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Effects of Anoxia on Immune Functions in the Surf Clam *Mactra veneriformis*

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Jin-Ha Yu, Min-Chul Choi, Kyung-Il Park, and Sung-Woo Park (2009) Effects of anoxia on immune functions in the surf clam *Mactra veneriformis*. *Zoological Studies* **49**(1): 94-101. The surf clam *Mactra veneriformis* is one of the important fishery resources in Korea. Its yields have recently been decreased due to environmental changes such as water levels, temperature, salinity, dissolved oxygen, pH, etc. In particular, exposure to air during low tides may have been a contributing factor. This study was carried out to examine the immune functions of the surf clam when it undergoes anoxic stress from air exposure for 24, 48, and 72 h. Some of the air-exposed clams were also examined after recovering for 24 h in aerated seawater. The total hemocyte count (THC), phagocytic activity, lysozyme activity, phenoloxidase (PO) activity, and nitroblue tetrazolium (NBT) reduction were assessed in both air-exposed and exposed-recovered groups. Air exposure, regardless of the period, induced significant decreases in the THC, phagocytic rate, lysozyme activity, PO activity, and NBT reduction. However, air exposure elicited no significant change in the THC. Clams exposed for 24 h and returned to seawater had almost fully recovered within 24 h. However, partial recovery was observed only in the THC and PO levels after 48 h of exposure followed by a 24 h recovery period. The present study demonstrates that air-exposure-induced anoxia compromises immunological functions in *M. veneriformis*, and the effects cannot be ameliorated if the stress exceeds a certain point. http://zoolstud.sinica.edu.tw/Journals/49.1/94.pdf

Key words: *Mactra veneriformis*, Surf clam, Immune function, Stress, Anoxia.

The surf clam *Mactra veneriformis* represents one of the important cultured species in the Korean fisheries industry. Its yield from the western coast of Korea was estimated to be 71 tons in 2003, but the figure dramatically decreased to about 6 tons in 2007 (MLTM, 2007). This reduction was ascribed to environmental pollution, overfishing, and land reclamation in the area.

Different forms of stresses are known to impair immune defense systems in bivalves (Ottaviani et al. 1992, Vetvicka and Sima 1998). For example, environmental changes such as salinity, temperature, and air exposure can result in increased disease outbreaks in bivalves due to compromised immune functions (Lacoste et al. 2002, Pampanin et al. 2002, LeBlanc et al. 2005, Gaganire et al. 2007, Ottaviani et al. 2007). Tsuchiya (1983) reported that mussels frequently exposed to air until the harvest season tended to show high mortality, especially during the hot season, although they are intertidal organisms.

Hemocytes play key roles in the defense strategy of bivalves, being involved in the recognition and uptake of foreign particles (Park and Oh 2006). Thus, their number can be an index to evaluate immune function. Lytic enzymes in the hemolymph also play important roles. For instance, lysosomal hydrolases within cytoplasmic granules of hemocytes are released during defensive immune responses (Cheng and Dougherty 1989).

Various hemolymph parameters are used as indices of the bivalve health status following

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exposure to different stress conditions such as diseases, pharmaceutical products, and anoxia (Peters and Raftos 2003, Marin et al. 2005, Gagné et al. 2006). Marin et al. (2007) demonstrated that total hemocyte numbers and lysozyme activity of hemocytes from *Chamelea gallina* were affected by high temperatures. Chu and Peyre (1989) observed that hemolymph lysozyme activities and protein concentrations fluctuated with seasonal changes in the American oyster *Crassostrea virginica*. The phagocytic index of hemocytes in *Chamelea gallina* exhibited a significant reduction in anoxic conditions (Marin et al. 2005). In *Tapes decussates*, Esteban et al. (2006) found that phenoloxidase (PO) activity was elevated when parasitized by *Perkinsus atlanticus*.

The objective of the present study was to examine changes in the immunological function of *Mactra veneriformis* when undergoing anoxic stress due to exposure to ambient air. We also attempted to estimate the extent of stress from which the bivalve maintains the ability to recover.

MATERIALS AND METHODS

Surf clams and hemolymph

Clams were sampled from the intertidal zone off the Gochang seashore located in Jeollabukdo Province, Korea (Fig. 1). The height and length of the shells (mean ± S.E., n = 50) were 28.7 ± 1.3 and 33.5 ± 1.6 mm, respectively. The temperature and salinity of the site were 18.0 ± 1.0°C and 33.5‰ ± 0.5‰, respectively, measured for 5 d during sampling. Hemolymph was collected from the posterior adductor muscle using a 1 ml disposable syringe.

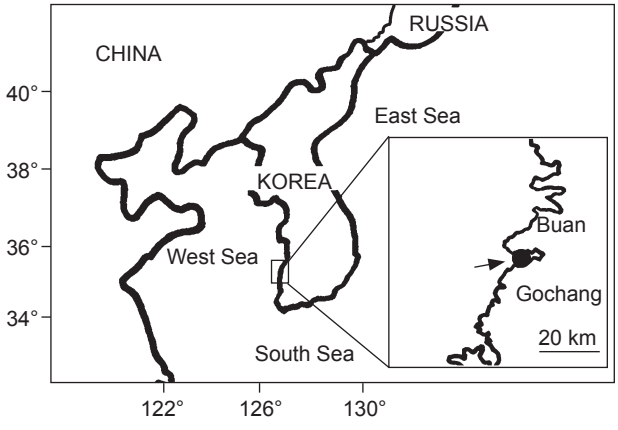

Fig. 1. Map showing the sampling site (arrow), Gochang, Korea.

Air exposure

One thousand clams were collected from the site, and maintained in a 650 L FRP tank supplied with filtered sea water in a laboratory for 7 d for acclimation. During acclimation, the clams were fed ad libitum on alternate days with a mixed algal diet consisting of *Isochrysis galbana*, *Nannochloris oculata*, and *Monochrysis luther*, but were starved for 24 h prior to the analysis in order to standardize their dietary status. Acclimated clams were divided into 3 groups of 250 clams and placed on flat trays (32 cm long × 42 cm wide × 8 cm high) with wet fine sand (0.5-2.0 mm in diameter) collected from the seashore where the clams were sampled. The sand was covered with a paper towel, and the clams were placed on top of that. Seawater was added to keep the paper towel wet, and to maintain constant humidity around the clams. The experiment was carried out at 18.0 ± 1.0°C. Clams were checked once a day, and dead clams were immediately removed from the tray. Clams were regarded as dead if the shell opened when the mantle and foot were touched with a syringe. For the recovery experiments, half of the air-exposed clams were kept in well-aerated seawater for 24 h. Clams for the control (250 individuals) were maintained in well-aerated seawater. The stress tests were carried out in triplicate.

Mortality in an anoxic condition

After 24, 48, and 72 h of anoxic exposure, 100 clams from each experimental condition were kept in 40 L aerated seawater aquaria to recover for a 24 h period. Those clams were then returned to an anoxic condition, and the mortality was recorded daily. The median lethal time (LT_{50}) was determined. For the control, 100 clams kept in a well-aerated tank were placed in a humidity-saturated condition.

Total hemocyte count (THC)

One hundred microliters of pooled hemolymph was mixed with 300 μl of cold modified Alsever's solution (MAS: 2.8 g dextrose, 0.8 g sodium citrate, 0.34 g EDTA, and 2.25 g sodium chloride; pH 7.5). A drop of the mixture was placed in a hemocytometer (Marienfeld, Lauda-Koenigshofen,

Germany), and the number of hemocytes was counted under a phase-contrast microscope (CK40, Olympus, Tokyo, Japan).

Total hemolymph protein

The total hemolymph protein concentration was quantified using a protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's protocol.

Phagocytic activity

Phagocytic activity was evaluated following a method of Park et al. (2000). Briefly, hemolymph collected in MAS (1: 3) was centrifuged at 300 ×*g* for 10 min at 4°C, and the supernatant was decanted. Hemocytes were resuspended in filtered seawater (FSW) at a final concentration of 10^6 cells/ml. To prepare the zymosan solution, zymonsan (Sigma, St. Louis, MO, USA) was suspended in fresh FSW at a concentration of 2 mg/ml, heated for 30 min to 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 10^6 particles/ml. One-half milliliter of the hemocyte suspension was mixed with 1 ml of the zymosan suspension and then incubated at 25°C for 60 min. Five milliliters of cold MAS (4°C) was added to the mixture to stop the phagocytic activity, and then this was 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 centrifugation in a cell-collection apparatus (Hanil, Gyeonggido, Korea) at 96 ×*g* for 3 min. The smear was stained with May-Grünwald Giemsa dye. Two replicas were prepared for each smear, and the phagocytic activity of 100 hemocytes was determined for each replicate.

Lysozyme activity

Hemolymph was centrifuged at 780 ×*g* for 10 min to obtain cell-free pooled hemolymph. The cell-free hemolymph was stored at -80°C until being analyzed. Forty microliters of cell-free hemolymph was added to 760 μl of a 0.15% suspension of *Micrococcus lysodeikticus* in 66 mM phosphate-buffered saline (PBS; pH 6.2) in a cuvette. The change in absorbance at 450 nm was read using a spectrophotometer (Jasco, V-530, Tokyo, Japan) for 2 min. One unit of lysozyme activity in each sample was defined as an absorbance change of 0.001/min.

PO activity

PO activity was measured by the method of Raftos et al. (2007). Briefly, hemolymph was mixed with an equal volume of cold marine anticoagulant (MAC; 0.1 M dextrose, 15 mM trisodium citrate, 13 mM citric acid, and 0.45 M NaCl; pH 7.0) and centrifuged at 400 ×*g* for 5 min at 4°C. After decanting the supernatant, the pellet was resuspended in MAC and then adjusted to a final concentration of 10^6 cells/ml. The hemolymph was centrifuged at 400 ×*g* for 5 min at 4°C. After decanting the supernatant, the pellet was resuspended in 0.01 M PBS (pH 7.4). To prepare the hemocyte lysate, the hemolymph was frozen at -80°C and thawed at 25°C 3 times. The lysate was centrifuged at 1500 ×*g* at 4°C for 10 min to remove cellular debris. Eighty microliters of the hemocyte lysate was pipetted into 96 well plates, and then 80 μl of L-dihydroxyphenylalanine (4 mg/ml in FSW) was added. Eighty microliters of 1 mM 3-methyl-2-benzothiazolinone was added to the mixture, and the absorbance was immediately measured at 490 nm in the dark with a microplate reader (BioRad, Model 680, Hercules, CA, USA) for 1 h at 37°C.

Nitroblue tetrazolium (NBT) reduction test

NBT reduction was measured with some modifications from Shiau and Lee (2002) and Verghese et al. (2007). Briefly, 100 μl of hemolymph was diluted with 400 μl of MAS and centrifuged at 250 ×*g* for 10 min and 4°C. The supernatant was removed, and the hemocyte pellet was resuspended in modified complete Hank's balanced salt solution (MCHBBS: 10 mM $CaCl_2$, 3 mM MgCl, 5 mM $MgSO_4$, and 24 mg/ml Hank's balanced salt). One hundred microliters of the hemocyte suspension was transferred to 96 well microplates and centrifuged at 250 ×*g* for 10 min at 4°C. After elimination of the supernatant, 100 μl of trypsin (2 mg/ml, BD, Franklin Lakes, NJ, USA) was added and allowed to react for 30 min at 37°C. MCHBBS was added to the remaining hemocyte suspension for the control reading. One hundred microliters of NBT (0.3% in MCHBBS) was added to the hemocyte-trypsin mixture and incubated at 37°C for 30 min. The reduction response was terminated by adding 100 μl of absolute methanol. Hemocytes were

washed 3 times with 70% methanol and air-dried. For coating, 120 μl of 2 M KOH and 140 μl of dimethyl sulfoxide were added to dissolve the cytoplasmic formazan. The concentration of the dissolved cytoplasmic formazan was measured at 630 nm with a microplate reader. The absorbance ratio between trypsin-elicited hemocytes and the control was used as an index to compare the effects of different treatments on reactive oxygen species production.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). All data were analyzed to test the statistical significance among groups by analysis of variance (ANOVA) followed by Duncan's test. p values of < 0.05 were accepted as being statistically significant.

RESULTS

Mortality in an anoxic condition

The mortality of control clams reached 100% in 18 d (Fig. 2). There was no difference between recovered clams after 24 h of air-exposure and the control. Clams exposed to the air for 48 and 72 h showed high mortality compared to the 24 h exposure and control groups. The LT_{50} values of 24, 48, and 72 h air-exposed and control clams were about 7, 4, 2, and 8 d, respectively.

THC

The THC in the 24 h air-exposed group decreased with respect to the control. However, the decrease in this group completely recovered when the clams were returned to the seawater tanks (Fig. 3). Clams exposed to air for 48 h showed significantly reduced THC, and the count partially recovered after placing them in seawater for 24 h. Clams exposed to air for 72 h had significantly decreased levels of THC, and the levels did not recover.

Total hemolymph protein concentration

There were no significant changes in total hemolymph protein concentrations after air exposure. The concentrations also did not change following recovery treatments.

Phagocytic activity

The phagocytic rate in the 24 h exposure group was significantly reduced compared to the control. In the recovery group after 24 h of air exposure, the phagocytic rate was significantly higher compared to the pre-recovery period, but its level was still somewhat lower than the control (Figs. 4, 5). In the 48 h exposure group, the

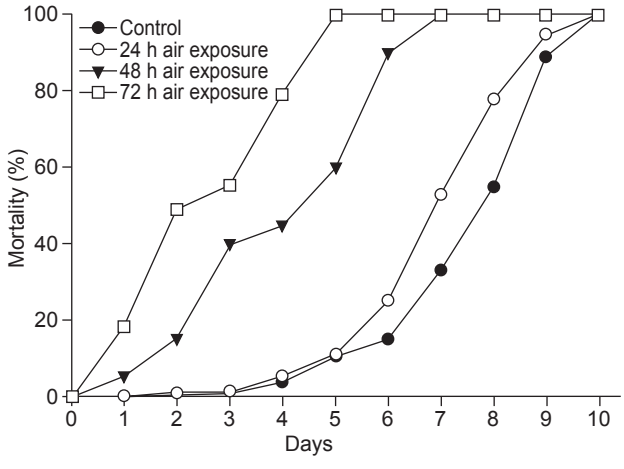

Fig. 2. Mortality of air-exposed *Mactra veneriformis*. The median lethal time (LT_{50}) of the clams in the control group was measured at 8 d. The LT_{50} values with 24, 48, and 72 h of exposure were measured at 6.8, 4.3, and 2.2 d, respectively (n = 100).

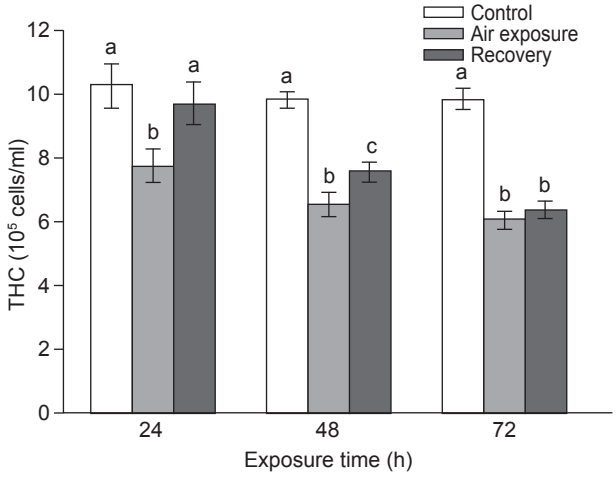

Fig. 3. Total hemocyte count (mean ± SEM) of air-exposed *Mactra veneriformis*, expressed as 10^5 cells/ml. Column bars with different letters indicate significantly different values (p < 0.05) (n = 4).

phagocytic rate was further reduced, and the rate did not recover during the 24 h seawater recovery procedure. Similarly, 72 h of air exposure led to an irrecoverable reduction in phagocytic activity.

Lysozyme activity

Clams exposed to air for 24 h exhibited significantly lower levels of lysozyme activity, but it completely recovered after 24 h in seawater (Fig. 6). In contrast, the enzyme levels did not recover after reduction following 48 or 72 h of air exposure.

PO activity

After exposure to air for 24 or 48 h, significant reductions in PO were observed. The reduction tended to only partially recover when the clams were placed in seawater for 24 h (Fig. 7). However, with 72 h of air exposure, the reduction in PO did not recover.

NBT reduction

Exposure to air for 24 h produced a slight decrease in the NBT-reducing property, and the reduction completely recovered after 24 h in seawater (Fig. 8). Longer periods of air exposure, of 48 and 72 h, resulted in dramatic decreases in the NBT-reducing ability. These decreases, however, did not recover after the clams were returned to seawater.

DISCUSSION

As is the case with all aquatic organisms, bivalves are susceptible to environmental changes such as dissolved oxygen, temperature, and salinity (LeBlanc et al. 2005, Marin et al. 2005, Gagnaire et al. 2006, Ottaviani et al. 2007). Bivalves residing in intertidal habitats are intermittently exposed to air owing to ebb tidal changes. To evaluate the effects of oxygen deficiency derived from lower tidal levels, we examined the influence of long-term air exposure on hemolymph parameters involved with immune functions in *Mactra veneriformis*, an intertidal bivalve.

Changes in THC indicate the status

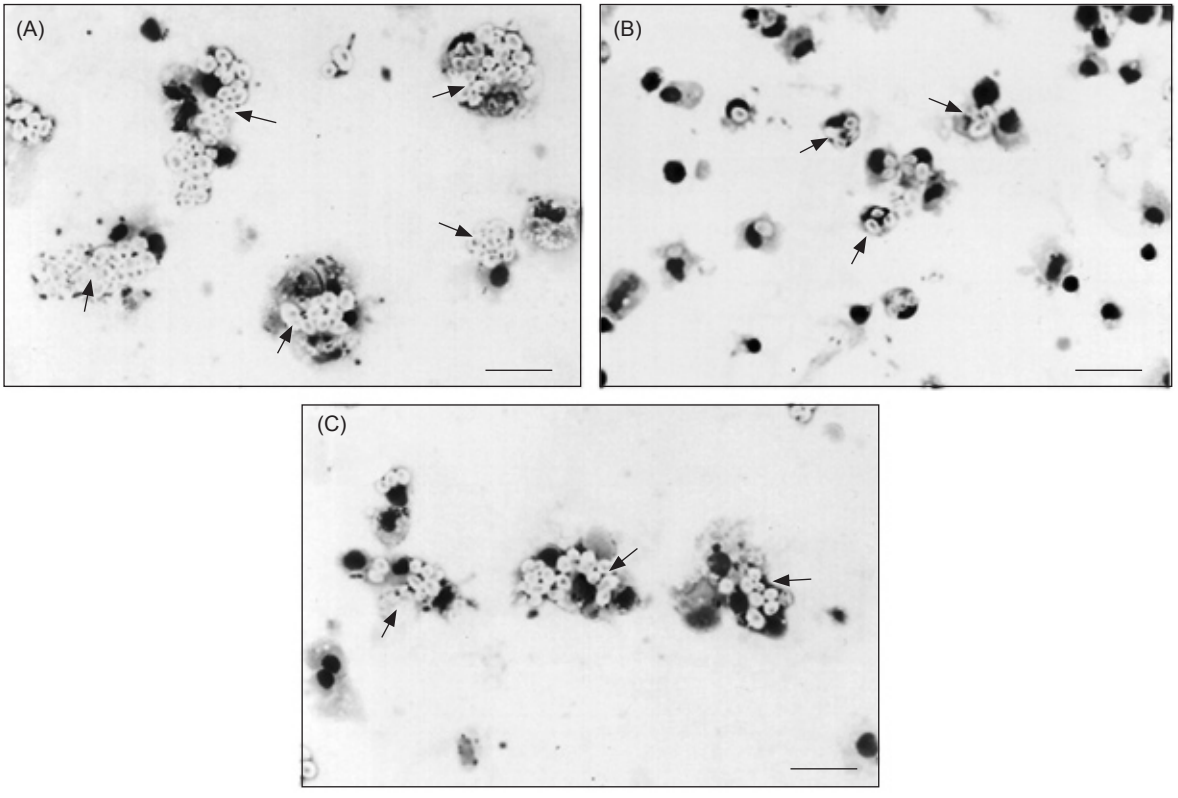

Fig. 4. Hemocytes of *Mactra veneriformis*. Hemocytes in control (A) and recovery clams (C) showing numerous ingested zymosans (arrows). Otherwise, hemocytes from air-exposed clams (B) showing a few engulfed zymosans (arrows). Scale bar = 10 μm.

of alterations in hemocyte proliferation or inflammatory mobilization to peripheral tissues (Pipe et al. 1995, Park and Oh 2006). We observed that THC decreased after air exposure for periods of 24-72 h. The bivalve retained the ability to recover when the exposure was short, e.g., 24 h, but the ability seemed to deteriorate if the period was prolonged beyond that. Similar to our results, it was reported in *Chamelea gallina* that the THC was considerably reduced by 1 d of air exposure, and this reduction slowly recovered over a 2-4 d period (Pampanin et al. 2002, Marin et al. 2005). Such air-exposure-induced THC reductions were reversible after 24 h of exposure, but not after 48 h (Marin et al. 2005). THC reduction in the hemolymph can be attributed to an accelerated movement of hemocytes to peripheral tissues. Excess mobilization of hemocytes out of the hemolymph was demonstrated in *C. gallina* where hemolymph moved to the gonads to remove cell debris produced during spawning (Suresh and Mohandas 1990b, Matozzo et al. 2003, Marin et al. 2005). It is not clear yet whether anoxic stress induced by air exposure stimulated hemocyte

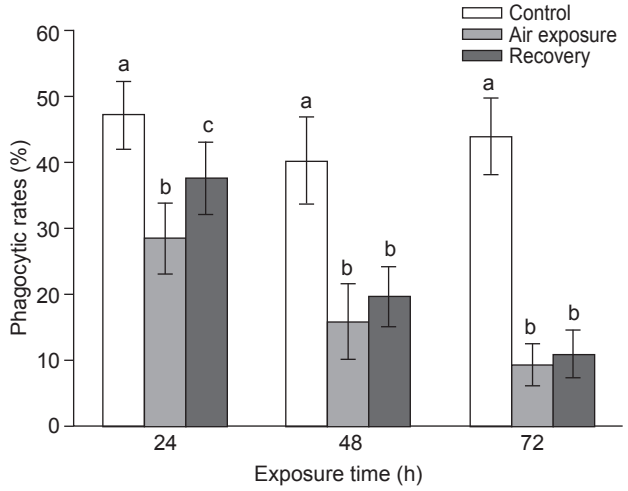

Fig. 5. Effects of air exposure on the phagocytic activity of hemocytes in *Mactra veneriformis*. Results are expressed as the mean ± SEM; $n = 6$. Column bars indicate significant differences ($p < 0.05$).

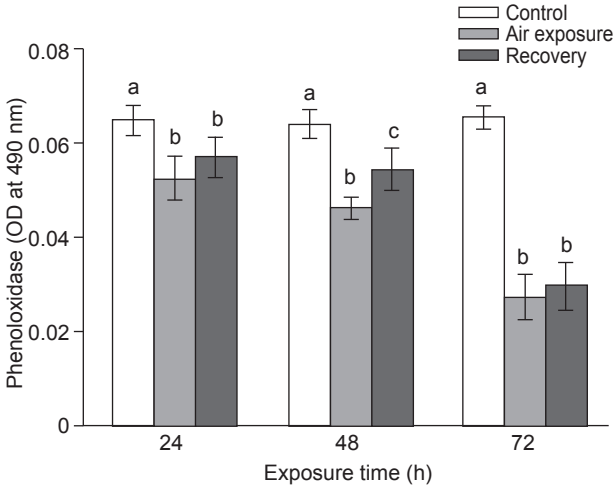

Fig. 7. Effects of air exposure on phenoloxidase activity (mean ± SEM) of the hemolymph lysate from *Mactra veneriformis*. Column bars with different letters significantly differ ($p < 0.05$) from the control group ($n = 6$).

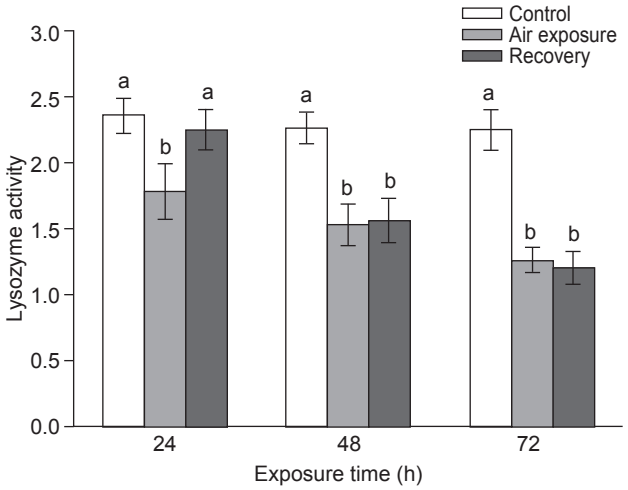

Fig. 6. Lysozyme activity (mean ± SEM) of the hemolymph of air-exposed *Mactra veneriformis*. Column bars with different letters significantly differ ($p < 0.05$) from the control group ($n = 6$).

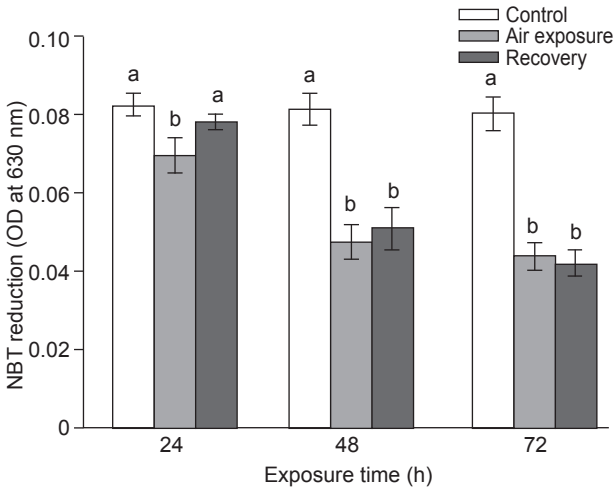

Fig. 8. Nitroblue tetrazolium (NBT) reduction (mean ± SEM) of hemocytes from air-exposed *Mactra veneriformis*. Column bars with different letters significantly differ ($p < 0.05$) from the control group ($n = 6$).

leakage. In this study, oxygen deficiency affected the THC level of *Mactra veneriformis*.

Phagocytic activity was also diminished in *M. veneriformis* when clams were exposed to air for longer than 24 h. Pampanin et al. (2002) reported that phagocytic activity decreased in *Chamelea gallina* with 1 d of air exposure, and the decrease recovered over a 3 d period. In *Mytilus edulis*, a reduction was induced by only 30 min of air exposure (Ottaviani et al. 2007). Phagocytosis by bivalve hemocytes is a crucial mechanism for immune function (Cheng and Sullivan 1984, Pipe and Coles 1995). This mechanism can be compromised in adverse conditions, such as with exposure to toxicants (Marin et al. 2005). Although the mechanism for the decline in phagocytosis is not clear, phagocytic function seems to be markedly influenced by anoxia, and its recovery seems to be extremely slow.

Lysozyme is the main bacteriolytic agent against several species of gram-positive and -negative bacteria (Marin et al. 2005 2007). The enzyme is synthesized in bivalve hemocytes and then secreted into the hemolymph during phagocytosis (Cheng et al. 1975). Its activity can be used as an indicator of the health status and the vitality of the defense system in bivalves (Chu and La Peyre 1989). Similarly, *Mercenaria mercenaria* showed a reduction in lysozyme under anoxic stress (Hawkins et al. 1993). Marin et al. (2005) suggested that the secretion of lysozyme from hemocytes to the hemolymph occurs as a consequence of higher phagocytosis in *C. gallina*. We found that lysozyme activity decreased with 24, 48, and 72 h of air exposure, whereas recovery of the activity was only observed in the clams exposed for 24 h. Anoxia also induced a reduction in lysozyme in *M. mercenaria* (Hawkins et al. 1993). Because lysozyme activity was measured in this study after removal of hemocytes, its activity reflects the enzyme activity produced by the preexisting hemocytes. As discussed above, the THC was reduced by air exposure. As the extents of reductions in THC and lysozyme were very similar, it could be deduced that the reduction in lysozyme was directly related to the decrease in hemocytes in anoxic conditions.

PO is the last component of a reaction cascade in mollusks consisting of a pool of defensive molecules released from immune-competent cells into the hemolymph during body injury (Coles and Pipe 1994, Hernandez-Lopez et al. 1996, Esteban et al. 2006, Raftos et al., 2007). Both increases and decreases in its activity were reported in bivalves infected by pathogens (Esteban et al. 2006, Peters and Raftos 2003). In the present study, clams exposed to an anoxic state exhibited low PO activity. This response suggests that in anoxic conditions, *M. veneriformis* may experience compromised immune function, and thus the host would be more susceptible to pathogens.

Phagocytosis is involved in the generation of reactive oxygen intermediates (ROIs) by the process called respiratory bursts. The degree of ROI generation during respiratory bursts depends on the number of hemocytes circulating in the hemolymph (Verghese et al. 2007). NBT reduction is a method to assess the rate of ROI production. In this study, the patterns of NBT reduction in anoxic conditions were well correlated with THC levels. This result suggests that NBT reduction can be a parameter to evaluate bivalve immune function under anoxic stress.

The present study showed that a lack of oxygen during air exposure may induce alterations in the immune function of *M. veneriformis* by decreasing the THC, lysozyme activity, phagocytosis, PO activity, and NBT reduction. These alterations may be associated with compromised immune functions which could lead to detrimental consequences such as reduced disease resistance, growth impairment, and lower survival rates. Yields of *M. veneriformis* have been decreasing over the past decade but the cause has been unclear. Although this study did not explore all factors that affect the western coast of Korea, it is feasible to suspect a single culprit for the drop in clam yields. Large quantities of dead clams including *M. veneriformis* and *Meretrix lusoria* were observed in the sampling area. Specifically, the area suffered dramatic sea level changes due to an extensive reclamation project, called the Saemangeum Reclamation. Therefore, future studies should ascertain the various environmental factors that affect immune function of *M. veneriformis* in this area.

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REFERENCES

Cheng TC, WJ Doughetty. 1989. Ultrastructural evidence for the destruction of *Schistosoma mansoni* sporocysts associated with elevated lysosomal enzyme levels in *Biomphalaria glabrata*. J. Parasitol. **75:** 928-941.

Cheng TC, GE Rodrick, DA Foley, SA Koehler. 1975. Release of lysozyme from haemolymph cells of *Mercenaria mercenaria* during phagocytosis. J. Invertebr. Pathol. **25:** 261-265.

Cheng TC, JT Sullivan. 1984. Effects of heavy metals on phagocytosis by molluscan hemocytes. Mar. Environ. Res. **14:** 305-315.

Chu FE, JF La Peyre. 1989. Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*). J. Invertebr. Pathol. **54:** 224-232.

Chu FL, JF La Peyre. 1993. Development of disease caused by the parasite *Perkinsus marinus* and defense-related hemolymph factors in three populations of oysters from the Chesapeake Bay, USA. J. Shellfish Res. **12:** 21-27.

Coles J, RK Pipe. 1994. Phenoloxidase activity in the haemolymph and haemocytes of the marine mussel *Mytilus edulis*. Fish Shellfish Immunol. **4:** 337-352.

Esteban MÁ, P Muñoz, J Moseguer. 2006. Phenoloxidase activity in three commercial bivalve species. Changes due to natural infestation with *Perkinsus atlanticus*. Fish. Shellfish Immunol. **20:** 12-19.

Gagnaire B, H Frouin, K Moreau, H Thomas-Guyon, T Renault. 2006. Effects of temperature and salinity on haemocytes activities of the Pacific oyster, *Crassostrea gigas*. Fish. Shellfish Immunol. **20:** 536-547.

Gagnaire B, M Gay, A Huvet, JY Daniel, D Saulnier, T Renault. 2007. Combination of a pesticide exposure and a bacterial challenge: *in vivo* effects on immune response of Pacific oyster, *Crassostrea gigas* (Thunberg). Aquat. Toxicol. **84:** 92-102.

Gagné F, C Blaise, M Fournier, PD Hansen. 2006. Effects of selected pharmaceutical products on phagocytic activity in *Elliptio complanata* mussels. Comp. Biochem. Physiol. **143:** 179-186.

Hawkins LE, LD Brooks, S Brooks, S Hutchinson. 1993. The effect of tidal exposure on aspects of metabolic and immunological activity in the hard calm *Mercenaria mercenaria* (Lineaeus). Comp. Biochem. Physiol. **140(Supplement A):** 225-228.

Hernandez-Lopez J, T Gollas-Galvan, F Vargas-Albores. 1996. Activation of the prophenoloxidase system of the brown shrimp *Penaeus californiensi*s Holmes. Comp. Biochem. Physiol. **113(Supplement C):** 61-66

Lacoste A, SK Malham, F Gélébart, A Cueff, SA Poulet. 2002. Stress-induced immune changes in the oyster *Crassostrea gigas*. Dev. Comp. Immunol. **26:** 1-9.

LeBlanc N, T Nandry, H Stryhn, R Tremblay, M McNiven, J Davidson. 2005. The effect of high air and water temperature on juvenile *Mytilus edulis* in Prince Edward Island, Canada. Aquaculture **243:** 185-194.

Marin MG, V Matozzo, M Monari, J Foschi, T Papi, O Cattani. 2005. Exposure to anoxia of the clam *Chamelea gallina*. I. Effects on immune response. J. Exp. Mar. Biol. Ecol. **325:** 163-174.

Marin MG, M Monari, V Matozzo, J Foschi, O Cattani, GP Serrazanetti. 2007. Effects of high temperature on functional responses of haemoyctes in the clam, *Chamelea gallina*. Fish Shellfish Immunol. **22:** 1-17.

Matozzo V, L Da Ros, L Ballarin, F Meneghetti, MG Marin. 2003. Functional response of haemocytes in the clam *Tapes philippinarum* from the Lagoon of Venice: fishing impact and seasonal variations. Can. J. Fish. Aquat. Sci. **60:** 949-958.

Ministry of Land, Transport and Maritime Affairs (MLTM). 2007. Statistics and annual report. Available at http://www.mltm.go.kr/USR/statistics/m_53/lst.jsp

Ottaviani E, E Caselgrandi, F Petraglia, C Franceschi. 1992. Stress response in the freshwater snail, *Planorbarius corenus*: interaction between CRF, ACTH and biogenic amines. Gen. Comp. Endocrinol. **87:** 354-360.

Ottaviani E, D Malagoli, L Casarini, S Sacchi. 2007. Stress and immune response in the mussel *Mytilus galloprovincialis*. Fish. Shellfish Immunol. **23:** 171-177.

Pampanin DM, LB Ballarin, L Carotenuto, MC Marin. 2002. Air exposure and functionality of *Chamelea gallina* haemocytes: effects on haematocrite, adhesion, phagocytosis and enzyme contents. Comp. Biochem. Physiol. **131:** 605-614.

Park SW, SG Kang, YG Kim. 2000. The morphology of the hemocytes of the clam, *Ruditapes philippinarum* (Mollusca: Bivalvia). J. Fish. Pathol. **13:** 129-136. (in Korean with English summary)

Park SW, MJ Oh. 2006. Fish and shellfish hematology. Seoul, Korea: Daekyung, pp. 175-191. (in Korean)

Peters R, DA Raftos. 2003. The role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). Aquaculture **223:** 29-39.

Pipe RK, JA Coles. 1995. Environmental contaminants influencing immune function in marine bivalve molluscs. Fish. Shellfish Immunol. **5:** 581-595.

Pipe RK, JA Coles, ME Thomas, VU Fossato, AL Pulsford. 1995. Evidence for environmentally derived immuno-modulation in mussels form the Venice Lagoon. Aquat. Toxicol. **32:** 59-73.

Raftos DA, S Aladaileh, PM Rodney, SV Nair. 2007. Characterization of phenoloxidase activity in Sydney rock oysters (*Saccostrea glomerata*). Compar. Biochem. Physiol. **148:** 470-480.

Ryou DK, SC Chung. 1994. Ecological studies on the population of surf clam, *Mactra veneriformis*. PhD dissertation, Cheju National Univ., Cheju, Korea. (in Korean with English summary)

Shiau SY, MH Lee. 2002. Dietary vitamin C and its derivatives affect immune responses in grass shrimp, *Penaeus monodon*. Fish. Shellfish Immunol. **12:** 119-129.

Suresh K, A Mohandas. 1990. Number and types of hemocytes in *Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis* (Bivalvia), and leukocytosis subsequent to bacterial challenge. J. Invertebr. Pathol. **55:** 312-318.

Tsuchiya M. 1983. Mass mortality in a population of the mussel *Mytilus edulis* L. caused by high temperature on rocky shores. J. Exp. Mar. Biol. **30:** 101-111.

Verghese B, EV Radhakrishnan, A Padhi. 2007. Effects of environmental parameters on immune response of the Indian spiny lobster, *Panulirus homarus* (Linnaeus, 1758). Fish. Shellfish Immunol. **23:** 928-936.

Vetvicka V, P Sima. 1998. Evolutionary mechanisms of defense reactions. Basel, Switzerland: Birkhauser Verlag, pp. 70-86.