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FERTILIZATION METHOD QUANTIFYING GAMETE CONCENTRATIONS AND MAXIMIZING LARVAE PRODUCTION IN *CRASSOSTREA GIGAS*

Most workers obtain oyster larvae by using experimental methods similar to those reported by Galtsoff (1964). Although useful in most hatchery or laboratory investigations, these methods do not quantify gamete concentrations. To obtain specific larval concentrations, most researchers dilute dense postfertilization concentrations.

This paper reports on a method of estimating sperm concentrations of Pacific oyster, *Crassostrea gigas*, using colorimetric techniques, and on a method of fertilization using small volumes of seawater and known gamete concentrations. We also present an index which may be useful in evaluating the efficiency of fertilization. These methods were developed during 1973 and should prove useful in the study and production of cultured oysters.

Materials and Methods

Pacific oysters were obtained from Fowler Oyster Co. on Yaquina Bay, Newport, Oreg. Sand-filtered seawater of 25-32‰ salinity and pH 7.0-8.1 was collected at the Oregon State University Marine Science Center (MSC) at Newport, exposed to ultraviolet light (3.785 liters/min), diluted (when necessary) to 25‰ with distilled water, and stored in Nalgene carboys. This salinity is within the range recommended for *C. virginica* by Davis and Calabrese (1964), and was used for maintenance of oysters and for experiments on fertilization and early larval development. In laboratory procedures, all glassware was initially acid-washed; used glassware was carefully cleaned and rinsed several times first in tap water and then in distilled water; all polyethylene tubing was Tygon[1] R3606 (nontoxic by bioassay, Breese, MSC, unpubl. data); gametes and larvae were confined in glass containers only (except for momentary exposure to stainless steel syringe needles and nylon screen); all seawater used in fertilization experiments was Millipore-filtered (0.47 μm) and stored in glass screw-cap bottles with Parafilm-lined caps (nontoxic by bioassay, Breese unpubl. data).

Procurement of Gametes

To enhance gonad development, we conditioned mature oysters in seawater at 16.0° ± 1.0°C for 3-6 wk (Loosanoff and Davis 1963). To identify test oysters, we drilled a 0.8-mm (1/32-inch) hole in the umbo and attached a 6.4- × 15.9-mm numbered plastic tag (Howitt Plastics Co., Mollala, Oreg.) with monofilament. After conditioning, access to the gonads was made by drilling a 1.2-mm (3/64-inch) hole in the posterodorsal region of the right valve. We extracted gametes with a 2.5-cm^3 glass syringe fitted with a 20-gauge 38-mm needle containing about 0.5 ml of seawater (Lannan 1971). Oysters containing either intensively motile sperm or eggs greater than or equal to 36 μm were kept for fertilization experiments. To prevent spawning after extractions, we isolated individual oysters for 12-24 h in 3-liter beakers containing seawater at 12°C.

Prior to gamete extraction we raised the temperature of all donor oysters to 27.0° ± 0.5°C, a temperature within the range recommended by Davis and Calabrese (1964) for fertilization and larval development. Oysters were transferred from the conditioning tray to an 18.9-liter (5-gallon) tank containing 11.4 liters (3 gallons) of seawater at 16.0° ± 1.0°C; a 100-W aquarium heater

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

connected to a thermostat slowly increased the temperature to 27.0° ± 0.5°C. After 30-60 min at this temperature, we extracted gametes by syringe (as above) for use in fertilization experiments. To protect gametes from large pressure changes during gonad extractions, we maintained a gentle and constant negative pressure in the syringe; gametes drawn abruptly into the syringe were discarded. Only extractions which entered the syringe as dense white cords were used; more diffuse or cloudy extractions were also discarded. Samples of gonad extractions and all seawater and glassware used were stored at 27.0° ± 0.5°C.

Gamete Concentrations and Fertilization

Extractions from the gonads of 3-5 males were transferred to and lightly agitated in a Klett-Summerson sample tube containing 5-8 ml of seawater. Using a Klett-Summerson colorimeter with a green (#54) filter, we measured light diffusion through diluted extractions. We then diluted subsamples, placed them in a hemacytometer, and counted the number of sperm. Comparisons were then made between the Klett reading (K) and the actual sperm counts.

Gonad extractions from 3-5 females were similarly pooled, transferred to a Nytex screen (36-μm mesh), and rinsed with seawater to remove small debris and reduce the possibility that some component of the eggs (released from any ova broken during extraction) would cause sperm to agglutinate (Galtsoff 1964). We then rinsed the cleaned eggs into a 250-ml beaker containing 20-50 ml of seawater, counted samples of eggs using a dissection microscope, diluted samples with seawater until reaching the desired concentration, and maintained the egg-seawater suspension at 27.0° ± 0.5°C. We discarded eggs remaining in seawater for more than 1 h to reduce the possibility of sperm agglutination resulting from secretions.

Using a Pasteur pipette (45 drops of seawater/ml), we transferred various sperm concentrations (Table 1) to numbered Syracuse watch glasses, then with an automatic pipette added 0.2 ml of egg-seawater suspension containing 100 ± 4 eggs. The pH of 7.8 ± 0.1 was in the range recommended by Humphrey (1950). After introduction of the egg-seawater suspension, we added 7 ml of seawater at different time intervals (flooding time, Table 2) to dilute the sperm concentrations and to reduce the possibility of polyspermy. The

TABLE 1.—Range and mean of percent fertilization (%Z) and percent of larvae developing to the D-shape stage (%D) resulting from different sperm concentrations combined with fresh eggs (100 ± 4/0.2 ml) of *Crassostrea gigas*.[1]

Sperm		%Z		%D			
Concn.	Vol (ml)	Range	Mean	Range	Mean	$L_{\bar{X}}$[2]	CI[3]
1.1 × 10^4	0.02	0- 58	27.6	0-56	25.0	2.60	0.74
3.3 × 10^4	0.07	10- 55	34.4	10-50	29.2	5.20	0.64
5.5 × 10^4	0.11	41- 86	57.4	36-73	48.0	9.40	0.99
1.1 × 10^5	0.22	55-100	77.2	50-93	58.6	18.60	0.91
2.4 × 10^5	0.02	60-100	72.6	47-88	58.2	14.40	1.05
7.3 × 10^5	0.07	75-100	87.0	51-94	68.8	18.20	1.16
1.2 × 10^6	0.11	78-100	89.0	35-70	45.2	43.80	0.33
2.4 × 10^6	0.22	80-100	89.2	13-72	29.0	60.20	0.12
5.0 × 10^6	0.50	80-100	92.4	8-43	21.9	70.50	0.06
1.1 × 10^7	1.00	87-100	96.0	0-23	15.0	81.00	0.03

[1]Seawater of salinity 25‰ and pH 7.8 ± 0.1; temperature 27° ± 0.5°C; gametes diluted with 7 ml of seawater at 10 min postfertilization; 5 repetitions per sperm concentration.

[2]$L_{\bar{X}}$ = Mean percent larvae losses = (%$Z_{\bar{X}}$ minus %$D_{\bar{X}}$).

[3]CI = Compatibility index = $\frac{(\%D_{\bar{X}})^2}{\sqrt{L_{\bar{X}}(\%Z_{\bar{X}})}} \times 10_{\bar{X}}^2$.

TABLE 2.—The range and mean of percent fertilization (%Z) and percent of larvae developing to the D-shaped stage (%D) obtained by different flooding times[1] after combining the gametes of *Crassostrea gigas*.[2]

Flooding time[1] (min)	%Z		%D		
	Range	Mean	Range	Mean	$L_{\bar{X}}$[3]
1	41- 88	67.2	41-72	62.0	5.2
5	73-100	85.4	65-94	73.0	12.4
10	75-100	87.8	51-94	68.2	19.6
15	78-100	89.8	0-68	47.0	42.8
30	82-100	96.4	0-20	8.8	87.6

[1]Flooding time = time (min) between the combination of gametes and the addition of 7 ml of seawater to the gamete mixture.

[2]Using seawater of salinity 25‰ and pH 7.8 ± 0.1, 100 ± 4 eggs in 0.2 ml of seawater were added to 7.3 × 10^5 sperm in 0.07 ml of seawater; 5 repetitions/flooding time were used.

[3]$L_{\bar{X}}$ = Mean percent larvae losses = (%$Z_{\bar{X}}$ minus %$D_{\bar{X}}$).

watch glasses were stacked to reduce evaporation and incubated at 27.0° ± 0.5°C. Because the number of swimming lavae did not increase after 6 h postfertilization time, the number of fertilized eggs was obtained by counting unfertilized eggs remaining on the bottom at 6 h and subtracting this figure from 100 (the number of eggs originally present). After 24 h we transferred the watch glasses to a 4°C refrigerator; within 30 min the D-shaped (straight-hinged) larvae settled to the bottom and were easily counted.

Although none of the 460 oysters examined appeared hermaphroditic, sperm-free controls were used in all experiments. We did not observe fertilization in any of the controls.

Results and Discussion

The relationship between K and the number of

sperm counted is linear (r = 0.996) from about K = 10 to about K = 80 (Figure 1). Because this method of estimation is sufficiently precise and accurate and because attempts to minimize gonadal debris (and thus minimize a variable in colorimetric evaluation) by gravity filtration or centrifugation usually resulted in broken tails and agglutination, respectively, we consider our methods of sperm procurement and estimation useful. Measuring light diffusion through a sample of *C. gigas* eggs did not accurately estimate egg numbers because they settled rapidly.

We estimate that about one-half the number of sperm counted had little or no observable motility; we may have withdrawn immature sperm or damaged mature sperm during extraction. Inactive sperm were not agglutinated, an indication that the acrosome reaction was not the major cause of immotility. Although not directly equating fertilization capacity with high motility, our assumption is that relatively immotile sperm are incapable of fertilizing viable eggs. Similarly, extractions from females often included small and presumably immature eggs (Galtsoff 1964). Sperm concentrations reported in Figure 1 and Tables 1 and 2 are observed values and do not reflect estimates of immotile cells; only "mature-sized" eggs were used because eggs less than 36 μm were rinsed through the cleaning screen.

Within the limits of this investigation, mean percent fertilization ($\%Z_{\bar{T}}$) increased as the number of sperm/100 eggs increased (Table 1). The mean percent of larvae developing to the D-shape stage ($\%D_{\bar{T}}$) increased until 7.3×10^5 sperm were used; $\%D_{\bar{T}}$ decreased with further increases of sperm concentration (Table 1). Because Glatsoff (1964) reported that high sperm concentrations may result in polyspermy and because in our experiments resulting in large losses of larvae ($L_{\bar{T}}$ [where $L_{\bar{T}} = \%Z_{\bar{T}}$ minus $\%D_{\bar{T}}$]) aberrant forms were observed (e.g., swimming chains of cells, and trochophores persisting beyond 48 h), we assume polyspermy was responsible for the increasing $L_{\bar{T}}$.

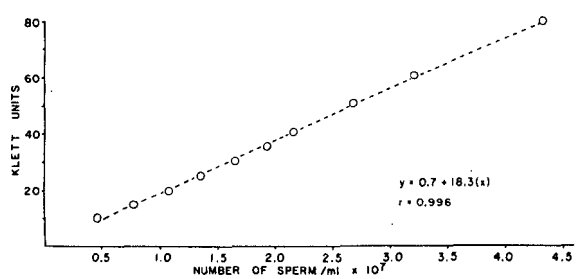

FIGURE 1.—Correlation between mean (N = 5) number of *Crassostrea gigas* sperm and Klett units (light diffusion readings) on a Klett-Summerson colorimeter.

Using 7.3×10^5 sperm/100 eggs, we observed that $\%Z_{\bar{T}}$ increased as flodding time increased (Table 2). $L_{\bar{T}}$ also increased as flooding time increased, and maximum $\%D_{\bar{T}}$ was obtained using a flooding time of 5 min.

Although most workers need only to maximize $\%D_{\bar{T}}$ without regard to $L_{\bar{T}}$, some investigators may need to minimize $L_{\bar{T}}$ due to limited spawning stock or other problems. Thus, to achieve maximum efficiency it is necessary to maximize $\%D_{\bar{T}}$ and minimize $L_{\bar{T}}$. Under different conditions (e.g., water quality and gamete viability may differ at different locations or at different times), the optimal sperm concentration and flooding time will vary in response to the environment. Increases in $\%D_{\bar{T}}$ (by increasing sperm concentration or flooding time) also produce undesirable increases in $L_{\bar{T}}$, thus a subjective decision usually is made to evaluate the efficiency of fertilization and larvae production. To reduce the subjectivity of this evaluation, we suggest the following formula reflects a compatability between maximum $\%D_{\bar{T}}$ and minimum $L_{\bar{T}}$:

$$\text{Compatability index (CI)} = \frac{(\%D_{\bar{T}})^2}{\sqrt{L_{\bar{T}}\ (\%Z_{\bar{T}})}} \times 10^{-2}.$$

In our lab, values greater than or equal to 1 were desirable, and 1.16 was the maximim value obtained (Table 1). CI values can be high for relatively low $\%D_{\bar{T}}$ if $L_{\bar{T}}$ is unusually low (e.g., where $\%Z_{\bar{T}} = 30$, and $\%D_{\bar{T}} = 28$, CI = 1.01). Low $L_{\bar{T}}$ values will normally be associated with low $\%D_{\bar{T}}$; however, if a low $L_{\bar{T}}$ occurs concommittantly with a "reasonable" $\%D_{\bar{T}}$, we assume that the evaluation would be based more on the desired $\%D_{\bar{T}}$ rather than on CI. Further, due to the often dramatic differences in conditions at different labs and hatcheries, or at different times, attempts to establish a desirable CI value or range under specified conditions may prove useful.

During a 4- to 6-wk period we made 8-12 extractions from individual oysters, but did not observe a deterioration of gametes. Data from experiments using gametes from initial extractions were consistent with those of later extractions. The pooling of extractions may have reduced observable changes. After about 8 wk, eggs were easily broken and we noticed free yolk in extrac-

tions. Although deterioration of male gonads was less evident, we noted that the sperm concentration decreased after about 8 wk, presumably as a result of resorption. The mortality rate for oysters repeatedly used for gamete extractions and maintained without food or biological filters in 113.6-liter (30-gallon) tanks containing recirculating seawater (25‰) at 16.0° ± 1.0°C was about 10% during the 8-wk period.

Because high concentrations and large numbers of gametes can repeatedly be extracted from the gonads of individual oysters without apparent detriment and because gamete extraction obviates artificial spawning and its inherent problems, we suggest our method of gamete procurement can be useful in many investigations and hatchery situations. Our method also permits repeated use of the gametes of selected oysters, and this together with the possible use of cryopreserved sperm (Staeger 1974) reduces variability and increases control and management of hatchery production or biological investigations.

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