

## Polyploidy in the Manila clam, *Ruditapes philippinarum*. II. Chemical induction of tetraploid embryos

Alain Diter and Christian Dufy

IFREMER, Laboratoire de Pathologie et de Génétique des Invertébrés Marins,  
BP 133, 17390 La Tremblade, France.

Received November 24, 1989; accepted March 7, 1990.

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Diter A., C. Dufy. *Aquat. Living Resour.*, 1990, 3, 107-112.

### Abstract

Tetraploidy was induced in embryos of *Ruditapes philippinarum* by treating the eggs with cytochalasin B (CB). Two treatment times were found to be effective in inducing tetraploidy: 0 to 10 minutes after insemination (before formation of the first polar body) and 45 minutes after insemination (just before the first cleavage). Treatments, started 5 and 45 minutes after insemination, produced  $64.4 \pm 7.8\%$  and  $28.3 \pm 2.3\%$  tetraploid embryos, respectively, after 6 hours (on the average over 3 experiments). However, no tetraploids were detected in 4-month-old spat. Sperm entry delayed by the CB could be involved in induction of tetraploidy by early treatment.

**Keywords :** Cytochalasin B, tetraploidy, bivalve, *Ruditapes philippinarum*.

*Polyploïdie chez la palourde japonaise, Ruditapes philippinarum. II: Induction chimique d'embryons tétraploïdes.*

### Résumé

La tétraploïdie a été induite par traitement des œufs par la cytochalasine B (CB). Deux moments d'application du traitement se sont révélés efficaces pour l'induction de la tétraploïdie: de 0 à 10 minutes après insémination (avant l'émission du premier globule polaire) et 45 minutes après insémination (juste avant le moment du premier clivage). Les traitements débutant 5 et 45 minutes après insémination ont produit respectivement  $64,4 \pm 7,8\%$  et  $28,3 \pm 2,3\%$  d'embryons tétraploïdes à 6 heures (en moyenne sur 3 expériences). Aucun tétraploïde n'a été détecté dans le naissain de 4 mois. Un retard de la pénétration du sperme provoqué par la CB pourrait être impliqué dans l'induction de la tétraploïdie par traitement précoce.

**Mots-clés :** Cytochalasine B, tétraploïdie, bivalve, *Ruditapes philippinarum*.

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### INTRODUCTION

Induced triploidy was shown to increase growth, glycogen content and subsequent meat quality throughout the year in several commercially important mollusc species (reviewed by Allen, 1987). Triploid shellfishes so far have been produced by treating the eggs during the maturation division. Different

agents have been employed for this: pressure, shock, cold or heat shock and cytochalasin B (CB) exposure. None of these treatments routinely produces all-triploid populations, although CB treatment can occasionally produce 100% triploids (Allen, 1987). The relative unpredictability of treatment effectiveness means that commercial hatcheries must assess the triploid percentage in each treated batch. Moreover,

the existence of even a few diploids in triploidized populations makes these methods inappropriate for protection of endemic species from competition and/or genetic introgression by closely related foreign species. Another method, the mating of diploid and tetraploid breeders, which results in all-triploid populations in fish (Chourrout *et al.*, 1986), would be better for these perspectives.

Up to now, no successful induction of tetraploid adult bivalves has been reported in the literature. This paper reports on attempts to induce tetraploidy in *Ruditapes philippinarum* (Adams and Reeve), through CB treatment of eggs. Triploidy has already been successfully induced in this commercially important species by several authors using CB and/or heat shock (Beaumont and Contaris, 1988; Gosling and Nolan, 1989; Dufy and Diter, 1990).

## MATERIALS AND METHODS

### Collection and fertilization of gametes

The techniques used are described in details in Dufy and Diter (1990). Spawning and fertilization were done at 25°C. Gametes from 10 to 15 individuals of each sex were pooled in each experiment. Zero development time was the time of insemination. The egg suspension was then divided into as many equal batches as were necessary for experimental designs.

### Treatments

Three experiments were carried out to determine the optimal treatment for induction of tetraploidy. Eggs were treated with 1 mg/l cytochalasin B (CB). Controls were only treated with DMSO (1 ml DMSO/l seawater). Technique details are given in Dufy and Diter (1990). The treatment was applied to newly fertilized eggs, at 25°C and lasted 15 minutes. Preliminary observations showed that first and second cleavages appeared respectively at 50 and 70 minutes, at 25°C. Different treatment times were tested from 0 to 60 minutes after insemination, at 3 or 5 minutes intervals. Treated and control larvae were left to develop for 26 hours, at a density of 100 ind/ml in 1.5l aerated cylinders. They were held in suspension by bubbling to avoid abnormalities described by Gruffydd and Beaumont (1970). Percentages of D-shaped shell larvae, normal and abnormal trochophore larvae were estimated by microscopic examination of 300 26-hour-old larvae for each batch.

### Larval rearing

Two treatments, previously found to be effective, were applied to eggs in an attempt to produce large numbers of tetraploids. Eggs were exposed to CB

at 10 minutes (early) and 45 minutes (late) post-insemination, respectively. In this experiment, the control was not treated with DMSO. The two treated batches and the control were all derived from the same pool of inseminated ova. After treatment, the 3 batches were duplicated for rearing. Larvae were reared as described in Dufy and Diter (1990). Total bacteria and presumptive vibrios were surveyed using plate counts on daily seawater samples from each tank. After metamorphosis, all the batches were kept duplicated until karyological examination at the spat stage. Tetraploid percentages were estimated for 6-hour-old embryos and for 4-month-old spat.

### Karyological examination

Chromosome preparations from 6-hour-old embryos were made following the method of Dufy and Diter (1990). A cell suspension was prepared from a population of over  $10^4$  embryos. Thirty metaphases were counted from each cell suspension.

In 4-month-old spat, chromosome preparations were individually prepared according to Thiriou-Quévieux and Ayraud (1982). Metaphase spreads showing 36 to 38 and 72 to 76 chromosomes were considered to have come from diploid and tetraploid cells, respectively.

### Statistical analyses

The percentages of tetraploid embryos were arc-sine transformed and compared by ANOVA, and optimal treatment times were outlined by a Newman-Keuls test using STATITCF software. The growth of each treated batch was individually compared with that of the control by a two-way ANOVA on Log-transformed shell lengths (60 data per batch and per day). The age of larvae and the type of batch (treated vs control) were the two effects analysed by the two-way ANOVA (Statgraphics software). The data from duplicates were pooled since preliminary ANOVAs showed no significant differences between duplicates.

## RESULTS

### Treatments

The percentage of D-shaped shell larvae at 26 hours varied with the time when CB treatment was started (*fig. 1*). The percentage of D-larvae was the lowest (4 to 26%) and the percentage of abnormal trochophore larvae was the highest (5 to 25%) when treatment was started between 0 and 13 minutes or between 46 and 52 minutes after insemination. The DMSO control was composed of 84% D-larvae and 16% normal trochophore larvae. The abnormal trochophore larvae induced by the CB were either large,

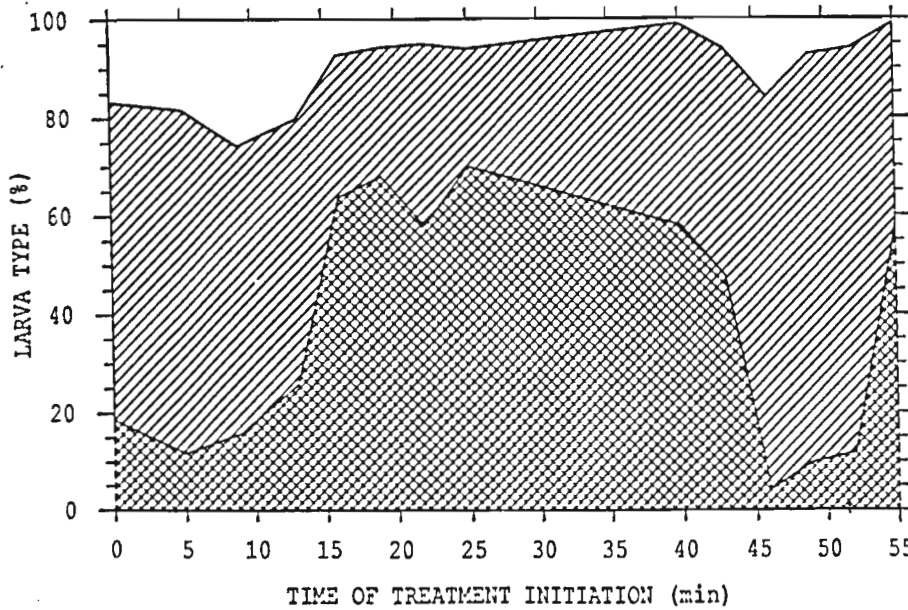


Figure 1. — Effects of CB treatment time on the proportion of D-shaped shell (cross-hatched), normal trochophore (simple-hatched) and abnormal trochophores (open) 26-hours-old larvae. The DMSO control was composed of 84% D-shaped shell and 16% normal trochophore larvae.

translucent and entirely ciliated, or showed malformations of the shell field.

CB induced tetraploidy at significantly different percentages ( $F = 5.82, p < 0.02$ ) depended on the treatment time (fig. 2). No polyploid metaphases were

found in the DMSO controls. The highest percentage of induced tetraploidy, 80%, was observed by a treatment beginning 9 minutes after insemination. However, over all three experiments, the highest mean percentage of tetraploidy ( $64.4 \pm 7.8\%$ ) resulted from

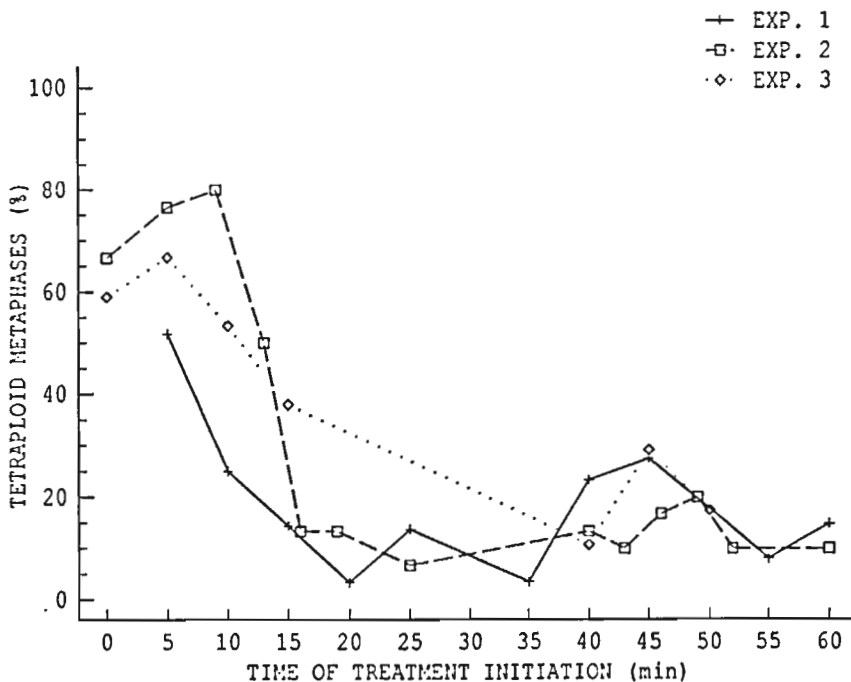


Figure 2. — Percentages of induced tetraploidy in 6-hour-old embryos, depending on the CB treatment time in 3 recurrent experiments.

treatments applied 5 minutes after insemination. A Newman-Keuls test revealed an optimum for the times 0 to 10 minutes. The yields of later treatments were not significantly different from each other but, among them, treatment beginning at 45 minutes induced the highest mean percentage of tetraploidy ( $28.3 \pm 2.3\%$ ).

### Larval rearing

The two treatments used here for tetraploid production (10 and 45 minutes post-insemination) were chosen before knowing the overall results from the three previous experiments. The percentages of tetraploid metaphases derived from 6-hour-old embryos were 73.3% and 33.3% in the early and late treated batches, respectively. The control was composed exclusively of diploids. No significant difference was evident between the mean shell length (Log-transformed) of either the early treated ( $F=0.19$ ,  $p>0.6$ ) or the late treated batch ( $F=3.63$ ,  $p>0.05$ ) versus that of the control from day 3 to day 11. In each batch, larvae began to metamorphose on day 13.

Between days 1 and 13, the mean survival of larvae (average for two replicates) was 0.9% and 14%, respectively, for the early and late treated batches, compared to 34% for the control. No abnormal bacteriological proliferation was detected in the rearing seawater. Total bacteria and presumptive vibrio counts ranged respectively from 0 to  $10^4$ /ml and from 0 to 10/ml in each tank. From 30 4-month-old spat examined per batch, none were tetraploid.

## DISCUSSION

The treatments which induced the highest tetraploid percentages in 6-hour-old embryos (0 to 10 minutes and 45 minutes after insemination) also produced the lowest percentages of D-larvae and simultaneously the highest proportions of abnormal trochophore larvae. Furthermore, the survival of veliger larvae was very poor. This and the non-detection of tetraploid spat indicated that tetraploidy induced by these common CB treatments is probably inviable, at least for the Manila clam.

Yamamoto *et al.* (1988), for *Crassostrea gigas*, and Wada *et al.* (1989), for *Pinctada fucata martensii*, reported that the highest abnormality percentages were produced by the earliest CB treatments, presumably interfering with meiosis I. For *Pecten maximus*, Beaumont (1986) found that CB, used at lower concentrations (0.1-0.5 ml/l), did not significantly increase abnormalities at the D-larva stage compared to the untreated control. However, the DMSO control had significantly lower larval abnormalities than the untreated control (Beaumont, 1986).

Stanley *et al.* (1981) observed, for *Crassostrea virginica*, some CB-induced tetraploids in embryos but

none in the resulting spat. Downing and Allen (1987) also reported, for *C. gigas*, the absence of tetraploid spat in batches resulting from CB-treated eggs at the time of first cleavage. However, their treatments were not designed for the induction of tetraploids. The different treatments used by these authors were separated by long intervals (15 minutes) and might therefore be far from optimal. Shorter intervals were used in our experiments (only 3 or 5 minutes) and, thus missing the effective treatment times initiation was improbable.

In Manila clam, no tetraploid embryos were reported by Beaumont and Contaris (1988), although a CB treatment closed to ours (1 mg/l, 0-15 minutes, 23°C) was used. Gosling and Nolan (1989) did not report tetraploid embryos in this species but the times of treatment were not given.

In contrast, in *C. gigas*, Yamamoto *et al.* (1988) observed that their earliest CB treatment (1 mg/l, 10-30 minutes, 19°C) induced the highest percentage of tetraploid embryos (40% at 8-32-cell stages) but had a very low survival rate (3.2% by 24 hours). Recently, in this species, Stephens and Downing (1989) induced a high percentage of 1-day-old tetraploid larvae (91%) by a similar CB treatment at PB1 formation. They noted the decrease in percentage of tetraploids during the larval rearing and the absence of tetraploid spat.

### Mechanisms involved in the induction of tetraploidy

We cannot strictly ascertain the tetraploid nature of the embryos produced here, since our technique of ploidy determination does not permit the detection of mosaic or aneuploid embryos.

Tetraploidy was induced at two different times and therefore, probably by two different mechanisms. The late treatment, started just before the 2-cell stage, produced a low percentage of tetraploids (33% at the best) which presumably resulted from the suppression of the first egg cleavage.

Unexpectedly, the highest percentages of tetraploids were obtained with treatments starting at the time of insemination or just after it, *i.e.* earlier than those inducing triploids (15-30 minutes and 20-35 minutes post-insemination; Dufy and Diter, 1990). At 25°C, meiosis I and II are completed respectively, between 15 and 20 minutes and between 30 and 35 minutes (Beaumont and Contaris, 1988; Gosling and Nolan, 1989). Consequently, early treatments (0-15 minutes to 10-25 minutes, post-insemination) are supposed to retain a high proportion of first polar bodies (PB1), but not second polar bodies (PB2). Stephens and Downing (1989) observed that similar treatments retained PB1 but not PB2, in *C. gigas*.

Allozymic evidences have demonstrated that inhibition of meiosis I results in triploids (Stanley *et al.*, 1984). It is therefore presumed that a supplementary mechanism is required to produce tetraploids. This mechanism might be the suppression (or delay) of

syngamy, followed by the inhibition of the first mitosis as a consequence of delayed sperm penetration.

CB, applied at the time of fertilization or soon after it, has been shown to inhibit sperm penetration into egg cytoplasm without inhibiting egg activation, in several marine invertebrates including molluscs (Gould-Somero *et al.*, 1977; Longo, 1978; Schatten and Schatten, 1981). When CB is removed, sperm entry can resume normally, just resulting in a delayed penetration (Gould-Somero *et al.*, 1977). In invertebrates, sperm brings centrioles around which forms the sperm aster involved in pronuclear migration and syngamy (Schatten and Schatten, 1981; Longo, 1983; Sawada and Schatten, 1988, 1989). The sperm aster participates also in the organization of mitotic asters required for egg cleavage (Vogel and Angenmann, 1967; Sawada, 1988; Sawada and Schatten, 1989). Consequently, if sperm entry is delayed by CB, formation of the mitotic apparatus would also be delayed.

Thus, it would not be available at the time when the chromosomes were ready for the first mitosis. It would then result in an inhibition of the mitotic separation. The delay induced by CB would be as long as the treatment duration (Gould-Somero *et al.*, 1977), *i.e.* 15 minutes, and the mitotic spindle would be thus available for the next mitosis which occurs 20 minutes after the first one.

Different genomes (e.g. pentaploid, tetra-pentaploid mosaics, gynogenetic tetraploids) could result from this mechanism, depending on whether or not the haploid male pronucleus fuses with the tetraploid female nucleus. Evidence could be provided by histological analysis of treated eggs.

We observed, in early treatments, a few hyper-tetraploid metaphases which could be aneuploid or pentaploid cells. Yamamoto *et al.* (1988) also reported pentaploid cells produced by similar treatments. These observations could provide a clue for understanding the present inviability of the embryos derived from early treatments.

#### Acknowledgements

We thank Drs. H. Grizel and A. Beaumont for improving the manuscript and Dr. D. Szöllösi (INRA, Jouy-en-Josas, France) for discussing the data. We also thank Dr. C. Bacher for his statistical advice, T. Noël for the bacterial survey and J. P. Cadoret for supplying cultured algae. Adult clams were kindly provided by Y. Boisard (Artouan, France) and SATMAR (St-Just, France). This work was partially supported by a Poitou-Charentes regional grant.

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