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RESEARCH PAPER

Tetraploid induction of *Crassostrea hongkongensis* and *C. sikamea* by inhibiting the polar body 1 release in diploid fertilized eggs

Yanping Qin[1,2,4] · Zohaib Noor[1,2,3] · Xingyou Li[1,2,3] · Haitao Ma[1,2,4] · Jun Li[1,2,4] · Yinyin Zhou[1,2,3] · Riguan Mo[5] · Yuehuan Zhang[1,2,4] · Ziniu Yu[1,2,4]

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Abstract

The production of an all-triploid population by mating tetraploid males with diploid females is the best and most fundamental method for the large-scale production of triploid oysters. Obtaining a stable tetraploid population is essential for guaranteed production in industrialized triploid cultivation. *C. hongkongensis* and *C. sikamea* are important oyster breeding species in southern China, and have great economic value. However, there are not any published data on inducing tetraploid *C. hongkongensis* or *C. sikamea*. Therefore, we investigated tetraploid induction in these two oyster species by inhibiting the PB1 release in diploid fertilized eggs using Cytochalasin B (CB) under 31 °C, 15 ‰ salinity. The results confirmed that the optimal tetraploid induction conditions for *C. hongkongensis* were a CB concentration of 0.50 mg/L with induction starting at 9.0 min after fertilization, and stopping at 21.0 min after fertilization; the induction efficiency index reached 0.123 under these conditions. The optimal tetraploid induction conditions for *C. sikamea* were a CB concentration of 0.50 mg/L, with induction starting at 7.5 min after fertilization and stopping at 18 min after fertilization; the induction efficiency index could be as high as 0.281 under these conditions. However, we confirmed that the tetraploid rate decreased with larval growth, and no tetraploids were detected in the juvenile period of either *C. hongkongensis* or *C. sikamea*. This may be attributed to the very low survival of the tetraploid larvae induced by this method, especially as most tetraploid larvae died during the first three days. In summary, it is simple to directly induce tetraploid *C. hongkongensis* and *C. sikamea* larvae by inhibiting the PB1 release of diploid zygotes, but the low survival rate makes it challenging to obtain viable juvenile tetraploids.

Keywords *Crassostrea hongkongensis* · *Crassostrea sikamea* · Tetraploid · Diploid · Optimal induction conditions

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✉ Yuehuan Zhang
yhzhang@scsio.ac.cn

✉ Ziniu Yu
carlzyu@scsio.ac.cn

1 CAS Key Laboratory of Tropical Marine Bio-Resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China

2 Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou 510301, China

3 University of Chinese Academy of Sciences, Beijing 100049, China

4 Innovation Academy of South China Sea Ecology and Environmental Engineering, Chinese Academy of Sciences, Guangzhou 510301, China

5 Guangxi A Bang-Ding Marine Technology Company, Nanning 530000, China

Introduction

Since Stanley et al. (1981) first successfully induced triploid *Crassostrea virginica* by inhibiting polar body 2 (PB2) release of diploid zygotes, many experts have demonstrated that triploid shellfish show fast growth, poor fertility, high survival rates, and good meat quality (Barreto-Hernández et al. 2018; Degremont et al. 2012; Hand et al. 2004; Qin et al. 2019; Wu et al. 2019). Based on these advantages, triploid breeding has become an important part of shellfish breeding (Callam et al. 2016; Francesc et al. 2009; Nell 2002). However, the physical and chemical methods (i.e., CB, 6-DMAP, caffeine and cold shock.) for directly inducing triploid oysters often result in low triploid rates, low D larval rates and unstable ploidy levels, and are complex to execute (Allen and Bushek 1992; Gerard et al. 1994; Qin et al. 2017). So, the production of 100% triploids by the interploid crossing of tetraploid sperm and diploid eggs is the most

practical method for obtaining large numbers of triploids (Francesc et al. 2009; Guo 1991; Guo and Allen 1994; Nell 2002). Therefore, tetraploid breeding stocks are critical for the commercial production and breeding of triploid oysters. However, viable tetraploid oysters are particularly difficult to obtain. Since Guo and Allen (1994) successfully induced tetraploid *C. gigas*, many scientists have made substantial efforts to induce tetraploid shellfish, but few have obtained viable juvenile tetraploids (Benabdelmouna and Ledu 2015; Eudeline et al. 2000b; Peachey and Allen 2016; Tan et al. 2017; Yang and Guo 2006b; Yang et al. 2000).

To date, there are three recognized methods for inducing tetraploid shellfish, although the commercial application of tetraploid technology is still limited to *C. gigas* and *C. virginica*. The first method of inducing tetraploid shellfish is to inhibit PB1 release of diploid fertilized eggs. This method was first proposed by Guo (1991), but he did not obtain viable tetraploid juvenile oysters (Guo 1991; Guo et al. 1992a, b). The lack of viable tetraploids was attributed to the "cell-number deficiency" hypothesis, which was based on the fact that a tetraploid cell would have to contain twice as much nuclear material as a diploid cell. However, the abnormal nucleus/cytoplasm ratio in a diploid cell would cause abnormal mitosis and a reduction in cell number (Francesc et al. 2009; Guo 1991; Tan et al. 2017; Yang et al. 2019). Since then, many scientists have used this method to induce tetraploids in other shellfish, but the number of tetraploids surviving to the juvenile stage is typically low (Allen et al. 1994; Peruzzi and Guo 2002; Tan et al. 2017; Yang and Guo 2006a; Yang et al. 2000). However, based on the above method, Benabdelmouna and Ledu (2007, 2015) succeeded in generating tetraploid larvae that survived to the larval period and produced viable and fertile autotetraploid *C. gigas*. These workers did not believe that egg size was a limiting factor for successful tetraploid induction. The mechanism of this method is that diploid fertilized eggs undergo multipolar division after the PB1 release is successfully inhibited, and then, some eggs form triploids (Fig. 1), with others forming tetraploids (Benabdelmouna and Ledu 2015; Guo 1991; Guo et al. 1992a; McCombie et al. 2009; Peachey and Allen 2016; Yang et al. 2019).

Subsequently, Guo and Allen (1994) proposed a method to induce tetraploids using eggs from triploids that were fertilized with sperm from diploids along with PB1 inhibition. These workers obtained viable tetraploid juvenile *C. gigas* using this method. However, there are two difficulties: the poor fertility of triploids and the low tetraploid rate (Allen et al. 1994; Brake et al. 2004; Eudeline et al. 2000b; Francesc et al. 2009; Peachey and Allen 2016). The third method is a complementary approach that enriches tetraploid genetic diversity. The indispensable condition for using this method is to first confirm the presence of tetraploid sperm. In this method, tetraploids are induced by inhibiting PB2 release after crossing tetraploid males and diploid females. McCombie et al. (2005) used this method to obtain viable tetraploid juvenile *C. gigas*, but other researchers considered that inducing tetraploids by this method was a challenge because of the low survival rate of the tetraploids originated from diploid eggs (Francesc et al. 2009; Yang et al. 2019).

C. hongkongensis and *C. sikamea* are native estuarine species in southern China, and the animals have high economic value. Previously, we confirmed the growth and survival advantages of triploid *C. hongkongensis* and *C. sikamea*, but not any published papers were available regarding tetraploid induction in these species (Qin et al. 2019; Wu et al. 2019). Moreover, the effectiveness of tetraploid

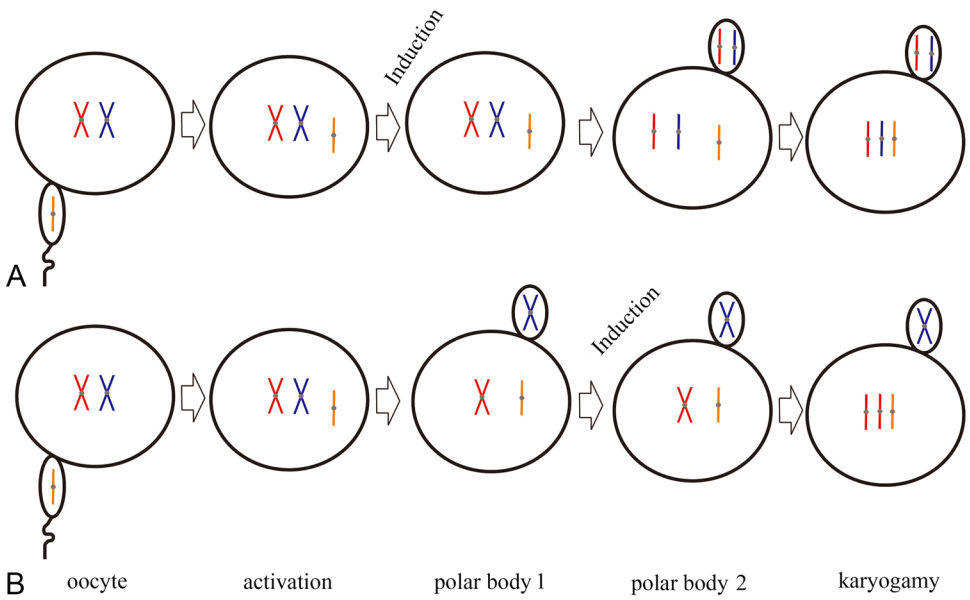

Fig. 1 Schematic diagram of triploid formation by inhibiting the polar body 1 or the polar body 2 release of diploid fertilized eggs during tetraploid induction. **A** Schematic diagram of triploid formation by inhibiting the PB1 release of diploid zygotes. **B** Schematic diagram of triploid formation by inhibiting the PB2 release of diploid zygotes

induction methods varies depending on the species, and determining which method will be most effective usually requires trials and optimization experiments (Francesc et al. 2009; Gerard et al. 1999; Yang and Guo 2006b).

The application of an appropriate and highly effective method is a key requirement for obtaining a successful breeding population of tetraploids. In this study, we designed different CB concentrations and duration time gradients to confirm the optimal induction conditions for tetraploid *C. hongkongensis* and *C. sikamea* by inhibiting PB1 release from diploid fertilized eggs. Furthermore, we assessed the variations in the ploidy profiles of larvae and juvenile oysters under optimal conditions. Based on these experiments, we provided a basis for the successful induction of tetraploid *C. hongkongensis* and *C. sikamea*.

Results

Optimization of tetraploid induction by inhibiting the polar body 1 release of diploid zygotes

Since the parent oysters have a substantial impact on the results of the experiments, it is recommended to compare the results that originated from the same batch of parental oysters. The cleavage rate was measured at 1.5 h; the corresponding D larval rate was measured at 18.0 h, and the tetraploid rate was identified at 36.0 h all after fertilization. Based on the results, we confirmed that the optimal induction conditions for tetraploid *C. hongkongensis* was a CB concentration of 0.50 mg/L with induction starting at 9.0 min and stopping at 21.0 min both after fertilization. Under these conditions, the induction efficiency index was highest (0.123), the cleavage rate was 78.71 ± 13.56%, the D larval rate was 67.43 ± 0.71%, and the tetraploid rate was 18.22 ± 0.68% (Table 1). Different treatment durations produced different proportions of tetraploids. Delayed induction start and stop times led to decreases in the cleavage rate and the D larval rate, which led to a reduction in the induction efficiency index. The lowest induction efficiency index was 0.15, and most eggs were stunted and deformed under these conditions (Table 1). The cleavage rate and D larval rate of the control group were significantly higher than those of the experimental groups.

The optimal induction condition for tetraploid *C. sikamea* was a CB concentration of 0.50 mg/L with induction starting at 7.5 min and stopping at 18 min after fertilization. The cleavage rate was 65.09 ± 5.23%, the D larval rate was 53.29 ± 0.94%, the tetraploid rate was 52.81 ± 4.33%, and the induction efficiency index (0.281) was highest under the optimal conditions (Table 2). Similarly, a delayed treatment start time and extended induction time caused decreases in the cleavage rate, D larval rate, and induction efficiency index. The lowest induction efficiency index was 0.022 (when the induction start and stop times were extended by 3 min and 6 min, respectively); whereas, the cleavage rate (21.55 ± 1.41%) and tetraploid rate (10.33 ± 1.44%) were significantly lower than those in the other experimental groups (Table 2). After CB treatment, partially induced eggs suffered from abnormal development (some fertilized eggs did not develop normally to the D larvae), and all experimental groups showed significantly lower cleavage rates and D larval rates than the control group. In addition, the optimal CB treatment concentration for the two oysters was the same, but the optimal start induction and stop induction times were quite different between *C. hongkongensis* and *C. sikamea*.

Variations in the tetraploid rate after optimal induction treatment

After determining the optimal conditions for tetraploid induction by inhibiting the PB1 release of *C. hongkongensis* diploid zygotes, we induced four batches of tetraploid groups under these conditions (the same source but different batches of parent oysters were used), followed by the identification of their DNA ploidy levels on the first, third, fifth, seventh, tenth, thirteenth and thirtieth days after fertilization. Based on the experimental results, we found that the ploidy composition of the larvae changed considerably over time, and the tetraploid rate gradually decreased. On the first day, the diploid rate was 39.84 ± 1.91%, the triploid rate was 32.62 ± 6.64%, the tetraploid rate was 22.82 ± 6.56% and the pentaploid rate was 4.72 ± 0.96%. Moreover, on the thirteenth day, the diploid rate was 57.37 ± 1.01%, the triploid rate was 40.79 ± 1.59% and the tetraploid rate was 1.84 ± 0.64%. However, on the thirtieth day, we identified 120 individual oysters, and could not detect the presence of any tetraploids; only 59.87 ± 4.01% diploids and 40.13 ± 4.01% triploids were identified (Fig. 2, Table 3).

As with *C. hongkongensis*, we identified the ploidy profiles of *C. sikamea* on the first, second, fifth, seventh, ninth, fifteenth and thirtieth days after fertilization (Fig. 3). The ploidy profiles of *C. sikamea* larvae were analyzed by a CyFlow Ploidy Analyser. All larvae were diploid in the control group. The ploidy profile of the larvae varied greatly over time, and the decrease in the tetraploid rate was obvious. On the first day after fertilization, the larval ploidy profile consisted of 2 N 24.34 ± 5.78%, 3 N 10.51 ± 5.09%, 4 N 55.49 ± 8.66%, and others 8.74 ± 2.99%. On the second day, the proportion of cells in each ploidy class was classified as 46.82 ± 5.40% diploid, 20.57 ± 6.48% triploid, 28.02 ± 6.18% tetraploid, and 3.61 ± 3.90% other. However, on the fifteenth day, the diploid rate increased to 71.96 ± 5.62%, the triploid rate increased to 26.33 ± 4.50%, and the tetraploid rate decreased to 1.71 ± 1.43%. In addition, on the thirtieth day, there were 80.00 ± 6.15% diploids and 19.99 ± 6.15%

Table 1 Optimal induction conditions for tetraploid *Crassostrea hongkongensis* by inhibiting the polar body 1 release of diploid fertilized eggs

Groups	Concentration (mg/L)	Start induction time (min)	Stop induction time (min)	Cleavage rate	D larval rate	Tetraploid rate	Induction efficiency index
A1	0.50	6.0	12	47.69 ± 9.25^{a}	39.09 ± 5.32^{l}	7.27 ± 3.47^{A}	0.028
A2	0.50	6.0	15	77.51 ± 5.95^{b}	56.98 ± 3.66^{m}	5.63 ± 1.97^{A}	0.032
A3	0.50	6.0	18	46.30 ± 11.57^{a}	30.35 ± 2.36^{n}	6.58 ± 2.24^{A}	0.020
A4	0.50	6.0	21	30.45 ± 5.69^{a}	21.07 ± 1.18^{o}	7.66 ± 0.96^{A}	0.016
A5	0.50	6.0	24	71.96 ± 5.57^{b}	46.25 ± 1.55^{l}	15.10 ± 2.34^{B}	0.070
B1	0.50	7.5	12	$54.22 \pm 4.42^{c,d}$	42.67 ± 2.30^{p}	5.58 ± 1.60^{C}	0.024
B2	0.50	7.5	15	$45.77 \pm 3.75^{c,d}$	$35.91 \pm 1.76^{p,q}$	$6.59 \pm 2.37^{C,D}$	0.024
B3	0.50	7.5	18	$51.25 \pm 8.20^{c,d}$	29.25 ± 1.81^{q}	5.31 ± 1.40^{C}	0.016
B4	0.50	7.5	21	62.10 ± 4.78^{c}	40.91 ± 0.85^{p}	10.47 ± 1.32^{D}	0.043
B5	0.50	7.5	24	40.30 ± 12.74^{d}	19.82 ± 4.54^{r}	$8.11 \pm 1.87^{C,D}$	0.016
C1	0.50	9.0	12	$50.00 \pm 10.00^{e,f}$	40.49 ± 1.44^{s}	5.97 ± 1.96^{E}	0.024
C2	0.50	9.0	15	74.92 ± 7.32^{g}	51.01 ± 3.22^{t}	5.87 ± 1.70^{E}	0.030
C3	0.50	9.0	18	$71.59 \pm 11.67^{f,g}$	51.40 ± 4.92^{t}	8.08 ± 2.97^{E}	0.042
C4	**0.50**	**9.0**	**21**	$\mathbf{73.13 \pm 5.54^{g}}$	$\mathbf{64.23 \pm 2.29^{t}}$	$\mathbf{14.73 \pm 3.28^{F}}$	**0.095**
C5	0.50	9.0	24	34.72 ± 4.11^{e}	28.91 ± 1.44^{u}	$9.13 \pm 1.25^{E,F}$	0.026
D1	0.50	10.5	12	$39.73 \pm 5.60^{h,i}$	34.39 ± 1.76^{v}	5.84 ± 0.14^{H}	0.020
D2	0.50	10.5	15	$40.27 \pm 4.05^{h,i}$	34.99 ± 2.37^{v}	4.38 ± 0.33^{I}	0.015
D3	0.50	10.5	18	$34.26 \pm 1.61^{h,i}$	29.59 ± 0.77^{w}	5.81 ± 0.08^{H}	0.017
D4	0.50	10.5	21	31.78 ± 1.36^{h}	26.28 ± 1.81^{w}	5.79 ± 0.17^{H}	0.015
D5	0.50	10.5	24	46.52 ± 9.56^{i}	40.80 ± 0.93^{x}	$5.39 \pm 0.79^{H,I}$	0.022
E1	0.25	9.0	21	78.89 ± 6.74^{j}	67.29 ± 0.95^{y}	5.24 ± 1.60^{J}	0.035
E2	**0.50**	**9.0**	**21**	$\mathbf{78.71 \pm 13.56^{j}}$	$\mathbf{67.43 \pm 0.71^{y}}$	$\mathbf{18.22 \pm 0.68^{K}}$	**0.123**
E3	0.75	9.0	21	47.92 ± 1.94^{k}	40.67 ± 0.79^{z}	14.78 ± 0.22^{L}	0.060
E4	1.00	9.0	21	44.07 ± 5.78^{k}	38.08 ± 2.36^{z}	6.82 ± 0.24^{J}	0.026
Control group	–	–	–	91.50 ± 6.00^{M}	83.92 ± 3.53^{N}	–	–

Three replicates were established for each group, M±SD represent the mean and standard deviation of the three replicates, respectively. M ± SD followed by different letters within a vertical column represent significant differences ($P < 0.05$); the start induction and stop induction times are the time after fertilization. The bold lines represent the optimal induction conditions

triploids of the 120 juvenile individuals, and no tetraploids were detected in any of the replicates (Fig. 3, Table 4).

Discussion

The tetraploid rate was significantly affected by the variations of induction conditions

Polyploid breeding in oyster species has been a popular topic of study since Stanley et al. (1981) successfully induced triploid *C. virginica* with CB. Researchers have developed and honed many polyploid induction methods for oysters (such as CB, 6-DMAP, caffeine, salinity stimulation, heat shock), and majority researchers consider CB to be an efficient inducing reagent (Allen and Downing 1986; Arai et al. 1986; Benabdelmouna and Ledu 2015; Gerard et al. 1999; Guo et al. 1992a, b; Qin et al. 2017; Tan et al. 2017). Some researchers confirmed the feasibility of inducing tetraploid oysters by inhibiting PB1 release from diploid fertilized eggs (Guo et al. 1992a; Peruzzi and Guo 2002; Tan et al. 2017), and Benabdelmouna and Ledu (2015) successfully produced a breeding population of tetraploid *C. gigas* by this direct method. However, no papers have been published on inducing tetraploid of *C. hongkongensis* and *C. sikamea*. Therefore, we considered it worthwhile to study tetraploid induction in these two oyster species by inhibiting PB1 release of diploid crosses with CB.

CB mainly affects actin polymerization, which is particularly important for forming the cleavage furrow of the polar body. The effect on fertilized eggs is almost immediate, so the induction time needs to be precisely controlled (Allen et al. 1989; Barber et al. 1992; Barreto-Hernández et al. 2018). Our results confirmed that both the CB concentration and the induction duration strongly influenced the normal development of eggs and the tetraploid rates. In both *C. hongkongensis* and *C. sikamea*, compared with that at 0.50 mg/L, the tetraploid rate was much lower when

Table 2 Optimal induction conditions for tetraploid *Crassostrea sikamea* by inhibiting the polar body 1 release of diploid fertilized eggs

Groups	Concentration (mg/L)	Start induction time (min)	Stop induction time (min)	Cleavage rate	D larval rate	Tetraploid rate	Induction efficiency index
A1	0.50	6.0	12	$61.19 \pm 7.81\%^{a}$	$56.50 \pm 1.54\%^{m}$	$20.31 \pm 1.23\%^{D}$	0.115
A2	0.50	6.0	15	$58.63 \pm 10.27\%^{a}$	$49.83 \pm 2.93\%^{n}$	$19.52 \pm 2.76\%^{D}$	0.097
A3	0.50	6.0	18	$53.33 \pm 5.77\%^{a}$	$43.20 \pm 1.92\%^{o}$	$21.20 \pm 3.06\%^{D}$	0.092
A4	0.50	6.0	21	$63.29 \pm 1.30\%^{a}$	$43.25 \pm 6.59\%^{o}$	$29.29 \pm 3.46\%^{E}$	0.127
A5	0.50	6.0	24	$63.09 \pm 7.20\%^{a}$	$38.90 \pm 4.82\%^{o}$	$32.90 \pm 3.18\%^{E}$	0.128
B1	0.50	7.5	12	$55.23 \pm 6.28\%^{c}$	$49.33 \pm 2.31\%^{p}$	$17.29 \pm 3.15\%^{F}$	0.085
B2	0.50	7.5	15	$65.88 \pm 8.48\%^{c,d}$	$57.32 \pm 5.39\%^{q}$	$20.20 \pm 2.57\%^{F}$	0.116
B3	**0.50**	**7.5**	**18**	$\mathbf{76.06 \pm 7.57\%^{d}}$	$\mathbf{62.11 \pm 5.81\%^{q,r}}$	$\mathbf{52.48 \pm 2.08\%^{G}}$	**0.326**
B4	0.50	7.5	21	$68.29 \pm 4.37\%^{c,d}$	$51.90 \pm 5.42\%^{r}$	$46.88 \pm 3.02\%^{H}$	0.243
B5	0.50	7.5	24	$76.13 \pm 7.52\%^{d}$	$60.40 \pm 4.59\%^{q,r}$	$24.97 \pm 2.10\%^{I}$	0.151
C1	0.50	9.0	12	$77.94 \pm 3.76\%^{e}$	$66.25 \pm 3.90\%^{s,t}$	$12.24 \pm 1.49\%^{J}$	0.081
C2	0.50	9.0	15	$75.40 \pm 5.05\%^{e}$	$58.56 \pm 1.90\%^{t}$	$13.25 \pm 1.26\%^{J}$	0.078
C3	0.50	9.0	18	$73.21 \pm 10.38\%^{e}$	$54.66 \pm 3.30\%^{s}$	$14.41 \pm 2.68\%^{J}$	0.079
C4	0.50	9.0	21	$65.51 \pm 9.51\%^{e}$	$42.14 \pm 3.36\%^{s}$	$17.69 \pm 2.74\%^{K}$	0.075
C5	0.50	9.0	24	$62.98 \pm 11.58\%^{e}$	$40.10 \pm 2.38\%^{u}$	$26.53 \pm 1.84\%^{L}$	0.106
D1	0.50	10.5	12	$71.34 \pm 7.10\%^{f}$	$51.03 \pm 2.42\%^{v}$	$17.80 \pm 2.13\%^{M}$	0.091
D2	0.50	10.5	15	$68.53 \pm 7.64\%^{f}$	$50.93 \pm 2.65\%^{v}$	$17.05 \pm 1.47\%^{M}$	0.087
D3	0.50	10.5	18	$51.19 \pm 9.12\%^{g}$	$33.91 \pm 3.17\%^{w}$	$14.19 \pm 1.79\%^{M}$	0.048
D4	0.50	10.5	21	$51.12 \pm 10.45\%^{g}$	$29.16 \pm 1.82\%^{x}$	$12.67 \pm 2.62\%^{N,O}$	0.037
D5	0.50	10.5	24	$41.05 \pm 3.94\%^{h}$	$21.55 \pm 1.41\%^{y}$	$10.33 \pm 1.44\%^{O}$	0.022
E1	0.25	7.5	18	$76.02 \pm 6.54\%^{i}$	$59.40 \pm 2.77\%^{z}$	$19.67 \pm 3.98\%^{P}$	0.117
E2	**0.50**	**7.5**	**18**	$\mathbf{65.09 \pm 5.23\%^{j}}$	$\mathbf{53.29 \pm 0.94\%^{A}}$	$\mathbf{52.81 \pm 4.33\%^{Q}}$	**0.281**
E3	0.75	7.5	18	$54.20 \pm 5.14\%^{k}$	$29.17 \pm 4.65\%^{B}$	$43.00 \pm 6.27\%^{R}$	0.125
E4	1.00	7.5	18	$44.72 \pm 6.28\%^{l}$	$22.47 \pm 0.81\%^{C}$	$28.72 \pm 4.90\%^{P}$	0.065
Control group	–	–	–	$87.77 \pm 5.76\%^{M}$	$78.05 \pm 5.10\%^{N}$	–	–

Three replicates were established for each group, M±SD represent the mean and standard deviation of the three replicates, respectively. M ± SD followed by different letters within a vertical column represent significant differences ($P < 0.05$); the start induction and stop induction times are the time after fertilization

the CB concentration was 0.25 mg/L, and the cleavage rate and D larval rate decreased sharply when the concentration was 0.75 or 1.00 mg/L (Tables 1, 2). If the CB concentration is excessive low, the release of PB1 is not inhibited successfully, so the induction efficiency index is low. Also, if the CB concentration is excessive high, some eggs cannot develop normally, and the deformity rate begins to rise (Barreto-Hernández et al. 2018; Ledu and McCombie 2003; Qin et al. 2017). CB has some toxic effects on developing eggs, so the D larval rates of all experimental groups are significantly lower than that of the control group (Peachey and Allen 2016; Yang and Guo 2006b).

According to previous studies, proper initiation and duration of induction times were particularly important for improving polyploid rates (Francesc et al. 2009; Nell 2002; Qin et al. 2017; Wadsworth et al. 2019). By inhibiting the PB1 release of diploid fertilized eggs to induce tetraploids, if the start induction time is early, some eggs will become deformed. If the start induction time is late, the PB1 of some eggs will have already been released, and further induction would lead to an increase in diploid and triploid rates. Also, we found that both *C. hongkongensis* and *C. sikamea* had low tetraploid rates when the treatment stop time was excessively early because most of the PB1 release was not inhibited successfully. In addition, when the stop induction time was delayed, the deformity rate and the pentaploid rate increased because both PB1 and PB2 were inhibited in some eggs. The same phenomena were also found in polyploid induction of other shellfish (Arai et al. 1986; Gerard et al. 1994; Ledu and McCombie 2003; Peachey and Allen 2016; Qin et al. 2017; Tan et al. 2017; Verdugo et al. 2000). Triploid production during induction was mainly due to the inhibition of the PB1 or PB2 release (Fig. 1).

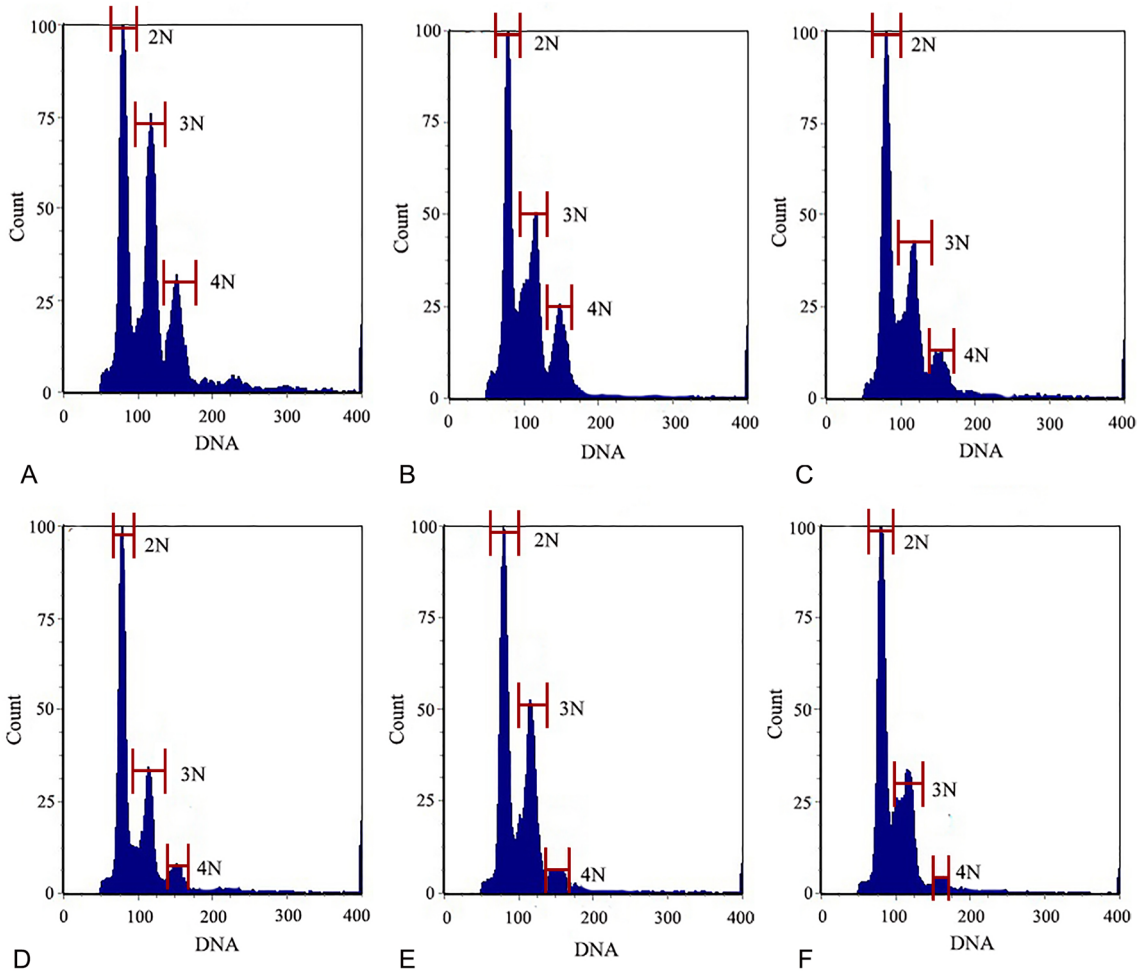

Fig. 2 The representatives of variations in the ploidy proportional composition of *Crassostrea hongkongensis* over time. **A** The first day; **B** the third day; **C** the fifth day; **D** the seventh day; **E** the tenth day; **F** the thirteenth day

Table 3 The variations in ploidy proportional composition of *Crassostrea hongkongensis* under the optimal induction conditions

	2 N	3 N	4 N	5 N and others
The first day	39.84 ± 1.91%	32.62 ± 6.64%	22.82 ± 6.56%	4.72 ± 0.96%
The third day	48.74 ± 3.25%	36.09 ± 7.12%	14.67 ± 4.64%	0.56 ± 0.65%
The fifth day	54.77 ± 1.91%	35.94 ± 5.57%	9.30 ± 4.14%	0
The seventh day	55.82 ± 1.32%	38.05 ± 1.84%	6.14 ± 2.20%	0
The tenth day	56.90 ± 1.41%	39.37 ± 0.55%	3.74 ± 1.82%	0
The thirteenth day	57.37 ± 1.01%	40.79 ± 1.59%	1.84 ± 0.64%	0
The thirtieth day	59.87 ± 4.01%	40.13 ± 4.01%	0	0

Four batches of tetraploid were induced under the optimal conditions (the same source but different batches of parent oysters were used)

The differences in the optimal induction conditions for tetraploid *C. hongkongensis* and *C. sikamea*

The experimental results showed that the optimal CB concentration for tetraploid induction was the same for both *C. hongkongensis* and *C. sikamea*. The results were consistent with the optimal CB concentrations for triploid induction in *C. gigas*, *C. virginica*, *C. sikamea,* and *C. hongkongensis*, suggesting that this concentration is sufficient to inhibit polar body release (Downing and Allen 1987; Qin et al.

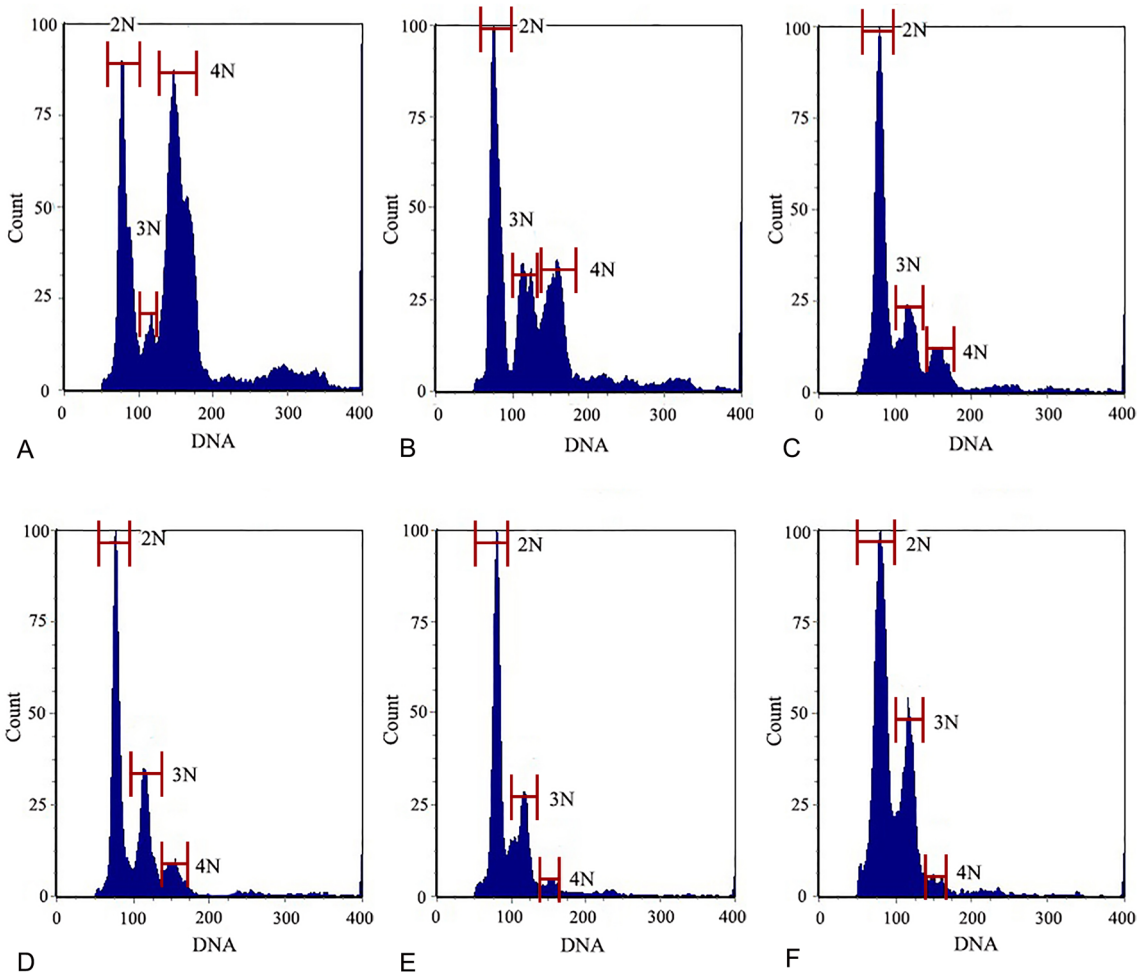

Fig. 3 Representatives of variations in the ploidy proportional composition of *Crassostrea sikamea* over time. **A** the first day; **B** the second day; **C** the fifth day; **D** the seventh day; **E** the ninth day; **F** the fifteenth day

Table 4 The variations in ploidy proportional composition of *Crassostrea sikamea* under the optimal induction conditions

	2 N	3 N	4 N	5 N and others
The first day	24.34 ± 5.78%	10.51 ± 5.09%	55.49 ± 8.66%	8.74 ± 2.99%
The second day	46.82 ± 5.40%	20.57 ± 6.48%	28.02 ± 6.18%	3.61 ± 3.90%
The fifth day	59.85 ± 1.94%	24.51 ± 5.13%	15.69 ± 4.18%	0
The seventh day	63.74 ± 5.59%	27.58 ± 4.64%	8.68 ± 2.93%	0
The ninth day	68.64 ± 5.74%	27.07 ± 3.68%	4.28 ± 3.23%	0
The fifteenth day	71.96 ± 5.62%	26.33 ± 4.50%	1.71 ± 1.43%	0
The thirtieth day	80.00 ± 6.15%	19.99 ± 6.15%	0	0

Four batches of tetraploid were induced under the optimal conditions (the same source but different batches of parent oysters were used)

2017; Stanley et al. 1981; Wu et al. 2019). However, the optimal start induction and stop induction times were different between *C. hongkongensis* and *C. sikamea*, which might be attributed to the difference in the releasing rate of PB1. According to previous studies, the temperature, salinity, hydration time, serotonin level, oyster source, and species of parent oysters all have effects on the dynamics of the polar body release (Eudeline et al. 2000a; Qin et al. 2018). In this experiment, the temperature, salinity and parental source were all consistent during fertilization, so the notable differences in the PB1 release could be attributed mainly to the difference in oyster species. Also, other researchers

demonstrated that the polar body release in different species was quite different (Barber et al. 1992; Gerard et al. 1994; Mallia et al. 2010; Tan et al. 2017; Wu et al. 2019). Through the observation of PB1 release in *C. hongkongensis* and *C. sikamea*, we found that although the optimal start induction and stop induction times of the two species were different, the corresponding polar body release ratios were consistent (Qin et al. 2018; Wu 2019). The optimal start induction times for the two oysters both corresponded to approximately 5% of the PB1 release, and the optimal stop induction times both corresponded to approximately 60% of the PB1 release. Also, this indicated that the polar body release rate of *C. sikamea* was faster than that of *C. hongkongensis* in the same environment (Qin et al. 2017; Wu 2019; Wu et al. 2019). These two polar body release ratios may be used as initiation and termination markers of tetraploid induction by inhibiting PB1 release of diploid zygotes.

After the optimal induction treatment, the tetraploid rate decreased with larval development

We confirmed that the tetraploid rates decreased with larval growth, and the presence of tetraploids was not detected in the juvenile period in either *C. hongkongensis* or *C. sikamea*. This may be due to the death of tetraploids during growth, especially during the first three days of the larval stage. The low survival rate might be the result of tetraploidy and the culture conditions (Eudeline et al. 2000b; Francesc et al. 2009; Tan et al. 2017; Yang and Guo 2006a). Tetraploid cell architecture and numbers have changed, which could impose developmental and physiological hardships on larvae, and a lack of careful breeding of tetraploid larvae aggravates the disadvantages of tetraploids (Luca 2005; Francesc et al. 2009; Peachey and Allen 2016). The mechanism of inducing tetraploids by inhibiting the PB1 release of diploid fertilized eggs is that inhibiting the PB1 release will lead to the occurrence of biopolar and tripolar divisions at the PB2, resulting in the production of partial tetraploids (Benabdelmouna and Ledu 2007, 2015; Guo 1991; Guo et al. 1992a). During the induction process, triploidy is produced at the same time, which is also due to the inhibition of the PB1 or PB2 of some diploid zygotes (Fig. 1). However, the direct induction of tetraploids by inhibiting the PB1 release of diploid fertilized eggs has proven to be difficult in shellfish. Thus, many researchers attributed the failure of this method to the cell-number deficiency due to the small volume of the diploid eggs (Francesc et al. 2009; Guo and Allen 1994; Guo et al. 1992b; Miller et al. 2014; Yang et al 2019). The mismatch between a normal-volume diploid shellfish egg and a large tetraploid nucleus was more likely to lead to cell-number deficiency compared to fish eggs, where the cell-number deficiency could be compensated at the later development of zygotes (Francesc et al. 2009; Guo and Allen 1994). Also, Guo (1991) attempted to directly select large diploid eggs to induce tetraploids. However, diploid egg size varies only slightly, so it would be difficult to induce tetraploids using diploid eggs with this method.

However, Benabdelmouna and Ledu (2015), Mccombie et al. (2005, 2009) and Tan et al. (2017) obtained tetraploid oysters through diploid eggs. Moreover, Benabdelmouna and Ledu (2007, 2015) confirmed that oocyte size was not a limiting factor for the success of tetraploid induction, and they obtained a patent for successfully inducing tetraploids by inhibiting the PB1 release in diploid zygotes. McCombie et al. (2005) were the first workers to demonstrate that viable tetraploid oysters could be produced using large diploid eggs. Consequently, they obtained a patent for inducing tetraploids by inhibiting PB2 release after diploid eggs were crossed with tetraploid sperm. The optimal breeding environment and the careful cultivation of the tetraploid larvae derived from the diploid eggs might be the reasons that they were able to obtain viable tetraploid oysters (Benabdelmouna and Ledu 2007; Benabdelmouna et al. 2007; Francesc et al. 2009). In this study, we tried many times with the same source but different parental batches of *C. hongkongensis* and *C. sikamea,* and found that although tetraploids could always be identified before metamorphosis, juvenile tetraploids were never detected. The treatment effect was highly variable from egg batch to egg batch in terms of the tetraploid percentage (Tables 3, 4). In conclusion, we confirmed that tetraploid larvae induced by inhibiting PB1 release of diploid fertilized eggs had difficulty reaching metamorphosis and attachment, so it was difficult to obtain tetraploid juvenile *C. hongkongensis* and *C. sikamea* directly using diploid zygotes. Moreover, no stable tetraploid oyster population originated from diploid zygotes has yet been obtained and applied to commercial promotion, and we have decided to abandon further exploration of this method.

Materials and methods

Preparation of parent oysters

The sexually mature diploid *C. hongkongensis* and *C. sikamea* used in this study were collected from Zhulin in Beihai, China. The parent oysters were artificially cultivated in an open circulating system (temperature 29.0–32.0 °C, salinity 15 ‰ salinity, pH 7.8–8.1) for at least one week before being used for later experiments. During the temporary rearing period, the parent oysters were fed twice daily with plenty of *Isochrysis zhanjiangensis* and *Chaetoceros calcitrans*, and stimulation was minimized to prevent gamete discharge (Qin et al. 2019).

Gamete preparation and fertilization

The oysters selected for the experiment were dissected, and the gametes were subsequently collected. The parent oysters were carefully shucked using a scalpel knife, sexed under a light microscope, and then segregated by sex. Before fertilization, all containers used in this study were cleaned with fresh water to prevent any accidental fertilization. Eggs were separated from faeces and large tissue debris by passing through a 48 μm nylon screen, and then rinsed on a 25 μm nylon screen to wash out broken eggs and other small impurities. Then, the eggs were soaked in seawater under optimum conditions (31 °C, 15 ‰ salinity) until germinal vesicle breakdown was observed, which was a sign that meiosis was ready to resume initiation (Qin et al. 2018).

Sperm were separated from impurities by passing through a 40 μm screen and were resuspended in seawater (31 °C, 15 ‰ salinity) for activation 10 min before fertilization. Just before fertilization, the most active sperm group was selected, and the fertilization procedure was performed following the protocol of Qin et al. (2019). According to a previous study, the release of polar bodies varies significantly under different environmental conditions (Qin et al. 2018). Therefore, before the formal experiment, pre-fertilization was performed to investigate the polar body release pattern at 31 °C with 15 ‰ salinity seawater.

Experimental protocol

The optimal CB treatment conditions for inducing polyploid bivalves are usually characterized by different concentrations, start induction times and stop induction times. CB was dissolved in dimethyl sulfoxide (DMSO) and stored at − 20 °C (Qin et al. 2019). Based on the previous studies (Qin et al. 2018; Wu 2019) and the polar body release pattern from pre-fertilization, the CB concentration gradient was set to 0.25, 0.50, 0.75, and 1.00 mg/L, the gradient of start induction time was set to 6.0, 7.5, 9.0 and 10.5 min after fertilization, and the gradient of stop induction time was set to 12.0, 15.0, 18.0, 21.0 and 24.0 min after fertilization (Barber et al. 1992; Peachey and Allen 2016; Qin et al. 2017, 2018). Then, induced combinations were established as shown in Tables 1 and 2. Three replicates were established for each group, and a control group was also established.

Briefly, the soaked eggs of five females were pooled and divided into corresponding groups, then fertilized with equal and sufficient sperm from one male. Then, based on Table 1 or Table 2, the corresponding CB concentration was used at the corresponding start induction time to the corresponding stop induction time. After the induction treatment, the drugs were washed off with a 25 μm nylon screen and the eggs were cultured at 31.0 °C in 15 ‰ salinity seawater and supplied with slow aeration. Then, the cleavage rate, D larval rate and tetraploid ratio of all groups were measured. In addition, after determining the optimal CB treatment conditions for inducing tetraploids, four batches of tetraploids (the same source but different batches of parent oysters were used) were further induced using these conditions, and the variations in the tetraploid rate were tracked and measured.

DNA ploidy determination

The DNA ploidy level was identified using flow cytometry with a CyFlow Ploidy Analyser (Sysmex, Japan) according to Qin et al. (2018). In the planktonic larval stage, about 200–300 larvae of free-swimming larvae were collected on a 48 μm nylon screen, and the larval suspensions were centrifuged at 3000 r/min for 3 min. After the removal of the supernatant, 0.20 ml of nuclear extraction buffer and 0.80 ml of DAPI staining buffer (Sysmex, Japan) were added to each tube. The larvae were resuspended by gentle mixing and stained for 10 min at room temperature. Then, the samples were filtered through a 42 μm nylon screen and immediately analyzed using flow cytometry. In addition, the DNA ploidy level of juvenile oysters was individually identified by flow cytometry using a small tube of lymph extracted from each oyster and the method described above.

Statistical analysis

The cleavage rate is defined as the proportion of cleaving eggs of the total fertilized eggs, and the D larval rate is defined as the proportion of D larvae of the total fertilized eggs. The induction efficiency index is the product of the D larval rate and the tetraploid rate (Qin et al. 2017). All data in this paper are presented as the mean ± standard deviation (M ± SD). Multiple comparisons of the cleavage rate, D larval rate and tetraploid ratio of all groups were performed using one-way analysis of variance (ANOVA) followed by Duncan test using SPSS18. $P < 0.05$ was considered significant, while $P < 0.01$ was considered highly significant.

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Author contributions YQ, ZY, and YZ designed experiments. YQ carried out all of the experiments with the help of ZN, XL, JL, HM, YZ and RM. YQ analyzed the data and wrote the paper. ZY and YZ

critically revised the manuscript and approved the final version to be published.

Declarations

Conflict of interest The author declares that there is no conflict of interest.

Animal and human rights statement This study was conducted in accordance with the Institutional Animal Care and Use Committee of South China Sea Institute of Oceanology, Chinese Academy of Sciences, and it does not contain any studies with human participants.

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