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Abstract

Cytochalasin B (CB) has been used to induce tetraploidy in oysters since the practice began in 1993. However, CB is toxic and presents health risks to hatchery workers who administer the treatment. 6-dimethylaminopurine (6-DMAP) is also an effective cytokinetic inhibitor, and does not carry the health risks of CB. We examined the relative effectiveness of 6-DMAP vs CB for producing tetraploids in the Eastern oyster (*Crassostrea virginica)*. Survival and yield of tetraploids varied widely among the 15 experiments. Larvae resulting from 6-DMAP treatment had higher survival in 11 of the 14 trials on day two and day six/ seven. For yield of tetraploids, 10 of 13 6-DMAP treatments had higher proportions of tetraploids on day two and at the second sampling – day six, seven, or nine – 7 of 10 had higher proportions of tetraploids. Tetraploid spat were obtained from the majority of surviving cultures. Based on these results, 6-DMAP can effectively replace CB for inducing polyploidy in *C. virginica,* and probably other *Crassostrea spp.,* due to the success of the treatment, the ease of application, and the reduction in health risk to hatchery workers. This study set the precedent for the use of 6-DMAP on *C. virginica* and

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established a new procedure for inducing tetraploids using triploid eggs. It might be possible to refine the treatment to further optimize yield of tetraploids.

Keywords: Tetraploid; Cytochalasin B; 6-dimethylaminopurine; *Crassostrea virginica*; Oyster; Aquaculture

1. Introduction

Thirty years after their introduction to commercial hatcheries, polyploid oysters remain important for oyster aquaculture industry around the world. For example, about 15% of seed production in Australia (S. Parkinson, Shellfish Culture Ltd., Pipe Clay, Tasmania), about 50% in the Pacific northwest US (pers. comm., J. Davis, Taylor Shellfish, Shelton, WA), and nearly all commercial seed production in both the Chesapeake Bay area (Hudson and Murray, 2015) and France (Dégremont et al., 2014) are triploid, the latter comprising nearly 3 billion seed. Triploid oysters are popular because of reduced gonadal development (Allen and Downing, 1986, 1990) and the opportunity to harvest them year-round, even during the spawning season (Allen et al., 1989; reviews by Beaumont and Fairbrother, 1991; Nell, 2002; Piferrer et al., 2009). The reduced gonadal development of triploid oysters also allows them to apply more energy to growth than fertile diploid oysters. In favorable conditions, triploid oysters grow larger than diploids (Allen and Downing, 1986; Dégremont et al., 2012). Triploid oysters may also be used as a method of population control due to their sterility (Guo et al., 1996; Piferrer et al., 2009; Jouaux et al., 2010). Initially, triploids were chemically induced (Stanley et al., 1984) until Guo and Allen (1994b) developed a method to produce viable tetraploids that led to the production of so-called natural triploids (Guo et al., 1996) – a cross between diploids and tetraploids that produces 100% triploid progeny. Tetraploids have found wide ranging use in oyster aquaculture (Piferrer et al., 2009; Dégremont et al., 2012), with possibly more potential

for future breeding innovations. For example, tetraploid oysters could be used to "bridge taxa" (to combine otherwise incompatible species such as *C. gigas* and *C. virginica*) using a technique derived from plant genetics (Guo and Allen, 1994a) or could be used to make custom triploid hybrids among *Crassostrea* species.

The Guo and Allen method of creating tetraploid oysters involves inhibiting the extrusion of the first polar body (PB1) in triploid eggs that have been fertilized with sperm from a diploid male (Guo and Allen, 1994b). Although the majority of triploid oysters are effectively sterile, there are some that produce enough eggs (Allen and Downing, 1990; Jouaux et al., 2010) to use for tetraploid induction (Guo and Allen, 1994b). Cytochalasin B (CB) is a fungal antibiotic that inhibits the polymerization of actin filaments (Theodoropoulos et al., 1994) and, in shellfish zygotes, inhibits the extrusion of polar bodies, thereby creating polyploid embryos. Chemical treatment yields variable results for a number of reasons (Allen et al., 1989). CB is labeled a toxin and a potential workplace hazard; it can be fatal through ingestion, inhalation, or through contact with skin (Sigma-Aldrich, 2015b) and is suspected of damaging fertility (Desrosiers et al., 1993). In 2000, Eudeline et al. optimized a tetraploid induction technique using CB. In order to compensate for differences in egg quality, Eudeline et al. (2000) developed the biological clock method that relies on observation of the extrusion of polar bodies over time in order to effectively administer treatments.

Desrosiers et al. (1993) examined an alternative to CB for inducing triploidy using the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) (Szöllösi et al., 1993) instead of CB. Comparisons between *C. gigas* triploids induced by CB and by 6-DMAP revealed that the treatments yield similar numbers of triploids and comparable survival rates (Desrosiers et al., 1993; Gérard et al., 1999). Handling 6-DMAP is significantly safer for hatchery workers than

handling CB. Where CB comes with numerous hazard statements warning of its toxicity, 6-DMAP is hazard free (Sigma-Aldrich, 2015a). Curiously, no papers thus far have investigated the efficiency of 6-DMAP in tetraploid induction. In this paper we report the efficiency of 6-DMAP and CB in tetraploid induction of *Crassostrea virginica*.

2. Methods

2.1 Source oysters

Diploid and triploid oysters were produced at the Aquaculture Genetics and Breeding Technology Center (ABC) hatchery in Gloucester Point, VA in June and July 2010. Broodstock were from two groups of selectively bred, disease resistant (DR) lines – 2006-year class (four lines) and 2008-year class of Superlines (four lines) – from ABC's breeding program. For the 2010 spawning, eggs were obtained from at least 10 dams per line by strip spawning and pooled in plastic beakers. The pools of eggs were then divided into two groups containing 3×10^6 eggs each, one for diploids and one for triploids. To produce diploids, one group of eggs was fertilized with sperm pooled from at least 10 sires of the same line. To produce triploids, the remaining groups of eggs were fertilized with sperm pooled from 10-11 sires from a single tetraploid family (Table 1). The diploid and triploid oysters produced above were grown in the Rappahannock River, the subject of a three year study (Callam et al., submitted). In the summer of 2013, we used triploid females to attempt tetraploid induction (crossed with the diploid males from the same line), with the overall purpose of producing new tetraploid lines and with the hypothesis that we could replace CB with 6-DMAP as an agent of induction. Average size of the various brood stock used for tetraploid inductions is recorded in Table 1.

2.2 Tetraploid induction

Putative triploid *C. virginica* were opened and checked for eggs. If eggs were found, a small piece of gill tissue from each female was prepared for flow cytometry (FCM) to verify ploidy (Allen, 1983); female triploid oysters were set aside for strip-spawning. Sex was determined in diploid oysters corresponding to the same line as the triploids (e.g., DBSL, hANA, Lola, etc.). The diploid males were used to fertilize the triploid eggs. Diploid males and females were also crossed to establish control cultures. Each triploid female was strip-spawned separately and the eggs were screened on a 63 micron Nytex screen to remove somatic tissue, catching eggs on a 20 micron screen. Triploid eggs were suspended in filtered sea water and a visual determination of egg number was made. Some triploid females possessed insufficient numbers of eggs alone to warrant a tetraploid treatment, so eggs from multiple females were pooled, with between two and twelve females in some test crosses. Eggs were left in 28°C water for at least 45 minutes before fertilization, but no longer than 120 minutes, to encourage final maturation. Water temperature was kept between 27° and 28°C from time of fertilization through the completion of treatments to maintain the best possible synchrony among the eggs (Eudeline et al., 2000).

Sperm from individual diploid males was assessed under a microscope and rated according to density and activity. Sperm from virile males was then pooled. Eggs were fertilized and immediately checked for an adequate quantity of sperm (~10-15 sperm around each egg) to achieve uniform, rapid fertilization among eggs. Just after fertilization, the volume of eggs was split evenly into two containers, one for treatment with CB, the other for treatment with 6-DMAP. A subsample of eggs was taken from the CB volume to use as a control population to judge the timing of CB treatment (Eudeline et al., 2000).

2.3 Treatments with 6-DMAP and CB

The optimum 6-DMAP treatment for *C. virginica* was determined ahead of time by testing different concentrations (250, 350, 466 µmol/L) as well as different treatment durations (5, 10, 15 minutes) and several treatment start times (at fertilization and at the appearance of polar bodies). Cultures treated with 250 µmol/L 6-DMAP had higher survival but fewer triploids than cultures treated with 466 µmol/L 6-DMAP. Therefore, a concentration of 350 µmol/L 6-DMAP was used to ensure adequate survival while still producing tetraploid embryos. For treatment duration, five minutes resulted in little to no triploids, while 10 or 15 minute treatments were comparable for both survival and percent triploidy. For start time, treatments commencing at fertilization and at the appearance of the first polar bodies both had similar survival but the treatments starting at fertilization resulted in more triploids. These experiments with 6-DMAP were performed on diploid eggs so as not to waste valuable triploid stock.

For tetraploid induction, two minutes after fertilization, a pre-made solution of 6-DMAP was added to achieve a final concentration of 350 µmol/L in the egg suspension. The suspension was stirred for 10 seconds to ensure dispersion of 6-DMAP. After 10 minutes, eggs were screened and rinsed on a 20 micron screen then resuspended in filtered sea water.

Treatment with CB depended on the timing of meiotic events as judged by the control eggs. Control eggs were kept at the same temperature (~27-28°C) as the eggs for treatment and monitored periodically for the appearance of polar bodies. Once polar bodies began to appear, the eggs were treated with CB at a final concentration 0.25 mg/L in the egg suspension. Observation of control eggs continued until polar bodies were evident in 60% of them (Eudeline et al., 2000). Treatment was ended by screening and rinsing the eggs on a 20 micron screen then resuspending zygotes in filtered sea water. The CB/water solution was disposed of in an appropriate waste container.

Each experiment, then, consisted of a pair of treatments originating from the same triploid females and diploid males. Control diploid spawns were used primarily to monitor larval culture conditions in the hatchery. After both treatments, the eggs were counted and incubated in larval culture tanks. A separate diploid control culture was raised alongside each set of treatments.

2.4 Larval culture

Larvae were raised at a density not exceeding 20 larvae/mL. Larvae were fed daily with *Pavlova sp.*, and as the larvae grew, *Chaetoceros neogracile* (Day 4) and *Tetraselmis* sp. (Day 8) were included in the diet. Larval tanks were drained and cleaned every two days. The larvae were collected on two screen sizes according to their age and size (e.g., a 48 µm mesh screen nested on top of a 35 µm screen on day two). Larvae caught on each screen were observed, and the larvae on the smaller screen were either kept or discarded, depending on their overall health. Healthy larvae were counted, their length determined, and their general condition noted.

For setting, eyed larvae were screened on a 250 µm mesh screen for the first two harvests, and on a 236 µm mesh screen for subsequent harvests. Harvest screens were chosen to match the largest eyed larvae in the culture, presumed to be tetraploids. Average size of this population of larvae was 364.0 ± 8.4µm (SD). Therefore, we probably enriched the proportion of tetraploids in the culture by favoring larger larvae. All larvae were set on micro-cultch in individual downwellers for each cross.

2.5 Flow cytometry

On day two and on either day six, seven, or nine, 3,000 larvae from each culture were subsampled from the larval population for analysis by flow cytometry (FCM). First, the

158 remaining larval population was estimated by counting an appropriate dilution. A proportional
159 volume from the larval container was removed and poured through a 20 µm screen to obtain
160 approximately 3,000 larvae. Total volume of the sample was reduced to <1.5mL and placed in a
161 microcentrifuge tube, which was then centrifuged to pellet the larvae. Supernatant seawater was
162 removed and about 1 mL DAPI (4', 6'-diamino-2-phenylindole) stain (Allen and Bushek, 1992)
163 was added. Larvae were resuspended and disaggregated by repeated aspiration with a 1-ml
164 syringe fitted with a 26G needle. Cell suspensions were passed through a 25-mm screen
165 immediately before FCM analysis, accomplished on a Partec CyFlow® Space cytometer.
166 Analysis of larval samples yielded data from cells obtained from a population of larvae.

167 2.6 Data analysis

168 The proportion of cells in each ploidy class was calculated relative to the proportion of
169 observations in all ploidy classes, after curve fitting with Modfit Verity Software House,
170 Topsham, ME, USA. (Allen and Bushek, 1992). Modfit analysis allowed more accurate
171 estimation of raw data for mean DNA content, coefficient of variation, and proportion of
172 observations among ploidy classes.

173 For determination of differences in survival and percent ploidy between CB and 6-DMAP
174 treatments, the Wilcoxon's signed-ranks test for two groups (Sokal and Rohlf, 1995) was used
175 after arcsine transformation of percentages. Correlation analysis between survival and
176 tetraploidy was also performed after arcsine transformation.

177 **3. Results**

178 We opened a considerable number of triploids to find females for tetraploid inductions
179 (Table 2). The percentage of triploid females encountered ranged from 21% (in the DBY line) to

only 5% (in the XB line). Across all lines, 99 of 693 (14%) opened oysters were female and the rest could best be categorized as non-female because it was difficult to determine the difference between underdeveloped triploid males and sterile oysters. The average number of eggs per triploid female (n=90) was 371,000, ranging from 11,500 eggs in XB females (they were pooled and the count averaged over females) to 4,430,000 in a DBSL female, although some females were rejected straight away because they lacked sufficient numbers of eggs. Only eight females had egg numbers exceeding 1,000,000: two individuals from the DBY and XBSL lines, one each from hANA, LGT, OBOY, and DBSL triploid lines (Table 2). Only crosses in which larvae survived in both CB and 6-DMAP treatments are shown in Table 3, which is why there are more crosses shown in Table 2 (all crosses made) than in Table 3.

The treatments were highly variable from egg batch to egg batch for both percent survival and percent tetraploid (Table 3, Figure 1). For CB treatments, percent survival on day two ranged from 3% to 33% (Table 3), with an average of 14%. Survival for 6-DMAP treatments on day two ranged from 7% to 35%, averaging 24%. Control survival at day two averaged 62%. By day six or seven, survival varied between 0% and 11% for CB treatments, averaging 4% (Table 3). Survival for 6-DMAP treatments on day six or seven ranged from 4% to 36%, averaging 10%, compared to an average control survival at day six/seven of 47%. Survival of 6-DMAP treatments was significantly higher than CB treatments on day 2 ($p<0.02$) and day six/seven ($p<0.02$).

Percent tetraploid in CB cultures on day two varied from 9% to 65%, averaging 33%, and from 0% to 85% at the second sampling (day six, seven, or nine), with an average of 34% tetraploid (Table 3). On day two, percent tetraploid in 6-DMAP cultures ranged between 11% and 89%, averaging 43%, and at the second sampling percent tetraploid ranged between 10% and

83%, with a 45% average. The proportion of tetraploids in 6-DMAP cultures was significantly higher on day two than in CB treatments ($p<0.05$) and also at day six/seven/nine, but this was not a statistically significant difference ($p>0.05$).

The relationship between percent survival and percent tetraploid was examined at the second sampling. Percentages were arcsine transformed. No significant correlation was found between the percent survival and the percent tetraploid in the cultures when the percentages from CB and 6-DMAP cultures were combined ($R=0.01$) (Figure 2). However, separately, 6-DMAP cultures showed a negative correlation between percent survival and percent tetraploid ($R= -0.53$) (Figure 3). CB cultures, on the other hand, did not have significant correlation between percent survival and percent tetraploid ($R= 0.04$) (Figure 4).

Between the second sampling and when the larvae were ready to set, between 99 and 100% of larvae died. The actual number of setters obtained for CB treatments was between 1 and 10,000, with a mean of 1192 and, for 6-DMAP cultures, between 16 and 1243, averaging 395 (Table 4). The tetraploid percentage in the setters from 6-DMAP cultures ranged between 9% and 90%, with a mean of 56% (Table 4). Setters from CB cultures contained 0% to 90% tetraploid individuals, averaging 31%. Overall, from all the pairs of treatments over all the cultures we obtained an estimated 6577 tetraploid spat from CB treatments and 2577 spat from 6-DMAP treatments. However, the preponderance of spat from CB treatments were from one successful culture out of five that yielded tetraploids, whereas eight cultures from 6-DMAP treatments yielded tetraploids (Table 4). For CB, 6577 spat were obtained from 13,397,000 eggs treated (0.049%) to make tetraploids; for 6-DMAP, 2577 spat obtained from 10,671,000 eggs treated (0.024%).

Finally, we developed an index to gauge efficiency of each treatment, as follows:

$$\frac{\%\ \text{survival day 6,7} \times \%\ \text{tetraploid day 6,7,9}}{10{,}000}$$

Results show that 8 of 15 CB treatments yielded tetraploids while 13 of 14 6-DMAP treatments did so (Figure 5). The highest yielding culture was from 6-DMAP treatment and 7 of the highest ten yields were also 6-DMAP treatments.

4. Discussion

There are now three recognized methods of producing tetraploid molluscan shellfish, although commercial application of tetraploid technology is still limited to *C. gigas* and *C. virginica*. The first viable tetraploids were reported in 1994 by Guo and Allen, based on pioneering work on chromosome set manipulation by Guo et al. (1992a, b). Guo et al.'s work set the course for all subsequent methods. Guo et al. found that inhibiting PB1 had numerous effects on meiotic chromosome segregations, one of which was to produce triploid embryos and another was to produce tetraploid embryos. The tetraploid embryos made by inhibiting PB1 from a diploid cross were inviable for Guo et al., but, 15 years later, that exact same method of making tetraploids was patented by Benabdelmouna and Ledu (2007) probably because they were able to keep the tetraploids embryos alive through the larval period and produce a breeding population of tetraploids from them. The Benabdelmouna and Ledu protocol is called the "direct" method. As yet, there is no published account of the direct method of tetraploid induction.

Elucidation of the cytogenetic mechanism leading to triploids after PB1 inhibition (Guo et al., 1992a, 1992b) led to the hypothesis that inhibiting PB1 in *triploid* eggs could lead to tetraploid also. It was only after the discovery that triploids were not entirely sterile (Allen and Downing, 1990) – meaning that it was possible to obtain eggs from "sterile" triploids – that experiments to make tetraploids via the Guo and Allen method commenced and viable

tetraploids were obtained (Guo and Allen, 1994b). The third method of producing tetraploids is a variation on the theme for inducing triploids, but requires having tetraploids in the first place, so-called the "indirect" method. McCombie et al. (2005) fertilized eggs from diploid females with sperm from tetraploid males in *C. gigas* and induced the retention of the second polar body (PB2), thus adding a fourth chromosome set to the zygote. Tetraploids produced in this way are easy to obtain as larvae, but difficult to rear to setting (S. Allen, unpubl. data; B. Eudeline, Taylor Shellfish, Washington; X. Guo, unpubl. data; S. Parkinson, Shellfish Culture Ltd, Tasmania).

Careful husbandry of tetraploid larvae that are derived from diploid eggs (versus triploid eggs) is the common feature for both the "direct" and "indirect" methods developed at the French Research Institute for Exploitation of the Sea (IFREMER) and likely the reason that patents now exist on both methods (Benabdelmouna and Ledu, 2007; Benabdelmouna et al., 2007). As yet, these methods have not been successful at other labs and, therefore, the Guo and Allen method remains the only recourse to tetraploid induction for many.

Chromosome set manipulation in oyster species has been a popular subject since the practice began (Stanley et al., 1981), with numerous methods of inducing polyploidy (CB, 6-DMAP, caffeine, heat shock) developed and honed. There are quite a few reports on tetraploid induction in shellfish species (*cf.* Guo et al., 2009). There has been no published work on inducing tetraploidy in *C. virginica* except an abstract (Guo et al., 2002), although three labs (ours, Rutgers University's Haskin Shellfish Research Laboratory, Louisiana State University's hatchery in Grand Isle, LA) are involved in this activity occasionally. In the experiments reported here, we used 6-DMAP for *C. virginica* tetraploid induction for the first time. These experiments have established the norm for what is to be expected from induced tetraploidy in *C.*

virginica, for both CB and 6-DMAP. Overall, the expectation is that, using either chemical, the process is difficult.

The major hurdle for making tetraploid *C. virginica* is obtaining eggs from triploid females. For this work, we used three year old individuals that were up to 100mm long, far past market size of 76mm. Larger sized females increased the odds that we would find some triploid females. Nonetheless, we observed low fecundity with the average egg count (from females that had enough to count) of about 371,000 (n = 90). This is about 15% of the fecundity of triploid *C. gigas* (Guo and Allen, 1994a, average of 19 triploid females = 2.3 million). Compared to diploid *C. virginica* that may release 23-85 million eggs/ female (Sellers and Stanley, 1984), triploids have only 2% to 0.4%, respectively, of the fertility. For *C. gigas* – capable of producing 25-105 million eggs – the same comparison of the relative fecundity of triploids is between 9% and 2%, respectively (Guo and Allen, 1994b). Gong et al. (2004) reported fecundity of triploid *C. gigas* females as high as 13% that of diploids. More recently, Jouaux et al. (2010) supposed that "un-locked" triploids, those that were able to attain near normal looking gametogenesis despite being triploid, should be considered as fertile as diploids.

Another major hurdle in making tetraploids using the Guo and Allen method is low survival, which in this work ranged from 7 tetraploids for every 13,000 eggs (CB) to 3 tetraploids for every 11,000 eggs (6-DMAP), compared to an expectation of 1 spat per 1000 eggs in diploid cultures (conservatively). Yet, survival of tetraploids in non-oyster species is decidedly worse, mostly 0% (Guo et al., 2009). Low survival could be a result of the tetraploid condition, which certainly must impose developmental and physiological hardships on larvae, or low survival could be a result of lack of sophistication in larval rearing of these disadvantaged polyploids. That the latter may be the case is indicated by the success of the "direct"

(Benabdelmouna and Ledu, 2007) and "indirect" (McCombie et al., 2005) methods where others have failed. Unfortunately, neither of these accounts for the "direct" or "indirect" methods reported larval survival.

By developing 6-DMAP as an alternative to CB, we have actually decreased the difficulty in the induction method, and this relates to the mode of action of the two chemicals. The original protocol for CB by Guo and Allen (1994b) was optimized by Eudeline et al. (2000) by the inclusion of biological markers for initiating and timing CB treatments. Briefly, this calls for initiating treatment at the first sign of PB1 extrusion and continuing until 50% PB1 extrusion, which has to be done with a control set of eggs set aside specifically for that purpose. CB affects actin polymerization (Maclean-Fletcher and Pollard, 1980) necessary to form the cleavage furrow of the polar body (Allen et al., 1989). Its effect on the fertilized egg is nearly immediate, hence the need for precise timing of the treatments. One advantage of CB stems from the fact that it does not affect karyokinesis, just cytokinesis. Therefore, migrations of chromosome to the poles can continue even under the influence of CB, acting as a virtual road block to elimination of the polar body after segregations have ceased. Other treatments, like heat shock or caffeine affect spindle fibers, thus arresting chromosome migrations to the poles. Any such divisions caught at the wrong time will fail to yield induced polyploids. As efficient as CB is for polyploidy induction, it is toxic and a workplace hazard to hatchery workers. Also, CB is dissolved in dimethyl sulfoxide (DMSO) as a carrier, a universal solvent that easily penetrates the skin and carries CB along with it. In a stock solution of 1mg CB/ mL of DMSO, the risk is high. Therefore, hatchery workers must take numerous precautions when using this chemical, such as, gloves, plastic sleeves, an apron, goggles, and a mask. After CB administration to the

oyster eggs, CB should be placed in a biological waste container and transferred to an appropriate biohazard waste site.

The mechanism of action for 6-DMAP, and the implications for treatments to induce polyploidy in oysters, is different. 6-DMAP is an inhibitor of protein phosphorylation and protein kinase activity (Dufresne et al., 1991; Simili et al., 1997; Szöllösi et al., 1993). 6-DMAP was first demonstrated to block polar body extrusion in starfish oocytes (*Asterias rubens* and *Marthasterias glacialis*) (Desrosiers et al., 1993). When applied soon after fertilization, 6-DMAP reversibly inhibits nuclear envelope breakdown and the mitotic apparatus (Dufresne et al., 1991). In various experiments, 6-DMAP has been found to act on specific protein kinases, promote chromatin decondensation, and act on microtubules and metaphase spindles in mouse oocytes and sea urchin embryos (Desrosiers et al., 1993; Gérard et al., 1999). In mammals, 6-DMAP has been used to activate pronuclear formation in fertilized eggs (Leal and Liu, 1998). 6-DMAP disturbs M-phase specific phosphorylation and histone H1 kinase in most species, although it has exhibited cell apoptosis in specific cell types (HL-600 cells and porcine embryos) (Meijer and Raymond, 2003; Ock et al., 2003). Also, 6-DMAP-mediated destruction of microtubules prohibits the extrusion of the polar body (Leal and Liu, 1998). The destruction of the microtubules is reversible once 6-DMAP is rinsed from the system thereby allowing the cells to continue development. Studies by Desrosiers et al. (1993) and Gérard et al. (1999) found that the survival and triploidy levels in 6-DMAP treated cultures were comparable with those found in CB cultures. Desrosiers et al. (1993) also determined that increasing the concentration of 6-DMAP, within a reasonable limit, increased the proportion of triploid larvae in a treatment. They touted 6-DMAP treatment as “the most simple ever reported for producing triploid bivalves.”

The effect of 6-DMAP is clearly a delayed response compared to CB, judging from our experiments. While CB treatments were carefully choreographed to PB extrusions, 6-DMAP was simply added two minutes after fertilization and removed ten minutes later – both starting and ending before the CB treatment, sometimes starting and ending before CB treatment even began. Yet results were comparable between the two methods. Therefore, for both ease of administration and for safety concerns, we suggest that 6-DMAP is clearly the choice for tetraploid inductions in *C. virginica*, and probably other *Crassostrea* species as well, although some ground truthing will likely be needed in the other species. At the same time, throughout the paired comparisons between CB and 6-DMAP, variation was high, and this variance did not seem to be correlated between CB and 6-DMAP treatments (Figure 1). Thus, whatever caused the variance in tetraploid production among the CB treatments did not seem to be the same factors that caused variation among the 6-DMAP treatments.

In summary, compared to the CB treatment protocol, the 6-DMAP treatment procedure used for inducing tetraploidy in *C. virginica* was much simpler. 6-DMAP is safer than CB; it can be dissolved in deionized water, and does not carry any of the toxic risks of CB. Also, 6-DMAP is substantially less expensive than CB. From Sigma-Aldrich®, 6-DMAP costs $0.34/mg (about $19.38/ treatment) compared to CB, $60.20/mg ($30.10/ treatment) (Sigma-Aldrich, 2015a, 2015b). Efficacy of 6-DMAP treatment may improve with further experimentation, for example, by basing initiation or completion of 6-DMAP treatments on timing of biological events, as in the work by Eudeline et al. (2000).

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Figure 1

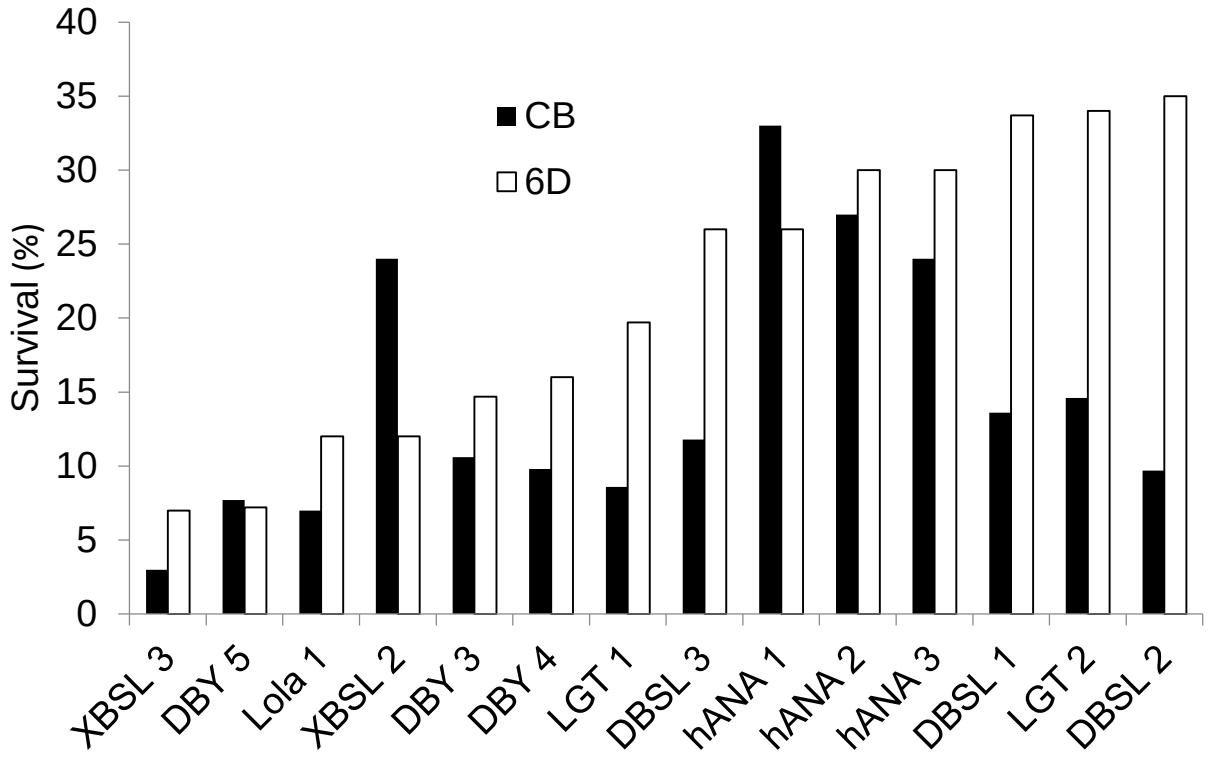

Figure 1: Percent survival of paired comparisons between CB and 6-DMAP (6D) treatments on larval cultures of *C. virginica* at day two after fertilization, arranged in ascending order of percent survival in 6-DMAP treatment. The x-axis denotes various lines of oysters used in the experiment.

Figure 2

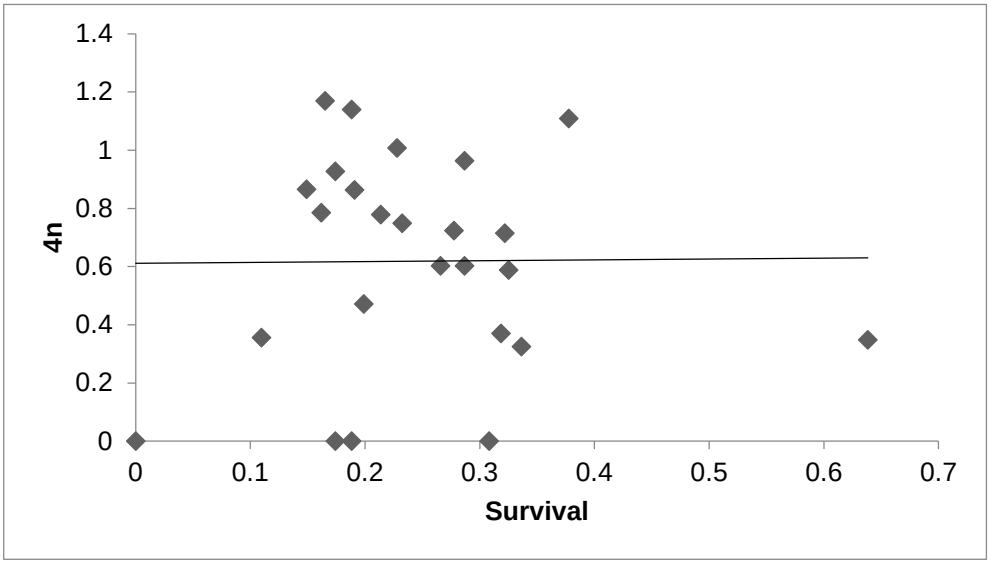

Figure 2: Correlation between percent survival and percent tetraploid for CB and 6-DMAP treatments on *C. virginica* on days six, seven, or nine. Arcsine transformed. (R=0.01)

Figure 3

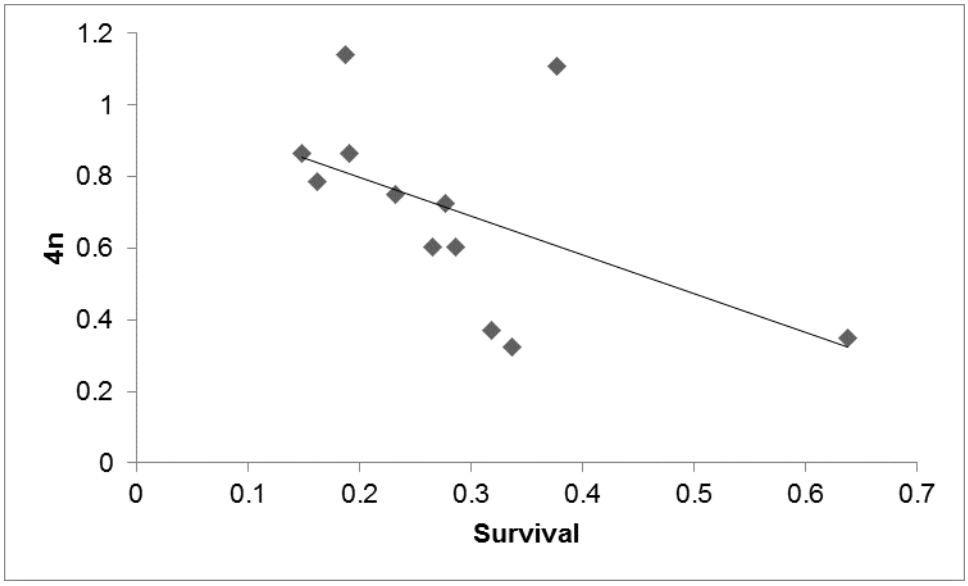

Figure 3: Correlation between percent survival and percent tetraploid for 6-DMAP treatments on *C. virginica* on days six, seven, or nine. Arcsine transformed. (R=-0.53)

Figure 4

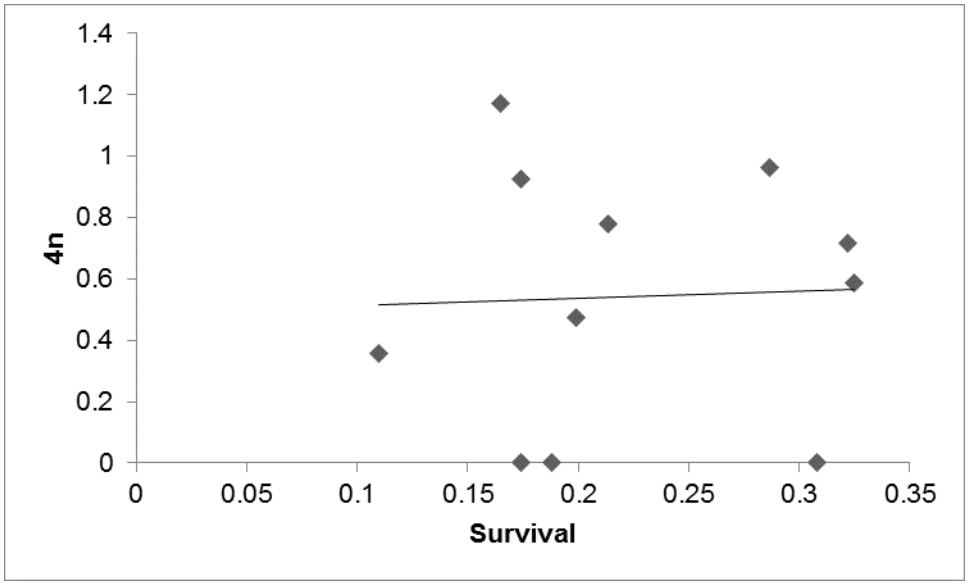

Figure 4: Correlation between percent survival and percent tetraploid for CB treatments on *C. virginica* on days six, seven, or nine. Arcsine transformed. (R=0.04)

Figure 5

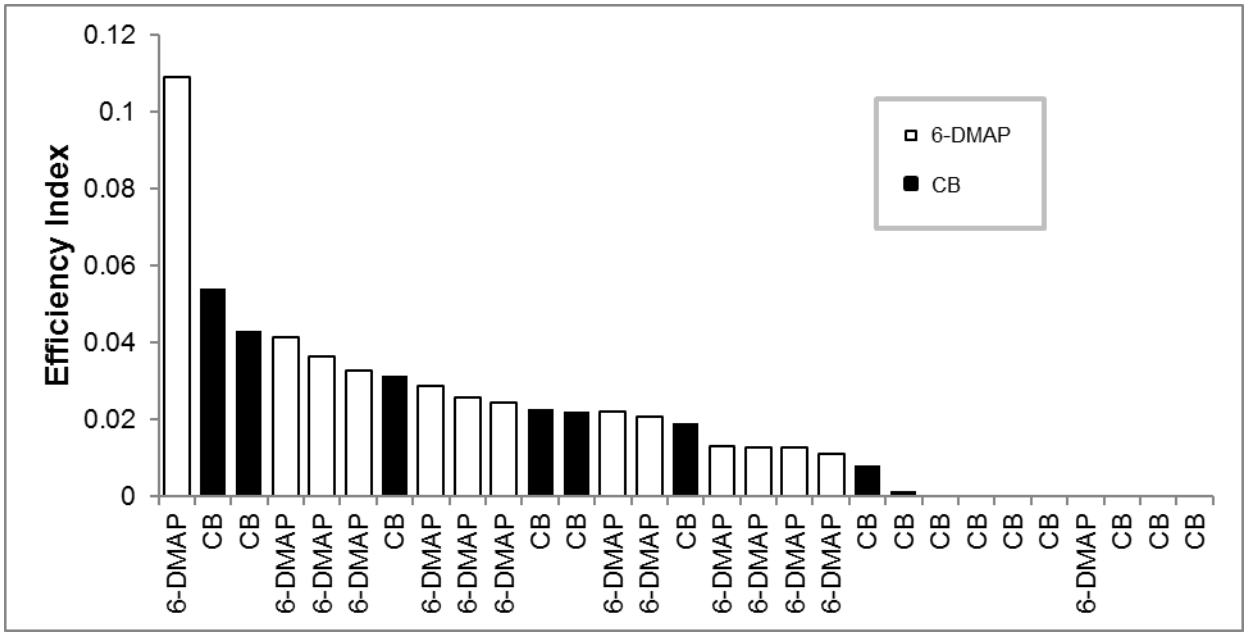

Figure 5: Efficiency index ((% survival day 6,7 x % tetraploid day 6,7,9)/10,000) of tetraploid crosses to compare treatments for production of tetraploids.

Table 1

	Females	LGT	OBOY	DBY	XB	Lola	hANA	DBSL	XBSL
Males					*Diploid*				
06 Lines	LGT	82.7 ±4.1							
	OBOY		89.9 ±3.3						
	DBY			80.2 ±2.8					
	XB				80.7 ±3.1				
08 Superlines	Lola					92.7 ±4.3			
	hANA						88.7 ±3.1		
	DBSL							83.9 ±3.7	
	XBSL								80.6 ±3.1
					Triploid				
Tetraploid	4B	95.2 ±2.7	93.3 ±3.5	91.2 ±3.5	94.6 ±2.5	92.1 ±2.9	90.9 ±3.0	86.3 ±3.6	85.3 ±2.6

Table 1: Summary of crosses made in 2010 that comprised the brood stock for this study. Eight lines, four from the 2006 year class and four from the 2008-Superline spawns were produced as diploid and triploid (using di-haploid sperm from tetraploid family 4B). These crosses were grown together (Callam et al., submitted) until Summer 2013. Average sizes of brood stock (±SD) are shown for each diploid and triploid line (mm).

Table 2

	Sex			Number of eggs (M)					
Line	F	"M"	Total	1	2	3	4	5	*Mean*
LGT	13	81	94	1.17 (12)	1.33 (1)				*0.192 (13)*
OBOY	8	33	41	0.66 (7)	3.34 (1)				*0.500 (8)*
DBY	24	89	113	1.96 (2)	1.10 (8)	0.88 (5)	2.15 (1)	2.74 (1)	*0.519 (17)*
XB	4	78	82	0.46 (4)					*0.115 (4)*
Lola	16	66	82	0.63 (11)	3.45 (5)				*0.255 (16)*
hANA	13	91	104	0.24 (1)	1.90 (1)	0.20 (10)			*0.195 (12)*
DBSL	12	64	76	4.43 (1)	1.03 (5)	0.66 (5)			*0.556 (11)*
XBSL	9	92	101	1.78 (1)	2.60 (1)	0.67 (7)			*0.560 (9)*
Total	**99**	**594**	**693**					**Overall**	***0.371 (90)***

Table 2: Sex ratio and egg yield from triploid ABC lines used in this study. F = female, "M" = non-female, males not always being distinguishable in triploids. Lines refer to those in Table 1. Egg counts, in millions (M), were made after deciding to pool eggs, or not. Number in parentheses is number of females in those counts. Numbered columns refer to the treatment number.

Table 3

	Survival (%)				Tetraploidy (%)			
	Day 2		Day 6,7		Day 2		Day 6,7,9	
Spawn	CB	6D	CB	6D	CB	6D	CB	6D
DBSL1	14	34	8	36	65	11	67	12
DBSL2	10	35	4	11	10	24	21	10
DBSL3	12	26	1	10	15	19	12	13
DBY3	11	15	3	5	9	30	0	46
DBY4	10	16	4	8	20	36	0	32
DBY5	8	7	3	4	50	48	64	58
hANA1	33	26	10	7	48	66	31	32
hANA2	27	30	4	14	23	28	nd	nd
hANA3	24	30	9	8	36	41	0	44
LGT1	9	20	0	5	30	52	nd	71
LGT2	15	34	5	14	27	70	49	80
Lola1	7	12	2	4	nd	nd	nd	83
Lola2	8	nd	3	nd	nd	nd	85	nd
XBSL2	24	12	11	4	47	89	43	58
XBSL3	3	7	1	4	49	45	nd	50
Mean	***14.2***	***23.7*****	***4.4***	***10.3*****	***33.0***	***43.0****	***33.8***	***45.3***
St. dev.	***8.5***	***9.5***	***3.2***	***8.7***	***18.0***	***18.9***	***31.5***	***26.8***
Control (2n)	**62±14 (7)**		**47±12 (7)**		**--**		**--**	

Table 3: Comparison of the percent survival and percent tetraploidy in *C. virginica* on day two and day six, seven, or nine resulting from either cytochalasin B (CB) treatments or 6-dimethylaminopurine (6D) treatment. nd = no data. * $p<0.05$, ** $p<0.02$

Table 4

	Setters (#)		Tetraploid (%)			Number of 4n setters	
LINE	CB	6D	CB	6D		CB	6D
DBSL1	8	388	0	40		0	155
DBSL2	23	16	8	9		2	1
DBSL3	0	0	nd	nd		--	--
DBY3	15	173	0	20		0	35
DBY4	29	1038	30	80		9	830
DBY5	620	1243	90	90		558	1119
hANA1	0	0	nd	nd		--	--
hANA2	0	0	nd	nd		--	--
hANA3	0	0	nd	nd		--	--
LGT1	0	201	nd	70		--	141
LGT2	28	79	30	70		8	55
Lola1	1	0	nd	nd		--	--
Lola2	2	74	nd	nd		--	--
XBSL2	10000	344	60	70		6000	241
XBSL3	0	0	nd	nd		--	--
Mean	***1191.8***	***395.1***	***31.1***	***56.1*****	***Total***	***6577***	***2577***
St. dev.	***3309.2***	***442.8***	***33.6***	***29.4***	***St. dev.***	***2101.2***	***417.0***

Table 4: Comparison of the number (#) of setters of *C.virginica* between cytochalasin B (CB) and 6-dimethylaminopurine (6D) cultures. nd=no data. Groups consist of lines of oysters selected by the Aquaculture Genetics and Breeding Technology Center's program. ** $p<0.02$