

CHEMICAL AND PHYSICAL METHODS OF TRIPLOIDY INDUCTION IN *Crassostrea gigas* (THUNBERG, 1793)*

Emílio Mateus Costa MELO¹; Carlos Henrique Araújo de Miranda GOMES¹; Francisco Carlos da SILVA¹; Simone SÜHNEL¹; Claudio Manoel Rodrigues de MELO¹

ABSTRACT

Three methods of triploidy (3N) induction were tested in diploid (2N) oysters *Crassostrea gigas*: two chemical methods cytochalasin-B (CB) and 6-dimethylaminopurine (6-DMAP), and one physical method with temperature shock. The objective was to evaluate the triploidy induction technology using flow cytometry as a tool to check the results of induction. The experiments were performed in separate and a seawater temperature in the tanks was maintained at 25 °C for all experiments. In the experiment I, the efficacy of triploidy induction was evaluated using CB (0.5 mg L⁻¹) and 6-DMAP (390 µmols L⁻¹). In the experiment II, the efficiency of triploidy induction was tested using CB (0.5 mg L⁻¹) and 6-DMAP (450 µmols L⁻¹). In the experiment III, the efficiency of triploidy induction was evaluated using CB (0.5 mg L⁻¹) and temperature shock (25 to 36 °C). In all three experiments, viable triploid larvae were obtained. However, in the experiments I and II (with chemical methods), high mortality of larvae was observed, especially for the treatment CB. From these results, it is suggested the replacement of CB by other methods of triploidy induction, due to its high cost and high toxicity to humans and to the environment.

Keywords: temperature; 6-dimethylaminopurine; cytochalasin-B; oyster

MÉTODOS QUÍMICO E FÍSICO DE INDUÇÃO À TRIPLOIDIA EM *Crassostrea gigas* (THUNBERG, 1793)

RESUMO

Três métodos de indução à triploidia (3N) foram testados em ostras diplóides (2N) (*Crassostrea gigas*); dois métodos químicos, citocalasina-B (CB) e 6-dimetilaminopurina (6-DMAP), e um método físico, com choque de temperatura. O objetivo foi avaliar a tecnologia de indução à triploidia, utilizando a técnica de citometria de fluxo como ferramenta para verificação dos resultados de indução. Os experimentos foram realizados em separado, sendo que a temperatura da água do mar foi mantida em 25 °C em todos os tanques. No experimento I, foi avaliada a eficácia da indução à triploidia com CB (0,5 mg L⁻¹) e 6-DMAP (390 µmols L⁻¹). No experimento II, foi testada a eficiência da indução à triploidia com CB (0,5 mg L⁻¹) e 6-DMAP (450 µmols L⁻¹). No experimento III, foi avaliada a eficiência da indução à triploidia com CB (0,5 mg L⁻¹) e choque de temperatura (25-36 °C). Nos três experimentos, foram obtidas larvas triplóides viáveis. Entretanto, nos experimentos I e II (com métodos químicos), observou-se elevada mortalidade das larvas, especialmente para o tratamento CB. A partir destes resultados, a substituição de CB por outros métodos de indução à triploidia é sugerida, devido ao seu elevado custo e elevada toxicidade para os seres humanos e para o meio ambiente.

Palavras chave: temperatura; 6-dimetilaminopurina; citocalasina-B; ostra

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¹ Federal University of Santa Catarina (UFSC), Laboratory of Marine Molluscs. Rua dos Coroaes, 503 - Barra da Lagoa - CEP: 88061-600 - Florianópolis - SC - Brazil. e-mail: efwp@yahoo.com.br (corresponding author)

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INTRODUCTION

Global aquaculture produced more than 90.4 million tons in 2012 (FAO, 2014), presenting an average annual growth of 8.9% since 1970. Advances in cultivation technology and biotechnology contributed to this growth in the recent decades. Biotechnology research, as well as chromosomes manipulation (polyploidy) has shown potential to improve the production in the aquaculture industry, especially for bivalve molluscs (RASMUSSEN and MORRISSEY, 2007).

Polyploidy is an important and successful technique for oyster farming (MATT and ALLEN, 2014). During the reproductive seasons of bivalve molluscs, it is observed a reduction in meat quality (DESROSIERS *et al.*, 1993) due to the spawning process. Triploid technique can be an alternative for this problem, since it affects the reproductive capacity of the animal. Consequently, the energy normally used for the reproductive effort might be available for the somatic growth and survival in sterile bivalves (DESROSIERS *et al.*, 1993; RASMUSSEN and MORRISSEY, 2007).

Another positive effect of triploidy is the quality of the final product. The flavor of the oyster may be related to the glycogen content (NELL, 2002). In the general, consumers do not appreciate the flavor of thin oysters (post-spawning) or fatty oysters (pre-spawning) (RASMUSSEN and MORRISSEY, 2007). Therefore, triploidy may favor the organoleptic characteristics of the product throughout the year, preventing the maturation and consequently the transformation of glycogen into lipids.

Studies on production of triploid bivalve larvae, by inhibiting the release of the second polar body during the process of meiosis, have been developed by different research groups. This inhibition can be achieved by the use of chemical methods, such as cytochalasin B (CB), 6-dimethylaminopurine (6-DMAP), and physical methods, such as heat shock and hydrostatic pressure, or even by the combination of both methods.

There are reports of chemical methods using CB tested in oysters, such as *Crassostrea virginica* (STANLEY *et al.*, 1981; MATT and ALLEN, 2014) *Saccostrea glomerata* (NELL *et al.*, 2004) *Crassostrea madrasensis* (MALLIA *et al.*, 2006), and *Crassostrea*

gigas (ALLEN and DOWNING, 1986; DESROSIERS *et al.*, 1993; GUO and ALLEN, 1994; GUO *et al.*, 1996; HUBERT *et al.*, 2009). In addition, CB was tested on the mussels *Mytilus galloprovincialis* (SCARPA *et al.*, 1994) and *M. edulis* (DESROSIERS *et al.*, 1993), and on the pectinid *Placopecten magellanicus* (DESROSIERS *et al.*, 1993). Studies concerning the method 6-DMAP were performed on *C. gigas* (DESROSIERS *et al.*, 1993) and *S. glomerata* (NELL *et al.*, 2004), and on the mussel *M. edulis* (DESROSIERS *et al.*, 1993; GÉRARD *et al.*, 1994) and scallop *P. magellanicus* (DESROSIERS *et al.*, 1993). The triploid larvae of bivalves induced with calcium and caffeine were tested on *C. gigas* (YAMAMOTO *et al.*, 1990) and on *M. galloprovincialis* (SCARPA *et al.*, 1994). The physical method of heat shock in order to induce triploidy was tested on *C. gigas* (QUILLET and PANELAY, 1986), *M. galloprovincialis* (SCARPA *et al.*, 1994) and on the clam *Tapes semidecussatus* (GOSLING and NOLAN, 1989).

Both CB and 6-DMAP affect the microtubules during the cell division, preventing the extrusion of the polar body. The physical methods, such as temperature shock, act breaking the meiotic spindle microtubules and changing the cell density (PIFERRER *et al.*, 2009).

Among these methods and combinations, in order to produce viable triploid animals, the chemical stress with CB is the most used method. However, due to its toxicity to the operator, the high cost (GUO *et al.*, 1992) and the instable responses obtained with this chemical method, the physical methods, although less efficient, also became prominent (GUO *et al.*, 1996). Therefore, the present study evaluated the effectiveness of cytochalasin-B, 6-dimethylaminopurine and temperature shock in order to obtain triploid individuals of oyster *C. gigas*.

MATERIAL AND METHODS

Animals

Two years old diploid broodstock oysters *C. gigas*, produced at the Laboratory of Marine Molluscs (LMM) (27°35'04''S; 48°26'29''W) and maintained at the experimental cultivation area of the LMM, Sambaqui Beach (27°29'23''S; 48°32'15''W), were used in the study.

Gametes and fertilization

In order to obtain gametes, the broodstocks were opened, sexed and then the mature animals were selected. The verification of the animal maturity was performed by observing the gamete shape of the females, number of oocytes and sperm motility. Male gametes (cells) movement was used to measured sperm motility.

The selected females were washed with seawater at 24-26°C and the excess of water was removed from the shells with paper towels. The oocytes were removed from the female tissues, simultaneously with a blade, and then stored in dry containers. After scraping, the material (oocytes and impurities) was separated using 70 µm (to retain impurities) and 18 µm (to retain gametes) screens. The obtained gametes were placed in a container of 20 L, with the volume adjusted to 14 L in seawater, and then hydrated for 1 h at 25 °C with salinity 35. The counting of oocytes was performed using a Sedgwick-Rafter chamber. During the counting, the presence/absence of cells was registered in the embryonic development, which characterizes fertilization before the appropriate time. It was discarded the batch in which the presence of cell in division process was detected.

As for the oocytes fertilization, a spermatozoa pool from the selected males was created. The male gametes were obtained through the germinal tissue scraping, subsequently added to the female gamete solution, in a ratio of approximately seven sperms per oocyte (7:1 sperms:oocytes), as a single dose and then homogenized.

After five minutes of fertilization, two slides containing 1 mL of fertilized solution were prepared (slide, fertilized solution and coverslip) for the assessment of the embryonic development, under light microscope. When approximately 50% of the fertilized oocytes presented the second polar body, the treatment of shock inducer of triploidy was started immediately.

Experiments on the ploidy induction

Three independent experiments were performed by testing two separate chemical methods: Cytochalasin-B $C_{29}H_{37}NO_5$ (CB) at a concentration of 0.5 mg L⁻¹ and 6-dimethyl aminopurina $C_7H_9N_5$ (6-DMAP) at concentrations

of 390 and 450 µmol L⁻¹; and a physical method of temperature shock from 25 to 36 °C, in triplicate and completely in a random design. As control group for each experiment, fertilized oocytes that were not subjected to any type of induced triploidy were used. The control group was kept separate from the treatment groups for further comparisons.

For the treatments with CB, a solution of 0.5 mg of CB and 1 mL of dimethyl sulfoxide (DMSO - C_2H_6SO) was prepared and stored at a temperature of -20 °C (ALLEN *et al.*, 1989). For the shock induction, the fertilized oocytes were placed into a cylinder tube with screen (18 µm) and submerged in 1 L of CB solution for 15 min. After this period, the material retained on the screen (embryos, unfertilized gametes and sperm, among others) was suspended and immersed in a solution of 0.05% DMSO in seawater, for 15 min.

For the treatments with 6-DMAP, solutions of 390 and 450 µmol L⁻¹ were prepared and stored at the temperature of -20 °C (DESROSIERS *et al.*, 1993). For each concentration tested, the shock induction occurred by transferring the fertilized oocytes to a cylinder tube with screen (18 µm) that was submerged in 1 L of 6-DMAP solutions, for 15 min.

After the triploidy induction, embryos from each treatment were transferred to larviculture tanks (2,500 L), where they remained for 48 hours. After this period, the water from the tanks was removed and the larvae from each treatment were concentrated in containers of 20 L with the volume adjusted to 14 L for subsequent counting.

All waste generated during the processes of induction were separated into suitable containers for disposal of chemicals.

Experiment I: shock induction with CB (0.5 mg L⁻¹) and 6-DMAP (390 µmol L⁻¹)

In this experiment, four male and eight female animals were used that produced 90 million of oocytes. They were divided into two treatments: CB (0.5 mg L⁻¹) and 6-DMAP (390 µmol L⁻¹) and a control group. A density of 10 million oocytes L⁻¹ was used. The oocytes were then hydrated for 1 h and the shock induction occurred after 26 min of fertilization at 25 °C.

Experiment II: shock induction with CB (0.5 mg L⁻¹), 6-DMAP (450 µmol L⁻¹)

In this experiment, six males and 17 females were used in order to obtain 440 million of oocytes, from which 135 million were distributed in two treatments: CB (0.5 mg L⁻¹), 6-DMAP (450 µmol L⁻¹) and a control group. A density of 15 million oocytes L⁻¹ was used and the remaining larvae were discarded. The oocytes were then hydrated for 1 h and the shock induction occurred after 32 min of fertilization at 25 °C.

Experiment III: shock Induction with CB (0.5 mg L⁻¹) and temperature (from 25 to 36°C)

In the experiment of triploidy induction through temperature shock, five males and 18 females were used, generating 213 million of oocytes. From these, 135 million were distributed into two treatments: CB (0.5 mg L⁻¹) and temperature shock (25-36 °C), and a control group. It was used a density of 15 million oocytes L⁻¹. The remaining oocytes were discarded. The hydration process lasted for 1 h and 25 min, and the shock induction occurred 35 min after the fertilization at 25 °C. The heat shock was performed with the rapid transfer of fertilized oocytes from a container (1 L) at 25 °C to another container (1 L) at 36 °C, where they remained for 15 min (QUILLET and PANELAY, 1986). After this period, the oocytes were transferred from the container with the temperature at 36 °C to another at 25 °C.

Polyploidy analysis

The ploidy test for the larvae of *C. gigas* was performed after 48 h of fertilization, using a Flow Cytometer (Partec). For this test, 2,000 larvae per treatment, collected after the counts, were used. The larvae were concentrated and added to a 0.5 mL extraction solution (HCl+NaOH) of the nucleus and 1.5 mL of a specific dye, DAPI (4,6-diamidino-2-phenylindole). The diploid organisms were used as standard for ploidy comparison for all experiments.

Larviculture of the Experiment II

Regarding the experiment II, after the ploidy evaluation (48 h after fertilization), the larviculture was continued and it was evaluated the larvae survival of pediveliger after 25 days. The larviculture was performed with a continuous flow of food and seawater in a 12 L tanks with a constant aeration system. The culture density was 19 larvae mL⁻¹.

The microalgae species provided during all the larviculture period were *Isochrysis galbana* (ISO) and *Chaetoceros muelleri* (CM). These microalgae grew in a modified Guillard F/2 medium (with addition of silica) with filtered (0.2 µm) and sterilized (UV) seawater, with continuous aeration, at 22 °C and 24 h of light regime. The microalgae were used in the exponential growth phase (Table 1).

Table 1. Microalgae diet provided during the larviculture period of the experiments I and II. ISO = *Isochrysis galbana* and CM = *Chaetoceros muelleri*.

Microalgae	Days of larviculture	Ratio	Concentration (x 10 ⁴ cell mL ⁻¹)
ISO: CM	2	70:30	1
ISO: CM	3-5	50:50	2
ISO: CM	6-12	30:70	4
ISO: CM	13-15	30:70	5
ISO: CM	16-18	30:70	6
ISO: CM	18-end	30:70	8

Statistical analysis

In each experiment, it was evaluated the efficiency of the triploidy induction (%) for each treatment, as well as the number of well-formed D-larvae, and the relationship between the well-

formed D-larvae and the total number of larvae after 48 h of fertilization. The evaluation of well-formed D-larvae was performed under light microscopy based on the morphological description of GALTISOFF (1964). In addition, in

the experiment II, the survival of pediveliger larvae was evaluated after 25 days.

The percentages of triploid larvae obtained in the different treatments, as well as larval survival and the numbers of well-formed D-larvae were subjected to analysis of variance (ANOVA). When the ANOVA presented significant differences among means, the Tukey test was applied. The analyses were performed using the software SAS®.

RESULTS

Experiment I

The peaks of fluorescence absorption in the cytometry analysis, for the treatments CB and 6-DMAP, were 78.01 and 79.24, respectively. The diploid (2N) individuals from the control groups reached a peak of 51.15 of fluorescence absorption (Figure 1). The treatment CB showed 1.53 times higher fluorescence than the control, and the treatment 6-DMAP showed 1.55 times.

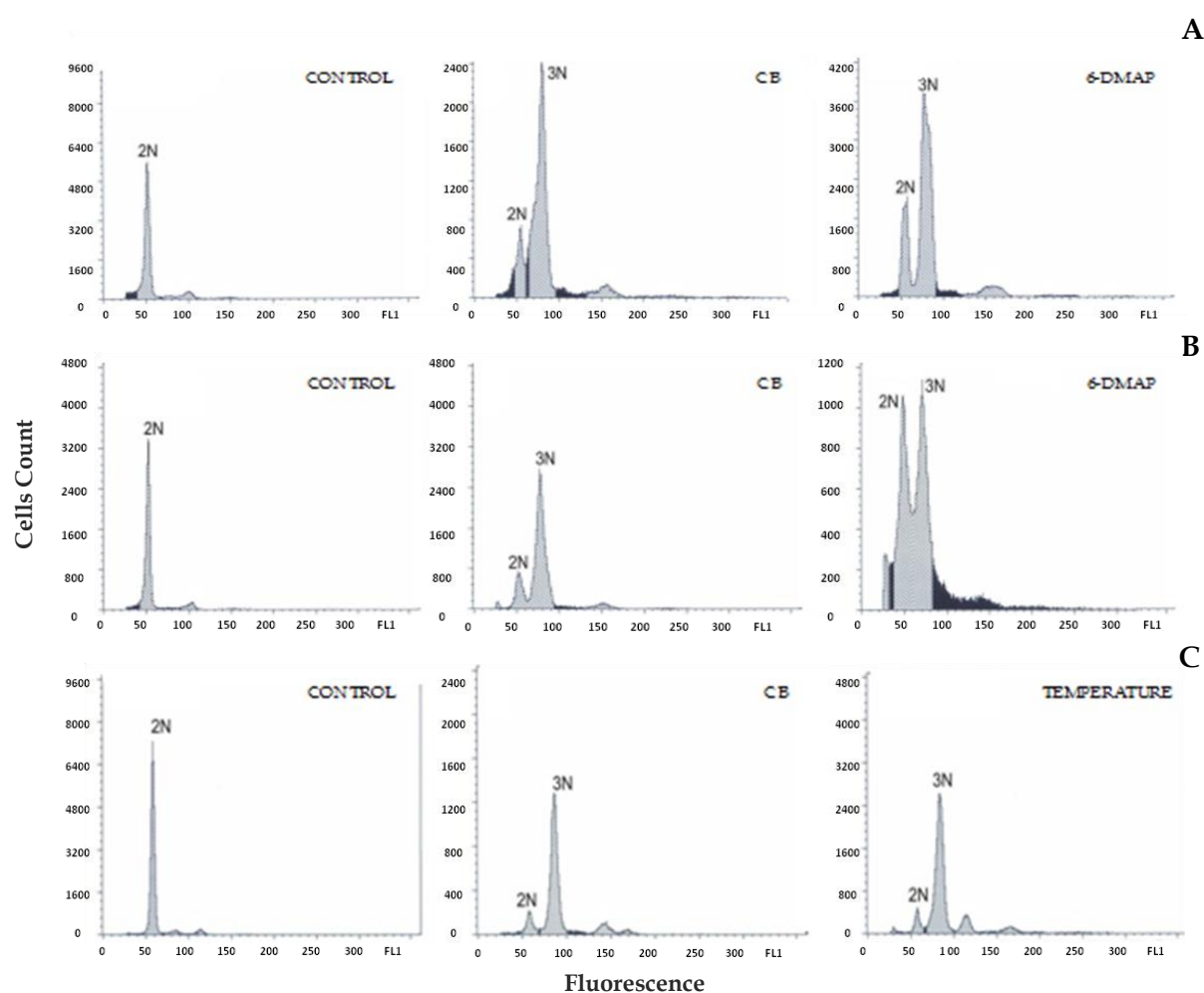


Figure 1. Flow cytometry analysis of 48 h old larvae, resulting from the blocking of the second polar body, 26, 32 and 35 min after the fertilization, in the experiment I(A), II(B) and III(C), respectively. The X-axis represents the fluorescence of the cell nucleus and the Y-axis represents the number of cells counted. 2N = diploid; and 3N = triploid.

The mean percentage of triploids larvae of *C. gigas* obtained with the CB was statistically ($P < 0.05$) higher than the average obtained with the 6-DMAP. The percentage of diploid (2N) larvae in the control group and the treatment

6-DMAP were significantly different ($P < 0.05$) from the treatment CB (Table 2).

The average number (\pm SD) of well-formed D-larvae after 48 h of fertilization did not differ

statistically between the experiments CB (7.77 ± 2.78 larvae mL⁻¹) and 6-DMAP (36.44 ± 40.00 larvae mL⁻¹). The average number (\pm SD) of well-

formed D-larvae from the control group (449.77 ± 88.51 larvae mL⁻¹) was significantly ($P < 0.05$) higher than in the treatments CB and 6-DMAP.

Table 2. Average percentage (\pm standard deviation) of the number of 48 h old diploid (2N) and triploid (3N) larvae.

Experiment	Treatment	2N (%)	3N (%)
I	Control	76.35 \pm 5.38 ^a	-
	CB (0.5 mg L ⁻¹)	23.24 \pm 8.83 ^b	57.12 \pm 11.28 ^a
	6-DMAP (390 μ mol L ⁻¹)	56.10 \pm 18.07 ^a	22.32 \pm 14.36 ^b
II	Control	73.14 \pm 2.45 ^a	-
	CB (0.5 mg L ⁻¹)	15.66 \pm 9.79 ^b	55.08 \pm 6.82 ^a
	6-DMAP (450 μ mol L ⁻¹)	23.11 \pm 3.89 ^b	56.49 \pm 10.09 ^a
III	Control	76.68 \pm 11.62 ^a	-
	CB (0.5 mg L ⁻¹)	17.11 \pm 6.40 ^b	57.31 \pm 9.57 ^a
	Temperature (from 25 to 36 °C)	29.28 \pm 11.10 ^b	45.29 \pm 18.11 ^a

Averages in the same column followed by the same letter do not differ significantly by Tukey test ($P < 0.05$).

The proportion of well-formed D-larvae (mean \pm SD) in relation to the total larvae after 48 h was not significantly different between the treatments CB ($60.07 \pm 3.56\%$) and 6-DMAP ($51.14 \pm 23.97\%$) and the control group ($12:47 \pm 51.82\%$).

Experiment II

The peaks of fluorescence absorption for the triploid individuals (3N) in the treatments CB and 6-DMAP were 78.14 and 70.89, respectively. The diploid individuals (2N) in the control group reached a peak of 51.64 (Figure 1). The triploid larvae from the treatment CB showed 1.51 times higher fluorescence than the control, and the treatment 6-DMAP showed 1.37 times.

No statistical differences in the efficiency of triploidy induction were observed in the experiment II between the treatments CB and 6-DMAP. The percentage of diploid larvae (2N) in the control group was significantly different ($P < 0.05$) from the treatments 6-DMAP and CB (Table 2).

The survival of the final larvae (mean \pm SD) after 25 days presented a significant difference ($P < 0.05$) between the control group (56.99 ± 15.63 larvae mL⁻¹) and the treatments with chemicals. However, no difference in the larvae survival was

observed between the treatments CB (20.90 ± 12.9 larvae mL⁻¹) and 6-DMAP (17.43 ± 10.60 larvae mL⁻¹).

The average number (mean \pm SD) of well-formed D-larvae from the treatment CB (35.99 ± 4.66 larvae mL⁻¹) was statistically different ($P < 0.05$) from the treatment 6-DMAP (177.10 ± 17.82 larvae mL⁻¹). Additionally, the average number of well-formed D-larvae in the control group (462.22 ± 85.72 larvae mL⁻¹) was statistically different ($P < 0.05$) when compared to the treatments CB and 6-DMAP.

There was no difference between the treatments CB ($71.27 \pm 1.51\%$) and 6-DMAP ($61.97 \pm 9.05\%$) and the control group ($76.93 \pm 5.4\%$) concerning the ratio of well-formed D-larvae and total larvae after 48 h (mean \pm SD).

Experiment III

In the cytometry analyzes, triploid individuals (3N) presented the absorption peak in the fluorescence ranges of 85.91 and 83.19 for the treatment CB and temperature, respectively. The diploid individuals (2N) in the control group reached a peak at 57.94 (Figure 1). The treatments CB and temperature presented higher fluorescence than the control group (1.48 and 1.51 times higher, respectively).

No statistical differences were observed among the percentages of triploid larvae using the

CB and temperature induction in the experiment III. The percentage of diploid larvae (2N) in the control group was significantly different ($P < 0.05$) than the percentage in the treatments CB and 6-DMAP (Table 2).

The average number of well-formed D-larvae (mean \pm SD) did not differ between the treatments CB (33.99 ± 24.11 larvae mL⁻¹) and temperature (87.77 ± 74.48 larvae mL⁻¹). However, the average number of well-formed D-larvae in the control group (471.00 ± 204.04 larvae mL⁻¹) was significantly different than the treatment with temperature and CB.

The ratio of well-formed D-larvae and the total number of larvae (mean \pm SD) did not differ between the treatments CB ($55.95 \pm 20.93\%$) and temperature ($56.20 \pm 23.15\%$) and the control group ($83.63 \pm 15.89\%$).

Low variation coefficient observed in this studied showed the quality of analyzed samples.

DISCUSSION

The fluorescence absorption for triploid and diploid larvae obtained in all experiments ranged from 1.37 (experiment II; 6-DMAP) to 1.55 (experiment I; 6-DMAP). These values are within the expected, since that, the stained nuclei of triploid cells emit around 1.5 times the fluorescence of diploid nuclei (GOSLING and NOLAN, 1989).

The percentage of triploid larvae induced with CB [0.5 mg L^{-1}] in the experiments I, II and III, with start of exposure at 26, 32 and 35 min after the fertilization, respectively, and lasting for 15 min, is below that observed for the same species and other bivalves.

Using a CB 0.5 mg L^{-1} concentration and applying shock induction 23 min after the fertilization, for 20 min, NELL *et al.* (1994) obtained better results for the oyster *S. glomerata*, with 81% of triploid individuals. ALLEN and DOWNING (1986), obtained 90% of triploid individuals (*C. gigas*) applying shock induction with CB [1.0 mg L^{-1}] 15 min after the fertilization that lasted for 15 min. Using the same concentration of CB [1.0 mg L^{-1}], GENDREAU and GRIZEL (1990) obtained 70 and 68% of triploid oysters (*Ostrea edulis*), applying the shock

induction at 30 and 90 min, respectively, after the fertilization, for 20 min. However, for the clam *T. semidecussatus*, GOSLING and NOLAN (1989) obtained a lower yield, with 50% of triploid larvae using the CB [0.5 mg L^{-1}] and the same exposure time (15 min), when compared to the present study.

The processes of triploidy induction are affected by several factors such as the refining of the technique, gamete quality, synchronism between time of induction shocks and embryonic development, intensity and lifetime of the induction shock, and even tropical characteristics could explain why the results were lower and different from the literature.

In this study the best result obtained for 6-DMAP was within the concentration of $450 \mu\text{mol L}^{-1}$ (experiment II), reaching an average of 56.49% of triploid larvae, after 32 min of fertilization and 15 min of exposure. However, this result also presented a lower percentage of triploid larvae when compared to the same and to other species. By studying *C. gigas*, GÉRARD *et al.* (1994) obtained, with the same concentration [$450 \mu\text{mol L}^{-1}$], 85% of triploid larvae, applying the shock induction 15 min after fertilization for 10 min. However, DESROSIERS *et al.* (1993), using a concentration of $300 \mu\text{mol L}^{-1}$ of 6-DMAP, achieved higher induction efficiency (90%) for triploid larvae of *C. gigas*, applying the shock 15 min after fertilization with 20 min of exposure. The same author mentioned above observed 95% of triploid larvae for the scallop *P. magellanicus*, when exposed to 6-DMAP $400 \mu\text{mol L}^{-1}$ for 15 min after 70 min of fertilization.

The concentration of 6-DMAP in the experiment II improved the efficiency of the shock induction, when compared to the treatment CB in the same experiment. DESROSIERS *et al.* (1993), by using the same substance in order to inhibit the polar masses of the scallop *P. magellanicus*, mussel *M. edulis* and of the oyster *C. gigas*, reported that the increased concentrations of 6-DMAP in the shock induction is directly related to the improvement of the induction outcome. Corroborating with the results of DESROSIERS *et al.* (1993), VADOPALAS and DAVIS (2004), by studying the geoduck *Panopea abrupta*, identified that the gradual increase of 6-DMAP concentrations in the triploidy induction increased both the efficiency of induction and the mortality rate of larvae.

The efficiency of the induction with temperature shock in the present study showed a lower triploid larval percentage, when compared to other species. YAMAMOTO and SUGAWARA (1988) and YAMAMOTO *et al.* (1990), obtained better results of triploid larvae (97.4%) for *M. edulis*, by applying the induction shock 20 min after the fertilization with a temperature of 32 °C for 10 min. On the other hand, for *T. semidecussatus*, with the same temperature shock (32 °C) and exposure time (10 min), GOSLING and NOLAN (1989) achieved only 55% of triploid larvae. GUO and YANG (2006), by studying the clam *Mulinia lateralis*, obtained from 86.3 to 98.5% of triploid larvae using heat shock at 35 °C, initiating from 8 to 11 min after the fertilization. YAMAMOTO *et al.* (1990) obtained 83% of triploid larvae of *C. gigas* applying the shock induction at a temperature of 37 °C, 45 min after the fertilization with duration of 15 min. The variation among the aforementioned results may be related to the improvement of the induction techniques by the research laboratory. In addition, the sexual stage of the broodstocks may affect the triploidy induction process.

The techniques for triploidy induction have many details, and each one acts decisively to the success of the procedure. Among the factors that directly affect the efficiency of triploidy induction and larval survival, the quality of the gametes, the synchronism between embryonic development and induction time of the shock application, the duration and intensity of the shock, the temperature of the processes and the techniques used in the larviculture are of the utmost importance (PIFERRER *et al.*, 2009).

The total number of well-formed D-larvae obtained in the chemical treatments (CB and 6-DMAP) was much lower than the amount obtained in the control treatment, demonstrating the harmfulness of chemical treatments. In the treatments using the CB as a triploidy inducer, high larvae mortality rate was recorded after 48 h of fertilization. This result corroborates those reported by other authors (STANLEY *et al.*, 1981; ALLEN *et al.*, 1989; GÉRARD *et al.*, 1994). STANLEY *et al.* (1981), who reported high mortality in *C. virginica* after 48 h of fertilization caused by the use of CB [0.5 mg L⁻¹] with the intention of inducing the triploidy. GÉRARD *et al.*

(1994) observed 64% of mortality using CB and 36% using 6-DMAP, 48 h after fertilization. According to ALLEN *et al.* (1989), mortalities between 70 and 80% indicate that the treatment was strong enough to cause the induction effect, but not harmful enough as to cause total mortality of the larvae. Thus, high mortalities are expected in the polyploidy induction process in bivalves. The mortality measured after 48 h demonstrated the harmful effects of triploidy induction with chemicals, mostly with CB, as observed in the present study where the treatments with 6-DMAP showed lower mortality when compared to CB.

Furthermore, the results suggest the use of an alternative method to CB, due to its high cost, high larval mortality in the shock induction, and especially to its high toxicity for both humans and the environment.

This study presented, as its main result, the production of the first triploid oyster larvae and seeds (*C. gigas*) in Brazil. It also indicates that the combined treatment of temperature and 6-DMAP at a concentration of 450 µmol L⁻¹ has similar potential to induce triploidy when compared to CB. These results collaborate with the improvement and choice of the technique of triploidy induction in order to be used in future studies. However, for the establishment of a commercial production of triploid seeds, more studies are required for evaluating the production cost and the demand and acceptance of triploid seeds by the market. It is also necessary to evaluate the seed yield of these organisms exposed to the growing conditions at sea. Studies that verify the best moments after the fertilization in order to apply the induction shocks should be performed to improve the survival rates and to increase the efficiency of triploidy induction.

The treatments with 6-DMAP [450 µmol L⁻¹] and temperature were effective for the triploidy induction and they could be successfully used to replace the method CB.

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