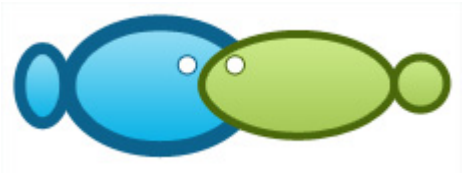

Temporal change of salinity stress in Manila clam *Ruditapes philippinarum*: implication for biodefense mechanism in response to climate change

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Abstract. Scientific consensus confirmed that coastal marine system is being threatened by anthropogenic global climate change. Aside from temperature rising and ocean acidification, increasing evens of low salinity stress due to extreme heavy rains events and freshwater runoff may be affecting marine organisms. The objective of the present study was to investigate the effect of temporary change in salinity on survival and lysozyme activity of Manila clam, *Ruditapes philippinarum.* The animal were exposed to short time periods (3 hours per day for 30 days) of hyposaline stress (14 psu and 24 psu), followed by recovery period mimic conditions typical for culture site at bays or estuaries experiencing heavy freshwater input, with a quick return to initial salinity (34 psu) with incoming tides and mixing seawater. One-way ANOVA showed that clams exposed temporary to hyposaline stress of 14 psu had a lower lysozyme activity compare to control and clam that kept at a temporary salinity of 24 psu ($p < 0.05$). There was no significant difference on survival between treatments. This result indicates that the experimental conditions were optimal to sustain the survival of the clam. However, under the temporary hyposaline stress, Manila clam might be susceptible by threat of disease due to low lysozyme activity.
Key Words: Manila clam, *asari*, salinity, lysozyme activity, climate change.

Introduction. Coastal marine systems are among the most ecologically and socio-economically important for aquaculture (Harley et al 2006; Somero 2012). Marine aquaculture provided 73.8-million-ton production (including fish, crustaceans, mollusks, and aquatic plants) with an estimating value of US$160 billion worth, and contributed up to 10 kg fish per capita for 7 billion human population (FAO 2016). However, scientific consensus confirmed that coastal marine system, a place for aquaculture practice, is being threatened by anthropogenic global climate change (IPCC 2014). The range impacts of climate change on aquaculture have been well described (Cochrane et al 2009). Several drivers 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.

The drivers of climate change have also affected marine culture bivalves. For example, the climate warming triggers a shift in distribution of the mussel *Mytilus edulis* (Jones et al 2010) and *Mytilus californiaus* (Harley 2011), as a species moves to occupy areas within its metabolic temperature tolerance (Root et al 2003). The ocean acidification due to the increasing levels of atmospheric CO_2 (pCO_2) decreased calcification and distorted shell ultrastructure on oyster species on larval stages (Gazeau et al 2007; Gazeau et al 2011; Kurihara 2008; Talmage & Gobler 2011). Elevated pCO_2 on bivalves also impacts the clapping performance in scallops (Schalkhausser et al 2013) and byssus attachment in mussels (O'Donnell et al 2013).

Aside from temperature rising and ocean acidification, cumulative events of extreme heavy rains and freshwater runoff might be also affecting marine bivalve organisms (Tomanek et al 2012). It has well documented that increasing extreme precipitation events were linked with climate change (Groisman et al 2005; Min et al 2011). However, in our best knowledge, studies on the potential impact on marine

organisms are still rare. The investigation on the oyster species showed that lower salinities due to extreme precipitation events disrupted the important thresholds for survival and metapopulation dynamics (Levinton et al 2011; Octavina et al 2015). Hyposaline stress also contributed to the shifting distribution range in blue mussel congeners, *Mytilus trossulus* and *Mytilus galloprovincialis* (Tomanek et al 2012). Yet, there are still limited information on how sudden change of hyposaline stress impacts on bivalve immune systems.

In order to comprehend the potential impact of the increasing of extreme precipitation events on bivalve immune system, we decided to investigate the physiological responses to hyposaline stress of Manila clam, *Ruditapes phillippinarum*. Manila clam is an intertidal species, that is a commercially important shellfish in Japan, as well as in other regions along the eastern coastlines of Asia, the north-west coast of North America and the northern coastline of the Mediterranean (Jones et al 1993; Shahabuddin et al 2015). Intertidal species such as Manila clam has been proven to be excellent study systems for evaluating climate change (Somero 2012). In this study, temporary salinity changes were exposed on Manila clams to evaluate the impact survival and lysozyme activity.

Material and Method

Specimen collection and experimental design. The study was conducted in January to February, 2016. Manila clams (mean of shell length × height x width = 31.06±1.62 x 21.65±1.82 x 14.18±1.67 mm; length:width:depth ratio = 2.19:1.53:1; individual weight = 6.30±1.58 g) were purchased from local market, supplied by Koryo Suisan, Co., Ltd., and originaly cultured in Mikawa Bay, Japan. The experimental conditions were set up to imitate temporary hyposaline stress conditions as occured in estuaries and bays during the heavy rains, followed by a quick return due to incoming tides and dilution of freshwater (Tomanek et al 2012).

Clams were kept at the indoor laboratory (Laboratory of Shallow Sea Aquaculture, Mie University, Japan) using recirculating clear polystyrene aquariums (18 x 60 x 25 cm), with density of 1 individual L^{-1}. Artificial sea water was used by dilluted sea salt (LIVESea® Salt, DELPHIS, Japan) into tap water with 24 h aeration prior to use. Seven days acclimatization period at salinity level of 34 psu were conducted before the treatment. All treatments were kept at 18^{o}C, set by immersing the aquarium into plystyrene foam (act as a thermostat) that filled with water as high as water level in the aquarium and equiped with both chiller and heater. Sea water was aerated to keep the oxygen supply, and also to keep food suspended and homogenous. Live feed, *Chaetoceros calcitrans* (Yanmar Co., Ltd., Japan) were given 1 mL individual^{-1} day^{-1} (cell density 100 x 10^6 cell mL^{-1}). Sea water was changed as needed, by measuring the phosphorus (PO_4^{3-}) and nitrogen (NH_3^-, NH_2^-, NH_3) content in sea water using handheld colorimeter for water quality measurement of aquaculture (HACH DR890, HACH Co., Ltd., USA).

Two experimental treatments were employed, 14 psu and 24 psu, and one control treatment 34 psu. Animals were exposed for 3 h target salinity (14 and 24 psu) by taking out appropriate amount of seawater in the aquarium and change it with fresh water added by degrees. After 3 h hyposaline exposed, concentrated seawater solution were added to maintain the salinity into the initial state (34 psu). The actual salinities were checked using handheld refractometer (NEW S-100α, Tanaka Sanjiro Co., Ltd., Japan). The threatment were continously carried out after 24 h recovery period, for the duration of 30 days experiment.

Growth and survival. In order to determine the growth rate, clam shell length and width were measured weekly. Each clam's treatment was put in the white paper towel. Only one clam was measured using digital caliper, and pictures that shown the value in the caliper and the whole clam were taken. The pictures were used to measure the rest of the clam shell length and width using image processing and analysis software (ImageJ 1.49v, NIH Image, USA). As for the survival, any mortality of clam in each treatment was

recorded daily. Clams were considered dead when their shells gaped and did not shut again after external stimulus (Munari et al 2011). The survival rate was calculated based on Muchlisin et al (2016).

Fluid collection. Hemolymph from the whole body was collected by homogenized the carcass. The flesh body of the clams were put in the centrifuge tube and were homogenized using tissue homogenizer. The equal volume of phosphate buffer (0.1 M, pH 7) was added into the homogenized tissue, then stirred firmly. The mixture was centrifuged at 4000 rpm for 30 min. The supernatant was collected into sterile storage tube, and stored at 10^{o}C for further analysis.

Lysozyme activity. Determination of lysozyme activities followed the method from Shugar (1952). As much as 1 mg mL^{-1} cells of *Micrococcus lysodeikticus* (MP Biomedicals, LLC., France) were prepared using 0.1 M phosphate buffer pH 7.0, shortly before the assay. In each of the microplate wells (Greiner Bio-One GmbH, Germany) was placed 50 µl sample, and 200 µl of the *Micrococcus* cells suspension was added quickly. The plate was gently shaken for 1 min and the sample was read using Bio-Rad Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Japan). As a standard, lysozyme from chicken egg white (Wako Pure Chemical Industry Ltd., Japan) with the range concentrations of 1.6, 0.8, 0.4, and 0.2 mg mL^{-1} were used. Measurement of the absorbance at 450 nm vavelength was initiated continuously every 1 min interval for 5 min at 25^{o}C. The lysozyme activity was calculated using the calibration curve of standard lysozyme vs. decrease in absorbance of the bacterial suspension after 1 minute.

Statistical analysis. Data were checked for normal distribution (Shapiro-Wilk's test) and homogeneity of variance (Bartlett's test). Results were compared using one-way ANOVA followed by a post-hoc test (Fisher's LSD test). All results are expressed as means±SD. SPSS® Statistics version 23 (IBM®, New York, US) software was used for statistical analysis. Prism version 7.0 (GraphPad Software, Inc., La Jolla, CA, US) was used for analyzing survival rate (Gehan-Breslow-Wolcoxon test). The alphabetical superscript were used to mark the differences at significant level of alpha 0.05. All research was conducted at the Laboratory of Shallow Sea Aquaculture, Department of Life Science, Faculty of Bioresources, Mie University, Tsu, Japan.

Results and Discussions. There was no visible effect of temporary change in salinity on growth rate of Manila clams. Shell length, width and depth did not increase over the course of the study. Clams acclimated to temporary salinity of 14 psu and 24 psu did not show significantly lower survival rates (14 psu: 85.71%; 24 psu: 85.7%) compared to clams in the control group (85.71%, Gehan-Breslow-Wolcoxon test, p = 0.05, Figure 1a). During the experimental period, water quality for rearing Manila clams were under the threshold (Table 1).

Table 1

Mean of water quality parameter on the experiment of 30-day exposure a temporary salinity on Manila clam, *Ruditapes philippinarum*

Parameter	*Control*	*14 psu*	*24 psu*
NO_3^- (mg L^{-1})	2.20±0.09	0.80±0.12	1.50±0.02
NO_2^- (mg L^{-1})	0.14±0.01	0.03±0.01	0.09±0.01
NH_3 (mg L^{-1})	0.04±0.01	0.00±0.00	0.00±0.00
PO_4^{3-} (mg L^{-1})	0.47±0.02	0.27±0.04	0.16±0.01
pH	7.99±0.11	8.14±0.15	8.08±0.10

Figure 1b shows the course of the reaction of the enzyme with the bacteria for 5 minutes for four different enzyme concentrations. The calibration curve of lysozyme concentration vs. decrease of absorbance is shown in Figure 1c. The linear regression equation and the correlation coefficient of this standard curve were: y = 0.0391x + 0.1958 and R^2 =

0.99496, respectively. Under our experimental condition (pH 7, 25°C), activity of lysozyme for Manila clam exposed to temporary salinity of 14 psu was significantly lower (5.803±0.158 mg mL^{-1}, $p < 0.05$, Table 2) than the control treatment (6.210±0.128 mg mL^{-1}) and clam that kept at a temporary salinity of 24 psu (6.068±0.243 mg mL^{-1}). However, there was no significant difference between Manila clam exposed to temporary salinity 24 and control.

Table 2

Mean of lysozyme concentration (mg mL^{-1}) on 30-days experiment of Manila clam, *R. philippinarum* exposed to temporary hyposaline stress

Control	*14 psu*	*24 psu*
3.105 ± 0.64^{b}	2.902 ± 0.079^{a}	3.034 ± 0.122^{b}

The values with different superscript are significantly different ($p < 0.05$).

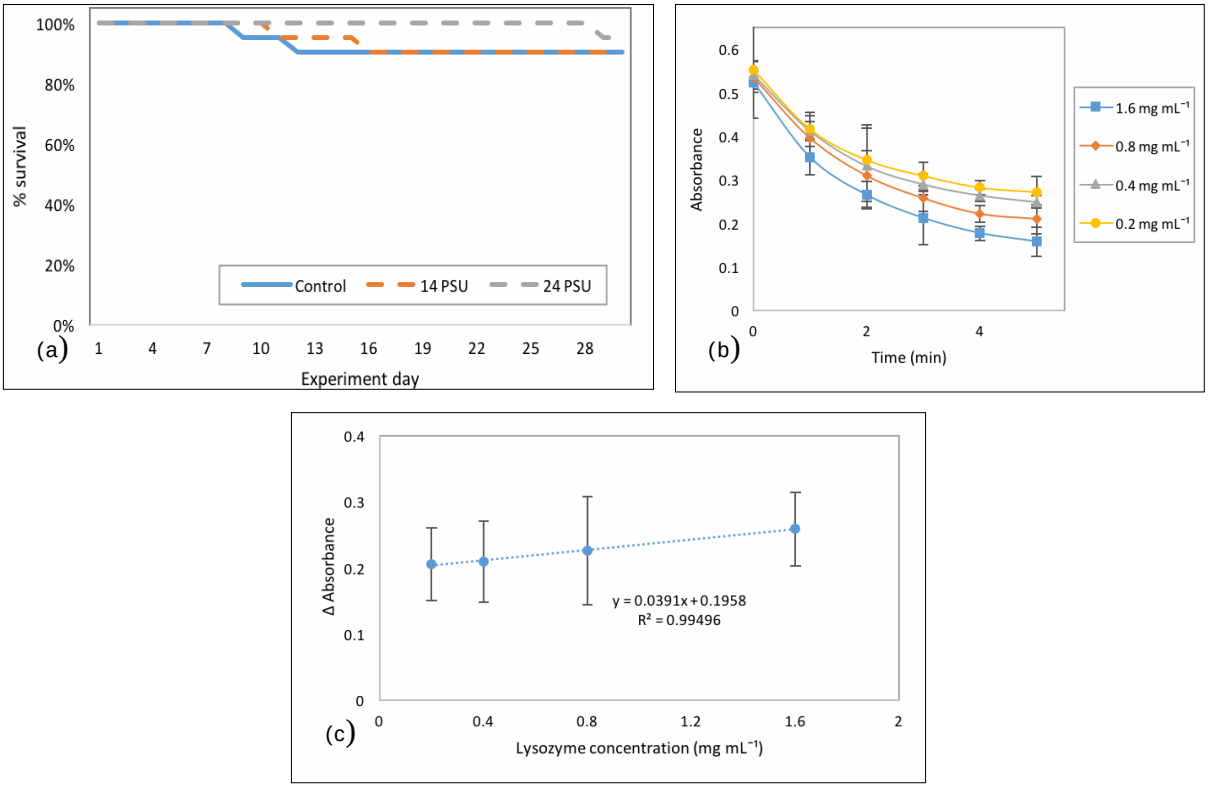

Figure 1. (a) Survival of R. philippinarum, express of percentage of clams surviving at a temporary hyposaline stress (control, 14 and 24 psu for 3 hours). Gehan-Breslow-Wolcoxon test: no significant difference ($p > 0.05$), (b) Decrease with the time of absorbance of suspensions of M. lysodeikticus, following addition of 1.6, 0.8, 0.4 and 0.2 mg mL^{-1} lysozyme from chicken egg white at 25°C and pH 7 in 0.1 M phosphate buffer, (c) Calibration curve of lysozyme concentration vs decrease in absorbance of bacteria suspension (in 0.1 M phosphate buffer, pH 7 at 25°C), produced during the first minute of reaction.

Study concerning the environmental factor induced by climate change, especially the temporary hyposaline stress due to increasing precipitation events on the biodefense mechanism on Manila clam was firstly revealed, in our best knowledge at least. Manila clam, one of the intertidal species is potentially impacted by climate change in term of lower lysozyme activity. Concerning the growth of the Manila clams, as suggested by the study on the other bivalve species (Paganini et al 2010), under environmental salinity stress the metabolic demands was increased without increasing the biomass. However, high survival rate was related to the optimal environmental condition (Table 1) to sustain the Manila clam.

External stress factors such as temperature and salinity are known to be able to affect the immune parameters of marine bivalve species (Fisher et al 1987; Park et al 2012; Rahmawati et al 2015). Under the scheme of environmental factors alteration due

to climate changes, our result showed that the immune parameter of Manila clam was affected by the temporary change on salinity. Immune parameter that used in this study is the lysozyme activity. Lysozyme on the immune system acts as an internal defense mechanism in order to protect the body against invading organisms or foreign substance (Gosling 2015). This biologically active molecule is secreted from the circulating haemocytes into the haemolymph, and plays an important role in humoral (cell product) defense system (Gestal et al 2008). Decreasing lysozyme activity in the digestive gland haemocytes would be a serious problem for the physiology and health status of marine bivalves, especially Manila clam. Considering the importance of bivalve species in ecosystems and aquacultures, this result will be valuable information to develop the adaptation and mitigation program in the future under climatic change scenarios.

Conclusions. The present study was designed to determine the effect of temporary change in salinity on survival and lysozyme activity of Manila clam, *Ruditapes philippinarum*. This study has shown that 3 hours hyposaline stress (14 psu and 24 psu) for 30 days did not affect the survival of Manila clams. However, Manila clams exposed temporary hyposaline stress of 14 psu had a lower lysozyme activity. Overall, this study strengthens the idea that under the temporary hyposaline stress, Manila clam might be susceptible by threat of disease due to low lysozyme activity. Moreover, further work could assess the effect of temporary hyposaline stress challenged with inoculation viral disease and the long-term effects of temporary change in salinity on Manila clams.

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