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1 Evaluation of cytochalasin B and 6-dimethylaminopurine for tetraploidy induction in the Eastern
2 oyster, *Crassostrea virginica*

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8 **Abstract**

9 Cytochalasin B (CB) has been used to induce tetraploidy in oysters since the practice began in
10 1993. However, CB is toxic and presents health risks to hatchery workers who administer the
11 treatment. 6-dimethylaminopurine (6-DMAP) is also an effective cytokinetic inhibitor, and does
12 not carry the health risks of CB. We examined the relative effectiveness of 6-DMAP vs CB for
13 producing tetraploids in the Eastern oyster (*Crassostrea virginica*). Survival and yield of
14 tetraploids varied widely among the 15 experiments. Larvae resulting from 6-DMAP treatment
15 had higher survival in 11 of the 14 trials on day two and day six/ seven. For yield of tetraploids,
16 10 of 13 6-DMAP treatments had higher proportions of tetraploids on day two and at the second
17 sampling – day six, seven, or nine – 7 of 10 had higher proportions of tetraploids. Tetraploid
18 spat were obtained from the majority of surviving cultures. Based on these results, 6-DMAP can
19 effectively replace CB for inducing polyploidy in *C. virginica*, and probably other *Crassostrea*
20 *spp.*, due to the success of the treatment, the ease of application, and the reduction in health risk
21 to hatchery workers. This study set the precedent for the use of 6-DMAP on *C. virginica* and

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

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

26 **1. Introduction**

27 Thirty years after their introduction to commercial hatcheries, polyploid oysters remain
28 important for oyster aquaculture industry around the world. For example, about 15% of seed
29 production in Australia (S. Parkinson, Shellfish Culture Ltd., Pipe Clay, Tasmania), about 50%
30 in the Pacific northwest US (pers. comm., J. Davis, Taylor Shellfish, Shelton, WA), and nearly
31 all commercial seed production in both the Chesapeake Bay area (Hudson and Murray, 2015)
32 and France (Dégremont et al., 2014) are triploid, the latter comprising nearly 3 billion seed.
33 Triploid oysters are popular because of reduced gonadal development (Allen and Downing,
34 1986, 1990) and the opportunity to harvest them year-round, even during the spawning season
35 (Allen et al., 1989; reviews by Beaumont and Fairbrother, 1991; Nell, 2002; Piferrer et al.,
36 2009). The reduced gonadal development of triploid oysters also allows them to apply more
37 energy to growth than fertile diploid oysters. In favorable conditions, triploid oysters grow
38 larger than diploids (Allen and Downing, 1986; Dégremont et al., 2012). Triploid oysters may
39 also be used as a method of population control due to their sterility (Guo et al., 1996; Piferrer et
40 al., 2009; Jouaux et al., 2010). Initially, triploids were chemically induced (Stanley et al., 1984)
41 until Guo and Allen (1994b) developed a method to produce viable tetraploids that led to the
42 production of so-called natural triploids (Guo et al., 1996) – a cross between diploids and
43 tetraploids that produces 100% triploid progeny. Tetraploids have found wide ranging use in
44 oyster aquaculture (Piferrer et al., 2009; Dégremont et al., 2012), with possibly more potential

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

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

63 Desrosiers et al. (1993) examined an alternative to CB for inducing triploidy using the
64 protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) (Szöllösi et al., 1993) instead of CB.
65 Comparisons between *C. gigas* triploids induced by CB and by 6-DMAP revealed that the
66 treatments yield similar numbers of triploids and comparable survival rates (Desrosiers et al.,
67 1993; Gérard et al., 1999). Handling 6-DMAP is significantly safer for hatchery workers than

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

72 **2. Methods**

73 2.1 Source oysters

74 Diploid and triploid oysters were produced at the Aquaculture Genetics and Breeding
75 Technology Center (ABC) hatchery in Gloucester Point, VA in June and July 2010. Broodstock
76 were from two groups of selectively bred, disease resistant (DR) lines – 2006-year class (four
77 lines) and 2008-year class of Superlines (four lines) – from ABC’s breeding program. For the
78 2010 spawning, eggs were obtained from at least 10 dams per line by strip spawning and pooled
79 in plastic beakers. The pools of eggs were then divided into two groups containing 3×10^6 eggs
80 each, one for diploids and one for triploids. To produce diploids, one group of eggs was
81 fertilized with sperm pooled from at least 10 sires of the same line. To produce triploids, the
82 remaining groups of eggs were fertilized with sperm pooled from 10-11 sires from a single
83 tetraploid family (Table 1). The diploid and triploid oysters produced above were grown in the
84 Rappahannock River, the subject of a three year study (Callam et al., submitted). In the summer
85 of 2013, we used triploid females to attempt tetraploid induction (crossed with the diploid males
86 from the same line), with the overall purpose of producing new tetraploid lines and with the
87 hypothesis that we could replace CB with 6-DMAP as an agent of induction. Average size of the
88 various brood stock used for tetraploid inductions is recorded in Table 1.

89 2.2 Tetraploid induction

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

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

112 2.3 Treatments with 6-DMAP and CB

113 The optimum 6-DMAP treatment for *C. virginica* was determined ahead of time by
114 testing different concentrations (250, 350, 466 $\mu\text{mol/L}$) as well as different treatment durations
115 (5, 10, 15 minutes) and several treatment start times (at fertilization and at the appearance of
116 polar bodies). Cultures treated with 250 $\mu\text{mol/L}$ 6-DMAP had higher survival but fewer triploids
117 than cultures treated with 466 $\mu\text{mol/L}$ 6-DMAP. Therefore, a concentration of 350 $\mu\text{mol/L}$ 6-
118 DMAP was used to ensure adequate survival while still producing tetraploid embryos. For
119 treatment duration, five minutes resulted in little to no triploids, while 10 or 15 minute treatments
120 were comparable for both survival and percent triploidy. For start time, treatments commencing
121 at fertilization and at the appearance of the first polar bodies both had similar survival but the
122 treatments starting at fertilization resulted in more triploids. These experiments with 6-DMAP
123 were performed on diploid eggs so as not to waste valuable triploid stock.

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

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

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

141 2.4 Larval culture

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

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

155 2.5 Flow cytometry

156 On day two and on either day six, seven, or nine, 3,000 larvae from each culture were
157 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 μ m 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

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

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

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

203 83%, with a 45% average. The proportion of tetraploids in 6-DMAP cultures was significantly
204 higher on day two than in CB treatments ($p < 0.05$) and also at day six/seven/nine, but this was not
205 a statistically significant difference ($p > 0.05$).

206 The relationship between percent survival and percent tetraploid was examined at the
207 second sampling. Percentages were arcsine transformed. No significant correlation was found
208 between the percent survival and the percent tetraploid in the cultures when the percentages from
209 CB and 6-DMAP cultures were combined ($R = 0.01$) (Figure 2). However, separately, 6-DMAP
210 cultures showed a negative correlation between percent survival and percent tetraploid ($R = -$
211 0.53) (Figure 3). CB cultures, on the other hand, did not have significant correlation between
212 percent survival and percent tetraploid ($R = 0.04$) (Figure 4).

213 Between the second sampling and when the larvae were ready to set, between 99 and
214 100% of larvae died. The actual number of setters obtained for CB treatments was between 1
215 and 10,000, with a mean of 1192 and, for 6-DMAP cultures, between 16 and 1243, averaging
216 395 (Table 4). The tetraploid percentage in the setters from 6-DMAP cultures ranged between
217 9% and 90%, with a mean of 56% (Table 4). Setters from CB cultures contained 0% to 90%
218 tetraploid individuals, averaging 31%. Overall, from all the pairs of treatments over all the
219 cultures we obtained an estimated 6577 tetraploid spat from CB treatments and 2577 spat from
220 6-DMAP treatments. However, the preponderance of spat from CB treatments were from one
221 successful culture out of five that yielded tetraploids, whereas eight cultures from 6-DMAP
222 treatments yielded tetraploids (Table 4). For CB, 6577 spat were obtained from 13,397,000 eggs
223 treated (0.049%) to make tetraploids; for 6-DMAP, 2577 spat obtained from 10,671,000 eggs
224 treated (0.024%).

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

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

226
227
228
229 Results show that 8 of 15 CB treatments yielded tetraploids while 13 of 14 6-DMAP treatments
230 did so (Figure 5). The highest yielding culture was from 6-DMAP treatment and 7 of the highest
231 ten yields were also 6-DMAP treatments.

232 4. Discussion

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

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

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

258 Careful husbandry of tetraploid larvae that are derived from diploid eggs (versus triploid
259 eggs) is the common feature for both the “direct” and “indirect” methods developed at the
260 French Research Institute for Exploitation of the Sea (IFREMER) and likely the reason that
261 patents now exist on both methods (Benabdelmouna and Ledu, 2007; Benabdelmouna et al.,
262 2007). As yet, these methods have not been successful at other labs and, therefore, the Guo and
263 Allen method remains the only recourse to tetraploid induction for many.

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

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

275 The major hurdle for making tetraploid *C. virginica* is obtaining eggs from triploid
276 females. For this work, we used three year old individuals that were up to 100mm long, far past
277 market size of 76mm. Larger sized females increased the odds that we would find some triploid
278 females. Nonetheless, we observed low fecundity with the average egg count (from females that
279 had enough to count) of about 371,000 (n = 90). This is about 15% of the fecundity of triploid
280 *C. gigas* (Guo and Allen, 1994a, average of 19 triploid females = 2.3 million). Compared to
281 diploid *C. virginica* that may release 23-85 million eggs/ female (Sellers and Stanley, 1984),
282 triploids have only 2% to 0.4%, respectively, of the fertility. For *C. gigas* – capable of producing
283 25-105 million eggs – the same comparison of the relative fecundity of triploids is between 9%
284 and 2%, respectively (Guo and Allen, 1994b). Gong et al. (2004) reported fecundity of triploid
285 *C. gigas* females as high as 13% that of diploids. More recently, Jouaux et al. (2010) supposed
286 that “un-locked” triploids, those that were able to attain near normal looking gametogenesis
287 despite being triploid, should be considered as fertile as diploids.

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

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

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

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

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

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

353 In summary, compared to the CB treatment protocol, the 6-DMAP treatment procedure
354 used for inducing tetraploidy in *C. virginica* was much simpler. 6-DMAP is safer than CB; it
355 can be dissolved in deionized water, and does not carry any of the toxic risks of CB. Also, 6-
356 DMAP is substantially less expensive than CB. From Sigma-Aldrich[®], 6-DMAP costs \$0.34/mg
357 (about \$19.38/ treatment) compared to CB, \$60.20/mg (\$30.10/ treatment) (Sigma-Aldrich,
358 2015a, 2015b). Efficacy of 6-DMAP treatment may improve with further experimentation, for
359 example, by basing initiation or completion of 6-DMAP treatments on timing of biological
360 events, as in the work by Eudeline et al. (2000).

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Figure 1

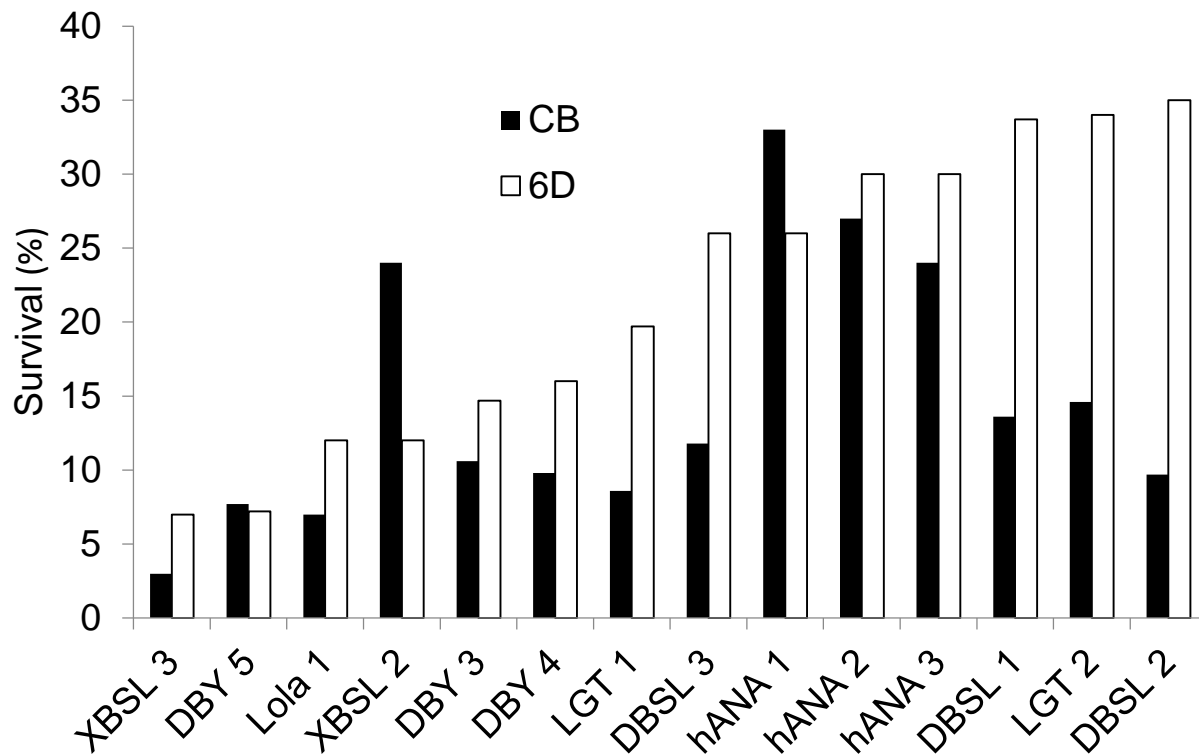


Figure 1: Percent survival of paired comparisons between CB and 6-DMAP (6D) treatments on larval cultures of *C. virginica* at day two after fertilization, arranged in ascending order of percent survival in 6-DMAP treatment. The x-axis denotes various lines of oysters used in the experiment.

Figure 2

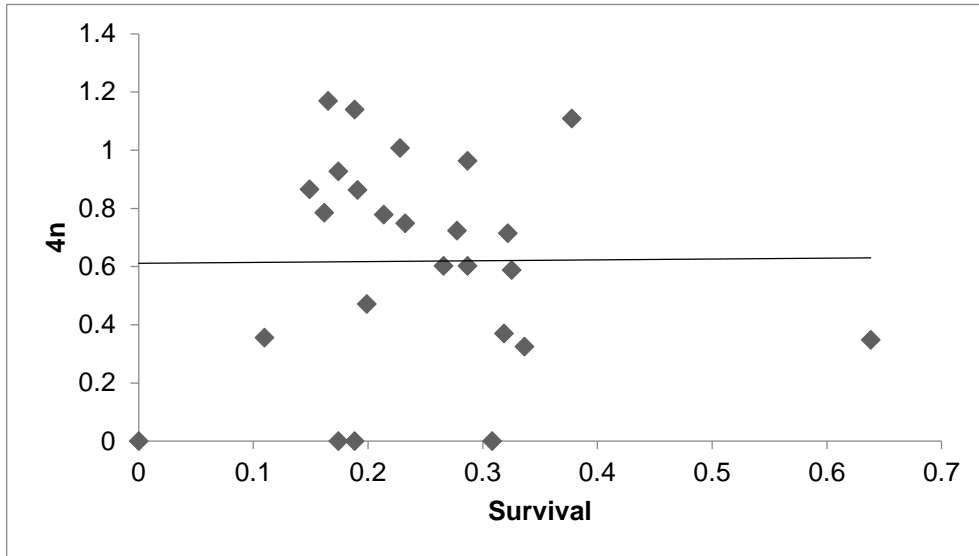


Figure 2: Correlation between percent survival and percent tetraploid for CB and 6-DMAP treatments on *C. virginica* on days six, seven, or nine. Arcsine transformed. ($R=0.01$)

Figure 3

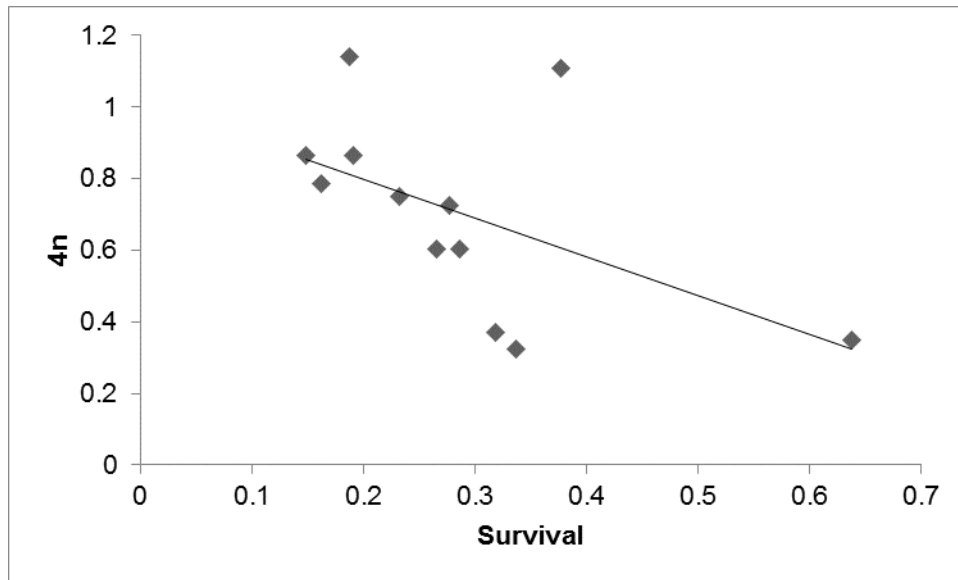


Figure 3: Correlation between percent survival and percent tetraploid for 6-DMAP treatments on *C. virginica* on days six, seven, or nine. Arcsine transformed. (R=-0.53)

Figure 4

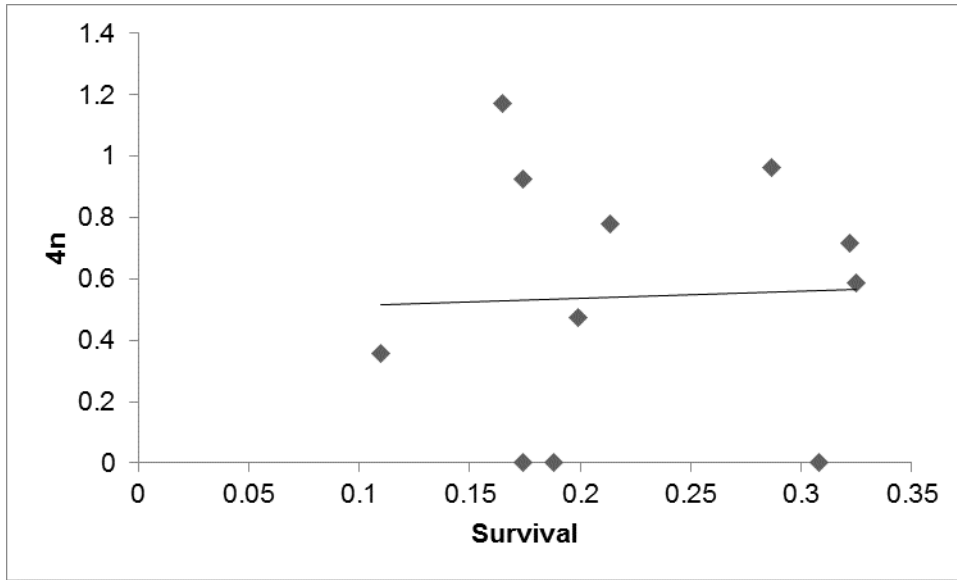


Figure 4: Correlation between percent survival and percent tetraploid for CB treatments on *C. virginica* on days six, seven, or nine. Arcsine transformed. (R=0.04)

Figure 5

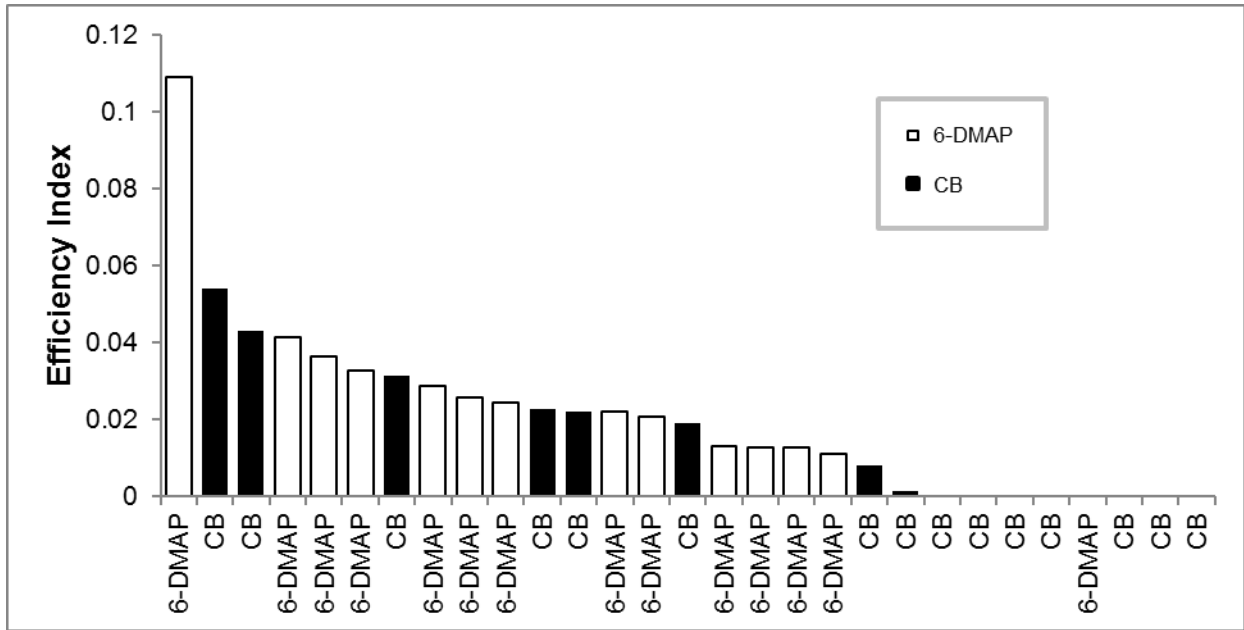


Figure 5: Efficiency index $((\% \text{ survival day } 6,7 \times \% \text{ tetraploid day } 6,7,9)/10,000)$ of tetraploid crosses to compare treatments for production of tetraploids.

Table 1

		Females	LGT	OBOY	DBY	XB	Lola	hANA	DBSL	XBSL	
Males		<i>Diploid</i>									
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	
		<i>Triploid</i>									
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 (\pm SD) are shown for each diploid and triploid line (mm).

Table 2

Line	Sex			Number of eggs (M)					
	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

Spawn	Survival (%)				Tetraploidy (%)			
	Day 2		Day 6,7		Day 2		Day 6,7,9	
	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

LINE	Setters (#)		Tetraploid (%)			Number of 4n setters	
	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