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Induction to tetraploidy in Pacific oysters (*Crassostrea gigas*)

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ABSTRACT. As an alternative to the use of cytochalasin B (CB), 6-dimethylamino-purine (6-DMAP) and thermal shock (heat shock by increasing the temperature from 25 to 36ºC) could be used to induce tetraploidy in Pacific oyster (*Crassostrea gigas*) diploids. Induction was performed by applying shocks after elimination of the first polar corpuscle at the end of meiosis I. Ploidy rates were verified using flow cytometry. Tetraploid larvae were obtained using all inductor (6-DMAP, thermal shock and CB) treatments. No difference in the efficiency of tetraploidy induction was noted among 6-DMAP, thermal shock and CB. The number of D-larvae and their yield, determined by calculating the percentage of well-formed D-larvae in relation to the total number of larvae, was similar ($p > 0.05$) among the evaluated induction methods. We suggest that 6-DMAP and thermal shock should be used in tetraploidy induction protocols, thereby avoiding the use of CB, which is a harmful agent for both humans and the environment.

Keywords: Polyploidy; pacific oysters; reproduction; biotechnology; genetic enhancement.

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Introduction

Polyploid individuals can be defined as organisms that have one or more sets of chromosomes in relation to those that occur naturally in the same species (Piferrer et al., 2009). In aquaculture, tetraploid organisms are used as broodstock, and crossed with diploid organisms to obtain triploids, which, in turn, are used for cultivation and commercialization (Guo, DeBrosse, & Allen Jr., 1996).

The interest in triploid organisms is directly related to their low reproductive capacity (Nell, 2002; Muthiah, Thomas, & Mallia, 2006). Among the advantages of triploidy are better growth rates (Normand, Ernande, Haure, McCombie, & Boudry, 2009), increased survival (Nell & Perkins, 2005) and genetic containment in the event of escape into the environment (Guo et al., 2002; Piferrer et al., 2009). However, in bivalve mollusks, triploidy does not necessarily produce complete sterility, but rather a reduction in gametogenesis processes (Piferrer et al., 2009). Melo et al. (2020) observed that diploid and triploid *C. gigas* oysters cultivated in southern Brazil showed a similar reproductive pattern when induced. In addition, triploidy can influence product quality. The flavor of oysters is related to the concentration of glycogen in oyster meat, and, in general, thin oysters (post-spawning), or very mature oysters, do not have a distinctive flavor. Nell (2002) suggests that oysters with medium-to-high glycogen have better flavor. In addition, Allen and Downing (1990, 1991) and Nell, O'Riordan, and Ogburn (2006) suggested that triploidy favors product quality throughout the year.

Thus, the production of tetraploid organisms is of great interest to laboratories for the reproduction of marine bivalve molluscs, as they are crossed with diploid specimens, generating natural triploid organisms (Peachey & Allen Jr., 2016). In this case, reproduction occurs naturally (without induction), making it much easier to obtain triploid oysters (Guo et al., 1996).

The first production of tetraploid *C. gigas* oysters occurred in 1993, with the crossing of triploid females (with reproductive capacity) with diploid males, followed by the blockade of the first polar corpuscle, using cytochalasin-B (CB) ($C_{26}H_{33}O_5$) (Guo & Allen Jr., 1994b). One of the negative aspects of this technique is the mandatory formation of a broodstock of triploid oysters to meet the need for viable oocytes. Another negative aspect would be induction shock with CB, which is recognized as a harmful agent, both to human beings and the environment (Guo & Allen Jr., 1994a; Eudeline, Allen, & Guo, 2000; Peachey & Allen Jr., 2016). Tetraploidy can also be obtained by inducing oocytes from diploid animals or by inhibiting mitosis or meiosis

I and/or II (Beaumont & Fairbrother, 1991; Benabdelmouna & Ledu, 2015). Tetraploidy rates obtained through this method are usually low, but the need for triploid breeders is obviated.

Another alternative method to produce tetraploid organisms is the crossing between diploid females and tetraploid males (McCombie et al., 2005), applying CB induction shock during the elimination of the second polar corpuscle from the oocyte. However, this method is not viable in Brazil owing to the absence of tetraploid oysters, even though it is more efficient with *C. gigas*.

Guo et al. (1996) confirmed the reproductive viability of *C. gigas* tetraploids in the production of triploids, demonstrating that both sexes are capable of producing 100% of triploid organisms when crossed with diploid individuals. The crossing between tetraploid individuals will produce new tetraploid breeders, thereby guaranteeing constant renewal of the broodstock (Guo & Allen Jr., 1994b).

In view of the high toxicity of cytochalasin B, some researchers (Desrosiers et al., 1993; Melo, Gomes, Silva, Sühnel, & Melo, 2015) have evaluated the use of 6-dimethylamino-purine (6-DMAP) ($C_7H_9N_5$) as an alternative to CB in the induction of polyploidy in bivalve molluscs and identified that both chemicals produce similar numbers of triploid organisms with comparable survival rates.

Therefore, in this study we evaluated the induction of tetraploidy in diploid Pacific oysters, *C. gigas*, with the use of 6-DMAP and thermal shock as an alternative to the use of CB.

Material and methods

Animal production

In this study, we used diploid females (3 years old) and diploid males (1 year old) from broodstock at the Laboratório de Moluscos Marinhos da Universidade Federal de Santa Catarina (LMM-UFSC) (27º35'04''S; 48º26 '29''W) and kept in the LMM experimental cultivation area at Sambaqui Beach (27º29'23''S; 48º32'15”W).

Gamete production and fertilization

To obtain the gametes, we opened the oysters to identify the sex and selected the best individuals (females: greater amount of oocytes; males: sperm with greater motility).

The spawning occurred in two previously selected females. The oocytes of two female oysters were removed simultaneously using blades and stored in clean and dry containers. For oocyte fertilization, a pool of three male sperm was obtained by scraping their reproductive tissue in seawater (filtered at 1µm and sterilized with UV; 25ºC and salinity 35). Fertilization was carried out in a single dose, in which the sperm solution was added to the female gamete solution at a ratio of seven sperm per oocyte (7M: 1F).

After this stage, two slides containing 1 mL of the fertilized oocyte solution were prepared for the evaluation of embryonic development under a light microscope.

Induction to tetraploidy

When about 50% of the fertilized oocytes had the first polar corpuscle at the end of meiosis I, tetraploidy induction treatments were started.

Three methods were used to induce tetraploidy, two chemical (6-DMAP or 6-dimethylamino-purine, 450 µmol L^{-1}; and CB or cytochalasin B (1.0 mg L^{-1}) and one physical (thermal shock with water temperature increased from 25 to 36ºC). In addition to these treatments, a group of fertilized oocytes (2N control) was maintained without any exposure to inductors to be used as a basis for comparison between ploidy levels obtained in flow cytometry samples. The experimental design was completely randomized with three replications. For each repetition, 1 million fertilized oocytes were used. Induction shocks occurred 14 minutes after fertilization, according to the methodology described by Melo et al. (2015) for triploidy induction.

For CB treatment, a solution of 1.0 mg of CB in 1 mL of dimethyl sulfoxide (DMSO - C_2H_6SO) was prepared and stored at a temperature of 20°C (Allen, Downing, & Chew, 1989). For induction shocks, the fertilized oocytes were placed in cylinders with an 18 µm mesh and immersed in 1 L of the CB solution for 15 min. After this period, the material retained on the mesh (embryos, nonfertilized oocytes and sperm, among others) was suspended and immersed in a solution of 0.05% DMSO in seawater for another 15 min. In the 6-DMAP treatment, a solution of 450 µmol of 6-DMAP was prepared and stored at a temperature of -20°C, following the methodology described by Desrosiers et al. (1993) for triploidy induction. The fertilized oocytes were transferred to cylinders with an 18 µm mesh and immersed in 1 L of the 6-DMAP solution for 15 min. In the

heat shock treatment, the induction was carried out by transferring the fertilized oocytes from a container (1 L) at 25°C to another container (1 L) at 36°C where they remained for 15 min. according to Quillet and Panelay (1986). After this period, the induced oocytes were transferred to a container at 25°C.

After inducing tetraploidy, the embryos in each treatment were moved to larviculture tanks (2,500 L), containing filtered seawater (1 µm) and sterilized with UV at a temperature of 25ºC and salinity of 35, where they remained for 48h.

Ploidy analysis

The percentage of triploid larvae was calculated 48h after fertilization, using flow cytometry (PARTEC-PA). Approximately 2,000 larvae from each repetition were sampled. The larvae were placed on Petri plates to which 0.5 mL of nuclei extraction solution (HCl + NaOH) was added for 5 min. After this procedure, 1.5 mL of DAPI-specific dye solution (4,6-diamidino-2-phenylindole) was added to the sample, which was taken to the cytometer (PARTEC-PA) for analysis. A group of diploid embryos that did not undergo any induction process was kept separate from treatments for ploidy comparisons.

Statistical analysis

The percentage of triploid larvae, the number of well-formed D-larvae [larva stage: larvae with the characteristic D shape and the presence of active velum and a healthy aspect (Galtsoff, 1964)], and the yield (ratio between well-formed D-larvae and the total number of larvae after 48h of fertilization) were analyzed using Kruskal-Wallis test and the computational package SAS® Institute Inc. (2003).

Results

The percentage of tetraploid larvae obtained varied from 17.18 to 55.73%, from 22.37 to 40.28%, and from 0 to 22.06%, respectively, for induction with 6-DMAP, heat shock and CB. The differences among average percentage of tetraploid larvae obtained with 6-DMAP (32.78 ± 20.30%), heat shock (29.17 ± 9.70%) and CB (10.28 ± 11.11%) were not significant ($p > 0.05$).

In flow cytometry, the fluorescence absorption peaks for 6-DMAP, heat shock and CB treatments were 101.33, 104.00 and 94.67, respectively (Figure 1). The diploid oysters in the control treatment showed fluorescence absorption of 49.12. The animals submitted to 6-DMAP, heat shock and CB treatments showed, respectively, 2.06, 2.11 and 1.93 times greater absorption of fluorescence than that of diploid oysters.

The number of well-formed D-larvae did not show statistical differences among the 6-DMAP (27.01 ± 11.07 D-larvae mL^{-1}), heat shock (32.17 ± 15.87 D-larvae mL^{-1}) and CB (13.14 ± 11.46 D-larvae mL^{-1}) treatments. Similar results were obtained for larvae yield obtained in the 6-DMAP (88.27 ± 4.29%), heat shock (67.70 ± 13.47%) and CB (61.34 ± 33.08%) treatments, among which no significant difference was noted after 48h of fertilization ($p > 0.05$).

Discussion

The production of tetraploid oysters was promising. The fluorescence rates of the samples from the diploid control group and from the tetraploidy induction treatments were as expected, according to Gosling and Nolan (1989), who stated that the nucleus cell of a tetraploid organism would have to emit twice the fluorescence of the nucleus cell of a diploid organism. Since diploid organisms emitted average fluorescence absorption of 49.12, it was expected that tetraploid nucleus cells would be found at around 100 in fluorescence absorption.

The percentages of tetraploid larvae obtained with the inductors 6-DMAP, heat shock and CB were similar to those reported in the literature (Yang & Guo, 2006a, b; McCombie, Cornette, & Beaumont, 2009; Peachey & Allen Jr., 2016).

Peachey and Allen Jr. (2016) evaluated the induction of tetraploidy in *Crassostrea virginica* and obtained superior results with the use of 6-DMAP compared to CB, with ploidy confirmed 48 h after fertilization. As an alternative to CB, this study pioneered the use of 6-DMAP to induce tetraploidy in *C. virginica*.

McCombie et al. (2009) induced *Mytilus edulis* diploids to tetraploidy, applying CB 5 min. after fertilization, obtaining similar results, with the percentage of tetraploid larvae varying from 18 to 60%. These authors found that the best results were observed using oocytes from individual females crossed with a pool of sperm from two males.

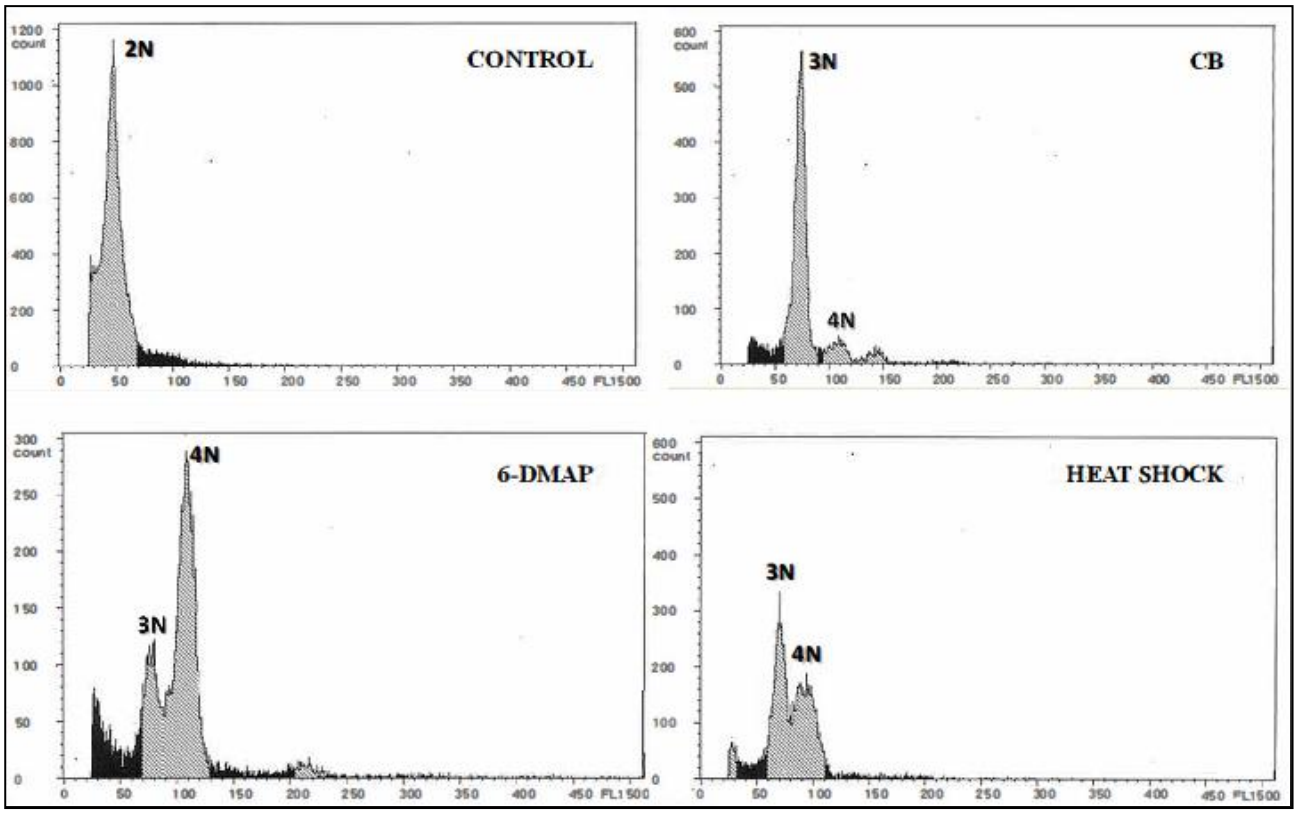

Figure 1. Flow cytometry analysis for larvae at 48h of age, resulting from blocking the first polar corpuscle, 14 min. after fertilization. The X axis corresponds to the fluorescence of the nucleus cell (DNA content), and the Y axis represents the number of cells counted. 2N = diploid; 3N = triploid; and 4N = tetraploid.

In the treatment of tetraploidy induction by temperature, our results were even more promising, considering that no chemical products or special care was needed during the process of induction.

Our results are similar to those of Yang and Guo (2006a, b), who evaluated the use of temperature. In their first study (Yang & Guo, 2006a), the authors verified the success of inducing tetraploidy in the clam *Mulinia lateralis* with thermal shocks 10 min after fertilization. The treatment started at 23-24ºC, and heat shock was raised to 32, 35 and 38ºC. The best result was observed at 35ºC, with 82.8% of tetraploid larvae. In their second study (Yang & Guo, 2006b), the authors applied thermal shock to diploid embryos of the clam *Mercenaria mercenaria*, with temperatures ranging from 23ºC to 32ºC, 35ºC and 38ºC, obtaining 77.3% and 67.2% of larvae tetraploids with temperatures of 35 and 38ºC, respectively.

The total number of well-formed D-larvae per mL (27.01 ± 11.07; 32.17 ± 15.87; and 13.14 ± 11.46, and the yield of 88.28 ± 4.30; 67.70 ± 13.47; and 61.34 ± 33.08, for 6-DMAP, heat shock and CB, respectively) after 48h of fertilization was high, considering that induction processes generally cause high mortality owing to the harmful effects caused by the induction shocks (Guo & Allen Jr., 1994b).

To produce tetraploid oysters, it is unnecessary to use indirect chemical or thermal induction processes to obtain triploid oysters, which are obtained from the crossing between tetraploid and diploid oysters (Guo & Allen Jr., 1996; Eudeline et al., 2000), enabling the production of triploid organisms on a commercial scale. It is worth mentioning that the use of CB is prohibited in several countries (Food and Drug Administration, USA and European Union) based on potential harm to both operator and the environment (Guo & Allen Jr., 1994a; Eudeline et al., 2000; Nell, 2002; Piferrer et al., 2009; Peachey & Allen Jr., 2016). Therefore, its use should be avoided in later induction processes.

We obtained the first tetraploid larvae of *Crassostrea gigas* oysters in Brazil, a potential boon to the future of oyster farming with the possibility of changing the form of production and commercialization of oyster seeds. However, future studies need to be carried out to determine survival in larviculture and nursing and to evaluate the production and cultivation of tetraploid oysters until sexual maturity.

In addition, we have added to knowledge of the reproductive aspects of tetraploid animals in Brazil, as well as the potential for using these animals in the production of triploid organisms.

Conclusion

No statistical difference was noted among the 6-DMAP, thermal shock and CB inductors; however, we suggest the use of 6-DMAP or heat shock as inductors for tetraploidy in Pacific oysters because they are safe for both humans and the environment.

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