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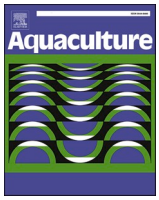

Growth, survival and gonad development of diploids, triploids and tetraploids of 'Haida No. 3' line of the Pacific oyster *Crassostrea gigas*

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ABSTRACT

Selection and polyploidization are two practical tools for improving productive traits of oysters. To evaluate the aquaculture traits of diploids, triploids and tetraploids of 'Haida No. 3' line of the Pacific oyster *Crassostrea gigas*, four mating combinations (HD- diploid 'Haida No. 3' line ♀ × diploid 'Haida No. 3' line ♂, HTR- diploid 'Haida No. 3' line ♀ × tetraploid 'Haida No. 3' line ♂, HTE- tetraploid 'Haida No. 3' line ♀ × tetraploid of 'Haida No. 3' line ♂, WD- wild diploid ♀ × wild diploid ♂) were established. At both Rongcheng and Rushan sites, HD had a larger shell height and wet weight than WD. HTR outperformed HD in growth and survival, with a consistently positive value of triploid advantage (*TA*). On the day 480 at Rushan site, the triploid advantages in terms of shell height, wet weight and cumulative survival were 20.70%, 40.28% and 29.27%, respectively. HTE were the lowest in growth and survival compared to the other three groups. Histomorphometric analysis revealed that HTR were partial highly sterile, indicating its gonadal atrophy and gamete abnormalities. In contrast, HD and HTE were fully fertile and contained functional gametes. During the whole culture period, HTR remained 100% triploid rate while HTE maintained a tetraploid rate of over 80%. HTR and HTE exhibited rapid growth and stable black shell color, suggesting that these traits were stably inherited by the ploidy change. Overall, our results demonstrated that polyploidization based on selective line had favorable performance for aquaculture, which would have vital practical interest for oyster culture in northern China.

1. Introduction

Selective breeding is a basic tool to improve important morphological and physical characteristics of stocks (Bentsen and Olesen, 2002; Gjedrem et al., 2012). The selective breeding of aquaculture animals has been commercially used to improve growth, disease resistance, morphology and yield (Chavanne et al., 2016) in Atlantic salmon, rainbow trout, shrimp, carps, oysters, etc. (Gjedrem and Baranski, 2009). Meanwhile, polyploidy is the heritable condition in which the cells of an organism possess more than two complete sets of chromosomes (Comai, 2005). Triploidy technology has widely been utilized for commercial seed cultivation with the aim of producing high yield, organoleptic quality and sterile animals (Nell, 2002). For instance, the triploidization in Atlantic cod (*Gadus morhua* L.) had addressed the biological bottlenecks of early maturation in commercialize and genetic introgression between hatchery and wild populations (Otterå et al., 2016; Puvanendran et al., 2019). Dégremont et al. (2016) demonstrated that triploid *C. virginica* were superior to diploids in terms of growth, survival and yield. However, triploidy technology also has some commercial problems, such as, deformations in salmonids (Sadler et al., 2001; Myers and Hershberger, 1991) and reversion to diploidy in oysters (Allen et al., 1999; Chandler et al., 1999).

Excellent performance of triploids obtained from selected diploid strains has been reported in several aquatic animals. Genetic gain in growth of the growth-selected line was maintained in triploid rainbow trout (*Oncorhynchus mykiss*), thus illustrating that the performance of triploids can be improved through direct selection for diploids (Leeds and Weber, 2019). In Atlantic salmon (*Salmo salar*), the superior performance of diploid strains could be transferred to triploid offspring (Sacobie et al., 2012). In Sydney rock oyster (*Saccostrea glomerata*), the triploids from selective population were 74% heavier in whole weight than control diploids (Hand et al., 2004). Triploid oysters exhibited a lower mortality rate than those produced with unselected or susceptible populations (Lionel et al., 2010; Dégremont et al., 2016). As these examples illustrated, the superior traits of diploids could be translated to triploids by ploidy manipulation.

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The Pacific oyster, *Crassostrea gigas*, naturally distributing along the coast of northeast Asia, is an aquatic species of considerable economic importance due to its fast-growing and strong environmental adaptability (Martinez-Garcia et al., 2022; Dundon et al., 2011). In previous work, we developed a selected line of *C. gigas* 'Haida No. 3' line with rapid-growth and black-shell traits (Xu et al., 2019). However, diploids of the 'Haida No. 3' line generally exhibit unpleasant taste and less marketability after oviposition in summer (Breese and Malouf, 1977; Nell, 2002). To overcome this problem, creating quasi-sterile triploid oysters may be a good solution. Compared to diploid oysters, sterile triploids can maintain preferable meat quality and sometimes higher growth rates during the spawning season, allowing for year-round marketing (Allen and Downing, 1986). In addition, producing sterile triploids is an effective way to conserve regional biodiversity (Piferrer et al., 2009; Guo et al., 1996) and intellectual property rights of new diploid varieties (Weber et al., 2014).

In this study, the growth performance of diploid, triploid and tetraploid of *C. gigas* 'Haida No. 3' line in Rongcheng and Rushan was investigated. Rongcheng and Rushan are two major aquaculture areas of *C. gigas* in northern China with distinct seawater conditions. The objectives of this study were: (1) to assess the differences in growth, survival and gonad development between diploid, triploid and tetraploid of *C. gigas* 'Haida No. 3' line; (2) to assess the inherited stability of the rapid growth and black shell characteristics in triploid and tetraploid of 'Haida No. 3' line; (3) to identify whether polyploidization based on selective line of oysters could further improve economically useful traits and realize the great potential for commercial production.

2. Materials and methods

2.1. Broodstocks and conditioning

The broodstocks of diploid, triploid and tetraploid were originated from the same selected line of *C. gigas* 'Haida No. 3', with black-shell color and rapid-growth as breeding targets, developed through four generations of family selection and two generations of mass selection (Xu et al., 2019). The tetraploid parents were produced by blocking the release of polar body 1 (PB1) in eggs from triploid 'Haida No. 3' line fertilized with the haploid sperm of 'Haida No. 3' line.

In May 2021, the one-year-old diploid and tetraploid broodstocks of 'Haida No. 3' line were collected from Rushan, Shandong Province, China, and transported to an oyster hatchery in Laizhou, Shandong Province for three-week conditioning. The wild population broodstocks were collected from Rongcheng, Shandong Province. All broodstocks were maintained separately in a concrete tank with the temperature at 24 ± 1 °C, salinity of 30 ± 1 psu (Practical Salinity Units), continuous aeration, 50% daily water changes, and daily feed a mixed diet of live algae including *Platymona* sp. and *Chaetoceros calcitrans*. Prior to the experiment, all tetraploid oysters were detected ploidy by flow cytometry to ensure that they were purely tetraploid.

2.2. Fertilization, larval rearing

The mature diploids and tetraploids were individually dissected, and the sex was determined under a light microscopy (Olympus, Japan). For each group, eggs from 10 females were pooled into 5-L bucket. Each bucket of eggs was fertilized with the mixing of sperm from each group of 10 males. Therefore, four mating combinations were created: HD- diploid 'Haida No. 3' line ♀ × diploid 'Haida No. 3' line ♂, HTR- diploid 'Haida No. 3' line ♀ × tetraploid 'Haida No. 3' line ♂, HTE- tetraploid 'Haida No. 3' line ♀ × tetraploid 'Haida No. 3' line ♂, WD- wild diploid ♀ × wild diploid ♂ (Fig. 1).

The larvae were reared following standard culture protocols as described by Li et al. (2011), the rearing conditions for four combinations were the same. Briefly, 24 h after fertilization, the upper D-larvae were collected with 38-μm nylon screen and transferred to the new 20 m^3 tank. The larvae density of each group was initially adjusted to 1–2 ind./mL and the feeding volume of *Isochrysis galbana* and *C. calcitrans* gradually increased with larval growth. The temperature and salinity of the rearing water were maintained at 25 ± 1 °C and 30 ± 1 psu, and 30% of the water was changed daily. When 40% of the larvae presented eyespots and feet, strings of scallop shells were suspended in the tanks as attachment substrates to induce larval settlement.

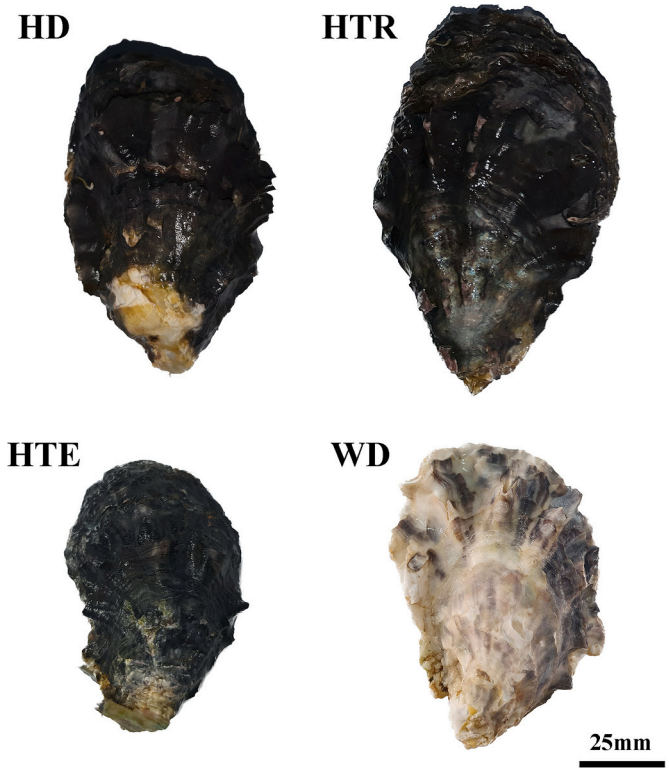

Fig. 1. The four experimental groups of diploids (HD), triploids (HTR) and tetraploids (HTE) of 'Haida No. 3' line and wild diploids (WD) of *C. gigas* on 480 day.

2.3. Spat nursery and grow-out

After most larvae metamorphosed into spats, newly settled spats were moved to an outdoor rearing pond (22–26 °C, 30–31 psu) for three weeks to protect them from wild spat contamination. In July 2021, the juvenile oysters from each group were transferred and cultivated in two sites along the coast of Shandong Province: Rongcheng (37.11° N, 122.35° E), Rushan (36.45° N, 121.42° E). In Rongcheng, farming area has an average annual water temperature of 12.95 °C, salinity of 32.20 psu, pH of 8.15, wave height of 0.3 m and tidal range of 0.7 m; while in Rushan, farming area has an average annual water temperature of 14.20 °C, salinity of 30.00 psu, pH of 8.00, wave height of 0.5 m and tidal range of 2.4 m (Han et al., 2020). In two culture environments, oyster spats were initially fixed with nylon ropes and cultured by long-line method for three months. In October, three replicate cages with 10 layers were deployed for each group in two environments, with 30 oysters per layer for field grow-out. Each cage was cleaned regularly, and the dead oysters were discarded afterward. Otherwise, the density of experimental cages was reduced monthly so that each replicate would have a similar volume and biomass.

2.4. Sampling and measurements

During the grow-out period, shell heights of 30 oysters were measured by a digital caliper (0.01 mm) for each group on 120 d, 210 d,

300 d, 390 d and 480 d, and wet weights were determined using electronic scales (0.01 g) on 210 d, 300 d, 390 d and 480 d. The triploid (or tetraploid) rate of each group was the ratio of triploid (or tetraploid) individuals to the total number of samples. The cumulative survival rate of each group was calculated at measurement time points according to the follow formula (Qin et al., 2019):

$$Z_t\ (\%) = (N_t/N_0) \times 100$$

Z_t (%) represents the cumulative survival rate of oysters at sampling time t; N_t represents the number of survived oysters at time t; N_0 represents the total number of oysters per layer in October 2020.

2.5. Ploidy analysis

The DNA ploidy levels of tetraploid parents, D-larvae, pediveliger, juvenile and adult oysters were detected using flow cytometer (Beckman Coulter CytoFLEX). A non-lethal hemolymph and gills sample was used for detecting tetraploid parental ploidy. The first ploidy test was performed using hemolymph extracted with a syringe from the adductor muscle. The second ploidy examine was carried out using gills. The tetraploid parents with consistent ploidy results from the two tests were used for breeding. For flow cytometry, 4000–8000 larvae from each group were collected to identify their composite ploidy, and gills of juvenile or adult progeny were randomly selected for detecting DNA ploidy. Larvae, hemolymph or gill samples were taken and placed in 0.1 M phosphate buffer solution (PBS) into 1.5 mL centrifuge tubes. The larval, hemolymph or gill suspensions were disaggregated by repeated aspiration through a 26G needle-equipped 1-mL syringe. Then, samples were filtered through a 42-μm nylon screen, and stained with DAPI solution (2 μg/mL, Sigma) for 15 min (Jiang et al., 2022). Finally, the ploidy level of each group was determined by flow cytometer. The relative DNA content of the diploids was used as a control for analyzing progeny ploidy levels (Fig. 2).

2.6. Comparison of sex ratio, fertility and gonad development

To evaluate the sex ratio and fertility of each group, fifty oysters were chosen randomly from the HD, HTR and HTE during the breeding season at two sites. The sex ratio of each group was determined by the optical microscope. Oysters were classified as female, male, hermaphrodite or asexual basing on the types of germ cells present. Each sample's gonad tissue was fixed in Bouin's solution for 18–24 h and then preserved in 70% ethanol. Afterward, the samples were dehydrated with series of ethanol, embedded in paraffin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin. The finished gonad sections were observed and photographed by a microscopy (Olympus BX53).

2.7. Data analysis

The phenotypic trait data (shell height, wet weight and survival rate) of all groups were expressed as mean ± standard deviation and analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test by SPSS 25.0 software. Differences with $P < 0.05$ are considered statistical significance. A two-factor analysis of variance (ANOVA) was conducted to determine the effects of the ploidy (diploid, triploid and tetraploid) and the environments (Rongcheng and Rushan) and their interaction on growth and survival rate with the following statistical model (Callam et al., 2016):

$$Y_{ijk} = \mu + E_i + P_j + (E_i \times P_j) + \delta_{ijk}$$

where Y_{ijk} = dependent variable (shell height, wet weight or cumulative survival rate), μ = overall mean, E_i = sites effect (Rongcheng and Rushan), P_j = ploidy effect (diploid, triploid or tetraploid), $E_i \times P_j$ = interaction effect between environments and ploidy, and δ_{ijk} = residual error.

To estimate the aquaculture traits of triploid 'Haida No. 3' (HTR) compared to diploid 'Haida No. 3' (HD), the triploid advantage (*TA*) was calculated using the follow formula (Qin et al., 2019):

$$TA\ (\%) = (\text{HTR–HD})/\text{HD} \times 100$$

where HTR and HD indicate the phenotypic value (shell height, wet weight or cumulative survival rate) of the diploid and triploid of 'Haida No. 3' line, respectively.

To assess the farmed characters of HD compared to WD, the selective advantage (*SA*) was calculated with the following equation:

$$SA\ (\%) = (\text{HD–WD})/\text{WD} \times 100$$

where HD and WD indicate the phenotypic value (shell height, wet weight or cumulative survival rate) of the diploid of 'Haida No. 3' line and wild diploid oysters, respectively.

3. Results

3.1. Growth traits at the two sites

The changes of shell height and wet weight of each group at two sites were shown in Fig. 3. At Rongcheng site, the shell height of HTR was always the highest from day 120–480 and had a significant difference

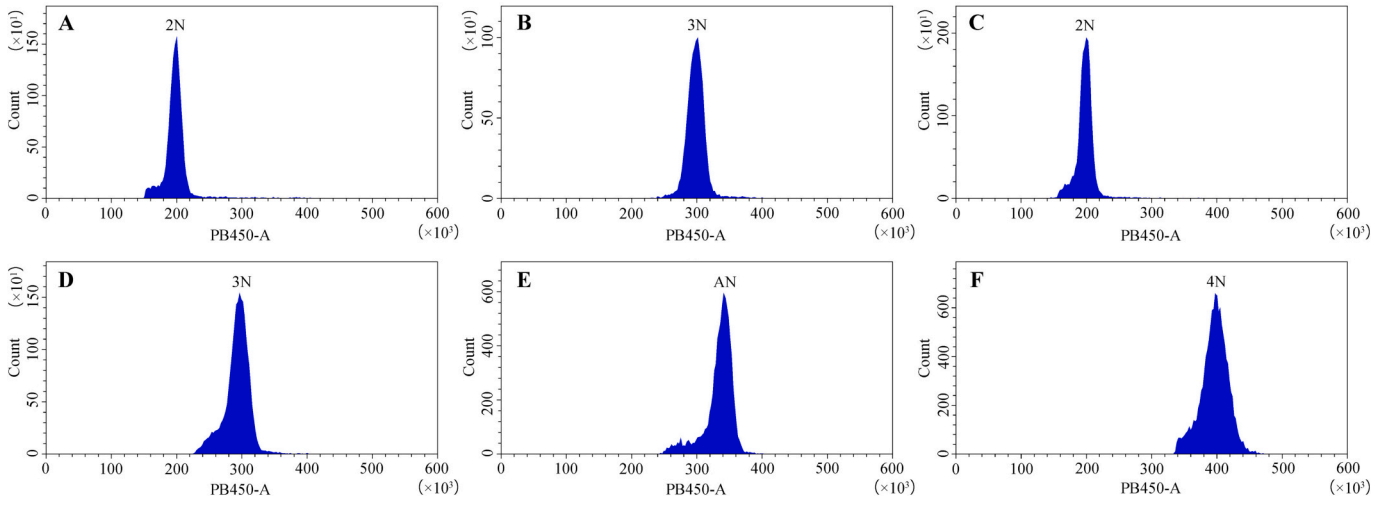

Fig. 2. Relative DNA content and ploidy level of each experimental groups detected by the flow cytometry. Note: A, HD group; B, HTR group; C—F, HTE group.

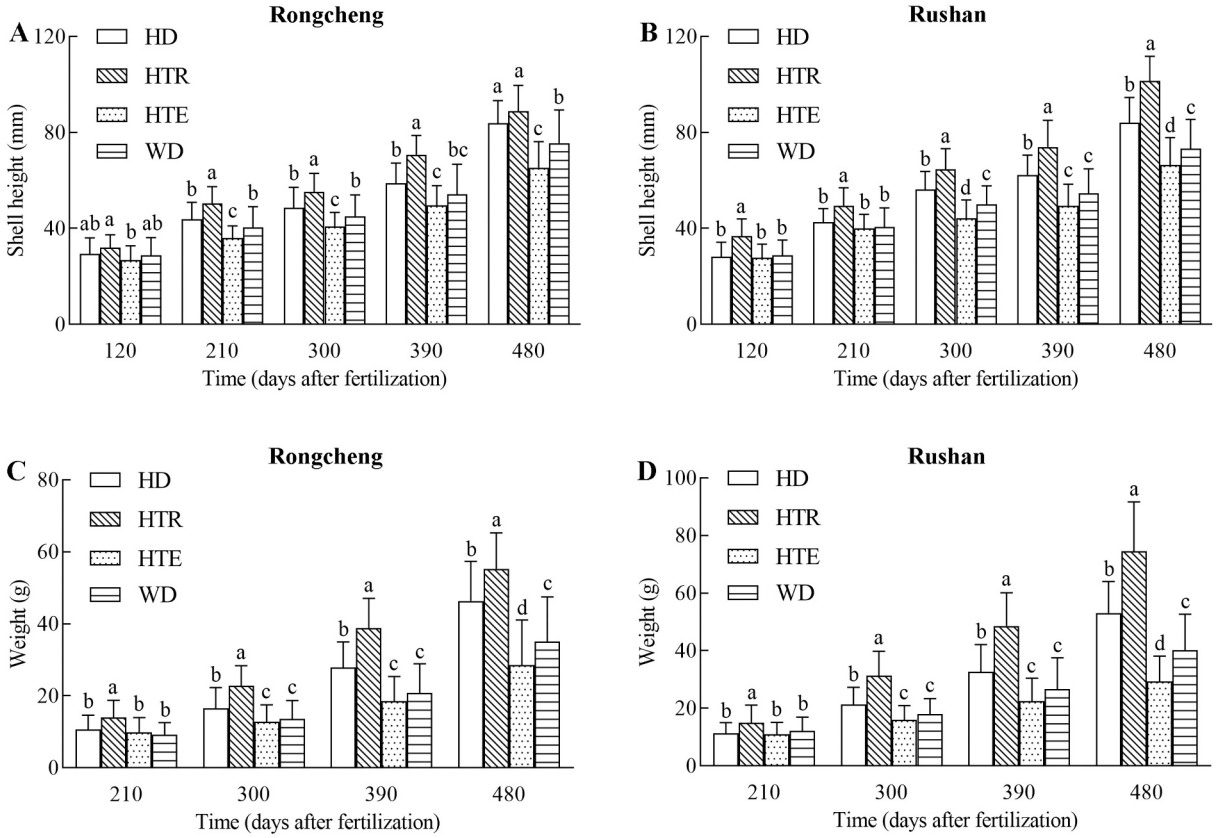

Fig. 3. Shell height and wet weight for four experimental groups from the day 120 to 480 at Rongcheng and Rushan. Different superscript letters at the same time indicate significant difference ($P < 0.05$).

from other groups on days 210, 300, and 390 ($P < 0.05$). Conversely, HTE was the lowest and significantly lower than the other 3 groups ($P < 0.05$). The shell heights of HD and WD were ranked between HTR and HTE, and HD was always higher than that of WD. The triploid advantage of shell height was consistently positive, ranging from 5.96% to 20.28%. At Rushan site, the shell height of HTR was also always significantly higher than others ($P < 0.05$), and HTE had always the lowest shell height. On day 480, the significant decreasing order of shell height was HTR (101.48 ± 10.11 mm) > HD (84.08 ± 10.34 mm) > WD (73.20 ± 12.03 mm) > HTE (66.48 ± 11.18 mm) ($P < 0.05$, Fig. 3B). The triploid advantage and selective advantage of shell height increased with time, reaching 20.70% and 14.86% at day 480, respectively (Table 1). The effect of ploidy on shell height was significant from day 210 to 480, but the interaction effect between environment and ploidy was only significant at day 210 and day 480 ($P < 0.05$, Table 2).

Wet weight measurements were taken from day 210 onwards. At Rongcheng site, the wet weight of HTR was significantly larger ($P < 0.05$), reaching 55.30 g ± 9.18 g at day 480, with the triploid advantage of 19.22%. The wet weights of HD and WD were heavier than HTE from day 210 to 480. At Rushan site, the wet weight of HTR was also always significantly higher than others ($P < 0.05$) and that of HTE was always the lowest. On day 480, the significant decreasing order of wet weight was HTR (74.50 ± 16.92 g) > HD (53.11 ± 10.75 g) > WD (40.25 ± 12.24 g) > HTE (29.40 ± 8.61 g) ($P < 0.05$, Fig. 3D). The triploid advantage and selective advantage of wet weight reached 40.28% and 31.96% at day 480 (Table 1). The effects of environment and ploidy on wet weight were highly significant from day 300 to 480 ($P < 0.001$), but the interaction effect between environment and ploidy was not significant ($P > 0.05$, Table 2).

3.2. Survival traits at the two sites

The survival rates for all groups continued to decrease throughout the entire period, with Rongcheng showing the highest rates. At Rongcheng site, the survival rates of HD, WD and HTR were always significantly higher than HTE ($P < 0.05$). On day 480, the decreasing cumulative survival rates of WD, HTR, HD and HTE were 73.33%, 71.11%, 68.89% and 48.89%, respectively (Fig. 4A). Meanwhile, the triploid advantage and selective advantage of cumulative survival rate were 3.23% and − 6.06% (Table 1). At Rushan site, the cumulative survival rate of HTE was the lowest in all periods. The difference of cumulative survival rate varied over time, with the cumulative survival rate of HTR being lower than the two diploid groups on days 210 and 300, but higher on days 390 and 480. Thus, although generally, the

Table 1
Triploid advantage (*TA*) and selective advantage (*SA*) for shell height, wet weight and cumulative survival in HD, HTR and WD from the day 210 to 480.

Items	Sites	Shell height				Wet weight				Cumulative survival
		210th	300th	390th	480th	210th	300th	390th	480th	
TA (%)	Rongcheng	15.28	13.59	20.28	5.96	32.22	38.01	39.50	19.22	3.23
	Rushan	15.99	15.03	18.59	20.70	33.20	47.70	48.64	40.28	29.27
SA (%)	Rongcheng	8.18	8.15	8.60	11.01	16.11	22.24	33.79	32.07	−6.06
	Rushan	5.13	12.32	14.43	14.86	−7.25	18.06	22.62	31.96	−6.82

Table 2
Two-way analyses of variance testing for environment by ploidy interaction effects on shell height, wet weight and cumulative survival.

Time	Effect	Shell height				Wet weight				Cumulative survival			
		d.*f.*	MS	*F*-value	*P*-value	d.*f.*	MS	*F*-value	*P*-value	d.*f.*	MS	*F*-value	*P*-value
210th	Environment	1	0.004	0.979	0.324	1	0.055	2.052	0.154	1	0.006	2.941	0.112
	Ploidy	2	0.215	51.775***	0	2	0.372	13.874***	0	2	0.067	31.853***	0
	Environment × ploidy	2	0.015	3.691*	0.027	2	0.005	0.174	0.84	2	0.003	1.441	0.275
	Error	174	0.004	–	–	174	0.027	–	–	12	0.002	–	–
300th	Environment	1	0.138	32.013***	0	1	0.630	36.53***	0	1	0.025	8*	0.015
	Ploidy	2	0.343	79.3***	0	2	1.194	69.216***	0	2	0.052	16.94***	0
	Environment × ploidy	2	0.006	1.376	0.255	2	0.003	0.146	0.865	2	0	0.14	0.871
	Error	174	0.004	–	–	174	0.017	–	–	12	0.003	–	–
390th	Environment	1	0.008	1.698	0.194	1	0.307	19.161***	0	1	0.076	11.194**	0.006
	Ploidy	2	0.423	91.153***	0	2	1.766	110.196***	0	2	0.039	5.711*	0.018
	Environment × ploidy	2	0.003	0.742	0.478	2	0.004	0.226	0.798	2	0.006	0.886	0.437
	Error	174	0.005	–	–	174	0.016	–	–	12	0.007	–	–
480th	Environment	1	0.022	5.866*	0.016	1	0.227	14.951***	0	1	0.113	8.870*	0.012
	Ploidy	2	0.411	107.577***	0	2	2.081	137.291***	0	2	0.076	5.933*	0.016
	Environment × ploidy	2	0.015	3.978*	0.02	2	0.039	2.589	0.078	2	0.006	0.494	0.662
	Error	174	0.004	–	–	174	0.015	–	–	12	0.013	–	–

The *P*-value associated with each *F*-value are indicated by asterisks (* – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$; NS – not significant).

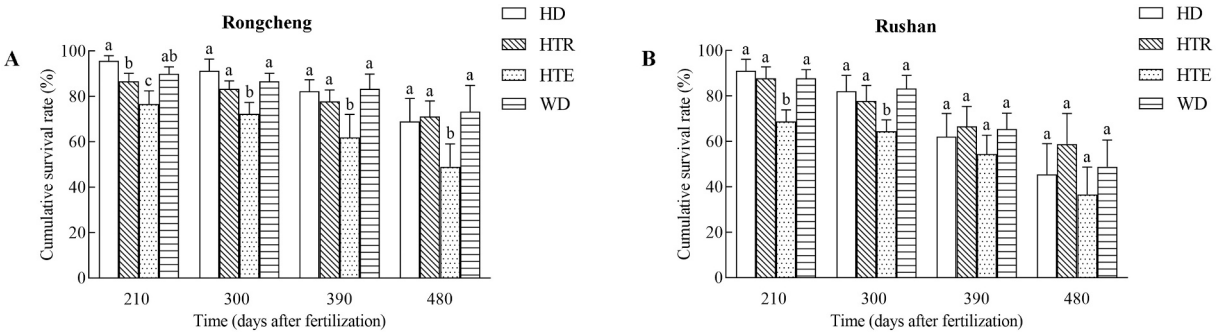

Fig. 4. Cumulative survival rate for four experimental groups from the day 210 to 480 at Rongcheng and Rushan. Different superscript letters at the same time indicate significant difference ($P < 0.05$).

survival rate of HTR was similar to HD, on the day 480, HTR showed an advantage over HD in terms of cumulative survival, with the triploid advantage rate of 29.27% (Table 1). The cumulative survival was significantly affected by environment and ploidy ($P < 0.05$) from day 300 to 480, but the interaction effect between environment and ploidy was not significant ($P > 0.05$, Table 2).

3.3. Comparison of sex ratio, fertility and gonad development

According to sex identification, HTR contained more females than males, while HTE contained more males than females (Table 3).The sexes of HTR and HTE were presented at two sites in the histological analysis: HTR in Rongcheng site (female: 32%; male: 6%; hermaphrodite: 4%; asexual: 58%), HTR in Rushan site (female: 36%; male: 4%; hermaphrodite: 6%; asexual: 54%), HTE in Rongcheng site (female: 36%; male: 62%; hermaphrodite: 2%), HTE in Rushan site (female: 40%; male: 60%) (Table 3).

Histomorphometric analysis revealed HD and HTE were fully fertile at one year of age and contained functional oocytes (Fig. 5A, G) or spermatocytes (Fig. 5B, H). The size of HTE eggs was greater than that of diploids. However, the majority of HTR displayed abnormal gametogenesis or few gametes in the gonia. A few HTR had morphologically normal germ cells, but fewer than those of diploid oysters (Fig. 5C, D). In addition, hermaphrodite HTR contained vitellogenic oocytes and spermatogenic cells in the gonads (Fig. 5E).

3.4. Variations in ploidy composition

The flow cytometry analysis indicated that the diploid and triploid rates were 100% at both sites and the ploidy composition remained constant throughout the life cycle. Meanwhile, the ploidy level of HTE was 100% tetraploid until day 120. From day 120 to 480, the tetraploid proportion in HTE gradually decreased at two sites. On day 480, the ploidy composition of HTE in Rongcheng site was 3.33% diploid, 5%

Table 3
The sex composition of HD, HTR, HTE and WD at gamete maturity phase at two different sites.

Site	Group	Female	Male	Hermaphrodite	Asexual	Total
Rongcheng	HD	24 (48%)	26 (52%)	–	–	50 (100%)
	HTR	16 (32%)	3 (6%)	2 (4%)	29 (58%)	50 (100%)
	HTE	18 (36%)	31 (62%)	1 (2%)	–	50 (100%)
	WD	26 (52%)	24 (48%)	–	–	50 (100%)
Rushan	HD	28 (56%)	22 (44%)	–	–	50 (100%)
	HTR	18 (36%)	2 (4%)	3 (6%)	27 (54%)	50 (100%)
	HTE	20 (40%)	30 (60%)	–	–	50 (100%)
	WD	29 (58%)	21 (42%)	–	–	50 (100%)

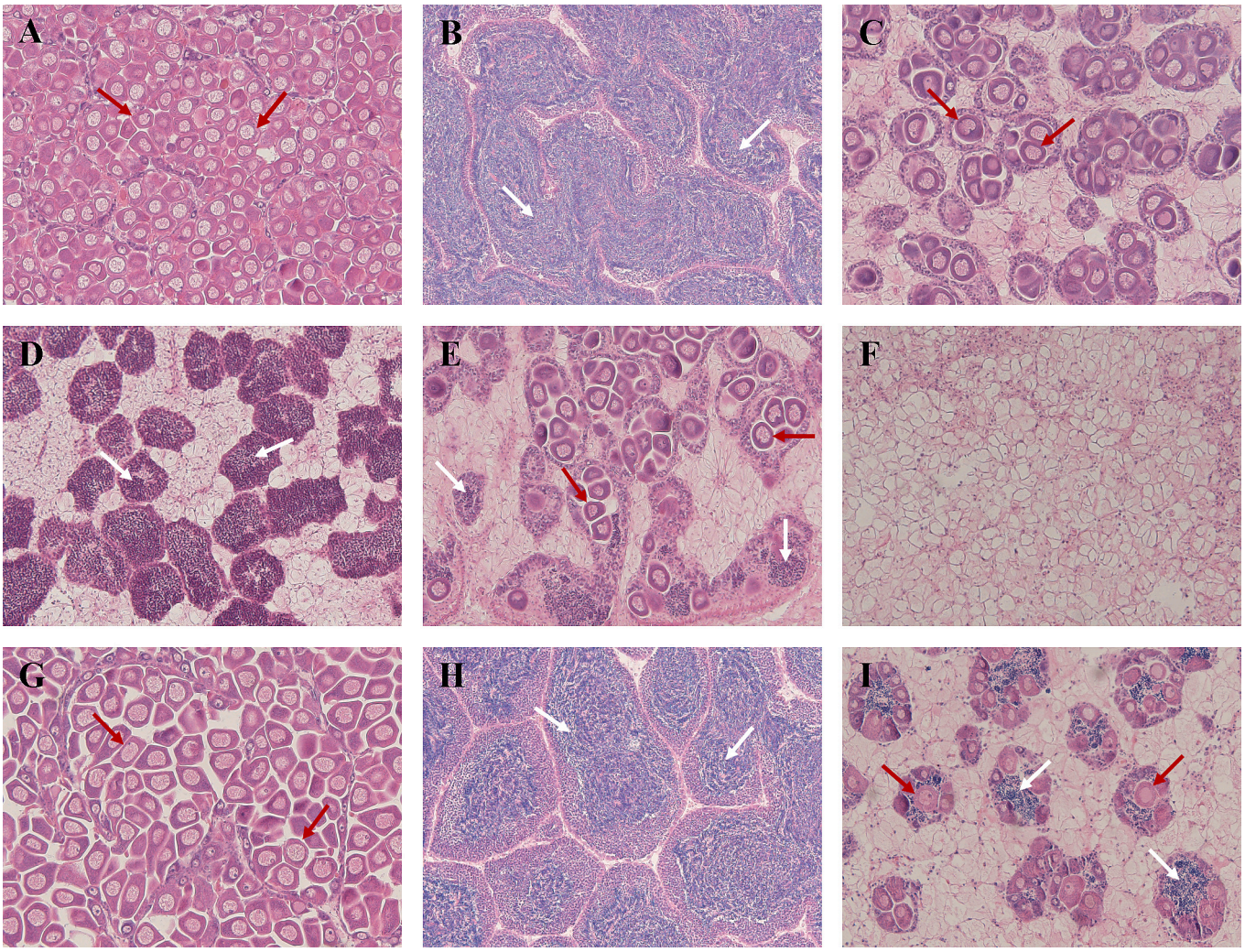

Fig. 5. Gonadal structure of diploid, triploid and tetraploid oysters under 20 × objective lens. Note: A, Female in HD group; B, Male in HD group; C, Female in HTR group; D, Male in HTR group; E, Hermaphrodite in HTR group; F, Asexual in HTR group; G, Female in HTE group; H, Male in HTE group; I, Hermaphrodite in HTE group. Therein, red arrows and white arrows indicate eggs and sperm, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

triploid, 85% tetraploid and 6.67% aneuploid, while that of Rushan site was 6.67% diploid, 5% triploid, 82% tetraploid and 6.67% aneuploid (Fig. 2, Table 4).

4. Discussion

Selective breeding and polyploid breeding have been used to improve performance and organoleptic quality in aquaculture, thus artificially producing offspring with morphologic and genotypic improvements (Nell, 2002; Gjedrem and Rye, 2018). In previous studies, polyploid offspring can inherit the superior traits from the selected population (Leeds and Weber, 2019; Li and Li, 2022). In this study, we explored the possibility of breeding a novel line with rapid-growth and black-shell characteristics, using polyploid breeding basing on selective populations.

4.1. Comparative growth and survival traits

4.1.1. Comparison with HD and WD

HD were bred through 4 generations of family selection and 2 generations of mass selection for black-shell and rapid-growth characteristics (Xu et al., 2019). In this study, the shell height and wet weight of HD at both sites were higher than those of WD from day 210 to 480. On the day 480, the selective advantage (*SA*) for shell height and wet weight was 11.01% and 32.07% at Rongcheng, 14.86% and 31.96% at Rushan, respectively. Similarly, after one or two generations of mass selection, selected Sydney rock oysters had greater whole weight than nonselected controls by 4% and 18%, respectively (Nell et al., 1999). However, both sites showed lower cumulative survival rate for HD than for WD at days 390 and 480, but not significant ($P > 0.05$). This is due to the fact that the breeding program for the 'Haida No. 3' line only focused on growth rate and black shell color, rather than survival rate (Han et al., 2020).

4.1.2. Comparison with diploids and triploids of 'Haida No. 3'

The majority of studies observed significant growth advantages for triploid oysters when compared to diploid oysters (Nell, 2002; Jiang et al., 2022; Wadsworth et al., 2019; Guo et al., 1996). This study also examined whether there was evidence of triploid advantage in growth under different cultural conditions. We found no significant ploidy-environment interaction, possibly indicating stable triploid performance in 'Haida No. 3' line. At any time point, the shell height and wet weight of HTR were larger than HD, with the triploid advantage of 5.96–20.7% and 19.22–48.64%, respectively. Theoretically, the faster growth of triploids may be attributed to three main factors. Firstly, increased heterozygosity was suggested as a reason for triploid advantage (Stanley et al., 1984; Comai, 2005). Heterozygosity levels were

Table 4
The ploidy composition of HD, HTR and HTE at different phases at two different sites.

Site	Group	DNA content	2th	20th	120th	210th	480th
Rongcheng	HD	2 N	100%	100%	100%	100%	100%
		3 N	–	–	–	–	–
		4 N	–	–	–	–	–
		AN	–	–	–	–	–
	HTR	2 N	–	–	–	–	–
		3 N	100%	100%	100%	100%	100%
		4 N	–	–	–	–	–
		AN	–	–	–	–	–
	HTE	2 N	–	–	–	–	3.33%
		3 N	–	–	–	6.67%	5%
		4 N	100%	100%	100%	90%	85%
		AN	–	–	–	3.33%	6.67%
Rushan	HD	2 N	100%	100%	100%	100%	100%
		3 N	–	–	–	–	–
		4 N	–	–	–	–	–
		AN	–	–	–	–	–
	HTR	2 N	–	–	–	–	–
		3 N	100%	100%	100%	100%	100%
		4 N	–	–	–	–	–
		AN	–	–	–	–	–
	HTE	2 N	–	–	–	–	6.67%
		3 N	–	–	–	6.67%	5%
		4 N	100%	100%	100%	87%	82%
		AN	–	–	–	6.67%	6.67%

2 N, 3 N, 4 N, AN indicate diploids, triploids, tetraploids and aneuploids, respectively.

confirmed to be strongly and positively correlated with the overall body sizes in diploids, 3nCB, and 3nDT triploids (Wang et al., 2002). A breeding strategy that increases heterozygosity may be beneficial if heterozygosity is essential to triploids. Secondly, the cell gigantism hypothesis proposed that the increase of triploid cell volume was not compensated by reduced cell numbers. With the constant number of cells, the increase in cell size inevitably led to rapid growth of triploids (Guo and Allen, 1994; Piferrer et al., 2009). Thirdly, triploids are considerably less fertile than diploids, meaning that the energy was saved and diverted to growing shells and somatic cells rather than to energy for gonadal development (Piferrer et al., 2009; Kesarcodi-Watson et al., 2001). According to this hypothesis, triploids would benefit during the breeding season when diploids were reduced the growth rate. In addition, the culture environment (food availability, temperature and salinity) and parental origin factors could also affect triploid oyster growth (Wadsworth et al., 2019; Qin et al., 2022; Brake et al., 2004).

The cumulative survival of diploid and triploid Pacific oysters has been reported to be conflicting. Some studies found that the cumulative survival of triploids was higher than that of diploids (Gagnaire et al., 2006; Nell and Perkins, 2005), but some studies have reported lower cumulative survival for triploid oysters (Houssin et al., 2019; Ibarra et al., 2017). In this study, the survival rates for HTR at both sites were higher than that of HD. It is demonstrated that the cumulative survival of diploids and triploids varies greatly, depending on the culture environment and the stage of gonad development (Gagnaire et al., 2006; Matt et al., 2020). Summer mortality in oysters was related to water temperature above 20 °C, which may also provide suitable condition to the growth of pathogens (Petton et al., 2013; Gagnaire et al., 2006). Diploid oysters with high mortality allocated the majority of energy to gamete production and a minimal amount to the defense system. Since triploids used relatively small amount of energy for gamete synthesis, which may allocate energy to hemocyte activities and defense mechanisms (Jouaux et al., 2013; Gagnaire et al., 2006).

4.1.3. Comparison with diploids and tetraploids of 'Haida No. 3'

The tetraploids of *C. gigas*, *C. angulata* and *C. virginica* were smaller than their diploid counterparts (Qin et al., 2022; Guo et al., 2002; Zhang et al., 2022). It was observed that HTE were lower than HD in terms of shell height, wet weight and cumulative survival at both sites. Despite the fact that the sizes of HTE were small, the rationale remained unclear. The researchers proposed that the poor performance could be the result of inbreeding, since the parental tetraploid oysters were siblings (Guo et al., 1996; Piferrer et al., 2009). In addition, the smaller individuals may also be caused by an increase in growth energy for the development of giant cells. (Qin et al., 2022). Generally, neopolyploids had lower survival or viability in synthetic populations, while tetraploid aquatic animals generally had more vigorous than diploid progenitors. Generally, neopolyploids had lower survival or viability in synthetic populations due to rapid adaptation to novel environments, while tetraploid animals generally had an advantage in heterosis, gene redundancy and asexual reproduction. Heterosis caused polyploids to be more viable than their diploid counterparts, whereas gene redundancy protected polyploids from the mutations' adverse effect (Comai, 2005).

4.1.4. Different performance in two culture sites

It is well known that the performance of oysters is highly influenced by environmental differences (Brown and Hartwick, 1988; Qin et al., 2022). In this study, the growth characteristics of oysters in Rushan were mostly better than those in Rongcheng. Numberous studies confirmed that the growth rate of *C. gigas* was highly site-specific and correlated with food availability and temperature (Brown and Hartwick, 1988; Callam et al., 2016). Compared with Rongcheng, Gao et al. (2006) found higher phytoplankton abundance and primary productivity in Rushan. A combination of large waves from the Yellow Sea and high average annual temperature may have contributed to the high primary productivity and richness of nutrients in Rushan (Jiang et al., 2022). In contrast, for all groups, the cumulative survival rates in Rongcheng were higher than in Rushan at day 480. A possible explanation is that high average annual temperature and large waves reduce the survival rate of oysters in Rushan. Gagnaire et al. (2006) showed that elevated temperature induced increased mortality of haemocyte in *C. gigas*. Ibarra et al. (2017) reported that the survival of *C. gigas* was low at high temperatures regardless of ploidy levels.

4.2. Polyploid breeding basing on selective populations

Selective breeding was an effective method of obtaining organisms with genetically desirable characteristics through controlled mating using selected parents (Chavanne et al., 2016; Gjedrem et al., 2012). The Pacific oyster 'Haida No. 3' line was obtained through four generations

family selection to fix black-shell color and two generations mass selection to promote growth rate (Xu et al., 2017). Improved traits (growth, disease resistance, etc.) could be transferred from selected diploids to polyploids (Dégremont et al., 2016; Hand et al., 2004). In this study, HTR and HTE still preserved the same black-shell color and rapid growth as *C. gigas* 'Haida No. 3' line, indicating that these traits were not affected by the ploidy change. The growth and shell color of tetraploids could be improved by selective breeding, which would require additional breeding time. Undoubtedly, it was preferable to use selective diploid populations with rapid growth, disease resistance or other important features as base populations for creating tetraploids (Li and Li, 2022). Once the tetraploid breeding lines had been established, tetraploid males could contribute two-thirds of the genetic complement of their triploid offspring (Nell, 2002).

4.3. Ploidy stability

Tetraploid oysters were created artificially and had been shown to possibly undergo chromosome loss and transform into triploids, diploids, aneuploids or mosaics (McCombie et al., 2005; Comai, 2005). Some researchers proposed that the mechanism of chromosomes loss in tetraploid *C. gigas* called chromosome clumping, and the unnatural clumping of chromosomes may be caused by uneven chromosomes segregation (Zhang et al., 2010a; Zhang et al., 2014). A higher percentage of aneuploid and mosaic oysters was observed among individuals with more chromosome clumps in the cells (Zhang et al., 2010a). The phenomenon increased with time and environmental stress and might lead to some or all cells reverting to diploidy (20 chromosomes) or triploidy (30 chromosomes) (Hand et al., 1999). In this study, HTR remained unchanged at 100% triploid throughout the culture period, but tetraploid proportion in HTE gradually decreased after day 120, which formed a small number of diploids, triploids and aneuploids except for tetraploids at day 480. The stable tetraploid population played a crucial role in oyster culture, while aneuploid tetraploid oysters may result in a tendency for their progenies to lose chromosomes and revert to lower ploidy levels (McCombie et al., 2005). In this study, the ploidy of the parental tetraploids was detected twice (hemolymph and gills) to obviate the influence of aneuploids or mosaics on the offspring. Possibly, if the reversion process occurred as a result of mitotic errors, reversion would be greatest in tissues with the highest mitotic rate (Zhang et al., 2010b). Hemolymph tissues with high mitotic activity exhibited the greatest levels of reversion (Matt and Allen, 2014), thus the ploidy of hemolymph tissue was essential to detect. Consequently, haemolymph (non-lethal method) was taken from the internal collecting muscle with a syringe for primary screening of tetraploid parents. Gills were examined after dissection and used to review the tetraploids (Li and Li, 2022). Tetraploid parents with consistent results from both ploidy tests were used in this study. The true tetraploid parents with large size of broodstock and a balanced sex ratio would help to increase the effective population size and maintain a stable tetraploid population.

4.4. Fertility and gonad development analysis

Consistent with previous reports (Guo and Allen, 1997; Qin et al., 2022; Zhang et al., 2022), the histological sections of HTE provided convincing evidence for normal gonad development in this study. Tetraploid females had similar gonadal output as normal diploid oysters, considering the increased volume of the eggs produced by tetraploid oysters (Guo and Allen, 1997). The high fecundity of tetraploids may be caused by the "high-fecundity genes" inherited from their parents and the presence of checkpoints to ensure an even-numbered chromosome set before spawning (Guo and Allen, 1997). The "checkpoint hypothesis" was supported by the observation in rainbow trout that tetraploid females produced large numbers of eggs while triploids did not (Chourrout and Nakayama, 1987; Chourrout et al., 1986).

Generally, triploid oysters have diminished reproductive capacity owing to problems with gonad development and chromosome pairing (Normand et al., 2008; Yang et al., 2022; Stanley et al., 1981). In this study, the majority of HTR presented gonad atrophy and gamete abnormalities, and a low proportion of triploids were fertile individuals with some normal male and female gametogenesis, indicating that triploid offspring could greatly decrease the reproductive ability of diploid parents. The high variation of gonad development in triploids was exacerbated by a lack of synchrony between follicle development and gamete production (Matt and Allen, 2021). Several factors affected the gonad development of triploids, including parental origin, temperature, food availability and culture time (Suquet et al., 2016; Gong et al., 2004; Houssin et al., 2019; Nell, 2002; Julien et al., 2009). Hence, the sterility of triploid oysters was generally considered the energy reallocation from gametogenesis to body growth (Guo and Allen, 1994), allowing them to increase growth rate, improve meat quality and permit year-round marketability. In addition, the proportion of males was higher in tetraploid oysters than females in this study, but lower in triploid oysters than females. The phenomenon could result from "disruption of sex differentiation mechanisms" and "environmental control of sex determinism" (Dheilly et al., 2014; Julien et al., 2009; Jouaux et al., 2013).

5. Conclusion

The growth, survival and gonad development in diploids, triploids and tetraploids of *C. gigas* 'Haida No. 3' line were compared under two different culture environments, and the interaction between site and ploidy was analyzed. HD had higher shell height and wet weight than WD at both sites. HTR showed growth and survival advantages compared with HD during the harvest season. Gonad development of HTR exhibited gonad atrophy and gamete abnormalities during the breeding season. Furthermore, HTE had lower growth and survival rates than diploids, but were similar to diploids in terms of fertility. The stable tetraploid population played a vital role in oyster farming, with exceeding 80% of tetraploids after two ploidy detections on parental tetraploids. HTR and HTE still retained the same black-shell color and rapid growth as diploid *C. gigas* of 'Haida No. 3' line, indicating that these traits could be stably inherited through the ploidy change. In summary, polyploidization based on selective lines of oysters can further improve economically important traits and realize great potential for commercial cultivation.

CRediT authorship contribution statement

Jianmin Zhou: Investigation, Conceptualization, Formal analysis, Writing – original draft. **Gaowei Jiang:** Investigation. **Chengxun Xu:** Supervision. **Xianchao Bai:** Investigation. **Qi Li:** Supervision, Conceptualization, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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