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Effects of water temperature change on immune function in surf clams, *Mactra veneriformis* (Bivalvia: Mactridae)

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ABSTRACT

Surf clam, *Mactra veneriformis* is one of the crucial fishery resources in Korea. This study was performed to examine the immune functions of the surf clam under the stress of water temperature changes at 10 °C, 20 °C or 30 °C for 24 h. Viable bacterial counts (VBC), total haemocyte count (THC), phagocytic activity, lysozyme activity, NRR times and SOD activity were assessed in three different water temperature groups. Clams held at 10 °C decreased in THC, lysozyme activity and NRR times, but phagocytic activity was increased. The highest temperature (30 °C) significantly increased in THC, whereas it decreased in phagocytic activity, lysozyme activity and NRR times. In clams maintained at 20 °C, phagocytic activity, lysozyme activity and NRR times were increased whereas THC was somewhat decreased with respect to clams held at 30 °C. However, water temperature changes did not elicit any alteration of VBC and SOD activity. The present study demonstrates that acute water temperature change affects the haemocytic and haemolymphatic functions, reducing immunosurveillance in stressed surf clam, *M. veneriformis*.

1. Introduction

Surf clam, *Mactra veneriformis* (Bivalvia: Mactridae), is one of the commonly cultured bivalve species on sandy beaches and tidal flats along the western coast of Korea. The clam was treated as a worthless species in the past, but it has been widely utilized recently as a nutritive food source owing to development of a new food processing technology (Lee, 1994). Its yield from the western coast of Korea was estimated to be 71 tons in 2003, but the figure has been decreasing dramatically to about 6 tons in 2007 (MLTM, 2007). Causes of its reduction are unclear, yet.

The innate defense system of bivalves consists of both humoral and cellular immunity (Marin et al., 2007; Yang et al., 2007). Haemolymph and haemocytes are the major components in this immune system. Haemocytes are responsible for recognition, ingestion and elimination of non-self particles in the haemolymph of invertebrate animals (Cheng, 1981; Fisher, 1986; Fisher et al., 1987; Chu, 2000; Wikfors et al., 2003). Especially, phagocytosis is considered as the primary defense mechanism of bivalves, so, it has been used as an index to evaluate the health status in bivalves. Lytic enzymes in haemolymph also play an important role. Lysosomal hydrolases within cytoplasmic granules of haemocytes are released during defensive immune responses (Cheng and Doughetty, 1989).

Several parameters have been employed to evaluate defensive capacity in bivalves. Lysomal membrane stability could be assessed by measuring the uptake of neutral red dye (NRU) (Li et al., 2006). Reader et al. (1989) modified the method using neutral red retention (NRR) to a simpler, and more convenient and economical one. The NRR assay has been widely applied to evaluate the effects of environmental and physiological changes and mechanical stresses in mollusks (Lowe and Pipe, 1994; Lowe et al., 1995a,b; Ringwood et al., 2002; Cho and Jeong, 2005; Li et al., 2006). Increase in reactive oxygen species (ROS) production in bivalves indicates the effort to degrade pathogens during infection (Cheng et al., 2004; Marin et al., 2007). Although the production of ROS may be aimed to remove foreign substances, ROS produced in bivalves can also cause damage to self cells depending upon the effectiveness of associated antioxidant defense system (Michiels and Remacle, 1998; Marin et al., 2007; Yang et al., 2007). For instance, superoxide dismutase (SOD) is a very important enzyme involved in ROS detoxification. It is known that SOD activity in *Chamelea gallina* depends on water temperature (Marin et al., 2007).

Several authors have reported immune functions in mollusks are influenced by seawater temperature variations (Abele et al., 2002; Wikfors et al., 2003; Paillard et al., 2004; Renault et al., 2006; Marin et al., 2007). However, immunological studies on surf clam, *M. veneriformis*, in relation to water temperature have not been performed yet, despite its importance as a culture species.

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This study attempted to assess the alteration of immune functions in the surf clam under the stress of acute temperature change in laboratory conditions.

2. Materials and methods

2.1. Surf clams

Clams were sampled from the intertidal zone off Taean seashore situated in Chungnam Province, Korea (Fig. 1.). Their height and length of the shells were 31.3 ± 1.4 mm and 35.7 ± 1.9 mm (mean ± SE, $n = 50$), respectively. The temperature, salinity and specific gravity of the sampling site were 19.5 ± 2.5 °C, 32.5 ± 1.0‰ and 1.025 ± 0.002, respectively, measured for 5 days when sampled.

2.2. Exposure to experimental temperatures

For laboratory acclimation, clams were maintained in individual 800 L sand-bottomed (approx. 10 cm deep) fiber glass reinforced plastic (FRP) tanks supplied with filtered sea water (temperature 20–21 °C, salinity 32–33‰, pH 8.1–8.2, specific gravity 1.024–1.026) for 12 days. During the acclimation, the clams were fed every other day as libitum with a mixed microalgal diet consisting of *Isochrysis galbana*, *Nannochloris oculata*, *Monochrysis lutheri* and *Phaeodactylum tricornutum*. Clams were starved for 24 h prior to the commencement of acclimation so as to standardize the dietary status. Dead clams were quickly removed from the tanks and the sea water was changed. The acclimated clams were divided into three groups and maintained in separate FRP tanks at 10 °C, 20 °C or 30 °C for 24 h. Clams were analyzed after the maintenance at there three different temperatures. Salinity, pH and specific gravity in the tanks were 32.5–33.5‰, 8.2–8.4 and 1.025–1.027, respectively, during the experiments. The stress tests were performed in triplicate tanks each containing 80 clams.

2.3. Haemolymph

Haemolymph was collected from the posterior adductor muscle using a 1-ml disposable syringe. For each analysis, pooled haemolymph from 5 clams was used.

2.4. Viable bacterial count (VBC) in haemolymph

VBC was performed following the method of Kim et al. (1992) and Lee (2005). Briefly, 1.0 ml of haemolymph from each group was diluted in 10-fold series with sterilized saline (0.85%). One hundred microlitre of each dilution was spread on Marine Agar 2216 (BD, US) plates. The plates were incubated at 25 °C for 24 h and then colony numbers were counted to express in colony forming units (CFUs).

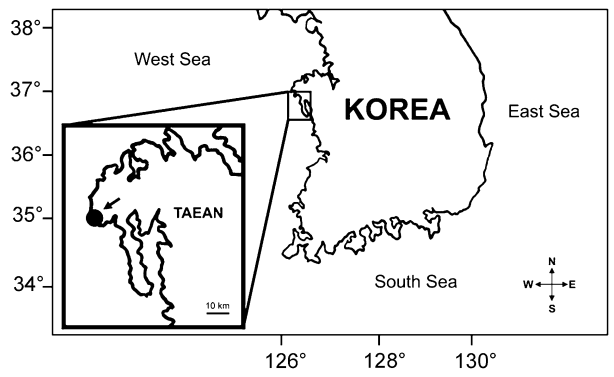

Fig. 1. Map showing a sampling site (arrow), Taean.

2.5. Total haemocyte count (THC)

One hundred microlitre of haemolymph was mixed with 300 μl of cold modified Alsever's solution (MAS: dextrose 2.8 g, sodium citrate 0.8 g, EDTA 0.34 g, sodium chloride 2.25 g, pH 7.5). A drop of the mixture was placed in haematocytometer (Marienfeld, Germany) and the number of haemocytes was counted under a phase contrast microscope (CK40, Olympus, Japan).

2.6. Phagocytic activity

Phagocytic activity evaluation was performed following the method of Park et al. (2000). In short, haemolymph diluted to 1:3 (v:v) in MAS was centrifuged at 300*g* for 10 min at 4 °C and the supernatant was decanted. Haemocytes were resuspended in 0.45 μm filtered sea water (FSW) at a final concentration of 1×10^6 cells/ml. To prepare zymosan solution, zymosan (Sigma, USA) was suspended in FSW at a concentration of 2 mg/ml, heated for 30 min at 100 °C and centrifuged at 250*g* for 10 min. After removing the supernatant, the pellet was resuspended in FSW. The final concentration of the zymosan suspension was adjusted to 1×10^6 particles/ml. One ml of haemocyte suspension was mixed with 1.0 ml of zymosan suspension and then incubated at 25 °C for 50 min. Eight ml of cold MAS (4 °C) was added to the suspension and centrifuged at 230*g* for 10 min. The supernatant was removed and the pellet was resuspended in 1.5 ml of MAS. The pellet suspension was smeared on slide glasses by centrifuging in a cell-collection apparatus (Hanil, Korea) at 96*g* for 3 min. The smear was stained with May-Grünwald Giemsa dye. Two replicas were prepared for each slide and 200 haemocytes were counted for phagocytic activity in each replica.

2.7. Lysozyme activity

Haemolymph was centrifuged at 780*g* for 10 min to obtain cell-free haemolymph. The cell-free haemolymph was stored at −80 °C until analyses. Fifty microlitre of cell-free haemolymph was added to 750 μl of 0.05% suspension of *Micrococcus lysodeikticus* in 0.06 M PBS (pH 6.5) in a spectrophotometer. The change in absorbance at 450 nm was read using a spectrophotometer (Jasco, V-530, Japan) for 2 min. The lysozyme activity in each sample was defined as 1 unit when absorbance change was 0.001/min.

2.8. Neutral red retention (NRR) assay

The NRR assay was performed following modified methods of Hauton et al. (1998) and Li et al. (2006). Briefly, the neutral red stock solution was made by dissolving 2.0 mg of the neutral red powder in 1 ml of dimethyl sulphoxide (DMSO). For working solution, 10 μl of defrosted stock solution was diluted with 5 ml of *Ostrea* saline solution (0.2% potassium chloride in filtered sea waster). Two hundred microlitre of haemolymph was placed into a tube containing 200 μl of the *Ostrea* saline solution and gently mixed. Forty microlitre mixture of haemolymph was smeared on slide glasses by centrifuging in a cell-collection apparatus at 96*g* for 2 min. Sixty microlitre of the working solution was added to the slides and then incubated in a humidity chamber at 10 °C for 15 min. The slides were covered with a coverslip (22 × 22 mm) and then granulocytes were examined under a microscope (CH2, Olympus) at low light intensity. The slides were examined every 10 min for the first 60 min and then every 20 min. Thereafter, 20 granulocytes were examined each time for an individual slide.

The endpoint of the assay was defined as the time at which 50% of the granulocytes had lost dye from their lysosomes.

2.9. Superoxide dismutase (SOD) activity assay

Haemolymph was centrifuged at 800*g* for 10 min at 4 °C and then supernatant was collected for cell-free haemolymph. For hemocyte lysate, 100 μl of haemolymph diluted with 200 μl of MAS was centrifuged at 800*g* for 10 min at 4 °C and the supernatant was decanted. Haemocytes were resuspended in MAS at a final concentration of 1×10^7 cells/ml and centrifuged at 800*g* for 10 min at 4 °C. After removing the supernatant, the pellet was suspended in saline (0.85%). This step was repeated twice. The pellet was suspended with 4 ml of deionized distilled water (ddH_2O), 1 ml of ethanol and 0.6 ml of chloroform and then mixed vigorously for 20 min. The haemocyte suspension was centrifuged at 600*g* for 10 min at 4 °C and transferred the upper water–ethanol phase to a new tube. One hundred microlitre of the upper phase was mixed with 700 μl of ddH_2O and diluted with 0.25% ethanol. The SOD activity was determined using a SOD assay kit-WST (Dojindo Co, Japan) following the manufacturer's protocol.

2.10. Statistic analysis

Results were expressed as means ± SD. All data were analyzed to test statistical significance among groups by analysis of variance (ANOVA) followed by Duncan's test. *p*-values less than 0.05 were used for declaration of significance.

3. Results

3.1. Viable bacterial count (VBC) in haemolymph

VBC in haemolymph of clams kept at 10 °C, 20 °C and 30 °C was approximately 1.5×10, 1.8×10, and 1.6×10 CFU/ml, respectively (Fig. 2). There was no statistical difference among temperatures.

3.2. Total haemocyte count (THC)

THC was significantly affected by temperature changes (Fig. 3). Clams held at 10 °C significantly decreased THC, with respect to animals held at 20 °C and 30 °C. The highest level in THC was observed in clams kept at 30 °C.

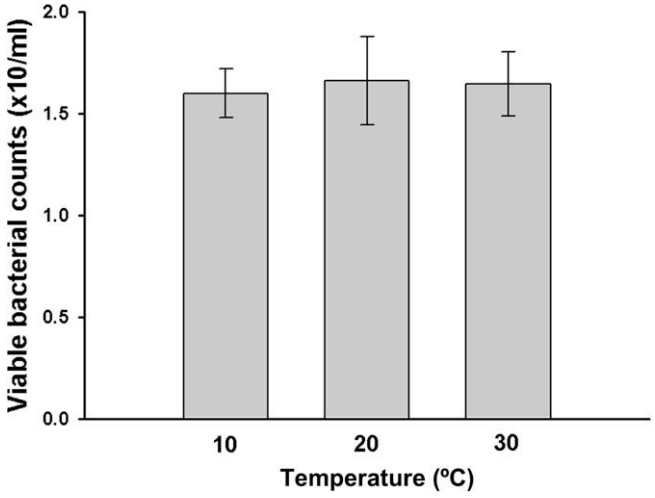

Fig. 2. Viable bacterial counts (VBC) in haemolymph ($n = 5$ pools).

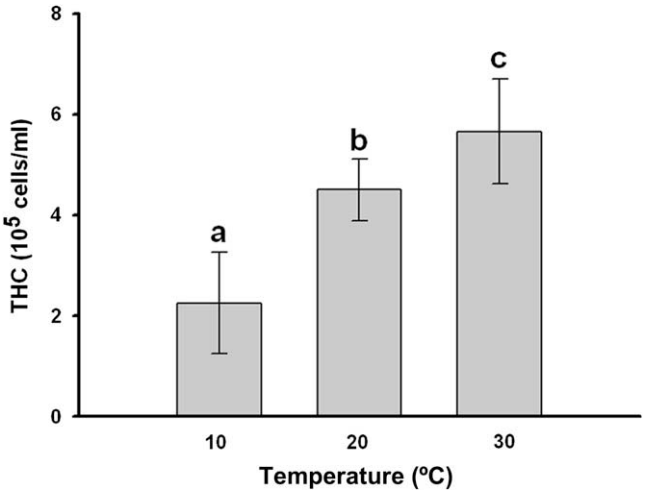

Fig. 3. Effects of water temperature changes for 24 h on total haemocyte counts (mean ± SD) in *M. veneriformis*. Column bars with different alphabet indicate significant difference ($p < 0.05$) among each group ($n = 5$ pools).

3.3. Phagocytic activity and lysozyme activity

The phagocytic rate in clams held at 30 °C was significantly reduced compared with those at 10 °C and 20 °C (Fig. 4). No differences in phagocytic activity were observed between clams kept at 10 °C and those at 20 °C. In lysozyme activity, clams at 30 °C exhibited low levels compared with those held at 10 °C and 20 °C, while lower activity was observed with 10 °C (Fig. 5).

3.4. Neutral red retention (NRR) times and superoxide dismutase (SOD) activity

Clams kept at 20 °C showed NRR value for approximately 90 min which was significantly longer than those held at 10 °C and 30 °C (Fig. 6).

No significant effect of temperature variation was observed in total SOD activity in both haemocyte lysates and cell-free haemolymph (Fig. 7). The activity values in cell-free haemolymph were slightly lower than those of haemocyte lysates, although mean statistical significance was not achieved.

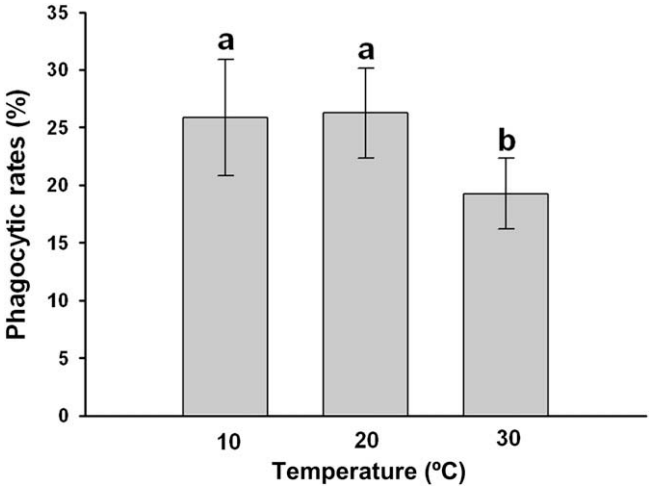

Fig. 4. Effects of water temperature changes for 24 h phagocytic rates (mean ± SD) of haemocytes in *M. veneriformis*. Column bars with different alphabet indicate significant difference ($p < 0.05$) among each group ($n = 5$ pools).

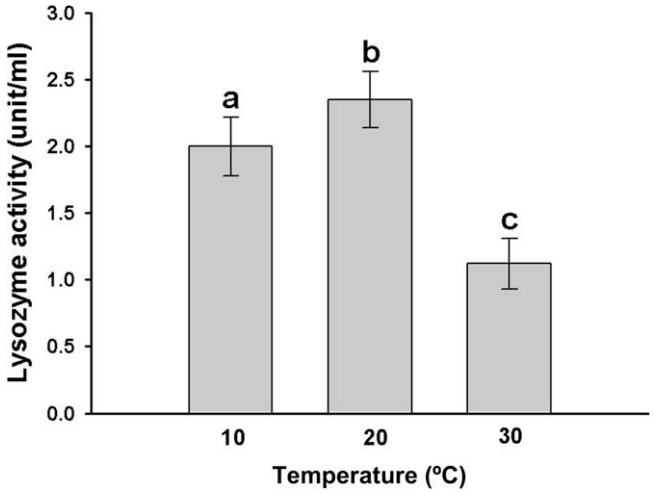

Fig. 5. Effects of water temperature changes for 24 h on lysozyme activity (mean ± SD) of cell-free haemolymph in *M. veneriformis*. Column bars with different alphabet indicate significant difference ($p < 0.05$) among each group ($n = 5$ pools).

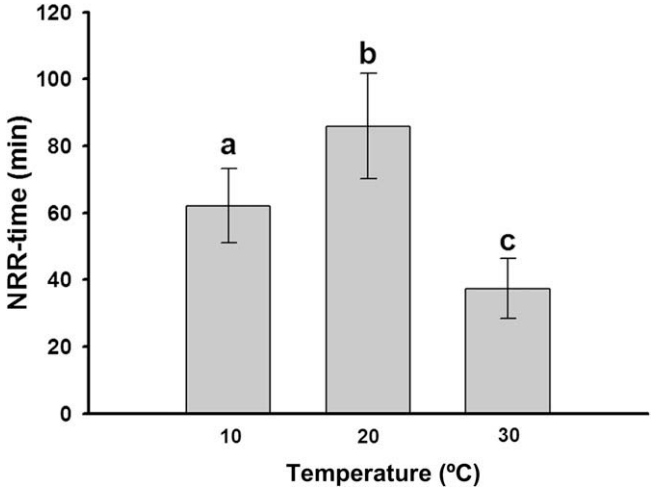

Fig. 6. Effects of water temperature changes for 24 h on NRR-time (min) (mean ± SD) of haemocytes in *M. veneriformis*. Column bars with different alphabet indicate significant difference ($p < 0.05$) among each group ($n = 5$ pools).

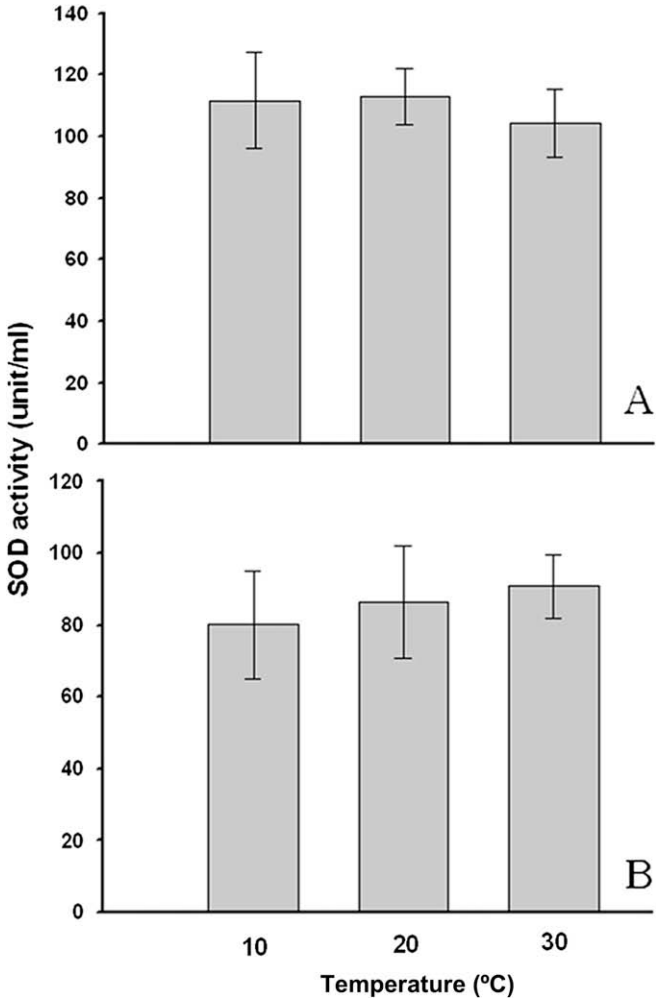

Fig. 7. Effects of water temperature changes for 24 h on SOD activity in haemocyte lysate (A) and cell-free haemolymph (B) (mean ± SD) of *M. veneriformis* ($n = 5$ pools).

4. Discussion

Like all aquatic organisms, bivalves are sensitive to environmental changes such as dissolved oxygen, temperature and salinity (Camus et al., 2000; LeBlanc et al., 2005; Marin et al., 2006; Gagnaire et al., 2006; Ottaviani et al., 2007). Temperature changes, especially to high temperature conditions, can suppress immune functions in bivalves (Ford and Tripp, 1996; Shumway, 1996; Marin et al., 2007; Yang et al., 2007). In the present study, we found that *M. veneriformis* is susceptible to water temperature alteration, particularly to high temperature.

Alteration in THC reflects the status of haemocyte proliferation or inflammatory haemocyte mobilization to peripheral tissues (Pipe and Coles, 1995; Gagnaire et al., 2006; Park and Oh, 2006; Marin et al., 2007). In *Ruditapes philippinarum* and *Chamelea gallina*, THC was significantly increased at higher temperature (Paillard et al., 2004; Marin et al., 2007). Similarly to their results, we observed in surf clam, *M. veneriformis* that THC levels considerably increased at 30 °C. Carballal et al. (1998) reported that THC levels are fluctuating in association with water temperature, the lowest levels being found in winter and the highest in summer. THC increase in haemolymph can also be attributed to an accelerated mobilization of haemocytes to the haemolymph component responding to the bacterial invasion to the haemolymph (Oubella et al., 1994; Carballal et al., 1998; Marin et al., 2007). In these studies (Oubella et al., 1994; Carballal et al., 1998), the authors measured haemocyte movement in clams infected with pathogenic agents. In the present study, we could not find any difference in viable bacterial counts in haemolymph among different temperature groups. Accordingly, THC increment does not seem to be associated with bacterial counts. Rather, we suppose that THC increase in surf clams at high temperature could be caused by haemocytes migration from tissues into haemolymph space. In a tendency qualitatively similar to our results, it was suggested that THC level reduces at low temperature which may be owing to cell lysis or accelerated movement of cells from haemolymph to peripheral tissues (Pipe and Coles, 1995; Carballal et al., 1998; Marin et al., 2007). Although the mechanism in surf clam for declined THC level is not clear yet, it is clear that temperature is a crucial factor to regulate its level.

Phagocytosis is one of the main defense mechanisms against non-self materials in bivalves (Marin et al., 2007). Oliver and Fisher (1999) have reported that phagocytosis is a temperature-dependent process in bivalves. Temperature change also affected to phagocytic activity in surf clam in this study. Clams kept at 30 °C showed lower level of phagocytosis when compared with those at 10 °C and 20 °C. It is know that the effect of temperature on phagocytic activ-

ity vary depending on bivalves. In *Chlamys farreri*, *Chamelea gallina* and *Crassostrea virginica*, phagocytosis was inhibited under high temperature conditions (Hégaret et al., 2003; Marin et al., 2007; Yang et al., 2007). In contrast, *Mytilus galloprovincialis* kept at 10 °C shows lower phagocytic activity than that at 20 °C and 30 °C (Carballal et al., 1997). However, Ordas et al. (1999) failed to find differences in phagocytosis between low and high temperatures in *Ruditapes decussates* and *Mytilus galloprovincialis*. In this study, high temperature significantly reduced phagocytic activity in *M. veneriformis*. There seems to be chemical mediators that link the temperature variation signals to phagocytic activity. Lacoste et al. (2001a,b) found that noradrenaline (catecholamine in mollusks) is synthesized by bivalve haemocytes and released into haemolymph during stress. In consequence, noradrenaline suppresses immune responses in bivalves such as phagocytosis (Lacoste et al., 2001c). In this work, the relationship between noradrenaline and phagocytosis inhibition was not examined.

Temperature change affects lysozyme activity in *M. veneriformis*: higher activity was observed at 20 °C with respect to those at 10 °C or 30 °C. In bivalves, lysozyme is the main bacteriolytic enzyme against Gram-positive and negative bacteria (Marin et al., 2005, 2007). Lysozyme activity is strongly related to the phagocytosis by haemocytes, and it is proposed that a decrease in lysozyme activity is a result of degeneration and/or functional alteration occurring in haemocytes under the stress of high temperature (Cheng et al., 1975; Park and Oh, 2006). In contrast to THC level elevation as discussed above, phagocytosis was reduced in high temperature condition. From these opposite changes, we speculate that high temperature might have reduced the secretion of lysozyme from unit number of haemocytes to haemolymph. The net result could be of lower phagocytosis despite the augmentation of haemocyte mobilization. Marin et al. (2007) suggested that clams release lysozyme to defend against the increase in bacterial number in haemolymph. Its activity thus could be an indication of physiological condition and the vitality of the defense system in bivalves (Chu and La Peyre, 1989). However, we could not observe any difference of bacterial counts in haemolymph in *M. veneriformis* in this study, because we assume that alteration in bacterial number was not a stimulus for the lysozyme activity at least under out experimental conditions. Clams kept at 20 °C showed higher lysozyme activity and phagocytic activity. These results probably indicate that 20 °C is the optimal water temperature to maintain adequate lysozyme activity and phatocytosis which will support the optimal immune function for survival of *M. veneriformis*. However, it is necessary to test the clams in natural environmental conditions without acute changes of temperature.

Neutral red is a lipophilic dye that can permeate the cell membrane quite freely (Lowe et al., 1992; Li et al., 2006). NRR assay has been employed because of the property that stressed cells do not take up as much of the dye as normal cells do (Borenfreund and Puerner, 1985; Hauton et al., 1998). Water temperature change leads to metabolic stress (Shpigel et al., 1992; Li et al., 2007). Harding et al. (2004) suggested that lysosomes can be a parameter for the earliest detectable stress response. We observed that NRR values were reduced at low and high temperatures, but the reduction was more significant in high temperature condition. Similarly to our results, it was reported in *Ostrea edulis* that NRR of haemocytes significantly decreased at low and high temperatures (Hauton et al., 1998). Our results suggest that lipid membrane of haemocytes in surf clam is unstable at low or high temperature and release of neutral red from the lysosome is rapid, particularly in high temperature condition. From this result, we can also conclude that low or high temperature causes instability of cell membrane of heamocytes which in turn reduces cellular immune function.

Bivalves under the stress of temperature change produce reactive oxygen species (ROS) and activate anti-oxidative enzymes (Abele et al., 2002). SOD is the most important defense factor in the antioxidant system (Downs et al., 2001; Yang et al., 2007). Marin et al. (2007) reported that high temperature led to alteration of SOD activity in haemocyte lysate or cell-free haemolymph in *Chamelea gallina*. However, in this study, no difference of the activity was observed among the three different temperatures either in haemocyte lysate or cell-free haemolymph. Yang et al. (2007) also failed in *C. farreri* to detect the effect of temperature on total SOD activity in haemocyte lysate and cell-free haemolymph. We postulate that the acute temperature challenge performed in the present study was not severe enough to affect SOD activity in haemocytes and haemolymph in surf clam.

In conclusion, water temperature alteration can induce immunosuppression in *M. veneriformis* that is detectable as the reduction of THC, phagocytosis, lysozyme activity and NRR time. Such compromise then can lead to detrimental consequences such as disease resistance, growth impairment and lower survival rates. In the west coast of Korea, yields of *M. veneriformis* have been declining over the past decade but its cause is not clear, yet. The area shows a wide fluctuation in seasonal water temperatures, especially, in between summer (average temperature 24–29 °C) and winter (average temperature range 2–5 °C) (Lee, 2005; Jang et al., 2008). Thus, in the area, surf clam might be suffering temperature change stresses although the bivalve is poikilothermic. Still, it is hard to conclude that the temperature alteration in the area caused the yield reduction of surf clam because this study was carried out only under a laboratory condition. Although the present study does not explore all possible factors that might have caused the reduction, it is feasible to suspect it as one culprit.

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