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Annual Changes in Maturation of the Gonad and Phagocytic Activity in Hemocytes of the Pacific Oyster, *Crassostrea gigas*, in Onagawa Bay, Miyagi Prefecture

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Abstract: We investigated the annual reproductive cycle and seasonal changes of hemocytic density and phagocytic activity in hemocytes of the Pacific oyster, *Crassostrea gigas*, from May 1996 to February 1997. Phagocytic activity in the hemocytes was estimated to evaluate the phagocytic rate and the phagocytic index. The gonadal condition of each oyster was classified into one of four stages: the developmental stage, the mature stage, the recovery stage, and the resting stage. From May to June, hemocytic density and phagocytic activity in the hemocytes increased corresponding to gonadal development, and higher values were therefore detected in the mature gonad group. The group of oysters at the mature stage in June showed the highest values of hemocytic density and phagocytic activity in the hemocytes. Contrastively, in the group collected in September, which was soon after the spawning period, hemocytic density and phagocytic activity were at decreased levels, the phagocytic index being markedly decreased. These results suggest that gonadal maturation and spawning affect density and phagocytic activity in the hemocytes and that reproduction is one major factor affecting the cellular host-defense system mediated by hemocytes.

Key words: Pacific oyster; Gonadal maturation; Phagocytic activity; Host-defense

In marine bivalve molluscs, cellular immunity is mediated by hemocytes, which participate in a variety of internal defense mechanisms. In particular, phagocytosis of hemocytes plays an important role in elimination of invading microorganisms such as bacteria[1,2], or viruses[3,4]. It is well known that environmental factors such as water temperature and salinity influence defense capabilities in the hemocytes of bivalve molluscs[5]. However, little is known about internal factors which influence the density and phagocytic activity in hemocytes.

The effort of reproduction is associated with a considerable consumption of energy, and in the Pacific oyster, *Crassostrea gigas*, the physiological activity and glycogen content decrease during the period of spawning[6]. It has therefore been assumed that gonadal maturation and spawning influence defense activities such as phagocytic activity of hemocytes, in marine bivalve molluscs.

In this study, we investigated the annual reproductive cycle and seasonal changes of hemocytic density and phagocytic activity in hemocytes of *C. gigas*. Results of this study suggest that both hemocytic density and phagocytic activity in the hemocytes are markedly enhanced by physiological activation in response to gonadal maturation, and that these functions in hemocytes are greatly lowered by the consumption of energy due to spawning.

Materials and Methods

Animals

Specimens of 2-year-old oysters, *C. gigas*,

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hanging-cultured at Matsushima Bay, Miyagi Prefecture, were obtained in March 1996. They were then kept by hanging in Takenoura Inlet, Onagawa Bay, Miyagi Prefecture until sampling. At each of 9 sampling, five oysters were collected during the period of May 1996 to February 1997. Fig. 1 shows the changes of water temperature and salinity in the area where the specimens were hanging during the experimental period.

Classification of Gonadal Condition of C. gigas

The gonad of each oyster used in this study was histologically observed to classify its condition. Gonads were dissected and fixed with Bouin solution for 3-7 days. Fixed samples were dehydrated with ethanol and embedded in paraffin. Six-micrometer transversal sections were stained with hematoxylin and eosin, and then observed using a light microscope.

Preparation of Hemocyte Suspension

Hanks' balanced salt solution (BSS, Flow Laboratories) for hemocytes of *C. gigas* was prepared by adding 20.3 g/*l* NaCl and 3.5 g/*l* glucose to adjust pH to 7.4. The BSS was filtered through a 0.22 μm membrane filter (MILEX GS, Millipore Co.). Hemolymph was withdrawn from blood sinus in the adductor muscle by a sterile syringe (23 G × 1), and then diluted with the BSS. The diluent was centrifuged at 600 × *g* for 10 min at 20°C and harvested hemocytes were resuspended in BSS at a concentration of 5×10^5 cells/m*l*.

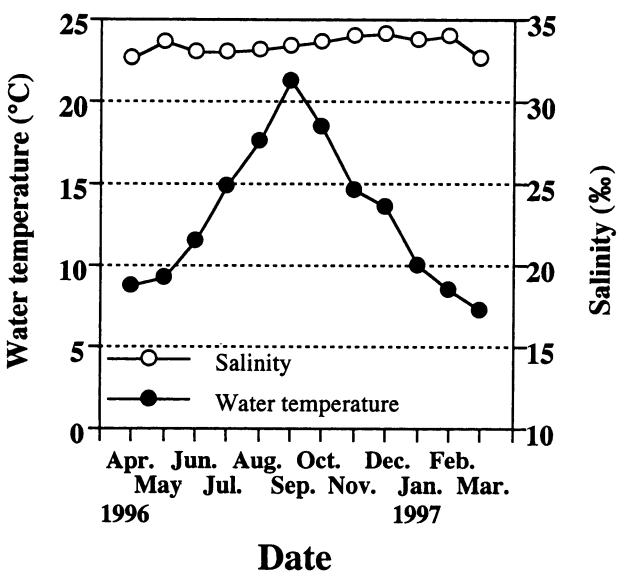

Fig. 1. Changes in water temperature and salinity at a depth of 5 m in Takenoura Inlet, Onagawa Bay from April 1996 to March 1997.

Preparation of FITC-yeast Suspension

Baker's yeast, *Saccharomyces cerevisiae* (IAM 4178), was cultured in yeast extract-malt broth (YM broth) at room temperature for 3 days. The grown cells were harvested by centrifugation and washed twice with 10 mM phosphate buffered saline (PBS). The washed cells were autoclaved at 121°C for 15 min, and suspended in carbonate buffer (pH 9.4) containing 2% fluorescein isothiocyanate (FITC) for 3 hours. After the labeling reaction, yeast cells were washed 4 times with PBS, and FITC-yeast cells were prepared and stored at -80°C until use.

Assay for Phagocytic Activity

Ninty μl of hemocyte suspension (5×10^5 cells/m*l*) was mixed with 10 μl of suspension of FITC-yeast cells (1×10^8 cells/m*l*, suspended in BSS) on a slide, and incubated at 20°C for 1 h in dark. After incubation, 100 μl of citric acid-NaOH buffer (pH 4.6) containing 0.2% trypan blue was mounted onto the slide for fluoresence-quenching for 5 min. After quenching, the slide was washed 3 times with PBS, and then fixed with 10% Formalin. The extent of phagocytosis was determined by randomly counting more than 200 hemocytes from each slide using a fluorescent microscope. Each experiment was performed in triplicate. Phagocytic activity was estimated as phagocytic rate (PR) and phagocytic index (PI), each value being calculated as follows:

PR = number of hemocytes exhibiting phagocytosis/total number of hemocytes

PI = number of FITC-yeast ingested by hemocytes/number of hemocytes exhibiting phagocytosis.

To evaluate capability in clearance of invading organisms by the oyster, we defined clearance index (CI) as follow:

CI = Density in hemocytes × PR × PI.

Results

Classification of Gonadal Condition of C. gigas

The gonadal condition was classified into four stages: the developmental stage, the mature stage, the recovery stage, and the resting stage (Fig. 2).

Developmental stage: Gametocytes were found in primary and secondary genital tubules, and sexual differentiation between males and females was possible under light microscopy. Spermatids and spermatocytes were observed, but these cells did not develop into sperm in males (Fig. 2-A). On the other hand, oocytes existed but were immature in females (Fig. 2-B). Secondary genital tubules developed accompanied by multiplication of gametes, and such development of genital tubules resulted in degradation of interstitial connective tissue (ICT).

Mature stage: In males, secondary genital tubules were full of mature sperm (Fig. 2-C).

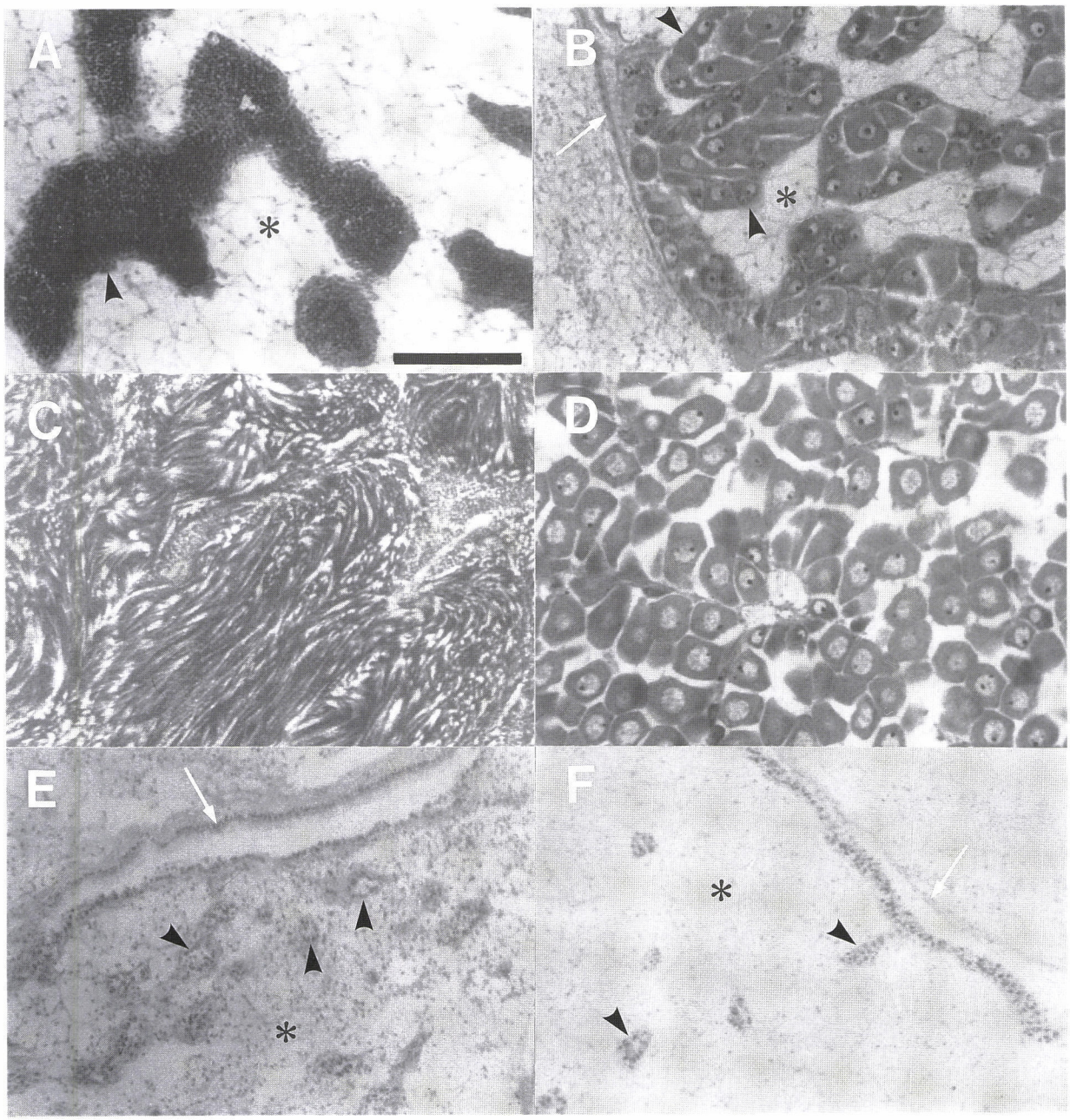

Fig. 2. Microscopic photographs of transversal sections of the gonad of *C. gigas*. A, male developmental stage; B, female developmental stage; C, male mature stage; D, female mature stage; E, recovery stage; F, resting stage. Bars indicate a scale of 200 μm. Asterisks indicate interstitial connective tissue (ICT). Arrows and arrowheads indicate primary and secondary genital tubules, respectively.

Oocytes sufficiently maturated and the germinal vesicle increased in volume in the female (Fig. 2-D). Secondary genital tubules developed sufficiently, and ICT was seldom found.

Recovery stage: At the end of spawning, secondary genital tubules and ICT began to recover (Fig. 2-E). Sexual distinction under light microscopy was impossible except for individuals which had residual gametes.

Resting stage: Although recovery of secondary genital tubules and ICT was completed, gametogenesis was dormant (Fig. 2-F). Sexual distinction was completely impossible under light microscopy.

The spawning period was estimated to be between late August and middle September, because all of the oyster samples had reached the recovery stage when observed on September 26.

Seasonal Change of Hemocytic Density

Hemocytic density (HD) exhibited a remarkable seasonal change (Fig. 3). From May to June, HD increased due to gonadal development; more mature oysters exhibited a higher value at the same sampling time. The group of oysters which were in the mature stage in June showed a maximum value of 2,813 cells/mm^3, then HD remained at a level of approximate 1,500 cells/mm^3 from July through August. In the group collected in September, soon after the spawning period, HD slightly decreased, thereafter recovering to a value of 1,338 cells/mm^3 in the October sampling group. Groups of oysters in the resting stage in December and February showed a lower value ($< 1,000$ cells/mm^3); the minimum value of 675 cells/mm^3 being recorded in February.

Seasonal Change of Phagocytic Activity in Hemocytes

Phagocytic activity also displayed remarkable seasonal changes (Figs. 4, 5). The change profile of the phagocytic rate (PR) was similar to that of HD (Fig. 4). From May to June, PR increased accompanying gonadal development, and more mature oysters exhibited a higher value at the same sampling time. The group of oysters at the mature stage in June showed a maximum value of 60.3%. Thereafter, PR greatly decreased to

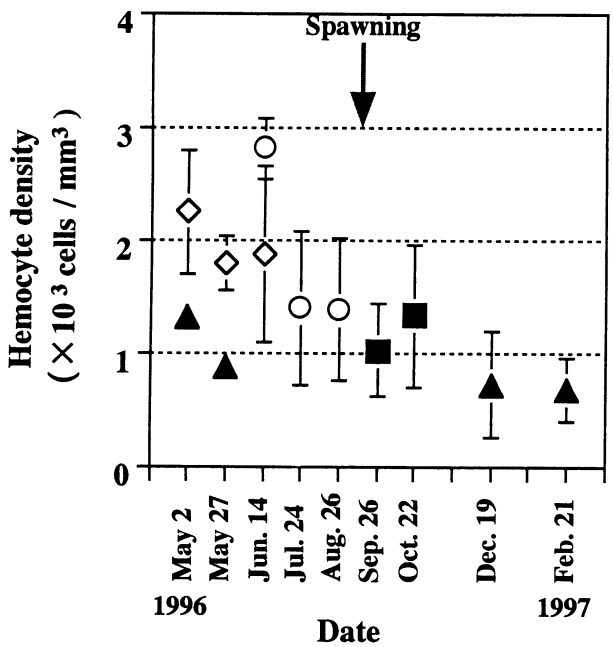

Fig. 3. Density of hemocytes in the hemolymph from *C. gigas* at each sampling time. ◇, developmental stage; ○, mature stage; ■, recovery stage; ▲, resting stage. Data show means ± SD. Five individuals were collected at each sampling time. The arrow indicates spawning time.

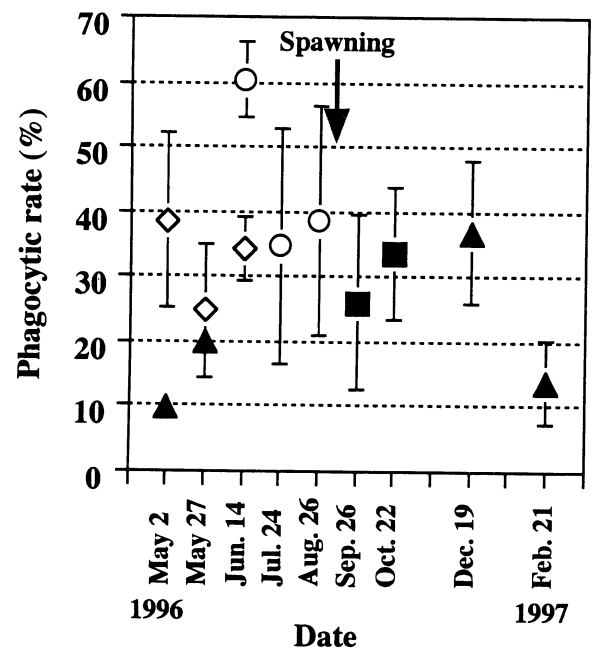

Fig. 4. Phagocytic rate of hemocytes from *C. gigas* at each sampling time. ◇, developmental stage; ○, mature stage; ■, recovery stage; ▲, resting stage. Data show means ± SD. Five individuals were collected at each sampling time. The arrow indicates spawning time.

34.8% in July, and maintained a value of approximate 35% throughout August. Moreover, in September, PR decreased to 25.9%, but slightly increased in the period of October through December. The group of oysters at the resting stage in February showed a minimum value of 13.8%.

Seasonal change of the phagocytic index (PI) was different from that of PR (Fig. 5). Of the sample collected in May, the group of oysters at the developmental stage revealed a higher PI value in comparison with the value of the resting stage group. The mature stage group in June had a PI value of 4.78, much higher than the value of the group of oysters at the developmental stage (3.10) at this same sampling time. Therefore, the PI increased concomitant with gonadal development until June. Then the PI maintained high values (>4.00) in the period of July through August; however, it rapidly decreased to 3.07 in September, soon after the spawning period. Contrastively, the PI sharply increased from October, and reached its maximum value of 5.15 in December. Thereafter, the PI of oysters at the resting stage greatly decreased again to a minimum value of 2.34 in February.

Seasonal Change of Clearance Ability in the Hemolymph

The change profile of the clearance index (CI) was similar to that of the PR (Fig. 6). From May to June, the CI increased accompanying gonadal development, and more mature oysters exhibited a higher value at the same sampling time. The CI of the group of oysters at the mature stage in June was markedly higher, a maximum value reaching $8,269 \times 10^3$ particles/mm^3. Then the CI rapidly decreased to $2,452 \times 10^3$ particles/mm^3 in July. In September, soon after spawning, the CI further decreased and reached a value of $1,066 \times 10^3$ particles/mm^3. The group of oysters at the resting stage in February showed a minimum value of 208×10^3 particles/mm^3, which was one fortieth of the maximum value.

Discussion

In this study, we demonstrated that gonadal

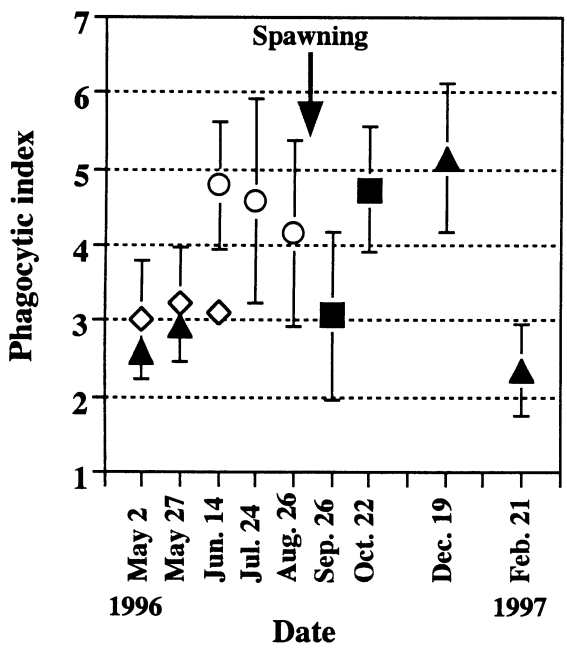

Fig. 5. Phagocytic index of hemocytes from *C. gigas* at each sampling time. ◇, developmental stage; ○, mature stage; ■, recovery stage; ▲, resting stage. Data show means ± SD. Five individuals were collected at each sampling time. The arrow indicates spawning time.

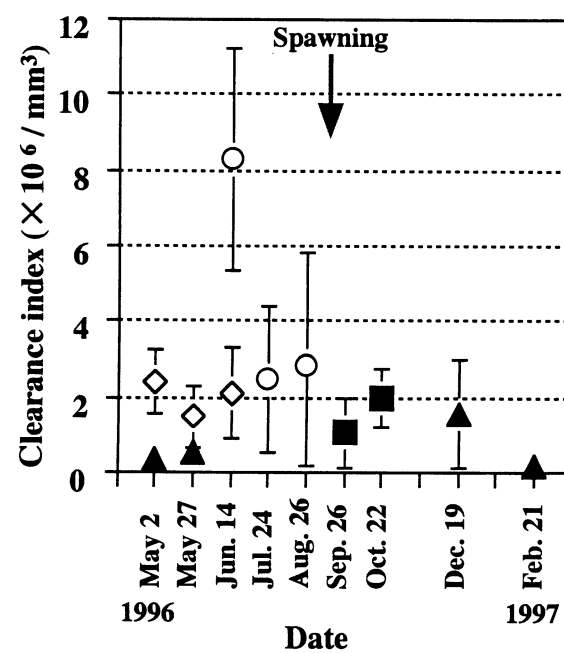

Fig. 6. Clearance index of the hemolymph from *C. gigas* at each sampling time. ◇, developmental stage; ○, mature stage; ■, recovery stage; ▲, resting stage. Data show means ± SD. Five individuals were collected at each sampling time. The arrow indicates spawning time.

maturation and spawning caused seasonal changes of hemocytic density and phagocytic activity in the hemocytes in *C. gigas*. Both hemocytic density and phagocytic activity in the hemocytes were markedly enhanced by physiological activation involving gonadal maturation, and these functions in hemocytes were greatly lowered by the energy consumption involved in spawning.

The influence of gonadal maturation was found in the group collected from May to June: hemocytic density and phagocytic activity in the hemocytes increased concomitant with gonadal development, and more mature oysters exhibited a higher value at the same sampling time (Figs. 3-6). Soon after the spawning period, hemocytic density and phagocytic activity in the hemocytes decreased; the phagocytic index, in particular, dropped markedly (Fig. 5), probably as a result of spawning. A reasonable hypothesis is that these decreases were due to the consumption of energy as indicated by the decrease in glycogen content during the period of spawning[6]. Another hypothesis is that hemocytes which possess high phagocytic activity are mobilized into secondary genital tubules for phagocytosis of decomposed egg residues and for healing of inflammation after spawning; the infiltration of hemocytes into the genital tubules has often been observed after the spawning period in *C. gigas*[7].

It is well known that environmental factors such as water temperature and salinity influence the defense capabilities in the hemocytes of bivalve molluscs[5]. Salinity in the area where specimens were hung in this study was within the range of 32 to 36‰ (Fig. 1), so the effect of salinity on hemocytic density and phagocytic activity in the hemocytes was concluded to be nil. On the other hand, water temperature affected hemocytic density and phagocytic activity in the hemocytes during the period when gonadal maturation was dormant, as it shown that the changes of hemocytic density and phagocytic activity were corresponded with changes of water temperature from December to February. In spite of the high water temperature in September, hemocyte density and phagocytic activity decreased after the spawning period (Figs. 3-6). Although it has been reported that water temperature affects on hemocyte density[8] and phagocytic activity in the hemocytes of bivalve molluscs[9], in the present study, seasonal changes in hemocytic density and phagocytic activity did not always correspond with the changes of water temperature. Therefore, to understand the seasonal changes of hemocytic density and phagocytic activity in the hemocytes, in addition to environmental factors such as water temperature, consideration must be given to gonadal maturation and spawning.

Results of this study suggest that not only environmental factors such as water temperature, but also gonadal maturation and spawning affect hemocytic density and phagocytic activity. To precisely clarify the effects of gonadal maturation and spawning on phagocytic activity in the oyster hemocytes, further experiments employing infertile triploid oysters and artificial spawning should be carried out.

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宮城県女川湾における垂下養殖マガキの生殖周期と血球の貪食能の季節変動

石川春彦・高橋計介・森　勝義

宮城県女川湾に垂下したマガキ，*Crassostrea gigas* の血球密度，血球の貪食能および生体内の異物排除能を示すクリアランス指数の季節変動について調べ，それらの変動におよぼす性成熟と産卵の影響について検討した。各個体の性成熟段階を組織学的に，発達期・成熟期・回復期・休止期の4段階に区分した上で，血球密度および血球の貪食能を測定した結果，同一時期に採取された個体間において，より性成熟が進行した個体の方が血球の密度，貪食能ともに高くなる傾向が認められた。さらに，クリアランス指数の差は顕著であった。また，産卵直後の回復期では，すべての個体の血球密度および貪食能が成熟期と比べて大きく低下した。以上のことから，マガキの血球密度，貪食能および生体内の異物排除能は，性成熟の進行と産卵によって大きく変動することが明らかとなり，生殖周期は細胞性防御能に影響する重要な要因であることが示唆された。