Induced triploidy in Pacific oyster Crassostrea gigas, and performance of triploid larvae

Tohoku Journal of Agricultural Research
Vol. 39, No. 1, October 1988
Printed in Japan

Induced triploidy in Pacific oyster *Crassostrea gigas*, and performance of triploid larvae

Satoshi YAMAMOTO*, Yoshio SUGAWARA*, Tadashi NOMURA*
and Akio OSHINO**

**Department of Fishery Science, Faculty of Agriculture, Tohoku University, Sendai 980, Japan*
***Oyster Research Institute, 211, Higashimohne, Karakuwa-cho Miyagi 988-05, Japan*

(Received, July 25, 1988)

Summary

Triploidy was induced in the Pacific oyster, *Crassostrea gigas* by cytochalasin B treatment or temperature treatment. Cytochalasin B (0.1, 0.5, 1.0 mg/*l* with 0.005% DMSO, duration 20 min) was administered to the zygotes incubated at 19° C 10 min or 30 min after fertilization, and the highest triploid induction was 67.2% when the treatment at 0.5 mg/*l* started at 10 min. Higher dose of CB induced higher ratios of polyploid (including tetra and pentaploids) and significantly decreased the ratios of 2 hr segmentation or 24 hr survival.

In the two series of temperature shocks at 0° or 35°C, duration 10 min, initiated at 5 min intervals from 15-60 min after fertilization, the most effective treatment was that at 20 min (68.3%) for heat shock and that at 15 min for cold shock (66.7%).

In 5 different temperature treatments (at 0, 32, 35, 37, 40°C ; duration 10 or 15 min, only 3 min for 40°C), heat shock at 37°C applied from 45 min with a duration of 15 min was the most effective (triploid, 83.3%).

The mean shell length of triploid larvae induced by heat shock (35°C, duration 10 min) applied at meiosis-I was greater than that of control or triploid induced by meiosis-II treatment, 11 and 25 days after rearing. In meiosis-II treatment group, mean shell length was less than that of control through the rearing. Significant differences have not been observed about their abilities to ingest or digest the algal food, *Isochrysis galbana*, by observation with a fluorescence microscope at the first larval feeding.

It has been suggested that gonadal development was also retarded in induced triploid bivalves (1-3) as found in various fish species (4-9). Retardation of the gonad development seems to prevent the utilization of glycogen in the bivalves which utilize the accumulation of glycogen reserves for gametogenesis, and also the energy not used for the sexual maturation is expected to accelerate the growth of triploids as found in the bay scallop, *Argopecten irradians* (1) and the Pacific

oyster, *Crassostrea gigas* (3). The glycogen rich oyster meat in the reproductive summer season is expected to increase its commercial value. We are going to investigate the physiological nature of the triploid bivalves and the possibility of their commercial exploitation. Triploids are also very interesting animals for the study of reproductive physiology because of their lack of reproductive capability. They will give us useful information as negative control. Furthermore, we believe that the study for the mechanisms of triploid induction from the stand point of cell motility and the cytoskeleton will provide us with a useful new tool for cell technology.

The purpose of the present investigation was two-fold : first, to examine the best method for inducing triploid ; second, to determine the nature of the triploid larvae.

We have tried to induce triploid *C. gigas* by cytochalasin B (CB) treatment or temperature treatment, and have made a study about a simple and effective method for triploid induction. Triploidy has been induced successfully in a number of marine molluscan species, and CB treatment was the most common and effective treatment (1, 10-13). Triploid induction by temperature treatment was also effective for some molluscs. Triploidy had been induced effectively in Pacific abalone, *Haliatis discus hannai* or in the mussel, *Mytilus edulis* by heat or cold shock (14, 15). And in *C. gigas*, Quillet and Panelay reported tripolid induction by heat shock (16), but their triploid inducing efficiency was less than that of CB treatment reported by Downing and Allen (13). Hydrostatic pressure shock also inducted triploid in *C. gigas* (17) or *H. discus hannai* (14), though the efficiency in *C. gigas* was moderate. What might be the most useful method for triploid induction was investigated.

Next, we made experiments about the growth of triploid *C. gigas* larvae induced by heat shock, and their abilities in ingestion and digestion of the algal food. The nature of triploid *C. gigas* larvae has never been reported on. This is the first report about it.

Materials and Methods

Gametes

Two-year-old *C. gigas* from the cultures in Matsushima bay and Mohne inlet were used. Oocytes and sperm were obtained by dissecting ovaries and testes in filtered sea water (FSW). Oocytes were rinsed three or four times.

Triploid induction

1. Cytochalasin B treatments

The optimum dose of CB was examined in this experiment. Oocytes (2×10^5/treatment) were placed into 50 ml FSW at 19°C and fertilized. A CB solu-

tion was added to this FSW at the beginning of the treatment period : at 10 or 30 min after fertilization, the time of meiosis-I or meiosis-II. Final concentration of CB was 0.1, 0.5 or 1.0 mg/l. After 20 min, the zygotes were filtered onto a 20 μm mesh, and then resuspended into a 0.005% dimethyluslfoxide (DMSO) for another 20 min to remove the residual CB. After treatment, zygotes were placed in 500 ml FSW at 19°C and incubated for 24 hr. Control zygotes were exposed to 0.005% DMSO instead of CB at the appropriate time.

2. Temperature treatments

Experiment 1

The optimum starting time of the treatment was examined. Two temperatures, 0°C and 35°C, were tested, with a duration of 10 min. Treatments were started every 5 min from 15 to 60 min after fertilization.

Oocytes (5×10^5/treatment) were placed into a cylinder (3 cm in diameter and 5 cm in depth) with 20 μm mesh in 60 ml FSW at 20°C and fertilized. A cylinder containing zygotes was soaked in warmed or chilled FSW at the beginning of a treatment, and then, it was returned to FSW at 19°C and incubated.

Experiment 2

The optimum temperature and duration was examined. Five temperatures were tested : 0, 32, 35, 37, and 40°C. From the results of Exp. 1, a treatment was initiated at 20 or 40 min after fertilization for inhibiting meiosis-I or meisis-II. The duration of treatment was 10 or 15 min (except 40°C : 3 min). The same experimental procedures as Exp. 1 was used.

Ploidy determination

Chromosome numbers were counted for determining the ratios of induced triploid. For CB treatment 8-32 cell-stage embryos and for temperature treatment swimming trochophore larvae were used for chromosome preparations. Chromosome preparation from the 8-32 cell-stage embryos was made by the aceto-orcein squashing method (Fig. 1), and that from the trochophore was made according to the air-drying method (Fig. 2). For CB treatment 38-68 zygotes and for temperature treatment 60 cells were observed. *C. gigas* has $2n=20$ chromosomes (18-21). Because many aneuploids were observed, we decided to classify the ploidy of cells as : triploid, for 25-34 chromosomes : tetraploid, for 35-44 chromosomes : pentaploid, for 45-64 chromosomes.

Ratios of 2 hr segmentation and 24 hr survival

Specimens from the treated zygotes were fixed by 4% formaline at 2 hr after fertilization and inspected under a microscope to determine the ratios of 2 hr

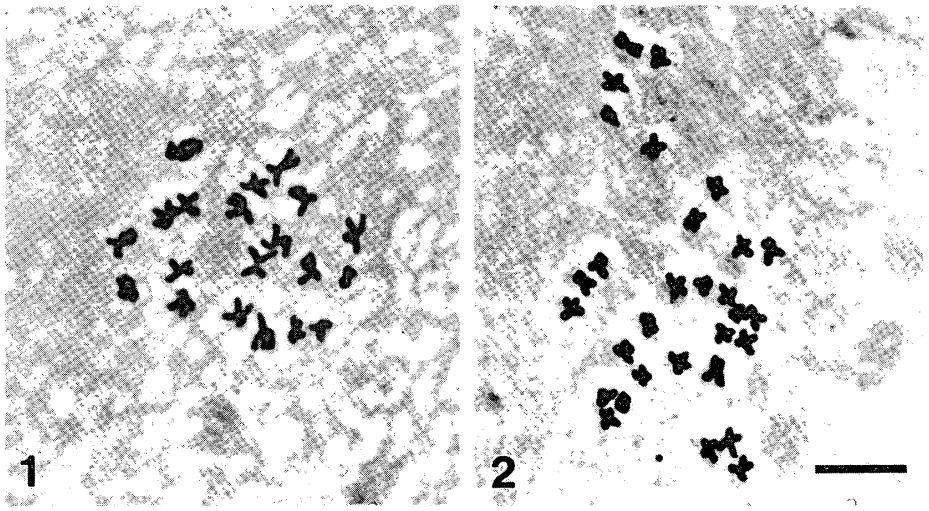

FIG. 1. Mitotic metaphase chromosomes from the embryonic cells of *C. gigas*. (Bar= 10 μm) 1 : 2 n=20 ; diploid 2 : 3 n=30 ; triploid

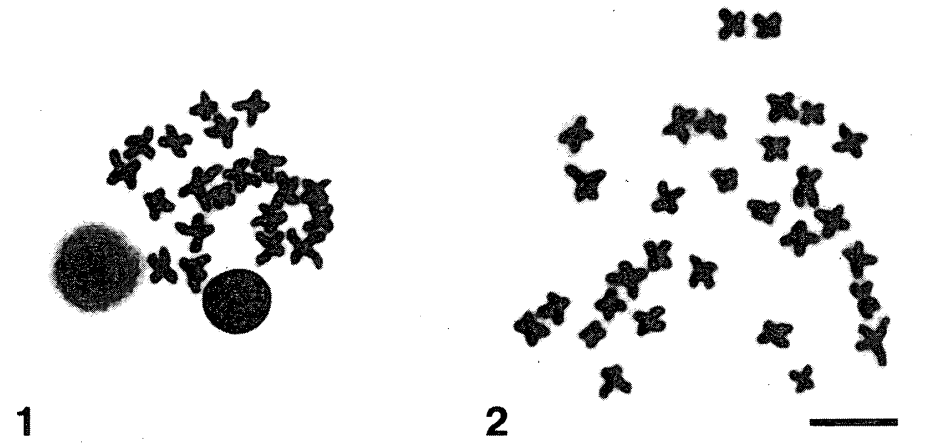

FIG. 2. Mitoctic metaphase chromosomes from larval cells of *C. gigas*. (Bar=10 μm) 1 : diploid 2 : triploid

segmentation : the ratios of zygotes which had made segmentation.

A portion of each treatment group was reared until 24 hr in FSW at 19°C and then inspected under a stereomicroscope to determine the ratios of 24 hr survival : the ratios of free swimming larvae to the total number of zygotes observed.

Comparison for the performance of triploid larvae

Triploid oyster larvae were induced by heat shock at 35°C, duration 10 min, applied at meiosis-I (from 10 min) or meiosis-II (from 30 min), using oocytes and sperm obtained by temperature spawning induction. The results are shown in Table 4. Early larval growth was measured, and observation for the feeding capability was made with fluorescence microscopy as described later.

Results

Triploid induction

1. Cytochalasin B treatments

CB treatment was effective for the induction of triploid in *C. gigas* (Table 1). The effect of CB treatment was dose dependent, and a higher dose of CB induced more polyploid including tri-, tetra- and pentaploid. Also 1.0 mg/l CB tratment at meiosis-I induced more polyploid but fewer triploid than lower dose treatments. It suggests that the effects of CB administered at meiosis-I is not restricted to the inhibition of the lst polar body extrusion but also affects the 2nd polar body extrusion and lst cell division by residual CB or other factors, or all of them combined. The most effective treatment for triploid induction was 0.5 mg/l CB at meiosis-I (67.2%), and that for the polyploid induction was 1.0 mg/l CB at meiosis-II (86.7%).

CB treatments caused toxicity on the zygotes dose dependently on meiosis-I treatment especially (Table 1). In 1.0 mg/l treatment at meiosis-I, only 3.2% of the zygotes survived. We did not make an observation about the viabilities of those CB treated zygotes which survived for 24 hr, but we suppose it should be low since we observed high ratios of abnormalities among them (data not shown).

2. Temperature treatments

Experiment 1

Heat and cold shocks were effective for triploid induction (Fig. 3). Two peaks of triploid induction could be obtained, concerned with the lst and 2nd

TABLE 1. *Effects of cytochalasin B on development and ploidy of C. gigas. Ratios of 2 hr segmentation and 24 hr survival denote the average of triplicate observations on more than 100 zygotes*

Dose og cytochalasin B (mg/*l*)	Control	0.1		0.5		1.0	
Time (min	—	10-30	30-50	10-30	30-50	10-30	30-50
2 hr segmentation (%)	78.2	69.1	63.8	26.9	61.9	12.0	54.8
24 hr survival (%)	56.0	43.9	47.8	18.6	34.7	3.2	13.8
Polyploid (%)	2.2	70.6	61.9	79.3	79.0	80.0	86.7
Triploid (%)	2.2	45.6	50.8	67.2	44.7	16.7	63.3
Tetraploid (%)	0.0	22.1	11.1	6.9	7.9	40.0	16.7
Pentaploid (%)	0.0	2.9	0.0	5.2	26.3	23.3	6.7
※	(44)	(68)	(63)	(38)	(58)	(30)	(35)

※ Numbers in parenthesis indicate the number of zygotes observed for the estimation of the ratios of polyploid induction.

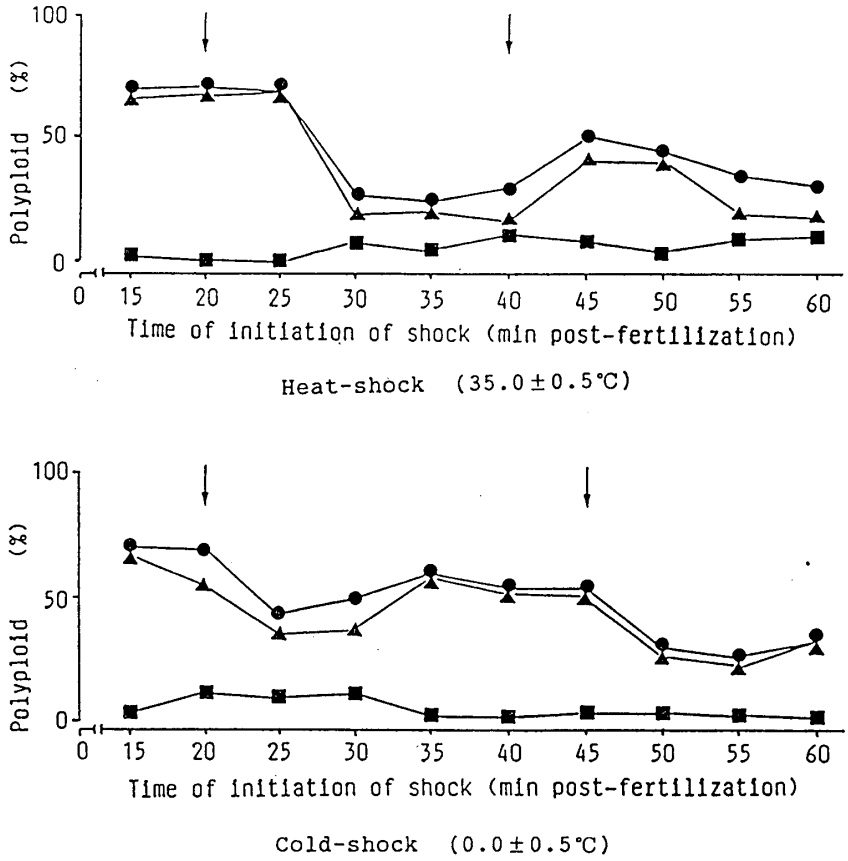

FIG. 3. Effects of temperature shocks on polyploidization of *C. gigas* in relation to the time of initiation of shocks (Exp. 1). Duration was 10 min in both treatments. Arrows indicate the time when 1st or 2nd polar body extrusion was first observed by microscopic observation of the specimens preserved with 4% formaline.

polar body extrusion. Treatments at meiosis-I was more effective than at meiosis-II in both treatments. Effective time of cold shock at meiosis-II was longer than that of heat shock, and the effect rose again soon after the lst polar body extrusion. Cold shock may be effective at early~later stages of polar body extrusion while heat shock may be effective at the later stage. This may suggest something about the difference of the mechanisms of the inhibition between them.

The highest triploid induction for heat shock was 68.3% by the treatment at 20 min after fertilization, and that for cold shock was 66.7% by the treatment at 15 min after fertilization.

No difference was detectable in the ratios of 2 hr segmentation and 24 hr survival (Table 2).

Experiment 2

The triploid inducing effect by heat shock was increased with the rise of a treatment temperature at the same duration and with a elongation of the duration

TABLE 2. *Effects of temperature treatments on ratios of 2 hr segmentation and 24 hr survival of C. gigas* (Exp. 1). Heat shock (35°C) orcold shock (0°C) were initiated at 5 min intervals from 15-60 min after fertilization. Duration was 10 min. Each ratios indicate the average of triplicated observations on more than 100 zygotes. Ratios of induced polyploids in this experdment re shown in Fig. 3.

Time after insemination	Heat shock (35.0°C)		Cold-shock (0.0°C)	
	2 hr segmentation (%)	24 hr survival (%)	2 hr segmentation (%)	24 hr survival (%)
15-25	71.2	58.3	76.1	72.3
20-30	73.0	56.2	76.3	72.3
25-35	66.9	44.0	73.8	70.6
30-40	67.2	56.4	74.4	67.5
35-45	67.3	55.8	78.3	71.5
40-50	62.0	48.8	70.4	63.8
45-55	68.4	57.0	81.0	71.4
50-60	69.3	55.5	78.6	68.8
55-65	67.9	54.9	73.6	73.8
60-70	69.4	62.7	81.3	75.7
Control	78.5	72.6	76.9	81.0

at the same temperature. But in cold shock, elongated duration did not improve the efficiency (Table 3).

The higest triploid induction was 83.3% at 37°C (duration 15 min, at meiosis-II), and the highest polyploid induction was 90.0% also at 37°C (duration 15 min, at meiosis-I).

There was almost no difference in ratios of 2 hr segmentation and 24 hr survival.

Comparison for the performance of triploid larvae

1. Growth of the larvae

Treated and control zygotes (1.4×10^6/group) were placed into 30 *l* FSW sterilized by UV irradiation (SFSW) in a plastic container, and free swimming larvae were transferred to another by decantation at 12 hr after fertilization. Three days later, metamorphosed "D" larvae were filtered onto a 20 μm mesh and resuspended into an another SFSW, and begun to be fed a cultured algae *Chaetooceros calcitrans*. SFSW was changed once a day or every two days, and larvae were fed once a day. The survival ratios from zygotes to "D" larvae were especially low in meiosis-I treatment group (Table 4)

TABLE 3. *Effects of temperature treatments on delopment and polyploidization of C. gigas* (Exp. 2). Heat or cold shock was applied at 20 or 40 min after fertilization. Duration of each treatment was 10 or 15 min except 40°C (3 min). Ratios of 2 hr segmentation were estimated from the triplicate observations and ratios of 24 hr survival were estimated from the duplicated observations on more than 100 zygotes.

Treatment temp. (°C)	Time (min)	2 hr segmentation (%)	24 hr survival (%)	Ploidy (%)			
				Tri	Tetra	>tetra	Poly
0	20-30	87.0	74.5	35.0	0.0	0.0	35.0
	20-35	85.2	75.6	33.0	10.0	1.7	45.0
	40-50	91.8	80.4	61.7	1.7	0.0	63.3
	40-55	92.6	67.9	60.0	5.0	0.0	65.0
32	20-30	79.4	82.6	26.7	3.3	0.0	30.0
	20-35	89.7	72.8	35.0	10.0	0.0	45.0
	40-50	87.2	70.8	38.3	3.3	0.0	41.7
	40-55	83.0	77.2	48.3	5.0	1.7	55.0
Control	(0, 32°C)	96.3	93.4	8.3	0.0	0.0	8.3
35	20-30	78.4	79.5	80.0	0.0	0.0	80.8
	20-35	88.5	74.9	76.7	1.7	0.0	78.3
	40-50	89.8	88.4	35.0	1.7	0.0	36.7
	40-55	90.4	72.7	53.3	11.7	1.7	66.7
Control	(35°C)	93.8	91.1	16.7	0.0	0.0	16.7
37	20-30	83.1	65.0	65.0	15.0	1.7	81.7
	20-35	84.5	54.2	68.3	11.7	10.0	90.0
	40-50	85.6	78.9	70.0	1.7	1.7	73.3
	40-55	84.8	71.9	83.3	0.0	0.0	83.3
Control	(37°C)	95.1	95.0	1.7	0.0	0.0	1.7
40	20-23	81.8	74.5	51.7	6.7	0.0	58.3
	40-43	89.3	84.6	45.0	0.0	0.0	45.0
Control	(40°C)	94.8	92.5	0.0	0.0	0.0	0.0

Shell length of the larvae (50/group) was measured on 3rd, 11th, and 26th days after breeding. The results are shown in Fig. 4. On the 26th day, all larvae in all experimental groups died of some unknown cause (infection of a bacteria or protozoa may have been responsible), so measurement was performed on the dead larvae which were still kept soft body stained with a lugol-eosin aqueous solution (I_2 0.2%, KI 0.33%, NaCl 0.2% and eosin 0.33%). We were unable to determine the final survival ratios.

On the 3rd day, mean shell length of the control group ($69.9 \pm 0.4\ \mu m$:

TABLE 4. *Induction and early development of triploid C. gigas.* Heat shock (35°C, 10 min) was applied to the zygotes at 10 or 30 min after fertilization. Ratios of 2 hr segmentation and 24 hr survival were estimated from the triplicate observations on more than 100 zygotes. Ratios of survival to "D" larvae were estimated from the observations at 72 hr after fertilization as percentage of the "D" larvae to the initial number of the oocytes.

Treatment time (min)	Control	10-30 Meiosis-I treatment	30-40 Meiosis-II treatment
2 hr segmentation (%)	95.6	81.0	81.0
24 hr survival (%)	95.9	74.6	84.5
Survival to "D" larvae (%)	82.0	24.7	75.6
Triploid (%)	6.7	68.3	38.3
Polyploid (%)	6.7	75.0	53.3

mean$\pm$S.E.) was greater than that of the meiosis-I treatment group (67.8$\pm$0.4 μm) (Cochran-Cox test; $P<0.002$) or meiosis-II treatment group (65.4$\pm$0.3 μm) (Cochran-Cox test; $P<0.001$). These differences may be due to the treatment stresses of triploidization. On the 25th day, mean shell length in the meiosis-I treatment group (212.6$\pm$2.7 μm) was significantly greater than that of the control (190.3$\pm$2.3 μm) or of the meiosis-II treatment group (184.9$\pm$2.4 μm) (student t-test; $P<0.001$). That in meiosis-II treatment group however, was less than the control (Student t-test; $P<0.05$).

2. Abilities in ingesting and digesting algal food

A comparison was performed at the first larval feeding, 72 hr after fertilization. Ingestion and digestion of *Isochrysis galbana* by the larvae was observed by a modified procedure of Babinchak and Ukeles (22), and Lucas and Rangel (23). The larvae (2.4×10^4) from each group were placed in 600 ml SFSW (4 larvae/ml). Cultured *I. galbana* (1.0×10^4/ml) was added to the SFSW, and the larvae were fed for 10 min. Later, the larvae were filtered onto 20 μm mesh for removing residual algae, then, the larvae were resuspended in 600 ml FSW at 20°C and cultured. Specimens were sampled at 0.5, 1, 1.5, 2, 2.5, 3, 6, 12, and 24 hr after feeding. Specimens were preserved with 2% formalin and stored at 4°C, and were examined under a Nikon fluorescence microscope equipped with FITC exciter filter and a 515W barrier filter. For each specimen 50 larvae were examined. The following scale was adapted to characterize the digestive stages.

Stage 1: all ingested algal cells kept their shape. (stomach entirely or almost entirely filled.)

Stage 2: parts of the algal cells lost their shape.

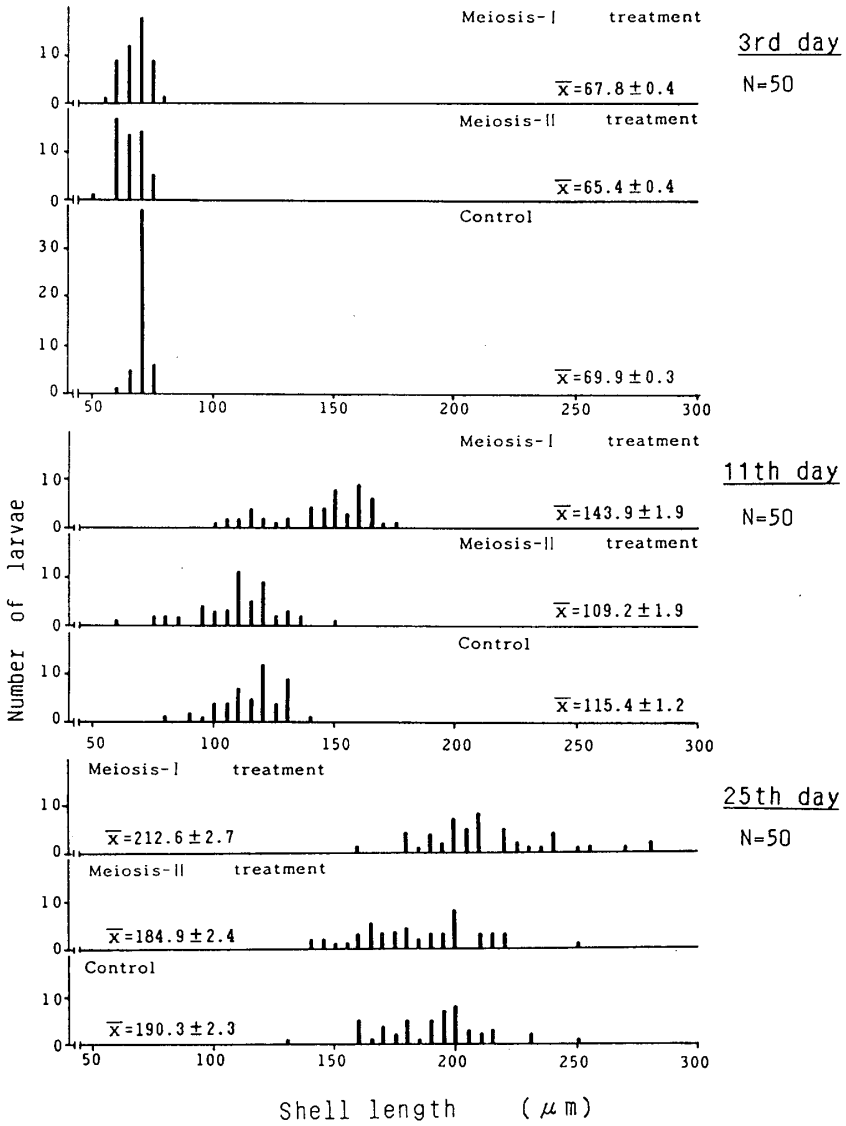

FIG. 4. Shell length distributions of the triploidized and control *C. gigas* larvae. Underlined numbers indicate the days after insemination. N: number of the larvae observed, $\bar{x}$: mean shell length±standard error (μm)

Stage 3: all algal cells lost their shape. (only cloud like fluorescence can be seen.)

Stage 4: stomach empty, no fluorescence.

Results are shown in Fig. 5, and no significant differences could be observed. The treated larvae had enough viability in both ingestion and digestion of algal food.

DISCUSSION

Triploidy could be induced in *C. gigas* at high efficiency by both CB treatment and temperature treatment. The highest triploid induction was 83.3% for

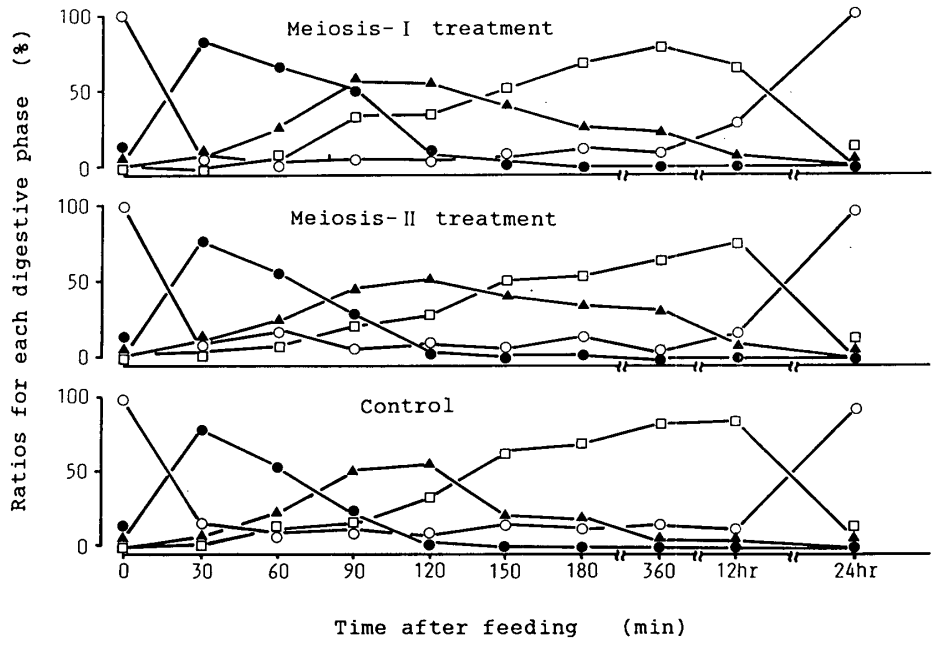

FIG. 5. A comparison of the abilities of triploidized *C. gigas* larvae to ingest and digest algal diet, *I. galbana*, observed with fluorescent microscope. Triploidy was induced by heat shock (35°C, duration 10 min) applied at meiosis-I or meiosis-II.
● : phase 1 ▲ : phase 2 □ : phase 3 ○ : phase 4

temperature treatment (37°C), and 67.2% for CB treatment (0.5 mg/l), and the highest polyploid induction was 90% for temperature treatment (37°C), and 86.7% for CB treatment (1.0 mg/l). It is difficult to compare the efficiency of the two, but these results may suggest that the effect of heat shock in appropriate conditions was comparable to that of CB treatment. Heat shock seems useful for commercial use, because it is highly efficient and needs no special divices or chemicals. Survivability of the heat shocked zygotes was not high, but it is easy to obtain a large number of triploid larvae due to the exceptional fecundity of *C. gigas*.

It is very important to induce triploid animals at low cost and at high efficiency as near as 100% as possible for the commercial culture of marine bibalves. The cost of heat shock is low enough to accomplish this purpose, but triploid inducing efficiency is not yet high enough. Downing and Allen reported that the optimal condition of CB treatment depends on the zygote incubating temperature in *C. gigas*. Their highest triploid induction was 100% (13). We believe that a comparable efficiency to optimal CB treatment could be obtained by improved optimal heat shock. Currently, we are making a study about an effective co-effecter of heat shock. Heat shock may cause effects on the cytoskeleton like microtubule, inferred from Inoué's dynamic equilibrium model (24), so regulator factors of assembly-disassembly of microtubules (ex. Ca^{2+}) might be hopeful. Improvement of triploid inducing efficiency by heat shock may also be

able to be accomplished by the synchronization of the development of zygotes depending on the incubation temperature as in CB treatment.

The triploid inducing efficiency of heat shock in our experiments was apparently higher than that of Quillet and Panelay (16) with the same treatment conditions. This my be due to some difference in shock application procedure or difference of ploidy determining developmental stage or shock sensitivity of animal stocks used.

The growth of triploidized larvae with the meiosis-I treatment was greater than that of the control or the meiosis-II treatment group on the 11th and 25th days. Stanley *et al.* reported the increased growth of triploid American oyster, *Crassostrea virginica* induced by meiosis-I treatment (25). And they explained that increased growth by the increased heterozygocity in the meiosis-I treatment group. The result in our experiment should be explained in the same way; triploid larvae showed no advantage in the capabilities of ingestion and digestion of algal food as we reported in this paper, so if the meiosis-I treated triploid larvae grew faster than the diploid there must be another difference which incresed the viability of the meiosis-I treated triploid. We are planning another experiment about this difference.

It is very important to determine the nature of adult or juvenile triploid bivalves for commercial purposes. Now there are only a few reports about the performance of the adult triploids whose most useful feature was sterility. The triploid Pacific oyster's sexual maturity is retarded and keeps its growth during the normal reproductive season and its glycogen contents are higher than that of the diploid (3). Triploid bay scallop also have retarded sexual maturity and shell inflation, with glycogen contents and weight of adductor muscle grater than that of the diploid (1). The triploid soft-shell clam did not mature, and an abnormal sex ratio was observed (2). These reports seem to promise the values of triploid bivalves in aquaculture. We must know more about the merits and demerits of triploids at the larval stages and at adulthood, so broader research on the nature of triploid marine bivalves is now required. In control groups, we have observed many polyploids, particularly triploid. Ahmed and Sparks (18), and Quiévreux (26) reported the polyploid or aneuploid in natural *C. gigas*. We also observed polyplod or aneuploid cells in artificially fertilized larvae of *M. edulis* (15). We suppose this kind of abnormal development occurs frequently in some bivalves, especially under artificial conditions. It is also of considerable interest that studies about triploid or aneuploid in natural environment are being undertaken.

References

1) Tabarini, C.L., *Aquaculture*, **42**, 151 (1984)
2) Allen, S.K., Jr., Hidu, H., and Stanley, J.G., *Biol. Bull.*, **170**, 198 (1986)
3) Allen, S.K., Jr., and Downing, S.L., *J. Exp. Mar. Biol. Ecol.*, **102**, 197

(1986)
4) Wolters, W.R., Libey, G.S., and Chrisman, C.L., *Trans. Am. Fish Soc.*, **111**, 102 (1982)
5) Lincoln, R.F., and Scott, A.P., *Aquaculture*, **30**, 375 (1983)
6) Lincoln, R.F., and Scott, A.P., *J. Fish Biol.*, **25**, 385 (1984)
7) Ueno, K., *The Aquiculture*, **10**, 37 (1985) (in Japanese)
8) Taniguchi, N., Kijima, A., Tamura, T., Takegami, K., and Yamasaki, I., *Aquaculture*, **57**, 321 (1986)
9) Johnson, O.W., Dickhoff, W.W., and Utter, F.M., *Aquaculture*, **57**, 329 (1986)
10) Stanley, J.G., Allen, S.K., Jr., and Hidu, H., *Aquaculture*, **23**, 1 (1981)
11) Allen, S.K., Jr., Gagnon, P.S., and Hidu, H., *J. Heredity*, **73**, 421 (1982)
12) Beaumont A.R., *Aquaculture*, **57**, 99 (1986)
13) Downing, S.L., and Allen, S.K., Jr., *Aquaculture*, **61**, 1 (1987)
14) Arai, K., Naito, F., and Fujino, K., *Bull. Jap. Soc. Sci. Fish.*, **52(3)**, 417 (1986)
15) Yamamoto, S., and Sugawara, Y., *Aquaculture*, **72**, 21, (1988)
16) Quillet, E., and Panelay, P.J., *Aquaculture*, **57**, 271 (1986)
17) Chaiton, J.A., and Allen, S.K., Jr., *Aquaculture*, **48**, 35 (1985)
18) Ahmed, M., and Sparks, A.K., *J. Fish. Res. Board. Canada*, **24 (10)**, 2155 (1967)
19) Nadamitsu, S., and Shinkawa, H., *Chromosome Information Service*, **15**, 29 (1973)
20) Ieyama, H., and Inaba, A., *VENUS*, **33 (3)**, 129 (1974)
21) Naruse, R., Sugawara, Y., and Sato, R., *Medicine and Biology*, **90(5)**, 269 (1975) (in Japanese)
22) Babinchak, J., and Ukeles, R., *Marine Biology*, **51**, 69 (1979)
23) Lucas, A., and Rangel, C., *Aquaculture*, **30**, 369 (1983)
24) Inoué, S., Fuseler, J., Salmon, E.D., and Ellis, S.W., *Biophysical Journal*. **15**, 725 (1975)
25) Stanely, J.G., Hidu, H., and Allen, S.K., Jr., *Aquaculture*, **37**, 147 (1984)
26) Quiévreux, C.T., *Genetica*, **70**, 225 (1986)