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Polyploidy in the Manila clam *Ruditapes philippinarum*. I — Chemical induction and larval performances of triploids

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Abstract

An effective treatment using cytochalasin B (1 mg CB/ml dimethylsulfoxyde in 1 litre of sea water) was developed to induce triploidy in the Manila clam *Ruditapes philippinarum* (Adams and Reeve). The percentage of induced triploidy was assessed by karyological examination of embryos and spat. The treatment was found to be optimal between 20 to 35 minutes post-insemination exposure to CB, at 25 °C, and produced 75.8 ± 5.7% triploid embryos on the average over four experiments. Growth and survival of larvae to metamorphosis were recorded for two different rearings each split into two replicates. Both larval rearings showed similar survival up to settlement: 19.3 and 27.0%, on average over two replicates, in the treated batches relative to the controls. Mean shell length of treated larvae was significantly smaller than that of the controls in one of the larval rearings, where mortality of triploids relative to diploids within the treated group was low (21.0%). No significant differences in shell length were observed in the other trial where a high rate of differential mortality (64.5%) was recorded.

Keywords : Triploidy, cytochalasin B, bivalve, larval growth, larval survival.

Polyploïdie chez la palourde japonaise Ruditapes philippinarum. *I — Induction chimique et performances larvaires de triploïdes.*

Résumé

L'induction de la triploïdie chez la palourde japonaise *Ruditapes philippinarum* (Adams et Reeve) a été mise au point en traitant les œufs à la cytochalasine B (1 mg de CB/ml de diméthylsulfoxyde pour 1 litre d'eau de mer). Le taux de triploïdie induite a été estimé par examen caryologique des embryons et du naissain. Le meilleur traitement (bain de 20 à 35 minutes après insémination, à 25 °C) a produit en moyenne, sur quatre expériences, 75,8 ± 5,7 % d'embryons triploïdes. La croissance et la survie ont été suivies pendant deux élevages larvaires. Ceux-ci ont présenté des taux de survie comparables avant la métamorphose: 19,3 et 27,0 % en moyenne sur deux réplicats dans les lots traités par rapport aux témoins. La taille individuelle moyenne des larves traitées était significativement inférieure à celle des témoins dans l'un des deux élevages où la mortalité des triploïdes rapportée à celle des diploïdes dans la population traitée a été faible (21,0 %). Dans l'autre élevage, où cette mortalité différentielle des triploïdes a été forte (64,5 %), aucune différence significative n'a été décelée entre la taille individuelle moyenne des larves traitées et celle des témoins.

Mots-clés : Triploïdie, cytochalasine B, bivalve, croissance larvaire, survie larvaire.

INTRODUCTION

Aquatic molluscs usually undergo growth reduction during their reproductive seasons. Over the last 10 years, research has been undertaken on commercially important shellfish to develop efficient techniques to sterilize them. Induction of triploidy achieves this goal successfully. Induced triploids of several species have been studied at the adult stage and have revealed better growth, and in certain cases a higher survival rate than diploids even when sterilization was not complete (Allen and Downing, 1986). Tabarini (1984) recorded a gain in weight of 73% in adductor muscle due to triploidization of the bay scallop *Argopecten irradians*. Allen and Downing (1986) also reported a 172% dry weight increase during the reproductive season for triploid *Crassostrea gigas* compared to 34% for diploid siblings. Moreover, the glycogen content of such improved animals was high all year round. This results in constant high meat quality and large energy reserves.

Goulletquer *et al.* (1987) recorded a post-spawning dry weight loss of 31 to 44% for *Ruditapes philippinarum* (Adams and Reeve).

Marketability of this cultured clam is considerably reduced during the reproductive season due to its susceptibility to the slightest stress and consequent poor holding qualities.

Preliminary work has been carried out by Beaumont and Contaris (1988) and Gosling and Nolan (1989) on the triploidization of this species. Both temperature and cytochalasin B (CB) treatment were tested but in neither case were the resulting larvae reared. The present paper reports the development of an effective treatment using CB, suitable for a larger scale production of triploids. Performance of triploid veliger larvae and changes in the proportion of triploids during early life were recorded.

MATERIALS AND METHODS

Gamete collection and fertilization

Manila clams (35 to 55 mm long) were allowed to mature either under artificial conditions (21 ± 1 °C) following Loosanoff and Davis (1963) from March to May or in natural ponds between June and August 1988. The seawater used for spawning induction, fertilization, experimental treatment and larval rearing was filtered to 1 µm, sterilized by ultraviolet irradiation and thermoregulated at 25 °C, except for the second polyploid larval rearing (23 °C).

Spawning was induced by emersion overnight at about 20 °C, followed by re-immersion and addition of ripe stripped spermatozoa destroyed by sonication. After careful rinsing, each spawning clam was isolated in a beaker and allowed to spawn fully. Eggs and sperm were separated from debris and faeces by sieving them through 75 and 25 µm meshes respectively. Gametes from 10 to 15 individuals of each sex were pooled. Only ova released within the first hour were fertilized only with motile sperm. An ova sample was taken prior to insemination and examined 1 hour later to estimate the percentage of uncontrolled natural fertilization. Egg numbers were estimated from three 100 µl-samples obtained after having thoroughly mixed the egg suspension in a 2 litre-volume.

After insemination, the ratio of spermatozoa/ova was assessed and ranged from 5/1 to 10/1. Eggs were then stirred and divided into as many equal batches as necessary for later experimentation. Batch volumes were always adjusted to 250 ml before treatment.

Experiments

To induce polyploidy, eggs were held for 15 minutes in a 2 µmol/l cytochalasin B (CB) seawater solution (1 mg CB dissolved in 1 ml dimethylsulfoxide (DMSO) diluted in 1 l seawater). They were then rinsed in a 0.1% DMSO seawater solution for 15 minutes. Eggs at a density 10×10^6 to 22×10^6/l were regularly stirred. All experiments were performed at a temperature of 25 °C. Three experiments were carried out to develop an efficient treatment time inducing triploidy. Three controls per experiment from 5, 30 and 60 minutes after insemination were held for 30 minutes in the 0.1% DMSO sea water solution. All batches were left to develop at a density of 100 larvae/ml in 1.5 litre-aerated plastic tanks until karyological preparation. Effects of CB treatment on the percentage of early trochophore larvae abnormality were also studied. Samples of 7 hour-old larvae were taken from each batch, fixed with formalin and examined under a microscope. Abnormal larvae percentages were recorded from 300 larvae per batch.

Larval rearing

Two successive triploid productions were made using CB-treatment beginning 20 minutes after insemination. Controls were not treated with DMSO. For both productions, treated and control batches were duplicated in rearing. Rearing of larval was done in 150 l cylindro-conical tanks using standard techniques (Loosanoff and Davis, 1963). Samples of 6-hour-old larvae were taken from each batch for chromosome preparations. Density was 100 larvae/ml until the D-shape stage (24 hours) and was then adjusted to 10 larvae/ml. Larvae were resuspended in fresh seawater every 2 days and fed daily with *Isochrysis galbana* (50 to 100 cell/µl). At each water renewal and for each tank, 30 larvae were measured along the longest shell axis, the number of live larvae was estimated, larval density was readjusted to 10/ml and antibiotics were added (alternately chloramphenicol 10 mg/l and gentamycin sulfate 5 mg/l). A survey

of total bacteria and presumptive vibrios was made using plate counts on daily samples of seawater for each tank.

When more than 50% of the population reached the pediveliger stage, larvae were allowed to metamorphose in shallow tanks of seawater. Spat were reared in running seawater and fed with cultured algae for 4 months until karyological examination. Duplicates were pooled after metamorphosis in the first rearing but not in the second.

Chromosome preparation

Six-hour-old embryos were transferred into a 0.02% colchicine seawater solution for 2 hours. They were then held for 20 minutes in 25% seawater diluted with distilled water. They were then fixed at 4 °C with three successive 30 minutes washes in Carnoy's solution (ethanol 3: acetic acid 1). A cell suspension was made from over 10^4 larvae per batch in 50% acetic acid (in distilled water) and small amounts were dropped onto a 45°C-prewarmed microscope slide. Preparations were stained for 10 minutes in 4% Giemsa solution (in pH 7 phosphate buffer). Four-month-old spat were prepared via the technique of Thiriot-Quiévreux and Ayraud (1982). Metaphasic spreads showing 36 to 38 and 54 to 57 chromosomes were identified as diploid and triploid cells respectively.

Statistical analyses

Triploid percentages were arc-sine transformed and analysed by ANOVA and optimal treatment times were outlined by a Newman-Keuls test using STATITCF software. Growth of the different batches was compared by a two-way ANOVA and, when useful, by multiple range analysis (95% confidence interval method) on Log-transformed shell lengths (STATGRAPHICS software). Slopes of growth regression lines were compared using a F-test.

RESULTS

The percentage of uncontrolled natural fertilization in all experiments ranged from 0 to 3±1.9%. The first three unequal cleavages of the egg were observed to occur without polar lobe formation, which is different from many molluscan species (Verdonk and Van Den Biggelaar, 1983).

CB treatments

Abnormal larvae were always significantly more numerous in CB-treated batches than in DMSO-treated controls (*fig.* 1). The percentage of abnormality was lowest when the CB-treatment was begun between the second meiotic division and the first mitosis. Percentages of induced triploidy resulting from three experiments and two productions were plotted together (*fig.* 2). Differences were significant between treatment times ($F=10.32$, $p<0.001$) but not between the three experiments ($F=0.68$, $p>0.5$). Although the difference between 15 and 20 minutes post-insemination times was not significant, the highest mean triploidy percentage (75.8±5.7%) was produced by the 20 minutes treatment time. No significant difference was detected between the four percentages of triploidy induced at 19 or 20 minutes after insemination ($\chi^2=6.32$, 3 *df*, $p>0.05$).

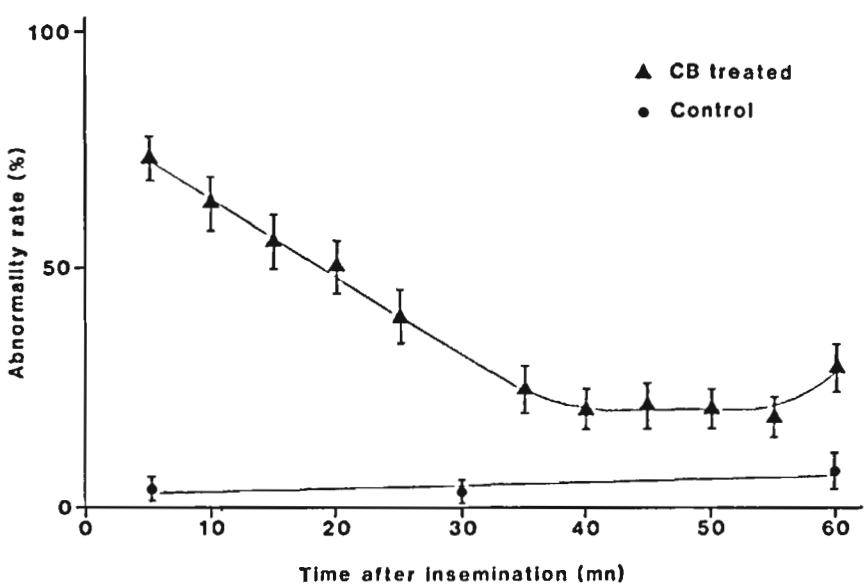

Figure 1. – Abnormality percentages of 7-hour-old larvae in relation to the time when the CB treatment was started. Treatment duration was 15 minutes. Controls were treated with DMSO only. Vertical bars represent 95% confidence intervals ($n=300$).

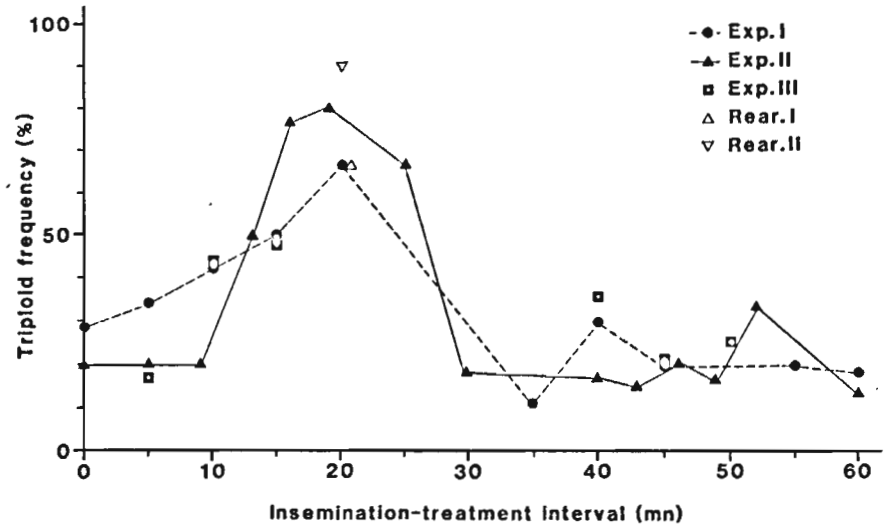

Figure 2. – Induced triploidy percentages in embryos versus the time of CB treatment initiation.

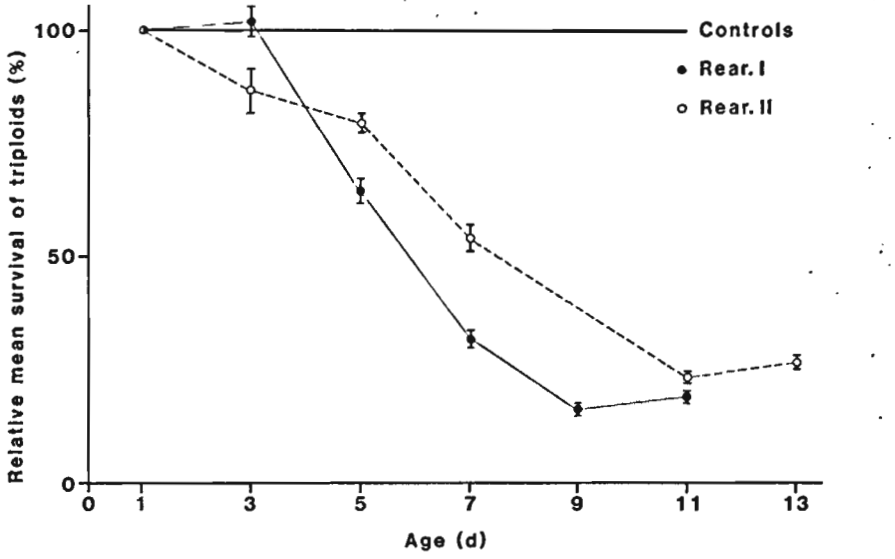

Figure 3. – Mean survival of CB treated batches relative to controls in rearings I and II. Absolute survival of each control at metamorphosis was 18.4 and 34.3%, respectively. Three estimates of survival were made for each tank. Points represent combined means of two replicates. Vertical bars are standard errors.

Larval rearings

A preliminary rearing of diploid larvae was done in order to select an optimal larval density among three tests: 5, 10 and 20 larvae/ml. ANOVA showed a significant effect of density on growth ($F = 55.89$, $p < 0.0001$) and multiple range tests revealed that the optimal density (10 larvae/ml) was significantly different from the other two. Furthermore, the variance of shell length was least and mortality was lowest at this density. Hence, further rearings were carried out at 10 larvae/ml from the D-shaped shell stage to metamorphosis.

Two different batches of triploids were reared with duplicates. Percentages of triploid embryos were estimated, respectively, at $66.7 \pm 17.6\%$ and $90.0 \pm 11.2\%$ for the two batches.

In both experiments, survival was much lower in the treated larvae than in controls (*fig.* 3). Between D larva and metamorphosis, relative mean survival of treated larvae were 19.3 and 27.0%, while absolute mean survival of controls were 18.4 and 34.3%, respectively in rearing I and rearing II. Bacterial survey did not reveal any abnormal phenomenon which could be correlated with mortalities. Total bacteria

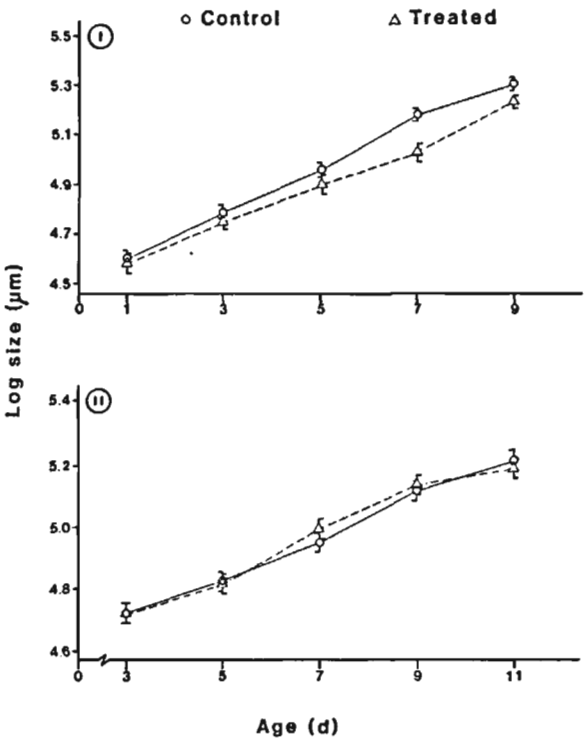

Figure 4. – Growth of treated and control populations (average of duplicates) in the first (I) and second (II) larval rearings. Vertical bars correspond to 95% confidence intervals from ANOVA.

counts ranged between 0 and 10^4/ml, and presumptive vibrios did not exceed 10/ml.

Mean larval shell length was significantly different from day 3 to metamorphosis between treated and control larvae in the first experiment ($F=46.3$, $p<0.0001$) but not in the second one ($F=0.120$, $p>0.7$) (*fig.* 4). A comparison of regression line slopes showed that the growth rate of treated populations was not significantly different from controls.

Table I. – Comparison of the triploid percentages between embryos and 4-month-old spat in rearings I and II. Estimates were made on the two replicates (spat 1 and spat 2) in rearing II.

	Rearing I		Rearing II		
	Embryos	Spat	Embryos	Spat 1	Spat 2
Triploid	20	19	27	9	5
Diploid	7	17	2	15	14
Tetraploid	3	0	1	0	0
TOTAL	30	36	30	24	19
Triploid %	66.7	52.7	90.0	37.5	26.3
χ^2, 1 *df*	1.3 ($p>0.05$)			0.6 ($p>0.05$)	
χ^2, 2 *df*			24.2 ($p<0.001$)		

The two rearings also differed in the way that the triploidy percentage changed with time. In the first rearing, no significant difference was found (*table* I) between early and later estimations of triploid percentages. In the second rearing, the triploid percentages changed from 90% at the embryo stage to 32% on the average from two replicates in the spat. Differential mortality of triploids within the treated population was (66.7-52.7)/66.7 = 21.0% for the first rearing, and (90.0-32.0)/90.0 = 64.5% for the second.

DISCUSSION

Optimal treatment for inducing triploidy seems reliable based on early karyological estimations since percentages of triploids resulting from four experiments were not significantly different. The results given here are different from those presented by Beaumont and Contaris (1988) for this species, since they reported no significant difference in early triploidy percentages between treatment times of 0, 15 and 30 minutes after insemination, and larger differences were found between two recurrent experiments than between the tested times. However, here, the best treatment gave an average of 75.8% triploids which is comparable to the best percentage obtained by the cited authors: 81.8%, though they used lower concentrations of DMSO (10-fold) and CB (2-fold). Gosling and Nolan (1989), using the same low concentrations of DMSO and CB, induced a lower percentage of triploids (50%), but this was assumed to be due to a certain proportion (not assessed) of uncontrolled fertilization.

The mean percentage of triploidy found for embryos was close to those obtained in other molluscan species either with CB or with thermal or pressure shocks (Stanley *et al.*, 1981; Arai *et al.*, 1986; Yamamoto *et al.*, 1988; and Baron *et al.*, 1989). However, it is rather difficult to effectively compare the different treatments reported in the literature, particularly in embryos or young larvae, since each author used their own particular technique, applied at different stages, in estimating the yield of triploids. Although the different techniques used for counting chromosomes in spat or adults may give similar results on a given sample (Allen, 1983; Komaru *et al.*, 1988), the differences observed between studies cannot be attributed to the efficiencies of different treatments. Indeed, when culturing conditions are not optimal, an attrition of either diploids or triploids may occur (Fujino *et al.*, 1987).

Moreover, depending on whether it was the first or second polar body which was retained (PB1 and PB2, respectively), the mean heterozygosity of these two types of triploids is different (Stanley *et al.*, 1984). Their viability may therefore differ since differential mortalities have been shown to be multiple locus heterozygosity-dependant in mussels (Diehl and

Koehn, 1985). Yamamoto *et al.* (1988) reported for *C. gigas*, at the straight-hinge stage, a much lower survival for PB1 triploids (24.7%) versus PB2 triploids (75.6%).

No evidence was found in our data for two distinct types of triploids. This might be expected since the treatment duration (15 minutes) was as long as the release lapse between the two polar bodies at 25°C (Diter and Dufy, unpubl.) According to these polar body release times, the optimal treatment, used in rearings I and II, is expected to inhibit the first maturation division in about half of the egg population and the second division in the remaining eggs. As discussed above, due to the differential mortality of PB1 and PB2 triploids, this initial ratio of the different triploids is not excepted to persist at older stages.

Yamamoto *et al.* (1988) reported for *C. gigas* that PB1 triploid larvae had a greater mean shell length than diploid controls, which were themselves larger than PB2 triploids. In this respect, the differences in growth observed between our two rearings could be related to differences in the changes observed in the ratio of triploids to diploids with time: in the second rearing, the high differential mortality of triploids (64.5%) might have occurred very soon after treatment, e.g. at the D-shaped shell stage. Thus, this larval population would have consisted of 70% diploids and might be expected to show no significant difference in growth compared to the controls.

Success of adult triploid shellfish in terms of increased growth (during gametogenesis), survival due to triploidy *per se* (genetic sterilization) or to overall increased heterozygosity (improved homeostasis) has already been demonstrated (Tabarini, 1984; Stanley *et al.*, 1984; Allen and Downing, 1986). It provides a promise for an eventual increase in growth and survival performance of triploid Manila clams. Nevertheless, the merits of such animals have yet to be demonstrated under commercial culture conditions (high densities and/or limited food availability).

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