

Sperm density required for inducing gynogenetic haploidy in scallop *Chlamys nobilis*

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Experiments conducted for standardizing sperm density for artificial insemination and inducing gynogenesis by ultra-violet rays irradiated sperms in *C. nobilis* showed that 10:1 ratio of sperms to eggs, gave maximum rate of fertilization and normal development of the veliger larvae. Insemination of eggs with sperms exposed to 5700 to 10260 ergs. mm^{-2} of UV irradiation induced gynogenetic haploidy.

Application of induced gynogenesis to attain maximum homozygosity for developing pure lines in the genetic improvement experiments was of recent origin. The male germ plasm was inactivated and the animals were developed with the maternal genotype. Exposure of sperms to ultra-violet ray irradiation before inseminating the eggs was widely accepted as a simple and safe method. In edible molluscs, informations on these aspects were available on *Crassostrea virginica*, *Haliotis discus* and *Mulinex lateralis*.

Present study was made to find the optimum limits of sperm density giving highest rate of fertilization of eggs, metamorphosis and normal development of the veliger larvae for artificial insemination and gynogenetic induction in scallop *Chlamys nobilis* (Reeve, 1852) (Bivalvia, Mollusca).

Materials and Methods

Scallops, *C. nobilis*, were collected from the field raft at Gokasho Bay, Mie prefecture, Japan during May/June 1988 and acclimatized to tank conditions for 48 h. 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 μm mesh size nets, transferred to beaker (2000 ml) 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 tank and the quality of milt was checked under the microscope. The selected animal was transferred to a tray (1000 ml) filled with seawater and allowed to breed. This was put in a beaker,

labelled 100% sperm stock suspension and maintained at 25°C.

Three series of experiments were designed with controls and replicates were maintained. In the first series 100% concentration of eggs was used. For sperms, 11 dilutions of the stock suspension (100%) 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 seawater and 1 ml of sperm suspension from respective dilution. In controls undiluted 1 ml of sperm stock suspension (100%) was used. The incubation was continued for 6 h at 25°C.

The sperms and eggs per ml in the 100% stock suspensions were counted using Thoma cell counter and Sedgewick rafter respectively. The number of sperms in each dilution was calculated accordingly. 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 6 h from the time of insemination and fixed in 5% formaline solution. The percentage of fertilized and unfertilized eggs was computed. Eggs with the germinal vesicle still intact were taken as unfertilized and the ones with germinal vesicle broken/polar bodies formed/under going cleavage divisions, were taken as fertilized.

The second series of experiments were same as series 1 but 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

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

Sperm dilutions of 100%	Sperms (S) (No. ml ⁻¹ in dilution)	S and E available in 50 ml of experimental sets		
		Sperms(S) (No. ml ⁻¹)	Eggs(E) (No. ml ⁻¹)	S:E ratio
Control (100%)	20,000000	400000	13672	30:1
3	6,660000	133000	13672	10:1
5	4,000000	80000	13672	6:1
10	2,000000	40000	13672	3:1
25	800000	16000	13672	1.20:1
100	200000	4000	13672	0.30:1
200	100000	2000	13672	0.15:1
500	40000	800	13672	0.06:1
1000	20000	400	13672	0.03:1
2000	10000	200	13672	0.015:1
4000	5000	100	13672	0.007:1
10000	2000	40	13672	0.003:1

* Number of eggs ml⁻¹ 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.

and 0.002 times of sperms to the egg ratio 1 were kept. The samples of developing zygotes were collected after 24 h and preserved in 5% formaline. 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 only the number of normal D-shaped larvae.

In the third series of experiments, the optimum limits of the basic parameters were maintained as deduced from results of the second series. In addition, the new factor of UV 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 h. In others sperms were exposed to various doses of UV irradiation by keeping a constant rate of 114 ergs. mm⁻².sec⁻¹ and changing the period of exposure from 4 to 90 sec. After 6 and 24 h of insemination the samples were collected for analyses of fertilization rate, normality in morula stage, metamorphosis, normality in D-shaped larvae and chromosomal analysis.

Results and Discussion

The results of first series of experiments indicated that in 10:1 and 30:1 sperm to egg ratio the fertilization rate was 100% (Table 2). It decreased in lower concentrations, however, the fall in rates of fertilization was gradual rather than an abrupt one. It may be due to the fact that though the actual number of sperms in the solutions decreased the small enclosed nature of the rearing environment (50 ml

Table 2—Rate of fertilization (%) in *C. nobilis*, 6 h after insemination with sperms in various ratios (series I)

Sperm:egg ratio	Fertilized eggs (no.)	Fertilization rate (%)
30.00:1	300(300)	100.0
10.00:1	200(200)	100.00
6.00:1	156(160)	97.0
3.00:1	95(100)	95.0
1.20:1	133(141)	94.32
0.30:1	83(100)	83.0
0.15:1	51(100)	51.0
0.06:1	66(187)	37.4
0.03:1	20(075)	26.66
0.015:1	21(164)	12.8
0.007:1	6(109)	05.5
0.003:1	3(141)	02.12

* (i) Actual number of sperms and eggs available for fertilization in 50 ml suspension is as given in table 1

(ii) Figures in parantheses are the number of eggs screened under the microscope

(iii) Egg ratio 1 = 13672 number of eggs

seawater) gave sufficient opportunities to most of the sperms for coming in close contact with eggs for fertilization. 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 study. 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 normal D-shaped larvae whereas in 100:1 ratio amongst 92 fertilized eggs only 16 developed normally (Table 3). The percentage of normality thus decreased with 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 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 3, the normality in 3:1 ratios was 84% whereas in 30:1 ratios it was 44%. It was further observed that in the ratios above 10:1 the number of zygotes developing up to morula stage and not growing further was higher (39 in 100:1 and nil in 10:1 ratios). The main abnormality seen in D-shaped larvae was in their swimming behaviour and shape of the larvae. Kikuchi and Uki³ had earlier reported adverse effects of too high and low concentrations of sperms on fertilization and normal development of the larvae in ablone hatcheries. The present experiments showed that higher concentrations of sperms than the optimum quantity were more harmful for normal development of 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 for regular gynogenetic experiments it was deduced that in *C. nobilis* an aliquot of 0.5 ml of stock sperm suspension with $20 \times 10^6 \text{ ml}^{-1}$ concentration should be used for fertilization of

2000-2500 eggs. ml^{-1} 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. min^{-1} of agitation of sperm suspension constant on the rotary shaker, most of the sperms will get a chance of exposure to U V rays.

The reliability of these assumptions was ascertained from results of preliminary gynogenetic induction experiments of series 3. There was 100% fertilization and 95% normality in control groups after 6 h of insemination and 98.05% metamorphosis and 96.12% normality after 24 h. The lower values of the same in the treated groups were due to the insemination with U V irradiated sperms. As shown in Table 4, in 90 sec exposed group, the rate of fertilization was 61.54% and metamorphosis and

Table 4—Percent rate of fertilization, normality and metamorphosis in *C. nobilis* eggs/zygotes after 6 and 24 h of insemination with the U V irradiated sperms

(Figures in parantheses give number of eggs/zygotes observed)

U V exposure	After 6 h		After 24 h	
	Fertilization (%)	Normality (%)	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(260)	04.00(26)	75.00(32)	35.89(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)

U V irradiation rate = 114 ergs. $\text{mm}^{-2} \cdot \text{sed}^{-1}$

Normality after 6 h = morula stage

Normality after 24 h = number of D-shaped larvae

Metamorphosis = normal + abnormal D-shaped larvae

Table 3—Number of fertilized, unfertilized eggs and various categories of developing zygotes of *C. nobilis* observed after 24 h of insemination with sperms in different ratios (series 2)

(Figures in parantheses are percentages)

Sperm:egg ratio	Total eggs/zygotes	Fertilized eggs	Unfertilized eggs	Stages up to Morula	D-shaped* normal larvae	D-shaped abnormal larvae	Metamorphosis ^f (%)
100.00:1 ^f	92	92(100)	—	39	16(31)	37	56.52
30.00:1	48	48(100)	—	17	21(44)	10	64.68
10.00:1	73	73(100)	—	—	73(100)	—	100.00
3.00:1	263	250(95)	13	40	220(84)	—	88.00
1.20:1	95	77(81)	18	20	57(60)	—	74.02
0.50:1	73	42(57)	31	18	24(32)	—	57.14
0.002:1	33	16(68)	17	9	7(21)	—	43.75

* Stages up to morula = 32-64 celled stage.

^f Number of eggs. ml^{-1} in egg ratio = 1, kept constant = 2000-2500.

Metamorphosis = total larvae metamorphosed to D-shaped larvae including both normal and abnormal larvae

normality were respectively 80.92 and 5.71% after 24 h. The chromosomal analysis showed gynogenetic haploidy induction from the 50-90 sec groups (5700-10260 ergs. mm^{-2} of U V irradiation).

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