported that at >18 psu salinity is a favourable range for normal growth of Akoya pearl oyster (Ohwada and Uemoto 1985) and better survival at 18 to 30 psu (Funakoshi et al. 1985). However, in the present study, the Akoya did not grow during the 30 days treatment period which was probably related to metabolic demand under increased environmental stress without increasing the biomass as has also been reported by Paganini et al. (2010). A high survival rate of Akoya pearl oysters was related to the optimal environmental condition (Table 2). The external factors are known to affect the immune parameter of marine shellfish (Fisher et al. 1987; Lacoste et al. 2001; Kuchel et al. 2011; Park et al. 2012). The immunological parameters measured in the present study were lysozyme activity and PO activity. Lysozyme is the main bacteriolytic agent against several species of Gram-positive and Gram-negative bacteria. The enzyme is widespread in nature (Jollès 1969) and the invertebrate, it is synthesised in haemocytes and then secreted into haemolymph during phagocytosis (Cheng et al. 1975), hydrolysing the bacterial cell wall (Salton 1957).

The PO is a key component of the shellfish and other invertebrate immune systems. It catalyses a cascade of oxidation reactions that convert tyrosine-based substrates to phenols and quinones, leading to the formation of the pigment, melanin. Melanin is an important component of capsules that sequester invasive pathogens (Butt et al. 2006), intermediates of the cascade, recruit cellular defences such as phagocytosis (Peters and Raftos 2003) or act as fungistatic and antibacterial agents (Sorrentino et al. 2002).

In the present study, lysozyme activity and PO activity of adult Akoya pearl oyster significantly declined when exposed to the temporary low salinity. A similar result was also reported in eastern oyster, *C. virginica* which showed a significant decrease in lysozyme activity when the oysters were exposed to low salinity (Chu et al. 1993). Kuchel (2011) reported that PO activity of Akoya, *P. imbricata* significantly decreased when animals were exposed to low salinity. A comparable effect of decrease of lysozyme and PO concentration was also reported in Manila clam, *R. philippinarum*, when exposed to lower salinity at 20 psu (Reid et al. 2003). Cheng et al. (2004) reported that PO activity of abalone *Haliotis diversicolor supertexta* Lischke 1870, decreased significantly when the abalone were transferred from 35 psu to 20, 25 and 35 psu. Moreover, Sydney rock oysters, *S. glomerata* exposed to water collected after heavy rain had lower PO activities than those held in water collected before rainfall (Butt et al. 2006). The combined stressor of salinity and temperature conducted on Manila clam, *R. philippinarum*, showed that there was no effect on lysozyme activity (Munari et al. 2011). The authors concluded that lysozyme response was optimal at the temperature of 25 °C.

Conclusion

Akoya pearl oyster *P. fucata* when exposed to hyposalinity that mimicked a similar situation causing stress which occurs in estuaries or bays during heavy rainfall, was shown to decrease the lysozyme and PO activity of the oyster. The lower level of lysozyme and PO activity indicate that it could affect the health status of the oyster by increasing their susceptibility to disease. The results obtained in this study can serve as important baseline information that can be used as a reference since there are limited studies on how temporary hyposalinity induced by climate change can affect marine shellfish. Further work is required to assess the effect of temporary hyposaline stress on the early life stages of other marine shellfish and in prolonged exposure to different levels of hyposaline conditions.

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Received: 01/05/2018; Accepted: 06/12/2018; (AFSJ-2018-0028)