Optimal Conditions for Artificial Fertilization, Embryonic Development, and Larval Growth of the Purple Clam, *Saxidomus purpuratus* from Southern Coast of Korea

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ABSTRACT

To obtain the basic information on culture conditions for the larvae of *Saxidomus purpuratus*, experiments were conducted on the population from southern coast for (1) the success in fertilization and development from artificial fertilization among different months of a year, (2) the viability of sperms after exposure to seawater, (3) and the effects of temperature, salinity, and food organism on the survival and growth of larvae. Gametes obtained from dissection showed high rate of fertilization at all months. But the rate of development was higher only May-July. Developmental success seemed to be related with the quality of eggs at the time of fertilization. Developmental times for 2-cell, 4-cell, 8-cell, blastula, trochophore larva, and veliger larva at 20°C were 1.5, 2, 4, 18, 24, and 32 hr, respectively. Sperms could survive for more than 8 hr, however, actively swimming sperms could be found within 1 hr after exposure to seawater. It is recommended that sperms should be used for fertilization as soon as possible when they are exposed to seawater. At temperature of 35°C, all the larvae died during 48 hr. Larval survival decreased when salinity was either lower than 20 psu or higher than 40 psu, and was 0% when salinity was 10 psu. Optimal range of temperature and salinity for rearing larvae of *S. purpuratus* were 20-25°C and 20-40 psu, respectively. Larvae grew from 111.5 to 235.3 μm during 21 days. Larvae fed mixed diets grew faster than unialgal diets. The fastest growth was observed when larvae were fed on the mixture of *Isochrysis galbana* and *Nannochloris oculata*.

Keywords: *Saxidomus purpuratus*, Optimal conditions, Artificial fertilization, Embryonic development, Larval growth.

INTRODUCTION

The purple clam, *Saxidomus purpuratus* (Class Bivalvia, Family Veneridae) is a local species inhabiting relatively restricted areas around Korea, Japan, and China (Choe *et al.*, 1999). The geographic distributions of *S. purpuratus* are southern and western coasts of Korea, from southern Hokkaido to Kyushu in Japan (Kishioka *et al.*, 1996), and coastal areas of Shandong and Liaoning provinces in China (Wei *et al.*, 1982). *S. purpuratus* is found from intertidal to 40 m depth of subtidal areas with mixed sediments of sand, silt, and clay (Kim *et al.*, 2000). This species is one of the most important shellfish resources for human consumption with high prices. Annual yield was 7,000-9,000 MT during 1995-1999 (Kim *et al.*, 2001b). Recently, the commercial yield from the traditional exploitation of natural fisheries by divers has been declining due to over-harvesting. So, much attention has been concentrated to the aquaculture of this species.

Previous studies on *Saxidomus purpuratus* have dealt with the basic ecological aspects (Kim, 1971),

natural yield and growth (Kim *et al.*, 2001b), reproductive biology (Kim, 1969; Ideo *et al.,* 1995; Chung *et al.*, 1999; Kim *et al.*, 2001a), feeding physiology (Lee *et al.*, 2002a), and aquaculture (Wei *et al.*, 1982; Kishioka *et al.*, 1996; Kim *et al.*, 2000; Choi *et al.*, 2001). The spawning periods of populations from various localities are reported differently: May-October for the population from southern coast of Korea (Chung *et al.*, 1999), May-September from western coast of Korea (Kim *et al.*, 2001a), October-November from south-western coast of Japan (Ideo *et al.*, 1995), and June-September from China (Wei *et al.*, 1982). In Korea, aquacultural studies were actively performed by Taean Marine Hatchery, National Fisheries Research and Development Institute (NFRDI) from 1999 to 2002 (Kim *et al.*, 2000; Choi *et al.*, 2001). Thanks to their efforts, basic techniques for culturing larvae and juveniles of *S. purpuratus* have been developed. However, the purpose of their studies was only successful production of seedlings as many as possible by artificial spawning. Optimal conditions for artificial fertilization, embryonic development, and larval growth have not been understood comprehensively yet.

This paper reports on some of fundamental conditions for artificial fertilization, embryonic development, and growth of larvae. We used specimens from southern coast to compare with the population from western coast. This study focused especially on the biological aspects of early life stage of *Saxidomus purpuratus*: (1) the success of fertilization and development from artificial fertilization among different months of a year, (2) the viability of sperms after exposure to seawater, and (3) the effects of physical and biological parameters (temperature, salinity, and food organism) on the survival and growth of larvae.

MATERIALS AND METHODS

1. Test organisms

Adult *Saxidomus purpuratus* were collected monthly or bimonthly from a subtidal area near Geoje Island by commercial divers from September 2001 to June 2002. Individuals with shell length of more than 7 cm

were selected and rinsed with 5-μm filtered seawater (salinity: 30 psu). They were reared in a 500 liter aquarium at 20℃ for 2 days before the artificial fertilization experiments. During this period, clams were fed 5 x 10^6 cells/ml of *Isochrysis galbana* everyday.

As food organisms for the larvae of *Saxidomus purpuratus*, unialgal cultures of *Isochrysis galbana* (Prymnesiophyceae), *Pavlova gyrans* (Prymnesiophyceae), and *Nannochloris oculata* (Chlorophyceae) were prepared. They were grown at 20℃ with f/2 medium (Guillard and Ryther, 1962) without silicate, with 14L: 10D cycle of 100 μE/m^2/sec provided by cool-white fluorescent lights. Only cultures in exponential growth phase were used for experiments.

2. Artificial fertilization

To evaluate the possibility to produce seedlings out of the natural spawning periods of *Saxidomus purpuratus,* trials on artificial fertilization were carried out on September, October, and December in 2001, and January, March, May, June, and July in 2002. Since *S. purpuratus* showed little significant spawning responses against physical, chemical, or biological stimuli from preliminary experiments, gametes were obtained by dissection (Kim *et al.*, 2000). Gonadal tissues were removed from shells and washed twice with GF/F-filtered seawater. Sex was determined by observing gonadal tissues under a compound microscope (× 400). Sperms or eggs were obtained by scrubbing gonads in filtered seawater. Sperm suspension was passed through a 20-μm screen, and was kept in a 100-ml beaker. Egg suspension was passed through a 100-μm screen to remove larger particles, and eggs were collected on a 40-μm screen for smaller eggs and particles to pass through. Eggs were transferred into diluted ammonia water (1/5000, pH = 9.1), allowed for 5 min, and then rinsed 3 times with GF/F-filtered seawater. Before fertilization, the density of egg was adjusted to 30 eggs/ml. Fertilization was achieved by injecting sperms into egg suspension. Successful fertilization and development was evaluated by observing 100 eggs after 2 and 48 hr from fertilization, respectively. For fertilization, eggs with fertilization membrane were

regarded as successfully fertilized. For development, embryos developed to the normal D-shaped veliger larvae were regarded as successfully developed.

3. Developmental time

The observation of embryonic development of *Saxidomus purpuratus* was conducted simultaneously with artificial fertilization experiment on June, 2002. To know the elapsed time to reach each developmental stages at 20℃ during embryonic development, 10-ml aliquots of subsamples were taken for 48 hr at 30 min interval during first 3 hr and 1 hr interval during the rest of period, then fixed with 10% buffered formalin. Subsamples were examined under a compound microscope (× 100) and the numbers of embryos developed to each developmental stages (2-cell, 4-cell, 8-cell, blastula, trochophore, and veliger) were counted. Elapsed time when more than 50% of embryos had reached each developmental stage was regarded as the developmental time for that stage.

4. Sperm viability

To know the viability of sperm, the survival and the swimming activity of sperm were measured. The procedure for preparation of sperm suspension was the same as described in the artificial fertilization experiment. After the sperms were exposed to seawater, 10-ml aliquots of subsamples were taken at 1 hr interval for 8 hr. The subsamples were then transferred into a counting chamber and the proportions of live and actively swimming sperms were enumerated from the video image through a CCD camera connected to an inverted microscope (× 320). Sperms with continuously moving tail were regarded as live sperms and those swimming forward or those with spinning movement were regarded as actively swimming sperms.

5. Effects of temperature and salinity on the survival of larvae

To know the effects of temperature and salinity on the survival at early larval stage of *Saxidomus purpuratus*, 5 days old larvae were incubated under the combinations of both 4 temperature (20, 25, 30, 35℃) and 4 salinity (10, 20, 30, 40 psu) regimes for 48

hr. For each treatment, larvae were transferred into a 500-ml beaker with a density of 2 larvae/ml. During incubation, larvae were fed 1×10^4 cells/ml of *Isochrysis galbana*. At the end of incubation, 10-ml aliquots were subsampled and the numbers of live and dead larvae were counted under a compound microscope (× 100). Larvae showing no ciliary movement in the velum for 10 sec were regarded as dead.

6. Effects of food organisms on the growth of larvae

Experiments for growth efficiency of food organisms for the larvae of *Saxidomus purpuratus* were conducted on June, 2002. Three unialgal culture and 3 combinations of dual mixture (50:50) of *Isochrysis galbana*, *Pavlova gyrans*, and *Nannochloris oculata* were used in experiments; i. e. (1) *I. galbana* only, (2) *P. gyrans* only, (3) *N. oculata* only, (4) *I. galbana* + *P. gyrans*, (5) *I. galbana* + *N. oculata*, and (6) *N. oculata* + *P. gyrans*. Three days old larvae (shell length: 111.5 ± 4.6 μm) were incubated in a 1000-ml beaker (initial density: 2 larvae/ml) for each treatment during 21 days. Food was supplied daily with final concentration of 1×10^4 cells/ml from day 1 to day 10, and 1×10^5 cells/ml from day 11 to day 21. Fifteen-ml aliquots of subsamples were taken from each treatment and fixed with 10% buffered formalin at 3, 7, 10, 15, 18, and 21 days after the experiment began. Shell length (the distance between both anterior- and posterior ends of the valve) of 20 larvae for each treatment was measured with a micrometer attached on the eyepiece of a compound microscope (× 100) to the nearest 1 μm. Shell length data among food conditions at the end of incubation were compared by one-way analysis of variance (ANOVA) with a significance level of $a = 0.05$. Multiple comparison was conducted using Tukey's HSD (Zar, 1984) to determine which means were significantly different from one another.

RESULTS AND DISCUSSION

1. Artificial fertilization

Fertilization was successful (> 90%) at all months with gametes obtained from dissected clams. The rate

of development to the veliger larvae was higher (78.3-90.2%) from May to July, but it was less than 30% during winter (Fig. 1). The natural spawning periods estimated from histological observations for the *Saxidomus purpuratus* were reported as May to October (Chung *et al.*, 1999), especially concentrated at late spring (May to June; KORDI, 2002) for south sea populations, and May to September for west sea populations (Kim *et al.*, 2001b). Our results indicate that successes in fertilization and development can be explained differently. That is, fertilization was successful irrespective of spawning period, while development was successful only in the midst of spawning period. This implies that high rate of fertilization does not always guarantee high rate of development. Presumably, developmental success is related with the quality of eggs at the time of fertilization.

However, the high rate of fertilization success during winter is still questionable. In this study, we determined the eggs were successfully fertilized when the fertilization membrane could be distinguished. But, in practice, it is somewhat confusing to determine whether egg was fertilized or not by the presence of fertilization membrane only, since the fertilization membrane is formed too closely to the edge of egg and eggs actually not fertilized also have membrane-like structure around eggs. Therefore, decision by fertilization membrane could lead to an overestimation of the rate of fertilization. Higher rate of fertilization during winter in our data should be proved by another indicator of fertilization success, for example, the success in first cleavage.

In this study, gametes were obtained only by dissection of gonads, since adults did not respond to any of other stimulations in previous work (Kim *et al.*, 2000). But, Kishioka *et al.* (1996) succeeded adults of *Saxidomus purpuratus* to spawn by raising water temperature. Therefore, we need more effort to develop techniques for artificial spawning besides dissecting, because gametes from dissection may be less healthy than those spawned from their parents.

2. Developmental time

The fertilized eggs of *Saxidomus purpuratus* were isolated and demersal, with the diameter of 75-80 μ
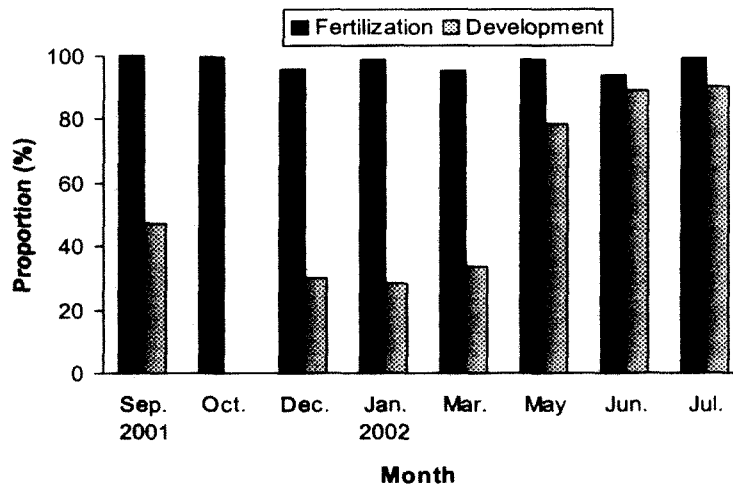

Fig. 1. The proportions of successes in fertilization and development to the veliger larvae of *Saxidomus purpuratus* for each month. Gametes were obtained by dissection of gonads.

Table 1. Comparison of elapsed times to reach each developmental stages of *Saxidomus purpuratus* among different populations.

Stage	South coast (20℃) (present study)	West coast (23℃) (Kim *et al.*, 2000)	China (23-24℃) (Wei *et al.*, 1982)
2-cell	90 min	78-88 min	50 min
4-cell	120 min	88-98 min	70 min
8-cell	240 min	115 min	110 min
Blastula	18 hr	6 hr	7-9 hr
Veliger	32 hr	23 hr	24-26 hr

m. The cleavage of *S. purpuratus* was total and spiral. The first cleavage was observed ca. 90 min after fertilization (2-cell stage). Embryos were divided into two blastomeres meridionally of which the one at animal pole and the other at vegetal pole. The second cleavage occurred ca. 120 min after fertilization (4-cell stage). The second cleavage was also meridional at right angles to the first and resulted in nearly equal sized 3 blastomeres and a large one. The third cleavage divided each quadrant at the equatorial plane ca. 240 min after fertilization (8-cell stage). The fourth and further cleavage were not distinguishable in the samples after 240 min, since the embryos showed various developmental stages even in the same sample, indicating that the developmental time after 8-cell stage is highly variable among embryos. After 18 hr from fertilization, more than half of embryos completed blastulation. Apical cilia were formed on the apical cells at the animal pole. With further development, cells in the region of the primary trochoblasts produced a complete ring of cilia to form the prototroch. With this ring of cilia, embryos swam freely by rotating movement. After blastula, embryos developed to the gastrula stage, and then became the trochophore larva 24 hr after fertilization. The trochophore larvae swam up and down by spiral movement. After 32 hr after fertilization, the embryo developed to the D-shaped veliger larva. The shell length of early veliger larvae was ca. 90-100 μm. Most larvae did not suspend to water surface and stayed near the bottom of rearing chamber.

The elapsed time to develop to the veliger larva was 32 hr in this study, which was 6-9 hr longer than populations from west coast and China (Table 1). Compared with other species, developmental time of

Saxidomus purpuratus is similar to *Crassostrea rivularis* (30.5 hr: Yoo and Kang, 1995), shorter than *Patinopecten yessoensis* (48 hr: Park, *et al.*, 2001), *Corbicula japonica* (48 hr: Kim *et al.*, 2002), and *Ostrea denselamellosa* (72 hr: Yang *et al.*, 1999), but is longer than *Crassostrea gigas* (24 hr: Hur and Hur, 2000). The difference in the duration of embryonic development among different regional populations may be due to the different rearing temperatures. In our study, embryos were incubated at 20℃, while those of west coast and China at 23-24℃. From these results, it is expected that slight increase (3-4℃) in water temperature can substantially shorten the period of embryonic development. Reduced developmental time can increase larval survival by decreasing the vulnerability of embryo to bacterial infection or protozoan attack. Therefore, seawater temperature of 23-24℃ is recommended to obtain more healthy larvae.

3. Sperm viability

The survival of sperm exposed to seawater decreased as the exposure time increased (Fig. 2). More than 80% of sperms stayed alive when they were exposed for 6 hr. After then, their survival decreased down to ca. 60% when they were exposed for 8 hr. After 17 hr, no live sperms could be observed. Thus, the longevity of sperm of *Saxidomus purpuratus* can be regarded as 17 hr. However, the proportion of actively swimming sperm decreased more rapidly as exposure time increased. The sperm activity was higher than 80% when exposed for 1 hr, but decreased to less than 10% after 4 hr. Even though sperms are alive, those lacking mobility cannot be regarded as viable. Successes in fertilization and development
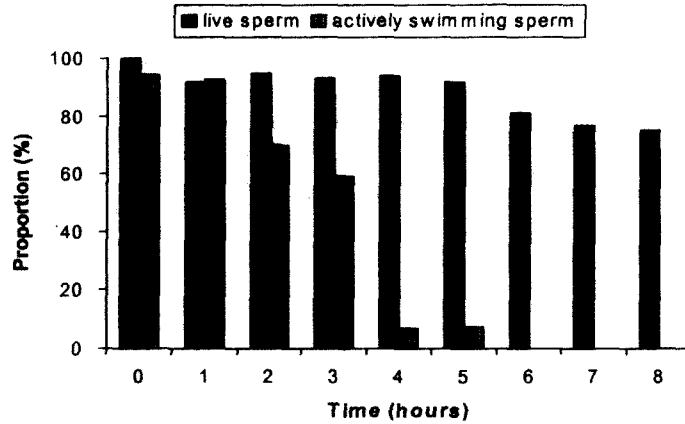
Fig. 2. Changes in the proportions of live- and actively swimming sperms of *Saxidomus purpuratus* with the time after they were exposed to seawater.

using sperms with reduced viability are still questionable. Lee *et al.* (2002b) observed the sperm viability of *Spisula sachalinensis*. They reported that the proportion of active sperm just after exposure was 85%, and it continuously decreased to 60% after 1 hr and then 10% after 3 hr. The active period of sperms of *S. purpuratus* is slightly longer than *S. sachalinensis*. Thus, to obtain healthy larvae, sperms should be used for fertilization as soon as possible (within 1 hr) when they are exposed to seawater.

4. Effects of temperature and salinity on the survival of larvae

Both temperature and salinity affected the survival of *Saxidomus purpuratus* larvae. There were no survivors at all temperatures when salinity was 10 psu (Fig. 3). When salinity was 20 psu, the survival of larvae at 20, 25, and 30℃ were 75, 88, and 44%, respectively. When salinity was 30 psu, more than 90%
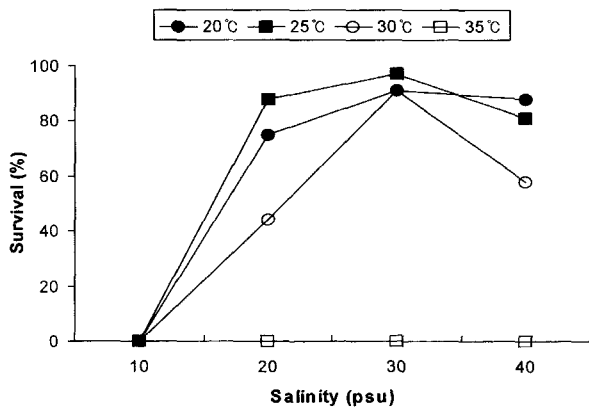

Fig. 3. The survival (%) of 5 days old larvae of *Saxidomus purpuratus* reared under different temperature and salinity conditions for 48 hr.
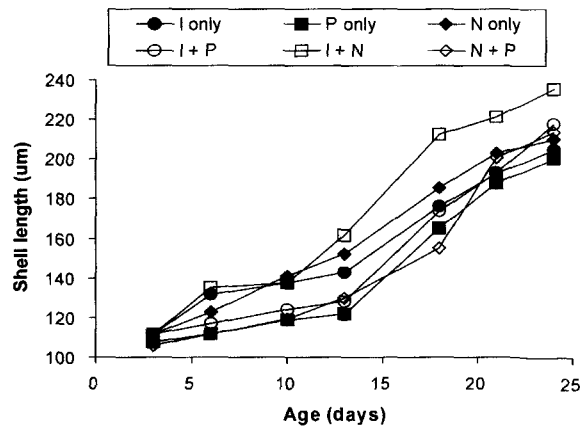

Fig. 4. Changes in shell length of the larvae of *Saxidomus purpuratus* reared with different conditions of food organisms. I: *Isochrysis galbana*, P: *Pavlova gyrans*, N: *Nannochloris oculata*.

of larvae survived within the temperature range of 20-30℃. When salinity was 40 psu, survival of larvae decreased to 58-88%. At 35℃, all the larvae died irrespective of salinity. Temperature of 35℃ seems too high and lethal to the larvae of S. purpuratus. At 3 0℃, temperature was either stressful or not, according to salinity. When salinity was 30 psu (similar to ambient water), more than 90% of larvae survived. However, when salinity was lower than 20 psu or higher than 40 psu, the survival of larvae was only 44-58%. At temperature of 20 and 25℃, survival of larvae were higher and least variable, except when salinity was 10 psu. Therefore, the optimal ranges of temperature and salinity were 20-25℃ and 20-40 psu, respectively. If the larvae of S. purpuratus were exposed to the low salinity regime due to heavy rains in summer, they would suffer physiological stresses. This might act as a factor to the annual fluctuation in recruitment of S. purpuratus.

5. Effects of food organisms on the growth of larvae

There were significant effects of food organisms on the growth of Saxidomus purpuratus larvae (F = 12.36, p < 0.001). The shell length increased from 111.5 to 235.3 μm during incubation period (Fig. 4). Larvae fed mixed diets grew faster than unialgal diets. The fastest growth was observed when larvae were fed on the mixture of Isochrysis galbana + Nannochloris oculata. The growth rates of the larvae fed either mixed diets of Pavlova gyrans + I. galbana or P. gyrans + N. oculata were not significantly different from any of unialgal diets (p = 0.111). Addition of P. gyrans did not enhance the growth of S. purpuratus larvae. The best combination of food for S. purpuratus larvae was the mixed diet of I. galbana and N. oculata. Kim et al. (2000) studied the effects of 5 species of food organisms on the growth of S. purpuratus larvae. They reported that the growth rates of larvae fed on unialgal diets were lower than mixed diets. They also found the highest growth rate when larvae were fed on the mixed diet of I. galbana + P. gyrans + N. oculata + Cyclotella cryptica. However, they emphasized that the differences in growth rates among dual, triple, and quadruple combinations of food organisms were not large.

Therefore, dual mixture of I. galbana + N. oculata will be sufficient for the S. purpuratus larvae. Not only the food organism but also the amount of food supplied is an important factor for the growth of larvae. Unfortunately, the scope of this study did not contain the effect of food quantity. To determine the optimal feeding regime for the larvae, it is necessary to know how much the larvae can uptake food organisms.

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