Growth, survival and gonad development of two new types of reciprocal triploid hybrids between *Crassostrea gigas* and *C. angulata*

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ABSTRACT

The allotriploid technology has been widely applied to aquaculture worldwide. To overcome the deficiency of low growth, poor taste and high mortality of diploid hybrids between *Crassostrea gigas* and *C. angulata* in summer, two new types of triploid hybrids were produced by inhibiting the polar body II using cytochalasin B. The growth, survival and gonad traits of triploid hybrids (TGA – *C. gigas* ♀ × *C. angulata* ♂ and TAG – *C. angulata* ♀ × *C. gigas* ♂), diploid hybrids (GA – *C. gigas* ♀ × *C. angulata* ♂ and AG – *C. angulata* ♀ × *C. gigas* ♂) and purebred crosses (GG – *C. gigas* ♀ × *C. gigas* ♂ and AA – *C. angulata* ♀ × *C. angulata* ♂) were evaluated. A high triploidy induction rate (> 95%) was found by ploidy analysis. The fertilization and hatch rates of triploid hybrids were significantly ($P < 0.05$) lower than that of diploid crosses. During the larval stage, the triploid advantage (*TA*) was positive in growth, despite was negative in survival. The triploid hybrids exhibited superior performance during the grow-out stage. Across three culture environments (Rongcheng, Rushan and Huangdao), triploid hybrids performed better than diploid hybrids in growth with an increasing value of *TA* over time. The incremental survival rates of triploid hybrids, especially that of TGA, were significantly ($P < 0.05$) higher than that of diploid hybrids at Rushan and Huangdao in summer. The *TA* in cumulative survival rate ranged from 13.80% to 36.04% among environments. A positive yield advantage for diploid and triploid hybrids was observed at Rongcheng (mid-parent heterosis *H*: 114.11%; *TA*: 35.76%), Rushan (*H*: 148.22%; *TA*: 48.78%) and Huangdao (*H*: 138.96%; *TA*: 60.22%). Additionally, most of the triploid hybrids exhibited high sterility during reproduction phase. These findings demonstrated that triploid hybrids between *C. gigas* × *C. angulata* had obvious advantage in growth, survival and yield, which have important application value for commercial oyster cultivation in northern China.

1. Introduction

Hybridization is an important method utilized in livestock breeding for altering the genotypes and phenotypes of the cultured species (Mallet, 2007). In aquaculture, interspecies hybrids have been commercially used to improve growth rate, disease resistance, environmental tolerance and nutrient value (Bartley et al., 2001). For example, the hybrid "bester", produced by hybridizing the beluga (*Huso huso*) ♀ and sterlet (*Acipenser ruthenus*) ♂, is characterized by fast growth, early sexual maturity and wide salinity tolerance (Fontana, 2002). Meanwhile, triploid breeding technology has been widely applied in aquaculture to produce sterile and high-yielding offspring. Currently, triploids have become an important part of seed production in various species, such as triploid crucian carp (*Carassius auratus*) (Hu et al., 2012) and triploid Pacific oysters (*C. gigas*) (Guo et al., 1996; Degremont et al., 2016; Yang et al., 2018). Triploids can be divided into autotriploids and allotriploids (triploid hybrids) based on the origin of chromosome sets. Interestingly, triploid hybrids are expected to exhibit enhanced heterosis in growth, survival and disease resistance relative to autopolyploids due to the combination of hybridization and polyploidization (Bingham et al., 1994; Yao et al., 2013; Zhang et al., 2014). Furthermore, the production of sterile triploids is an effective method to avoid the potential ecological risk resulting from hybrid escape and release into natural waters (Garcia-Abiado et al., 2002; Chen et al., 2009; Wang et al., 2020a, 2020b).

In aquatic animals, most studies on triploid hybrids have been reported in several fish species. As a taxon of vertebrates, fish have flexible chromosomal numbers and are therefore more likely to produce

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polyploids (Liu, 2010). Based on the crosses of female red crucian carp with male common carp (*Cyprinus carpio*), allotriploid crucian carp with faster growth rate and stronger disease resistance was produced (Liu et al., 2001). In salmonids, all-female triploid hybrids exhibited growth advantages at maturity and have been produced commercially (Galbreath and Thorgaard, 1995). However, induction and application of triploid hybrids is relatively rare in shellfish. Zhang et al. (2014) reported that the allotriploid between *C. hongkongensis* and *C. gigas* exhibited positive advantages in both growth and survival during grow-out. Similarly, growth advantage was also found in the allotriploid between *C. hongkongensis* and *C. ariakensis* (Qin et al., 2020). Nevertheless, high embryonic and larval mortalities of the triploid hybrids were found in both studies due to the poor gametic compatibility between the parental species (Hu et al., 2012; Qin et al., 2020). Hence, the hybridization of two closely related species may be a useful method to improve the early viability of triploid hybrids.

The Pacific oyster *C. gigas* is a global aquaculture species because of its rapid growth rate, strong disease resistance and high economic value (Dundon et al., 2011). In China, *C. gigas* was mainly cultured in the north, while its closely related species Portuguese oyster *C. angulata* farmed in the south due to its high tolerance to warm seawater (Ghaffari et al., 2019). In our previous studies, reciprocal hybrids between *C. gigas* and *C. angulata* showed high heterosis in growth, survival and thermotolerance (Jiang et al., 2021a, 2021b). Like diploid oysters, however, hybrid oysters usually exhibit low growth rate, poor taste and high mortality after spawning during summer months (Zhang et al., 2017), thereby failing to meet the industry's demand for year-round marketable oysters (Yang et al., 2018). The production of quasi-sterile triploid hybrids triploid hybrids may be an effective approach to solve this problem caused by sexual maturation. Meanwhile, *C. gigas* and *C. angulata* are two closely related species without fertilization barriers (Ren et al., 2016), thus there is great potential for hybridization to produce triploid hybrids with high early viability.

In the present study, we aimed to check the possibility of inducing triploid hybrids with high early viability by crossing *C. gigas* with *C. angulata* through inhibiting the release of polar body II after fertilization. We also test the potential utility of triploid hybrids by comparing growth, survival and gonad development of reciprocal diploid and triploid hybrids relative to their broodstocks under three different rearing environments in northern China.

2. Materials and methods

2.1. Parental stocks and conditioning

In May 2020, one-year-old *C. gigas* (shell height: 100.55 ± 11.29 mm) used as broodstock were collected from cultured stocks in Rongcheng, Shandong Province, China, and one-year-old *C. angulata* (shell height: 62.60 ± 12.33 mm) were obtained from wild stocks in Zhangzhou, Fujian Province (Fig. 1). *C. gigas* and *C. angulata* were identified based on cytochrome oxidase I as described by Wang and Guo (2008). Broodstocks were conditioned separately in a cement tank with seawater at 26.0–27.0 °C, salinity of 31 psu, constant aeration, daily water renewal of 30%, and daily feed a mixed diet of *Platymonas* sp. and *Chaetoceros calcitrans*.

2.2. Production of diploids and triploids

Triploid and diploid oysters were produced at the Haiyi hatchery in Laizhou, Shandong Province (Fig. 1). In June 2020, 45 mature females and 45 mature males of *C. gigas* and *C. angulata* were selected for this experiment. Eggs were obtained from 15 females of *C. gigas* or *C. angulata* by dissecting gonads and then divided equally into three 5-L beakers to produce diploid or triploid oysters. The eggs of each beaker were fertilized with a mixture of sperm from 15 *C. gigas* or 15 *C. angulata*. Triploid hybrids were induced by inhibiting the formation of the second polar body (PBII) of fertilized eggs using 0.5 mg/L cytochalasin B (CB) when about 30% of the hybrid zygotes extrude the first polar body (PBI) (Yang et al., 2018). After 15 min of CB treatment, the embryos be collected by filtering through a 15-μm screen, soaked in 0.005% DMSO solution for 40 min, and resuspended in fresh seawater for hatchery. The whole experiment was repeated three times, each of which consisted of two purebred crosses (GG – *C. gigas* ♀ × *C. gigas* ♂ and AA – *C. angulata* ♀ × *C. angulata* ♂), two diploid hybrid crosses (GA – *C. gigas* ♀ × *C. angulata* ♂ and AG – *C. angulata* ♀ × *C. gigas* ♂) and two triploid hybrid crosses (TGA – *C. gigas* ♀ × *C. angulata* ♂ and TAG – *C. angulata* ♀ × *C. gigas* ♂).

2.3. Larval rearing and setting

The culture of larvae was done following the methods described by Jiang et al. (2021b). The newly hatched D-larvae of each cross were collected by sieving and reared separately in 100-L buckets at 24–26 °C, ambient salinity (30–31 psu) and gentle aeration. Oyster seeds were fed
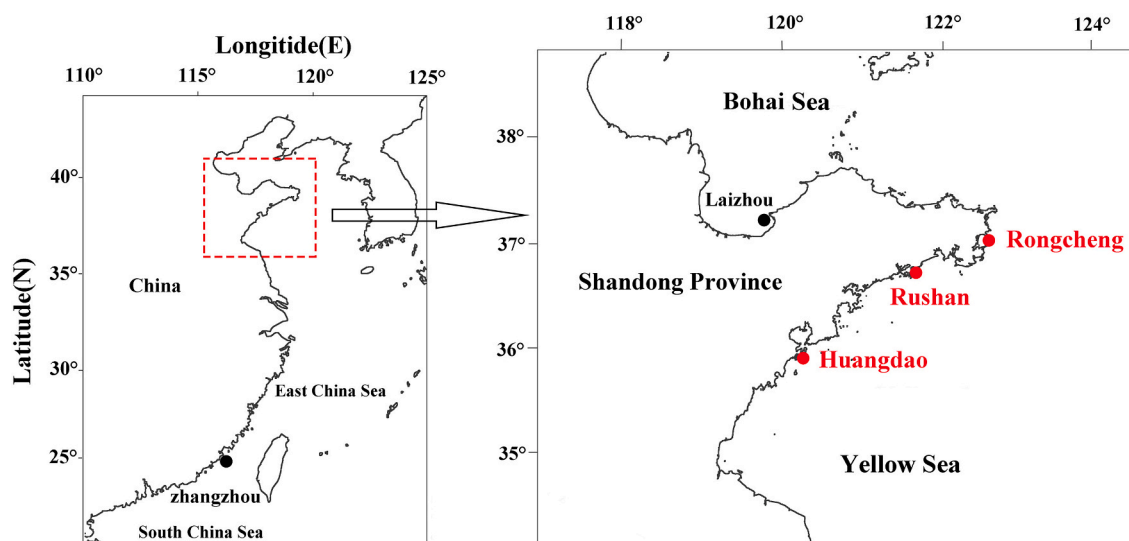

Fig. 1. Location of the broodstocks origin and oyster farming environments in China.

three times per day (~ 8 h apart) with *Isochrysis galbana* during the first 7 days and then with a 1:1 mixture of *Platymonas* sp. and *I. galbana* until metamorphosis. The larvae density of each cross was initially set to 1–2 larvae ml^{-1}. When 30% or more of the pediveliger larvae appear eye-spots, strings of scallop shells were placed in the buckets as substrate for settlement.

2.4. Spat nursery and grow-out

After all eyed larvae metamorphosed to spat, the collectors with attached spat were placed to an outdoor pond (24–27 °C, 30–31 psu) for two weeks to prevent contamination from wild oysters. In July 2020, seed oysters from each cross were transferred to three different culture environments along the coast of the Shandong Province (Rongcheng, Rushan and Huangdao) (Fig. 1). Environmental parameters for the three sea areas in this study were adopted from Han et al. (2020), Wang et al. (2021) or downloaded from National Marine Data Center, National Science & Technology Resource Sharing Service Platform of China (http://mds.nmdis.org.cn/) (Table 1). The average annual temperature was highest in Rushan, medium in Huangdao, and decreased to 12.95 °C in Rongcheng; the annual average wave height in Rushan is equal to that of Huangdao and greater than that of Rongcheng (Table 1). Oyster spat were initially cultivated using the long-line method at all culture environments, then artificially detached from scallop shells and counted in September 2020. Three replicate 10-layer cages were deployed for each cross at each environment and 30 individuals were placed in each layer for field grow-out. Only oysters that were confirmed to be triploids were selected to be placed in cages for growth and survival comparisons with diploids. As the juvenile grew, the cages were cleaned monthly and the dead oysters were discarded at every cage cleaning. Meanwhile, the density in each cage was reduced monthly to maintain the same volume and biomass of each replicate.

2.5. Sampling and measurements

Fertilization rate was calculated 1 h after fertilization by examining 150 eggs. The hatching rate was defined as the percentage of hatched D-larvae among zygotes. At 24 h postfertilization, the hatching rate was given by the ratio between number of hatched D-larvae and the total number of embryos, including fertilized but undeveloped embryos. During the planktonic larval stage, phenotypic traits were measured for each cross on days 4, 8, and 12 after fertilization. Shell height was recorded from 30 replicate specimens using an ocular micrometer fitted to a compound optical microscope. The number of larvae was counted in three randomly 50-mL samples. Cumulative survival rate was calculated as the percentage of the total numbers of live larvae on day 12 out of the number of D-shaped larvae on day 1. The incremental survival rate of larvae was calculated as described for spat. The incremental survival rate of larvae was defined as the percentage of the total numbers of live larvae on day 4, 8 and 12 out of the number of larvae on day 1, 4 and 8, respectively.

The size of individual oysters (wet weight and shell height) and mortality from each cross was monitored regularly in December 2020, March, June and September 2021. Dead oysters were recorded and cumulative survival was then assessed using the following formula (Qin et al., 2019):

Table 1
Annual mean parameters of surface seawater in Rongcheng, Rushan and Huangdao site.

Site	Temperature (°C)	Salinity (psu)	pH	Wave height (m)
Rongcheng	12.95	32.20	8.15	0.3
Rushan	14.20	30.00	8.00	0.5
Huangdao	13.51	31.10	8.03	0.5

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

where Z_t indicates that the cumulative survival rate at sampling point t; N_t is the number of live spat at sampling point t; N_0 is the total number of oysters per cage in September 2020.

The incremental survival rate was calculated by the following equation (Qin et al., 2019):

$$S_{t+1} \ (\%) = (N_{t+1}/N_t) \times 100$$

where S_{t+1} indicates the incremental survival rate of oyster at sampling point t + 1; N_{t+1} is the number of live spat at sampling point t + 1; t + 1 was the next sampling point after sampling point t.

Thirty oysters per cross were sampled randomly to measure the shell height (SH) with a vernier caliper (0.01 mm). The wet weight (TW) of 30 individuals per cage in September 2021 was randomly measured with an electronic scale (0.01 g). The final yield (Y) was calculated according to the following formula (Rawson and Feindel, 2012):

$$Y = Z \times N_0 \times TW$$

where Z is the cumulative survival rate of oyster at the last sampling point (September 2021).

2.6. Ploidy assessment

The ploidy levels of D-larvae at 24 h post-fertilization, larvae at day 12 and adult oysters in September 2021 were verified via a flow cytometer (Beckman Coulter). In each cross, 5000–6000 larvae were collected to determine their composite ploidy, and 100 gill fragments of adult oysters were randomly sampled to measure the relative DNA content. Larvae or gill samples of adult oyster from each cross were taken and placed in centrifuge tubes containing $1 \times$ phosphate buffer solution (solution contains 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4; pH = 7.4). The larval or gill suspensions were thawed and disaggregated by repeated aspiration with a 1-ml syringe fitted with a 26G needle, filtered through a 48-μm nylon sieve, stained with 2 μg/mL DAPI solution (Sigma) for 10 min. Subsequently, the ploidy of each cross was detected by flow cytometry. The relative DNA content of purebred crosses was used as controls in ploidy levels analysis of diploid and triploid hybrids.

2.7. Comparison of gonadal development and sex ratio

To evaluate the reproductive characteristics of diploid and triploid oysters, 80 adult oysters from purebred, diploid hybrid and triploid hybrid crosses were randomly selected in June 2021. The ratio of males to females in each cross was determined using an optical microscope. Gametogenesis levels during the sexual maturity stage were identified as previously reported (Allen and Downing, 1990). The gonad tissue slices were prepared according to the method adopted by Zhang et al. (2014) and photographed under a light microscope.

2.8. Statistical analyses

All growth-related metrics (shell height, survival rate and yield) are expressed as the mean ± standard deviation and analyzed using the SPSS 26.0 software. Homogeneity of variances among means was assessed using Levene's test for equality of variance errors. The fertilization rate, hatching rate and survival rate were arcsine transformed to stabilize the variances of errors. The shell height and yield were transformed to a logarithmic scale with base 10 to get normality and homoscedasticity. Statistical significance was accepted as $P < 0.05$. In each culture environment, growth-related parameter among each cross were analyzed via one-way analysis of variance (ANOVA) followed by multiple comparison Tukey test. A two-factor analysis of variance was conducted to test the effect of environment, genotype (six crosses) and

their interaction on growth-related parameter using the following formula (Ibarra et al., 2017):

$$Z_{ab} = M + E_a + G_b + (E_a \times G_b) + \delta_{ab}$$

where Z_{ab} = dependent variable (shell height, survival rate and yield); M = common mean; E_a = environments effect (Rongcheng, Rushan or Huangdao); G_b = genotypes effect (GG, AA, GA, AG, TGA or TAG); $E_a \times G_b$ = interaction effect between environments and genotypes; δ_{ab} = residual error.

Mid-parent heterosis (H) was calculated using the formula modified from that used in Cruz and Ibarra (1997):

$$H = \frac{(GA + AG)/2 - (GG + AA)/2}{(GG + AA)/2} \times 100(\%)$$

where GA and AG are the mean shell height (yield or survival rate) of the diploid hybrids; GG and AA are the mean shell height (yield or survival rate) of C. gigas and C. angulata.

To estimate the increase in growth-related traits of the triploid hybrids compared with that of the diploid hybrids, the triploid advantage (TA) was calculated by the formula modified from that used in Qin et al. (2019):

$$TA = \frac{(TGA + TAG) - (GA + AG)}{GA + AG} \times 100(\%)$$

where TGA and TAG are the mean shell height (yield or survival rate) of the triploid hybrids.

3. Results

3.1. Ploidy levels of hybrid oyster

24 h after fertilization, the diploid rates of purebred crosses and diploid hybrid crosses were 100%, whereas the triploid rates of TGA and TAG were 96% and 95%, respectively. At day 12, the ploidy assessment of GG, AA, GA and AG indicated 100% diploids, while TGA and TAG showed 92% and 90% triploids. In September 2021, the triploidy rate of both triploid crosses was 100% due to prior screening (Fig. 2).

3.2. Fertilization rates and hatching rates

High fertilization rates were observed in both two purebred crosses and their diploid hybrids: GG, 88.44%; AA, 87.78%; GA, 80.22%; AG, 79.55%; they were significantly higher than their triploid hybrids: TGA, 63.33%; TAG, 56.89% (P < 0.05) (Fig. 3A). Similarly, the hatching rates of diploid crosses (67.78–74.44%) were significantly higher than those of triploid crosses (41.78–50.00%) (P < 0.05).

3.3. Growth and early survival up to metamorphosis

At day 4, significantly higher incremental survival rates were observed in the two diploid hybrids, with 70.10% and 69.29% for GA and AG, respectively (Fig. 3A). Meanwhile, the incremental survival rates of triploid hybrids (TGA: 49.12%; TAG: 44.74%) were significantly lower than the two diploid hybrids (P < 0.05) (Fig. 3A). However, no significant differences (P > 0.05) in incremental survival (81.54–87.16%) have been found among all the crosses at day 12 (Fig. 3A). The cumulative survival rates of triploid hybrids at day 12 were significantly inferior (P < 0.05) to those of diploid hybrids but similar to those of purebred crosses in the following order: GA (48.29%) > AG (45.90%) > GG (39.80%) > AA (36.98%) > TGA (22.29%) > TAG (18.51%) (Fig. 3A). The mid-parent heterosis (H) for incremental survival varied from 4.52% to 10.09%, while the triploid advantage (TA) varied between −32.37% and −3.69% (Table 2).

The diploid and triploid hybrid crosses showed better growth up to metamorphosis (Fig. 3B). At day 4, no significant differences have been found in shell heights among all the crosses. At day 12, however, triploid hybrids were 10.58% larger than the diploid hybrids (P < 0.05), which themselves overturned the purebred crosses. Eyed larvae were observed in the triploid crosses at day 12, whereas in the diploid crosses they were not observed until day 20. The value of triploid advantage (TA) increased with larval growth, equal to mid-parent heterosis (H) at day 8 and exceeding it at day 12 (Table 2). At day 12, the triploid hybrids showed an approximately 18.51% increase in shell height relative to the purebred crosses (Fig. 3B).
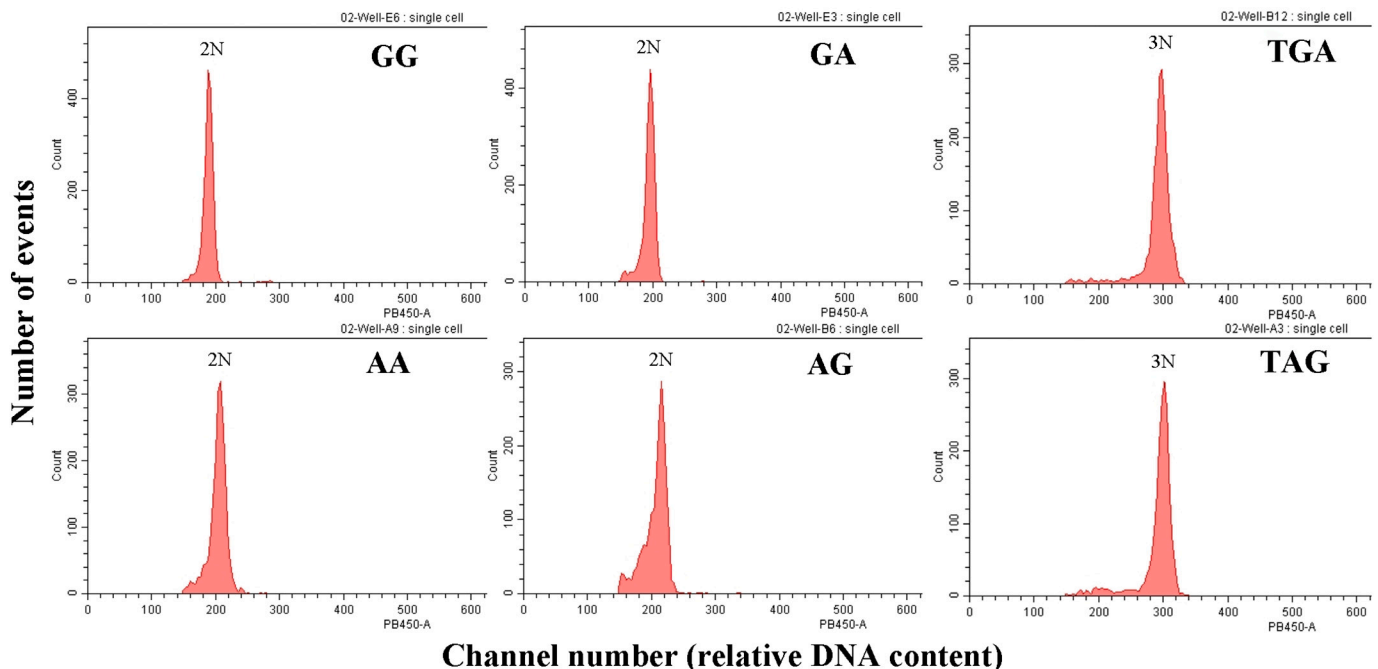

Fig. 2. Relative DNA content analysis for two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) at the adult stage.
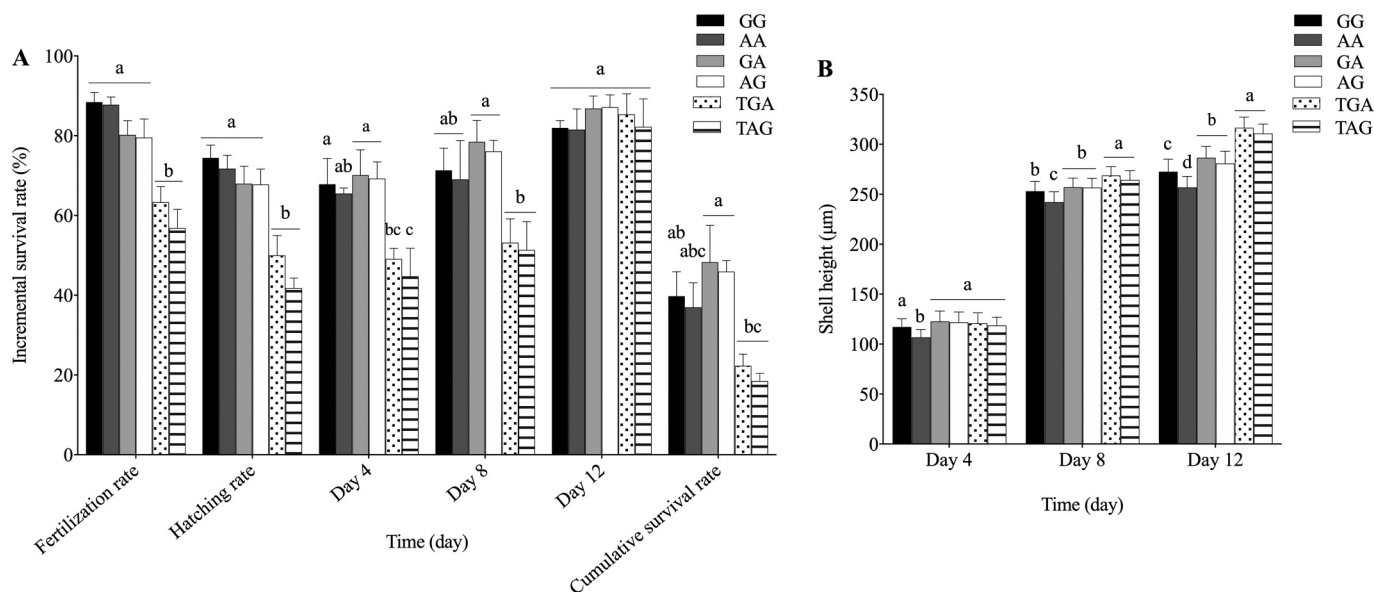
Fig. 3. Fertilization rate, hatching rate, larval survival rate and shell height for two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) in the planktonic larval stage. Different superscript letters at the same time indicate significant difference ($P < 0.05$).

Table 2
Triploid advantage (*TA*) and heterosis (*H*) for incremental survival rate and shell height in purebred groups (GG and AA) and their diploid and triploid hybrids (GA, AG, TGA and TAG) at the larval stage.

Items	Advantage rate	Fertilization rate	Hatching rate	Day 4	Day 8	Day 12
Incremental survival rate	*H* (%)	−9.33	−7.14	4.52	10.09	6.43
	TA (%)	−24.76	−32.41	−32.66	−32.37	−3.69
Shell height	*H* (%)	–	–	9.29	3.74	7.17
	TA (%)	–	–	−2.06	3.79	10.58

3.4. Growth and survival performance at juvenile and adult stages

3.4.1. Growth

In general, triploid hybrids had the best growth, diploid hybrids were intermediate and diploid purebred crosses were inferior at all three environments (Fig. 4). Therefore, growth performance in terms of shell height was ranked in the following order: TGA > TAG > GA > AG > GG > AA, except at Rushan where the growth of AG was better than GA (Fig. 4B). Two-way ANOVA showed that there was a significant genotype-environment interaction effect on shell height in December 2020 ($P < 0.001$), March 2021 ($P < 0.01$) and June 2021 ($P < 0.01$) (Table 3). During both juvenile and adult stages, growth of each cross was faster at Rushan than at Rongcheng and Huangdao, particularly the TGA (132.09 ± 9.61 mm), which exhibited a significantly ($P < 0.05$) higher average shell height than TAG (116.32 ± 9.14 mm) in September 2021 (Fig. 4B). Variation in shell height between triploid hybrids and diploid hybrids were little from September 2020 to March 2021 and tended to be significant ($P < 0.05$) afterwards, thereby the triploid advantage increased with time (Rongcheng: 8.47–24.53%; Rushan: 6.07–16.04%; Huangdao: 3.12–21.71%) (Table 4).

3.4.2. Survival

Throughout the field experiment, the best survival was recorded at Rongcheng for all crosses, despite AA performed slightly higher incremental survival at Huangdao (85.33%) than at Rongcheng (79.31%) in June 2021 (Fig. 5). An interaction between genotype and environment relative to incremental survival rate was observed in December 2020 ($P < 0.01$) and June 2021 ($P < 0.01$) (Table 3). In contrast, the cumulative survival advantages of the diploid and triploid hybrid crosses were greater in Rushan and Huangdao than in Rongcheng (Table 5). Specifically, performance in terms of cumulative survival rate was ranked in

the following order: TGA > TAG > GA/AG > GG/AA (Fig. 5D). Among the four monitored periods, the highest mortality rate was observed in summer months (September 2021) for the remaining five crosses, except for AA, where the maximum mortality occurred in winter months (March 2021) (Fig. 5). Meanwhile, significant heterosis and triploid advantage were also observed in summer months (September 2021) at Rongcheng, Rushan and Huangdao (Table 5).

3.4.3. Yield

The TGA had the highest yield at Rongcheng ($16,334.05 \pm 505.52$ g), Rushan ($14,256.66 \pm 209.16$ g) and Huangdao ($12,140.13 \pm 597.75$ g), and was larger than TAG, albeit without any statistical significance ($P > 0.05$); followed by diploid hybrids, GA performed better in Rongcheng and Huangdao, but slightly worse than AG in Rushan ($P > 0.05$); purebred crosses were inferior, with the most severe losses for GG in Huangdao and AA in Rushan (Fig. 4D). A positive yield advantages for diploid and triploid hybrids were observed in Rongcheng, Rushan and Huangdao (Table 4). The yield of the triploid hybrids at Rushan was increased by nearly 148.22% compared to the purebred crosses (Fig. 4D). Furthermore, after 15 months of growth, all crosses showed the highest yield in Rongcheng and lowest yield in Huangdao, except the AA, with lowest yield of 2887.77 ± 186.82 g in Rushan (Fig. 4D). On average, in Rongcheng, the best triploid hybrid TGA yielded $16,334.05$ g, whereas the best yield for purebred crosses was 6974.93 g for GG. A significant environment by genotype interaction effect was detected in two-way ANOVA for yield ($P < 0.001$) (Table 3).

3.5. Gonad development and sex ratio

In June 2021, *C. gigas*, *C. angulata* and reciprocal diploid hybrids were fully mature and presented two gonadal sexes in the histological
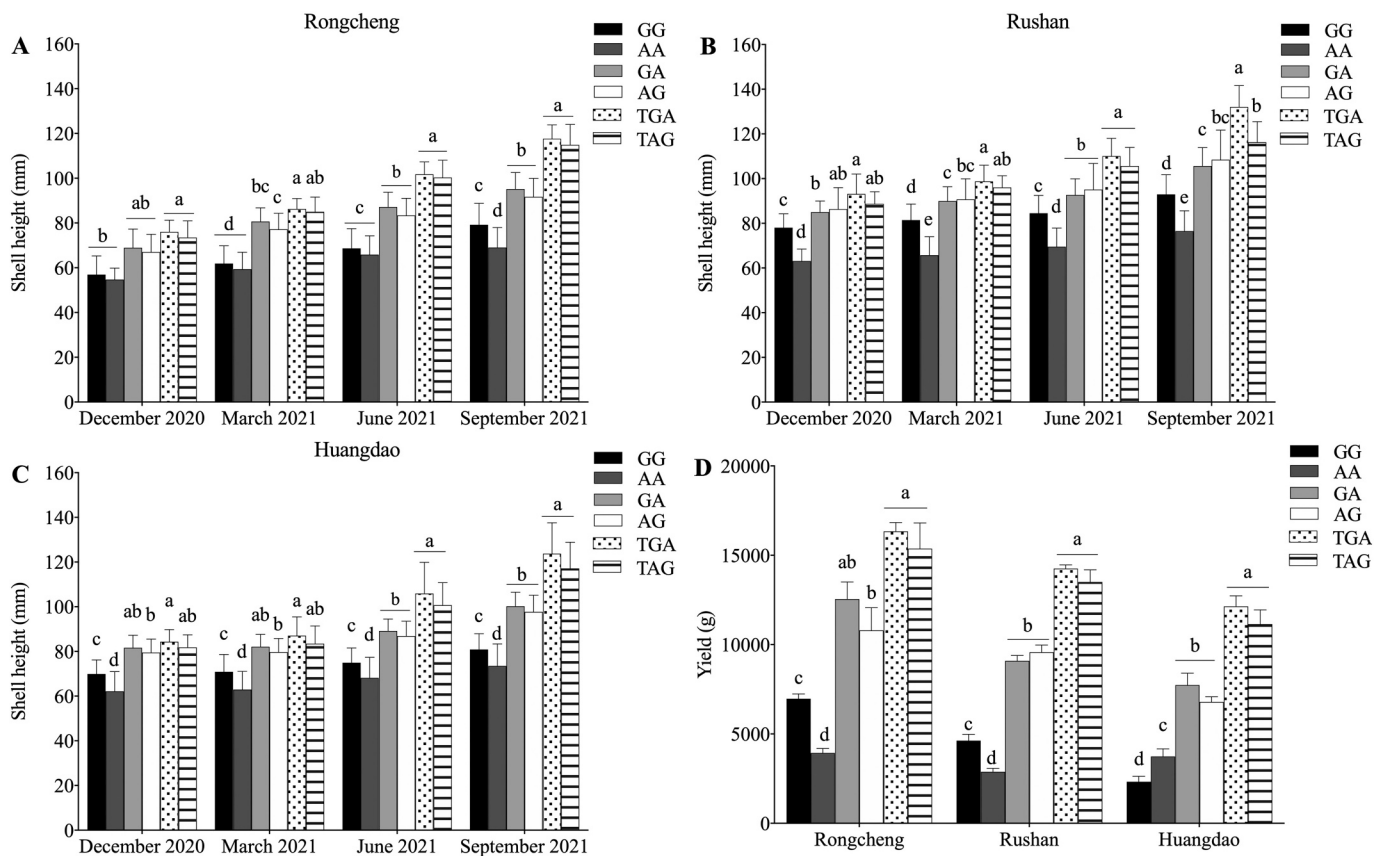
Fig. 4. Shell height and yield for two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) at Rongcheng, Rushan and Huangdao from December 2020 to September 2021. Different superscript letters at the same time indicate significant difference ($P < 0.05$).

Table 3
Two-way analyses of variance testing for site by line interaction effects on shell height, incremental survival rate and yield.

Time	Effect	Shell height			Incremental survival rate			Yield		
		d.f.	MS	*F*-value	d.f.	MS	*F*-value	d.f.	MS	*F*-value
December-2020	Line	5	0.286	163.394***	5	0.084	10.419***	–	–	–
	Site	2	0.432	246.697***	2	0.482	59.861***	–	–	–
	Line × Site	10	0.006	3.63***	10	0.022	2.756**	–	–	–
	Error	522	0.002	–	36	0.008	–	–	–	–
March-2021	Line	5	0.387	200.877***	5	0.244	26.597***	–	–	–
	Site	2	0.017	8.783***	2	0.336	36.611***	–	–	–
	Line × Site	10	0.006	3.010**	10	0.009	0.935[NS]	–	–	–
	Error	522	0.002	–	36	0.009	–	–	–	–
June-2021	Line	5	0.505	263.107***	5	0.059	5.886***	–	–	–
	Site	2	0.082	42.863***	2	0.160	15.849***	–	–	–
	Line × Site	10	0.006	3.034**	10	0.029	2.899**	–	–	–
	Error	522	0.002	–	36	0.010	–	–	–	–
September-2021	Line	5	0.732	395.711***	5	0.354	32.742***	5	0.584	374.726***
	Site	2	0.022	11.915***	2	0.141	13.046***	2	0.173	111.313***
	Line × Site	10	0.001	0.391[NS]	10	0.006	0.548[NS]	10	0.022	14.251***
	Error	522	0.002	–	36	0.011	–	36	0.002	–

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

analyses: female (GG: 48.75%; AA: 52.50%; GA: 53.75%; AG: 55.00%), male (GG: 51.25%; AA: 47.50%; GA: 46.25%; AG: 45.00%); while four gonadal sexes were observed in allotriploid hybrids: female (TGA: 7.50%; TAG: 10.00%), male (TGA: 26.25%; TAG: 18.75%), hermaphrodite (TGA: 5.00%; TAG: 15.00%), and no gametes (TGA: 61.25%; TAG: 56.25%).

Histological analyses showed that *C. gigas* and *C. angulata* diploid progenies were completely fertile and contained normally mature vitellogenic oocytes (Fig. 6a, c) or spermatozoa (Fig. 6b, d). Likewise, gonadal structures in histological sections of diploid hybrids (GA and

AG) were morphologically normal (Fig. 6e – 6h), suggesting that they had similar reproductive capacity compared to parents. On the contrary, most triploid hybrids (TGA and TAG) showed abnormal gonadal development and no gametes were found in the asexual type (Fig. 6l, p). A few TGA and TAG had morphologically normal reproductive cell, but the majority were still in the oogonia (Fig. 6i, m) or spermatocyte stage (Fig. 6j, n). Moreover, the gonad in hermaphrodite triploid hybrids was composed of vitellogenic oocytes and spermatozoa (Fig. 6k, o).

Table 4
Triploid advantage (*TA*) and heterosis (*H*) for shell height and yield in purebred groups (GG and AA) and their diploid and triploid hybrids (GA, AG, TGA and TAG) from December 2020 to September 2021.

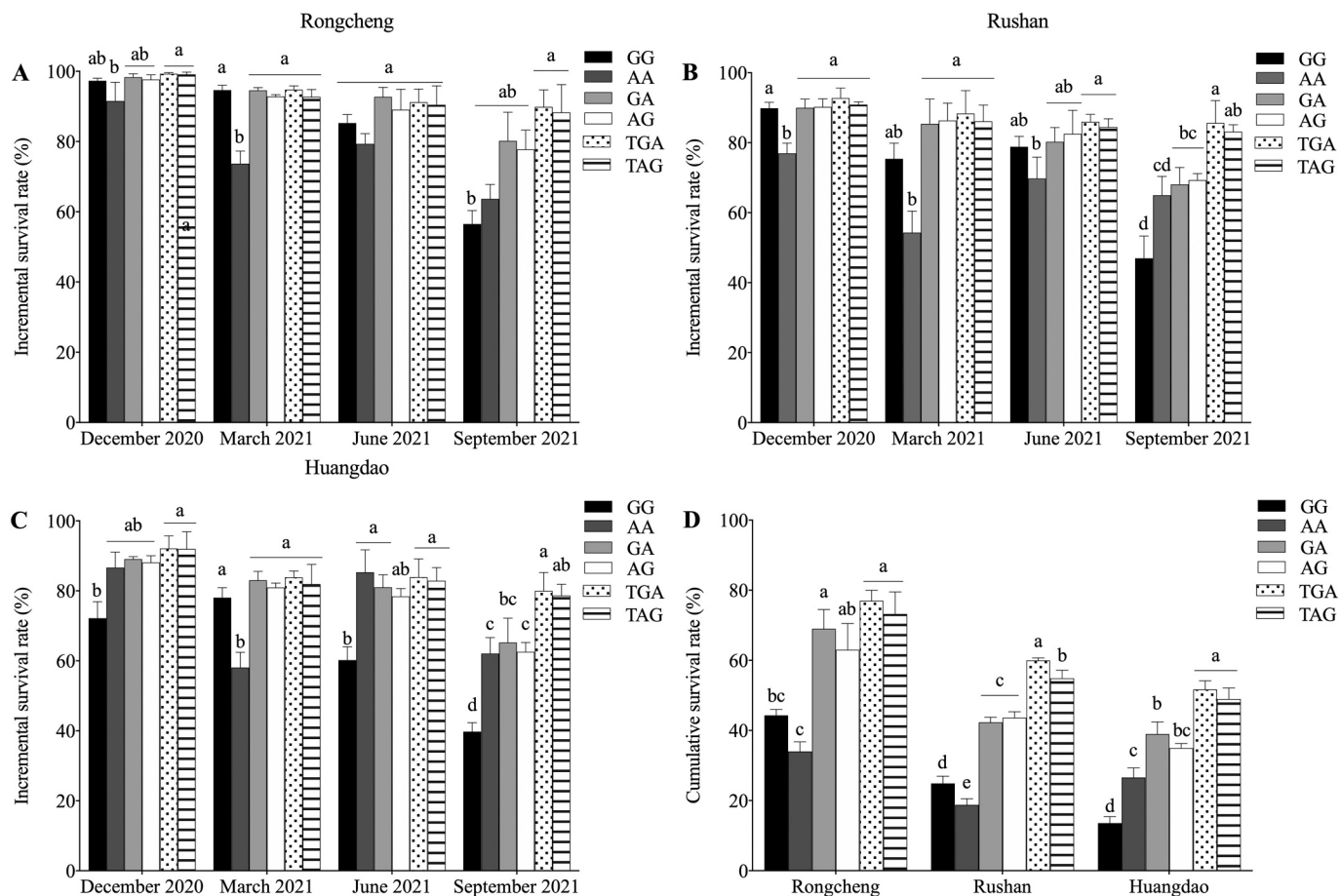| Site	Advantage rate	Shell height				Yield
		December 2020	March 2021	June 2021	September 2021	
Rongcheng	*H* (%)	21.75	30.25	26.73	25.97	114.11
	TA (%)	10.00	8.47	18.52	24.53	35.76
Rushan	*H* (%)	21.44	22.78	21.83	26.26	148.22
	TA (%)	6.07	7.88	14.88	16.04	48.78
Huangdao	*H* (%)	22.02	20.90	22.87	28.18	138.96
	TA (%)	3.12	5.39	17.41	21.71	60.22

Fig. 5. Incremental survival rate and cumulative survival rate for two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) at Rongcheng, Rushan and Huangdao from December 2020 to September 2021. Different superscript letters at the same time indicate significant difference (*P* < 0.05).

Table 5
Triploid advantage (*TA*) and heterosis (*H*) for incremental survival rate and cumulative survival rate in purebred groups (GG and AA) and their diploid and triploid hybrids (GA, AG, TGA and TAG) from December 2020 to September 2021.

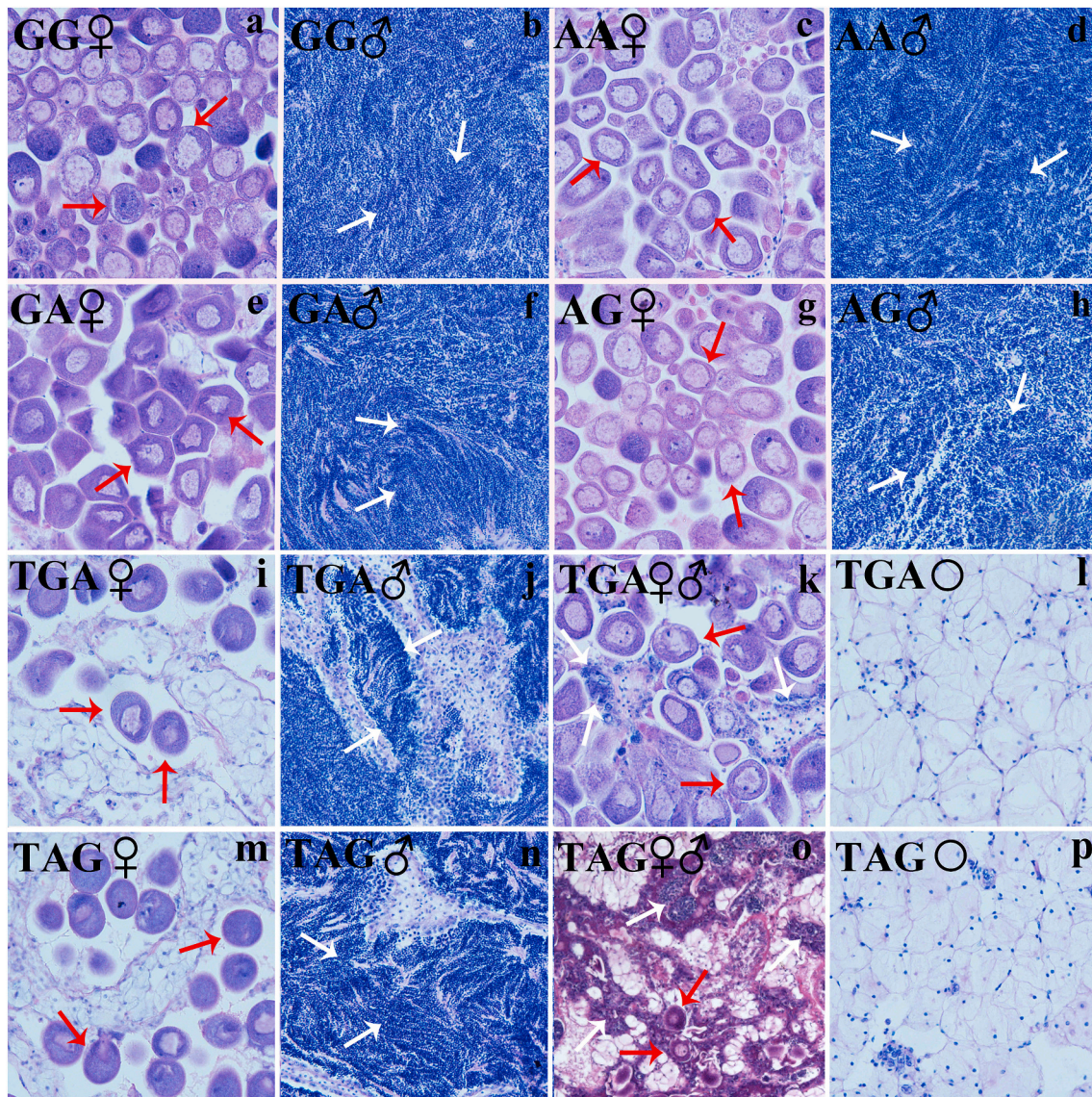| Site	Advantage rate	Incremental survival rate				Cumulative survival rate
		December 2020	March 2021	June 2021	September 2021	
Rongcheng	*H* (%)	3.76	11.33	10.45	31.43	68.51
	TA (%)	1.25	0.02	−0.08	12.77	13.80
Rushan	*H* (%)	7.99	32.41	9.50	22.68	96.95
	TA (%)	1.91	1.61	4.73	22.81	33.59
Huangdao	*H* (%)	11.54	20.44	9.56	25.50	84.49
	TA (%)	3.89	1.10	4.60	24.11	36.04

Fig. 6. The gonadal structure of two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) under 40× objective lens (female: ♀; male: ♂; hermaphrodite: ♀♂; asexuality: O). Note: red arrows and white arrows indicate vitellogenic oocytes and spermatozoa, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Hatching index and growth during the larval stage

A major focus of our study was to determine the possibility of inducing triploid hybrids with high early viability by crossing *C. gigas* with *C. angulata* through inhibiting the release of polar body II using cytochalasin B. High induction rate of triploid oysters has been reported in *C. hongkongensis* ♀ × *C. gigas* ♂ using hypotonic fresh seawater (Zhang et al., 2014) and *C. hongkongensis* ♀ × *C. ariakensis* ♂ using CB (Qin et al., 2020). In our study, a high triploidy rate was observed in triploid hybrid crosses at 24 h post-fertilization (TGA: 96%; TAG: 95%) and day 12 (TGA: 92%; TAG: 90%). Nonetheless, the results of triploid hybrids induction for oyster have generally been less successful than those of autotriploids induction, due to the poor early survival of triploid hybrids. Thus, one of the keys for successful production of useful triploid hybrids may be to improve early viability of embryos (or larvae) in diploid hybrid crosses. Based on this view, we used two closely related species (Wang et al., 2010; Ren et al., 2016), *C. gigas* and *C. angulata*, to induce allotriploid oysters, since interspecific hybrids of these two oysters had superior survival than parental species (Jiang et al., 2021b). The survival rates of TGA and TAG were comparable with that of self-crossing crosses in the late larval stage, even if still lower than that of the diploid hybrid crosses (Fig. 3A). This study explicitly demonstrates that triploid hybrids with high early viability can be obtained by crossing *C. gigas* with *C. angulata* through artificial induction. However, the lowered fertilization rate, hatching rate and incremental survival rate at day 4 was observed in TGA and TAG (Fig. 3A), which may be caused by the induction treatment rather than the hybridization itself. Previous study reported that CB reduces the survival rate of shellfish during early development (Stanley et al., 1981). To overcome this disadvantage, another method for producing all-triploid oysters is crossing tetraploids with diploids (Guo and Allen Jr, 1994; Yang et al., 2018). In addition, a positive growth advantage was observed in allo-triploid crosses from day 8 to day 12. Due to the low initial density at larval stage, the effect of density on growth in this study was likely to be small and did not bias our finding concerning triploid advantage.

4.2. Triploid advantage

This study also tested whether there was proof of triploid advantage under commercial culture environments. Triploid hybrids possess combinations of excellent parental characteristics and further exhibit heterosis and triploid advantage, thus having higher yield than diploid hybrids and parental species (Bingham et al., 1994; Bodt et al., 2005; Yao et al., 2013). Abundant evidence for the triploid advantage of phenotypic traits and nutritional value have been reported in allo-triploid fishes (Liu et al., 2001; Liu, 2010; Wu et al., 2019). At the end of the trial, the shell height of the triploid hybrids was significantly larger than that of the diploid hybrids with a triploid advantage of 16.04–24.53% at three culture environments. Other studies reported triploid advantage in shell height similar or only slightly lower than the present study (Zhang et al., 2014; Qin et al., 2020). The improved growth rate of triploids may result from increased genomic heterozygosity, the "gigantism" effect in cell size or more energy allocated to somatic cell growth because of the high sterility of triploid gonads (Comai, 2005; Piferrer et al., 2009).

At the three culture environments, there was no significant difference ($P < 0.05$) in shell height of triploid hybrids compared to diploid hybrids in December 2020 or March 2021, while the triploid hybrids exhibited superior growth over the period of June 2021 to September 2021, reflecting an increasing triploid advantage with age (Fig. 4; Table 4). This result may indicate the difference in energy allocation between triploids and diploids during gonad development and spawning (Downing and Allen Jr, 1987; Eversole et al., 1996). Akhan et al. (2011) reported that triploid hybrids between rainbow trout and black sea trout reached higher body weight compared with diploid hybrids and parental species after 170 days of growth. Similarly, triploid hybrids derived from *Megalobrama amblycephala* Yih ♀ × *Xenocypris davidi* Bleeker ♂ grew faster than both parents corresponding with sexual maturity (Hu et al., 2012).

A disease in aquaculture worldwide is "Pacific oyster mortality syndrome (POMS)" resulting in extensive mass mortalities of the *C. gigas* (De Lorgeril et al., 2018). Mortality associated with SMS coincide with the gonad maturation period (Mori, 1979; Soletchnik et al., 1997; Samain, 2011). Yang et al. (2021) reported that *C. gigas* losses of up to 60% from July to October during the SMS outbreaks in Shandong, China. In the present field trials, substantial mortality (55.56–60.21%) was observed during summertime (i.e., September 2021) in GG across environments. The incremental survival rates for the triploid hybrids were much higher than that of the purebred crosses, reaching a maximum 55.76% increase at Huangdao. Since triploid shellfish typically have reduced gametogenesis, the saved glycogen is diverted to physiological metabolism and somatic growth, triploid oysters may have a higher survival during summer months (Perdue et al., 1981). Another possible explanation for this survival advantage may be that heterosis makes triploids more robust than diploid purebred crosses, and gene redundancy protects triploids from the harmful effects of mutations (Launey and Hedgecock, 2001; Comai, 2005). Nevertheless, published results of comparative survival between diploid and triploid oysters were contradictory. For instance, the higher mortality was observed in triploid eastern oysters over diploid oysters along the Gulf of Mexico (Wadsworth, 2018).

At the end of the experiment, the triploid hybrids yielded the most biomass with a significant triploid advantage (Rongcheng: 35.76%; Rushan: 48.78%; Huangdao: 60.22%), indicating the superior commercial traits of triploid hybrids. The patterns in growth and survival of the crosses described above resulted in significant differences in yield. On the one hand, growth of diploids usually slowed down in later stages, especially in spawning period, which contributed to increase the differences between the allotriploid and diploid crosses. On the other hand, cumulative survival rates at all culture environments were significantly higher in triploid crosses than in diploid crosses ($P < 0.05$), except at Rongcheng where mean survival between diploid hybrids and triploid hybrids did not differ ($P > 0.05$). Heterosis could have also been estimated by comparing purebred and hybrid triploids. However, in the present study, we did not produce purebred triploids, preventing the possibility to compare purebred and hybrid triploids.

4.3. Different performance in the three culture environments

It has been well recognized that environmental difference has a substantial influence on performance in mollusks. In this study, the best growth was recorded at Rushan for all crosses, while the worst growth was observed in Rongcheng. The observed growth discrepancy may be attributed to the difference in food abundance between the two culture environments. Gao et al. (2006) verified that the Rushan present higher phytoplankton abundance and primary productivity compared with the Rongcheng and Huangdao. The larger waves from the Yellow Sea, together with the higher average annual temperature, may explain the high primary productivity and abundant nutrients around Rushan (Table 1). In contrast, the cumulative survival rate for all crosses was much better in Rongcheng than in Rushan or Huangdao, which was corroborated by Han et al. (2020). This contradictory result between growth and survival suggest that oysters do not necessarily exhibit a high survival rate in nutrient-rich environments. A possible explanation is that higher seawater temperature and larger waves reduce the survival rate of oysters in Rushan and Huangdao. Besides, Blanc and Maunas (2005) showed that less favorable conditions maybe more deleterious to weaker genotypes. Therefore, the higher heterosis and triploid advantage for cumulative survival rate of diploid and triploid hybrids grown in Rushan and Huangdao may be a consequence of divergent environmental adaptability.

The effect of genotype (G) × environment (E) interactions has been studied for some important aquaculture species, such as hard clam (Rawson and Hilbish, 1991), European seabass (Dupont-Nivet et al., 2010) and Pacific white shrimp (Tan et al., 2017). Evans and Langdon (2006) and many other authors (Swan et al., 2007; De Melo et al., 2018) reported significant differences in the growth performance of full-sib families of *C. gigas* in different farming environments. Likewise, in the current study, results of the two-way ANOVA indicate a significant effect of genotype × environment interaction on shell height in December 2020, March 2021 and June 2021. Meanwhile, significant genotype × environment interactions in incremental survival rate (December 2020 and June 2021) and final yield were also detected among the six different crosses at the three environments. Although the ability to quantify G × E interactions is affected by breeding methods (population or family), it can be inferred that differential behavior of GA and AG across environments may contributed most to the interaction effect for shell height, while the different performances of GG and AA contributed the most to the interaction effect for incremental survival. On the contrary, the relative genotype-specific performance of allotriploid hybrids was consistent and optimal across environments, suggesting that they have stronger environmental adaptability. This may be crucial for the popularization of oyster varieties, as it ensures that a steady and large harvest yield, irrespective of their farming environments. Our results clearly showed that genotype × environment interactions have an impact on phenotypic traits. An accurate evaluation of the effect of genotype × environment interactions on the performance of different crosses will assist in choosing appropriate oyster seed for specific culture environments.

4.4. Gonad development

Consistent with previous studies (Menzel, 1974; Huvet et al., 2002), the reciprocal diploid hybrids in this study showed convincing evidence for normal gonad maturation. Nevertheless, the production of fertile diploid hybrids carries the risk of affecting the genetic profile of wild populations (Wang et al., 2020a; Wang et al., 2020b). In fact, a total ban on the production of diploid hybrids may be difficult to implement given

its superior performance. A 'safe production' solution was thus presented, using sterile triploid hybrids. As expected, most of the triploid hybrids in this study displayed gonad atrophy and gamete abnormalities, highlighting that triploidy induction could significantly reduce the reproductive capacity of diploid hybrids. Studies concerning the sterility of triploid hybrids have also been reported in other aquatic animals, such as allotriploid trout (Blanc and Maunas, 2005), allotriploid carp (Liu, 2010) and allotriploid oysters (Zhang et al., 2014). Dheilly et al. (2014) suggested that disruption of sexual differentiation and mitosis may be the reason for the impaired gametogenesis of triploid *C. gigas*. Theoretically, sterility of triploids occurs because homologous chromosomes fail to form synapses during meiosis (Stanley et al., 1981). Hence, triploid oysters are typically thought to divert energy used for gonadal maturation to body growth, allowing them to increase body size and maintain flesh quality, especially in summer when diploid oysters are thin and watery (Yang et al., 2018). Furthermore, triploidization may affects the sex ratio of oyster hybrids between *C. gigas* and *C. angulata*. For example, histological studies showed GA were composed of 53.75% females and 46.25% males, while TGA consisted of 7.50% females and 26.25% males in this study. This phenomenon could result from incompatible genomes and abnormal expression and regulation of genes associated with gonad development (Hu et al., 2012).

5. Conclusions

We successfully obtained two new types of reciprocal triploid hybrids between *C. gigas* and *C. angulata* by inhibiting the polar body II using cytochalasin B. A high triploidy induction rate ($> 95\%$) was found by ploidy analysis using a flow cytometer. The survival rates of triploid hybrids were comparable to that of purebred crosses in the late larval stage. During the grow-out stage, triploid hybrids exhibited high triploid advantage in shell height, survival rate and yield compared to diploid crosses under three main commercial farming environments in northern China. Moreover, the yield was highest in Rongcheng than in Rushan or Huangdao, particularly in triploid hybrids, showing a strong genotype × environment interactions. Also, our study showed that the triploid hybrids displayed high sterility during reproductive season. In summary, the combination of heterosis and triploid advantage further enhances the adaptability of two new types of triploid hybrids, which enables rapid growth, high survival in summer, maximized yield and produces a high sterility permitting year-round marketability, thus presenting great potential for commercial cultivation in northern China.

CRediT authorship contribution statement

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

Declaration of Competing Interest

The authors declare no conflict of interest.

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