Development of an Artificial Fertilization Method for the Japanese Pearl Oyster *Pinctada fucata martensii* by Using a Microplate

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**Abstract:** Japanese pearl oysters are widely cultivated by artificial fertilization using gametes obtained from the ovary and testis. Using the gametes from the Japanese pearl oyster, we developed a method for conducting small-scale artificial fertilization tests in a 24-well microplate. Following induction of germinal vesicle breakdown (GVBD), we inseminated 20,000 eggs in 2.0 ml seawater in a single well of a microplate. The highest fertilization rates were obtained using a testicular semen volume of 1.25 \(\mu\)l when the concentration of NH\(_3\) in seawater was 0.75 mM during insemination. Changes in contact time (the time from commencement of insemination to rinsing the eggs) from 3 to 60 min did not affect the fertilization rate. However, a contact time of >12 min was necessary to ensure consistency in contact time among the 24 wells.

**Key words:** Japanese pearl oyster; Artificial fertilization; Microplate

Artificial fertilization of shellfish is typically conducted by adding semen to a fertilization vessel that contains eggs in seawater or freshwater. After allowing some contact time between the eggs and sperm, the gametes are rinsed with seawater and fresh water to eliminate excess spermatozoa. The fertilized eggs are then transferred to a hatching incubator.

Testing the success of artificial fertilization is essential for successful aquaculture management. These tests are used for a variety of purposes, such as estimation of gamete quality, development of gamete preservation techniques, and improvement of artificial fertilization techniques. These tests are typically carried out using a relatively small quantity of gametes under a wide range of conditions. Researchers have used a variety of vessels for artificial fertilization, including beakers (Iwata et al. 1989; Paniagua-Chavez et al. 2001; Aoki et al. 2007), flasks (Usuki et al. 1997), vials (Babcock and Keesing 1999; Powell et al. 2001; Narita et al. 2008), and plastic containers (Encena et al. 1998). However, microplates offer many advantages for conducting artificial fertilization tests. For example, it is possible to run many tests simultaneously using small quantities of gametes and water. Furthermore, microplates are easy to prepare and allow the researcher to maintain a homogeneous environment for the gametes. Therefore, microplates are also used for rearing the eggs and yolk-sac larvae to estimate the rates of fertilization, hatching, and survival in marine teleosts (Tagawa et al. 2004; Unuma et al. 2004). Recently, Adams et al. (2004) conducted artificial fertilization assays in the Pacific oyster using a 12 well-tissue culture plate. The eggs

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were artificially fertilized in a single well of the microplate and were fixed by adding formalin into each well at the 4-cell stage to determine the fertilization rate. In these experiments, the total volume of water in each well was 3 ml, and the total number of eggs/well was less than 600.

Most oocytes extracted from the ovaries of the Japanese pearl oyster *Pinctada fucata martensii* have a germinal vesicle in the cytoplasm. This indicates that they are in the prophase of the first meiosis (Wada 1963). The spermatozoa from the testis of the Japanese pearl oyster are immotile when suspended in seawater (Wada 1961; Ohta et al. 2007). The breakdown of the germinal vesicle of the oocyte is accelerated markedly, and immotile spermatozoa become active in seawater containing 0.5 – 1.0 mM NH₃. The success of fertilization also increases significantly if the oocytes are incubated in seawater containing NH₃ before insemination (Kuwatani 1965).

In Japan, the majority of pearl oyster hatcheries conduct artificial fertilization using oocytes from 1 female and spermatozoa from several males in a bowl (Hayashi and Seko 1986; Aoki et al. 2007). Before insemination, approximately 10 – 15 million oocytes from 1 female are incubated in 1 l of seawater containing 0.75 mM NH₃ for 30 – 60 min in a bowl. When most of the oocytes have undergone germinal vesicle breakdown, testicular semen is added to the bowl. Thus, the concentration of eggs at the time of fertilization is between 10,000 and 15,000 eggs/ml in seawater containing 0.75 mM NH₃.

Our goal was to develop a simple and rapid assay for assessing the fertilization success of the Japanese pearl oyster. Using a 24-well microplate, we evaluated the following parameters: the volume of testicular semen, NH₃ concentration in seawater during insemination, and contact time between eggs and spermatozoa.

**Materials and Methods**

**Collection of gametes**

We used mature (3-year old) Japanese pearl oysters reared in the Mie Prefectural Fish Farming Center and its offshore. Seawater used in this study was collected by 400 meters of Mie Prefectural Fish Farming Center offshore and was sand filtered. Before the experiments, seawater was further filtered with the mesh of 0.5 µm. The shells were opened with a shell-opener, and the gonads were dissected using scissors. Using a scalpel, several incisions were made on the surface of the ovary, and the tissue was covered with gauze (mesh size, 1 mm). The ovary was then soaked in 50 ml of 0.75 mM NH₃-seawater at 25°C in a plastic beaker. The oocytes were separated into the seawater by gently shaking the ovary. The egg density in seawater was adjusted to 40 eggs/µl by addition or removal of seawater containing 0.75 mM NH₃. When more than 80% of the oocytes had undergone germinal vesicle breakdown (approximately 40 – 60 min), they were used for fertilization. In addition, several pieces of testicular tissue were cut using a scalpel, and semen was collected with a micropipette. The semen was then transferred to a microtube. Only samples that contained more than 60% of motile spermatozoa were used in the experiments.

**Artificial fertilization**

The eggs were artificially fertilized in a 24-well microplate (catalog # 3820-024; well diameter, 16 mm; Iwaki, Asahi Glass Co. Ltd, Japan). On the basis of preliminary experiments, we adopted the following protocol for artificial fertilization. A 500-µl aliquot of the seawater/NH₃ mixture that contained 40 eggs/µl was gently transferred into a single well of the microplate using a micropipette. The semen was then diluted 500 times with raw seawater, and 500 µl of the dilute semen was gently transferred into a single well of the microplate using a micropipette. The semen was then diluted 500 times with raw seawater, and 500 µl of the dilute semen was gently mixed with 1,000 µl of seawater containing 1.125 mM NH₃. Immediately, the mixture of the dilute semen (1,500 µl) was added to the well containing the eggs, and gently mixed by pipetting with a micropipette (Fig. 1A). Thus, each well contained a total of 2.0 ml of 0.75 mM NH₃-seawater, 20,000 eggs, and 1 µl testicular semen. The mixture was incubated for 30 min, after which the contents were transferred to a 20-µm mesh sieve, placed on a small funnel, and gently rinsed with 5 ml raw seawater (Fig. 1B). The rinsing procedure took approximately
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20–30 s/well. Therefore, we staggered the introduction of semen into each well by 30 s to ensure the contact time was constant among wells. Thus, the commencement of insemination was delayed by 11.5 min from the first well to the last well. After rinsing, the eggs were transferred to a well of another 24-well microplate and incubated in 2 ml raw seawater at 25°C for 1.5–2.0 h (Fig. 1C).

Using this protocol, we evaluated the effects of changes in the following parameters on the fertilization rate: volume of testicular semen (Experiment 1), concentration of NH₃ in the seawater during insemination (Experiment 2), contact time (the time between addition of semen to eggs and rinsing of the eggs) (Experiment 3).

**Experiment 1: Volume of testicular semen necessary for artificial fertilization**

We serially diluted (2 times) semen with raw seawater to give 6 dilutions ranging from 100 to 3,200-fold. We then gently mixed 500 µl of the dilute semen with 1 ml of 1.125 mM NH₃-seawater, and immediately added the mixture to a well that contained 500 µl of the egg/0.75 mM NH₃-seawater mixture.

**Experiment 2: Concentration of NH₃ in the seawater during fertilization**

We gently mixed 1 µl of semen, which was diluted 500 times with raw seawater, with 1,000 µl of seawater that contained various concentrations of NH₃ (0, 0.375, 0.75, 1.125, 2.625, and 5.625 mM). The dilute semen/seawater (with NH₃) mixture (1,500 µl) was then immediately pipetted into a well that contained the eggs in 500 µl of 0.75 mM NH₃-seawater. The eggs, which were to be fertilized with the mixture of dilute semen containing 0 mM NH₃, were rinsed with 10 ml raw seawater just prior to insemination to remove the NH₃. The eggs were then transferred to a well containing 500 µl raw seawater and were mixed with 1,500 µl of dilute semen (1 µl of semen in 1,500 µl seawater). Thus, the final concentration of NH₃ in each treatment group during fertilization was 0, 0.375, 0.563, 0.75, 1.5, and 3.0 mM. The mixture was incubated for 30 min, after which the eggs were rinsed with raw seawater.

**Experiment 3: Contact time for eggs and spermatozoa**

We gently mixed 500 µl of dilute semen (500-times dilution) with 1,000 µl of seawater containing 1.125 mM NH₃. The mixture was then added to a well that contained the eggs and seawater (20,000 eggs in 500 µl of 0.75 mM NH₃-seawater). The eggs were rinsed with raw seawater for either 10 s or 1, 3, 5, 10, 30, or 60 min after the addition of semen. The fertilization rate was determined 90–120 min after the commencement of addition of semen.

**Measurement of fertilization rate**

The fertilization rate was evaluated by counting the number of cleaved eggs in a sample containing >100 eggs by using a transmission microscope. The measurement of the fertilization rate in each treatment group was performed by 2 observers, and the average result was used in the data analysis.

**Measurement of sperm density and sperm motility**

Testicular spermatozoa are immotile in seawater in the absence of NH₃ (Ohta et al. 2007).
Therefore, we diluted 5 μl of semen with raw seawater (2,500 times) to measure sperm density. The density was measured by using a disposable cell counter (CC0013, Matsunami Glass Ind. Ltd., Japan).

To measure sperm motility, 1 μl of testicular semen was diluted with 1,000 μl activating solution [seawater containing 2 mM NH3 and 0.5% bovine serum albumin (BSA)] in a plastic tube by using a vortex mixer. The mixture was incubated for 2 min, after which an 8-μl aliquot of the diluted spermatozoa was transferred to a chamber (2X-CEL Dual Sided Sperm Analysis Chamber, Hamilton Thorne Biosciences, USA) with a coverslip. BSA was added to the activating solution to prevent sperms from sticking to the glass surface of the chamber. The percent sperm motility was measured as previously reported (Ohta et al. 2007).

Statistical analyses
All data are presented as mean ± SEM. Percentage data were transformed by using an arcsine-square root transformation before analysis. We compared the fertilization rates among treatments using one-way analysis of variance (ANOVA) followed by Tukey’s pair-wise comparison test. Values of \( P < 0.05 \) were considered to be statistically significant.

Results
The mean sperm density and the percent sperm motility was \( 2.12 \pm 0.10 \times 10^7 / \mu l \) and 81.5 ± 5.1%, respectively (n = 14).

**Experiment 1: Volume of testicular semen necessary for artificial fertilization**
Greater than 60% of the eggs were fertilized in the treatment groups, in which the semen volume was between 1.25 – 5.0 μl (Fig. 2). Fertilization rates decreased gradually when the semen volume was below 0.63 μl. The lowest fertilization rate (48.0 ± 7.9%) was at 0.16 μl. However, there were no significant differences between any of the the experimental groups.

**Experiment 2: Concentration of NH3 in seawater during fertilization**
The fertilization rates were close to 0% at NH3 concentrations below 0.375 mM (Fig. 3). The rates increased rapidly as the concentration of NH3 increased above 0.563 mM (52.9 ± 4.6%), and peaked at a concentration of 0.75 mM (72.3 ± 3.1%). The fertilization rate then decreased at a NH3 concentration of 1.5 mM, although the differences were not significant.

**Experiment 3: Contact time for eggs and spermatozoa**
Very few eggs (1.2 ± 0.6%) were fertilized when the contact time was 10 s (Fig. 4).
time of contact increased, the fertilization rates also increased, reaching $33.2 \pm 6.9\%$ at 1 min and $70.8 \pm 2.5\%$ at 3 min. Further increase in the contact time from 3 min to 60 min did not result in any significant difference in the fertilization rates.

### Discussion

We developed a method for rapidly assessing the fertility of Japanese pearl oyster gametes using a 24-well microplate. Using this method, we tested the effect of 3 variables on fertilization success. Each test required the use of a single 24-well microplate and took approximately 3–4 h to complete. The test consists of the following steps: incubation of ovarian oocytes with $0.75 \text{mM NH}_3$-seawater for ca. 40–60 min to induce final maturation, insemination (contact time) for 30 min, egg-rinsing for 20–30 s, incubation of fertilized eggs with raw seawater for 1.5–2.0 hours, and measurement of percent cleavage. The amount of contact time between sperm and eggs had little effect on fertilization success within the range of 3–60 min, suggesting that the contact time could be shortened to 12 min when using a 24-well plate. Recently, Aoki et al. (2007) examined the effects of an extended contact time (60 min) during insemination in $0.75 \text{mM NH}_3$-seawater. The authors found no harmful effects on growth and feed intake ability in pearl oyster larvae when compared with larvae produced by artificial fertilization using a 5-min contact time. Together, these results suggest that contact time may be altered within a range of 3–60 min to suit the goal of a particular study.

We incubated ovarian oocytes in $0.75 \text{mM NH}_3$-seawater for induction of maturation. Thus, the maturational stage of the oocytes at the commencement of the insemination may differ between microplate wells depending on the time to commencement of insemination. However, the effects of prolonging the incubation time in $0.75 \text{mM NH}_3$-seawater on the fertility of oocytes have not been studied.

Researchers have made numerous attempts to determine the optimal sperm density and sperm-egg ratio for fertilization in a number of invertebrate species, including members of the Haliotidae family [see Beaker and Tyler (2001) for review]. The optimal conditions for artificial fertilization appear to differ for each species and each method of insemination. For an easy use of our method in a hatchery setting, we compared the effect of changing the semen volume, irrespective of the concentration of spermatozoa. Fertilization rates increased gradually as the volume was increased from $0.16 \mu\text{l}$ to $1.25 \mu\text{l}$. However, we found no significant differences in fertilization rates within the range of $0.16 - 5.0 \mu\text{l}$. An excess of spermatozoa often leads to a decrease in fertilization success and abnormal development of eggs due to polyspermy (Encena et al. 1998; Baker and Tyler 2001). Therefore, we conclude that the optimum volume of testicular semen for the fertilization of 20,000 eggs in 2 ml seawater is $1.25 \mu\text{l}$. Given that the mean sperm density of the testicular semen used in this study was $2.12 \times 10^7\text{sperm}/\mu\text{l}$, the optimum sperm density for fertilization is $1.3 \times 10^7\text{sperm}/\text{ml}$ (i.e., $2.12 \times 10^7\text{sperm}/\mu\text{l}$ multiplied by $1.25 \mu\text{l}$ and divided by $2 \text{ml}$), and the sperm-egg ratio is $1.3 \times 10^3\text{sperm/egg}$. In agreement with our results, Narita et al. (2008) reported that high fertilization rates (about 60%) in the Japanese pearl oyster were obtained following the addition of more than $3.5 \times 10^5\text{sperm/ml}$. Furthermore,
they reported that the fertilization rate gradually decreased to 40% or less when the numbers of spermatozoa were reduced to \(<0.12 \times 10^7\) sperm/ml.

We previously showed that spermatozoa of the Japanese pearl oyster were immotile in raw seawater. The motility increased as the NH₃ concentration was increased from 0.75 to 2.0 mM. High levels of motility were maintained up to 5.0 mM. In 0.75 mM NH₃-seawater, the percent motility after dilution increased with time from 10 s to 5 min, and then gradually decreased to 120 min (Ohta et al. 2007). Together, these results suggest that the increase in fertilization rate that we observed with time from 10 s to 5 min, and then gradually decreased to 120 min (Ohta et al. 2007). Together, these results suggest that the increase in fertilization rate that we observed with an increase in contact time from 10 s to 5 min, and then gradually decreased to 120 min (Ohta et al. 2007).

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In conclusion, we developed a rapid method for artificial fertilization of the Japanese pearl oyster using 24-well microplates. Maximal fertilization rates were obtained by using 1.25 μl of testicular semen to fertilize 20,000 eggs in 2 ml of seawater containing 0.75 mM NH₃. Changes in contact time in the range of 3–60 min did not affect the fertilization rate. However, to ensure consistency in the contact time between wells, we recommend allowing a contact time of at least 12 min. In the future, we intend to use this method for sperm cryopreservation in the Japanese pearl oyster.

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References


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マイクロプレートを用いたアコヤガイの人工授精方法

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アコヤガイの種苗生産技術の向上に関する研究の一環として、24穴のマイクロプレートを用いた多種類の人工授精実験を行う方法を検討した。アコヤガイから採集した卵を各ウェルに2万粒入れ、媒精精液量、媒精時のアンモニア濃度、媒精時間について検討した。媒精精液量については、受精率は1.25 μl以上では高く、それ以下では低下したが有意差は認められなかった。アンモニア濃度については、0.563 mM以上で高い受精率を示したが、0.375 mM以下では有意に低下した。媒精時間は3分以上60分まで高い受精率を示したが、それ以下では有意に低下した。これらの結果から得られた条件を用い、マイクロプレート上で最大24種類の受精条件を一度に検討することが可能となった。