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Induced triploidy in the European clam, *Ruditapes decussatus* (L.), and performance of triploid larvae

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Abstract. For the first time, effective treatments using cytochalasin B were developed to induce triploidy in the European clam, *Ruditapes decussatus* (L.). The percentage of triploid embryos was assessed by karyological or image analysis. Two treatments (0·5 or 1 mg of cytochalasin B (CB) per ml of dimethylsulfoxide in 1 litre of sea water) were applied at different times after fertilization (10 to 25 min), for two different periods (15 and 20 min). Best results were obtained for a CB concentration of 1 mg/l. When treatment was applied 15 min after fertilization for a 20 min period, 94% and 95% of triploid embyros were obtained in two repeated experiments. At metamorphosis, the treated larvae appeared to be no smaller than the control larvae in all experiments. However, in general, significantly higher mortalities for CB-treated batches were found when compared with the untreated batch.

Introduction

Triploidization is now a widespread technique for producing sterile aquatic animals. Over the last 10 years, research has been undertaken on commercially important shellfish, as the result of which cytochalasin B (CB) treatments have been found to be effective in producing triploid animals in the following species: *Crassostrea gigas* (Thunberg) (Allen, Downing, Chaiton & Beattie 1986), *Crassostrea virginica* (Gmelin) (Stanley, Allen & Hidu 1981; Barber, Mann & Allen 1992), *Ostrea edulis* (L.) (Gendreau & Grizel 1990), *Chlamys varia* (L.) (Baron, Diter & Bodoy 1989), *Chlamys nobilis* (L.) (Komaru & Wada 1989), *Argopecten irradiens* (L.) (Tabarini 1984), *Pinctada fucata martensii* (D.) (Wada, Komaru & Uchimura 1989), the Manila clam, *Ruditapes philippinarum* (Adams & Reeve) (Diter & Dufy 1990; Dufy & Diter 1990) or *Tapes semidecussatus* (Reeve) (alternative name for *R. philippinarum*, Beaumont & Contaris 1988; Utting & Doyou 1992), and see Beaumont & Fairbrother (1991) for review. In several experiments the triploid animals gained weight faster than diploid controls (Allen *et al.* 1986; Komaru & Wada 1989; Akashige & Fushimi 1992; Shpigel, Barber & Mann 1992). This is presumably as a consequence of the triploids being more or less sterile (Tabarini 1984; Allen & Downing 1986; Akashige & Fushimi 1992; Shpigel *et al.* 1992).

Until now in the south of Europe, the European clam, *Ruditapes decussatus* (L.), has been more valuable and more appreciated by consumers than the Japanese clam,

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R. philippinarum. In France, however, *R. decussatus* has seldom been reared and its commercialization has been reliant on wild clam fishing. One reason for its low use in mariculture is that the European clam grows more slowly than the Japanese clam, and usually takes one additional year to reach a commercial size. As triploidization can sometimes accelerate mollusc growth, triploidy induction in *R. decussatus* has been suggested as a way of improving its performance. This paper demonstrates the first development of an effective triploidization treatment using CB ever recorded for *R. decussatus*. This treatment would be suitable for the large-scale production of triploids and therefore could allow increased use of *R. decussatus* in mariculture. Growth of triploid veliger larvae was recorded and survival rates were studied during the early stages of clam life.

Materials and methods

Artificial ripening, gamete handling and fertilization

Gametogenesis in the clam broodstock was accelerated under artificial conditions: the sea water was warmed to 22 ± 1°C and complemented with a non-restrictive mixed diet of *Skeletonema costatum, Pavlova lutheri, Isochrysis galbana, Chaetoceros calcitrans* and *Tetraselmis suecica*. After 3 weeks, full ripening was obtained.

Spawning was subsequently induced by thermal shocks, achieved by transferring clams consecutively between filtered sea water (FSW – 3 μm) at 16°C and 28°C. Addition of ripe stripped spermatozoa was also used to help spawning. Each spawning clam was carefully rinsed and isolated in a beaker of FSW at 25°C, and allowed to spawn fully. Ova and sperm were separated from debris and faeces by sieving through a 100 μm mesh. Gametes from eight to ten individuals of each sex were pooled. Only ova released within the first hour were fertilized with motile sperm. An ovum sample was taken prior to insemination and examined 1 day later to estimate the percentage of uncontrolled natural fertilization. Ovum and spermatozoon numbers were evaluated and ovum density was adjusted to 100/ml. The ratio of spermatozoa/ova was fixed to 50:1 for fertilization. After fertilization, eggs were stirred and divided into as many batches as necessary for later experimentation. Batch volumes were always adjusted to 1 l before treatment.

Triploidy induction

Four separate experiments were conducted. The first and second were conducted in 1991 and studied the time of treatment application. The remaining two were conducted in 1992 with some different induction parameters, and their purpose was to study the effect of treatment duration (Table 1). For each experiment, an untreated batch was kept as control. All the experiments were conducted at 25°C, in 1 l of FSW. Cytochalasin B (CB, Sigma C-6762, Sigma, St Quentin, Falavier, France) concentrations were chosen following established protocols: 0·5 mg CB dissolved in 0·5 ml dimethylsulfoxide (DMSO, Lab-Osi D-770, Lab-Osi, Maurepas, France) diluted in 1 l FSW was used for experiments 1 and 2 (Stanley *et al.* 1981, 1984; Komaru & Wada 1989). Seeing that the resulting triploid percentages were not as good as expected, the CB concentration was increased to 1 mg/l

for experiments 3 and 4 (Allen *et al.* 1986; Downing & Allen 1987; Dufy & Diter 1990). The times of treatment after fertilization were 10, 15, 20 and 25 min for the first experiment, and 10 and 15 min for the second (Table 1). Time of application was fixed to 15 min for experiments 3 and 4. Two different treatment durations were tested: 15 min for experiments 1 and 2, 15 and 20 min for experiments 3 and 4. After treatment, embryos were washed in a 0·1% DMSO sea water solution for 15 min. The different batches were then transferred to 150-l tanks.

Larval culture

The subsequent effects of CB treatment on growth and survival rates were analysed in the resulting larvae. Following the treatments, the embryos were incubated for 24 h in 150-l tanks of FSW at a density of about 10 individuals/ml. The D-shaped larvae were then collected, counted and diluted to 3 larvae/ml. The sea water was changed every other day, and the larvae were counted and measured. Measurements of larval sizes and counts of survivors were made, up to metamorphosis. Sizes were measured on 30 individuals from each batch and survival rates were computed using larval density. Larvae were fed with a mixed diet of *Pavlova lutheri*, *Isochrysis galbana* and *Chaetoceros calcitrans* at a concentration of 20 cells/µl/day for each algae species.

Timing of first and second polar body extrusion

In order to follow the meiotic and mitotic stages reached at various times, embryo samples were taken from the control batches of experiments 3 and 4 every 5 min over a period of 55 min after fertilization, and fixed for 1 h in a 2% formaldehyde solution prepared in a glucosamine-acetate buffer. Embryos were then washed with fixative-free buffer and nuclear DNA was stained with 0·5 µg/ml of Hoechst 33258 (Sigma B2883) (Dubé, Schmidt, Johnson & Epel 1985). This chemical stains the DNA which then appears as white spots on a blue background when observed with an Olympus epifluorescence microscope.

Table 1. Induction parameters for the four experiments

Experiment	Temperature (°C)	Cytochalasin B concentration (mg/l)	Time of application (min)	Treatment duration (min)
1	25	0·5	10	15
			15	15
			20	15
			25	15
2	25	0·5	10	15
			15	15
3	25	1·0	15	15
			15	20
4	25	1·0	15	15
			15	20

Ploidy determination by the karyological method

This method was used only for the first experiment. Six-hour-old embryos were transferred into a 0·02% colchicine solution in sea water for 2 h, and then held for 20 min in 25% sea water diluted with distilled water. Embryos were subsequently fixed at 4°C with three successive 30 min washes in Carnoy's solution (ethanol:acetic acid, 3:1). A cell suspension was made from at least 10^4 embryos per batch in 50% acetic acid (in distilled water) and small amounts were dropped onto a microscope slide pre-warmed to 45°C. Preparations were stained for 10 min in a 4% Giemsa solution (in pH 7 phosphate buffer). Metaphasic spreads showing 36 to 38 or 54 to 57 chromosomes were identified as diploid (2N or 2C) or triploid (3N or 3C) cells respectively. Triploid percentage was computed from 50 metaphasic spreads.

Ploidy determination by image analysis

For experiments 2, 3 and 4, ploidy levels were determined using image analysis of pooled larvae. Slides were prepared following the Feulgen–Rosalin staining method. The program analyses the optical density of stained nuclei (Gérard, Peignon & Chagot 1991): by comparison with a diploid sample, it is possible to determine the percentage of triploid nuclei of any CB-treated sample. About 200 nuclei were analysed from each treated batch in experiments 2, 3 and 4.

Statistical analysis

Quantitative measurements (size of animals at different stages) were analysed by ANOVA, followed by a multiple-range t-test analysis. Triploid and diploid D-shaped larval percentages, as well as survival rates were compared following Schwartz (1991). The test consists of the calculation of an ε statistic:

$$\varepsilon = \frac{|p_1 - p_2|}{\sqrt{p \times q\,(1/n_1 + 1/n_2)}} \qquad (1)$$

with $p = (p_1 + p_2)/2$ and $q = (1 - p)$, p_1 and p_2 being the percentages to be compared, and n_1 and n_2 being the two sample sizes respectively. Significance of the ε test is given in the standard deviation table.

Results

Effect of time of treatment application: first and second experiments

For experiment 1, the highest triploid percentage (63%) was obtained for both first and second treatments, respectively, applied 10 min and 15 min after fertilization (Table 2). When treatment was applied 20 or 25 min after fertilization, triploid percentages decreased to 36% and 3% respectively ($\varepsilon_{(63\%,36\%)} = 2{\cdot}70$, $P < 0{\cdot}01$ and $\varepsilon_{(36\%,3\%)} = 4{\cdot}16$, $P < 0{\cdot}001$). The fourth treatment was terminated after 1 day because of a very low percentage of D-shaped larvae (only 9·9%). Larval sizes at metamorphosis were equivalent for the three

Table 2. Results for the four experiments

Experiment	Induction parameters*	Triploid percentage†	Normal D-larvae percentage†	Larval survival at metamorphosis† (%)	Larval size at metamorphosis† (μm)
1	0·5 (10,15)	63 a	–‡	52 a	194 a
	0·5 (15,15)	63 a	–	35 b	185 a
	0·5 (20,15)	36 b	–	29 b	190 a
	0·5 (25,15)	3 c	–	–	–
	Control	–	–	60 a	185 a
2	0·5 (10,15)	50 a	–	65 a	187 b
	0·5 (15,15)	63 a	–	68 a	194 a
	Control	–	–	66 a	169 c
3	1·0 (15,15)	95 a	76 b	31 b	209 a
	1·0 (15,20)	94 a	67 b	44 b	217 a
	Control	–	100 a	60 a	218 a
4	1·0 (15,15)	86 b	73 c	26 b	224 a
	1·0 (15,20)	95 a	87 b	24 b	226 a
	Control	–	100 a	58 a	191 b

* The first number gives the CB concentration. The two numbers in parentheses give time of treatment application and treatment duration respectively.
† Treatments with the same letter are not significantly different (with $\alpha = 0{\cdot}05$).
‡ Not recorded.

remaining treatments and the control ($F = 1{\cdot}38$, $P > 0{\cdot}05$), but survival rates differed greatly (Table 2 and Fig. 1): larval survival in the control (60%) and the first treatment (52%) were not significantly different ($\varepsilon = 1{\cdot}12$, $P > 0{\cdot}05$), but survival rates in the second and third treatments (35% and 29%) were significantly lower than in the control ($\varepsilon = 3{\cdot}54$, $P < 0{\cdot}001$) and first treatment ($\varepsilon = 2{\cdot}42$, $P < 0{\cdot}02$).

For experiment 2, the highest triploid percentage (63%) was obtained for the treatment applied 15 min after fertilization (Table 2). Triploid percentage of the second treatment (50%) was, however, not significantly different from the first one ($\varepsilon = 1{\cdot}78$, $P > 0{\cdot}05$). Survival rates were not significantly different for the two treatments and the control (mean of 66·3% and SD = 1·3%), but major differences were observed for size at metamorphosis ($F = 42{\cdot}72$, $P < 0{\cdot}0001$, Table 2 and Fig. 2). For both treatments, larvae were significantly larger than in the control ($t_{(169,187)} = 6{\cdot}27$ and $t_{(169,194)} = 9{\cdot}02$, $P < 0{\cdot}001$). Also, larvae were significantly larger in the 15 min compared with the 10 min treatment ($t_{(187,194)} = 2{\cdot}75$, $P < 0{\cdot}01$). However, 32 days after fertilization, the differences in larval size were no longer significant ($F = 0{\cdot}18$, $P > 0{\cdot}05$).

Effect of treatment duration: third and fourth experiments

For the third and fourth experiments, the mean percentage of triploid induction increased to 92·5% (SD = 3·8%, Table 2). Percentage of triploid embryos induced by the two treatment durations differed only in the fourth experiment (86% and 95%, $\varepsilon = 3{\cdot}21$, $P < 0{\cdot}01$). In experiment 3, the two treatment durations did not produce significantly different percentages of triploids (95% and 94%). Both 20 min treatment durations also

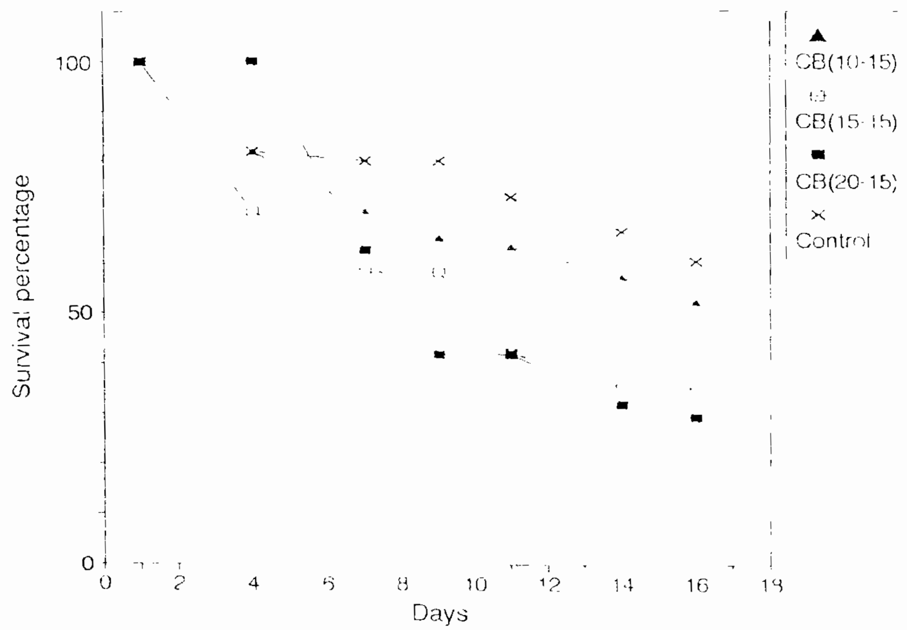

Figure 1. Larval survival for experiment 1 with a CB concentration of 0·5 mg/l. The two numbers in parentheses give time in minutes of treatment application and treatment duration.

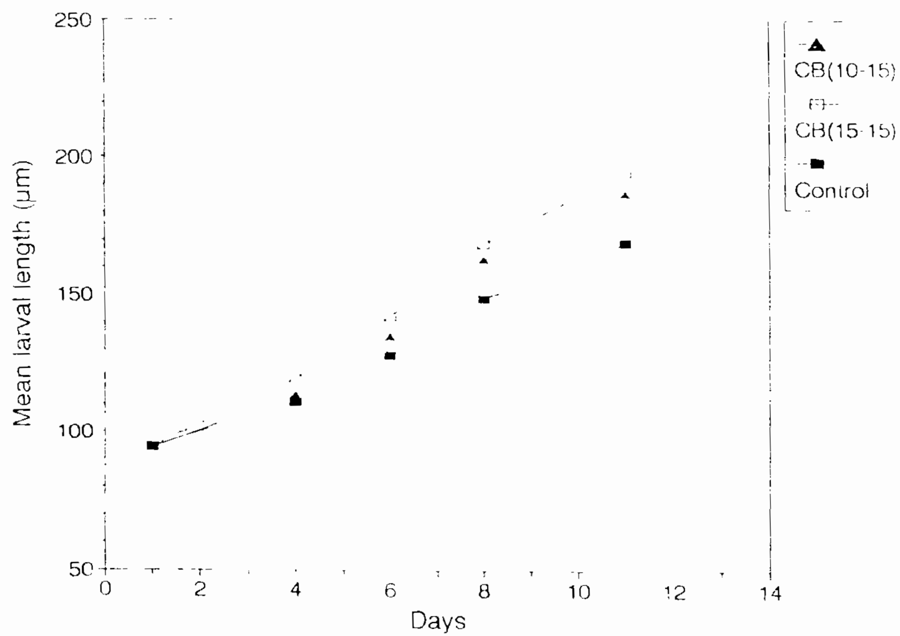

Figure 2. Larval growth for experiment 2 with a CB concentration of 0·5 mg/l. The two numbers in parentheses give time in minutes of treatment application and treatment duration.

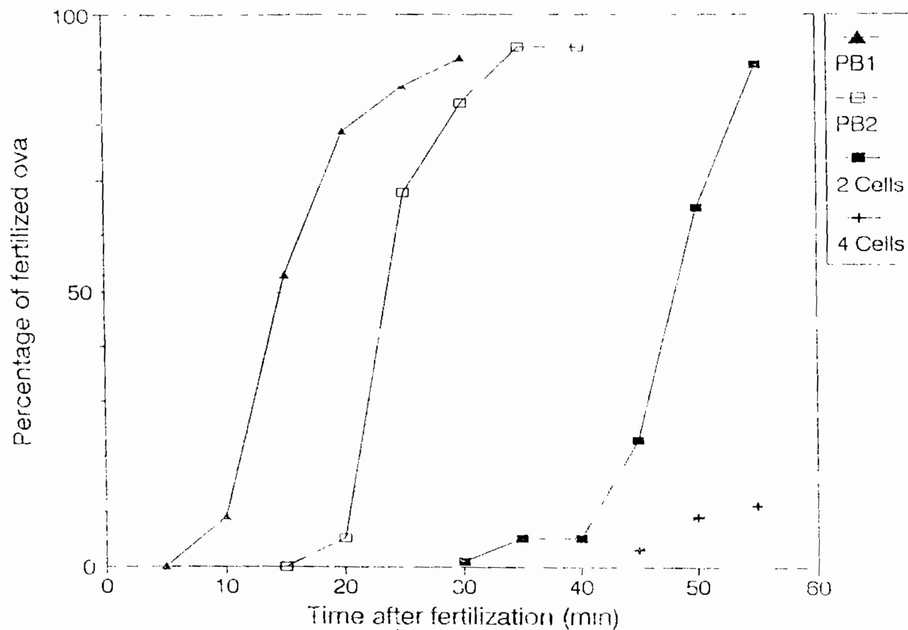

Figure 3. Timing of first and second polar body (PB1 and PB2) extrusion from fertilized ova in experiments 3 and 4.

gave the same result (94% and 95%). The timing of polar body extrusion (Fig. 3) indicates that second polar body (PB2) extrusion was just beginning when treatments were applied (15 min after insemination in experiments 3 and 4), and that between 80% and 85% of the cells had already expelled their first polar body (PB1).

A mean of 75·8% (SD = 7·3%) for morphologically normal D-shaped larvae was obtained for the four treated batches over the two experiments (Table 2). Within the four CB treatments, the 20 min treatment of experiment 4 (87%) was significantly different from the other treatments (76%, 73% and 67%, $P < 0{\cdot}05$ for the 15 min treatments of experiments 3 and 4 compared with the 20 min treatment of experiment 4 and $P < 0{\cdot}01$ between the two 20 min treatments). The percentage of normal larvae obtained decreased from the shorter treatment time to the longer one in the third experiment (76% and 67%) and increased in the fourth one (73% and 87%).

In experiment 3, metamorphosis occurred 16 days after fertilization and the treated samples reached an average larval size of 213 μm. There was no significant difference between the two CB samples and the control ($P > 0{\cdot}05$, Table 2 and Fig. 4). In experiment 4, metamorphosis occurred earlier, 13 days after fertilization. The CB-treated larvae reached an average size of 225 μm and were significantly larger than control larvae ($P < 0{\cdot}001$, Table 2 and Fig. 5). As in experiment 1, the treated samples were not measurably different from each other.

Larval survival in experiments 3 and 4 is presented in Table 2 and illustrated in Figs 4 and 5. For both experiments, there was no difference in survival rate between the two treated batches, but all treated batches presented significantly higher mortality than the control ($P < 0{\cdot}01$ to $P < 0{\cdot}001$).

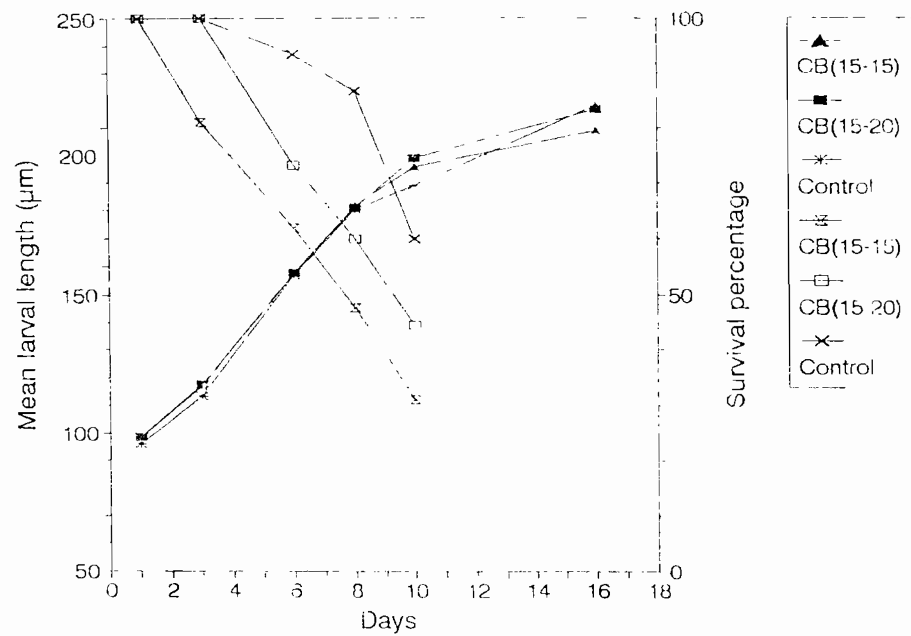

Figure 4. Larval growth and survival in experiment 3 with a CB concentration of 1 mg/l. The two numbers in parentheses give time in minutes of treatment application and treatment duration.

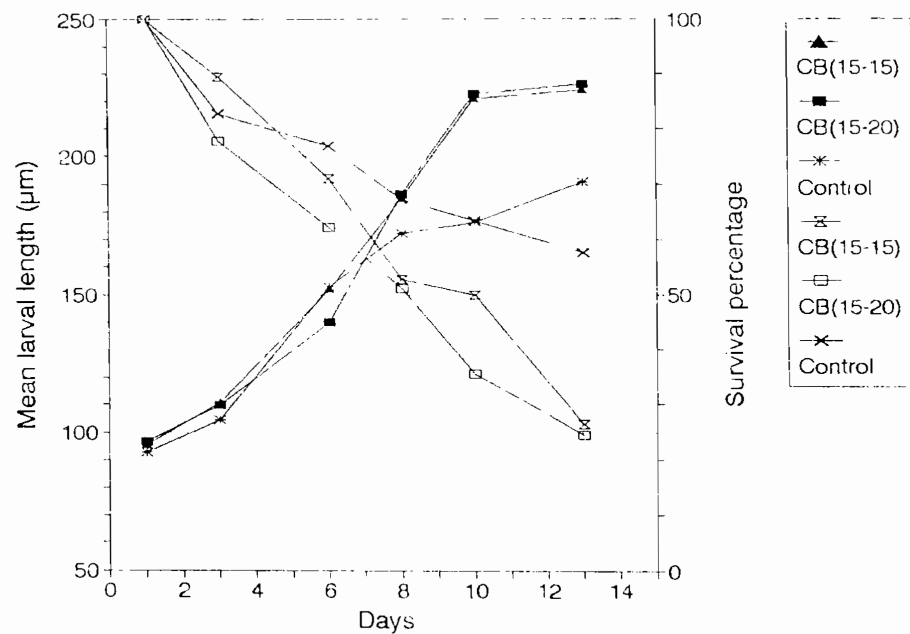

Figure 5. Larval growth and survival in experiment 4 with a CB concentration of 1 mg/l. The two numbers in parentheses give time in minutes of treatment application and treatment duration.

Discussion

Effect of CB concentration

In 1991 (experiments 1 and 2), the CB concentration used (0·5 mg/l) was not as efficient as expected: the resulting triploid percentages ranged between 50% and 63% for the more successful treatments. This is comparable to the 50% of triploids obtained in *Ruditapes philippinarum* with the same CB concentration by Gosling & Nolan (1989), but slightly lower than proportions obtained in other published experiments (73%, Utting & Doyou 1992; 77·7% and 81·8%, Beaumont & Contaris 1988; for *Crassostrea virginica* 73·9%, Stanley *et al.* 1981; 61% and 72%, Stanley *et al.* 1984; 100%, Barber *et al.* 1992). In 1992 (experiments 3 and 4), a CB concentration of 1 mg/l gave better induction rates: triploid percentages ranged between 86% and 95%, higher than published rates for *Ruditapes philippinarum* using the same CB concentration (45·8%, Beaumont & Contaris 1988; 40% Gosling & Nolan 1989; 75·8%, Dufy & Diter 1990). The present results with *Ruditapes decussatus* are at variance with published data on *Ruditapes philippinarum* (Beaumont & Contaris 1988) and *Pinctada fucata martensii* (Wada *et al.* 1989), which show, on average, better induction rates with low CB concentrations. The present results are, however, in agreement with published data in showing that increasing CB concentration usually leads to reduced survival rates (Barber *et al.* 1992).

Effect of application time

Experiments 1 and 2 show that the later the treatments are applied, the lower the resulting triploid percentages are. Treatments applied 10 or 15 min after fertilization show no significant difference in triploid percentage. For larval survival and larval size at metamorphosis the two experiments gave conflicting results, and it is not possible to deduce a clear effect of time of treatment application. However, the treatment applied 15 min after fertilization was chosen for further experiments as it gave the best results in experiment 2.

Effect of treatment duration

Experiments 3 and 4 gave no conclusive evidence for the effect of treatment duration on percentages of either triploids or normal D-shaped larvae: experiment 3 showed no significant differences between the two treatments but experiment 4 gave the highest triploid percentage as well as the highest percentage of normal D-shaped larvae with the longest treatment (20 min). It can be suggested that the two durations tested were not different enough to produce any significant difference in results.

Larval growth

Results presented here provide no clear evidence of increased growth rates for triploid larvae. Triploid animals were never significantly smaller than diploids as recorded in *Ruditapes philippinarum* by Dufy & Diter (1990). In experiments 2 and 4, triploids were

significantly larger than diploids at metamorphosis, but this difference had disappeared by several days after settlement.

Optimal triploid induction rates, were obtained with a CB concentration of 1 mg/l and treatments applied for 20 min, 15 min after fertilization. With such a treatment, most of the triploids must have been induced by retention of the second polar body. With earlier treatments, triploids would have been produced by suppressing meiosis I and a higher heterozygosity level among animals could have then been expected (Beaumont & Fairbrother 1991 for review). This would only be of interest if heterozygosity is proven to be related to better growth rates as suggested for other molluscs (Foltz, Newkirk & Zouros 1983; Stanley *et al.* 1984; Foltz & Chatry 1986).

Cytochalasin B is now proven to be as efficient in producing triploid larvae in *R. decussatus* as in other molluscan shellfish. However, assessment of the interest of triploids versus diploids and their potential impact on clam rearing and commercialization in France needs further experimentation.

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