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Evaluation of advantages in the growth, survival and reproductive aspects of triploid hybrids derived from *Crassostrea gigas* tetraploids and *C. ariakensis* diploids in northern China

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ABSTRACT

Crossbreeding and polyploid breeding are two important tools for the genetic improvement of oysters, and their cross-application is expected to provide new benefits to oyster breeding. To evaluate the aquaculture traits of allotriploids between *Crassostrea gigas* and *Crassostrea ariakensis*, two triploid hybrids (TAG (diploid *C. ariakensis* ♀ × tetraploid *C. gigas* ♂) and TGA (tetraploid *C. gigas* ♀ × diploid *C. ariakensis* ♂)) were established. Phenotypic traits including growth, survival and reproduction were analyzed in two diploid hybrids (AG (diploid *C. ariakensis* ♀ × diploid *C. gigas* ♂) and GA (diploid *C. gigas* ♂ × diploid *C. ariakensis* ♀)) as controls. The results showed that TAG presented triploid advantage in fertilization rates and hatching rates, up to 11.34% and 26.50% respectively, while TGA did not. The survival rate of TAG and the shell height of TGA exhibited triploid advantage throughout the larval stage and were significantly higher ($P < 0.05$) than those of AG and GA at day 25. During the grow-out stage, triploid advantages appeared in the shell height of TAG and TGA, but only TAG showed triploid advantages in the survival rate. The triploid rates of TAG and TGA began to decrease during the grow-out stage, and reached 96.11% ± 4.41% and 90.56% ± 3.47%, respectively, at day 350. It was also found that the reproduction of TAG and TGA on day 350 was poor, manifesting as a high proportion of individuals with no gametes or a low number of gametes. Overall, TAG always presents triploid advantages in terms of growth and survival throughout the grow-out stage and shows potential value as a new resource of oyster farming or genetic improvement.

1. Introduction

Five *Crassostrea* oyster species (*C. angulata*, *C. hongkongensis*, *C. gigas*, *C. ariakensis*, *C. sikamea*) are naturally distributed along China's long, broad coastline (Wang and Guo, 2008a; Zhang et al., 2012b; Zhang et al., 2016). Among these five oysters, *C. gigas* and *C. ariakensis* are the two most important species in northern China in terms of economic value and ecological value, respectively (Li et al., 2021). *C. ariakensis* naturally inhabits estuaries along the coastline, but its artificial breeding and cultivation have not been widely promoted due to its meat color (Qin et al., 2020; Wang et al., 2004). *C. gigas* is mainly distributed in the north of the Yangtze River; it is the most important aquaculture shellfish in northern China and is mainly farmed in Shandong and Liaoning provinces, which present the highest yield annually (Guo, 2009; Li et al., 2011; Zhang et al., 2016). As an important oyster culture species around the world, *C. gigas* shows superior aquaculture traits such as fast growth, a high meat yield and excellent taste (Jiang et al., 2021; Zhang et al., 2012b). Although *C. ariakensis* shows an advantage in terms of ecological value, it has long remained an undeveloped resource because of its lower aquaculture trait value, and genetic improvement is urgently needed. Interspecific hybridization is an effective way to transfer ideal characteristics and realize germplasm improvement (Bartley et al., 2000; Hulata, 1995; Rahman et al., 2013), which can be applied to *C. ariakensis* and *C. gigas* to achieve genetic improvement of *C. ariakensis*. Previous studies have shown that symmetrical fertilization occurs between these two species and that the hybrids can successfully hatch and survive; however, the aquaculture traits of the hybrids were not ideal, especially in hybrid from female *C. ariakensis* × male *C. gigas* (Allen and Gaffney, 1993; Li et al., 2021; Zhang et al., 2012a). Therefore, we attempted to carry out triploid breeding to improve the aquaculture

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traits of the hybrids.

Since the successful induction of polyploid oysters was initially reported in 1981 (Stanley et al., 1981), polyploid breeding of oysters has developed rapidly and created great economic value. Triploid oysters boast some advantages, such as rapid growth (Allen and Downing, 1986; Nell, 2002), strong disease resistance (Gagnaire et al., 2006; Hand et al., 1988), no risks of genetic pollution (Guo et al., 1996; Piferrer et al., 2009), and high nutritional value (Qin et al., 2018). Due to these economic advantages, triploid oysters are extremely popular in oyster farming and have been widely cultivated in some oyster farming countries such as China, the United States, France and Australia (Peachey and Allen, 2016; Suquet et al., 2016). The best way to produce triploid oysters on a large scale is by hybridization between tetraploid and diploid oysters, which can achieve high, stable triploid rates (Guo et al., 1996). The application of polyploid breeding in crossbreeding can produce allotriploids, which can help improve the aquaculture traits of diploid hybrid offspring (Bartley et al., 2000; Zhang et al., 2014a).

Among oysters, the establishment of allotriploids has been reported between *C. gigas* and *C. hongkongensis* (Zhang et al., 2014a) and between *C. ariakensis* and *C. hongkongensis* (Qin et al., 2020) via the methods of salinity or drug induction. Moreover, it is feasible to cross *C. gigas* tetraploids with *C. ariakensis* diploids to obtain allotriploids (Que and Allen, 2002), but there are no available data on the phenotypic traits of the offspring. Hence, in this study, allotriploids were generated by crossing *C. gigas* tetraploids with *C. ariakensis* diploids, and the phenotypic traits of the hybrids were analyzed to evaluate the production value of the aquaculture traits of the allotriploids generated from *C. gigas* tetraploids and *C. ariakensis* diploids.

2. Materials and methods

2.1. Oyster collection and artificial insemination

Two-year-old tetraploid (shell height, 5.94 cm ± 0.78 cm) and diploid (shell height, 9.46 cm ± 1.83 cm) *C. gigas* were obtained from an artificial breeding population located in Xuejiadao, Qingdao City, Shandong Province. Four- or five-year-old diploid *C. ariakensis* (shell height, 20.32 cm ± 5.13 cm) were harvested from wild populations in the Yellow River Delta in Dongying City, Shandong Province. All oysters were transported to the hatchery of Shuguang Fisheries LTD in April 2020.

Before the experiment, all tetraploid (4n) *C. gigas* were tested for ploidy to ensure that they were pure tetraploids. Specifically, gill tissue was collected from each oyster, used to generate a cell suspension, stained and tested for ploidy by flow cytometry (Beckman Coulter, Beckman Coulter CytoFlex S, America). Tetraploid *C. gigas*, diploid (2n) *C. gigas* and diploid *C. ariakensis* were dissected separately, and the sex was distinguished under a light microscope (NIKON, Eclipse E100, Japan). Then, sperm and eggs were collected as previously described by Xu et al. (2009), and a piece of adductor muscle from each oyster used for insemination was stored in 95% alcohol for subsequent molecular identification. After gamete collection, artificial insemination was carried out in a 15 L bucket according to Table 1. Four diploid insemination combinations (AA- 2n *C. ariakensis* ♀ × 2n *C. ariakensis*♂, AG- 2n *C. ariakensis* ♀ × 2n *C. gigas* ♂, GA- 2n *C. gigas*♀ × 2n *C. ariakensis*♂ and GG- 2n *C. gigas* ♀ × 2n *C. gigas* ♂) and two triploid insemination combinations (TAG- 2n *C. ariakensis* ♀ × 4n *C. gigas* ♂ and TGA- 4n *C. gigas* ♀ × 2n *C. ariakensis*♂) were obtained, and the fertilized eggs were transferred to a 20 m^3 tank for incubation.

Table 1
Design of hybridization experiment between *Crassostrea ariakensis* and *C. gigas*.

	4 nG♂$_{(1,2,3)}$	2 nG♂$_{(1,2,3)}$	2 nA♂$_{(1,2,3)}$
4 nG♀$_{(1,2,3)}$	–	–	TGA
2 nG♀$_{(1,2,3)}$	–	GG	GA
2 nA♀$_{(1,2,3)}$	TAG	AG	AA

2n, diploid; 4n, tetraploid; G, *C. giga*; A, *C. ariakensis*; GG, 2n *C. gigas*♀ × 2n *C. gigas*♂; AA, 2n *C. ariakensis*♀ × 2n *C. ariakensis*♂; GA, 2n *C. gigas*♀ × 2n *C. ariakensis*♂; AG, 2n *C. ariakensis*♀ × 2n *C. gigas*♂; TGA, 4n *C. gigas*♀ × 2n *C. ariakensis*♂; TAG, 2n *C. ariakensis*♀ × 4n *C. gigas*♂. The subscripted numbers (1, 2 and 3) represent three oysters , corresponding to one real replicate, and each replicate was conducted by mating three females with three males.

2.2. Larval rearing, spat nursery and grow-out

After 24 h of incubation, D-larvae were transferred to a 60 L bucket for breeding at a density of 3 larvae/mL. The methods of larval rearing, spat nursery and grow-out followed those described by Li et al. (2011). During larval rearing, the water temperature was maintained at 23–25 °C, with salinity of 29 psu. In simple terms, 40% of the water was changed twice a day. Larvae were fed with *Isochrysis galbana* at 5000 to 30,000 cells/mL on days 0–6 and a mixture of *I. galbana* and *Chaetoceros calcitrans* (1:1) at 30,000 to 80,000 cells/mL after day 6. When the proportion of eyespot larvae exceeded 30% after 20–25 days of incubation, spat collectors (scallop shell string) were placed in the bucket. On day 14 after fertilization, the collectors with settled spats were transferred to an outdoor nursery tank. On day 14 after fertilization, the collectors with settled spats were fixed to a nylon rope and transferred to the sea area of Xuejiadao, Qingdao City for culturing.

2.3. Molecular identification and ploidy analysis

The adductor muscles of offspring and parents were collected at day 120, and DNA was extracted by using a TIANamp Marine Animals DNA Kit (TIANGEN, DP324–03, China). Then, PCR amplification was conducted. PCR was performed in a 25 μL volumes, and the PCR cycling conditions were as follows: 94 °C for 2 min, then 35 cycles of 30 s at 94 °C, 40 s at 52 °C and 1 min at 72 °C, which was followed by a final extension at 72 °C for 5 mins. The PCR products were separated on 3% agarose gels containing ethidium bromide and then visualized under a UV transilluminator. Hybrid identification was carried out using the internal transcribed spacer 2 (ITS2) gene, and the primer sequences were TCTCGCCTGATCTGAGGTC G(5.8S forward) and GCAGGACACATTGAACATCG (18S reverse) (Wang and Guo, 2008b).

A total of 1000–2000 larvae were randomly collected to generate a cell suspension, which was then stained with 2 μg/mL DAPI (Sigma, D9542, America). Thereafter, ploidy was detected by flow cytometry. Ploidy analysis was performed on 80 individuals in each group during grow-out stage. The gill fragments collected from each individual were separately placed in centrifuge tubes containing phosphate buffer solution and repeatedly pumped with a syringe to produce a cell suspension. The cell suspension of each individual was stained with DAPI, and ploidy was detected by flow cytometry.

2.4. Analysis of reproductive parameters

The analysis of reproduction was performed at day 350 after fertilization. When the oysters were dissected, their gonads were collected to distinguish female, male, hermaphroditic and agametic individuals under a light microscope. Ninety individuals were taken from each experimental group (30 per replicate) for sex determination analysis. To determine egg numbers, the complete gonads of each oyster were dissolved in 1 L filtered seawater, and an aliquot of 100 μL was then taken for counting under the microscope after even stirring. Thirty individuals were taken from each experimental group for the determination of egg counts (10 per replicate). The complete gonads of each male oyster were dissolved in seawater, and the number of sperm was then counted by flow cytometry. Fifteen individuals were randomly taken from each experimental group (5 per replicate) for the determination of sperm counts.

2.5. Measurement and analysis

The fertilization rate of eggs, hatching rate of zygotes, shell height and survival rate of larvae were calculated according to the method described by Zhang et al. (2012). The ploidy analysis was carried out at 24 h and on day 25, day 120, day 180, day 300 and day 350 after fertilization, and shell heights and survival rates were measured on day 5, day 15, day 25, day 120, day 180, day 300 and day 350 after fertilization. The triploid rate is the ratio of the number of triploid individuals to the total number of samples, and the survival rate of spats or adults is the ratio of the number of individuals at corresponding ages to the initial number. Fifty individuals in the larval stage and thirty individuals in the grow-out stage were randomly collected from each replicate of each group to measure shell height.

To evaluate the aquaculture traits of diploid hybrids, mid-parent heterosis (H) was used (Cruz and Ibarra, 1997; Xu et al., 2019a) and was calculated by using the following equation:

$$H(\%) = [X_{F1} - (X_A + X_G)/2] \times 100/(X_A + X_G)/2$$

where X_{F1} indicates the mean phenotypic value of the hybrids, and X_A and X_G indicate the mean phenotypic values of the *C. ariakensis* and *C. gigas*, respectively.

To evaluate the aquaculture traits of triploid hybrids, the triploid advantage rate (*Td*) was used (Zhang et al., 2014a) and was calculated by using the following equations:

$$Td_{AG}\ (\%) = (\mathrm{TAG} - \mathrm{AG}) \times 100/\mathrm{AG}$$

$$Td_{GA}\ (\%) = (\mathrm{TGA} - \mathrm{GA}) \times 100/\mathrm{GA}$$

where Td_{AG} and Td_{GA} refer to the triploid advantage rates, and TAG, TGA, AG and GA indicate the phenotypic value.

All data analysis was performed by using SPSS19.0. The differences in each indicator among different experimental groups were analyzed by one-way ANOVA, and Tukey multiple comparisons were conducted, with the significance level set at 0.05.

3. Results

3.1. Fertilization, hatching and larvae development

The two intraspecific combinations showed significantly higher fertilization rates among all 6 groups ($P < 0.05$), with AA and GG showing rates of 77.35% and 79.84%, respectively. There were no significant differences in the fertilization rates between TAG and AG, or between TGA and GA ($P > 0.05$), and the fertilization rates of TAG and TGA were only 18.39% ± 4.67% and 5.13% ± 1.64%, respectively. The hatching rate of TGA was only 30.64% ± 2.86%, which was significantly lower than those of the other groups ($P < 0.05$) (Table 2).

The survival rate of TAG was significantly higher than that of AG during the larval stage ($P < 0.05$), with triploid advantage rates of 21.48% at day 5, 49.47% at day 15 and 144.79% at day 25. The survival rate of TGA was only 6.11% ± 3.44% at day 25 and was always significantly lower than that of GA ($P < 0.05$). The shell height of TGA was significantly higher than that of GA and AG at day 5 and day 25 ($P < 0.05$), with triploid advantage rates of 5.52% and 4.13%, respectively, while the shell height of TAG was always the lowest among the 6 groups (Table 2).

3.2. Genetic confirmation and ploidy analysis

The flow cytometry results showed that the relative DNA content of diploid *C. gigas* was basically the same as that of *C. ariakensis*. In *C. gigas*, the relative DNA content of tetraploids was twice that of diploids. The relative DNA content of the triploid crosses (TAG and TGA) was 1.5 times that of the diploid crosses (Fig. 1). *C. ariakensis* (parents and progeny) and *C. gigas* (parents and progeny) showed clear specific bands at 500–600 bp and 600–700 bp, respectively. There were two specific bands observed in both diploid (GA and AG) and triploid (TAG and TGA) crosses (Fig. 2).

3.3. Growth, survival and wet weight of spat

Shell height was not statistically different between the TAG and AG crosses ($P > 0.05$), but the *Td* (triploid advantage rate) values of TAG were always positive throughout the grow-out stage. The shell height of TGA was only lower than that of GG and was significantly higher than that of GA at days 180–350 ($P < 0.05$), with triploid advantage rates of 8.27% at day 120, 8.50% at day 180, 10.32% at day 300 and 6.16% at day 350. The heterosis values of diploid crosses (AG and GA) for shell height and the survival rate were always negative. A significantly greater wet weight ($P < 0.05$) was found in GG, reaching 30.96 g ± 3.65 g at day 350. The wet weights of TAG and TGA were greater than those of AG and GA, respectively, and the triploid advantage rates reached 5.37% and 4.26%, respectively, at day 350.

The survival rates of interspecific diploid (AG and GA) and triploid (TAG and TGA) crosses were always significantly lower than those of intraspecific combinations (AA and GG) ($P < 0.05$). The survival rate of TGA was always the lowest, only reached 8.16% ± 2.09% at day 350, and was always significantly lower than that of GA ($P < 0.05$). There was no difference in the survival rate between TAG and AG at days 120–300, but TAG displayed a significantly higher survival rate than AG at day 350, with a triploid advantage rate of 82.72% (Table 3).

3.4. Analysis of the triploid rate

The triploid rates of TAG and TGA were always 100% before day 25. The triploid rate of TGA began to decline between day 120 and day 180, and finally decreased to 90.56% ± 3.47% at day 350. However, the

Table 2
Fertilization rate, hatching rate, larval survival rate, larval shell height ($n = 3$ replicates, mean ± SD) of intraspecific crosses (AA and GG), diploid (AG and GG) and triploid (TAG and TGA) crosses between *Crassostrea ariakensis* and *C. gigas*, in addition to heterosis (*H*) values and triploid advantage rates (Td_{AG} and Td_{GA}).

Items	Fertilization rate (%)	Hatching rate (%)	Survival rate (%)			Shell height (μm)		
			Day 5	Day 15	Day 25	Day 5	Day 15	Day 25d
AA	77.35 ± 5.59^{c}	71.88 ± 13.75^{cd}	67.11 ± 14.33^{c}	52.78 ± 6.34^{b}	28.78 ± 7.82^{c}	92.12 ± 7.81^{d}	187.16 ± 31.32^{c}	300.13 ± 26.33^{c}
GG	79.84 ± 11.45^{c}	83.02 ± 2.83^{d}	81.67 ± 7.25^{d}	63.22 ± 7.85^{c}	47.89 ± 7.20^{d}	96.03 ± 6.89^{e}	202.40 ± 25.31^{d}	310.12 ± 28.44^{c}
AG	16.51 ± 2.27^{b}	50.93 ± 7.27^{b}	49.67 ± 9.12^{ab}	31.22 ± 8.58^{a}	10.67 ± 3.50^{a}	86.91 ± 6.11^{b}	163.44 ± 24.67^{a}	268.57 ± 40.20^{a}
GA	9.64 ± 2.17^{a}	56.24 ± 3.73^{b}	58.33 ± 11.32^{bc}	50.56 ± 13.21^{b}	19.33 ± 6.46^{b}	84.74 ± 5.95^{a}	177.62 ± 30.73^{b}	272.69 ± 32.45^{a}
TAG	18.39 ± 4.67^{b}	64.43 ± 9.85^{bc}	60.33 ± 6.98^{c}	46.67 ± 7.12^{b}	26.11 ± 6.86^{c}	84.39 ± 6.41^{a}	159.23 ± 33.33^{a}	263.28 ± 51.09^{a}
TGA	5.13 ± 1.64^{a}	30.64 ± 2.86^{a}	43.44 ± 7.63^{a}	26.78 ± 6.51^{a}	6.11 ± 3.44^{a}	89.42 ± 6.70^{c}	179.44 ± 25.52^{bc}	283.96 ± 22.63^{b}
H (%)	−83.37	−10.10	−27.41	−29.50	−60.87	−8.77	−12.45	−11.31
Td_{AG} (%)	11.34	26.50	21.48	49.47	144.79	−2.90	−2.58	−1.97
Td_{GA} (%)	−46.76	−45.52	−25.52	−47.03	−68.39	5.52	1.03	4.13

Different superscripted letters in each column indicate significant differences ($P < 0.05$). Heterosis (*H*) refers to diploid hybrids compared to intraspecific crosses (AA or GG). The triploid advantage rate (*Td*) refers to triploid hybrids compared to diploid hybrids.

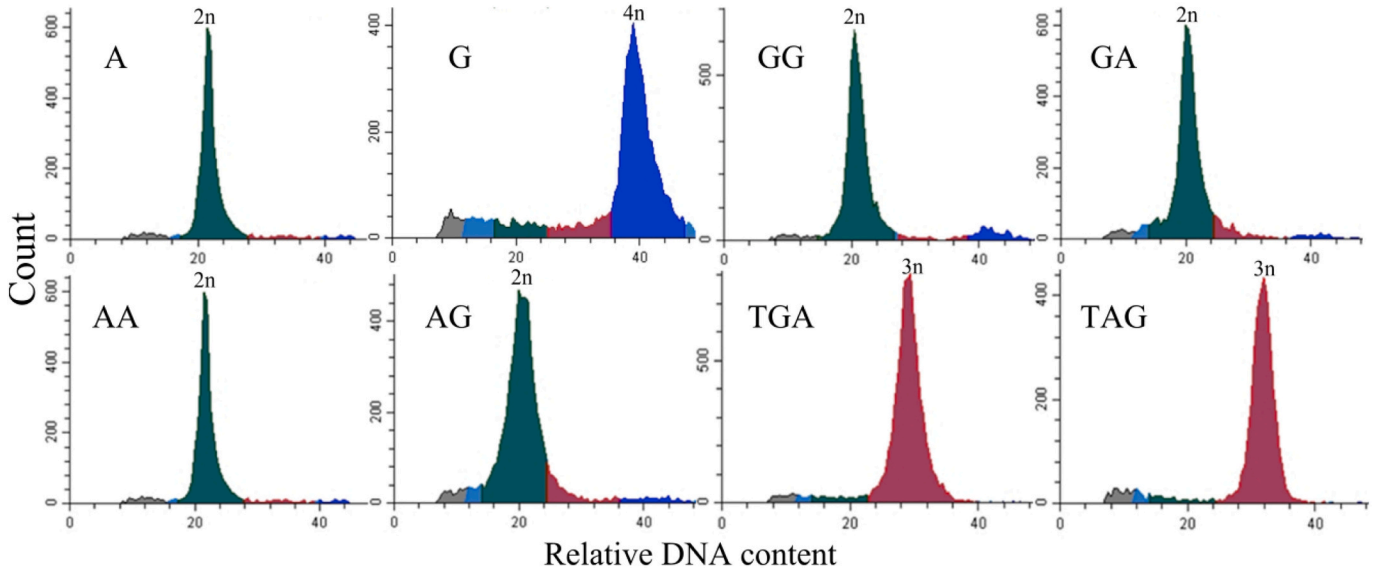

Fig. 1. Ploidy analysis of *Crassostrea ariakensis* (A), *C. gigas* (G) and their intraspecific crosses (AA and GG), diploid crosses (AG and GA), and triploid crosses (TAG and TGA).

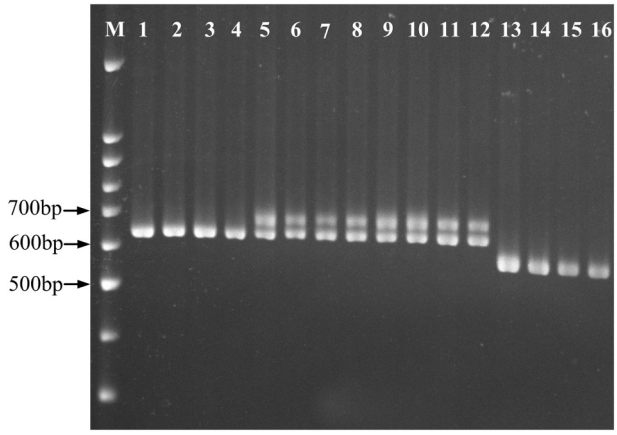

Fig. 2. PCR results for the ITS2 gene of *Crassostrea ariakensis*, *C. gigas* and their intraspecific crosses (AA and GG), diploid crosses (AG and GA) and triploid crosses (TAG and TGA) (M, 100 bp marker; 1–2, *C. gigas*; 3–4, GG; 5–6, GA; 7–8, AG; 9–10, TAG; 11–12, TGA; 13–14, *C. ariakensis*; 15–16, AA).

triploid rate of TAG remained 100% before day 180, and began to decline between days180 and day 300, and declined to 96.11% ± 4.41% at day 350. Overall, the triploid rate of TAG was always higher than that of TGA after day 120 (Table 4).

3.5. Analysis of reproduction

The male ratio was generally higher than the female ratio in all experimental groups except for TAG at day 350. Hermaphrodites were found only in AG, TAG and TGA, with ratios up to 2.2%, 12.2% and 14.4% respectively. A large number of agametic individuals appeared in two triploid hybrids, TAG and TGA, accounting for 55.6% and 39.0% of the oysters, respectively, while no similar phenomenon occurred in the two intraspecific crosses (AA and GG). GG and AA showed the highest numbers of eggs, up to $(83.37 \pm 8.31) \times 10^5$ eggs and $(61.69 \pm 6.32) \times 10^5$ eggs, respectively, while the two triploid hybrids TAG and TGA, showed the significantly lowest number of eggs ($P < 0.05$), only $(0.46 \pm 0.02) \times 10^5$ eggs and $(0.58 \pm 0.06) \times 10^5$ eggs respectively. The number

Table 4
Triploid rates (n = 3 replicates, mean ± SD) of triploid hybrids TAG and TGA.

Crosses	D-larvae (%)	Day 25 (%)	Day 120 (%)	Day 180 (%)	Day 300 (%)	Day 350 (%)
TAG	100	100	100	100	98.33 ± 2.89	96.11 ± 4.41
TGA	100	100	97.08 ± 4.02	95.83 ± 2.60	92.08 ± 4.02	90.56 ± 3.47

Table 3
Survival rate, shell height and wet weight (n = 3 replicates, mean ± SD) of intraspecific crosses (AA and GG), diploid (AG and GA) and triploid crosses (TAG and TGA) between *Crassostrea ariakensis* and *C. gigas*, in addition to the heterosis (*H*) values and triploid advantage rates (Td_{AG} and Td_{GA}).

Items	Survival rate (%)				Shell height (mm)				Wet weight (g)
	Day 120	Day 180	Day 300	Day 350	Day 120	Day 180	Day 300	Day 350	Day 350
AA	78.35 ± 4.87^{c}	59.75 ± 6.61^{c}	55.41 ± 4.97^{c}	52.13 ± 5.16^{d}	31.76 ± 5.44^{b}	40.55 ± 4.60^{abc}	51.42 ± 5.75^{ab}	53.37 ± 4.55^{ab}	23.01 ± 2.55^{ab}
GG	88.22 ± 4.64^{c}	71.16 ± 5.02^{c}	64.28 ± 3.32^{c}	51.69 ± 8.85^{d}	33.65 ± 2.90^{b}	47.73 ± 1.05^{c}	58.94 ± 2.97^{b}	62.43 ± 9.46^{d}	30.96 ± 3.65^{d}
AG	43.70 ± 11.76^{b}	30.00 ± 9.30^{ab}	24.04 ± 9.73^{ab}	17.98 ± 6.14^{ab}	23.23 ± 1.48^{a}	31.90 ± 5.25^{a}	44.18 ± 6.70^{a}	50.33 ± 6.82^{a}	21.61 ± 2.33^{a}
GA	52.73 ± 10.19^{b}	39.82 ± 5.45^{b}	28.55 ± 3.74^{b}	24.85 ± 2.88^{bc}	30.10 ± 1.45^{b}	42.33 ± 5.91^{bc}	51.51 ± 2.58^{ab}	55.73 ± 7.01^{bc}	25.92 ± 4.84^{bc}
TAG	53.25 ± 6.50^{b}	35.42 ± 7.92^{b}	33.90 ± 7.33^{b}	32.85 ± 7.58^{c}	23.84 ± 6.06^{a}	33.63 ± 4.85^{a}	45.62 ± 3.55^{a}	53.60 ± 7.43^{ab}	22.77 ± 4.15^{ab}
TGA	24.15 ± 5.62^{a}	21.11 ± 7.55^{a}	13.52 ± 3.76^{a}	8.61 ± 2.09^{a}	32.59 ± 4.36^{b}	45.93 ± 5.86^{c}	56.82 ± 4.03^{b}	59.17 ± 8.66^{cd}	27.02 ± 5.26^{c}
H (%)	−42.11	−46.67	−56.06	−58.75	−18.46	−15.91	−13.29	−8.41	−11.94
Td_{AG} (%)	21.84	18.08	50.00	82.72	2.63	5.43	3.25	6.49	5.37
Td_{GA} (%)	−54.21	−46.99	−52.63	−65.34	8.27	8.50	10.32	6.16	4.26

Different superscripted letters in each column indicate significant differences ($P < 0.05$). Heterosis (*H*) refers to diploid hybrids compared to intraspecific crosses (AA or GG). The triploid advantage rate (*Td*) refers to triploid hybrids compared to diploid hybrids.

of sperm in the intraspecific crosses (AA and GG) was highest, and significant so ($P < 0.05$), followed by the diploid crosses (AG and GA), whereas that in the triploid crosses (TAG and TGA) was the lowest, and this difference was significant ($P < 0.05$) (Table 5). According to the appearance of the gonads, AG and GA showed a fatter edible part than TAG and TGA, but less fatter than AA and GG (Fig. 3).

4. Discussion

The shell height of the triploid hybrid TAG was lower than that of the diploid hybrid AG throughout the larval stage; similarly slow growth of triploid larvae has been found in triploid *C. sikamea* (Wu et al., 2019) and allotriploid between *C. gigas* and *C. hongkongensis* (Zhang et al., 2014a). Wang et al. (2002) reported that the triploidization of diploid oysters leads to an increase in heterozygosity, thereby affecting their phenotype. The phenomenon of an increase in heterozygosity promoting growth mostly occurs after metamorphosis, while the increase in heterozygosity in the larval stage may harm growth (Mallet et al., 1985; Zouros et al., 1983), which might explain the slow growth of the triploid hybrid larvae in this experiment. Although TGA grew faster than GA, its fertilization rate, hatching rate and larval survival rate were all lower, which may result from egg source problems (Guo et al., 1996; Matt and Allen, 2014). Previous studies have shown that *C. gigas* tetraploids exhibit a lower number of eggs and lower sperm quality than diploids (Guo and Allen, 1997; Suquet et al., 2010), meaning that tetraploids may show slightly lower fecundity than diploids. In our experiment, we found that the gonad development of the female tetraploid *C. gigas* was far inferior to that of diploid *C. gigas* and *C. ariakensis*. Poor gonad development, characterized by a low number of eggs and a high proportion of deformed eggs, may directly reduce the hatching rate and larval survival rate of progenies with tetraploid *C. gigas* as the female parent. In fact, as long as the survival rate is high in the larval stage, it is valuable for mass production. However, if the survival rate is so low that it failure to obtain a large number of eyespot larvae, the aquaculture value will be zero. The survival rate of TGA was only 6.11% ± 3.44% at day 25, and such a low larval survival rate means that it is extremely unlikely that TGA will become a new breed for commercial production.

The bands obtained in the genetic identification of the ITS2 gene in *C. ariakensis* or *C. gigas* were consistent with previous studies (Yao et al., 2015; Zhang et al., 2012a). The hybrids showed two specific bands, indicating that they were true heterozygotes. During the grow-out stage, the shell heights of TAG and TGA were always greater than those of AG and GA, respectively, showing the rapid growth of triploid juveniles, which was consistent with the results of most studies on triploid oysters (Zhang et al., 2017; Zhang et al., 2014a). However, although the triploid hybrids employed in this experiment grew faster than the related diploid hybrids, the growth advantage was not significant. We propose two hypotheses based on previous research to explain this phenomenon. First, the heterosis value of shell height in the diploid hybrids (AG and GA) was consistently significantly negative, indicating that the two hybrids showed growth inhibition relative to their parents, and the growth inhibition resulting from hybridization may hinder the presentation of the triploid growth advantage. Second, it may be related to its environmental adaptation. The phenotypic characteristics of triploid oysters are usually affected by environmental factors (Callam et al., 2016; Melo et al., 2020; Qin et al., 2019). In addition, Zhang et al. (2012) reported that the hybrid derived from female *C. gigas* and male *C. ariakensis* showed obvious survival and growth advantages in larval and grow-out stages, which was different from our results. The differences in environmental salinity between the two studies may be the main reason that the hybrids exhibit different phenotypic traits. Considering the difference in salinity adaptability between *C. ariakensis* and *C. gigas* (Li et al., 2021), their hybrids may show completely different aquaculture traits under environmental salinities. Therefore, the advantages of triploid hybrids under different farming circumstances, especially in sea areas with different salinities, need to be further studied.

The triploid hybrids showed extremely poor reproduction, which specifically manifested as a high proportion of agametic and hermaphroditic individuals or a low number of gametes. This verified the observations of completely sterile or slightly fertile triploid oysters in previous studies (Dheilly et al., 2014; Jouaux et al., 2010). Moreover, it is the poor fertility of the triploid that accounts for its inability to reproduce normally and maintain excellent meat quality year round, which would allow the gap in oyster production to be filled during the breeding season. In addition, we observed an interesting phenomenon of higher hermaphrodite ratios were higher in the triploid hybrids. Hermaphroditism is a manifestation of a sexual differentiation disorder and has been reported in previous studies on triploid fertility (Gong et al., 2004; Jouaux et al., 2010). At the molecular level, the presence of an extra set of chromosomes blocks chromosome sex differentiation (Dheilly et al., 2014), resulting in a large number of hermaphroditic and agametic individuals.

The survival rates of the diploid hybrids (AA and GG) were always significantly ($P < 0.05$) lower than those of the two parents, showing an obvious hybrid disadvantage between *C. gigas* and *C. ariakensis*, which has also been found in the hybridization of other oysters (Huo et al., 2013; Xu et al., 2009; Xu et al., 2019b; Yao et al., 2015). Although F_1 hybrids show no growth and survival advantages, backcrossing with their parents will be of great valuable in further breeding because the offspring of backcrosses with parents often exhibit phenotypic traits completely different from those of F_1 hybrids (Zhang et al., 2016). In AG, the survival rate of its triploid TAG significantly increased, indicating that the triploid mutation could improve the survivability of AG and highlighting the high stress resistance of the triploid oyster (Dégremont et al., 2016; Gagnaire et al., 2006). However, the survival rate of TGA was significantly lower than that of GA, indicating that the survival rate of the triploid obtained from the 4n ♀ × 2n ♂ cross was lower than that of the common diploid, and a similar phenomenon has been reported in the triploid hybrids of *C. virginica* (de Sousa et al., 2017) and *C. gigas* (Guo et al., 1996). This also suggests that the generation of triploids from 4n ♀ × 2n ♂ cross is not a suitable approach for large-scale production.

The instability of ploidy is a complex problem in oyster polyploid breeding that has a serious impact on the commercial production of all-triploid oysters and the generation of stable tetraploid lines (Comai, 2005; McCombie et al., 2005; Zhang et al., 2010). In our experiment, we

Table 5
Sex ratio (n = 3 replicates, mean) and numbers of eggs and sperm (n = 3 replicates, mean ± SD) of intraspecific crosses (AA and GG), diploid crosses (AG and GA) and triploid crosses (TAG and TGA) between *Crassostrea ariakensis* and *C. gigas* at day 350.

Crosses	Female ratio (%)	Male ratio (%)	Hermaphrodite ratio (%)	Agametic ratio (%)	Number of eggs ($\times 10^5$)	Number of sperm ($\times 10^7$)
AA	41.1	58.9	0.0	0.0	61.69 ± 6.32^{e}	144.64 ± 22.70^{d}
GG	48.9	51.1	0.0	0.0	83.37 ± 8.31^{d}	156.95 ± 29.21^{d}
AG	39.0	54.4	2.2	4.4	27.71 ± 4.57^{b}	85.29 ± 5.75^{c}
GA	42.2	52.2	0.0	5.6	39.37 ± 3.42^{c}	48.13 ± 5.01^{b}
TAG	17.8	14.4	12.2	55.6	0.46 ± 0.02^{a}	2.03 ± 0.21^{a}
TGA	22.2	24.4	14.4	39.0	0.58 ± 0.06^{a}	3.37 ± 1.01^{a}

Different superscripted letters in the "Number of eggs" and "Number of sperm" columns indicate significant differences ($P < 0.05$). Not all crosses simultaneously have female, male, hermaphroditic and agametic individuals, so there are no multiple comparisons of sex ratios.

Fig. 3. Appearance of gonads in intraspecific crosses (AA and GG), diploid crosses (AG and GA) and triploid crosses (TAG and TGA) between *Crassostrea ariakensis* and *C. gigas* at day 350.

found that the two allotriploid groups showed varying degrees of decreases in triploid rates, which reached 96.11% ± 4.11% (TAG) and 90.56% ± 3.45% (TGA) at day 350. The reversion of ploidy caused by chromosome loss at the individual level is assumed to be the main reason for the decreases in triploid or tetraploid rates in a polyploid population. This phenomenon of chromosome loss usually occurs in triploid oysters (Zhang et al., 2010) and tetraploid oysters (Zhang et al., 2014b; Zhang et al., 2014c). Current studies indicate that chromosome reversion may be linked to a meiosis error, which means that the appearance of chromosome clumping during meiosis leads to a gradual reduction of the chromosome number (de Sousa et al., 2016; Zhang et al., 2010). However, there are relatively few studies on the external influence and internal mechanism of oyster chromosome loss, and the in-depth exploration of the molecular mechanism should be the main approach for fundamentally addressing polyploidy instability. In addition, female tetraploid oysters are more likely to produce aneuploid gametes than male tetraploids, thus increasing the proportion of aneuploid larvae in the triploids produced by female tetraploids (Guo and Allen, 1997). These aneuploid individuals are considered to be an unstable intermediate process of chromosome loss that will eventually reverse to the stable state of diploids (Zhang et al., 2010), which seems to explain why the triploid rate of TGA was lower than that of TAG. Therefore, the generation of a triploid oyster population by crossing male tetraploids with female diploids can reduce the decrease in the triploid rate to some extent.

In conclusion, we successfully produced diploid and triploid hybrids by hybridization between *C. gigas* and *C. ariakensis* and obtained growth, survival and reproductive data. The triploid hybrid TAG, obtained from a cross of 2n female *C. ariakensis* × 4n male *C. gigas*, showed advantages in growth and survival compared with the diploid hybrid AG, and it is expected to become a new cultured oyster resource in northern China; nevertheless, its advantages in terms of nutritional value, meat quality and salinity adaptation need to be further studied. Although the triploid hybrid TGA, obtained from the cross of 4n female *C. gigas* × 2n male *C. ariakensis*, showed a growth advantage, its lower survival rate remains bottleneck to its application in large-scale production. The results of this study will further enrich research on the crossbreeding and polyploid breeding of oysters, and the obtained diploid and triploid hybrids will become important resources for research on the genetic improvement of oysters.

Data availability statement

The data used to support the findings of this study are available from the first author upon request.

Ethics statement

The present study did not involve human subjects. All animal experiments in the present study complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). All animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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