

GYNOGENETIC INDUCTION IN MARINE BIVALVE MOLLUSCS FOR IMPROVEMENT OF STOCKS : STANDARDIZATION OF SOME IMPORTANT FACTORS

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ABSTRACT

Experiments conducted for standardizing sperm density for artificial insemination of *Chlamys nobilis* prior to inducing gynogenesis by ultra-violet rays irradiated sperms showed that 10 : 1 ratio of sperms to eggs should be maintained for getting maximum rate of fertilization and the normal development of the veliger larvae. The optimum quantity and density of sperm suspension recommended for irradiation exposure experiments in 10 ml capacity glass petri dish was 0.5 ml of stock sperm suspension with 20×10^6 per ml concentration so as to effectively inseminate 2000 - 2500 eggs in 50 ml of seawater. The results of the preliminary gynogenetic induction experiments performed ascertained the reliability of these parameter limits. In controls, it showed 100% rate of fertilization & 95.00% normality in the form of morulae after 6 hours of insemination and 98.05% metamorphosis and 96.12% normality in the form of normal D-shaped larvae, 24 hours after insemination. The lower values of fertilization, metamorphosis and normality in the other groups were due to the insemination of eggs with the ultra-violet ray irradiated sperms. The gynogenetic haploid larvae with 16 chromosomes were induced.

INTRODUCTION

Application of induced gynogenesis for improvement of stocks in cultivable aquatic organisms is of recent origin. Most of the earlier work done was on fishes using various types of chemical and physical agents (Onazato, 1982; Purdom, 1983). Amongst these exposure of sperms to ultra-violet ray irradiation before inseminating eggs was widely accepted as a simple and safe method. In edible molluscs, informations were available on *Crassostrea virginica*, *Haliotis discus* and *Mulina lateralis* (Arai et al., 1984; Allen, 1987). Presently studies were undertaken to find the optimum limits of the sperm density giving highest rate of fertilization of eggs, metamorphosis and normal development of the veliger larvae for artificial insemination and gynogenetic induction in *Chlamys nobilis* (Bivalvia : mollusca).

MATERIALS AND METHODS

Scallops *Chlamys nobilis* were collected from the field raft at Gokasho Bay, Mie prefecture, Japan during the month of May/June, 1988 and acclimatized to tank conditions for 48 hr. The male and female specimens were kept separately in 10 l tanks and induced to spawn individually by thermal stimulation (25° C). The eggs were screened through 20 μ m mesh size nets, transferred to 2000 ml beaker containing seawater, labelled as 100% egg stock suspension and were kept in a water bath at 25°C. The male specimens when showed signs of extruding milt, were immediately removed from the 10 litre tank and the quality of milt was checked under the microscope. The selected animal was transferred to a tray of 1000 ml capacity filled with seawater and allowed to breed. This was put in a beaker, labelled 100% sperm stock suspension and was maintained at 25° C.

Three series of experiments were designed and performed in glass beakers of 50 ml capacity. The controls and replicates were maintained. In the first series of experiments the concentration of eggs was used as present in the stock suspension without making any further quantitative adjustments. But for sperms, 11 dilutions of the stock suspension were prepared viz: 3, 5, 10, 25, 100, 200, 500, 1000, 2000, 4000 and 10000 times. Each experimental set up contained 10 ml of egg stock suspension, 39 ml of sea water and 1 ml of sperm suspension from respective dilution. In control experiments the undiluted 1 ml of sperm stock suspension was used. The incubation was continued for 6 hours at 25° C.

The number of sperms and eggs per ml in the 100% stock suspensions were microscopically counted using "Thoma cell counter" and "Sedgewick rafter" respectively. The number of sperms in each dilution was calculated accordingly by dividing the stock suspension's per ml value by the times of sperm dilution made. For artificial insemination the measure of sperm to egg relative ratios was used. The number of eggs per ml in stock suspension was taken as ratio 1 (Table 1).

Rate of fertilization was recorded from samples collected after 5 hour duration from the time of insemination and fixed in 5% formalin solution. The percentage of ferti-

Table 1. Number of sperms and eggs in different sperm to egg ratios of *Chlamys nobilis* available for fertilization in experimental sets of series-1.

Sperm dilutions	Number of sperms/ml in dilution	Number of sperms/ml	Number of eggs /ml	sperm: egg ratios
available in 50 ml of experimental sets				
stock sus- pension (C)	20.00 x 10 ⁶	400,000	13672	* 30 : 1
3	6.66 x 10 ⁶	133,000	13672	10 : 1
5	4.00 x 10 ⁶	80,000	13672	6 : 1
10	2.00 x 10 ⁶	40,000	13672	3 : 1
25	0.80 x 10 ⁶	16,000	13672	1.20 : 1
100	0.20 x 10 ⁶	4,000	13672	0.30 : 1
200	0.10 x 10 ⁶	2,000	13672	0.15 : 1
500	0.04 x 10 ⁶	800	13672	0.06 : 1
1000	0.02 x 10 ⁶	400	13672	0.03 : 1
2000	0.01 x 10 ⁶	200	13672	0.015 : 1
4000	0.005 x 10 ⁶	100	13672	0.007 : 1
10000	0.002 x 10 ⁶	40	13672	0.003 : 1

* Number of eggs / ml in 100% stock suspension = 68360
 Eggs in 10 ml of stock suspension added to experimental beaker = 683600
 Eggs available for insemination in 50 ml of seawater in the experimental beaker = 683600.
 Eggs per ml available for insemination in experimental sets = 683600/50 = 13672.

lized and unfertilized eggs was computed. The eggs with the germinal vesicle still intact were taken as unfertilized and the ones with germinal vesicle broken/polar bodies formed/undergoing cleavage divisions, were taken as fertilized.

The second series of experiments were conducted by keeping the general details of methodology the same as in series 1 but making amendments wherever felt necessary; viz; the number of eggs was reduced to 2000 - 2500 per ml as available for fertilization in the experimental beakers and was thereafter denoted as ratio 1. Some of the sperm dilutions were discontinued and only ratios of 100, 30, 10, 3, 1.2, 0.5, & 0.002 times to the ratio of eggs taken as 1 were kept. The samples of developing zygotes were collected after 24 hours and preserved in 5% formalin. The percent rates of fertilization, metamorphosis and normality were noted. The term metamorphosis was used here to the total number of veliger larvae formed whereas the normality was calculated taking into consideration only the number of normal D-shaped larvae.

In the third series of experiments, the optimum limits of the basic parametres were maintained as deduced from results of the second series. In addition, the new factor of ultra-violet ray irradiation to sperms for inducing gynogenetic haploidy was introduced. In control group, the eggs were inseminated with the unexposed sperms and allowed to develop for 24 hours. For other set ups the sperms were exposed to various dozes of U.V. irradiations by keeping a constant rate of 114 ergs /mm²/second and changing the different times of exposure between 4 sec. to 90 sec. After 6 and 24 hours of insemination the samples were collected for analysis of fertilization rate, normality in morula stage, metamorphosis, normality in the D-shaped larvae and chromosomal analysis.

RESULTS AND DISCUSSION

The results of the first series of experiments indicated that in 10 : 1 sperm to egg ratio the fertilization rate was 100%. In concentrations higher than this viz; 30:1 the same trend continued. But it decreased in the lower concentrations. For example in ratio 6: 1 the rate of fertilization was 97.50%, in 3 : 1 it was 95.00% and in the lowest concentration i.e. 0.003 : 1 it was 02.12% (Table II). It was difficult to understand the rather gradual fall noticed in rates of fertilization between 0.15 : 1 to 0.015. This may be due to the fact that actual number of sperms available in the limited space of 50 ml seawater for inseminating 13672 eggs got a good chance of coming in contact with the eggs. Like in 0.015 : 1 ratio 2000 sperms got a chance of fertilizing 13672 eggs. Out of which randomly only 100 were observed for analysing rate of fertilization. Infact where the values were less than 100% it was not of much interest keeping in view the objectives of the studies. But it was highly important to find out if amongst the ratios showing 100% fertilization any differences were present during the larval development. Hence the second series were performed.

The results of these series showed that though the rate of fertilization was 100% in the ratio 10:1 and above, the number of normal D-shaped larvae decreased rapidly. In 10 : 1 ratio out of 73 fertilized eggs observed all developed into the normal D-shaped larvae whereas in 100 : 1 ratio amongst 92 fertilized eggs encountered only 16 developed normally (Table III). The percentage of normality thus decreased with the increase of sperm to egg ratios higher than 10:1. The rate of actually metamorphosed D-shaped larvae including both normal and abnormal types were 100% in 10:1, 64.68% in 30:1 and 56.52% in 100:1 ratios. The ratios lower than 10:1 showed a decrease in the

Table II. Rate of fertilization (%) in *Chlamys nobilis*, 5 hours after insemination with sperms in various ratios, Series-I.

*Sperm Ratio: Egg Ratio	Number of fertilized eggs	Fertilization rate (%)
30.00 : 1	300 (300)*	100.00
10.00 : 1	200 (200)	100.00
06.00 : 1	156 (160)	97.50
03.00 : 1	95 (100)	95.00
01.20 : 1	133 (141)	94.32
00.30 : 1	83 (100)	83.00
00.15 : 1	51 (100)	51.00
00.06 : 1	66 (187)	37.40
00.03 : 1	20 (075)	26.66
00.015 : 1	21 (164)	12.80
00.007 : 1	6 (109)	05.50
00.003 : 1	3 (141)	02.12

- * 1. Actual number of sperms and eggs available for fertilization in 50 ml suspension is as given in table 1.
 2. Figures in parantheses are the number of eggs screened under the microscope.
 3. Egg Ratio 1 = 13672 number of eggs.

Table III. Number of fertilized, unfertilized eggs and various categories of developing zygotes of *Chlamys nobilis* observed after 24 hours of insemination with sperms in different ratios, Series - II.

Sperm: Egg ratio	Total Eggs/ Zygotes	Fertilized eggs	Unfertilized eggs	Stages upto Morula	*D-shaped normal larvae	D-shaped abnormal larvae	***Metamorphosis (%)
100.00 : 1**	92	92 (100.00)	—	39	16 (31.52)	37	56.52
30.00 : 1	48	48 (100.00)	—	17	21 (43.75)	10	64.68
10.00 : 1	73	73 (100.00)	—	—	73 (100.00)	—	100.00
3.00 : 1	263	250 (95.05)	13	40	220 (83.65)	—	88.00
1.20 : 1	95	77 (81.05)	18	20	57 (60.00)	—	74.02
0.50 : 1	73	42 (57.53)	31	18	24 (32.87)	—	57.14
0.002 : 1	33	16 (48.48)	17	9	7 (21.21)	—	43.75

Figures in parantheses means percentages.

*Stages upto morula = 32-64 celled stage.

**Number of eggs/ml in egg ratio = 1, kept constant, = 2000-2500.

***Metamorphosis = Total larvae metamorphosed to D-shaped larvae including both normal and abnormal larvae.

rate of fertilization. The percent normality also decreased but the fall in the lower concentrations was gradual whereas towards higher concentrations it was abrupt. As shown in Table III, the normality in 3:1 ratios was 83.65% whereas in 30:1 ratios it was 43.75%. It was further observed that in the ratios above 10:1 the number of zygotes upto morula stage and stopping thereby was higher (39 in 100 : 1 and nil in 10:1 ratios). The main abnormality seen in the D-shaped larvae was in their swimming behaviour and shape of the larvae (Fig. 1). Kakuchi and Uki (1974) had earlier reported adverse effects of too high and low concentrations of sperms on fertilization and normal development of the larvae on abalone hatcheries. The present experiments denoted that the higher concentrations of sperms than the optimum quantity were more harmful for normal development of the larvae than the lower concentrations. The latter influenced mainly the fertilization rate.

In context with the objectives of the present experiments for artificial insemination and as a pre-requisite to the regular gynogenetic experiments it was deduced that in *Chlamys nobilis* an aliquot of 0.5 ml of stock

sperm suspension with 20×10^6 per ml concentration should be used for fertilization of 2000 – 2500 per ml eggs in 50 ml seawater. When spread in a petri dish of 10 ml capacity, it formed a sufficiently thin film of 0.1 mm thickness. By keeping 100 rotations per minute of agitation of sperm suspension constant on the rotary shaker, most of the sperms will get a chance of exposure to ultra-violet rays.

The authenticity of these suggestions were ascertained from results of the actual preliminary gynogenetic induction experiments of series 3. It showed 100% fertilization and 95% normality in control groups after 6 hours of insemination and 98.05% metamorphosis and 96.12% normality after 24 hours. The lower values of the same in the treated groups were due to the insemination with U.V. irradiated sperms (Table IV). The chromosomal analysis made showed haploidy induction in the groups inseminated with sperms exposed for 50 sec. duration (5700 ergs/mm²/second). The chromosome number was 16 (1 n) as shown in fig. 2. Komaru and Wada (1985) had earlier described a diploid number of 32 chromosomes for this species.

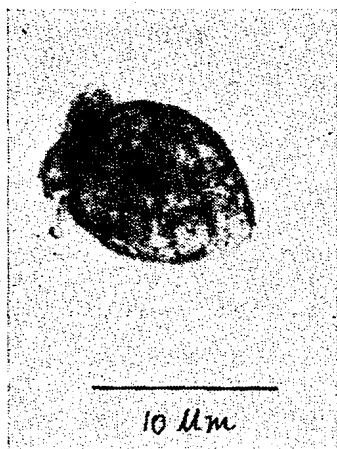


Fig. 1 Abnormal D-shaped larvae.

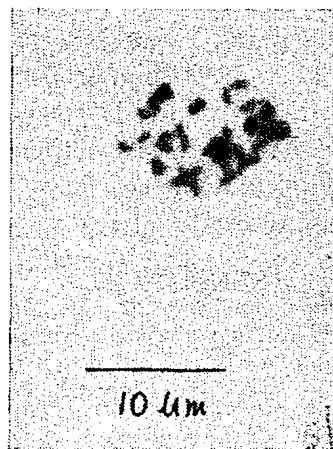


Fig. 2. Plate with 16 number of chromosomes as encountered in the gynogenetic haploid larvae

Table IV. Percentage rate of fertilization, normality and metamorphosis in *Chlamys nobilis* eggs/zygotes after 6 and 24 hours of insemination with the ultra-violet ray irradiated sperms.

U.V. exposure time (seconds)	after 6 hours fertilization (%)	normality (%)	after 24 hours metamorphosis (%)	normality (%)
Control	100.00 (40)	95.00 (40)	98.05 (100)	96.12 (100)
4	56.00 (50)	95.00 (40)	82.92 (82)	23.17 (82)
8	81.48 (27)	20.00 (50)	46.98 (83)	39.75 (83)
12	70.83 (25)	48.15 (27)	58.97 (39)	35.89 (39)
16	62.50 (26)	04.00 (26)	75.00 (32)	31.25 (39)
20	35.71 (28)	07.40 (28)	35.48 (31)	09.67 (31)
30	52.00 (25)	04.00 (25)	32.55 (43)	04.65 (43)
50	66.66 (36)	33.33 (36)	65.38 (26)	30.76 (26)
60	57.69 (26)	07.69 (26)	75.26 (93)	21.50 (93)
90	61.54 (26)	07.69 (40)	80.92 (178)	05.71 (178)

Figures in parantheses give number of eggs / zygotes observed.

U.V. irradiation rate = 114.00 ergs/mm²/second.

Normality after 6 hours = morula stage

Normality after 24 hours = number of D-shaped larvae.

Metamorphosis = normal + abnormal D-shaped larvae.

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