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Effects of spawning on immune functions in the surf clam *Mactra veneriformis* (Bivalvia: Mactridae)

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The production of surf clam, *Mactra veneriformis*, an important fishery resource in Korea, has recently been decreasing. This study was carried out to examine effects of spawning on immune functions of this species. Total hemocyte count (THC), phenoloxidase (PO) activity, phagocytic activity, neutral red retention (NRR) time and antibacterial activity were assessed. Spawned clams showed reduction in THC, PO, phagocytic activity and NRR times compared with unspawned ones. While spawning event did not elicit any change of antibacterial activity in both spawned and unspawned ones. This study indicates that spawning process decreases immune functions in the surf clams which could cause mortality increment and yield reduction.

Key words :Surf clam, *Mactra veneriformis*, Spawning, Immune function

The surf clam, *Mactra veneriformis* (Bivalvia: Mactridae), inhabits the western and southern coast of the Korean peninsula, and represents one of the crucial fishery resources in the region (Yu *et al*., 2009). The yield of surf clam from the western coast of Korea was estimated to be 1,444 tons in 2008, but the amount has been declining dramatically to about 698 tons by 2009 (FPS 2009). Until now, the causes of reduction are not well understood but some researchers suggested multifactor causes, including high temperature and anoxia (Yu *et al*., 2009 and 2010).

Spawning period of surf clam varies depending upon habitats and climate. In general, the spawning occurs in April through August; nearly all clams are being completely spent (Chung *et al*., 2008). During the period, moribund or dead clams are easily found. Considering the occurrence of mortality in surf clam during the spawning period, it has been hypothesized that spawning, to some extent, is responsible for the yield reduction. However, immunological studies on surf clam in relation to spawning stress have not been performed in spite of its importance as a culture species. Thus, the aim of this study was to assess the influence of spawning stress on total hemoyctes counts (THC), phenoloxidase (PO) activity, neutral red retention (NRR), phagocytic activity and antibacterial activity in surf clam.

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Materials and Methods

Surf calm

Three hundred of fully ripened clams were sampled from the inter-tidal zone off the Yeongheung seashore located in Incheon, Korea (37° 13' N and 126° 27' 20 E) in July (spawning period). The height and length of the shells (mean ± S.E., n = 50) were 29.8 ± 1.6 mm and 33.1 ± 1.2 mm, respectively. The temperature, salinity and specific gravity of the site were 24.5 ± 1.0°C, 32.5 ± 1.0 ppt and 1.028 ± 0.002, respectively, measured for 5 days when sampled. For laboratory acclimation, clams were maintained in 800 L fiber glass reinforced plastic (FRP) tanks supplied with filtered sea water (temperature 22-25°C, salinity 32-33 ppt, specific gravity 1.022-1.024) for a week. The clams were fed every day ad libitum with a mixed algal diet consisting approx. equal weight of *Isochrysis galbana*, *Monochrysis luther* and *Nannochloris oculata*.

Spawning induction

Regardless of sexes, half of the clams were kept at 25°C as the pre-spawning population, while the other half received a spawning stimulation through thermal escalation at 30°C to obtain post-spawning clams. Spawned clams were removed immediately and placed in different tanks at 25°C. Expelled gametes were determined by turbid water in tanks and gonads shrinkage when clams shucked. Unspawned clams were discarded. All tests were performed in triplicate tanks each containing 30 clams.

Hemolymph collection

Hemolymph was collected from the posterior adductor muscle using a 1-ml disposable syringe. For each analysis, pooled hemolymph from 3 clams was used.

Total hemocyte count (THC)

One hundred microliters of pooled hemolymph were 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).

Phenoloxidase (PO) activity

PO activity was measured following a modified method of Aladaileh *et al*. (2007). In short, hemolymph collected in MAS (1: 3) was centrifuged at 400 × g for 5 min at 4°C. After decanting the supernatant, the pellet was resuspended in 0.1 M phosphate buffered saline (PBS, pH 7.4) at a concentration of 1×10^6 mL^{-1}. To prepare hemocyte lysate, the hemolymph was frozen at -80°C and thawed at 25°C repeatedly for 3 times. The lysate was centrifuged at 1500 × g at 4°C for 10 min to remove cellular debris. One hundred microliters of the hemocyte lysate were pipetted into 96 well plates, and then 100 µl of 1 mM 3-methyl-2-benzothiazolinone were added. One hundred microliters of L- dihydroxyphenylalanine (2 mg mL^{-1} in filtered sea water) were added to the mixture, and absorbance was instantly measured at 490 nm with a microplate reader (BioRad, Model 680, Hercules, CA, US). Plates were then incubated for 1 hour at room

temperature in the dark before a second reading was taken. PO activity was calculated by subtracting the first readings from measurement made after 1 hour.

Neutral red retention (NRR) assay

The NRR assay was carried out by the method of Yu *et al*. (2009) which is modification from Hauton *et al*. (1998) and Li *et al*. (2006). Briefly, neutral red stock solution was made by dissolving 2.0 mg of 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 water). Two hundred microliters of hemolymph were placed into a tube containing 200 µl of the *Ostrea* saline solution and gently mixed. Forty microliters of the mixture were smeared on slide glasses by centrifuging in a cell-collection apparatus (Hanil, Korea) at 96 g for 3 min. Sixty microliters of neutral red working solution were 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 cm) and then granulocytes were examined under a microscope (CH2, Olympus, Japan) 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 (Pipe, 1990).

Phagocytic activity

Phagocytic activity was evaluated following the method of Park *et al*. (2000). In short, 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 sea water (FSW) at a final concentration of 10^5cells mL^{-1}. To prepare the zymosan solution, zymosan (Sigma, St. Louis, MO, USA) was suspended in FSW at a concentration of 2 mg mL^{-1}, 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 10^6 particles mL^{-1}. One-half millimeter of hemocyte suspension were mixed with 1 ml of the zymosan suspension and then incubated at 25°C for 60 min. Five millimeters of cold MAS (4°C) was added to the mixture, and then the mixture was centrifuged at 230 × g for 10 min. The supernatant was discarded, and hemocytes were 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. Two replicates were prepared for each smear, and the phagocytic activity of 100 hemocytes was determined for each replicate.

Anti-bacterial activity

Anti-bacterial activity was measured with a modified method of Ordás *et al*. (2000). Shortly, 25 µl of cell-free hemolymph were placed in 96 well plates and then 75 µl of *Vibrio harveyii* (ATCC 14126) (OD 0.9020～0.9050 at 600 nm) suspended in tryptone soy broth (TSB) were added to the plates. After incubation for 3 hours at 18°C, the plates were centrifuged for 10 min at 200 × g, the supernatant decanted and 100 µl of 3-(4,5-dimethylthiazole-2-yl) 2,5-diphenyltetrazolium bromide

(0.5 mg mL^{-1} in TSB) were added to the plates. After 15 min in the dark at 20°C, the absorbance at 600 nm was measured. In control, the cell-free hemolymph was replaced by TSB. The anti-bacterial activity index (BI) was calculated as below:

BI = sample ABS_{600}/control ABS_{600}

Statistic analysis

Results were expressed as the mean ± standard error of the mean (SEM). Statistical significance among groups was tested by analysis of variance (ANOVA) followed by Duncan's test. The *p*-values of < 0.05 were accepted as being statistically significant.

Results

Overall data show that spawning stress affected THC, PO activity, NRR and phagocytic activity. Spawned clams exhibited dramatically decreased THC (mean: 6.6 $\times 10^5$ cells mL^{-1}) in comparison with unspawned ones (mean: 12.0 $\times 10^5$ cells mL^{-1}) (Fig. 1). In PO activity, spawned clams exhibited significantly lower levels (mean: 0.10 ± 0.01) compared with unspawned ones (mean: 0.17 ± 0.03) (Fig. 2). Similar and significant reduction was observed in NRR, an indication of lysomal membrane stability. The mean time of NRR in the spawned clams was 92.1 ± 11.5 min whereas that for unspawned ones was 55.2 ± 9.6 min (Fig. 3).

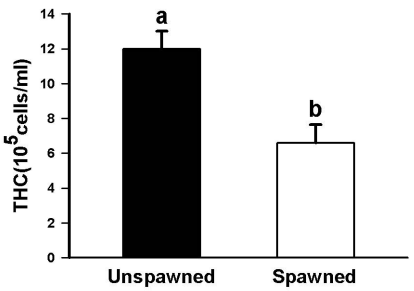

Fig. 1. Total hemocyte count (mean ± SEM) of unspawned or spawned *Mactra veneriformis*, expressed as 10^5 cells/ml. Column bars with different alphabet indicate significantly different values ($p < 0.05$) (n = 3, pooled from 3 individuals each trial).

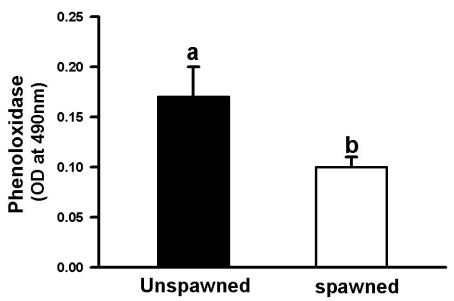

Fig. 2. Effects of spawning on phenoloxidase activity (mean ± SEM) of the hemolymph lysaste from *Mactra veneriformis*. Column bars with different alphabet indicate significantly different values ($p < 0.05$) (n = 3, pooled from 3 individuals each trial).

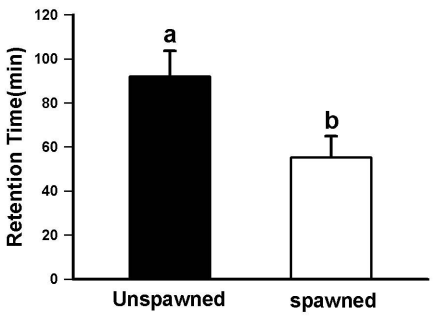

Fig. 3. Effects of spawning on neutral red retention time (mean ± SEM) of the hemocytes in *Mactra veneriformis*. Column bars with different alphabet indicate significantly different values ($p < 0.05$) (n = 3, pooled from 3 individuals each trial).

Phagocytic rate in the spawned clams (mean 24.4 ± 3.7) was also reduced compared with those in unspawned ones (mean 18.2 ± 3.0) (Fig. 4). There was no significant difference in anti-bacterial activity between unspawned (1.00 ± 0.07) and spawned (0.96 ± 0.16) clams (Fig. 5).

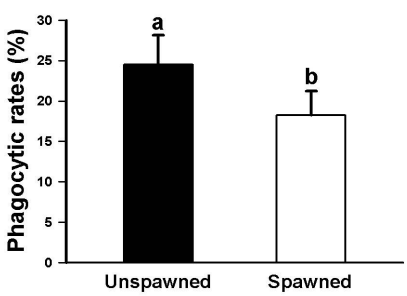

Fig. 4. Effects of spawning on the phagocytic activity (mean ± SEM) of the hemocytes in *Mactra veneriformis*. Column bars with different alphabet indicate significantly different values ($p < 0.05$) (n = 3, pooled from 3 individuals each trial).

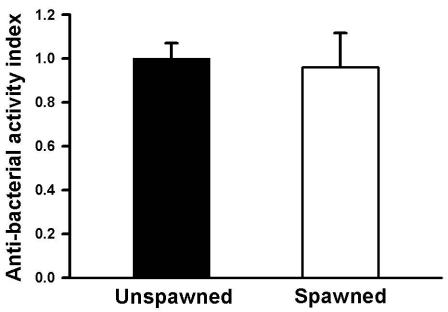

Fig. 5. Effects of spawning on anti-bacterial activity (mean ± SEM) of hemolymph in *Mactra veneriformis* ($p < 0.05$) (n = 3, pooled from 3 individuals each trial).

Discussion

Like other aquatic organisms, bivalves are susceptible to environmental and physiological changes such as temperature, salinity, pathogens, spawning or anoxia (Cho and Jeong, 2005; LeBlanc *et al*., 2005; Marin *et al*., 2005; Beatrice *et al*., 2006; Ottaviani *et al*., 2007; Yu *et al*., 2009, 2010). During the summer when water temperature reaches above 20°C and induces full sexual ripeness, high mortality of surf clams occurs. But the cause resulted in the high mortality is not yet clear. We hypothesized that summer mortality could be related with spawning events that are quite stressful to surf clams.

Changes in THC reflect the status of hemocyte proliferation or inflammatory mobilization to peripheral tissues (Pipe and Coles, 1995; Beatrice *et al*., 2006, Marin *et al*., 2007). In this study, we observed a significant reduction in THC after spawning. Similarly to our results, it was reported in *Crassostrea gigas* that the THC was reduced by spawning (Cho and Jeong, 2005). The current THC reduction in surf clam can be

attributed to an accelerated movement of hemocytes to peripheral tissues (Pipe and Coles, 1995; Carballal *et al*., 1998; Marin *et al*., 2007; Yu *et al*., 2009, 2010). Excess movement of hemocytes out of the hemolymph was demonstrated in *Chamelea gallina* where hemolymph circulated to the gonads to remove cell debris produced during spawning processes (Suresh and Mohandas, 1990; Matozzo *et al*., 2003; Marine *et al*., 2005, Yu *et al*., 2010). Whenever the mechanism for the THC reduction after spawning, it seems clear that THC is markedly by spawning.

A significant decrease in PO activity was also observed in the spawned clams. PO is the last component of a defensive reaction cascade in mollusks released from immune competent cells into the hemolymph during body injury (Peters and Raftos, 2003; Esteban *et al*., 2006; Aladaileh *et al*., 2007, Yu *et al*., 2010). In this study, spawned clams showed lower PO activity compared with unspawned ones. This response suggests that spawned clams may have been experiencing, compromised immune functions and thus the bivalves would be more sensitive to pathogens.

Neutral red is a lipophilic dye that can permeate the cell membrane fairly freely. NRR assay has been employed due to the property that stressed hemocytes do not take up as much of the dyes as normal cells do (Borenfreund and Puerner, 1985; Hauton *et al*., 1998; Li *et al*., 2006; Yu *et al*., 2009). The assay has been widely applied to evaluate the changes deriving from environmental, physiological and mechanical stresses in mollusks (Lowe and Pipe, 1994; Cho and Jeong, 2005; Li *et al*., 2006; Yu *et al*., 2009). We observed that NRR value was reduced in spawned clams. Similarly to our results, it was reported in *C. gigas* that NRR value of hemocytes significantly decreased after spawning. It was hypothesized that oysters were in weak condition during spawning period owing to the energetic cost of the spawning event and this led to summer mortalities (Bertheline *et al*., 2000; Perdue *et al*., 1981; Cho and Jeong, 2005). Our results suggest that lipid membrane of hemocytes in surf clam is unstable, may be due to energetic cost of the spawning, and such instability resulted in rapid release of neutral red from the lysosome.

Pahgocytosis is one of the main defense mechanisms against non-self materials in bivalves (Marine *et al*., 2007; Yu *et al*., 2009). In Pacific oyster, *C. gigas*, phagocytic activity was impaired during the spawning period (Beatrice *et al*., 2006; Duchemin *et al*., 2007). In this study, phagocytic activity in spawned clams decreased compared with those in unspawned ones. This result indicates that less energy supply for hemocyte phagocytosis due to diversion for spawning activity might have caused reduction of phagocytic activity as proposed by Delaporte *et al*. (2006).

Many cytotoxic molecules have been described in the hemocytes and plasma of bivalves including lysosomal hydrolases, reactive oxygen species, reactive nitrogen species, and antimicrobial peptides (Pipe, 1990; Adema *et al*., 1991; Anderson, 1996; Roch *et al*., 1996; Arumugan *et al*., 2000; Anderson and Beaven, 2001). These activities may be found as naturally-occurring components of the hemolymph, or they can be enhanced by infections and various experimental treatments. Cheng *et al*. (1975), Cheng (1992) and Anderson and Beaven (2001) described that endogenous antibacterial agents are synthesized and stored in the hemocytes, and

released into the plasma when the cells are stimulated by microbial infection. In this study, no difference in the antibacterial activity was observed in both unspawned and spawned clams. It is not demonstrated yet that surf clam can bio-synthesize antibacterial substances. However, we can also postulate that the spawning stress might be not severe enough to stimulate hemocytes to secret various antibacterial agents into the hemolymph.

The present study showed that spawning stress may induce changes in the immune function of surf clam in terms of decreased THC, PO activity, NRR and phagocytic activity. These changes may be related with compromised immune functions that led to detrimental consequences such as decreased disease resistance and lower survival rates. In the west cost of Korea, the spawning of surf clams usually begins in summer period (average temperature 24~29°C). Moribund or dead clams, a reflection of yield reduction, are easily identified during the period. It is difficult, however, to conclude that spawning causes the yield reduction of surf clam because this study was performed only under a laboratory condition. Nevertheless, we can speculate that spawning may at least increase susceptibility to various pathogens causing summer mortality and yield reduction, due to energy depletion required for competent immune functions.

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