PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

ARTICLES FOR FACULTY MEMBERS

Title/Author	A comparative analysis of the growth, survival and reproduction of Crassostrea hongkongensis, Crassostrea ariakensis, and their diploid and triploid hybrids / Qin, Y., Li, X., Noor, Z., Li, J., Zhou, Z., Ma, H., Xiao, S., Mo, R., Zhang, Y., & Yu, Z.
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Title/Author	Aquaculture performance comparison of reciprocal triploid C. gigas produced by mating tetraploids and diploids in China / Qin, Y., Zhang, Y., & Yu, Z.
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Title/Author	Effect of farming practices on growth and mortality rates in triploid and diploid eastern oysters Crassostrea virginica / Bodenstein, S., Walton, W. C., & Steury, T. D.
Source	*Aquaculture Environment Interactions* Volume 13 (2021) Pages 33–40 https://doi.org/10.3354/aei00387 (Database: Inter-Research)

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Title/Author	Effects of different aquaculture patterns on growth, survival and yield of diploid and triploid Portuguese oysters (Crassostrea angulate) / Sun, Y., Zhang, C., Liu, F., Zhang, H., Du, H., Zhang, Y., & Zheng, H.
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Title/Author	Growth, survival and gonad development of two new types of reciprocal triploid hybrids between Crassostrea gigas and C. angulata / Jiang, G., Li, Q., & Xu, C.
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Title/Author	Refinement of a classification system for gonad development in the triploid oyster Crassostrea gigas / Yang, Q., Yu, H., & Li, Q.
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Title/Author	Transcriptomic and metabolomic analyses provide insights into the growth and development advantages of triploid Apostichopus japonicus / Xie, J., Sun, Y., Cao, Y., Han, L., Li, Y., Ding, B., Gao, C., Hao, P., Jin, X., Chang, Y., Song, J., Yin, D., & Ding, J.
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A comparative analysis of the growth, survival and reproduction of *Crassostrea hongkongensis*, *Crassostrea ariakensis*, and their diploid and triploid hybrids

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ABSTRACT

Although the market demand for *Crassostrea ariakensis* is lower than *Crassostrea hongkongensis* in southern China, it has some advantages over *C. hongkongensis*, such as higher salinity tolerance and better stress resistance. Here, we present the first report of a systematic comparative study on the two native sympatric oysters of southern China, *C. hongkongensis* and *C. ariakensis*, and their diploid and triploid hybrids. Three replicates were successfully conducted, and each replicate contained two intraspecific crosses (AA–*C. ariakensis*♂ × *C. ariakensis*♀ and HH–*C. hongkongensis*♂ × *C. hongkongensis*♀), a diploid interspecific hybridization (HA–*C. hongkongensis*♀ × *C. ariakensis*♂) and a triploid interspecific hybridization (HHA). Fertilization rates of intraspecific crosses were significantly higher than those of interspecific crosses, but HA grew significantly faster than the two intraspecific crosses during the larval stage. The D larval rates of all diploids were significantly higher than that of HHA, and HHA larvae had significantly lower survival rates than diploids. At the high salinity site (Zhulin), the shell heights of HA were significantly higher than those of HH (90–600 days) and AA (270–600 days). The whole weights of HA were always significantly higher than those of the intraspecific crosses. At the low salinity site (Dafeng River), HA always had significantly higher shell heights than AA and had similar shell heights to HH. HA had higher whole weights than AA on the 360th and 480th days. In summary, data analysis revealed that HA always exhibited positive heterosis at both sites, indicating that it had growth advantages regardless of different environmental conditions. At both sites, the growth traits of HHA were significantly higher than those of HA. Our trials also demonstrated that HA had a greater salinity tolerance than the two pure species. HA was completely fertile, but 40%–48% of the HHA exhibited either significant sterility with atrophic gonads and no mature gametes or residual sterility with partly atrophic gonads and some mature gametes. Our study confirms the clear advantages of HA and HHA in terms of growth traits and viability, and provides promising evidence of their potential value as new variants for use in farming.

1. Introduction

Hybridization is an important and useful tool in animal genetic breeding and has created enormous economic benefits in many countries. Examples of this include: grass carp × bighead carp in America (Allen and Wattendorf, 1987), grass carp × topmouth culter in China (Wu et al., 2019a), and African × Thai catfish in Thailand (Bartley et al., 2000). Hybridization includes hybrid within a species and between species, which usually produces changes in genotype and phenotype as a result of the combination of parental genetics (Wu et al., 2019a). Hybrids are often used to combine advantageous characteristics of both parents, which often results in increased growth rates, a transference of desirable traits, and improved disease resistance and stress tolerance (Xu et al., 2019b; Zhang et al., 2016b). Triploid

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induction is used to solve the problems associated with sexual maturation in diploid such as lower growth rates, increased incidence of diseases and reduced survival rate (Guo and Allen, 1994a; Qin et al., 2019). Hence, allotriploid can combine heterosis and triploid advantage, which can further improve the growth rate and sterility. In addition, some triploid hybrids have increased disease resistance and viability compared to diploids, and triploid induction is considered as potentially useful method for the genetic improvement of farmed animals (Francesc et al., 2009; Hu et al., 2012). For example, diploid and triploid interspecific hybridization between grass carp and topmouth culter was carried out by Wu et al. (2019a), and the hybrid offspring exhibited higher total amino acid contents than the parental species. Zhang et al. (2014) confirmed that allotriploids (female *Crassostrea hongkongensis* × male *Crassostrea gigas*) had faster growth and higher survival rates than the parental species.

Many papers have been published on artificial interspecific hybridization of marine bivalves, including abalones (Hoshikawa et al., 1998; Lafarga de la Cruz and Gallardo-Escárate, 2011), scallops (Hu et al., 2013; Qing Yin and Li Qing, 2004) and oysters (Barton, 2010). Interspecific hybridization in oysters has mainly been reported in the genus *Crassostrea*. *C. gigas* × *C. hongkongensis* (Zhang et al., 2016b), *Crassostrea angulata* × *C. hongkongensis* (Zhang et al., 2016a) and *C. angulata* × *Crassostrea sikamea* (Yan et al., 2018) only exhibited unidirectional fertilization, but *C. gigas* × *C. angulata* (Soletchnik et al., 2002), *C. gigas* × *C. sikamea* (Xu et al., 2019a), *C. angulata* × *Crassostrea ariakensis* (Yao et al., 2015), *C. hongkongensis* × *C. sikamea* (Zhang et al., 2017b) and *C. gigas* × *Crassostrea nippona* (Xu et al., 2019b) showed bidirectional fertilization. The interspecific hybrids usually showed faster growth than one or both parents, but Zhang et al. (2016b) reported that hybrids between *C. gigas* and *C. hongkongensis* had slower growth than the parental species. Because of the poor fertility of triploids, it is difficult to carry out direct genetic improvement of triploids. However, the production of allotriploid may be an indirect means for triploid genetic improvement. In marine bivalves, there are few studies on allotriploid. Zhang et al. (2014) produced allotriploid between *C. gigas* and *C. hongkongensis* by inhibiting the second polar body release and Que and Allen (2002) produced a small number of *C. gigas* × *C. ariakensis* allotriploids by crossing tetraploid male *C. gigas* with diploid female *C. ariakensis*. Both of these displayed growth advantages.

C. hongkongensis and *C. ariakensis* are native estuarine species in southern China, and *C. ariakensis* is also distributed in northern China, while *C. hongkongensis* is endemic to southern china. The production and sales volume of *C. ariakensis* is gradually decreasing because of its yellow flesh color, but it has advantages in growth and suitability for higher salinity environments, while *C. hongkongensis* is popular because of its white flesh color, high-quality adductor muscle, and delicious taste but has disadvantages in disease resistance, stress tolerance and distribution areas (Xiao et al., 2018; Zhang et al., 2016a). Hence, interspecific hybridization between these two species may be a good method to minimize the disadvantages of each species and is potentially useful for the genetic improvement of these two important aquaculture species. Because of gametogenesis, diploid oysters usually exhibit slow growth, inferior taste, and high mortality during the reproductive stage (Qin et al., 2019). Triploid gonads are usually sterile, so triploid production may be a useful way to solve the problems related to gonad development (Francesc et al., 2009). Huo et al. (2013) have studied the growth of hybrids between *C. hongkongensis* and *C. ariakensis* in northern China, but no *C. hongkongensis* individual survived to adulthood because of low environmental temperatures. As *C. hongkongensis* is not distributed and cannot survive in northern China, it is incomplete and not suitable for study this interspecific hybridization in northern China.

Therefore, we intend to conduct a systematic comparative study of *C. hongkongensis*, *C. ariakensis*, and their diploid and triploid hybrids cultured at two sites with different salinities in southern China. The aims of this study are to investigate the fertilization, D larval rates, shell heights and cumulative survival rates of the four groups at the planktonic larval stage, and further compare the differences in shell height, whole weight, incremental survival rate and gonad development at the spat and adult oyster stages. The morphological distinction, molecular identification, and ploidy detection methods are used for rapid identification of *C. hongkongensis*, *C. ariakensis*, diploid and triploid hybrids.

2. Materials and methods

2.1. Preparation and mating of parent oysters

Gonadal mature diploid *C. hongkongensis* and *C. ariakensis* used in the experiment were collected from Beihai, Guangxi Province. The parent oysters were artificially cultivated in an open circulating system (temperature 28.0–30.0 °C, salinity 15 ppt) for at least one week before the experiments. The parent oysters were fed twice daily with plenty of *Isochrysis zhanjiangensis* and *Chaetoceros calcitrans* (Qin et al., 2018b). Before fertilization, *C. hongkongensis* and *C. ariakensis* were identified based on external morphology and inner structure as described in Fig. 4.

Setting up the experiments according to Table 1, three replicates were set for each group. Because *C. hongkongensis* sperm could not fertilize *C. ariakensis* eggs, each replicate consisted of the following crosses: HH — *C. hongkongensis* ♀ × *C. hongkongensis* ♂, AA — *C. ariakensis* ♀ × *C. ariakensis* ♂, HA — *C. hongkongensis* ♀ × *C. ariakensis* ♂, and HHA. One male and one female from each species were used in each replicate. The fertilization procedure was based on Qin et al. (2018b). Triploid hybrids were induced by blocking the release of the second polar body (PB2) in fertilized eggs using 0.5 $mg.L^{-1}$ CB according to Qin et al. (2019). Triploid rates were detected using flow cytometry and the triploid rates of 90%~100% were selected for use in the experiment. Diploid and triploid hybrids in the same replicate were offspring of the same parent oysters. After fertilization, a patch of adductor muscle from each parent oyster was fixed in absolute ethyl alcohol and used for subsequent molecular identification.

2.2. Morphological and molecular identification

In terms of morphological aspects, the shell mold, flesh color, and gills structure differed between *C. hongkongensis* and *C. ariakensis*. The shell mold of most pure *C. hongkongensis* was elongated, whereas the shells of *C. ariakensis* tended to be rounded. The flesh color of pure *C. hongkongensis* was white, whereas that of *C. ariakensis* was yellow or red. When compared the structure of gills, we confirmed that the central part of the gill was detachable from the soft tissue in *C. hongkongensis* and four rows of gill tubes were visible after pulling open the mantle. In *C. ariakensis*, the gills were attached to the soft tissue and

Table 1
Experimental design for hybridization and allotriploid production between female *C. hongkongensis* and male *C. ariakensis*.

	♂					
♀	A_1	H_1	A_2	H_2	A_3	H_3
A_1	AA_1	–	–	–	–	–
H_1	$HA_1(HHA_1)$	HH_1	–	–	–	–
A_2	–	–	AA_2	–	–	–
H_2	–	–	$HA_2(HHA_2)$	HH_2	–	–
A_3	–	–	–	–	AA_3	–
H_3	–	–	–	–	$HA_3(HHA_3)$	HH_3

Note: HH: *C. hongkongensis*; AA: *C. ariakensis*; HA: diploid hybrids; HHA: triploid hybrids. *C. hongkongensis* sperm could not fertilize *C. ariakensis* eggs, so there was no AH group in this study. The subscript number 1, 2, 3 denotes the 3 replications, and each replication consisted of one female and one male of each species.

when the mantle was opened, only one row of gills tube was visible. In addition, the holes in the gill water tubes were larger in *C. ariakensis* than in *C. hongkongensis* (Fig. 4).

The adductor muscle was used for hybrid molecular identification. The adductor muscles of the hybrids were sampled and fixed on the 90th day. DNA was extracted from the adductor muscles using a Magen tissue DNeasy kit (Magen). Polymerase chain reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP) was used to identify the hybrid status of individuals in the diploid and triploid hybrid groups. The cytochrome oxidase I (CO I) gene was used for maternal identification and the ITS-2 gene was used for hybrid identification. The universal primers for the CO I gene were 5′-GGTCAACAAATCATAAAGATAT TGG-3′ (forward primer) and 5′-TAAACTTCAGGGTGACCAAAAAA TCA-3′ (reverse primer) (Haiyan and Ximing, 2008; Park and Foighil, 2000). In addition, the species-specific primer was 5′-GGAGTAAGTGG ATAAGGGTGGATAG-3′ for *C. hongkongensis* and 5′-AAAAAAGATTAT AACTAATGCATGTCGTG-3′ for *C. ariakensis* (Folmer et al., 1994; Wang et al., 2004). The universal primers for ITS-2 gene amplification were 5′- GGGTCGATGAAGAACGCAG -3′ (forward primer) and 5′- GCTCTT CCCGCTTCACTCG -3′ (reverse primer) (Xia et al., 2009). The PCR amplifications were carried out in a total volume of 30 μL, with 3 μL of 10× PCR buffer, 0.3 μL rTaq enzyme, 5 μL dNTP, 0.50 μM forward and reverse primers, and 1.5 μL of the diluted DNA, with the remainder of the volume made up with sterile water. After amplification, the products of the ITS-2 PCR were digested using Hinc II to show species-specific RFLP patterns (Huo et al., 2013). All amplified fragments were separated on 1.5% agarose gels containing 0.2 μg/mL ethidium bromide and visualized under a UV transilluminator.

2.3. Ploidy detection

The DNA ploidy level was detected by a CyFlow Ploidy Analyser (Sysmex) using the method presented by Qin et al. (2019).

2.4. Growth-related data measurement

During the planktonic larval stage, all groups of the larvae were cultured at the same density in cement ponds. Fertilization rate was defined as the percentage of all eggs containing zygotes, and D larval rate was defined as the proportion of zygotes which developed into normal D-shaped larvae. A light microscope was used to measure the shell heights of the larvae, and cumulative survival rates from D-shaped larvae to eyespot larvae were calculated according to the following formula:

$$M_t\% = \frac{S_t}{S_1} \times 100$$

where M_t% is the cumulative survival rate of larvae at time n; S_1 is the number of normal D-shaped larvae on the first day, and S_t is the number of planktonic larvae at time t.

When metamorphosis occurred and the larval feet appeared, pediveliger were collected with a 180 μm nylon screen and immediately moved to a cement pond, in which had been hung numerous cement substrates for attachment. After attachment and growth to about 1 mm spat size in these ponds, the spat of all groups were simultaneously transported and deployed at 2 sites in Beihai, Guangxi Province: Dafeng River site and Zhulin pond site (Fig. 1). At each measuring time, a total of 60 individual oysters of each group from each site were randomly collected and measured. The shell height of oysters was measured using vernier calipers (accurate to 0.01 mm), and the whole weight was measured with an electronic scale (accurate to 0.01 g). In triploid hybrid groups, each oyster was labeled and their ploidy status assessed after measuring weight and shell height. At every measurement time, 5 incremental survival rates of each group were measured at each site. The incremental survival rate of the oysters was calculated according to the following formula:

$$N_{t+1}\% = \frac{S_{t+1}}{S_t} \times 100$$

where N_{t+1}% is the survival rate of oysters at time t + 1; S_t is the number of surviving oysters at time t; S_{t+1} is the number of surviving oysters at time t + 1; and t + 1 is the next measurement time point after time t.

2.5. Comparison of gonadal development and sex ratio

In the breeding season, 50 individuals were randomly selected from each group: *C. hongkongensis*, *C. ariakensis*, diploid and triploid hybrids. The gonadal development period was determined according to Ibarra et al. (2017) and Qin et al. (2018a). The oysters were opened with a knife, and a light microscope was used for sex ratio determination. The oysters were sorted into the following categories: male, female, hermaphrodite or no gametes. Gonad tissues were then sampled, placed in Bouin's fixing fluid for 20–24 h and then preserved in 70% ethyl alcohol. Subsequently, the samples were dehydrated with a series of concentrations of ethyl alcohol, rendered transparent with chloroform, and then embedded in paraffin. Finally, standard 6-μm sections were sectioned and stained with hematoxylin and eosin (Hu et al., 2012; Xu et al., 2018). The finished tissue slices were examined and photographed with a microscope.

2.6. Statistical analysis

The phenotypic characters data in this paper are presented as mean ± standard deviation (M ± SD). Multiple comparisons of the growth, whole weight and incremental survival rates of all groups were performed using a one-way analysis of variance (ANOVA) followed by a multiple comparison test using Statistical Product and Service Solutions 18.0 (SPSS18). Differences were considered significant when $P < .05$.

The heterosis (HT) is defined as the percentage difference in the growth, whole weight or survival rate between diploid hybrids (HA) and intraspecific groups (*C. hongkongensis*-HH, *C. ariakensis*-AA) and is calculated according to the following formula (Zhang et al., 2014):

$$HT\ (\%) = \left(\frac{2HA - (HH + AA)}{HH + AA}\right) \times 100$$

where a positive HT indicates that the diploid hybrids performed better than the intraspecific groups, and a negative HT indicates that the diploid hybrids performed worse.

The triploid advantage (TA) is defined as the percent difference in phenotypic characters (the growth, whole weight or survival rate) between the diploid hybrids (2 N) and the triploid hybrids (3 N) and is calculated using the following formula (Qin et al., 2019):

$$TA\ (\%) = \left(\frac{3N - 2N}{2N}\right) \times 100$$

where a positive TA indicates that the triploid hybrid oysters performed better than the diploids, and a negative TA indicates that the triploids performed worse.

3. Results

3.1. Environmental variation between grow-out sites

During the growth phase there was insignificant variations in the water temperature from the 90th day to the 600th day. Because both sites were in southern China and were close together, the seawater temperatures at the two sites were very similar (Fig. 2A). However, the salinity of seawater differed greatly between the two sites, ranging from 25 ppt to 32 ppt at the Zhulin site and from 15 ppt to 25 ppt at the Dafeng River site (Fig. 2B).

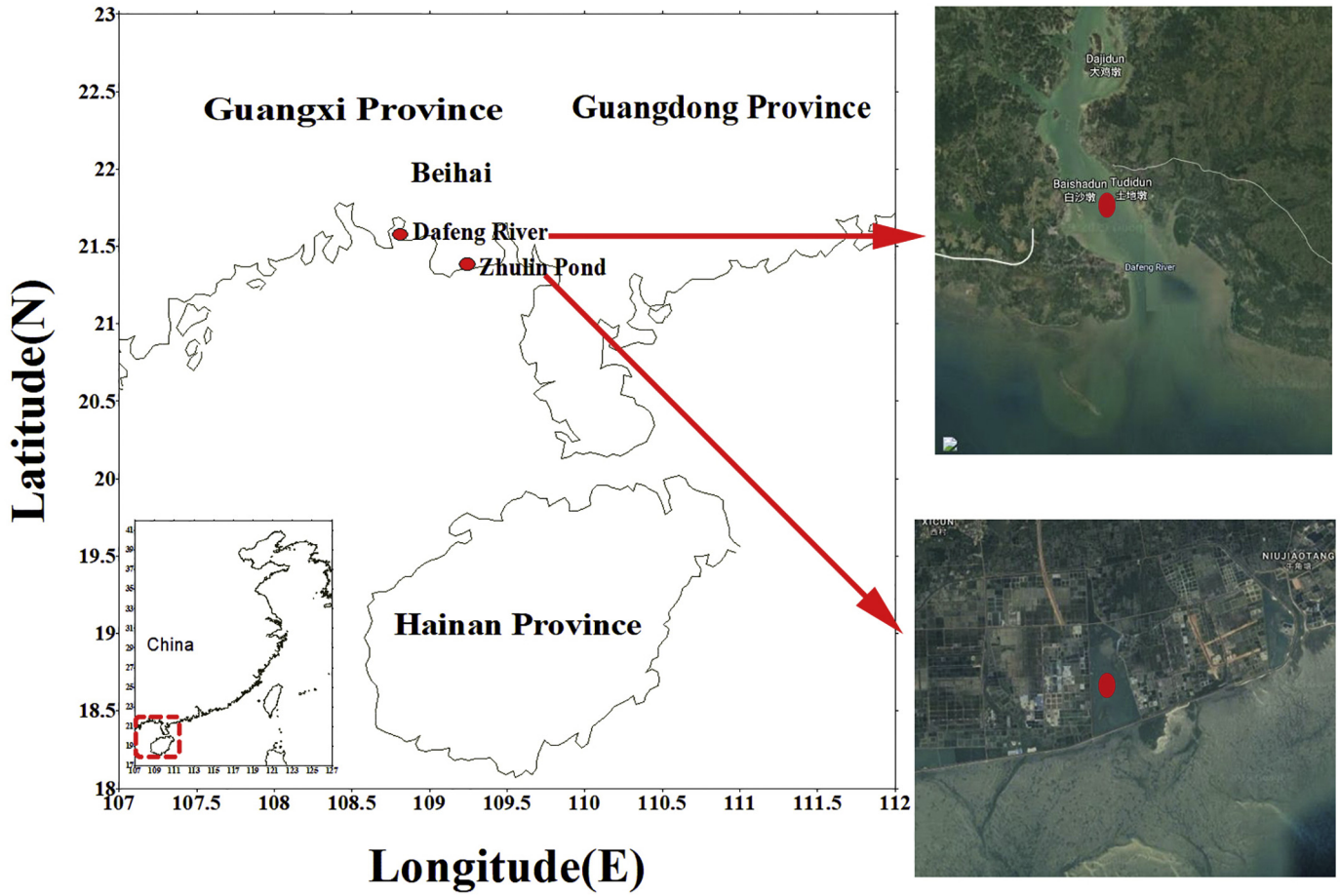

Fig. 1. Location of the 2 experimental grow-out sites in Beihai, Guangxi Province of China.

3.2. Phenotypic and molecular characteristics of hybrids

The majority of hybrids had elongated shells, which were similar to *C. hongkongensis* and obviously different from *C. ariakensis*. Moreover, the flesh color of the hybrids was similar to the white of *C. hongkongensis* than the yellow or red of *C. ariakensis*. However, when the mantle was lifted, only one row of gills water tube was exposed in hybrids, the gill water tubes were not free but tightly connected to the soft tissue. From a morphological perspective, the hybrids combined the characteristics of *C. hongkongensis* and *C. ariakensis* (Fig. 4).

The results of our molecular identification confirmed that the lengths of the COI amplicons of *C. hongkongensis* and *C. ariakensis* were 387 and 183 bp, respectively. The hybrids produced a CO I band of 387 bp similar to that of *C. hongkongensis*, and lacked the characteristic 183 bp band of *C. ariakensis* (Fig. 3B). On the ITS-2 gene, a Hinc II restriction site was found in *C. hongkongensis* and the hybrids, but not in *C. ariakensis*. Digestion of the *C. hongkongensis* ITS-2 amplicon with Hinc II yielded 2 fragments, whereas the *C. ariakensis* amplicon remained uncut and the hybrid amplicons were divided into three fragments. This allowed us to accurately distinguish between *C. hongkongensis*, *C. ariakensis* and the hybrids (Fig. 3C and D).

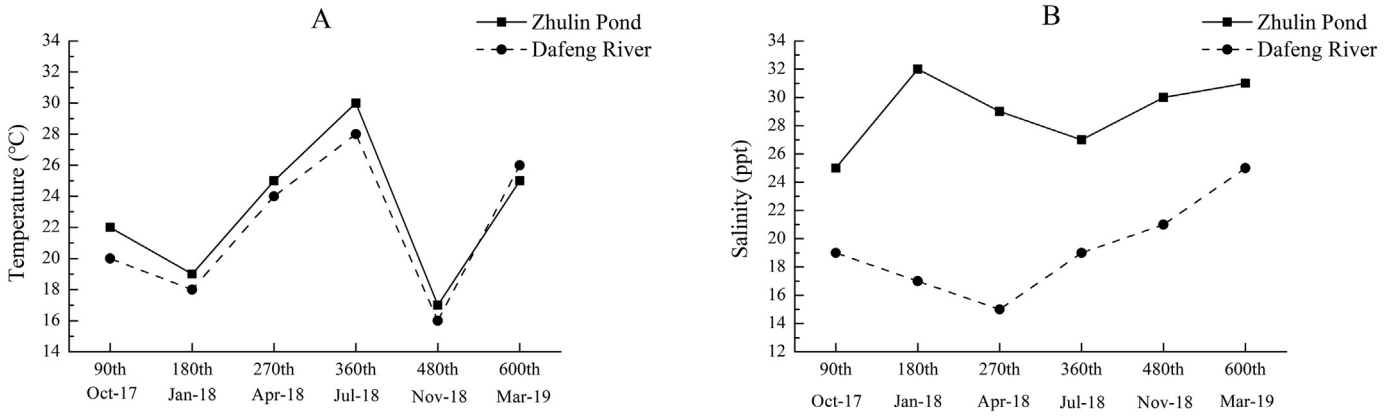

Fig. 2. The variations of temperature and salinity in 2 sites from the 90th day to the 600th day.

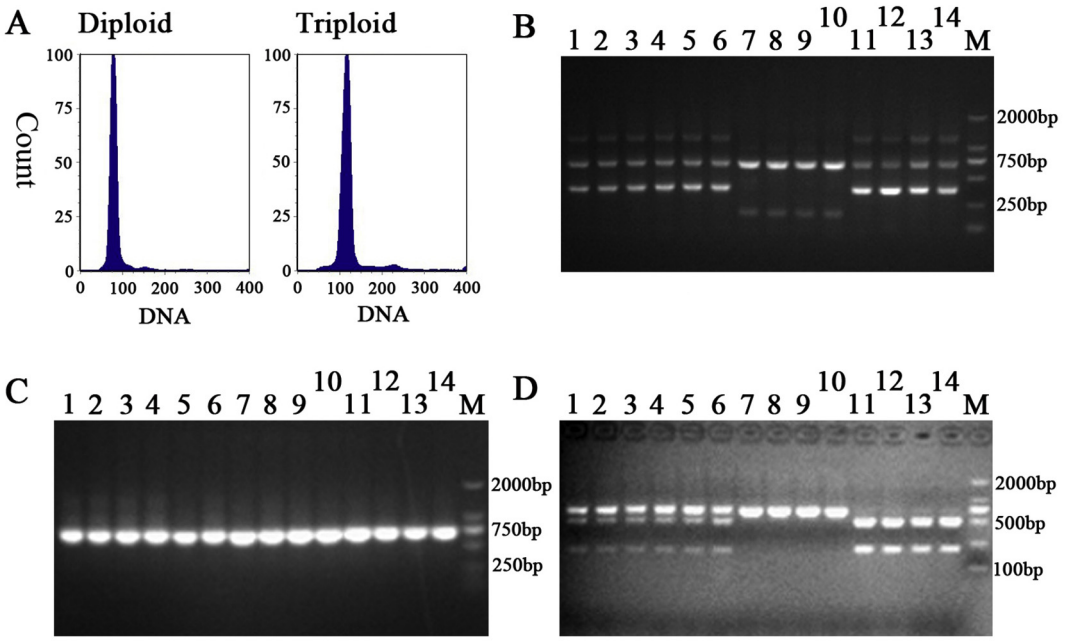

Fig. 3. The paternity testing and species identification of *Crassostrea ariakensis*, *Crassostrea hongkongensis* and their diploid and triploid hybrids using molecular and ploidy analytical methods.

Note: A: The identification of individual diploid and triploid hybrids using flow cytometry. The peak of the triploid oyster was 120, while the peak of the diploid was 80; B: Amplified fragment analysis of COI; C: Amplified fragment analysis of ITS-2, no enzyme digestion; D: Amplified fragment analysis of ITS-2, Hinc II enzyme digestion. 1–3: diploid hybrids; 4–6: triploid hybrids; 7–10: *C. ariakensis*; 11–14: *C. hongkongensis*.

3.3. Growth and survival rates of planktonic larval stage

The fertilization rates of the intraspecific groups were significantly higher than those of the hybrid groups ($P < .05$) (Fig. 5B). The D-larval rates of the diploid groups were significantly higher than that of the triploid group ($P < .05$) (Fig. 5B). Moreover, there was no significant difference between the cumulative survival rates of the diploid groups, but the cumulative survival rates of the triploid hybrids were significantly lower than those of the diploid groups ($P < .05$) in the planktonic larval stage (Fig. 5B). In general, the shell heights of hybrid larvae were significantly larger than those of intraspecific larvae ($P < .05$), and triploid hybrids were significantly larger than diploid hybrids ($P < .05$) (Fig. 5A).

3.4. Shell height, whole weight and survival rates at the two sites

3.4.1. Shell height

At the Zhulin site, there were significant differences between the shell heights of diploid hybrids and those of *C. hongkongensis* ($P < .05$) at all measurement time points. Moreover, the shell heights of triploid hybrids were significantly higher than those of diploid hybrids ($P < .05$) at all measurement times at the Zhulin site (Fig. 6A). On the 90th, 180th, 270th, 360th, 480th and 600th day, HTs were 15.07%, 9.66%, 19.99%, 25.54%, 24.72% and 26.20% (Table 2), and TAs were 21.30%, 10.33%, 5.40%, 6.14%, 6.42% and 1.37% (Table 3), respectively.

At the Dafeng River site, from the 180th day to the 480th day, there were no significant differences between the shell heights of *C. hongkongensis* and the diploid hybrids, but the shell heights of diploid hybrids were significantly higher than those of *C. ariakensis* ($P < .05$). Moreover, the mean shell heights of diploid hybrids were significantly lower than those of triploid hybrids at all measurement times except the 270th day ($P < .05$) (Fig. 6B). The HTs for shell height at the Dafeng River site ranged from 4.76% (the 90th day) to 5.20% (the 600th day) (Table 2) and the TAs ranged from 24.37% to 10.20% (Table 3).

3.4.2. Whole weight

Comparative analysis showed that the mean whole weights of *C. hongkongensis* were significantly lower than those of diploid hybrids ($P < .05$) and the mean whole weights of diploid hybrids were significantly lower than those of triploid hybrids ($P < .05$) (Fig. 6C). The HTs for whole weight were 13.75%, 13.32%, and 21.19% and the TAs were 10.43%, 26.03% and 20.06% for the 360th, 480th and 600th days, respectively (Table 2 and Table 3).

At the Dafeng River site, between the 360th and 600th days the mean whole weight of *C. hongkongensis* increased from 68.00 ± 5.65 g to 92.56 ± 12.81 g, that of *C. ariakensis* increased from 41.34 ± 5.33 g to 115.06 ± 19.29 g, that of the diploid hybrids increased from 68.66 ± 7.03 g to 109.20 ± 20.02 g and that of the triploid hybrids increased from 72.93 ± 6.60 g to 120.70 ± 25.21 g

Table 2
Heteroses (HT) for incremental survival rate, shell height and whole weight in *C. hongkongensis*, *C. ariakensis* and diploid hybrids at two sites.

		Site	90th day	180th day	270th day	360th day	480th day	600th day
HT (%)	Incremental survival rate	Zhulin pond	−0.23	4.68	9.66	−0.08	8.56	32.18
		Dafeng River	2.23	1.00	−0.56	0.90	0.16	9.07
	Shell height	Zhulin pond	15.07	9.66	19.99	25.54	24.72	26.20
		Dafeng River	4.76	4.96	12.09	11.19	12.09	5.20
	Whole weight	Zhulin pond	–	–	–	13.75	13.32	21.19
		Dafeng River	–	–	–	25.60	8.90	5.19

Table 3
Triploid advantage (TA; compared to diploid hybrids) for incremental survival rate, shell height and whole weight at two sites.

		Site	90th day	180th day	270th day	360th day	480th day	600th day
TA (%)	Incremental survival rate	Zhulin pond	−2.03	−0.40	0.12	7.48	0.42	−5.73
		Dafeng River	−1.91	1.15	−0.56	8.77	−0.89	−1.20
	Shell height	Zhulin pond	21.30	10.33	5.40	6.14	6.42	1.37
		Dafeng River	24.37	6.57	5.31	9.49	6.56	10.20
	Whole weight	Zhulin pond	–	–	–	10.43	26.03	20.06
		Dafeng River	–	–	–	6.22	20.86	10.53

(Fig. 6D). There were significant differences between the mean whole weights of *C. ariakensis* and the diploid hybrids on the 480th day and the 600th day ($P < .05$) and the mean whole weight of the diploid hybrids was significantly lower than that of the triploid hybrids ($P < .05$) at all measurement times (Fig. 6D). In addition, the HT ranged from 25.60% to 5.19% and the TA ranged from 6.22% to 10.53% (Table 2 and Table 3).

3.4.3. Incremental survival rate

At the Zhulin site, the incremental survival rates of *C. hongkongensis* were significantly lower than those of the other three groups on the 180th, 360th, 480th and 600th days ($P < .05$). On the 360th day, there was no significant difference among the incremental survival rates of *C. hongkongensis*, *C. ariakensis* and the diploid hybrids ($P > .05$), but that of the triploid hybrids was significantly higher than those of the other three groups ($P < .05$). On the 600th day, the mean incremental survival rate of *C. ariakensis* was significantly lower than those of the diploid and triploid hybrids ($P < .05$) (Fig. 7). The HTs for incremental survival rate were − 0.23%, 4.68%, 9.66%, −0.08%, 8.56% and 32.18% (Table 2) and the TAs were −2.03%, −0.40%, 0.12%, 7.48%, 0.42% and −5.73% (Table 3) on the 90th, 180th, 270th, 360th, 480th, and 600th days, respectively.

At the Dafeng River site, there was no significant difference among the mean incremental survival rates of the four groups on the 90th, 180th, 270th or 480th days ($P > .05$) (Fig. 7). The incremental survival rate of triploid hybrids was significantly higher than those of the other three groups on the 360th day ($P < .05$), and that of *C. hongkongensis* was significantly lower than those of the other three groups on the 600th day ($P < .05$). The HTs for incremental survival rate were 2.23%, 1.00%, −0.56%, 0.90%, 0.16% and 9.07% (Table 2) and the TAs were −1.91%, 1.15%, −0.56%, 8.77%, −0.89% and −1.20% (Table 3) on the 90th, 180th, 270th, 360th, 480th, and 600th days, respectively.

3.5. Sex ratio and fertility analysis

In the reproductive stage, observation of gonad development showed that *C. hongkongensis*, *C. ariakensis,* and diploid hybrid oysters were fertile and had functional eggs or sperm, but the majority of the gonads of triploid hybrids were mostly or completely atrophic (Fig. 4). Sex ratio determination using a light microscope and statistical analysis determined that the proportions of males were 42%, 30%, 34% and 14% for *C. hongkongensis*, *C. ariakensis*, diploid and triploid hybrids at the Zhulin site, respectively, and 42%, 46%, 56% and 16% at the Dafeng River site, respectively. Moreover, unlike the three diploid groups which only displayed male or female gonads, there were four gonadal sexes among the triploid hybrids: female, male, hermaphrodite and no gametes. At the Zhulin site, 30% of the triploids were female, 14% were male, 8% were hermaphrodites and 48% had no gametes, and at the Dafeng River site the proportions were 34%, 16%, 10%, and 40%, respectively (Table 4**)**.

Histological examination of the gonads revealed that *C. hongkongensis*, *C. ariakensis,* and the diploid hybrids were completely fertile, with mature and morphologically normal vitellogenic oocytes or spermatozoa, and there was no significant difference in quantity among the 3 groups. However, male triploid hybrids had a very small number of mature spermatozoa and most male germ cells remained in the spermatocyte stage. Female triploid hybrids had mature and morphologically normal vitellogenic oocytes but the quantity was considerably lower than in the diploid hybrids (Fig. 8).

4. Discussion

As is well-known, hybrid breeding can combine the genetic prepotencies of both parent species or produce favorable traits that neither parent species has, thus artificially producing offspring with phenotypic and genotypic improvements (Barton and Hewitt, 1989; Barton, 2010; Liu, 2010; Seehausen, 2004). The fundamental purpose of hybridization is to produce offspring with desirable features. In this study, we hybridized two oysters' species and confirmed that the hybrid offspring are characterized by fast growth and wide adaptability to salinity.

4.1. Differences between interspecific hybrids and pure species offspring

4.1.1. Fertilization

Fertilization rate is an important index to evaluate the commercial viability of interspecific hybridization. Our results confirmed that *C. ariakensis* sperm can fertilize *C. hongkongensis* eggs, but *C. ariakensis eggs* cannot be fertilized by *C. hongkongensis* sperm. Unidirectional fertilization results have been found in interspecific hybridization of other oyster species, such as *C. gigas* male × *C. hongkongensis* female (Zhang et al., 2016b), *C. angulata* male × *C. sikamea* female (Yan et al., 2018), *C. angulata* male × *C. hongkongensis* female (Zhang et al., 2016a), *C. gigas* male × *C. ariakensis* female (Allen Jr and Gaffney, 1993), and *C. ariakensis* male × *C. sikamea* female (Xu et al., 2011). Many scientists have also successfully demonstrated bidirectional fertilization in interspecific hybridization of some oyster species, such as *C. sikamea* × *C. hongkongensis* (Zhang et al., 2017a), *C. angulata* × *C. ariakensis* (Yao et al., 2015), *C. gigas* × *C. angulata* (Soletchnik et al., 2002), *C. gigas* × *C. sikamea* (Xu et al., 2019a) and *C. gigas* × *C. nippona* (Xu et al., 2019b). Many factors can affect the fertilization rate of interspecific hybrid oysters, including the quality of the gametes, gametic compatibility, sperm motility and density, environmental factors (temperature, salinity, pH, etc.) during fertilization, and gametic compatibility. While the gametic compatibility may be a determinant factor in the success of interspecific fertilization (Hu et al., 2012; Mallet, 2007; Xu et al., 2019b; Yao et al., 2015).

The molecular mechanisms mediating molluscan fertilization are complex, but large variations between the gamete recognition proteins (GRPs) of different oyster species may be the main reason for gametic incompatibility (Bhakta et al., 2019; Wu et al., 2011; Zhang et al., 2016a). When sperm and eggs of two different species correctly recognize each other through GRPs, sperm may acquire the ability to fertilize eggs through a process known as capacitation (Bhakta et al., 2019; Huo et al., 2013; Moy et al., 2008). The inability of *C. hongkongensis* sperm to fertilize the eggs of *C. ariakensis* may be related to their inability to recognize each other correctly.

4.1.2. Growth

Published results comparing the growth of interspecific hybrids to

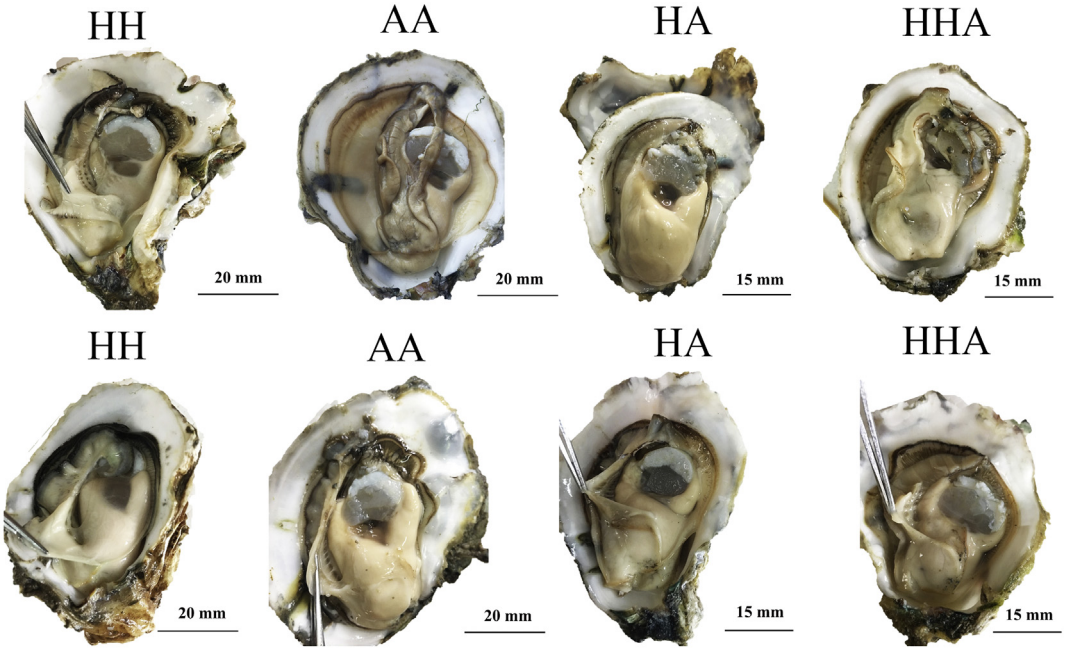

Fig. 4. Morphological identification of *Crassostrea ariakensis*, *Crassostrea hongkongensis* and their diploid and triploid hybrids. HH: *C. hongkongensis*; AA: *C. ariakensis*; HA: diploid hybrids; HHA: triploid hybrids.

intraspecific groups in marine bivalves vary, with not all studies finding growth advantages of interspecific hybridization. For instance, Yan et al. (2018) found that the growth of hybrids between *C. angulata* and *C. sikamea* was significantly higher than that of *C. sikamea* progeny; Xu et al. (2019b) demonstrated that the hybrids of *C. nippona* females and *C. gigas* males had a higher growth rate than intraspecific selfing groups, but also found that the growth of *C. nippona* male × *C. gigas* female hybrids was not significantly different from those of intraspecific groups; and Zhang et al. (2016b) found that the hybrids of *C. hongkongensis* and *C. gigas* showed significantly lower growth than *C. honkongensis*. Our results demonstrated that the shell heights of the hybrids were significantly higher than those of *C. hongkongensis* offspring at all measurement times at the Zhulin site, and the shell heights of the hybrids were significantly higher than those of *C. ariakensis* at the Dafeng River site. Many researchers have shown that environmental factors such as temperature and salinity have a strong influence on the growth of oysters. For example, Xu et al. (2011) reported that salinity affected the growth of *C. ariakensis*, *C. sikamea* and their hybrids; Nell and Perkins (2005) found that *C. gigas* exhibited faster growth and higher condition indices at elevated temperatures; and Hand et al. (1998) reported that high water temperatures at low latitudes in Australia promoted the growth of *Saccostrea commercialis*. In addition, Degremont et al. (2012) investigated the growth of *C. virginica* at three sites characterized by low or moderate salinity in the Chesapeake Bay and also demonstrated that *C. virginica* grew faster at higher salinity sites. In our study, the variations in results between the two sites may have been contributed by numerous environmental conditions, but considering the closeness of the locations and minimal temperature difference, we suggested that the large difference in salinity may be the main reason. Lam and Morton (2003) and Qin et al. (2019) reported that *C. hongkongensis* grew fast in medium-salinity conditions. The salinity of the Zhulin site was always over 25 ppt, while that of the Dafeng River site was always below 25 ppt, so there was no significant difference between the growth of *C. hongkongensis* and the diploid hybrids at the Dafeng River site. Meanwhile, analysis of growth data revealed that the HT was always positive at both sites, indicating that the hybrids had growth advantages regardless of the environmental conditions. This may be greatly related to the effects of parental oyster species (Akhan et al., 2011; Francesc et al., 2009).

4.1.3. Survival

There was no significant difference between the cumulative survival rates of *C. hongkongensis*, *C. ariakensis,* and the interspecific hybrids during the planktonic larval stage. Similar results have also been reported by many researchers in *C. gigas* × *C. hongkongensis* (Zhang et al., 2016b), *C. gigas* × *C. sikamea* (Xu et al., 2019a) and *C. hongkongensis* × *C. sikamea* (Zhang et al., 2017b) hybrids. However, different results were found in *C. gigas* × *C. nippona* (Xu et al., 2019b), *C. ariakensis* × *C. angulata* (Yao et al., 2015) and *C. gigas* × *C. angulata* (Soletchnik et al., 2002) hybrids, which exhibited lower survival rates in the larvae stage than their intraspecific crosses. The survival rates of *Crassostrea* genus larvae vary depending on the genetic subtypes of the parental species and the culture conditions (Zhang et al., 2017b).

According to our results, the incremental survival rates of *C.*

Table 4
The sex proportion and sex ratio of *C. hongkongensis*, *C. ariakensis*, diploid hybrids and triploid hybrids at two sites.

Items	Zhulin pond				Dafeng River			
	HH	AA	HA	HHA	HH	AA	HA	HHA
Female	29 (58%)	35 (70%)	33 (66%)	15 (30%)	29 (58%)	26 (52%)	22 (44%)	17 (34%)
Male	21 (42%)	15 (30%)	17 (34%)	7 (14%)	21 (42%)	23 (46%)	28 (56%)	8 (16%)
Hermaphrodite	0	0	0	4 (8%)	0	1 (2%)	0	5 (10%)
No gamete	0	0	0	24 (48%)	0	0	0	20 (40%)
Total	50 (100%)	50 (100%)	50 (100%)	50 (100%)	50 (100%)	50 (100%)	50 (100%)	50 (100%)

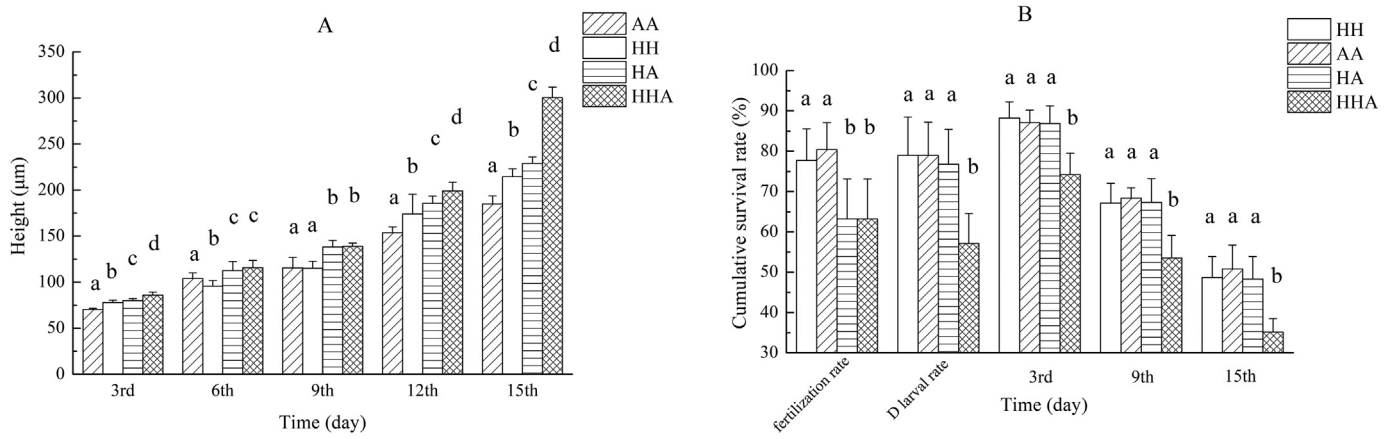

Fig. 5. Comparison of growth and cumulative survival rate among HH, AA, HA and HHA in the planktonic larval stage. HH: *C. hongkongensis*; AA: *C. ariakensis*; HA: diploid hybrids; HHA: triploid hybrids. At the same measurement time, the different letters indicate significant differences ($P < .05$).

hongkongensis were significantly lower than those of *C. ariakensis* and diploid hybrids at the Zhulin site (Fig. 7), this suggests that the hybrids might have an adaptability advantage over *C. hongkongensis* in adverse environmental conditions which is not present in good conditions. The survival rates in this study differed considerably depending on the environmental conditions, which has also been reported by many researchers; for instance, Qin et al. (2019) confirmed that the mortality rate of *C. hongkongensis* increased significantly in high seawater salinity, Paynter et al. (2008) reported that salinity affected the mortality rate of *C. ariakensis* in Chesapeake Bay, and Southworth et al. (2017) also reported that mortality of *C. virginica* increased with prolonged exposure to high temperature and low salinity. Our experiments also demonstrated that the resistance of *C. hongkongenis* to high salinity is poor and significantly worse than those of *C. ariakensis* and interspecific hybrids, which is consistent with the results of many researchers (Huo et al., 2014; Qin et al., 2019; Xiao et al., 2018).

4.1.4. Gonad development

Like *C. hongkongensis*, the diploid interspecific hybrids were completely fertile and could produce mature gametes, and there was no significant difference in sex ratio among three diploid groups at the same site. Similar results were also reported in *C. angulata* × *C.*

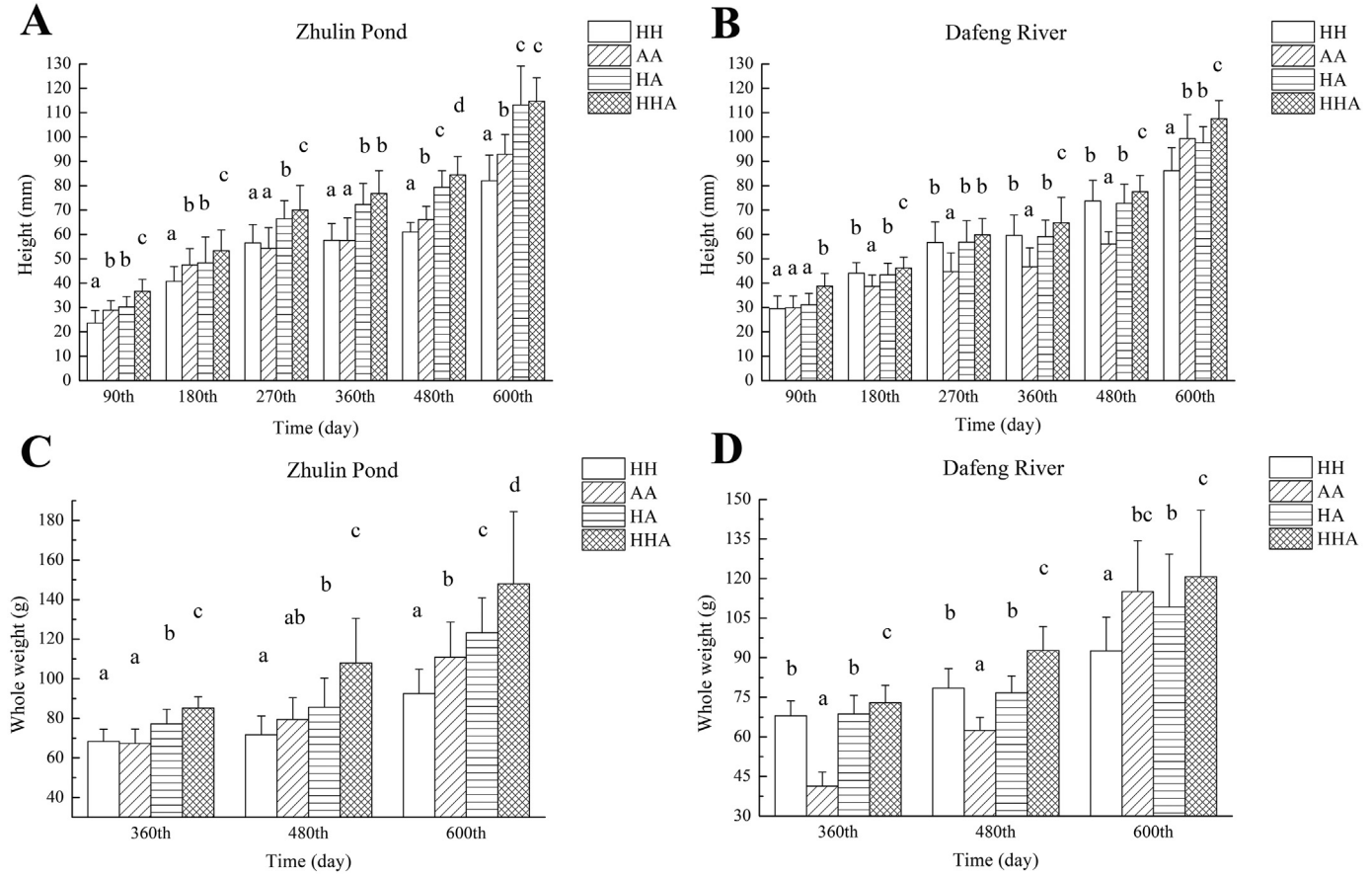

Fig. 6. Comparison of shell height (A, B) and whole weight (C, D) among HH, AA, HA and HHA at 2 sites from the 90th day to the 600th day. At the same measurement time, the different letters indicate significant differences ($P < .05$).

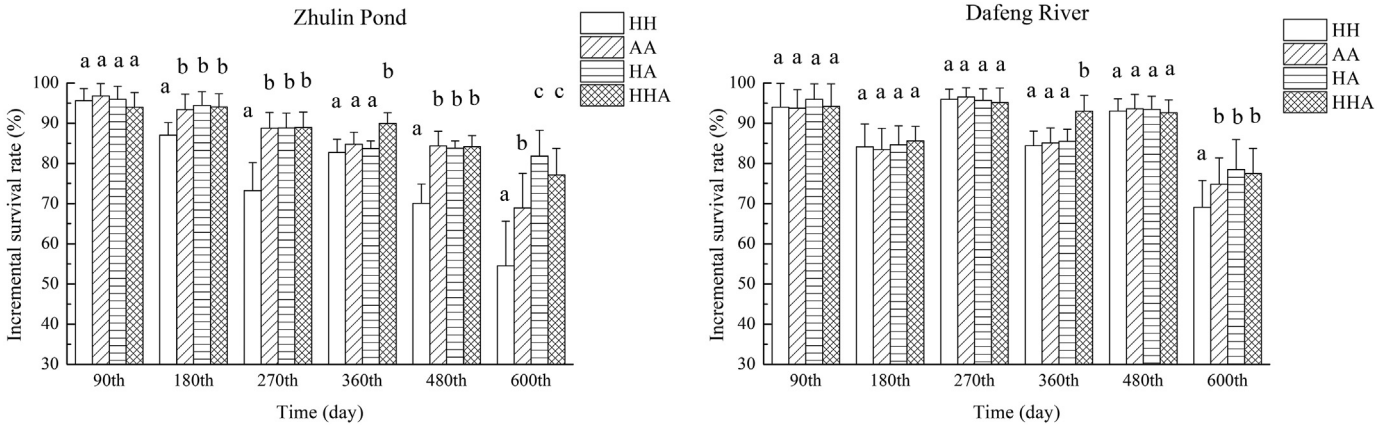

Fig. 7. Comparison of incremental survival rate among HH, AA, HA and HHA at 2 sites from the 90th day to the 600th day. At the same measurement time, the different letters indicate significant differences ($P < .05$).

hongkongensis (Zhang et al., 2016a), *C. angulata* × *C. ariakensis* (Yao et al., 2015), *C. hongkongensis* × *C. sikamea* (Zhang et al., 2017b) and *C. gigas* × *C. angulata* (Soletchnik et al., 2002). Complete or partial fertility is a common characteristic of interspecific hybrids of *Crassostrea* species, and cross-infertility has not been reported in interspecific *Crassostrea* hybrids (Zhang et al., 2017b). In this case, the reason for the complete fertility of the hybrids may be the relatively close phylogenetic relationship between *C. hongkongensis* and *C. ariakensis*. These two species were relatively recently identified as separate through mitochondrial DNA and morphological methods by Lam and Morton

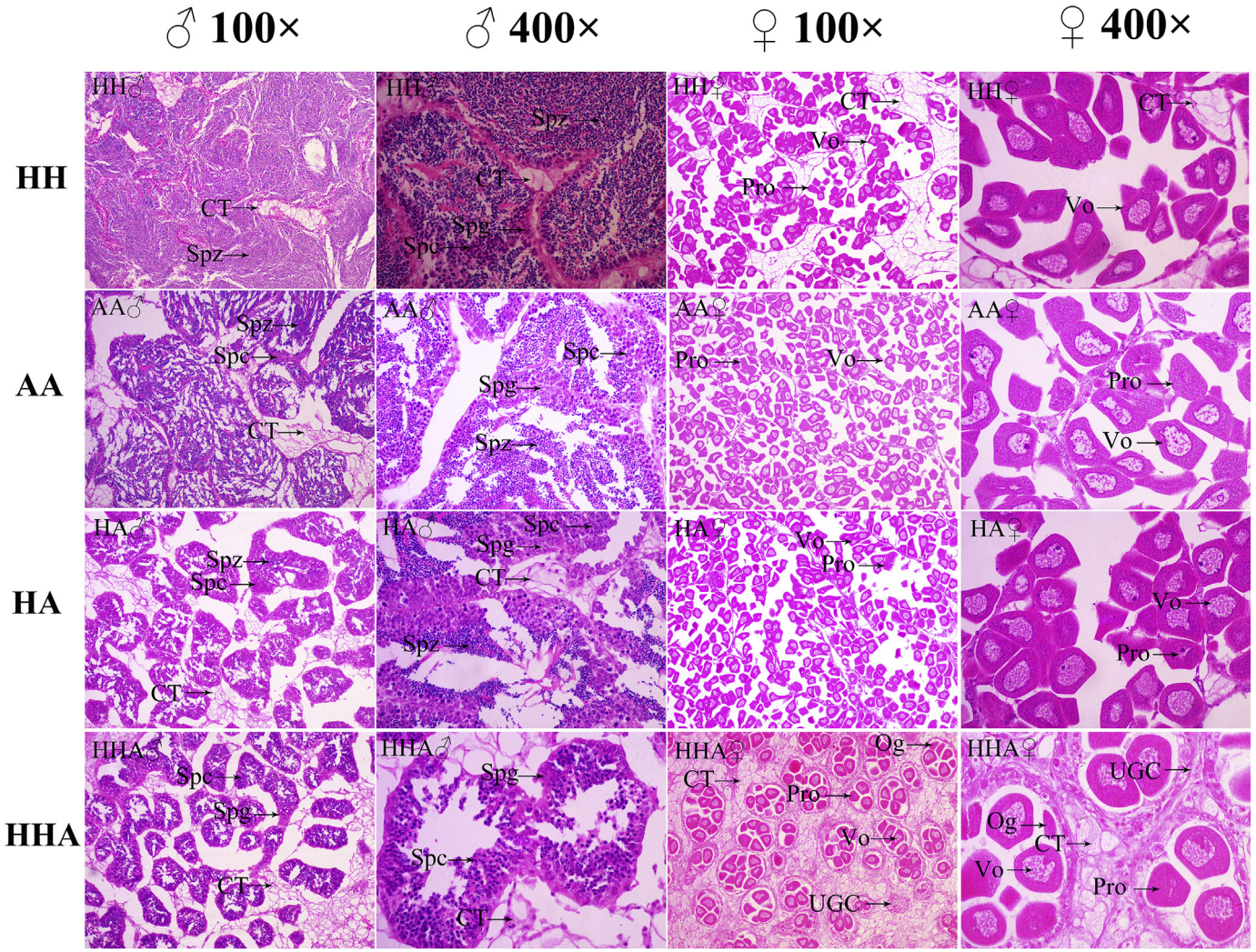

Fig. 8. The observation of the development of the gonads in the HH, AA, HA and HHA during the matured period (10× and 40× objective lens, respectively).
Note: UGC: undifferentiated germ cells; CT: conjunctive tissues; Og: oogonia; Pro: previtellogenic oocytes; Vo: vitellogenic oocytes; Spg: spermatogonia; Spc: spermatocyte; Spz: spermatozoa.

(2003) and have been found to have a large overlap of distribution range in southern China (Xia et al., 2009). Therefore, it is not surprising that the interspecific hybridizations between *C. hongkongensis* and *C. ariakensis* were completely fertile.

4.2. Differences between diploid hybrids and triploid hybrids

4.2.1. Growth and survival

Many studies have demonstrated the growth advantages of autotriploids over autodiploids, such as in *C. gigas* (Benabdelmouna and Ledu, 2015), *C. angulata* (Francesc et al., 2009), *C. virginica* (Degremont et al., 2012; Harding, 2007), *C. hongkongensis* (Qin et al., 2019) and *C. sikamea* (Wu et al., 2019b). But few studies have been done on allotriploids, just Zhang et al. (2014) reported that the allotriploids between *C. gigas* and *C. hongkongensis* exhibited positive growth advantages in the spat and adult stages. Theoretically, three main reasons have been proposed to explain the faster growth of triploids: extra energy allocated to somatic growth due to reduced gonad development in triploids; cell size gigantism in triploids due to their extra set of chromosomes; and a higher overall genomic heterozygosity in triploids than diploids (Comai, 2005; Francesc et al., 2009). We believe that the first two reasons may be the main reasons for the fast growth of allotriploids. In the reproductive stage, the gonad development of allodiploids requires a great deal of energy, causing growth stagnation (Que and Allen, 2002). Since allotriploids are poor fertility, the energy required for gonad development is greatly reduced and that energy is available for somatic growth, which is a strong explanation for the continued growth of allotriploids. Poor fertility may be an important reason for the faster growth of allotriploids than diploid hybrids. The second explanation is based on the idea that allotriploids' increased cell volume is not compensated by a reduction in cell number (Guo and Allen, 1994a; Guo and Allen, 1994b). An increase in cell size with the same number of cells would necessarily lead to faster growth of allotriploids.

Many previous studies have confirmed that the survival difference between diploid and triploid bivalves varies considerably depending on the aquaculture sites and gonadal development stage (Francesc et al., 2009; Nell and Perkins, 2005). According to our results, the incremental survival rate of allotriploids was significantly higher than that of allodiploids only on the 360th day, which may be related to gonad development. Due to gonad development, limited energy is available for disease resistance, leading to suppression of the immune system and increased mortality in diploids (Dégremont et al., 2010; Francesc et al., 2009; Li et al., 2007). Similar results have also been found in many autotriploids, such as *C. gigas* (Ibarra et al., 2017; Nell and Perkins, 2005), *C. virginica* (Degremont et al., 2012) and *C. sikamea* (Wu et al., 2019b).

4.2.2. Gonad development

According to our results, gonad development of allotriploid was reduced: 48% of allotriploids at Zhulin and 40% of those at Dafeng River exhibited sterility with no mature gametes and significantly atrophic gonads, and the number of mature gametes in fertile allotriploid was very low (Table 4). In particular, male allotriploids had few active sperm during the gonadally mature stage, with most gametes remaining in the early stages of gametogenesis (Fig. 8). These results were very similar to the gonad development of some autotriploid oysters, such as *C. gigas* (Ibarra et al., 2017), *C. virginica* (Degremont et al., 2012) and *C. sikamea* (Wu et al., 2019b), which all found that triploids were usually not totally sterile but had atrophic gonads and greatly reduced reproductive capacities compared with those of diploids. The sex ratio of HA and HHA was inconsistent and exhibited significant differences, which may result from different gametogenic patterns and incompatible genetic control (Houssin et al., 2019; Jouaux et al., 2010; Zhang et al., 2016a). Similar results were also reported in allotriploids between *C. hongkongensis* and *C. gigas* by Zhang et al. (2016b). Like autotriploids, it has been shown that fertile allotriploids tend to be highly feminized in many species (Wu et al., 2019b; Ibarra et al., 2017).

5. Conclusions

In conclusion, we present the first systematic and comprehensive comparative study on the two native oysters of southern China, *C. hongkongensis* and *C. ariakensis*, and their diploid and triploid hybrids cultured under two different environmental conditions. Interspecific hybrids were produced by unidirectional fertilization since the sperm of *C. hongkongensis* were unable to fertilize *C. ariakensis* eggs. Fertilization rates of the two pure species (HH and AA) were significantly higher than those of HA, but the mean shell heights of the HA larvae were significantly higher than those of HH and AA. The growth rates of HHA were significantly higher than those of HA, but HHA had a lower D larval rate and higher mortality rates than HA. Our results also confirmed that the interspecific hybrids generally had growth advantages although under different salinity conditions, and also indicated that the growth-traits of HHA were always significantly higher than those of HA. Another important finding is that interspecific hybrids had a higher salinity tolerance than the two intraspecific crosses. We also studied the gonad development of the four groups and found that HA oysters were completely fertile and HHA oysters were partially fertile with atrophic gonads and few mature gametes. Overall, these results provide powerful foundations for the commercial promotion of HA and HHA in the aquaculture industry.

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.

Acknowledgments

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ARTICLES FOR FACULTY MEMBERS

PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

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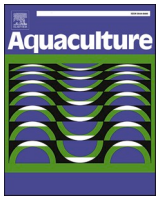

Aquaculture performance comparison of reciprocal triploid *C. gigas* produced by mating tetraploids and diploids in China

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ABSTRACT

Crassostrea gigas is an important species of oyster culture in northern China, which has important economic value. In recent years, triploid cultivation of *C. gigas* has gradually emerged because of the advantages of poor fertility and fast growth, but the comparative studies on tetraploid, triploid and diploid *C. gigas* are not comprehensive and systematic. In addition, forward or reverse mating tetraploids and diploids all can produce triploids, it is necessary to compare the performance of the two triploid groups. This study was conducted to understand the growth, survival, ploidy level and fertility of tetraploids in China, and to figure out how to mass-produce triploids. Aritificial hybridization was made between diploid (D) and tetraploid (T) to produce DD, TD, DT and TT groups (males are listed first). The results showed that the fertilization rate, cleavage rate, D larval rate and larvae survival rate of TD group were significantly higher than those of DT and TT groups, and the larval growth of TD group was significantly faster than that of the other three groups. In cultivation period, the growth and survival rate of TD in the 2 sites were significantly higher, and the 100% triploid rate was stable. In addition, some triploids in TD and DT groups were fertile and could produce functional gametes. But the proportion of female and hermaphrodite in fertile triploids was higher than that in diploids and tetraploids, and the fertility of triploids was still poor overall. However, some tetraploids in TT group would lose chromosomes during growth and become triploids, diploids or aneuploids. Tetraploid had the slowest growth and the lowest survival rate at the both sites, but its fertility was normal and could produce a large number of functional gametes, which could provide sufficient sperm for large-scale production of triploids. All these data support tetraploid ♂ × diploid ♀ is a good strain for commercial promotion in China. In conclusion, this study provides comprehensive data for understanding the performance of tetraploid and the aquaculture potential of triploid *C. gigas*, and also supports the commercial promotion of triploid *C. gigas* (tetraploid ♂ × diploid ♀) in China.

1. Introduction

C. gigas (Pacific oyster) is an important oyster in the coastal areas of northern China. It is mainly distributed in Liaoning, Shandong, Jiangsu, Zhejiang and part of Fujian provinces. In 2020, the production of *C. gigas* from aquaculture was about 1.57 million tons, accounting for 28.90% of total China's oyster production, with very important economic value (China Fishery Statistical Yearbook, 2021). Traditionally cultured Pacific oysters are usually diploids, with the disadvantages of slow growth, poor taste during gonad development, high mortality rate, and can't be marketed annually (Francesc et al., 2009; Melo et al., 2020; Nell and Perkins, 2005). However, triploid can make up for these disadvantages. Triploid has the advantages of sterility, good meat quality and fast growth, and the performance degrees of these advantages is affected by environmental conditions (Francesc et al., 2009; Gagnaire et al., 2006).

Since Stanley et al. (1981) successfully induced triploid *C. virginica* and proved its growth advantages, scholars have conducted detailed studies on the breeding of the triploid oyster and constructed different induction methods, such as cytochalasin B (CB), 6-dimethylaminopurine (6-DMAP), caffeine, temperature stress, hydrostatic pressure, etc. (Allen and Downing, 1986; Desrosiers et al., 1993; Francesc et al., 2009; Ledu and Mccombie, 2003; Paynter et al., 2008; Qin et al., 2017). However, these physical and chemical induction methods have disadvantages such as low triploid rate, high larval malformation rate, low hatchability rate,

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high mortality rate and complex operation, which limit the application of these methods in large-scale production of triploids (Eudeline et al., 2000; Guo et al., 1996; Qin et al., 2021b). In addition, the ploidy of induced triploid is unstable, with chromosome loss occurring during growth, and some triploid individuals are reversed to diploids, and researchers attribute it to the effects of environmental conditions, age and chemical reagents (Ibarra et al., 2017; Melo et al., 2020).

Since then, some scientists have optimized the conditions for triploid induction through physical and chemical methods, hoping to greatly reduce the defects of triploid induction. However, the results showed that although the induction effect was improved and the defects of inducing triploid were weakened to a certain extent, the satisfactory results of large-scale production of triploids were still not achieved (Qin et al., 2017; Wang et al., 2021; Wu et al., 2019; Yang et al., 2000). Guo et al. (1996) discovered a method of triploid production by mating tetraploids and diploids. This method is practical and suitable for commercial production of triploid. However, the production of tetraploid is a great technical difficulty, which restricts the promotion and cultivation of triploids in China. We successfully induced tetraploid C. hongkongensis (the Hong Kong oyster) for the first time in China (Zhang et al., 2017), and extended the method to *C. gigas* and *C. angulata*, and obtained three kinds of tetraploid oysters. However, little is known about the growth, survival and fertility of tetraploid Pacific oyster in China. Both forward and reverse mating of tetraploid and diploid can produce triploid, but which is more suitable for large-scale production in China still needs further research. Therefore, it is necessary to comprehensively compare the aquaculture potential of tetraploids, diploids and triploids (derive from reciprocal mating of tetraploids and diploids) in the main breeding areas of *C. gigas* in China.

In this study, the fertilization rate, cleavage rate, D larval rate, growth, survival rate, ploidy variation, fertility of diploid, tetraploid and triploids (both forward and reverse mating) of Pacific oyster were systematically compared, and the effects of sites and genotypes interactions on growth and survival of Pacific oyster were deeply analyzed. Through these studies, we hope to provide support and basic data for understanding the characteristics of tetraploids and the further commercial promotion and cultivation of triploid *C. gigas* in China.

2. Materials and methods

2.1. Preparation of parent oysters

The healthy and sexually mature diploid *C. gigas* used in this study were 2 years old and collected from Laizhou, Shandong province of China. The active and sexually mature tetraploid *C. gigas* used in this study were 1 year old and produced according to Zhang et al. (2017). All parent oysters were transported to the oyster hatchery and maintained in an open circulating system (temperature 25.0–27.0 °C, salinity 28–30 ppt) for at least one week. During the acclimatization period, oysters were fed with excessive *Chaetoceros calcitrans* and *Platymonas subcordiformis* twice a day. In order to avoid accidental discharge of gametes, stimulation should be minimized during temporary culture, such as reducing water flow rate, oxygen, and salinity and temperature changes (Qin et al., 2018a).

2.2. Experiment design, ploidy determination and fertilization

The parent oysters were carefully opened, and then the ploidy and sexed were identified under a light microscope. The ploidy level was identified using flow cytometry with a CyFlow Ploidy Analyser (Sysmex, Japan) according to Qin et al. (2021a, 2021b). Our goal was to obtain ploidy pure tetraploid parents, so for male tetraploid oysters, we needed to identify not only the ploidy of adductor muscle, but also the ploidy of sperm. For the female tetraploids, only the adductor muscle needed to be identified.

Male and female oysters were separated, and all containers were

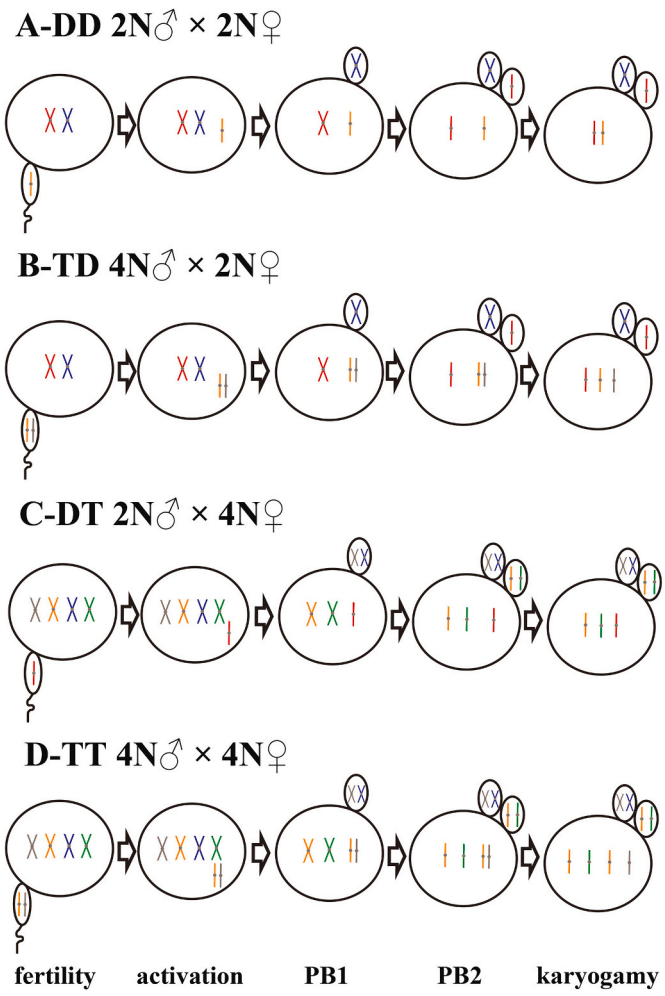

Fig. 1. Schematic diagram of embryogenesis in DD, TD, DT and TT groups. Note: 2 N represents diploid; 3 N represents triploid; 4 N represents tetraploid; AN represents aneuploid.

cleaned with fresh water to avoid accidental fertilization. Eggs were first filtered through a 48 μm nylon sieve to remove large tissue residues, and then filtered twice with 25 μm nylon sieve to remove broken eggs and other small impurities. The eggs were then hydrated in seawater under optimum conditions (temperature 29 °C, salinity 28 ppt) until most germinal vesicle breakdown, which was a sign that meiosis was ready to resume initiation and fertilization began (Qin et al., 2018a, 2018b). Ten minutes before fertilization, sperms were filtered through a 40 μm nylon sieve to remove impurities and then activated in 29 °C and 28 ppt seawater. The fertilization procedure was performed according to Qin et al. (2020). In this study, 4 oyster groups were established: DD-diploid *C. gigas* ♂ × diploid *C. gigas* ♀, TD-tetraploid *C. gigas* ♂ × diploid *C. gigas* ♀, DT-diploid *C. gigas* ♂ × tetraploid *C. gigas* ♀, TT- tetraploid *C. gigas* ♂ × tetraploid *C. gigas* ♀ (Fig. 1). 3 replicates were established for each group, and only 1 male and 1 female were fertilized in each replicate.

During fertilization, an appropriate number of sperms were added to the eggs, and 3–5 sperms were kept around each egg. After fertilization, the fertilization rate, cleavage rate and D larval rate were measured by a light microscope, and each replicate measured 3 data. Fertilization rate was defined as the ratio of fertilized eggs to total eggs, cleavage rate was defined as the proportion of the number of cleaved fertilized eggs to total fertilized eggs, and D larval rate was defined as the proportion of D-shaped larvae to total fertilized eggs. After 24 h of fertilization, D-stage larvae were collected with a 45 μm nylon sieve and then placed into 10,000 L cement ponds with temperature of 26–30 °C and salinity of

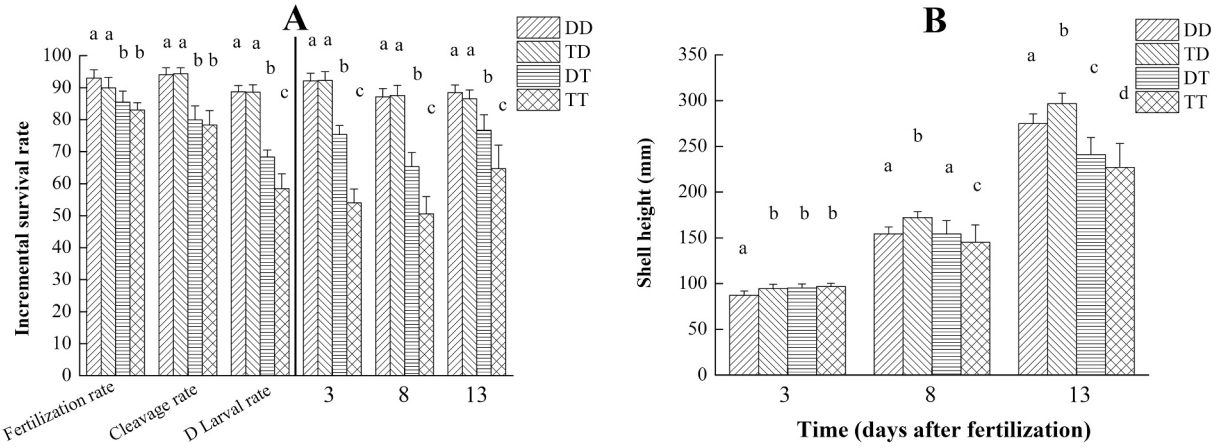

Fig. 2. Comparison of fertilization rate, cleavage rate, D Larval rate, incremental survival rate and shell height among DD, TD, DT and TT groups during the planktonic larval stage.
Note: Different letters indicate significant differences at the same measurement time ($P < 0.05$).

26–28 ppt.

2.3. Larval breeding and measurement of growth-related data

The ploidy of each group was identified according to the method in section 2.2. Larval breeding was carried out according to Qin et al. (2020). In order to ensure stable water quality and control seedling density, 20% of seawater was changed every three days. On the 3rd, 8th and 13th days, the shell height of larvae was measured by a light microscope (90 larvae of each group were measured at each time). The incremental survival rate of larvae was defined and measured according to Qin et al. (2020), mainly based on the variation of larval density and total quantity. When about 50% of the larval feet were present, the attachment substrates were suspended in the cement ponds for larval attachment. When the juvenile oysters grew to about 3 mm, 4 groups were simultaneously transported and cultivated in Laizhou, Shandong province of China and Ningde, Fujian province of China.

Shell heights and incremental survival rate of 4 groups were measured at 90th, 210th and 360th days after fertilization. At each measurement time point, the ploidy composition of each group was identified according to section 2.2, and the shell heights of 90 individuals and 9 incremental survival rates were measured in each group. In addition, only tetraploids were measured in TT group. The incremental survival rate was calculated according to the following formula:

$$IS_{t+1}\% = \frac{S_{t+1}}{S_t} \times 100$$

where $IS_{t+1}\%$ is the incremental survival rate at time t + 1; S_t is the number of surviving oysters at time t; S_{t+1} is the number of surviving oysters at time t + 1; and t + 1 is the next measurement time point of t.

2.4. Comparison of fertility and sex ratio

At the gonadal maturation stage, the ploidy was identified first, and then the sex composition of each group was identified and counted under a light microscope, including male, female, hermaphrodite and asexual. For the TT group, only the sex composition of tetraploids was counted. Gonadal development and gamete morphology were further analyzed with paraffin section. Paraffin section was performed according to the conventional methods (Qin et al., 2021a; Qin et al., 2020). After preparation, paraffin sections were observed and photographed under a light microscope.

2.5. Statistical analysis

All data (fertilization rate, cleavage rate, D larval rate, shell height and incremental survival rate) were expressed as mean ± standard deviation (M ± SD), and were compared by one-way analysis of variance (ANOVA) followed by a Duncan test using SPSS18. $P < 0.05$ was considered as significant difference, while $P < 0.01$ was considered as highly significant difference.

To investigate the effects of sites (Shandong and Fujian), genotypes (DD, TD, DT and TT) and their interactions on shell height and survival rate, a two-factor analysis model was established. The following model was used for further analysis (Cruz and Ibarra, 1997; Degremont et al., 2012; Ibarra et al., 2017; Zhang et al., 2016):

$$W_{ab} = M + S_a + G_b + (S_a \times G_b) + \delta_{ab}$$

where W_{ab} is the dependent variable (shell height and incremental survival rate), M is the mean value, S_a is the sites influence (Shandong, Fujian, a = 1, 2), G_b is the genotype effect (DD, TD, DT and TT, b = 1, 2, 3, 4), $S_a \times G_b$ is the interaction effects between the sites and genotypes, and δ_{ab} is the residual error. To ensure the normality and homogeneity of variance, shell height data was logarithmically transformed before analysis, incremental survival rate data was arcsine transformed before analysis, and statistical significance was evaluated using a two-factor ANOVA (Nell and Perkins, 2005; Zhang et al., 2016).

3. Results

3.1. Comparison of larval growth and survival

The results showed that the fertilization rate, cleavage rate and D larval rate of diploid maternal groups (DD and TD) were significantly higher than those of tetraploid maternal groups (DT and TT, $P < 0.05$). There was no significant difference in fertilization rate and cleavage rate between the two groups with the same ploidy egg source ($P > 0.05$), but the D larval rate of TT was also significantly lower than that of DT ($P < 0.05$, Fig. 2A). In the planktonic larval stage, there was no significant difference in the incremental survival rate between DD and TD ($P > 0.05$), but they survival rates were significantly higher than DT and TT ($P < 0.05$). In addition, the incremental survival rate of DT was always significantly higher than that of TT ($P < 0.05$, Fig. 2A). The shell height of DT was significantly lower than that of TD, but significantly higher than that of TT ($P < 0.05$) (Fig. 2B).

Table 1
Analysis of variance showing the effects of site (S), genotype (G) and their interaction (S × G) on shell height and incremental survival rate at different times.

			Shell height		Incremental survival rate	
Time	Parameters	df	MS	*P*	MS	*P*
90th day	S	1	0.159	<0.001***	0.004	0.394
	G	3	0.560	<0.001***	0.330	<0.001***
	S × G	3	0.004	0.400	0.019	0.017*
210th day	S	1	2.274	<0.001***	0.297	<0.001***
	G	3	0.610	<0.001***	0.119	<0.001***
	S × G	3	0.006	0.143	0.016	0.048*
360th day	S	1	1.188	<0.001***	0.020	0.055
	G	3	1.308	<0.001***	0.297	<0.001***
	S × G	3	0.009	0.064	0.022	0.008**

3.2. Shell heights and incremental survival rates at the two sites

The variations of shell height and incremental survival rate of 4 groups at 2 sites are illustrated in Fig. 4 and Fig. 5, respectively. At the Shandong site, the shell height of TT was always the lowest from 90th to 360th day, which was significantly lower than that of other 3 groups ($P < 0.05$). On the contrary, the shell height of TD was always the highest. On the 210th and 360th days, a similar and significant decreasing order of shell height was observed, that was TD > DT > DD > TT ($P < 0.05$, Fig. 4A). At the Fujian site, the shell height of TD was also always the highest and that of TT was always the lowest. On the 360th day, the significant decreasing order of shell height was TD (91.72 ± 8.63 mm) > DT (86.41 ± 9.25 mm) > DD (77.71 ± 9.11 mm) > TT (58.30 ± 10.10 mm) ($P < 0.05$, Fig. 4B). From 90th to 360th day, sites and genotypes significantly affected shell height, but their interaction had no significant effect on shell height (Table 1).

At the Shandong site, the incremental survival rate of TT was always the lowest and was significantly lower than that of the other 3 groups from 90th to 360th day ($P < 0.05$, Fig. 5A). On the 90th day, the incremental survival rate of DD, TD, DT and TT was 89.85 ± 2.54%, 93.07 ± 3.82%, 90.26 ± 2.85% and 80.16 ± 3.26, respectively. The decreasing order was TD ≥ DT ≥ DD > TT. On the 360th day, the descending order of incremental survival rate was TD (90.26 ± 3.04%) > DT (82.43 ± 3.93%) = DD (80.86 ± 3.40%) > TT (72.43 ± 2.38%). At the Fujian site, the difference of survival rate among 4 groups changed with time. The incremental survival rate of TT was significantly lower than that of other 3 groups on the 90th and 210th days ($P < 0.05$), but there was no significant difference between DD and TT on the 360th day ($P > 0.05$, Fig. 5B). Although site did not always have a significant effect on increased survival rate, genotype and the interaction between site and genotype always had significant positive effect on survival rate from 90th to 360th day (Table 1).

3.3. Variations in ploidy composition

The relative DNA content of diploid *C. gigas* was used as the controls for ploidy identification. On the 3rd and 13th days of larval stage, the ploidy composition of DD group was 100% diploids, TD and DT groups were 100% triploids, and TT group was 100% tetraploids (Table 2, Fig. 3). However, the ploidy compositions of DD, TD and DT did not change from the 90th to 360th days, but the ploidy composition of TT group changed greatly. From the 90th to 360th day, the tetraploid proportion in TT group decreased gradually at both sites. On the 90th day, the ploidy composition of TT group in Shandong site was diploid (2 N) 2.22%, triploid (3 N) 4.44%, tetraploid (4 N) 91.11% and aneuploids (AN) 2.22%, while that of Fujian site was 2 N 3.85%, 3 N 5.77%, 4 N 88.46% and AN 1.92% (Table 2, Fig. 3). However, on the 360th day, the ploidy composition of TT group in Shandong site changed to 4.50% 2 N, 43.25% 3 N, 47.75% 4 N and 4.50% AN, and that of Fujian site changed to 5.49% 2 N, 52.75% 3 N, 39.56% 4 N and 2.20% AN (Table 2, Fig. 3).

3.4. Fertility and sex ratio

The observation of gonadal development showed that DD and TT groups were fully fertile at stage of gonadal maturation, with full gonads, which could produce many mature and functional sperms or eggs, but most of the triploids in TD and DT groups were sterile and the gonadal tissue was filled with flesh (Fig. 6). According to the statistical analysis of the sex ratio of the DD in Shandong site and Fujian site was 62.86% males and 37.14% females, and 60.38% males and 39.62% females, respectively (Table 3, Fig. 7).

In addition, a small number of triploids in TD and DT groups were fertile and can only produce few mature gametes (Fig. 7), but the proportion of female individuals was higher, and the proportion of hermaphrodites was significantly higher than that in DD groups. In Shandong site, 16.82% of the triploids were fertile in TD group, including 13.64% females, 1.82% males and 1.36% hermaphrodites, and 18.78% of the triploids were fertile in the DT group, including 13.15% females, 2.82% males and 2.82% hermaphrodites. Similarly, in Fujian site, 22.05% individuals of the TD group were fertile, including 17.72% females, 2.76% males and 1.57% hermaphrodites, while 21.03% of the DT group were fertile, including 15.45% females, 3.00% males and 2.58% hermaphrodites (Table 3, Fig. 7). The tetraploids of TT group were fully fertile and could produce a large number of mature sperm or eggs, and a small number of hermaphrodite individuals still existed (Fig. 7). In Shandong site, the sex composition of fertile tetraploid was 80.33% males, 17.21% females and 2.46% hermaphrodites. In Fujian site, the sex composition of tetraploids in the TT group was 81.68% males, 16.79% females and 1.53% hermaphrodites (Table 3).

Table 2
Ploidy composition of DD, TD, DT and TT at different time points of two different sites.

Site	Group	Ploidy	3rd	13th	90th	210th	360th
Shandong	DD	2 N	100%	100%	100%	100%	100%
	TD	3 N	100%	100%	100%	100%	100%
	DT	3 N	100%	100%	100%	100%	100%
	TT	2 N	0	0	2.22%	2.91%	4.50%
		3 N	0	0	4.44%	23.30%	43.25%
		4 N	100%	100%	91.11%	71.85%	47.75%
		AN	0	0	2.22%	1.94%	4.50%
Fujian	DD	2 N	100%	100%	100%	100%	100%
	TD	3 N	100%	100%	100%	100%	100%
	DT	3 N	100%	100%	100%	100%	100%
	TT	2 N	0	0	3.85%	3.61%	5.49%
		3 N	0	0	5.77%	25.30%	52.75%
		4 N	100%	100%	88.46	66.27%	39.56%
		AN	0	0	1.92%	4.82%	2.20%

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

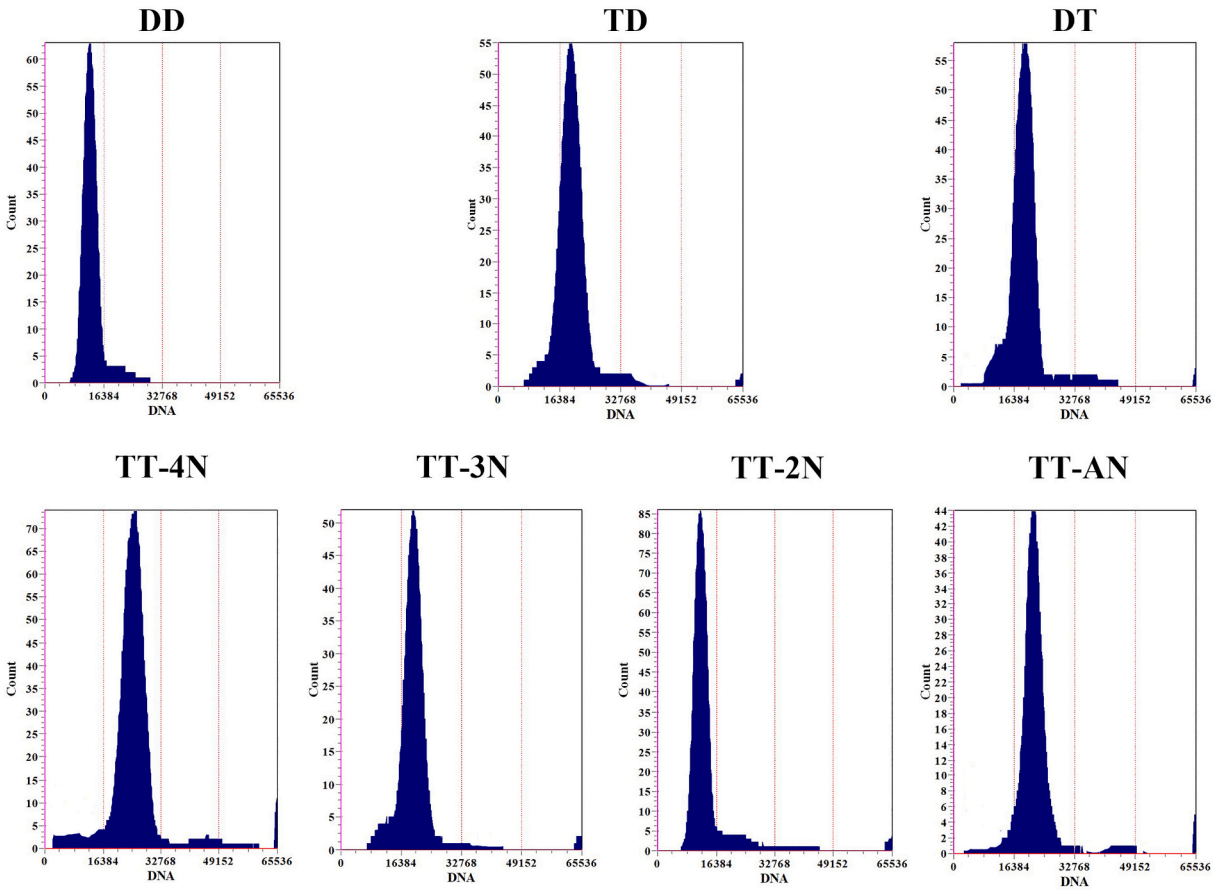

Fig. 3. Relative DNA content and ploidy level of DD, TD, DT and TT groups detected by flow cytometry.
Note: 2 N represents diploid; 3 N represents triploid; 4 N represents tetraploid; AN represents aneuploid.

Table 3
The sex ratio and composition of DD, TD, DT and TT during gonad maturity at two different sites.

Site	Group	Total	Male	Female	Hermaphrodite	Asexual
Shandong	DD	140 (100%)	88 (62.86%)	52 (37.14%)	0	0
	TD	220 (100%)	4 (1.82%)	30 (13.64%)	3 (1.36%)	183 (83.18%)
	DT	213 (100%)	6 (2.82%)	28 (13.15%)	6 (2.82%)	173 (81.22%)
	TT	122 (100%)	98 (80.33%)	21 (17.21%)	3 (2.46%)	0
Fujian	DD	313 (100%)	189 (60.38%)	124 (39.62%)	0	0
	TD	254 (100%)	7 (2.76%)	45 (17.72%)	4 (1.57%)	198 (77.95%)
	DT	233 (100%)	7 (3.00%)	36 (15.45%)	6 (2.58%)	184 (78.97%)
	TT	131 (100%)	107 (81.68%)	22 (16.79%)	2 (1.53%)	0

4. Discussion

4.1. Tetraploid ♂ × diploid ♀ can be used as a triploid superior line for commercial promotion and aquaculture

Since Stanley et al. (1981) successfully induced triploid *C. virginica* with CB in 1981, polyploid breeding has been an important research direction of shellfish genetic breeding. Many polyploid induction methods have been developed and perfected by researchers, such as CB, 6-DMAP, caffeine, heat shock, hydrostatic pressure, etc. (Allen and Downing, 1986; Arai et al., 1986; Desrosiers et al., 1993; Eudeline et al., 2000; Guo and Allen, 1994b; Ledu and Mccombie, 2003; Qin et al., 2017; Stephens and Downing, 1988; Yang and Guo, 2006). However, most researchers are not satisfied with the low ploidy rate and low larval hatching rate of shellfish induced by these methods, and have been looking for better triploid production methods (Eudeline et al., 2000; Francesc et al., 2009; Qin et al., 2020). According to our results, both forward and reverse mating of tetraploid and diploid (TD and DT) could produce triploids with triploid rates of 100%, and the triploid rates remained 100% during growth (Table 2, Fig. 1). The production of 100% triploid in TD and DT groups suggested that strict tetraploids could produce strict diploid gametes, and tetraploids should be carefully

identified DNA content before used. Similar results have also been demonstrated in other aquatic animals, including *C. virginica*, *Carassius auratus langsdorfii*, *Chlamys ferreri*, *C. angulata* (Guo and Allen, 1994b; Guo et al., 1996; Li et al., 2021; Sousa et al., 2016; Yamaguchi et al., 2021; Yang et al., 2000; Zhang et al., 2022). In addition, our results showed that the fertilization rates, cleavage rates, D larval rates and larval survival rates of TD and DD groups were all significantly higher than those of DT and TT groups. Many researchers have also confirmed that the high fertilization rate, high survival rate and stable triploid rate of triploids produced by tetraploid ♂ × diploid ♀ (Guo et al., 1996; Mccombie et al., 2005; Nell and Perkins, 2005; Que and Allen, 2002; Sousa et al., 2016). Higher fertilization rate, larval hatching rate and survival rate of TD indicated that the cross between male tetraploids and female diploids was better used for commercial production of triploid plankton larvae.

At both sites, the TD group had the largest shell heights, and the survival rate was also consistently higher, which was consistent with the results of previous studies (Francesc et al., 2009; Ibarra et al., 2017; Mallia et al., 2006; Nell and Perkins, 2005; Paynter et al., 2008). Triploid growth is significantly better than diploid growth in most shellfish, including oysters (Degremont et al., 2012; Ibarra et al., 2017; Nell and Perkins, 2005; Qin et al., 2020; Wu et al., 2019), abalone (Liu et al., 2009; Wang et al., 2021) and scallops (Ruiz et al., 2000). However, the mechanisms and reason of triploid rapid growth are still not clear. So far, three different mechanisms have been proposed. Firstly, some researchers believed that due to the poor fertility of triploid, the energy originally devoted to gonad development and gametogenesis was saved and diverted to shell and somatic growth (Barber and Mann, 1991; Francesc et al., 2009; Hand et al., 1998). Secondly, because triploids had one more set of chromosomes than diploids, the cell volume of the triploid must be larger, as can be seen from the larger diameter of triploid eggs than the diploid eggs. However, the increase of triploid cell size did not result in the decrease in cell number, so the increase in cell size inevitably leads to larger body and faster growth of triploids (Francesc et al., 2009; Guo and Allen, 1994a, 1994b). Thirdly, the genomic heterozygosity of triploids was higher than that of diploids, resulting in triploid showing certain heterosis (Francesc et al., 2009; He et al., 2002; Nell and Perkins, 2005). All the three mechanisms had certain limitations and could not well explain the underlying reasons, which could also be attributed to the high food availability and filter feeding efficiency of triploids (Racotta et al., 2008). Parental origin, especially maternal origin, could significantly affect the growth of offspring (Cruz and Ibarra, 1997; Francesc et al., 2009; Zhang et al., 2016). The eggs of tetraploid oysters had a retarding effect on the growth of progeny, which could be seen in the phenomenon of slower growth of DT and TT groups, and the similar result was also found in *C. angulata* and *C. virginica* (Guo, 2012; Zhang et al., 2022; Yang et al., 2019). So, although both groups were triploid, the growth of TD group was significantly faster than DT group due to the maternal effect.

The high survival rate of triploid adults might be related to their strong immune system, which was mainly related to the number and type of lymphocytes in shellfish. During gametes formation, release and reabsorption, oysters had a low abundance of circulating lymphocytes, which leaded to their low ability to resist bacterial infections, diseases, and external stimulations (Duchemin et al., 2007; Francesc et al., 2009; Gagnaire et al., 2006; Nell and Perkins, 2005). The fertility of triploid was obviously worse than that of diploid, so its stress resistance was stronger. Gagnaire et al. confirmed that triploids *C. gigas* had higher immune parameters and lower mortality rate than diploid (Gagnaire et al., 2006), and Duchemin et al. (2007) also demonstrated that immune paraneters of *C. gigas* varied seasonally with gonad development, and triploids was less sensitive to environmental stimulations than diploids. At both sites, the survival rates of the TT group were the lowest, but the survival rate was still significantly higher than the results of previous studies (Guo and Allen, 1994b; Guo et al., 1996; Guo, 2012; Yang et al., 2019). This difference was mainly due to the shortcomings of previous studies in the cultivation technology, cultivation methods and management methods. Through the comprehensive comparative analysis of hatching rate, ploidy stability, growth and survival, it was found that tetraploid ♂ × diploid ♀ could be used as an excellent triploid strain for large-scale promotion.

4.2. Partial triploids can produce few mature gametes

Traditionally, triploids were considered completely sterile because they have three sets of chromosomes and could not undergo normal meiosis (Carrasco et al., 1998). However, observations of gonad development in the TD and DT groups showed that few triploids were fertile and could produce some functional gametes (Table 3, Fig. 7). Fertile individuals and functional gametes were also been found in other triploid shellfish, such as *C. angulata* (Zhang et al., 2022), *C. sikamea* (Wu et al., 2019), *C. hongkongensis* (Qin et al., 2018a), *C. virginica* (Degremont et al., 2012), *Haliotis rubra* (Liu et al., 2009) and *Argopecten ventricosus* (Ruiz et al., 2000). The low proportion of fertile individuals, few gametes and high proportion of hermaphrodites all indicated that the gonadal development of triploids was difficult, and the development of triploid gonads was closely related to the food abundance, temperature, salinity and culture time (Degremont et al., 2012; Francesc et al., 2009; Guo and Allen, 1994a; Jouaux et al., 2010; Qin et al., 2020; Racotta et al., 2008). The proportions of fertile individuals in TD and DT groups at Fujian site were higher than that at Shandong site, which was related to the higher average water temperature at Fujian site (Supplementary Fig. S1), and high temperature could promote gonad development of marine bivalves (Francesc et al., 2009; Qin et al., 2020; Tan et al., 2021). The proportion of females in fertile triploid individuals of TD and DT groups was much higher than males at both sites (Table 3), which provided the possibility of inducing tetraploids used triploid eggs (Eudeline et al., 2000; Guo and Allen, 1994b; Zhang et al., 2017). Some triploids could produce functional gametes, which might be related to random segregation of additional set of chromosomes during meiosis (Allen and Downing, 1986; Guo and Allen, 1994b). For triploid oysters, fast growth is only one of their advantages, and another important advantage is their poor fertility, which makes them available all year round, and have better quality and nutritional value than diploid oysters (Qin et al., 2018b; Tan et al., 2021). Although some triploid shellfish may produce functional gametes, there are few fertile individuals and low gametes production limits, but it does not hinder their quality improvement and year-round marketing.

4.3. Chromosome instability of tetraploid oysters

Tetraploid oysters play a decisive role in the large-scale production of triploid oysters. So far, no natural tetraploid oysters have been found in nature. We found that the offspring of artificially induced tetraploids (TT) would suffer from chromosome loss and DNA content reduction, and some tetraploids would become diploids, triploids or aneuploids at both sites (Table 2), which was reported in few previous studies (Matt and Allen, 2014; Mccombie et al., 2005; Qin et al., 2021b; Zhang et al., 2022; Zhang et al., 2014). Sousa et al. (2016) reported aneuploid progeny in tetraploid × diploid crosses of *C. virginica*, demonstrating the chromosome instability of tetraploid oysters. Matt and Allen (2014) further confirmed that tetraploid *C. virginica* undergo reversion, losing entire set of chromosomes, and truned into heterploids. Zhang et al. (2014) attributed the chromosomes loss in tetraploid *C. gigas* to the formation of chromosome clumping, and it was confirmed that this reveral happened relatively quickly and began at a relatively early age. However, Guo et al. (1996) did not find ploidy level changes and chromosome loss in tetraploid *C. gigas*, because the sample size was too small to be representative, and chromosome loss was more likely to occur in the first to fourth generation tetraploids, whereas Guo et al. (1996) might detect and analyse subsequent generation of tetraploids.

Unlike the random segregation of an extra set of chromosomes in

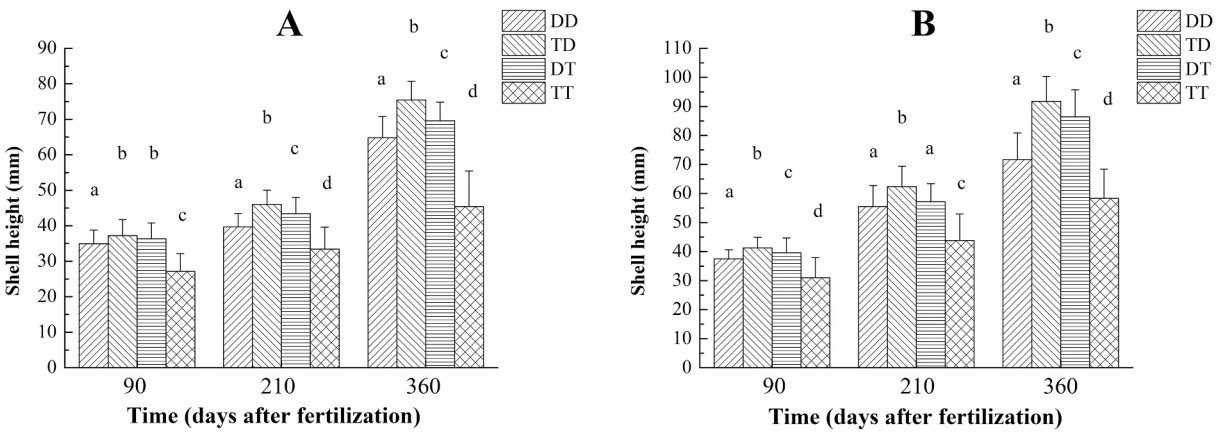

Fig. 4. Comparison of shell heights of DD, TD, DT and TT groups at Shandong (A) and Fujian (B) sites. Different letters indicate significant differences at the same measurement time ($P < 0.05$).

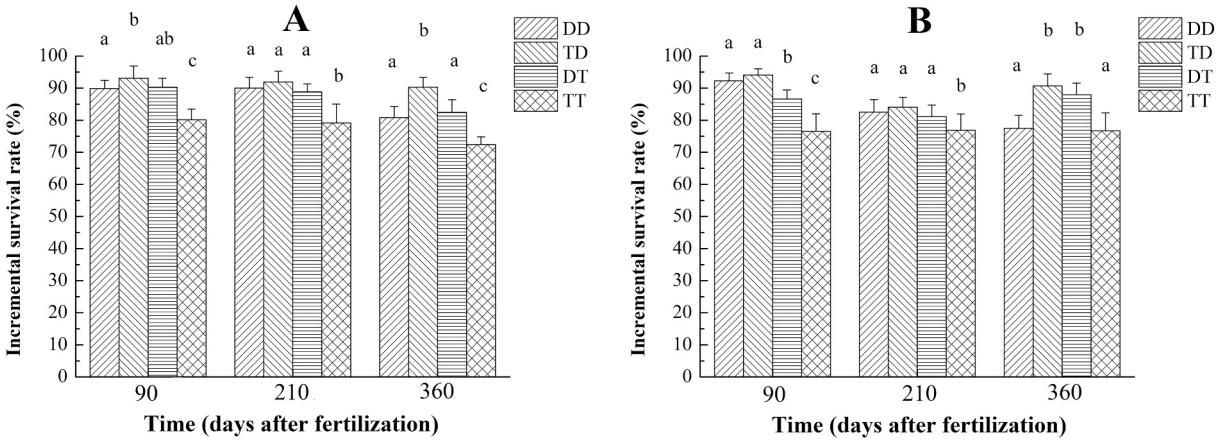

Fig. 5. Comparison of incremental survival rate of DD, TD, DT and TT groups at Shandong (A) and Fujian (B) sites. Different letters indicate significant differences at the same measurement time ($P < 0.05$).

triploids, the balanced co-segregation of the two sets of chromosomes in tetraploids occurs during meiosis, which is similar to the mechanism of chromosome segregation in normal diploid (Fig. 1). Defects and errors in chromosome balance function may result in disordered segregation, which is an important reason for the change of ploidy level changes of tetraploids (Guo and Allen, 1997). The early generation of tetraploids is more sensitive to environmental stimuli, the DNA content is easily reduced, and the ploidy level will become triploids. However, after several generations, the tetraploid tends to be stabilized and well-adapted to the ambient environment (Benabdelmouna and Ledu, 2015; Sousa et al., 2016). The 100% triploids in TD and DT groups remained unchanged, and several tetraploids tended into triploids, indicating that triploids was a relatively stable ploidy of *C. gigas* (Table 2) (Guo and Allen, 1997; Guo et al., 1996). In addition, aneuploid individuals in the TT groups may eventually transformed into triploids. Tetraploids and their dihaploid sperm are very important for the large-scale commercial production of triploids, but the loss of chromosomes may have certain restrictions on the commercialization of triploids in the future, especially tetraploids chromosomes loss will lead to the further loss of tetraploid in offspring. However, there was no detectable differences between triploids produced from mosain and non-mosaic tetraploid males (Matt and Allen, 2014), but had significant difference between triploids produced from mosain and non-mosaic tetraploid females, which once again supported the commercial production of triploids using tetraploid ♂ × diploid ♀.

4.4. Tetraploid has the characteristics of slow growth, low survival and full fertility

Through comprehensive comparative analysis, it was found that the hatching rate, growth and survival of TT at both sites were significantly lower than that of triploids and diploids (Figs. 2, 4, 5). The results of tetraploid growth and survival disadvantages were also reported in *C. virginica* (Guo, 2012) and *C. angulata* (Zhang et al., 2022). However, for *C. gigas*, Benabdelmouna and Ledu (2015) believed that the tetraploids grow slower and the individuals were smaller than triploids and diploids, while Guo and Allen (1994b) confirmed that the tetraploids grow faster than diploids and triploids. Guo and Allen (1994b) attributed the opposite result to the genetic defect of tetraploid, and the parents of TT group originated from the same family, which was actually the mating between brothers and sisters. The genetic diversity of tetraploid population would be less and less with the increase of tetraploid generation. But this explanation was very limited, and the growth of tetraploid was also directly related to the sampling quantity, chromosome stability of tetraploids, environmental conditions and food abundance (Francesc et al., 2009; Sousa et al., 2016; Yang et al., 2019; Zhang et al., 2022). In addition, we also believe that the slower growth of tetraploids is also related to the more energy required to segregate the four sets of chromosomes and develop giant cells during meiosis and mitosis (Francesc et al., 2009; Mccombie et al., 2005). The potential mechanism of smaller tetraploids needs to be further studied.

Guo et al. (1996) found that the mean survival rate of tetraploid

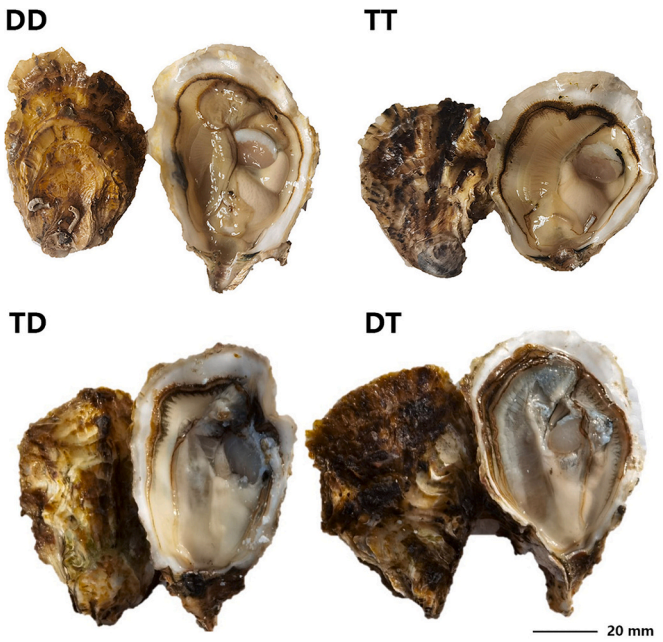

Fig. 6. Phenotypic characteristics of DD, TD, DT and TT at 360 days after fertilization.

C. gigas from larvae to spat was only 0.002%, which was much lower than that of the triploid and diploid groups, Eudeline et al. (2000) also confirmed that the average survival rate of tetraploid *C. gigas* was 4.4%. Guo (2012) further confirmed that the first two generations of tetraploid *C. virginica* suffered severe mortalities associated with early gametogenesis. In addition, Qin et al. (2021b) found the low survival rates of tetraploid *C. hongkongensis* and *C. sikamea*. Zhang et al. (2022) also confirmed that the cumulative survival rate of *C. angulata* on the 360th day after fertilization was only 1.16%. In this study, we also found that the survival rate of tetraploid (TT) *C. gigas* was significantly lower than that of TD, DT and DD at both sites, all of which suggested that the low survival rate of tetraploids had been confirmed. However, the survival rates of tetraploid *C. gigas* varied greatly among different studies, and the survival rate of tetraploid *C. gigas* in this paper was significantly higher than that reported by Guo et al. (1996). Some studies attributed this to the influence of tetraploid generation, and the survival rate of tetraploid increased significantly from the 4th generation, but this contrary to their statement that inbreeding within tetraploid group caused some regression in performance (Guo, 2012; Yang et al., 2019). We propose that the low survival rate of tetraploid is related to the need for more energy for giant cells division, the unsuitable farming conditions and the different cultivating techniques, but this requires further confirmation (Qin et al., 2021b).

As diploids, tetraploid *C. gigas* is comletely fertile, and can produce functional di-haploid gametes through normal meiosis in this study (Fig. 7, Fig. 1, Table 3). Guo and Allen (1997) confirmed that the fecundity of tetraploids was comparable to that of normal diploids, Zhang et al. (2022) found that the gonads of tetraploid *C. angulata* had a normal development, and Yang et al. (2019) found that the gametes of tetraploid *C. virginica* developed normally and spawn naturally. Further studies also found that the DNA content of gametes of some tetraploid individuals decreased, resulting in some aneuploid and mosaic gametes (Guo and Allen, 1997; Matt and Allen, 2014; Sousa et al., 2016). When applied to triploid production, mosaic sperm had little effect on larvae and juveniles, but mosaic eggs had significant effects on triploid growth and survival (Matt and Allen, 2014). Although tetraploid is completely fertile, its slow growth and low survival rate limit its large-scale artificial culture, but its sperm can be used for large-scale production of triploids.

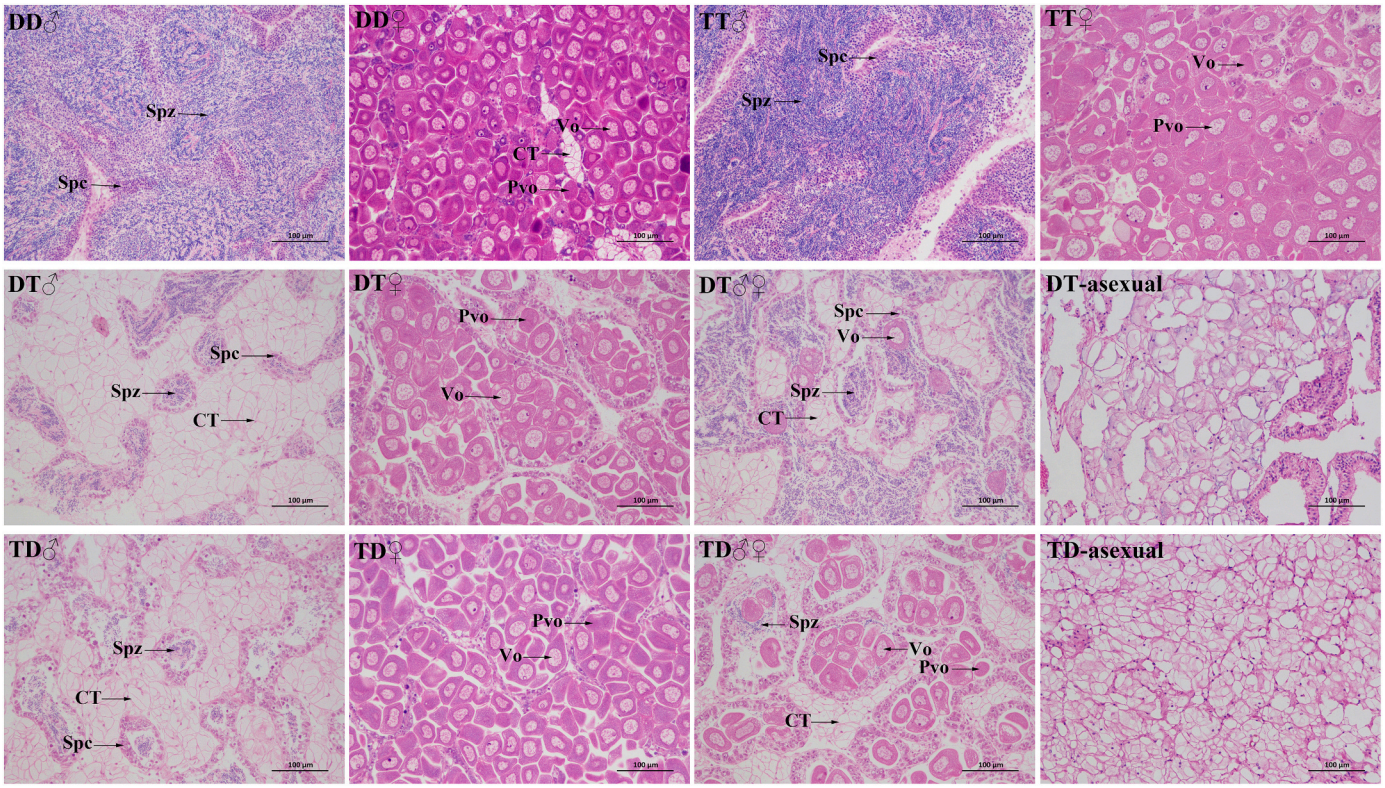

Fig. 7. The paraffin sections of gonadal tissues of DD, TD, DT and TT groups during the maturation period.
Note: ♂-male; ♀-female; ♂♀-hermaphrodite; Pro-previtellogenic oocytes; Vo-vitellogenic oocytes; Spc-spermatocyte; Spz-spermatozoa.

5. Conclusion

We conducted a comprehensive comparative analysis on the hatching, growth, and survival of DD, TD, DT and TT groups in China, and further analyzed the interaction between sites and genotypes. Triploid groups, especially TD group, showed significant growth and survival advantages in bote sites, and their 100% triploid rate remained unchanged from planktonic larvae to adults. In addition, the gonadal development of the triploid groups were still poor in general, although some individuals were fertile and produced few functional gametes. Moreover, we also demonstrated that tetraploid was completely fertile and can produce a large number of functional gametes. The ploidy of some tetraploids was unstable, some individuals turned into triploid, aneuploid and diploid, the chromosomes wre loss. Tetraploid was not suitable for large-scale cultivation because of its slow growth, low survival rate and unstable ploidy, but its sperm could be used for commercial production of triploids. All these indicated that tetraploid ♂ × diploid ♀ can be used as an excellent strain for large-scale promotion and aquaculture.

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Author statement

Yanping Qin: Designed the experiments, performed the experiments, collected and analyzed the data, wrote the paper and funding acquisition. Yuehuan Zhang: Collected the data, funding acquisition and Writing - review & editing. Ziniu Yu: Funding acquisition, supervision, Writing - review & editing.

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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ARTICLES FOR FACULTY MEMBERS

PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

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Effect of farming practices on growth and mortality rates in triploid and diploid eastern oysters *Crassostrea virginica*

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ABSTRACT: Commercial off-bottom aquaculture of the eastern oyster *Crassostrea virginica* is challenged by repeated summer mortality events that appear to affect triploid oysters disproportionally. Many farmers believe common farming practices, especially when performed during hot summer months, may increase triploid mortality. The goal of this study was to investigate how diploid and triploid oysters react to common stressors imposed by farmers: tumbling during size grading and desiccation to prevent biofouling. Triploid and diploid oysters were deployed in floating cages at 3 farm sites along the US Gulf of Mexico coast. In May and July, oysters in 7 cages were subjected to 1 of 6 stress treatments: 1 of 3 levels of desiccation (18, 24, or 48 h) mixed with 1 of 2 levels of tumbling (tumbled or not tumbled), along with a never handled, submersed control. The mortality and growth rate of oysters were assessed in June, August, and September. Growth rates of both ploidies were affected by compounding stressors; that is, treatments with both tumbling and the longest desiccation period exhibited slower growth. Triploid oysters exhibited a greater increase in mortality in response to farm stress than diploid oysters, and mortality increased in all oysters subjected to extreme stress treatments when compared to the control. Based on this study, farmers should limit the desiccation time of oysters (particularly triploids) in the summer months to avoid any mortality or reduced growth resulting from compounding stressor effects.

KEY WORDS: Oysters · Triploids · Mortality · Stress · Aquaculture · Desiccation · Tumbling

1. INTRODUCTION

Eastern oysters *Crassostrea virginica* are grown commercially in aquaculture operations throughout the east coast of North America and the Gulf of Mexico. The estimated value of aquaculture production for the US eastern oyster in 2015 was $173 million (NOAA Fisheries 2016). The industry is growing quickly, especially in the northern Gulf of Mexico along the coasts of Alabama, Mississippi, Florida, and Louisiana (Casas et al. 2017). In Alabama alone in 2016, oyster farmers harvested at least 2.6 million oysters with a farm-gate value of nearly $2 million (Grice & Walton 2017). However, a major problem limiting industry success is frequent summer mortality events (Casas et al. 2017, Wadsworth et al. 2019).

Summer mortality is a concern for both diploid and triploid oysters, both of which are commonly used in aquaculture. Triploidy is a condition in which the animal contains 3 sets of chromosomes (3n), rather than the usual 2 sets (2n or diploidy). Triploid oysters have reduced gametogenesis and therefore rarely spawn. Consequently, they expend less energy on reproduction and may grow faster and reach market size (75 mm) sooner than diploid oysters. For example, one study indicated that whole wet weight of

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triploids can be up to 70% more than diploids grown for the same amount of time under the same conditions (Ibarra et al. 2017). Reduced gonad production in triploids may also improve the meat quality (Housin et al. 2019). Thus, the use of triploid eastern oysters by commercial aquaculture has become increasingly popular in recent years. In 2014, triploids made up 91% of growers' plantings in Virginia (Hudson & Murray 2015). Similarly, the Auburn University Shellfish Laboratory (AUSL) produced and shipped 36 million triploid seed to off-bottom oyster farms in the Gulf of Mexico region in 2017, far outpacing diploid orders (F. S. Rikard unpubl. data).

Despite the benefits of triploid oysters for commercial aquaculture, farmers in the industry are increasingly concerned that triploid oysters are more sensitive to summer mortality events. Farmers in South Australia find triploid Pacific oysters *C. gigas* to be 'fragile' and are more careful when handling them during the summer months (S. Allen pers. comm.). In summer 2016, several farmers in Alabama noticed unexpectedly high levels of triploid oyster mortality in 1 to 2 yr old oysters at farm sites; mortality rates ranging from 19 to 100% were observed, with the majority of the mortality occurring over only a few weeks in early July (Wadsworth 2017). In early May 2018, a local commercial farm located in Grand Bay, Alabama reported triploid mortality of around 30% with no noticeable increase in diploid mortality (W. C. Walton pers. obs.). The cause of these mortality events remains inconclusive, but the need to reduce triploid (and diploid) summer mortality is vital to the continued success of this oyster industry.

Oysters are subject to a number of naturally occurring environmental stressors, as well as stressors imposed by aquaculture activities, that may increase risk of mortality. Furthermore, these farm and environmental stressors may contribute to differences in mortality rates between triploid and diploid oysters (Wadsworth et al. 2019). For example, low salinity (an environmental stressor) can cause triploid oysters to grow more slowly than diploid oysters (Callam et al. 2016). Such a stressor could also contribute to higher mortality rates. Desiccation and tumbling are 2 potential stressors imposed by aquaculture farms that may influence oyster mortality rates. Desiccation is the practice of exposing oysters to ambient air for extended periods of time to reduce biofouling and infestation by many marine parasites (Grodeska et al. 2017). A common farming practice in the US Gulf Coast region is to desiccate oysters for 18 to 24 h duration once a week. Tumbling is the process of running oysters through a rotary style mechanical grader, or tumbler, in order to sort the oysters by size (Ring 2012). Tumbling has the added benefit of chipping away fragile new shell growth, thus improving the shell shape of the oysters for market. It is common for farmers to tumble their oysters once per month (Ring 2012). Ultimately, the interaction of multiple stressors, both environmental and farm-based, must be observed to understand their effects on the growth and mortality of both diploid and triploid oysters.

In this experiment, we sought to test 2 possible causes of increased summer mortality by subjecting both diploid and triploid oysters to common stressors potentially imposed by farmers: tumbling and desiccation. We predicted that triploid oysters would exhibit higher mortality rates in response to these farm-based stressor events. Growth and mortality rates of diploid and triploid oysters subjected to different levels of these stressors were compared across 3 farm sites. Based on the results, we sought to recommend best farm management practices to reduce summer mortality.

2. MATERIALS AND METHODS

2.1. Experimental set up and sampling

This experiment was conducted at 3 farm sites across the northern Gulf of Mexico (Fig. 1). Sites in different US states were chosen so that results would reflect the variable growing conditions across the US northern Gulf of Mexico. The first site was at Grand

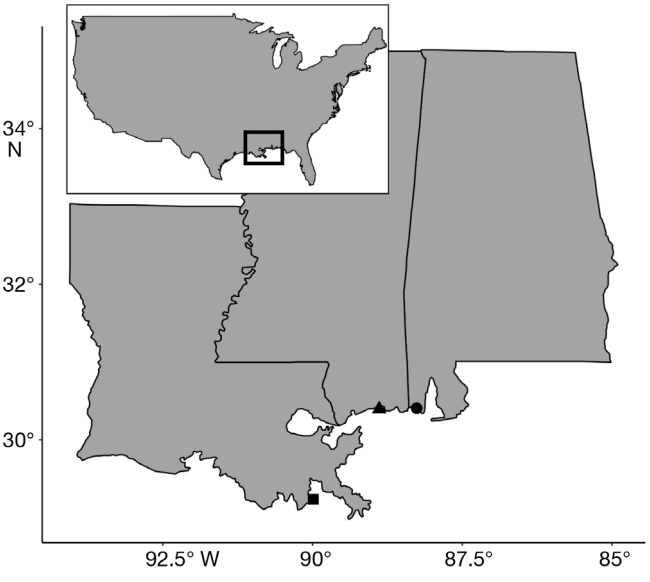

Fig. 1. The 3 farm sites used in this study: Grand Bay, AL (●), Deer Island, MS (▲), and Grand Isle, LA (■), USA

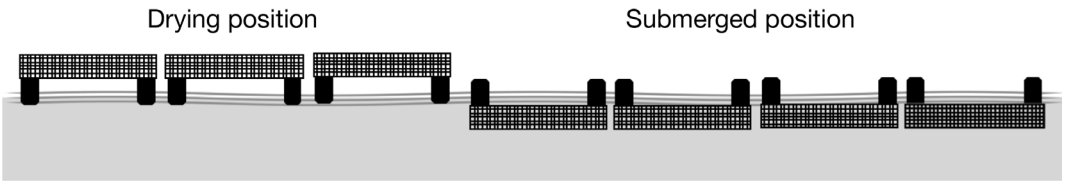

Fig. 2. The OysterGro set-up for a farm site. Three cages are in the drying position and 4 are in the submerged position. Image credit: Victoria Pruente, Auburn University Shellfish Laboratory, AL, USA

Bay Oyster Park in Grand Bay, Alabama, at an AUSL research site. The second site was at Deer Island in Biloxi, Mississippi, in cooperation with the Mississippi Department of Marine Resources. The third site was at the Michael Voisin Oyster Research Lab and Hatchery in Grand Isle, Louisiana, in cooperation with Louisiana Sea Grant and Louisiana State University.

At each of the 3 sites, 7 OysterGro® cages were deployed in early April 2018 (Fig. 2). Each cage held six 12 mm mesh grow-out bags (36 × 18.9 × 7.6 cm). Three bags in each cage contained diploid oysters and 3 bags contained triploid oysters (produced from crossing diploids and tetraploids), in an alternating pattern. Thus, 42 bags were deployed at each site, with a grand total of 126 bags across all 3 sites. All oysters were 12 mo old, half-sibling triploids and diploids spawned and raised at AUSL and grown out in Portersville Bay in 2017. Each bag was initially stocked with 75 oysters, well below the standard stocking density of 150, to ensure overcrowding did not contribute to stress or mortality. All oysters were allowed to acclimate for 1 mo after being deployed. In early May, the first stressor trial was imposed on oysters at all 3 sites because it was qualitatively observed in prior years that oyster mortality begins to rise at that time of year, putatively in response to warming water temperatures. Additionally, a commercial farmer near the Alabama study site was reporting high mortalities at that time (W. C. Walton pers. obs.).

At each site, each of 6 cages was randomly assigned to a stressor treatment based on 3 levels of desiccation (18, 24, or 48 h) mixed with 2 levels of tumbling (tumbled through a mechanical grader/not tumbled), while 1 cage served as a never handled, submersed control (Table 1). Two HOBO Pendant® MX Water Temperature data loggers were placed at each site to track temperature fluctuations. To ensure the rate of temperature change would more accurately reflect conditions inside an oyster shell, each logger was inserted between 2 oyster shells bound together with zip ties. One logger was placed inside a control bag (never desiccated, never tumbled) to monitor water temperature for the duration of the experiment. The other logger was placed inside a bag in the 48/T treatment to monitor temperature changes when oysters were taken out of the water to be tumbled and desiccated. At the Grand Isle and Deer Island sites, salinity data were collected from USGS sensors (United States Geological Survey 2018) and at Grand Bay, salinity data were collected from an Aqua TROLL sonde at the site.

Oysters that were desiccated and not tumbled stayed in their OysterGro cage, which was flipped to the drying position, with floats down. The cage was then flipped back over to the submerged position, floats up, once the appropriate amount of time had passed. Bags of oysters that were desiccated and tumbled were removed from their cage and taken to the mechanical grader at each site to be tumbled (≤20 min transport). Oysters from each bag were fed through the grader, one bag at a time in a random order, and all oysters were placed back in the bag before the next one was started. Tumbled oysters were left to desiccate on land overnight before being returned to their respective cages, already flipped to the drying position, the following morning. These cages were then flipped back over once the appropriate amount of desiccation time had passed, inclusive of the overnight desiccation on land.

Oysters at each site were allowed to recover *in situ* for approximately 1 mo after the stressor trial at that site had been completed. In June, samples were taken to assess oyster mortality and growth rate. Due

Table 1. List of the control treatment and the 6 stressor treatments that oysters were subjected to in the experiment

Stressor treatment	Abbreviation
0 h desiccation and no tumbling	0/NT
18 h desiccation and no tumbling	18/NT
18 h desiccation and tumbling	18/T
24 h desiccation and no tumbling	24/NT
24 h desiccation and tumbling	24/T
48 h desiccation and no tumbling	48/NT
48 h desiccation and tumbling	48/T

to very low overall mortality observed at the June timepoint, stressor trials were repeated in July to impose the stressors during a warmer period. Approximately 1 mo after the second round of stressor trials, in August, mortality and growth rates were again assessed. In September, one final round of sampling was performed to track any residual impacts that imposed stress had on mortality and growth. Analysis was performed on the sum of the mortality measurements collected in June, July, and August.

Mortality was measured by counting the number of dead oysters in each bag at the end of the study period and percent mortality for each bag was calculated by dividing this by 75 (initial planting density). Length of 5 haphazardly selected oysters from each bag were measured using calipers (nearest 0.01 mm). Length was measured from umbo to the tip of the bill. Average growth rates were calculated, in mm d^{-1}, using the change in length (mm) from the average initial size of the oysters in April to the final individual size in September 2018 (to account for initial differences in average size between ploidy treatments).

2.2. Data analysis

All analyses were completed using R v.1.0.153 (R Core Team 2018). The growth rate data were normally distributed but percent mortality data were not. Therefore, the percent mortality data were log transformed to restore normality. Linear mixed-effects models (lme4 package, Bates et al. 2015) were used to examine the 3 fixed factors (ploidy, treatment, and their interaction) across site and cage (blocked random factors) for the response variables (growth rate and mortality, Eqs. 1 & 2, respectively). Eqs. (1) & (2) were compared to corresponding linear mixed-effects models that did not contain an interaction term using an ANOVA for linear model fit. The more complicated model (containing the interaction term) was considered a significant improvement over the simpler model if $p < 0.05$ and the Akaike's information criterion (AIC) value was lower. If the interaction term was significant, then we analyzed diploid and triploid data separately to further elucidate the results.

$$\begin{aligned}\text{Growth} = {} & \mu + \beta_1(\text{ploidy}) + \beta_2(\text{treatment}) + \\ & \beta_3(\text{ploidy} \times \text{treatment}) + \\ & \varepsilon_{\text{Site/Cage}} \sim N(0, \sigma_{\text{Site/Cage}}) + \\ & \varepsilon_{\text{random}} \sim N(0, \sigma_{\text{random}})\end{aligned} \quad (1)$$

where growth is the dependent variable of growth rate in mm d^{-1}, μ is the overall mean, ploidy is the effect of ploidy (triploid or diploid), treatment is the effect of the stress treatment (0/NT, 18NT, 18/T, 24NT, 24/T, 48/NT, 48/T), $\varepsilon_{\text{Site/Cage}}$ is the random effect of cage nested within site, $\varepsilon_{\text{random}}$ is the random effect, and β, N, and σ are error terms.

$$\begin{aligned}\text{Mortality} = {} & \mu + \beta_1(\text{ploidy}) + \beta_2(\text{treatment}) + \\ & \beta_3(\text{ploidy} \times \text{treatment}) + \\ & \varepsilon_{\text{Site/Cage}} \sim N(0, \sigma_{\text{Site/Cage}}) + \\ & \varepsilon_{\text{random}} \sim N(0, \sigma_{\text{random}})\end{aligned} \quad (2)$$

where mortality is percent mortality and all other terms are as described for Eq. (1) above. Additionally, a linear model was used to analyze the relationships of temperature, salinity, and ploidy (fixed, independent variables) to the dependent variable of mortality over the duration of the study (Eq. 3). This linear model was compared to a corresponding linear model that did not contain interaction terms using ANOVA for linear model fit. The more complicated model (containing interaction terms) was considered a significant improvement over the simpler model if $p < 0.05$.

$$\begin{aligned}\text{Mortality} = {} & \mu + \beta_1(\text{temperature}) + \\ & \beta_2(\text{salinity}) + \beta_3(\text{ploidy}) + \\ & \varepsilon_{\text{random}} \sim N(0, \sigma_{\text{random}})\end{aligned} \quad (3)$$

where temperature is the effect of water temperature and salinity is the effect of salinity.

3. RESULTS

3.1. Growth rates

There was no significant interaction between ploidy and treatment in our analysis of oyster growth rates (ANOVA for linear model fit; $p = 0.45$, AIC = −482.0 as compared to AIC = −488.3 of simpler model) and thus the interaction term was removed. At the conclusion of the field study, the factors that significantly affected growth rate were ploidy and the stress treatments 24/T and 48/T (Table 2). Triploid oysters grew 0.06 mm d^{-1} (CI = 0.01) faster than diploid oysters across all stress treatments ($p < 0.01$). Triploids had an average growth rate of 0.23 mm d^{-1} (CI = ±0.01) compared to the average diploid growth rate of 0.16 mm d^{-1} (CI = ±0.03). The 2 stress treatments that affected growth caused oysters to grow more slowly than in other treatments (Table 2). Oysters (diploid and triploid) in the 24/T stress treatment grew 0.03 mm d^{-1} (CI = 0.03) slower than oysters in the control treatment ($p = 0.03$), and oysters in the 48/T stress grew 0.040 mm d^{-1} (CI = 0.03) slower than oysters in the control treat-

ment ($p < 0.01$). The growth rates of oysters in treatments 24/T and 48/T were not significantly different from each other or the remaining treatments (18/NT, 18/T, 24/NT, and 48/NT; all $p > 0.06$) (Fig. 3).

Table 2. Linear mixed-effects models for growth rate and mortality with 2 ploidies (triploid and diploid) under 6 stress treatments (18/NT, 18/T, 24/NT, 24/T, 48/NT, 48/T) and the control. Significant p-values are given in **bold**. See Table 1 for treatment abbreviations

	df	Estimate	SE	*t*	p
Growth (mm d^{-1})					
Intercept	104	0.16	0.01	11.64	**≤0.01**
Ploidy	104	0.06	0.01	11.57	**≤0.01**
Treatment 18/NT	12	−0.03	0.01	−2.08	0.06
Treatment 24/NT	12	−0.02	0.01	−1.95	0.08
Treatment 48/NT	12	−0.03	0.01	−2.04	0.06
Treatment 18/T	12	−0.02	0.01	−1.74	0.11
Treatment 24/T	12	−0.03	0.01	−2.44	**0.03**
Treatment 48/T	12	−0.04	0.01	−3.11	**0.01**
Diploid mortality (%)					
Intercept	41	0.01	0.05	0.24	0.81
Treatment 18/NT	12	0.02	0.08	0.21	0.84
Treatment 24/NT	12	0.03	0.08	0.37	0.72
Treatment 48/NT	12	0.15	0.08	1.94	0.08
Treatment 18/T	12	0.04	0.08	0.49	0.63
Treatment 24/T	12	0.10	0.08	1.29	0.22
Treatment 48/T	12	0.27	0.08	3.57	**≤0.01**
Triploid mortality (%)					
Intercept	41	0.05	0.07	0.71	0.48
Treatment 18/NT	12	<0.01	0.10	−0.01	0.10
Treatment 24/NT	12	0.03	0.10	0.33	0.75
Treatment 48/NT	12	0.23	0.10	2.40	**0.03**
Treatment 18/T	12	0.07	0.10	0.74	0.47
Treatment 24/T	12	0.08	0.10	0.83	0.42
Treatment 48/T	12	0.36	0.10	3.77	**≤0.01**

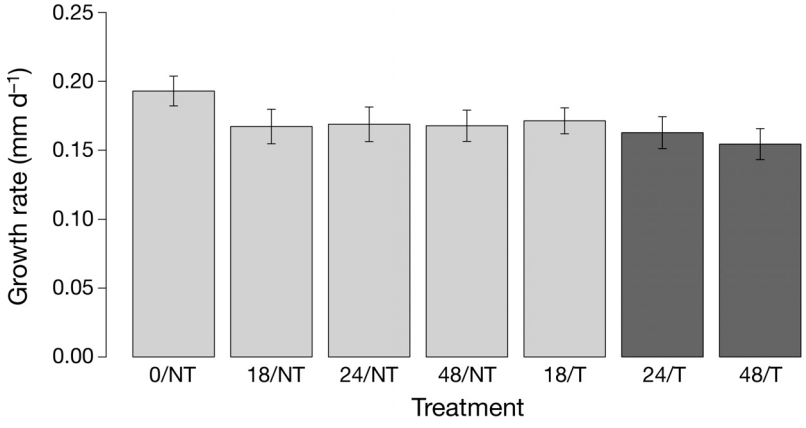

Fig. 3. Oyster growth rate for both ploidies from each of the 6 stress treatments and the control. Dark grey bars indicate growth rate of the stress treatment was significantly lower than the control (0/NT). Error bars indicate lower and upper 95% confidence limits. See Table 1 for treatment abbreviations

3.2. Mortality

Triploid oysters had a higher mortality rate than diploid oysters (type II sum-of-squares analysis $p < 0.01$). Specifically, on average, triploids had 6.79% (lower confidence limit [LCL] = 4.13%, upper confidence limit [UCL] = 9.43%) higher mortality than diploid oysters (back transformed from the model). Furthermore, stress treatment and ploidy had a significant interaction on oyster mortality (ANOVA for linear model fit; $p = 0.04$, AIC = −240.4 as compared to AIC = −239.4 of simpler model). Due to this interaction, the effect of treatment on mortality was analyzed separately for each ploidy using 2 models (Table 2). Diploid oysters in treatment 48/T had significantly higher mortality than diploids in the control treatment ($p < 0.01$), while triploid oysters in both 48 h desiccation treatments (48/NT and 48/T) had higher mortality than control triploids ($p < 0.01$ for all comparisons; Table 2, Fig. 4). Control diploid oysters experienced 1.32% (LCL = −9.19%, UCL = 13.05%) mortality, while diploid oysters in treatment 48/T experienced 33.06% (LCL = 11.22%, UCL = 55.06%) mortality at the conclusion of the field study. In contrast, control triploid oysters experienced 5.38% (LCL = −9.20%, UCL = 22.32%) mortality, and triploid oysters in treatments 48/NT and 48/T experienced 33.06% (LCL = 20.97%, UCL = 55.13%) and 51.62% (LCL = 16.59%, UCL = 77.54%) mortality, respectively (Table 2, Fig. 4). Triploid and diploid oysters in other treatments had levels of mortality that fell between those seen in the control and in treatments 48/NT and 48/T, although none of the mortality rates were significantly different from other treatments (all $p > 0.42$).

3.3. Environmental parameters

We did not find any significant interactions between ploidy, water temperature, and salinity in our analysis ($p = 0.63$), and thus the interaction terms were removed from the linear model. We did find that, as water temperature increased, oyster mortality (of both ploidies) increased (linear regression, $t = 3.74$, df = 326, $p \leq 0.01$). However, no significant relationship was found between salinity and mortality (linear regression, $t = 0.01$, df = 326, $p = 0.92$). Average water temperature and salinity across the 3 field sites from May through September can be seen in Table 3.

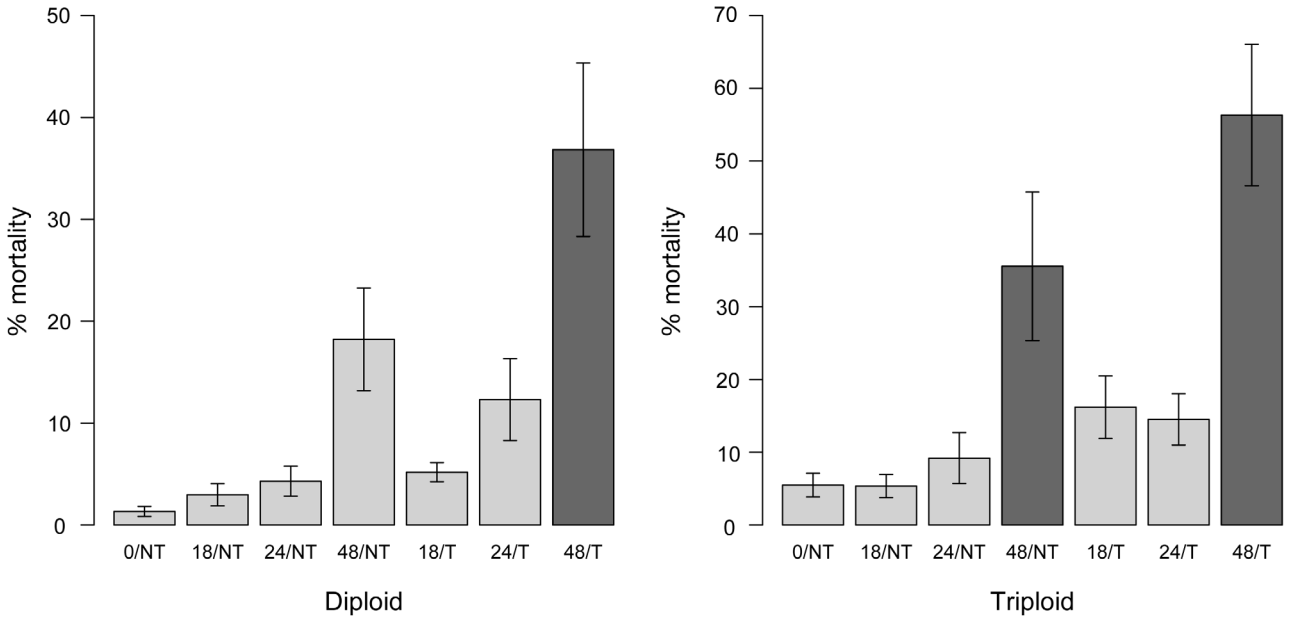

Fig. 4. Percent mortality of diploid and triploid oysters from each of the 6 stress treatments and the control (note different *y*-axis scales). Dark grey bars indicate the mortality of the stress treatment was significantly higher than the control (0/NT). Error bars indicate lower and upper 95% confidence limits. See Table 1 for treatment abbreviations

4. DISCUSSION

4.1. Growth rates

Triploid oysters, across all stress treatments, exhibited a significant growth advantage over their diploid counterparts. The faster growth of triploid oysters may be due to both reduced gametogenesis and/or higher heterozygosity of triploid oysters, leading to lower metabolic energy costs (Hawkins & Day 1996). Stress treatments also affected oyster growth rate; however, they did not interact with ploidy. Oysters (both diploid and triploid) subjected to any stress treatment tended to exhibit reduced growth relative to the control treatment, though only oysters in treatments 24/T and 48/T had significantly decreased growth when compared to the control (Fig. 3). Treatments 24/T and 48/T were 2 of the most severe stress treatments because they involved both extended periods of desiccation and tumbling combined. Prior studies have noted that compounding stressors, such as environmental stress and multiple induced farm stressors, can cause increased adverse effects to oysters (Cheney et al. 2000). Furthermore, intertidal oysters living in high-stress environments with more wave action have been shown to exhibit reduced growth when compared to oysters (of the same ploidy) living in lower stress environments (Ortega 1981). However, we note that in the present study, both stress treatments likely result in shell breakage as the oysters are handled during desiccation and tumbling and the observed responses may not have been explicitly slowed growth but rather greater loss of shell.

Table 3. Mean ± SD water temperature, and salinity across the 3 grow-out sites in the months of May through September. Water temperature data was collected from HOBO sensors, all other data were collected from USGS. The Deer Island water temperature for August and September was also taken from the USGS as the temperature sensors may have malfunctioned. Different letters indicate significant differences ($p < 0.05$) in temperature or salinity between each month

Experimental sites	Temperature (ºC)	Salinity (ppt)
May	28.96 ± 0.29^{a}	12.46 ± 0.80^{a}
June	29.92 ± 0.21^{b}	15.28 ± 0.84^{b}
July	30.20 ± 0.20^{b}	19.19 ± 0.78^{c}
August	29.99 ± 0.21^{b}	20.88 ± 0.78^{d}
September	28.51 ± 0.32^{c}	15.61 ± 1.94^{b}

4.2. Mortality

In the present study, we found that triploid oysters experienced higher mortality rates than diploid oysters. Results of previous studies comparing mortality between diploids and triploids vary, with some find-

ing mortality in triploid oysters to be much higher than in diploid oysters (*Crassostrea gigas*, Cheney et al. 2000; *C. virginica*, Wadsworth et al. 2019), some researchers finding mortality in triploids to be lower than in diploids (*C. gigas*, Gagnaire et al. 2006), and some finding no difference between ploidies (*C. gigas*, Dégremont et al. 2010).

Stress treatments affected the mortality of triploid oysters differently to that of diploid oysters. Regardless of tumbling, the 48 h desiccation treatment led to significantly higher mortality in triploids (relative to the control), while only diploids in the most severe treatment (48 h desiccation and tumbled) experienced elevated mortality (Fig. 4). Diploid and triploid oysters subjected to less severe farm stress (less than 48 h desiccation) tended to exhibit increased levels of mortality as compared to control oysters, though these differences were not significant and did not appear to vary between ploidies.

Furthermore, though differences were not statistically significant, mortality of triploid and diploid oysters in treatments with desiccation and tumbling were always higher than in treatments with matching desiccation times (Fig. 4). Additive farm stressors (such as tumbling and desiccation as opposed to desiccation alone) have been shown to increase oyster mortality (Ring 2012). Combinations of different stressors such as elevated water temperature, pathogens, low DO, salinity, aquaculture practices, and physiological stress associated with reproduction have long been thought to be the cause of summer mortality events (Cheney et al. 2000, Gagnaire et al. 2006, Soletchnik et al. 2007, Dégremont et al. 2012, Pernet et al. 2012). In our case, compounding stressor treatments (desiccation and tumbling) seemed to be more lethal than desiccation alone for both ploidies.

Environmental factors may have also played a role in driving oyster mortality, though both ploidies were affected similarly. As water temperatures increased, so did the mortality of both ploidies. Higher water temperatures have been correlated with increased oyster mortality before, though higher water temperature alone may not be lethal (Cheney et al. 2000). No relationship between mortality and salinity was observed in this study.

5. CONCLUSION

Across ploidies, the 2 most severe stress treatments (24/T and 48/T) significantly decreased growth rate. The act of putting oysters into a mechanical grader, tumbling, could have caused this reduction in growth rate due to increased shell breakage (Ring 2012). In turn, this shell breakage may have outweighed any physiological effects the stress treatments had on growth rate. However, physiological stress from high levels of desiccation and tumbling likely did play a role in reducing growth rate. All 3 tumbling treatments (18/T, 24/T, and 48/T) experienced the same intensity of tumbling stress (i.e. shell breakage), only desiccation times differed among the treatments. The treatments with higher levels of desiccation (24 and 48 h) displayed significantly reduced growth, indicating that a combination of tumbling and longer desiccation times led to slower growth.

The combination of tumbling and desiccation stress affected the mortality of each ploidy differently. The 2 stress treatments with the longest desiccation times (48/NT and 48/T) significantly amplified triploid oyster mortality, while diploid mortality was only significantly affected by the combination of 48 h desiccation and tumbling. However, this interactive effect was not significant at lower levels of stress treatments. Overall, triploid oysters experienced higher mortality than diploid oysters (~7%), and the mortality of both ploidies tended to increase compared to the control (though not always significantly) when oysters were subjected to any stress treatment. Additionally, the mortality of both ploidies increased as the water temperature increased. Many farmers already treat oysters with more care during the summer, reducing handling stress and desiccation time. It is impractical, however, for farmers to completely avoid imposing stress on oysters during the summer, as desiccation and tumbling are necessary practices in order to produce the highest grade of oyster (Ring 2012). Based on the present study, we recommend that farmers limit the desiccation time of oysters, particularly of triploid oysters, during the summer. In this study, mortality generally increased with desiccation time, with triploids and tumbled diploids exhibiting significantly higher mortality when desiccated for 48 h.

Another solution could be selectively breeding oysters that are more resistant to heat and environmental stress. Stock selection for the purpose of lowering oyster mortality due to stressors has been successfully performed. Casas et al. (2017) produced a line of oysters that were more resistant to dermo disease, caused by *Perkinsus marinus*, and exhibited lower mortality than unselected oyster stock. Additionally Dégremont et al. (2010) compared diploid oysters whose parents had been selected for resistance to summer mortality to triploid oysters with a diploid parent selected for resistance. A positive response to

survival was found for both these diploid and triploid lines, demonstrating the potential for selective breeding. However, the diploids still had higher survival than the triploids, possibly because the selected diploid parents only contributed a third of the chromosomes to the triploid offspring. Selecting for resistance in tetraploid, as well as diploid, parents would create a triploid line with higher resistance to summer mortality events (Callam 2013).

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ARTICLES FOR FACULTY MEMBERS

PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

Title/Author	**Effects of different aquaculture patterns on growth, survival and yield of diploid and triploid Portuguese oysters (Crassostrea angulate) / Sun, Y., Zhang, C., Liu, F., Zhang, H., Du, H., Zhang, Y., & Zheng, H.**
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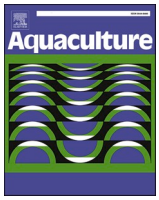

Effects of different aquaculture patterns on growth, survival and yield of diploid and triploid Portuguese oysters (*Crassostrea angulata*)

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ABSTRACT

Bivalve molluscs are increasingly being promoted as an alternative to land-based meat production due to their high levels of high-quality protein and lipids. The *Crassostrea angulata*, also known as the Portuguese oyster, has been a globally productive and renowned oyster species. In this study, we evaluated the aquaculture performance of *C. angulata* over two consecutive culture periods (pre-harvest phase, post-harvest phase) at different stocking densities and water depths, and compared the survival, growth and yield of diploid and triploid oysters. The experiment spanned 13 months, beginning in June 2022 and ending in June 2023. The results indicated that both stocking density and water depth significantly affected the aquaculture performance of *C. angulata* and exhibited distinct patterns in the two culture phases. Overall, compared to diploid *C. angulata*, triploid oysters showed significant culture advantages in the pre-harvest period, the growth rates and total yield of which were much higher than those of diploids. In the pre-harvest phase, the depth of 1 m and low-density culture (5 substrates/rope) favored the survival and growth of triploid oysters, while diploid *C. angulata* presented higher growth rates at high-density cultivation (15 substrates/rope), and the total oyster yield under 5 m culture reached a minimum for both diploids and triploids. In the post-harvest phase, the survival rate was stabilized, water depths of 1–3 m were optimal, and diploid oysters grew better at low (50 ind/cage) and medium (100 ind/cage) densities, whereas triploids had a higher growth rate of shell height at medium and high (150 ind/cage) densities. The findings of this research provide theoretical guidelines for exploring efficient *C. angulata* cultivation methods in the future, which will be beneficial to its commercial production.

1. Introduction

Bivalve molluscs have high nutritional quality of protein and lipids and are the main providers of high-quality meat diets for humans (Tan et al., 2020, 2022). Bivalve aquaculture is presently one of the fastest-growing food production industries in the world and plays a key role in food production to help sustainably feed the expanding population, delivering 15% of animal protein per capita to 1.5 billion people and sustaining the livelihoods of >200,000 people, primarily in developing nations (Gentry et al., 2017; FAO, 2021). Currently, bivalve molluscs make up 21.3% (equivalent to 17.5 million tons) of the overall global seafood production, with Asia, particularly China, being the major contributor (FAO, 2021). In terms of culture structure, oysters are the most productive species among many marine bivalves, with Chinese oysters accounting for 86% of global oyster production, which is of important economic and social value (Botta et al., 2020).

The Portuguese oyster, scientifically named *Crassostrea angulata*, is a valuable species in aquaculture due to its economic significance. Currently, it has become the largest aquatic oyster species in China, sometimes referred to as the "Fujian oyster", and is favored by farmers as it can be cultivated year-round (Qin et al., 2012). However, its aquaculture development still faces many problems. Traditionally farmed *C. angulata* are mainly diploids, but due to the excessive nutrients consumed in the reproduction process to promote the development of

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gonads, the glycogen content, fertility and quality are reduced, and with the continuous expansion of breeding density, the phenomenon of small breeding individuals, low resistance and mass mortality is serious (Buestel et al., 2009; Zeng and Ning, 2011; Zhang et al., 2022). The application and promotion of triploid oysters have alleviated these problems well. Due to their low fertility, the mass mortality caused by the discharge of sperms and spawns during the reproductive period has been avoided, and the problem that traditional diploid oysters cannot be marketed after the breeding season has been solved, thus effectively filling the gap in the sales market (Lin et al., 2023). These triploid oysters, usually obtained by artificial hybridization of tetraploids and diploids or induced by physical and chemical methods, are poor in reproduction and more focused on shell and meat growth, which greatly shortens the length of the culture cycle (Stanley et al., 1981; Guo et al., 1996; Francesc et al., 2009; Zhang et al., 2022). Driven by the significant benefits of culture, triploid oyster aquaculture has been developing rapidly in China in recent years, showing a broad prospect for development. Nonetheless, the blind development of aquaculture has led to the phenomenon of overloaded culture in the sea area, coupled with the imperfection of aquaculture technology as well as the imbalance of aquaculture, the development of triploid oyster aquaculture industry urgently needs to be guided (Lin et al., 2023). Therefore, how to optimize the culture technology and rationally plan the culture mode is what we urgently need to take into account at present.

The stocking density, culture water depth, as well as culture apparatus, are crucial influencing factors in bivalve aquaculture. Previous studies on aquaculture performance (including growth, survival and production) of bivalve molluscs in different culture conditions have been reported (Avendaño et al., 2008; Cassis et al., 2011; Capelle et al., 2020; Zorita et al., 2021; Tan et al., 2021, 2023). However, many authors only studied the effect of a single factor, stocking density or water depth, on bivalves and did not evaluate the cross-effects (Avendaño et al., 2008; Roncarati et al., 2017; Oliveira et al., 2019; Rusydi et al., 2021), and the time scales of the experiments were mostly focused on nursery or/and grow-out phases (up until common harvesting phase) of bivalve molluscs (Goslin, 2015; Roncarati et al., 2017; Tan et al., 2023). In fact, oysters are not only valued as seafood but also for the ecosystem services they provide, including improving water quality and reducing eutrophication. Oyster-mediated denitrification (as opposed to nitrogen removal by biomass extraction) is increasingly recognized as the primary pathway for in situ nitrogen reduction, and its inclusion in management plans may have important implications for a variety of conservation and resource management plans (DePiper et al., 2016; Ray et al., 2021; Rose et al., 2021). If denitrification is affected by stocking density, harvest size, ploidy, or culture strategy (e.g., off-bottom versus bottom culture), it may result in future oyster investments being biased toward specific types of inputs or production methods (Rose et al., 2021). Scientists have studied the relationship between denitrification rates and oyster biomass in reef restoration areas and have considered increasing (without harvesting) natural oyster biomass through reef restoration as a nitrogen reduction practice (Sisson et al., 2011; Reichert-Nguyen, 2018). Therefore, there is still a need to evaluate the cultural performance of oysters in the later stages of maturation, and having this information is critical to understanding growth patterns in restoration environments.

In this study, *C. angulata* was used as the experimental object, and the oyster culture period was divided into two phases, pre-harvest (from spats period to harvest period) and post-harvest, to evaluate the culture performance of diploid and triploid *C. angulata* under different culture modes by setting up three different stocking densities and water depths, respectively, with the aim of enriching the basic information and providing theoretical guidance for the *C. angulata* cultivation and management techniques.

2. Materials and methods

2.1. Experimental animals and study area

One-month-old spats of diploid and triploid *C. angulata* were obtained from an oyster hatchery in Zhangzhou, Fujian Province, China. These spats were attached to the empty shells of adult oysters at a density of over 50 spats per substrate. The oysters were brought back to Nan'ao Island (Shantou, China) with the polyethylene net bags, and culture experiments were conducted in the field of the Marine Biological Experiment Station of Shantou University (23.47°E, 117.11°N) (Fig. 1). Nan'ao Island is located on the Tropical of Cancer and has maritime characteristics (Table 1), is mild all year round, rich in heat, and has less frost. The average annual rainfall from 1981 to 2010 was 1417.1 mm (https://weather.cma.cn). The prevailing wind in Nan'ao Island is the Southwest monsoon (from May to August) and the Northeast monsoon (from September to April).

2.2. General experimental design

Experiments were conducted in two consecutive phases (pre-harvest: in June 2022–January 2023 and post-harvest: in February–June 2023) (following the local common oyster harvest cycle), with the same culture method for diploid and triploid *C. angulata.* The design and sampling of the two different phases of the experiment were completely randomized and controls were set up according to the local oyster farming practices. Temperature (Temp) and dissolved oxygen (DO) were recorded at the cultured sea area during each sampling event with a multi-parameter probe (Lohand Biological, LH-T600, China).

2.2.1. Phase 1: Effects of stocking density and water depth on survival, growth and yield of diploid and triploid C. angulata at the pre-harvest phase

In June 2022, empty oyster shells (substrates) with spats attached were assembled into oyster shell strings and oysters were cultured using the longline culture method (Yang et al., 2003) (Fig. 2). According to the number of substrates hanging on the 2.4 m nylon rope, three stocking density treatments were set up: group low-density (5 substrates/rope, represented by "L"), group medium-density (10 substrates/rope, represented by "M") (local common oyster farming practice) and group high-density (15 substrates/rope, represented by "H"), and the spacing of substrates was the same for each density group. Three different water depth treatments were established based on the water depth of the aquaculture area, namely 1 m (the typical depth for local oyster farming), 3 m, and 5 m. Subsequently, the oyster shell strings of each density group (L, M and H) were suspended at varying depths (1, 3 and 5 m) beneath the surface of the water respectively to create an interactive aquaculture (density × water depth) for 240 days. The experiment included nine treatments each with six replicates.

2.2.2. Phase 2: Effects of stocking density and water depth on survival and growth of diploid and triploid C. angulata at the post-harvest phase

Post-harvest phase oysters were taken entirely from the oysters in the pre-harvest phase, and were cultured using the single cage technique (Yang et al., 2003). Nine-month-old adult oysters from phase 1 were harvested, then separated from the substrates, and all individuals from the different treatment groups were mixed and then randomly and evenly distributed into lantern cages (diameter = 30 cm; number of layers = 10; interlamellar spacing = 10 cm; mesh size = 1.3 cm). Three different densities were set according to the number of adult oysters placed in each lantern cage: low-density group (50 ind/cage, represented by "L"), medium-density (100 ind/cage, represented by "M") and high-density group (150 ind/cage, represented by "H"), and the same number of oysters per layer. Subsequently, the lantern cages were hung at various depths below the water surface (1, 3, and 5 m) for a period of 150 days, respectively. The experiments were conducted including nine treatments each with three replicates.

Fig. 1. Mariculture area of Nan'ao Island (Shantou, China).

Table 1
Environmental parameters in the surface water of Nan'ao Island.

	Spring (Mar-May)	Summer (Jun-Aug)	Autumn (Sep-Nov)	Winter (Dec-Feb)
Temperature (°C)	17.46–25.24	27.48–27.79	22.20–27.80	15.48–18.29
Salinity (‰)	30.68–32.03	27.96–30.32	31.34–32.67	31.69–32.15
Dissolved Oxygen (mg/L)	5.89–6.87	4.60–5.93	4.81–6.17	6.80–7.20
pH	8.53–8.64	8.09–8.42	8.13–8.34	8.51–8.59

2.3. Sample collection

2.3.1. pre-harvest phase

Every 30 days, we used the Simple Random Sampling Method to randomly select 3 ropes from each treatment group in order to estimate the survival rate (%) of spats. To determine the total oyster yield, we weighed the total weight of live oysters on each rope with an electronic scale that was accurate to 0.005 kg. Afterward, 20 live oysters were randomly sampled from each treatment group. Length and height measurements of each oyster were made to the nearest 0.01 mm using a vernier caliper. According to Galtsoff (1964), shell length was defined as the maximum distance between the anterior and posterior margin measured parallel with the hinge axis and at a right angle to the height axis, and shell height was defined as the distance from the umbo to the distal margin of the shell. The individual weight was measured using a precision electronic balance (accurate to 0.01 g). The shell height daily growth rate and the daily weight gain rate were used to assess the growth of oysters.

2.3.2. post-harvest phase

The number of oysters that died in lantern cages in each treatment group was counted at 30-day intervals to estimate oyster survival rates (%) during the post-harvest phase. The purpose of this study was to evaluate long-term effects of stocking density and test for influences in late culture by maintaining a constant density. Therefore, dead oysters were removed and replaced with individuals of similar shell height and weight to maintain densities (Monteforte et al., 2005; Freites et al., 2020; Tan et al., 2021). The replacement oysters received the same treatments as the experimental oysters and were adapted to the identical culture environment. They were marked and excluded from subsequent

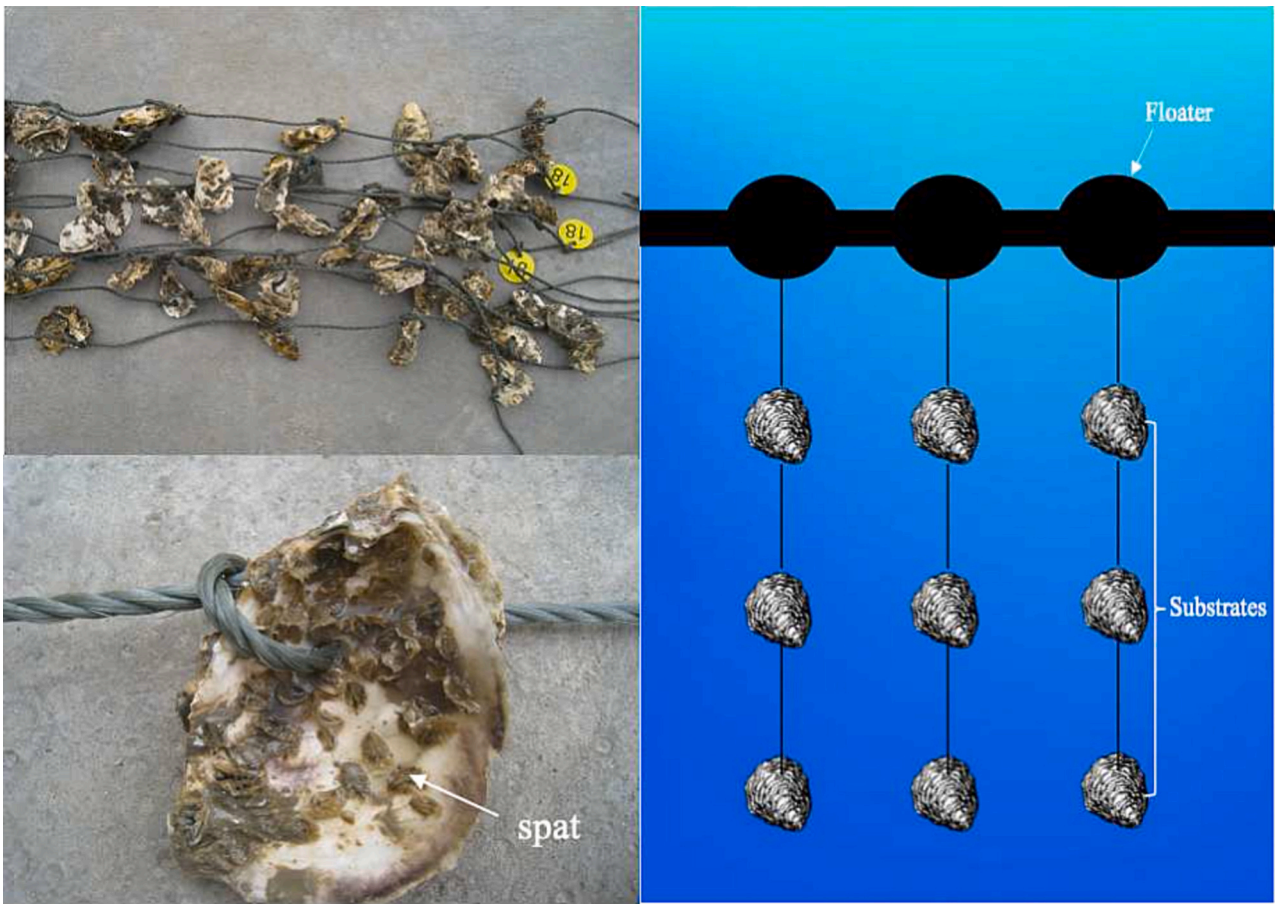

Fig. 2. Longline oyster culture method.

measurements (Underwood et al., 2002). Subsequently, a random sample of 20 live oysters was taken from each cage and the shell length and shell height were measured with a vernier caliper (accurate to 0.01 mm) and their wet weight was determined with a precision electronic balance (accurate to 0.01 g). Once the measurements were taken, the oysters were placed back into their respective lantern cages.

2.4. Statistical analysis

The Statistical Package for the Social Sciences (SPSS, version 26) was utilized for data analysis. First, the Kolmogorov-Smirnov test was used to check the normality of all variables, and Levene's test was utilized to examine the homogeneity of variance. A one-way ANOVA examined the effects of single variables (density and water depth) on initial growth (shell height and wet weight) in both phases and the differences across equal replicates to determine that no significant differences existed. Differences in survival rate, growth rate, and total yield of oysters among various stocking densities and water depths were analyzed by Two-way ANOVA and LSD multiple comparisons. Independent samples *t*-test was utilized to test the significant differences in the above indices between diploid and triploid oysters. Under non-normality and heterogeneous of variance conditions, the Kruskal-Wallis nonparametric test was applied followed by the Mann-Whitney U post hoc test for pair-wise comparison between groups (Zorita et al., 2021). The relationships between oyster survival and growth with environmental parameters (Temp and DO) were evaluated by the non-parametric Spearman correlation.

$P < 0.05$ indicates that the difference is statistically significant. All tests were two-tailed, and results were exhibited as mean ± standard deviation (SD).

3. Results

3.1. Trial 1: Effects of stocking density and water depth on survival, growth and yield of diploid and triploid C. angulata at the pre-harvest phase

The survival rates of diploid and triploid *C. angulata* at the pre-harvest phase cultured under different stocking densities and water depths are summarized in Fig. 3a and Fig. 3b, respectively. In general, water depth had a stronger effect on the survival rate of diploids than stocking density ($F = 16.302$, $P < 0.001$), with a significant interaction observed between these two factors ($F = 3.770$, $P = 0.026$). Diploid *C. angulata* cultured at 1 m (47.24 ± 2.25%) and 3 m (53.17 ± 4.39%) both had significantly higher survival rates compared to those cultured at 5 m (25.73 ± 5.20%) ($P < 0.001$). In terms of triploid oysters, they were not significantly affected by stocking density and water depth. However, triploids had a significantly higher survival rate compared to diploids at 5 m culture depths (43.52 ± 4.05% versus 25.73 ± 5.20%) ($P = 0.022$).

Growth of juveniles, expressed in terms of shell height and wet weight, was rapid during the first two months of cultivation (June–August 2022), but slower in the following months, and distinct differentiation of different treatment groups was observed over time (Fig. 4). The effects of stocking density and water depth on growth rates (in terms of the shell height daily growth rate and the daily weight gain rate) of *C. angulata* during the pre-harvest phase are shown in Fig. 5. Analysis of variance demonstrated that the growth rates of diploids were significantly affected by stocking density and water depth with significant interactions (shell height: $F = 8.994$, $P < 0.001$; weight: $F = 5.976$, $P < 0.001$). The growth rates of diploid oysters at high-density culture

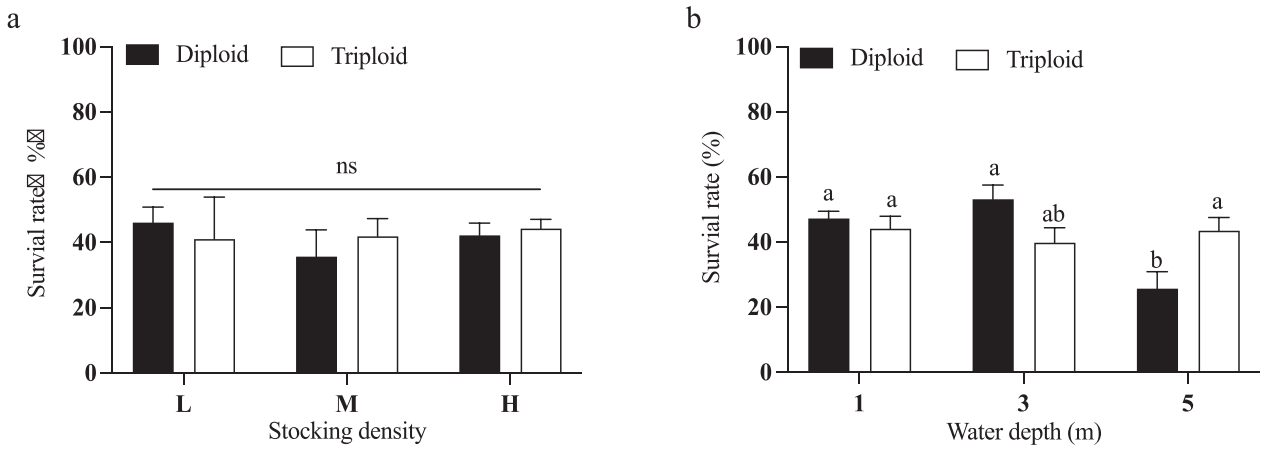

Fig. 3. Effects of stocking density (a) and water depth (b) on survival rate of diploid and triploid *C. angulata* at the pre-harvest phase. The presence of distinct letters indicates statistically significant differences ($P < 0.05$). ns represents no significant difference.

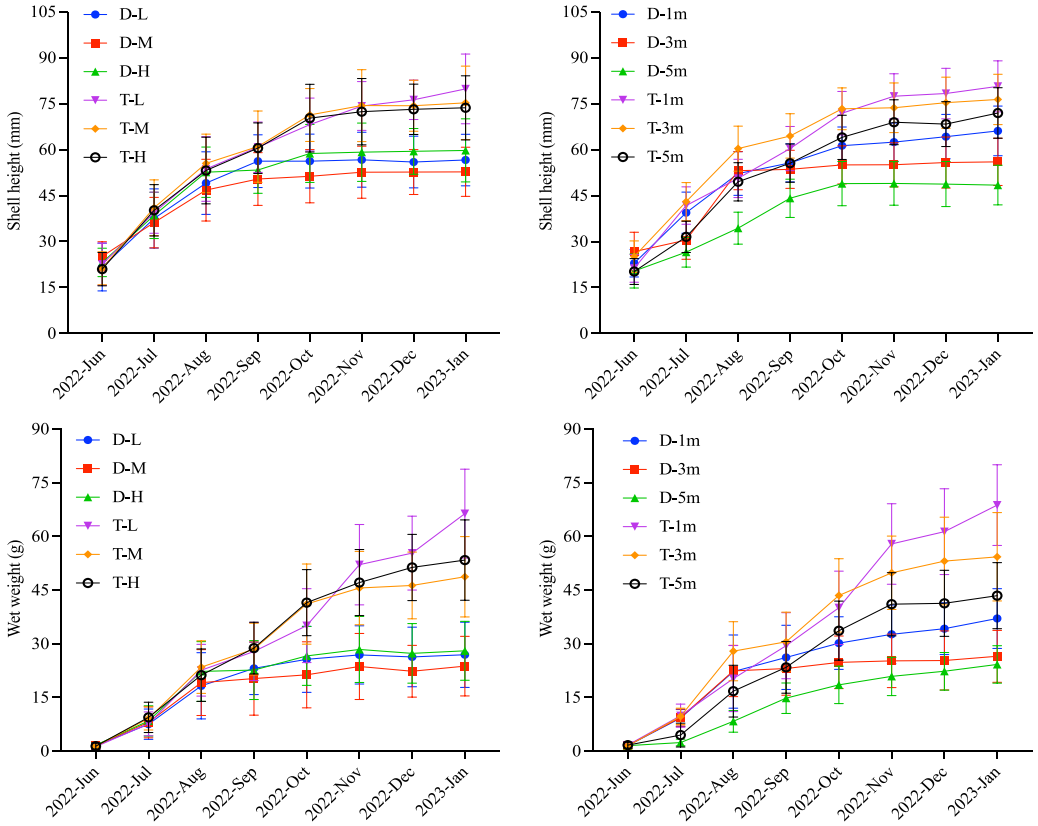

Fig. 4. Variation of shell height and wet weight of diploid (D) and triploid (T) oysters at different densities and water depths during the pre-harvest phase.

(shell height: 0.17 ± 0.01 mm/d, weight: 0.13 ± 0.01 g/d) were significantly higher than those cultured under low ($P = 0.015$) and medium-density ($P < 0.001$) and were significantly higher when diploids cultured at 1 m (shell height: 0.21 ± 0.01 mm/d, weight: 0.17 ± 0.01 g/d) than those cultured at 3 m and 5 m ($P < 0.001$). In triploid *C. angulata*, low-density and 1 m cultivations showed significantly higher growth rates (shell height, weight) than medium/high-density and 3 m/5 m cultivations, respectively ($P < 0.05$). In all treatment groups, triploids had significantly higher growth rates compared to diploids ($P < 0.05$).

The variations in total oyster yield of diploid and triploid *C. angulata* under different stocking densities and water depths are summarized in Fig. 6. Variance analysis revealed that both stocking density and water depth had significant effects on total yield of *C. angulata* with significant interactions (diploids: $F = 5.503$, $P = 0.007$; triploids: $F = 3.161$, $P = 0.048$). The total oyster yield at 5 m culture exhibited a significant decrease compared to that of 1 m and 3 m for both diploid and triploid oysters ($P < 0.001$). Moreover, with the exception of 1 m water depth, the total yields of triploid *C. angulata* at all density and depth treatments were significantly higher than those of diploid oysters ($P < 0.05$).

3.2. Trial 2: Effects of stocking density and water depth on survival and growth of diploid and triploid C. angulata at the post-harvest phase

During the post-harvest phase, the survival rate of *C. angulata* was less affected by stocking density (diploids: $F = 0.455$, $P = 0.644$;

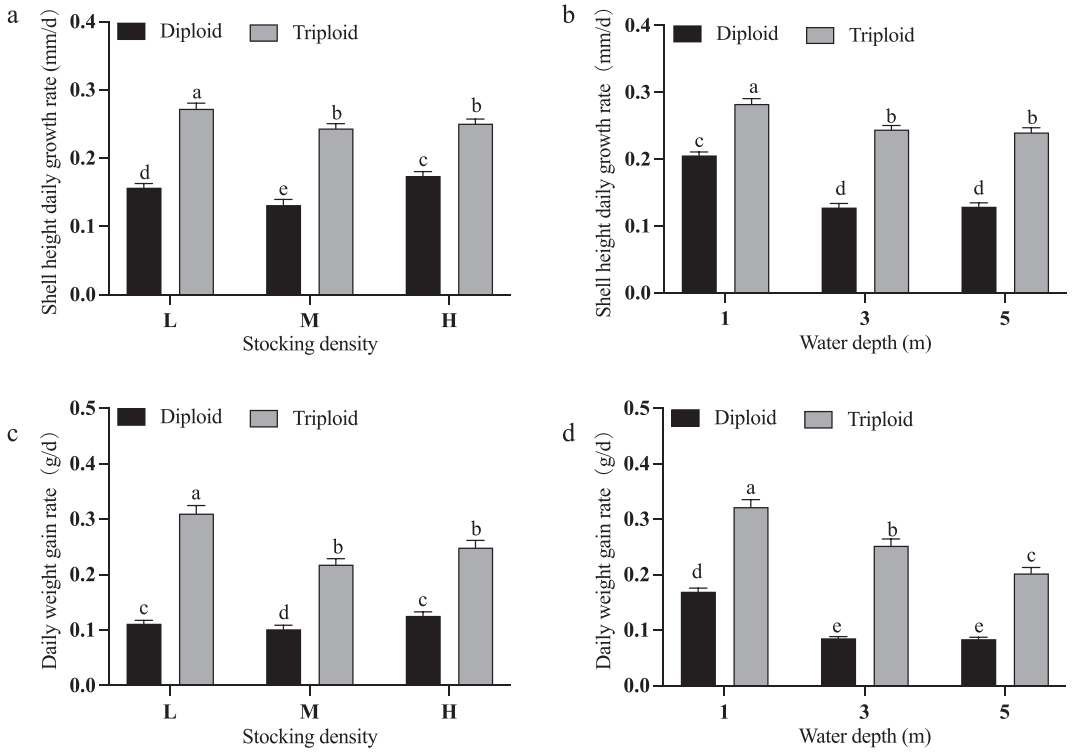

Fig. 5. Effects of stocking density (a, c) and water depth (b, d) on growth of diploid and triploid *C. angulata* at the pre-harvest phase. The presence of distinct letters indicates statistically significant differences ($P < 0.05$).

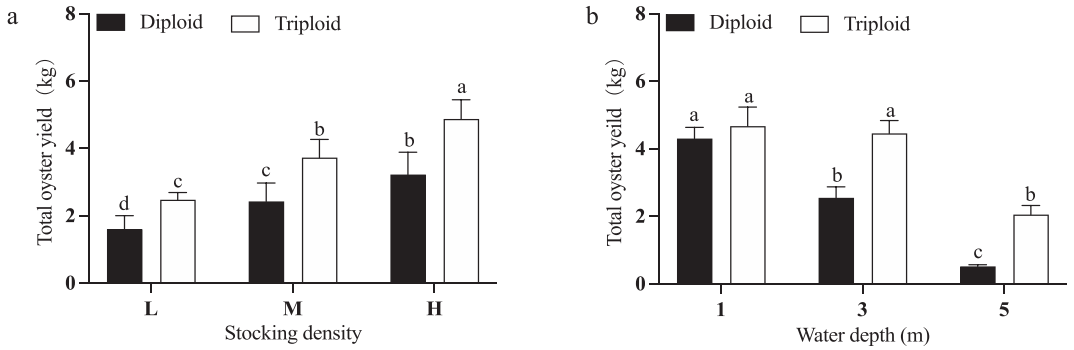

Fig. 6. Effects of stocking density (a) and water depth (b) on total oyster yield of diploid and triploid *C. angulata* at the pre-harvest phase. The presence of distinct letters indicates statistically significant differences ($P < 0.05$).

triploids: $F = 1.136$, $P = 0.351$) and water depth (diploids: $F = 0.981$, $P = 0.401$; triploids: $F = 0.385$, $P = 0.688$) and without a significant difference between diploid (87.40 ± 6.58%) and triploid (86.24 ± 7.06%) groups ($P = 0.564$).

On the other hand, although the shell height of oysters varied gently, there was a clear and continuous trend of increasing wet weight, which was evident in triploids (Fig. 7). In post-harvest phase, the shell height daily growth rate of triploid oysters under low-density cultivation was significantly lower than those of triploids cultured at medium ($P = 0.031$) and high density ($P = 0.039$), in contrast, in high-density culture, diploid oysters had a lower daily growth rate of shell height ($P < 0.001$) (Fig. 8). The diploid oysters cultured at 3 m had significantly higher shell height daily growth rate than those cultured at 1 m ($P = 0.035$) and 5 m ($P = 0.012$), while there was no significant difference in triploids at different water depths. For daily weight gain rate, both diploids and triploids were not significantly affected by stocking density but showed a significant effect by water depth, in which oysters cultured at 5 m were significantly lower than those cultured at 1 m (diploids: $P < 0.001$; triploids: $P = 0.005$) and 3 m (diploids: $P < 0.001$; triploids: $P = 0.033$).

3.3. Influence of environmental conditions on survival and growth of *C. angulata*

The temperature varied between the lowest point of approximately 15.47 °C in January/February and the highest point of 27.65 °C in September throughout the experimental period, however, dissolved oxygen exhibited an inverse pattern, reaching its peak in winter (7.20 mg/L) and declining to a minimum in summer (3.71 mg/L) (Fig. 9). Kruskal-Wallis test showed no significant differences in temperature and dissolved oxygen at different water depths (Temp: $P = 0.639$; DO: $P = 0.612$). However, strong fluctuations in both indicators were observed during the summer period (June–August), with Temp and DO in 5 m water depth significantly lower than 1 m water depth ($P < 0.05$) (Fig. 9).

During the pre-harvest phase, a significantly inverse correlation

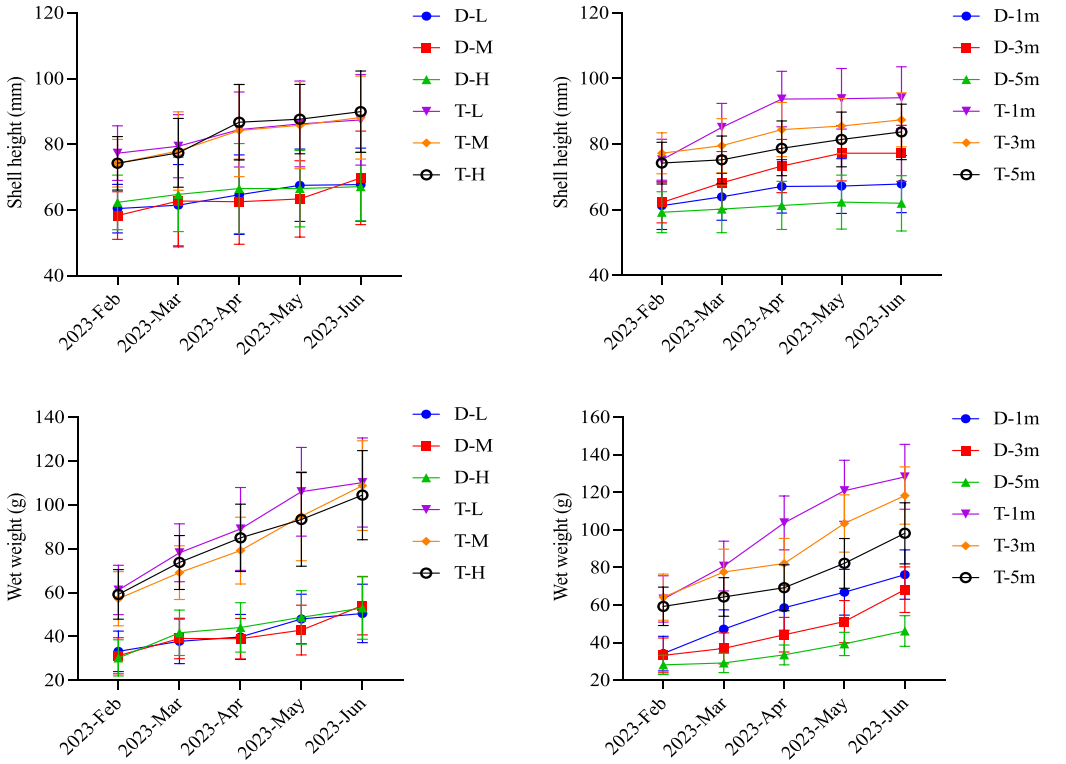

Fig. 7. Variation of shell height and wet weight of diploid (D) and triploid (T) oysters at different densities and water depths during the post-harvest phase.

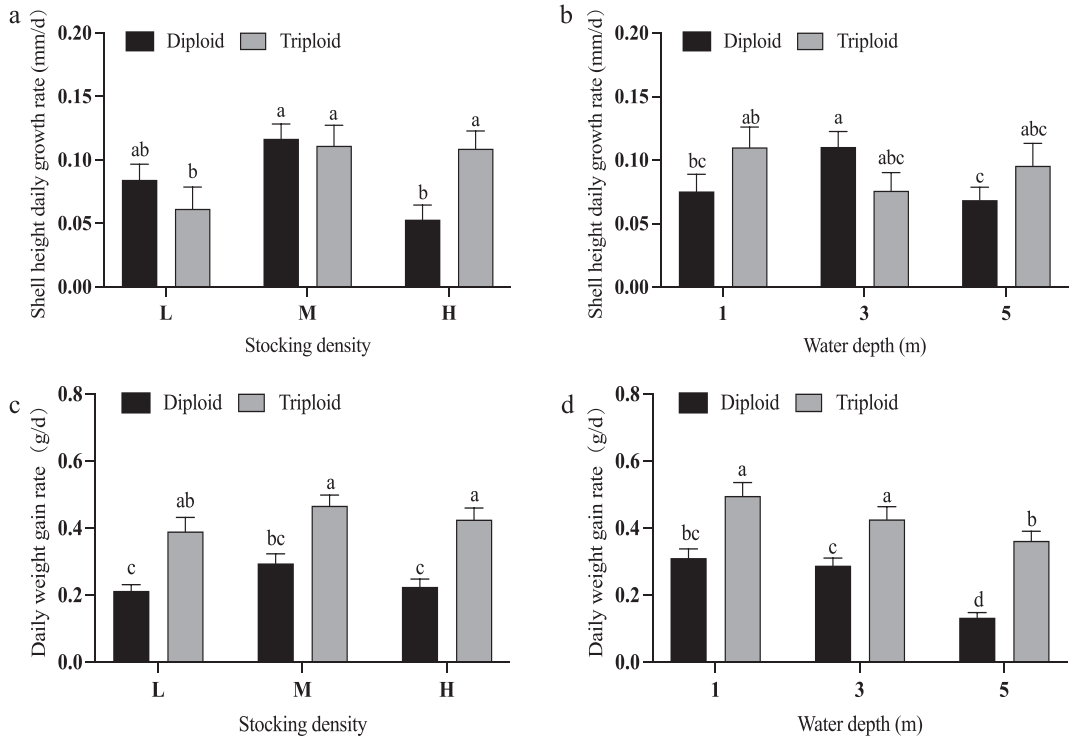

Fig. 8. Effects of stocking density (a, c) and water depth (b, d) on growth of diploid and triploid *C. angulata* at the post-harvest phase. The presence of distinct letters indicates statistically significant differences ($P < 0.05$).

between the survival rate and the water temperature was observed ($r = -0.373$, $P = 0.042$), whereas the relationship with dissolved oxygen was not significant ($r = 0.316$, $P = 0.089$). The growth rate of oysters was positively correlated with temperature (shell height: $r = 0.584$, $P < 0.001$; weight: $r = 0.414$, $P = 0.012$) and negatively correlated with dissolved oxygen (shell height: $r = -0.585$, $P < 0.001$; weight: $r =$

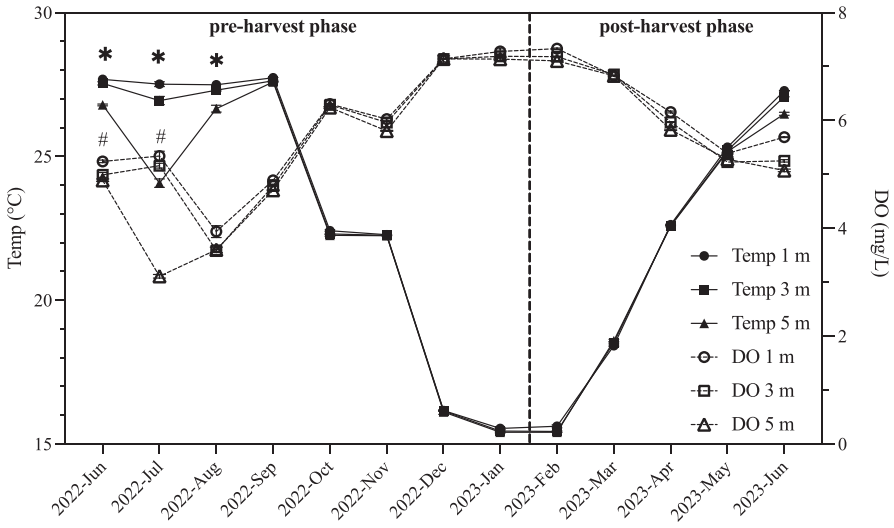

Fig. 9. Evolution of temperature (Temp) and dissolved oxygen (DO) over the experimental period at 1 m, 3 m and 5 m water depth. Note: * and # indicate significant differences between water depths.

-0.361, $P = 0.031$).

During the post-harvest phase, the survival rate was negatively correlated with water temperature ($r = -0.643$, $P = 0.001$) and insignificantly positively correlated with dissolved oxygen ($r = 0.400$, $P = 0.053$). For growth, oyster shell height growth rate was significantly negatively correlated with water temperature ($r = -0.591$, $P = 0.002$) and positively correlated with dissolved oxygen ($r = 0.449$, $P = 0.028$), while the rate of weight gain was not significantly related to these two environmental factors.

4. Discussion

4.1. Effects of stocking density on survival, growth and total yield of C. angulata at the pre-harvest phase

This study showed that stocking density strongly affected the growth of *C. angulata* during the pre-harvest phase, whereas had a lesser effect on their survival. Similar results were observed in the study of Tan et al. (2021), in which diploid and triploid *C. angulata* cultured at different stocking densities showed survival rates had no significant difference. Recently, Tan et al. (2023) conducted a trial to evaluate the culture performance of a new *C. angulata* variety at various stocking densities, which indicated that *C. angulata* survival rate was similar for 4 substrates/rope versus 7 substrates/rope culture (20–30 ind/substrate). However, different results were recorded in many other bivalve species, such as *Pinctada maxima* (Taylor et al., 1997), *C. gigas* (Tan et al., 2021), *Chlamys nobilis* (Liu et al., 2019) and *Ostrea edulis* (Zorita et al., 2021), all of which exhibited higher survival rates cultured at low density than those cultured at high density. Many scholars have attributed this phenomenon to density-dependent mortality due to intraspecific competition in bivalves (Stillman et al., 2000; Cubillo et al., 2012). In fact, however, changes in natural environmental conditions are also potential factors that should be taken into account to influence bivalve survival (Chávez-Villalba et al., 2010; Tan and Zheng, 2019; Cowan et al., 2023). For example, Chávez-Villalba et al. (2010) found that high stocking densities and seasonal high temperatures were both important causes of high mortality in *C. gigas*. We observed in our experiments that a mass mortality phenomenon of *C. angulata* during the summer coincided with sudden changes in temperature, which may indicate that dramatic changes in sudden temperature interfered with the effects of stocking density on oyster survival and that high temperatures led to mass mortality of oyster spats during the nursery phase, allowing for some degree of dilution of population densities. However, we cannot rule out the possibility that the density setting not yet reaching the threshold that would significantly affect the survival of *C. angulata*. Therefore, in future studies, it is strongly suggested to increase the stocking density of the experiment and consider the combined effects of environmental factors, especially some sudden environmental perturbations.

The variations in growth rates and total oyster yield at the pre-harvest phase reflected the culture advantage of triploid *C. angulata*. The results that triploid *C. angulata* at low culture densities grew better than medium and high culture densities are not surprising. This has been similarly found in many reports (Monteforte et al., 2005; Carlucci et al., 2010; Chávez-Villalba et al., 2010; Rinju et al., 2020). For example, Carlucci et al. (2010) found that *O. edulis* grew slower at high-density cultivation (90 ind/reply) compared to low-density cultivation (45 ind/reply) in the Taranto Sea, Mediterranean Sea. Rinju et al. (2020) found that low stocking density (400 nos. Per cage) of *P. imbricata fucata* facilitated resource optimization of 38–39 mm juveniles to 50–51 mm subadults. However, diploid oysters exhibited intriguing phenomena in this study (faster growth at high stocking density). This may be related to energy partitioning. High stocking density can cause oysters to partition energy into increasing shell weight and thickness rather than the growth of body tissues due to chronically inadequate food supply (Wilson, 1987). Therefore, it is essential to evaluate the parameter of the condition index of bivalves in future studies. Moreover, the relationship between water flow rate and bivalve filtration rate may explain this phenomenon to some extent (Wildish et al., 1992; Tan et al., 2023). Hydrodynamic patterns largely determine the amount of locally available food (Dame and Prins, 1997), while the impact of bivalve culture on local hydrodynamics depends on culture structures and spatial characteristics in this place (Cranford et al., 2014). Aquaculture on suspended structures especially at high stocking densities is a special case of aggregation, where the frictional effect of the body drag induced by bivalves themselves to water velocity affects food supply rates (Duarte et al., 2008). For instance, Cranford et al. (2014) developed a raft-scale food depletion model to explain the effect of mussel raft-induced reduction in current speed on food acquisition by cultured bivalves. The results revealed that chlorophyll-a depletion was inversely related to the current speed. Consequently, the aggregation of diploid *C. angulata* at high stocking density reduced localized water velocity, allowing them to more fully filter feed on particulate matter in the water column, thus enhancing growth.

4.2. Effects of water depth on survival, growth and total yield of C. angulata at the pre-harvest phase

In the present study, water depth had a strong effect on survival, growth and total yield of *C. angulata*. These results we observed are compatible with triploid superiority, which has been confirmed in *C. virginica* (Walton et al., 2013) and *C. gigas* (Nell and Perkins, 2005; Qin et al., 2022). The optimal water depth for *C. angulata* culture at the pre-harvest phase is 1 m, which is consistent with the results of previous studies (Thouzeau, 1991; Avendaño et al., 2008; Bao et al., 2012; Freites et al., 2020). For instance, according to Avendaño et al. (2008), suspension culture of *Argopecten purpuratus* at 1 m depth resulted in higher survival rates (79.2%–99.7%) and growth rates (1.7 times) compared to those cultured at 16 m depth (40.3%–78.6%). Freites et al. (2020) found that *Pteria colymbus* cultured at 2 m and 6 m had significantly higher survival rates and growth rates than those cultured at 10 m. The authors agreed that the main reason for these phenomena was that food availability was higher in surface waters than in deep waters. Bivalves, as poikilotherms, are highly susceptible to environmental fluctuations (Yukihira et al., 2006). The variation of environmental parameters in different water layers is very complex, the species and number of plankton, and the number of attached organisms are different, and the difference in water temperature variation and its change not only affects the biological bait but also the metabolism of the organisms, which may be an important reason that causes the differences in the growth and survival of shellfish (Bao et al., 2012). In temperate seas, bivalve species have shown links between temperature, food supply (from phytoplankton sources and/or seston), and growth rates (Freites et al., 2017). Temperature, food availability, and turbidity conditions have been reported to tend to present suboptimal vertical gradients below the critical depth, resulting in reduced bivalve growth (Fréchette and Daigle, 2002; Tomaru et al., 2002). It is noteworthy that the total yield of diploid *C. angulata* cultured at 5 m coincided with the lower survival rate they had at that water depth. Based on our results, it is reasonable to assume that this may be caused by a cascading effect of high temperatures and low dissolved oxygen. In addition to the dramatic perturbations in temperature, we observed an abrupt drop in dissolved oxygen levels in the summer water column that accompanied a sudden mass mortality event in diploid oysters. The effects of predation on survival rate of oysters were not assessed in this study, but aquaculture expansion into deeper waters should also take into account these types of risks that may compromise farm yields (Avendaño et al., 2008; Filgueira et al., 2018).

4.3. Effects of stocking density on survival and growth of C. angulata at the post-harvest phase

The results of this study showed the effect of stocking density on survival rate of *C. angulata* during the post-harvest phase was not significant. Other studies have also reported similar results (Carlucci et al., 2010; Chávez-Villalba et al., 2010), with Carlucci et al. (2010) suggesting that natural variability was the main reason for the lack of significant differences in survival and mortality of *O. edulis* at different densities in a culture period more than one year. In terms of growth, the growth rates of *C. angulata* during the post-harvest period showed an opposite variation pattern to that of the pre-harvest period. Compared to the pre-harvest phase, growth rates (shell height and weight) of diploid *C. angulata* cultured at high densities changed from highest to below or equal to the other treatments. Intraspecific competition induced by food and spatial constraints imposed by high population densities is a good explanation for this phenomenon (Gascoigne et al., 2005; Liu et al., 2019). In bivalves, crowding conditions negatively affect growth due to spatial constraints, especially as individuals reach larger sizes (Cubillo et al., 2012), leading to shell deformation (Bertness and Grosholz, 1985). In addition, mechanical interference between adjacent neighbors may affect valve openings and thus limit removal rates, resulting in reduced baiting and reduced bivalve growth (Jørgensen et al., 1988). In San José Gulf, Argentina, Orensanz (1986) found a negative correlation between individual size and population density of *C. tehuelcha*. A similar situation was observed in triploid *C. angulata* cultured at low density in this study, where the daily growth rate of shell height at this density shifted in comparison to the pre-harvest variability. It is possible that triploids cultured at this density were the first to approach or reach a growth bottleneck due to their high growth rates at the pre-harvest phase. This is because we found that triploid oysters showed a decrease (reduced by ~54% to 78%) in daily growth rates of shell height during the post-harvest period compared to pre-harvest, with levels similar to those of diploid oysters, even though they still maintained high daily weight gain rates. As mentioned by Cubillo et al. (2012), after 4–7 months of cultivation the shell lengths of the mussel *Mytilus galloprovincialis* reached 40–50 mm, and the growth slowed down. Recently, Qin et al. (2023) reported that culture performance deficiencies exhibited by triploid *C. angulata* in the late stages of growth (slow growth, high summer mortality, fragile and thin shells), may result in some losses during the farming process. It is important to emphasize that we conducted a study of the effects of sustained density pressure on oysters. The operation of replacing dead oysters to ensure a constant stocking density is common in previous studies (Holliday et al., 1991; Underwood et al., 2002; Monteforte et al., 2005; Chávez-Villalba et al., 2010; Freites et al., 2020; Tan et al., 2021), although there are still some uncertainties that may interfere with oyster growth to some extent. In the future, we need to combine culture experiments with different standards to better observe and explain variability in shellfish aquaculture performance.

4.4. Effects of water depth on survival and growth of C. angulata at the post-harvest phase

The mortality of *C. angulata* under each water depth treatment during the post-harvest phase was low and the survival rate stabilized, while the effects of water depth on growth continued from the pre-harvest to the post-harvest period. Overall, 5 m is still a sub-optimal culture depth, while 1–3 m below the water surface is the ideal culture depth for *C. angulata* during the post-harvest phase, similar to the results for the pre-harvest phase. It is important to note that the high culture efficiency at 3 m water depth was reflected in the growth performance of oysters in the post-harvest stage. Filgueira et al. (2018) utilized the theory of dynamic energy budget to merge a 1-D vertical resuspension model and a mussel bioenergetic model to determine that a depth of 3 m below the water surface is the optimal culture depth for mussels. It is well known that the effects of environmental factors on species depend on individual size and life stage (Kinne, 1970; Jansson et al., 2015). Abiotic factors such as dissolved oxygen, pH, temperature and salinity have been reported to have different effects on larval and adult bivalves (Jansson et al., 2015; Yuan et al., 2016). This extension of the growth range of the vertical gradient reflects the increased adaptability of bivalves cultured in the same water layer to environmental variables with age.

5. Conclusions

In a nutshell, both stocking density and water depth had important effects on growth of Portuguese oysters *C. angulata* and showed different patterns of variations in two consecutive culture phases, while the survival rate of oysters was more affected by water depth compared to stocking density. The suggested stocking density and depth for triploid oysters during the pre-harvest phase are 5 substrates/rope and 1 m, respectively, because this culture practice has the highest survival and growth rates, and diploid oysters are recommended to be cultured according to 15 substrates/rope and a depth of 1 m. For the post-harvest stage, some adjustments to the culture are recommended, with low to medium stocking densities (50 or 100 ind/cage) for diploids and medium to high stocking densities (100 or 150 ind/cage) for triploids, with water depths of 1 to 3 m clearly optimal. This work contributes to the

optimization of existing oyster culture techniques and clarifies the aquaculture performance of triploid *C. angulata*, with a view to providing a reference and basic information for healthy culture and production management in *C. angulata*.

CRediT authorship contribution statement

Yizhou Sun: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Chuanxu Zhang:** Investigation, Data curation. **Faxin Liu:** Investigation, Visualization. **Hongkuan Zhang:** Methodology. **Hong Du:** Resources. **Yuehuan Zhang:** Resources. **Huaiping Zheng:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

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.

Data availability

No data was used for the research described in the article.

Acknowledgments

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ARTICLES FOR FACULTY MEMBERS

PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

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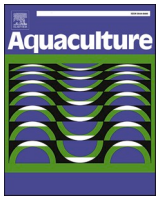

Growth, survival and gonad development of two new types of reciprocal triploid hybrids between *Crassostrea gigas* and *C. angulata*

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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

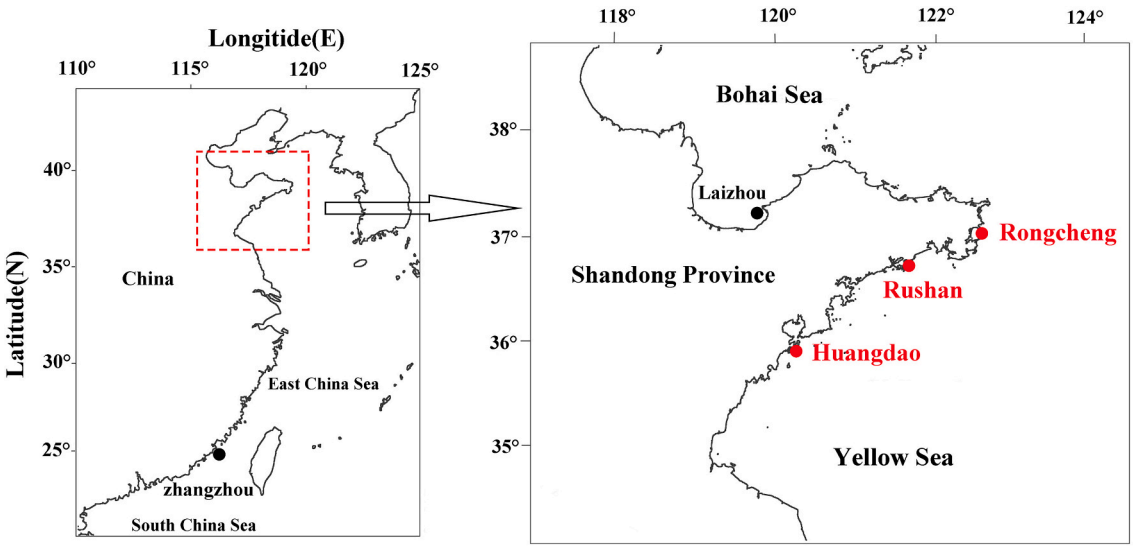

Fig. 1. Location of the broodstocks origin and oyster farming environments in China.

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 eyespots, 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 × 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).

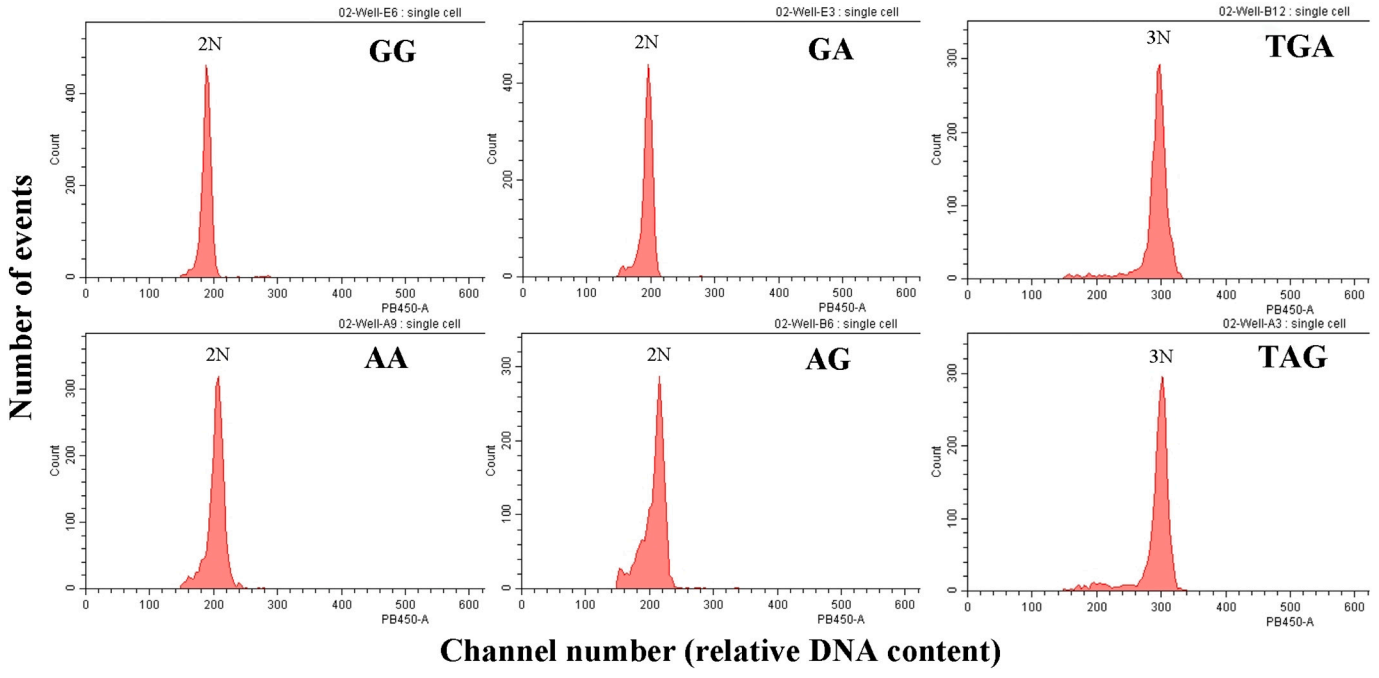

Fig. 2. Relative DNA content analysis for two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) at the adult stage.

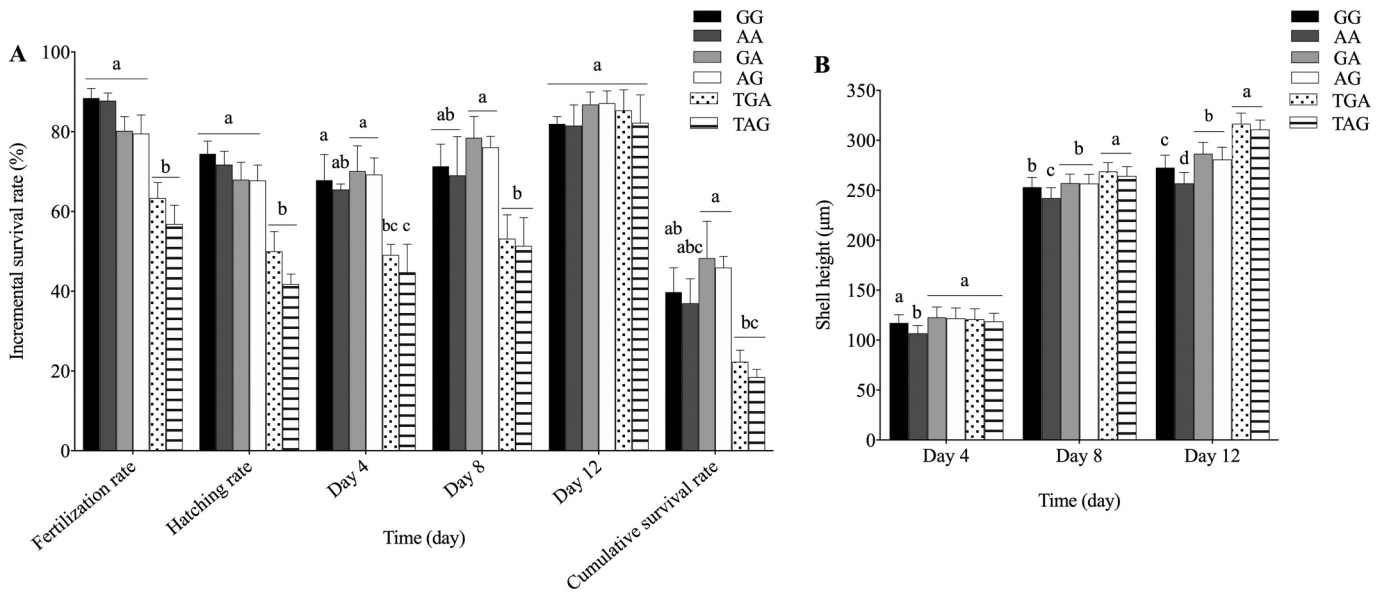

Fig. 3. Fertilization rate, hatching rate, larval survival rate and shell height for two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) in the planktonic larval stage. Different superscript letters at the same time indicate significant difference ($P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

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 ± 505.52 g), Rushan (14,256.66 ± 209.16 g) and Huangdao (12,140.13 ± 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

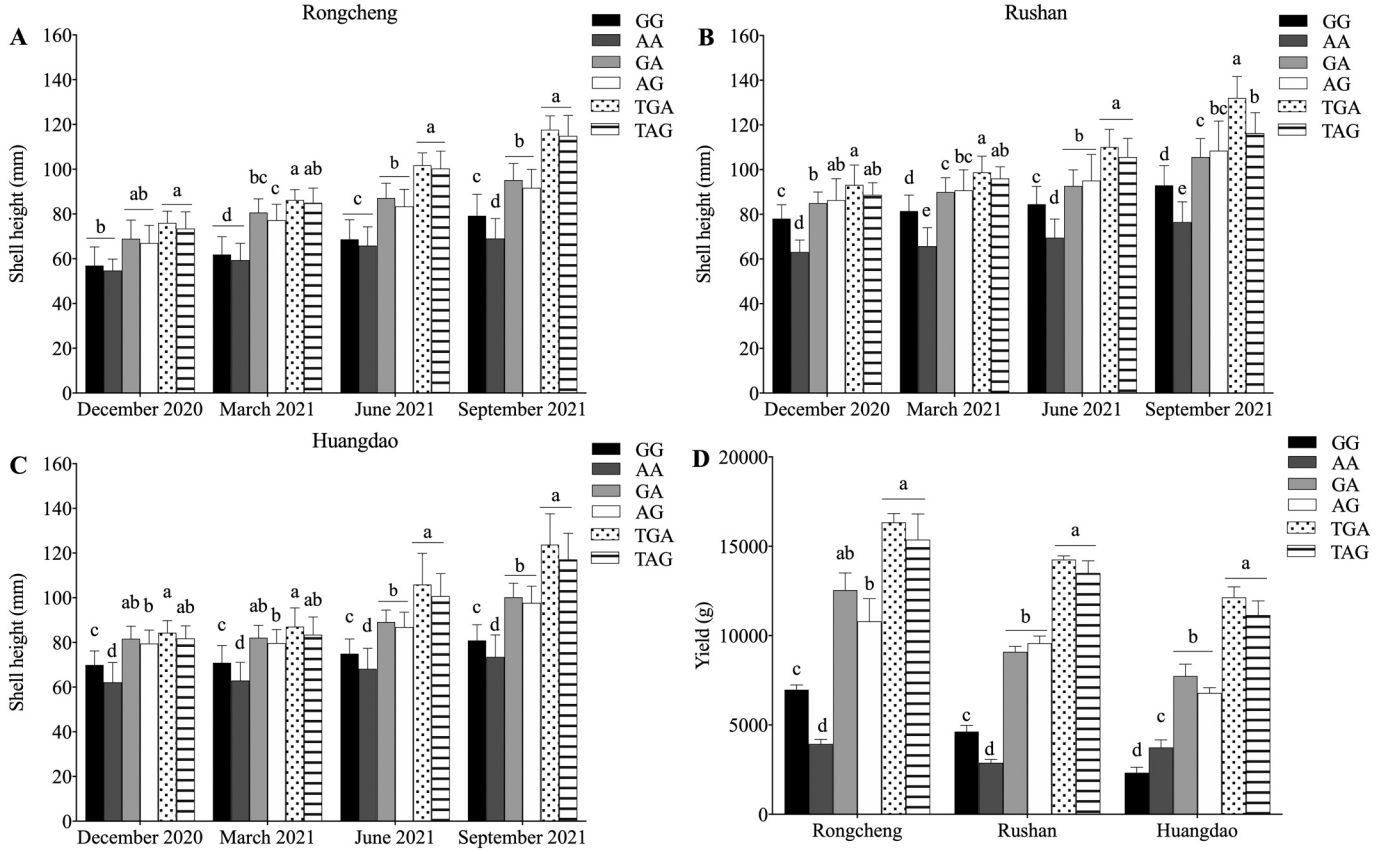

Fig. 4. Shell height and yield for two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) at Rongcheng, Rushan and Huangdao from December 2020 to September 2021. Different superscript letters at the same time indicate significant difference ($P < 0.05$).

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

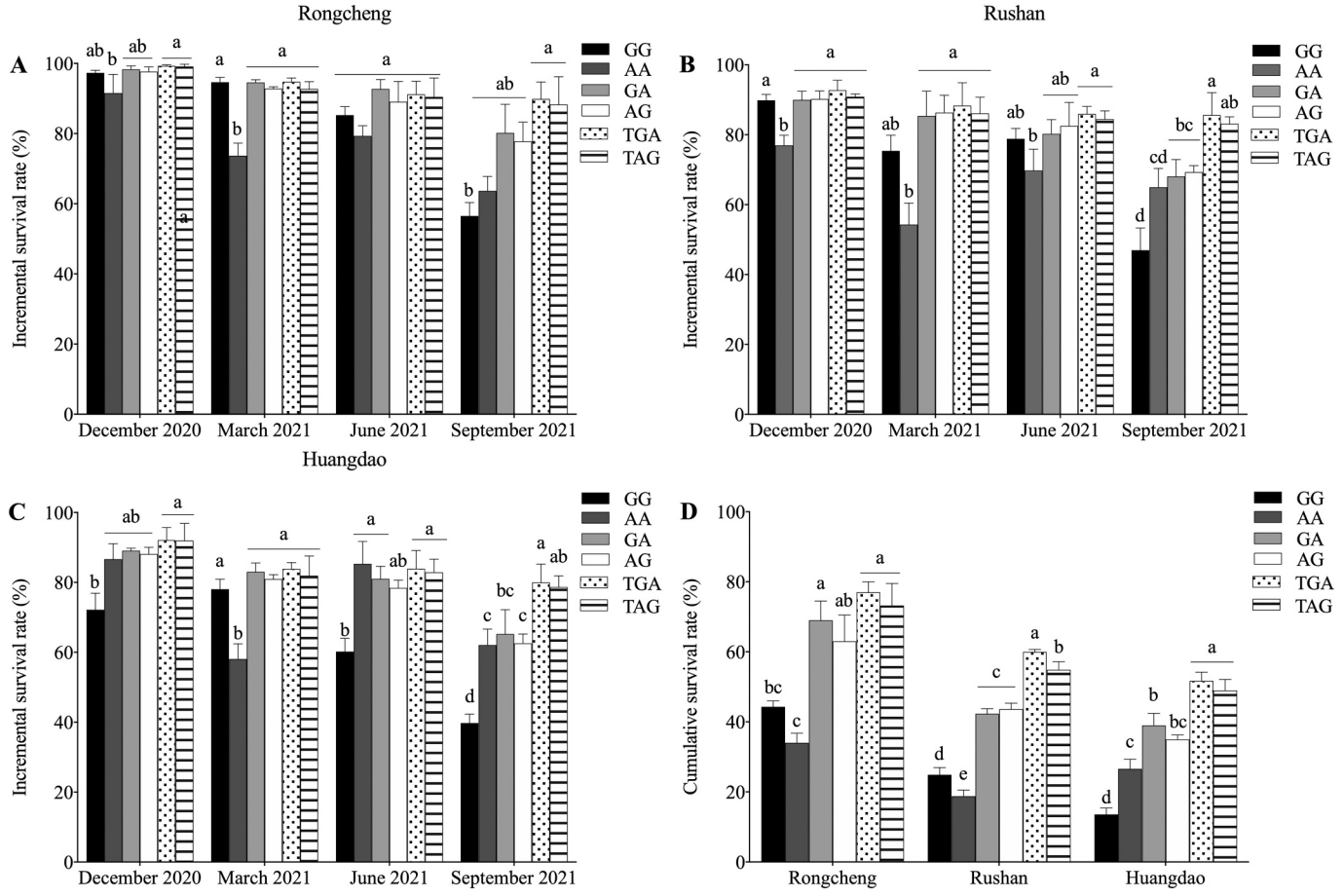

Fig. 5. Incremental survival rate and cumulative survival rate for two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) at Rongcheng, Rushan and Huangdao from December 2020 to September 2021. Different superscript letters at the same time indicate significant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

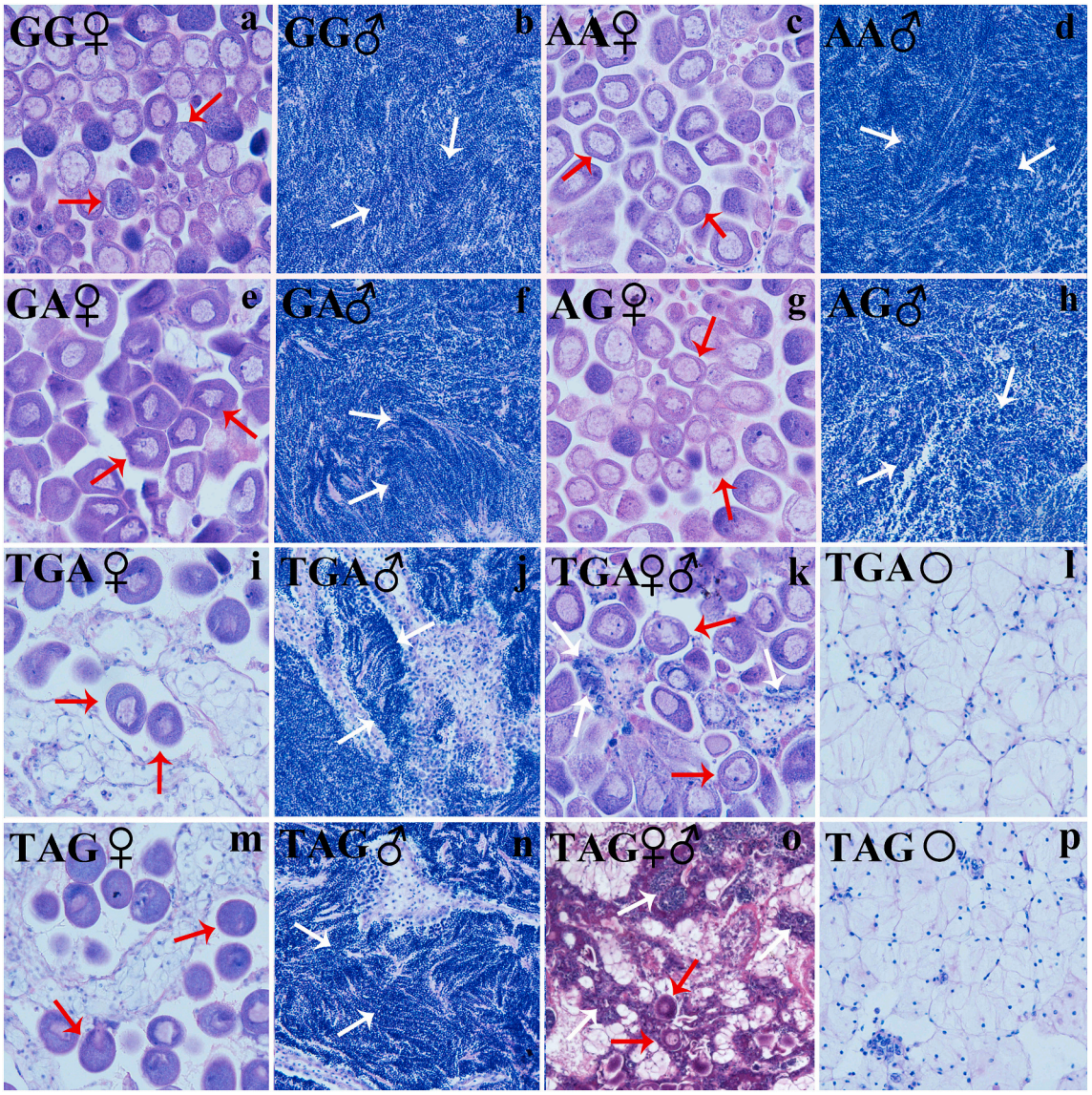

Fig. 6. The gonadal structure of two purebred crosses (GG and AA) and their diploid and triploid hybrids crosses (GA, AG, TGA and TAG) under 40× objective lens (female: ♀; male: ♂; hermaphrodite: ♀♂; asexuality: O). Note: red arrows and white arrows indicate vitellogenic oocytes and spermatozoa, respectively. (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 allotriploid 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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ARTICLES FOR FACULTY MEMBERS

PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

Title/Author	**Investigating the molecular mechanism of sterility in female triploid Pacific oyster (Crassostrea gigas) / Zhang, E., Li, Z., Li, B., Fu, J., Feng, Y., Sun, G., Xu, X., Cui, C., Wang, W., & Yang, J.**
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Investigating the molecular mechanism of sterility in female triploid Pacific oyster (*Crassostrea gigas*)

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ABSTRACT

The emergence of triploid oysters has freed the oyster market from the problem of interruptions in the breeding season, allowing for year-round availability. However, according to recent statistics, the phenomenon of fertility in triploid oysters has been increasing yearly, perhaps eventually leading to the loss of the sterility characteristic. This has in turn affected the oyster farming industry. Therefore, we attempted to mine key genes through transcriptomics to find the causes of triploid fertility. During our surveys and sampling, we found that most of the fertile triploid *Crassostrea gigas* were females, with a much smaller percentage of males. Therefore, the main objective of this study was to analyze the differences between fertile and sterile triploid female *C. gigas*. We performed transcriptome analysis of fertile female triploid *C. gigas* and sterile female triploid *C. gigas* and identified 2361 up-regulated differentially expressed genes (DEGs) and 1541 down-regulated DEGs, respectively. Enrichment analysis of these DEGs revealed that most GO terms and KEGG pathways were associated with mitosis. We then created a protein–protein interaction (PPI) network using genes enriched in mitosis-associated KEGG pathways. Finally, we screened and validated 26 key genes by quantitative RT-PCR and all were found to be downregulated in expression in sterile female triploids. The main cause of sterility is the suppression of the expression of genes that control the process of germ cell proliferation, the failure of the mitotic cycle to proceed smoothly, and the inability to produce an oogonium. These results can be used to explore ways to reduce the fertility of triploid *C. gigas* and to promote the breeding of higher quality triploid oysters.

1. Introduction

Polyploidy is considered to be a common mode of species formation, and it has had a profound influence on the evolution of species (Van de Peer et al., 2017). Polyploid plants are more common than animals due to differences in reproductive biology (Rodriguez and Arkhipova, 2018; Spoelhof et al., 2020). The development of polyploid induction techniques has led to the polyploidization of many aquatic organisms, including shrimps (Manan and Ikhwanuddin, 2021), grouper (Zhang. et al., 2022b), and scallops (Ma et al., 2018). Since the publication of the first research study on triploid oysters (Stanley et al., 1984), their emergence has benefited the aquaculture industry due to the triploids' rapid growth (Wadsworth et al., 2019; Walton et al., 2013), high quality meat (Garnier-Géré et al., 2002), and increase in output value (Li and Li, 2022). After decades of research, polyploidy technology has matured, with triploid oysters gradually increasing their commercial value. According to recent market statistics, the annual production of triploid *C. gigas* has increased significantly to satisfy growing consumer demand (Matt and Allen, 2021; Nell, 2002).

Sterility characteristics are important for oyster farming because most of the energy in diploid oysters is used for gonad production (Chen et al., 2021). The discharge of germ cells during the breeding season results in the oysters becoming thin, manifested as lesser quality flesh and meat texture, thus leading to an off-season or break in the market supply (Kang et al., 2013). Triploid oysters, due to their sterility and low fertility, consume little energy for gonad development during the breeding season, thereby allowing more energy to be allocated to growth rather than reproduction (Allen and Downing, 1986; Bi et al., 2023; De Decker et al., 2011). Eventually, the oyster market could be free from the break in the breeding season and could achieve a

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year-round supply (Zhou et al., 2023). However, according to recent statistics, the phenomenon of fertility in triploid oysters has been increasing yearly during the breeding process, and this has affected production in the oyster farming industry.

The developmental stages of diploid oysters change periodically with the seasonal temperature, and the maturation of gonads can be accelerated by warming (Maneiro et al., 2017). An increase in fertile female triploid oysters and a decrease in sterile female triploid oysters matching the seasonal development of oyster gonads have been observed in previous studies (Yang et al., 2022). The majority of fertile triploid oysters were found to be female in previous statistics, and male triploid oysters were rare (Yang et al., 2022). This phenomenon was also found in our sampling process, and thus, we focused on the sterility of female triploid oysters in this study. In recent years, global warming has led to an increase in seawater temperatures (Jiang et al., 2016). It is unknown whether the increase in the fertility phenomenon of triploid oysters during culture is due to the influence of the external environment or due to changes in internal mechanisms.

In this research, we investigated the differences in the intrinsic mechanisms between fertile and sterile female triploid *C. gigas* through transcriptomics to identify the causes of triploid oyster sterility. The main focus was on the screening of differentially expressed genes (DEGs), Gene Ontology (GO) analysis, and functional enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Subsequently, we produced a protein–protein interaction (PPI) network based on the KEGG pathway results in order to identify key genes. Finally, we screened these gene loci to obtain 26 key genes based on KEGG pathways and the PPI results and identified those genes using quantitative RT-PCR. In summary, the results provide insight into the mechanism of triploid oyster sterility and should help to solve the industrial challenge posed by fertile triploid oysters.

2. Materials and methods

2.1. Oyster collection

Oysters were collected in August 2022 in Changhai, Dalian, located in Dalian Province, China. These oysters were approximately 2 years old and had an average weight of 90.31 g (range: 85.92–100.42 g). Triploids were produced by mating tetraploid males with diploid females. The gonadal tissue of each individual was taken, and a portion was immediately frozen in liquid nitrogen and kept at − 80 °C.

2.2. Ploidy verification

All triploid *C. gigas* were verified for ploidy prior to sampling. The detailed process was as follows. Approximately 1 mm^3 of oyster gill tissue was placed in PBS buffer and cut up as much as possible. Then, 10 μl of 100 μg/ml DAPI solution was added for nuclear staining. After staining for 20 min and filtering using a 300 mesh sieve, ploidy assays were performed using a flow cytometer (CytoFlex Beckman Coulter, USA), with diploid oysters serving as controls. Triploid oysters peaked at 1.5 times the value of diploids. Diploid and triploid ploidy diagrams are shown in Fig. S1 and Fig. S2.

2.3. RNA extraction, library construction and Illumina sequencing

In this study, triploid oysters were divided into two groups: fertile female triploids (F-3Nα-1, F-3Nα-2, F-3Nα-3) and sterile female triploids (S-3Nβ-1, S-3Nβ-2, S-3Nβ-3). Total RNA from triploid oyster gonad tissue was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. A portion of RNA from the gonads of triploid oysters was used to construct a library and the remaining RNA was used for qPCR validation. The methods and steps for building the library followed previous studies (Bao et al., 2022; Ge et al., 2018). A total of six different libraries were constructed. Finally, the constructed libraries were sequenced using the Illumina Novaseq platform, yielding 150 bp paired-end reads.

2.4. Processing of data and bioinformatics analysis

Filtering of the raw data was required to ensure the accuracy of the following analyses. Clean reads were obtained by removing reads with adapters, reads containing poly-N, and low-quality reads. Meanwhile, we calculated the Q20, Q30, and GC content to assess the quality of the clean data. FPKM was applied to remove the impact of sequencing depth and gene length on the reads to obtain gene expression levels. Differential expression analysis was performed using DESeq2 software (1.20.0), and the filtering criteria for DEGs were: $|\log_2 \text{fold change}| \geq 1$ and q-value ≤ 0.01.

DEGs matching the threshold criteria were used to perform GO and KEGG enrichment analyses using DAVID v6.8. The enrichment analysis process parameter setting was an EASE score ≤ 0.05. DEGs enriched in the mitosis-associated KEGG pathways were used to construct PPI networks via the STRING V11.5 online tool.

2.5. Quantitative real-time PCR

To assess the accuracy of transcriptome sequencing, we validated a total of 26 DEGs using qPCR. The specific primers for 26 genes related to fertility were designed using Premier 5.0 software. qPCR was performed using the method described in our previous study (Zhang et al., 2022a). We verified the stability of EF-1a, GAPDH, RS18, RO21, and RL7 expression levels in each group. Finally, RO21, the gene with the highest stability, was selected as the reference gene. The relative expression levels of the 26 genes were measured using the $2^{-\Delta\Delta CT}$ method. Primer information for the 26 genes is shown in Table S1.

3. Results

3.1. RNA-seq results and ploidy verification

RNA-seq was performed for both F-3Nα and S-3Nβ groups, with three biological replicates for each sample group. The clean reads obtained by filtering are mapped to the reference genome of *C. gigas* (Qi et al., 2021). The numbers of genes detected in the F-3Nα and S-3Nβ groups were approximately 26,000 and 28,000, respectively (FPKM > 0). All the obtained genes were compared with the SwissProt database for functional annotation. The results of RNA-seq are shown in detail in Table 1. The correlation coefficients between the transcriptome data of the three biological replicates are shown in the Fig. S3.

3.2. Analysis of DEGs

To obtain a differential transcriptome profile between sterile and fertile triploid female *C. gigas*, we compared gene expression in the two groups F-3Nα and S-3Nβ and screened a total of 3902 DEGs. Specifically, there were 2361 upregulated DEGs and 1541 downregulated DEGs in F-3Nα compared to the S-3Nβ group. Fig. 1 shows the trend of DEG expression between F-3Nα and S-3Nβ samples. The clustering heatmap (Fig. 2) illustrates the variation in expression and clustering distribution of these DEGs. From the figure, it can be seen that there is strong agreement between the experimental biological replicates of this study.

3.3. GO and KEGG functional enrichment analysis of DEGs

In this study, we selected 3902 DEGs for GO and KEGG functional enrichment analyses. The GO function enrichment analysis identified 53 level-3 biological processes, 40 level-3 cellular components, and 27 level-3 molecular function subclasses. Fig. 3 shows the top 10 level-3 GO terms for biological processes, cellular components, and molecular functions. We performed KEGG enrichment analysis to gain further

Table 1
Sequencing results.

Time points	Read length (bp)	Raw reads	Clean reads	Clean Q20 (%)	Clean Q30 (%)	GC (%)	Mapped reads ratio (%)	Detected gene number
F-3Nα-1	150	45428830	44743960	97.73	93.37	39.63	54.47	25269
F-3Nα-2	150	43556884	42648504	97.59	93.05	39.72	61.7	27384
F-3Nα-3	150	45083312	44077584	97.59	93	39.65	56.79	25282
S-3Nβ-1	150	46653698	45286020	97.27	93.32	41.1	73.52	28347
S-3Nβ-2	150	45013522	43377680	97.58	93.46	41.08	73.89	28455
S-3Nβ-3	150	45469178	44141846	97.68	93.75	41.35	74.33	28770

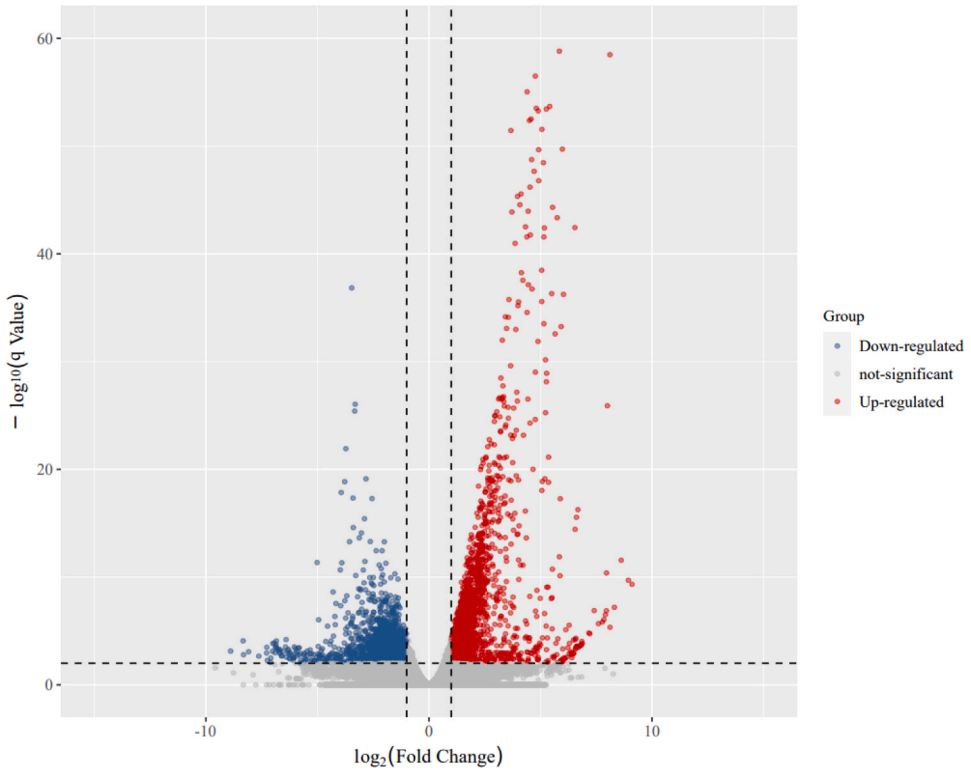

Fig. 1. Volcano plot of the distribution trend of DEGs between F-3Nα and S-3Nβ samples. Dots in this graph denote the genes. Red dots correspond to DEGs with upregulated expression; blue dots correspond to DEGs with downregulated expression, and gray dots are not DEGs.

insight into the functions of these DEGs. As shown in Fig. 4, DEGs were significantly enriched in DNA replication, Nucleotide excision repair, Metabolic pathways, Cell cycle, and other pathways. From the results of the enrichment analysis, we were particularly interested in genes that were enriched in two pathways, namely cell cycle and DNA replication. A total of 36 genes were enriched in both pathways; surprisingly, the expression levels of all 36 DEGs were suppressed in the sterile female triploid *C. gigas* (Fig. 5).

3.4. Construction of protein interaction network

Constructing the PPI network contributed to the identification of key genes affecting the fertility of triploid oysters. We constructed a PPI network using 36 DEGs enriched in the mitosis-associated signaling pathway (Fig. 6). As shown in Table 2, there were more protein interaction edges corresponding to the selected DEGs than expected. This result suggested that there were multiple complex interaction relationships between these DEGs.

3.5. Analysis of important DEGs associated with the sterility of triploid oysters

Those DEGs with many interaction relationships were likely to be key genes affecting gonadal development in triploid oysters. Hence, we screened and identified 26 DEGs based on the number of interaction relationships and the fold change of expression difference (Table 3). These 26 key genes were divided into five broad categories: Origin recognition complex, Cell division cycle, DNA polymerase, Anaphase-promoting complex and other important hub genes. As the three DEGs with the highest number of interactions, *CDC6*, *CDC20* and *PCNA* were most likely to be the key genes responsible for oyster fertility.

3.6. Quantitative RT-PCR validation

Detection of the expression levels of 26 gonadal development-related DEGs used qPCR to verify the results of the RNA-seq analysis. The results are shown in Fig. 7. The expression levels of the 26 DEGs were consistent with the transcriptome analysis, indicating that the transcriptome data were reliable. All 26 key genes were repressed in the sterile female triploid *C. gigas*.

4. Discussion

4.1. The purposes of transcriptome research

The triploid oyster offers many benefits to the modern oyster

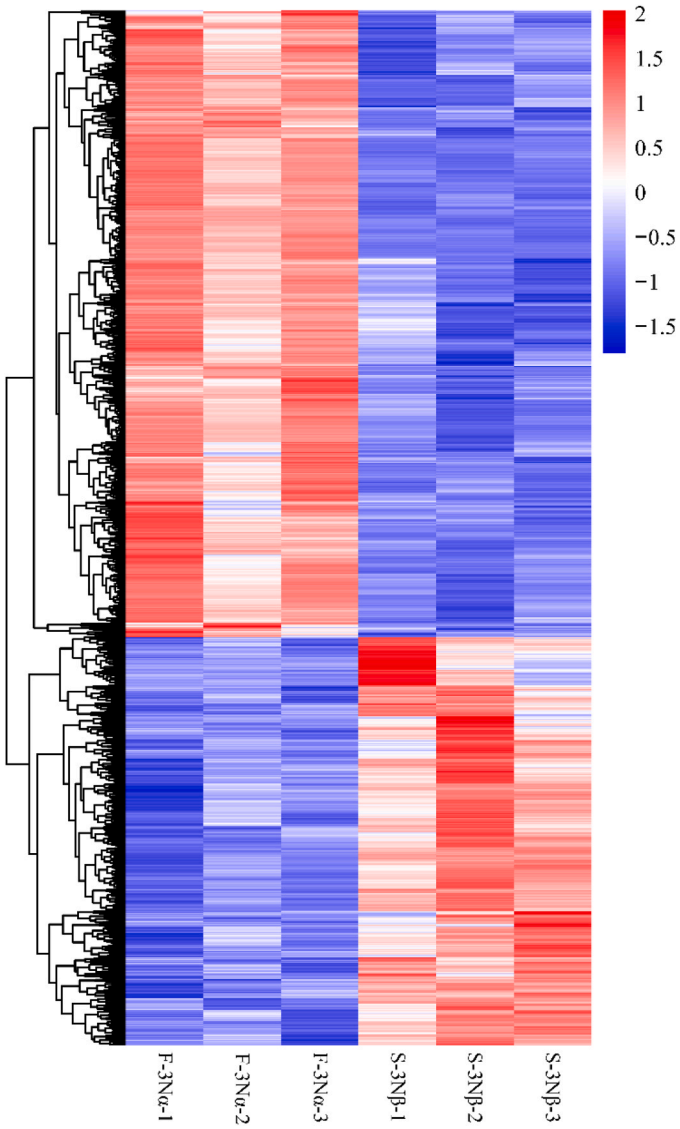

Fig. 2. Clustering analysis heatmap of all DEGs for the two sample groups. Expression levels of DEGs were normalized using the $\log_{10}$ FPKM method. Each row stands for one gene, and each column stands for a sample group. Blue to red in the figure indicated that the gene FPKM value is from low to high.

industry due to its low fertility (Li and Li, 2022; Mallia et al., 2009). Most triploid oysters are sterile, a phenomenon known as the β-pattern, and produce very few gametes at the mature stage. However, in recent years, there has been an increase in the fertility of triploid oysters, i.e., an increase in 3 N-α, during the breeding process. The majority of fertile triploid oysters were female in previous studies, and male triploid oysters were rare (Yang et al., 2022). This phenomenon was also found in our sampling process, and thus, we focused on the sterility of female triploid oysters in this study. Normally, triploid organisms are sterile because homologous chromosome pairing and synapsis are disrupted during meiosis. However, female oysters only complete meiosis in vitro (Qi et al., 2000), and thus, the reason for the sterility of triploid female oysters is that the mitotic process is blocked. In this study, we focused on the differences between the mitotic processes of fertile and sterile triploid female *C. gigas* to analyze the causes of sterility and potentially solve the industrial problem of fertile triploids.

4.2. GO terms and KEGG pathways associated with mitosis

Fig. 4 shows the top 10 (biological process, cellular component and molecular function) level-3 GO terms, many of which are related to mitosis, including as DNA replication, DNA replication initiation, DNA repair, cell cycle, and cell division. KEGG enrichment analysis identified two items associated with mitosis, namely DNA replication and Cell cycle. An in-depth study of these mitosis-related GO terms and the KEGG pathways was conducted to explore the key factors affecting fertility in triploid female *C. gigas*.

4.3. Speculation regarding hub genes

In general, proteins are the main catalysts, structural constituents, signaling molecules, and molecular mechanisms in living organisms (Liu et al., 2008). Protein interaction networks are composed of proteins that interact with each other to participate in various aspects of life processes such as biological signaling, regulation of gene expression, energy and material metabolism, and cell cycle (Bachman and Liu, 2009; Zhou et al., 2021). Those proteins that interact with multiple interactions may play an important role in the function and modular structure of the interactome. For instance, it seems reasonable to hypothesize that hub genes are essential in a certain biological regulatory process, since the consequences of their repressed expression are more influential compared to other proteins (Bertolazzi et al., 2013). Thus, systematic studies of protein interactions are useful for exploring fertility in the triploid oyster. Thus, we constructed a PPI network using 36 genes from the mitosis-related KEGG pathway. The number of interactions between the proteins encoded by our screened DEGs was greater than expected (Table 2). This result further validated the hypothesis that proteins interact to maintain germ cell production. Hence, those genes corresponding to proteins with numerous interactions were the hub genes that needed to be identified.

4.4. Functions of hub genes and KEGG pathway analysis

In this study, transcriptomic analysis of the gonads was carried out to study the molecular mechanisms responsible for the sterility of triploid oysters. From the results of the enrichment analysis, we were particularly interested in genes that were enriched in two pathways, namely cell cycle and DNA replication, as the proteins encoded by these genes had multiple interactions. Ultimately, we obtained a total of 26 hub genes based on KEGG enrichment and protein interaction analyses. Below, we discuss in detail the important hub genes related to fertility in female triploid *C. gigas*.

4.4.1. Origin recognition complex

The origin recognition complex (ORC) comprises six subunits that form a core component of eukaryotic pre-replication (Li et al., 2018; Popova et al., 2018). The ORC is required for eukaryotic cells to initiate DNA replication at the beginning of the S phase (Bielinsky et al., 2001). Other replication factors such as CDC6, Cdt1, and MCM are recruited by the ORC in the G1 phase to form pre-replication complexes (pre-RCs) that are loaded onto the DNA (Speck et al., 2005; Yuan et al., 2017). The ORC is not limited to its role in DNA replication; it has several other functions such as centrosome replication, chromosome segregation, and cytokinesis (Hossain and Stillman, 2012; Prasanth et al., 2002). In our study, three genes encoding ORC subunits were repressed in 3Nβ, namely *ORC2*, *ORC3*, and *ORC5*. The interaction between ORC2 and ORC3 forms the core of the ORC, which then recruits ORC5 and ORC4 (Dhar et al., 2001; Dhar, Suman Kumar et al., 2001; Vashee et al., 2001). The regulation of ORC2 SUMOylation at the G2/M phase is essential for the successful progression of the mitotic cycle (Popova et al., 2018). The present study indicates that the ORC has a significant impact on gonadal development in triploid oysters. The genes encoding the ORC subunit were repressed in sterile female triploids, thereby preventing normal initiation of DNA replication.

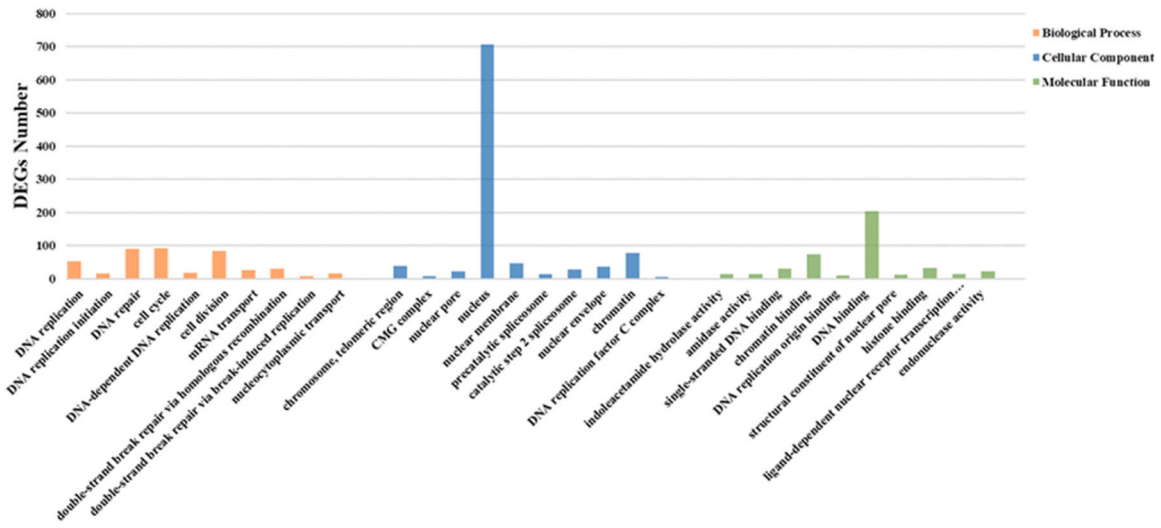

Fig. 3. GO enrichment analysis of DEGs. The horizontal coordinate stands for the enriched level-3 GO subclasses, and the vertical coordinate stands for the number of DEGs for the corresponding level-3 GO subclasses.

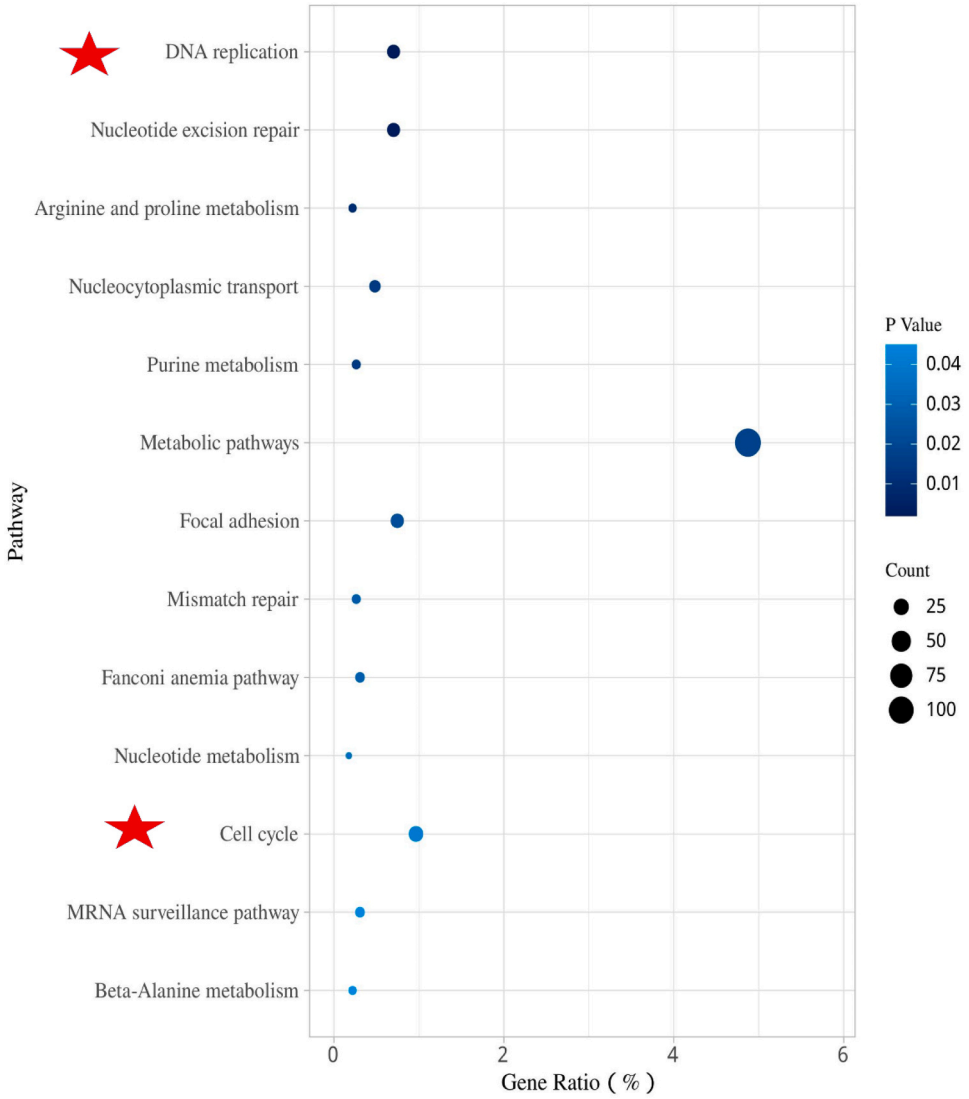

Fig. 4. KEGG enrichment analysis of DEGs in fertile and sterile triploid oysters.

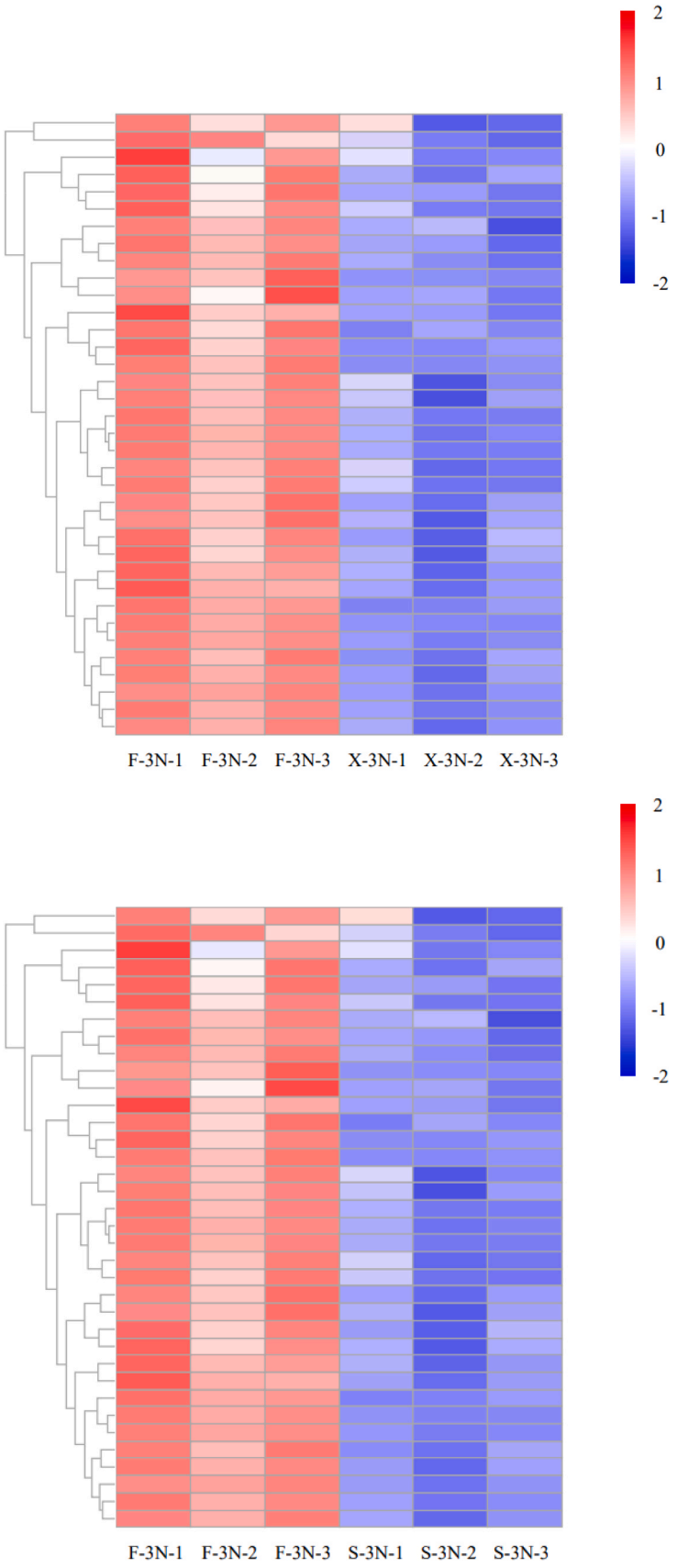

Fig. 5. Clustering analysis heatmap of 36 DEGs enriched in selected KEGG pathways.

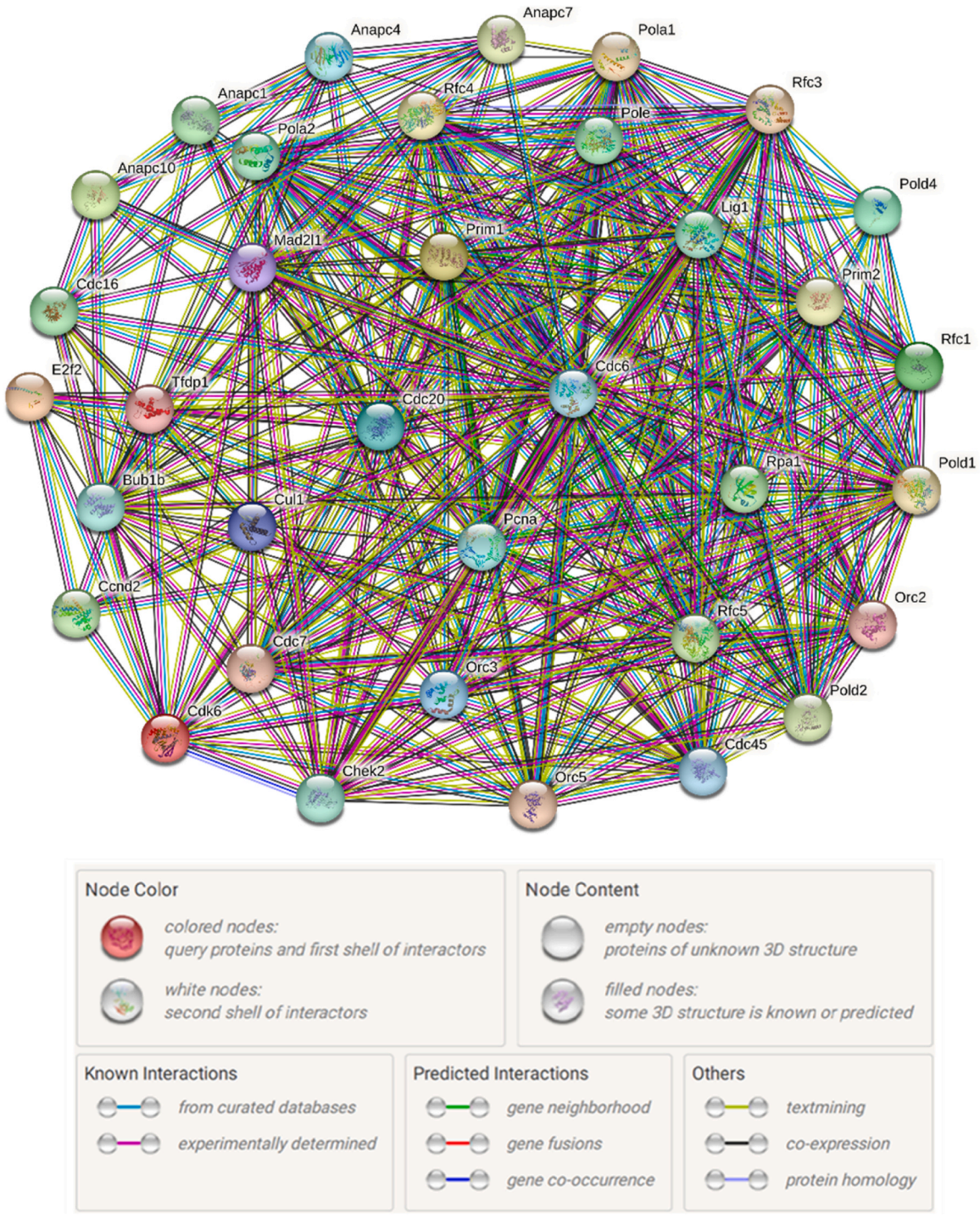

Fig. 6. Mitosis-related PPI network. The network nodes represent proteins and the degrees represent the interaction relationships between different proteins.

Table 2
Protein interaction network information.

Network Stats	
Number of nodes	35
Number of edges	352
Average node degree	20.1
Clustering coefficient	0.816
Expected number of edges	27
PPI enrichment p-value	1.0E-16

4.4.2. Cell division cycle

There were five cell division cycle proteins in our study, namely *CDC6*, *CDC7*, *CDC16*, *CDC20*, and *CDC45*, and they were all enriched in the cell cycle pathway. In this study, CDC6 and CDC20 were the top two genes in terms of the number of protein interactions, and we will focus on these two genes later. CDC7 is required for the G1/S transition in the cell cycle (Faul et al., 1999). Downregulation or inhibition of CDC7 decreases mitotic kinase activity in vivo, thereby delaying M phase progression (Ito et al., 2019). CDC16 is phosphorylated in the M phase; this is required for the anaphase-promoting complex to function as a ubiquitin ligase. CDC45 is a component of eukaryotic replicative

Table 3
Summary of 26 key DEGs.

Gene name (abbreviation)	Gene name (official full name)	Number of protein-protein interaction
CDC6	Cell division cycle 6	33
CDC20	Cell division cycle 20	29
PCNA	Proliferating cell nuclear antigen	27
POLA1	DNA polymerase alpha 1, catalytic subunit	27
CDC45	Cell division cycle 45	26
CHEK2	Checkpoint kinase 2	25
MAD2L1	Mitotic arrest deficient 2 like 1	25
POLA2	DNA polymerase alpha 2, accessory subunit	25
POLD1	DNA polymerase delta 1, catalytic subunit	25
PRIM1	DNA primase subunit 1	25
RFC5	Replication factor C subunit 5	24
CDC7	Cell division cycle 7	23
LIG1	DNA ligase 1	23
ORC5	Origin recognition complex subunit 5	23
RPA1	Replication protein A1	23
ORC2	Origin recognition complex subunit 2	21
POLD2	DNA polymerase delta 2, accessory subunit	20
CDK6	Cyclin dependent kinase 6	18
ORC3	Origin recognition complex subunit 3	18
RFC1	Replication factor C subunit 1	18
TFDP1	Transcription factor Dp-1	14
CDC16	Cell division cycle 16	13
ANAPC4	Anaphase-promoting complex subunit 4	11
ANAPC7	Anaphase-promoting complex subunit 7	10
ANAPC1	Anaphase-promoting complex subunit 1	9
ANAPC10	Anaphase-promoting complex subunit 10	8

helicase, an enzyme that ensures that chromosomal DNA is replicated only once in a cell cycle during DNA replication (Pollok et al., 2007; Yadav et al., 2020). Cell division cycle proteins act as components of many complexes in the cell cycle and promote normal cell cycling.

4.4.3. DNA polymerase

Mitosis is divided into prophase and interphase. The interphase prepares material for division, including DNA replication. DNA replication requires various enzymes to work together. The most important enzyme for achieving DNA replication is DNA polymerase that drives the synthesis of DNA strands to achieve DNA replication. In the present study, two classes of genes controlling DNA polymerase synthesis were identified as the key genes affecting sterility in triploid female *C. gigas* by KEGG and PPI analysis, namely *POLA1*, *POLA2*, *POLD1*, and *POLD2*. *POLA1* and *POLA2* are the catalytic subunits encoding DNA polymerase alpha (Polα). Polα plays an important role in DNA replication by synthesizing the RNA-DNA primers required for the replication of both DNA strands (Baranovskiy et al., 2018). *POLD1* and *POLD2* are the catalytic subunits encoding DNA polymerase delta (Polδ). Polδ is a highly conserved DNA polymerase, which performs a critical role in chromosomal DNA synthesis and DNA repair processes (Prindle and Loeb, 2012). Polδ can participate in the processing and extension of the lagging-strand using the RNA-DNA primer synthesized by Polα as a substrate (Coloma et al., 2016; Lujan et al., 2016). If cells lack Polδ, this will affect the function of the replication starting point and slow the replication of the lagging strand (Koussa and Smith, 2021). The gene controlling synthetic DNA polymerase was repressed in the 3Nβ group, suggesting that DNA replication carried out during the S phase of mitosis in the gonads of sterile female triploid oysters is hindered during development, resulting in the inability of germ cells to transition to the G2 phase.

4.4.4. Anaphase-promoting complex

The anaphase-promoting complex (APC) is an E3 ubiquitin ligase that coordinates the cell cycle process by influencing the degradation of cell cycle regulators (Alfieri et al., 2017). APC can ubiquitinate a variety of proteins, including A-type and B-type cell cyclins, to regulate the stability of cell division (Mossman et al., 2012). If there is no APC in the cell, the cell cannot separate sister chromatids during late division, meaning that two daughter cells cannot be produced (Pines, 2011). The APC consists of at least a dozen different subunits (Peters, 2006). In our study, four hub genes encoding APC subunits were enriched in the Cell cycle pathway, *ANAPC1*, *ANAPC4*, *ANAPC7*, and *ANAPC10*. Moreover, these genes were downregulated in 3Nβ, suggesting that APC expression was repressed in 3Nβ, resulting in a failure of the mid division to late division transition.

4.4.5. The top three key genes in terms of the number of interactions

In this study, we screened for important genes based on the interaction relationships between proteins. The hub genes with the highest number of interactions, *CDC6*, *CDC20*, and *PCNA*, were found to be the most likely genes to affecting the fertility of triploid female oysters. The CDC6 protein is a component of the pre-RC and is a regulatory factor for initiation of chromosome replication (Lee et al., 2017). In eukaryotes, CDC6 is required to bind to ORC and recruit the Cdt1-MCM2–7 complex to constitute the pre-RC (Fragkos et al., 2015). The absence of CDC6 causes cell cycle arrest in the S phase so that germ cells are unable to expand (Yao et al., 2017). In 3Nβ, CDC6 expression is suppressed in the gonads, and CDC6 protein cannot be formed, leading to the inability to initiate chromosome replication. CDC20 is a coactivator of APC and is essential for ubiquitin ligase activity (Du et al., 2022). An important function of CDC20 is to recognize APC substrates, facilitate their recruitment to the APC, and stimulate catalysis, processes that play key roles in regulating chromosome segregation and mitotic exit (Kapanidou et al., 2017; Richeson et al., 2020). PCNA acts on chromatin as a processing factor for DNA polymerase (Shiomi and Nishitani, 2017). The presence of PCNA increases the processing capacity of DNA polymerase from tens of nucleotides to thousands of nucleotides, thereby greatly enhancing the processing efficiency of DNA polymerase (Moldovan et al., 2007). From the results of the present study, we conclude that the top three genes in terms of the number of protein interactions were the most likely to affect gonadal development in female triploid oysters. Thus, there is a need to explore the specific roles of these three genes in the gonadal development of female triploid oysters in future studies.

4.4.6. Other hub genes associated with gonadal development of female triploid oysters

In addition to the key genes mentioned above, we also identified other hub genes associated with sterility in triploid female oysters, including *CDK6*, *MAD2L1*, *RFC1*, and *RFC5*. MAD2L1 is a component of the mitotic spindle assembly checkpoint (Wang et al., 2019). In the 3nβ group, downregulation of *MAD2L1* expression indicated that abnormal spindle assembly occurred, resulting in the failure of chromosome segregation. CDK6 drives the cell cycle transition from G1 to S phases by forming complexes with D-type cyclins (Nebenfuehr et al., 2020). RFC1 and RFC5 are two subunits of replication factor C. Replication factor C acts as a cofactor of Polδ and loads the PCNA onto the 3′ end of the nascent DNA strand (Reynolds et al., 1999; Yao et al., 2006). The biological processes and some hub genes identified in the present study will be verified and confirmed in future studies.

5. Conclusion

In this study, we conducted a transcriptomic comparison of the gene

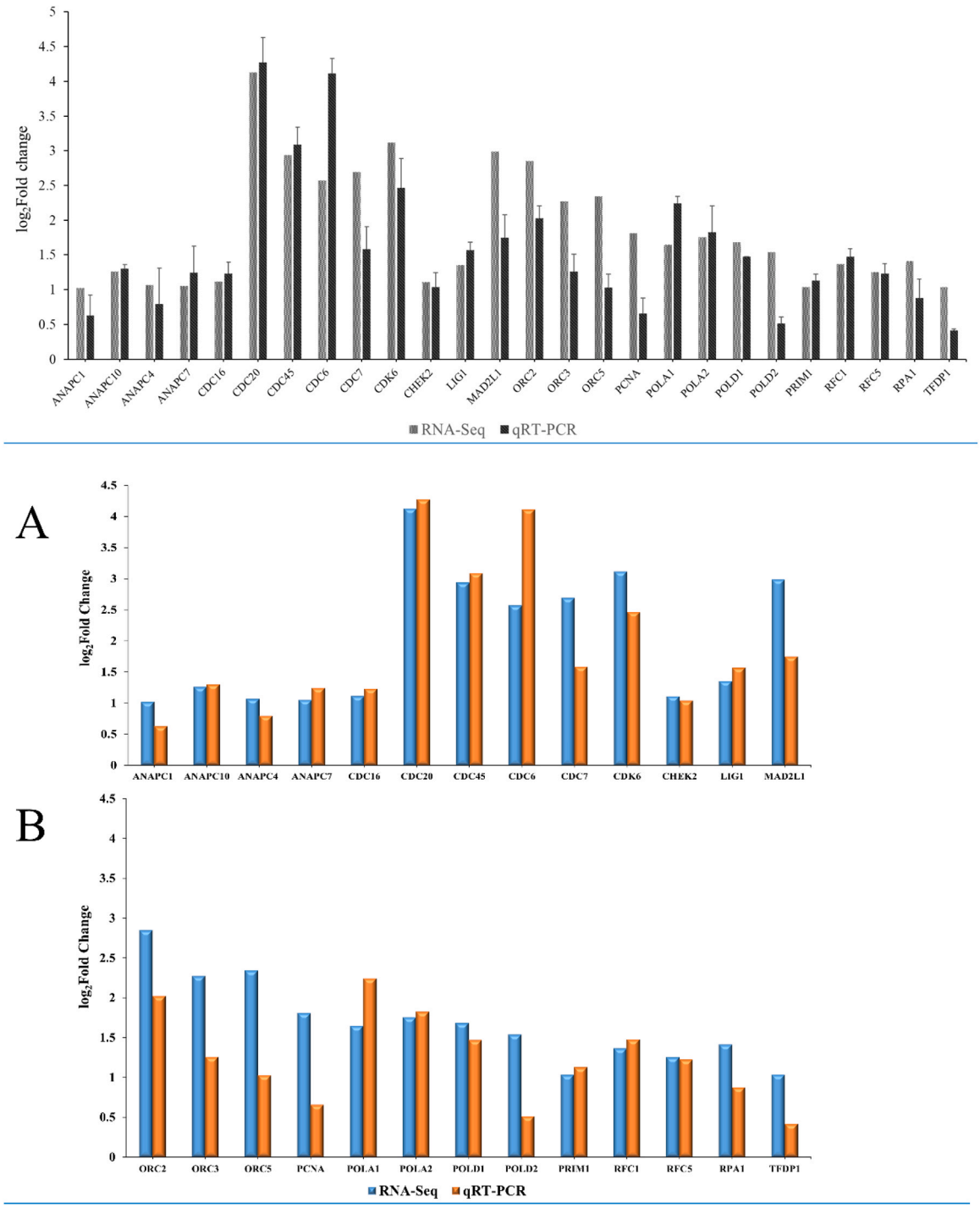

Fig. 7. Comparison of expression patterns of 26 hub genes by qPCR and RNA-Seq results.

expression profiles of fertile and infertile female triploid *C. gigas* and constructed a PPI network. Through our research, we identified four key gene families, namely the Origin recognition complex, Cell division cycle, DNA polymerase, and Anaphase-promoting complex. These genes primarily function in influencing DNA replication during mitosis, controlling the proliferation of reproductive cells, and exhibiting low expression in 3Nβ, which hinders the smooth progression of the mitotic cycle and the generation of a sufficient number of oogoniums. Ultimately, through analyzing the mechanism of infertility in female triploid *C. gigas*, our findings will contribute to addressing the industrial challenge of reproductive capacity in triploid *C. gigas* and promoting the breeding process of triploid oysters.

CRediT authorship contribution statement

Zhang Enshuo: Conceptualization, Writing – original draft. **Li Zan:** Conceptualization, Methodology, Project administration, Writing – review & editing. **Li Bin:** Methodology. **Fu Jingjing:** Methodology. **Feng Yanwei:** Methodology. **Sun Guohua:** Methodology. **Xu Xiaohui:** Methodology. **Cui Cuiju:** Methodology. **Wang Weijun:** Funding acquisition, Project administration, Writing – review & editing. **Yang Jianmin:** Funding acquisition, Project administration.

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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101885.

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ARTICLES FOR FACULTY MEMBERS

PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

Title/Author	**Male triploid oysters of Crassostrea gigas exhibit defects in mitosis and meiosis during early spermatogenesis / Maillard, F., Elie, N., Villain-Naud, N., Lepoittevin, M., Martinez, A. S., & Lelong, C.**
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Male triploid oysters of *Crassostrea gigas* exhibit defects in mitosis and meiosis during early spermatogenesis

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Keywords
Crassostrea gigas; meiosis; mitosis; oysters; spermatogenesis; triploid

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The Pacific oyster, *Crassostrea gigas*, is a successive irregular hermaphrodite mollusk which has an annual breeding cycle. Oysters are naturally diploid organisms, but triploid oysters have been developed for use in shellfish aquaculture, with the aim of obtaining sterile animals with commercial value. However, studies have shown that some triploid oysters are partially able to undergo gametogenesis, with numerous proliferating cells closed to diploids (3n alpha) or a partial one with an accumulation of locked germ cells (3n beta). The aim of our study therefore was to understand the regulation of spermatogenesis in both groups of triploid oysters (alpha and beta) from the beginning of spermatogenesis, during mitosis and meiosis events. Our results demonstrate that the reduced spermatogenesis in triploids results from a deregulation of the development of the germinal lineage and the establishment of the gonadal tract led by a lower number of tubules. Morphological cellular investigation also revealed an abnormal condensation of germ cell nuclei and the presence of clear patches in the nucleoplasm of triploid cells, which were more pronounced in beta oysters. Furthermore, studies of molecular and cellular regulation showed a down-regulation of mitotic spindle checkpoint in beta oysters, resulting in disturbance of chromosomal segregation, notably on spindle assembly checkpoint involved in the binding of microtubules to chromosomes. Taken together, our results suggest that the lower reproductive ability of triploid oysters may be due to cellular and molecular events such as impairment of spermatogenesis and disruptions of mitosis and meiosis, occurring early and at various stages of the gametogenetic cycle.

Abbreviations
2n, diploid; 3n, triploid; 4n, tetraploid; APC/C, anaphase-promoting complex; bub3, budding uninhibited by benomyl 3; cdc20, cell division cycle 20; EF1α, elongation factor 1α; GA, gonadal area; GAI, Gonadal Area Index; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. GT, gonadal tubules; GTI, Gonadal Tubule Index; H3S10p, H3-phosphoS10 antibody; HP1, heteroprotein 1; IHC, fluorescent immunohistochemistry; LBR, Lamin B receptor; mad2l1, mitotic arrest-deficient 2-like 1; MCC, mitotic checkpoint complex; mis12, kinetochore complex component; rad21, a component of the cohesin complex; SAC, spindle assembly checkpoint; ST, storage tissue. TAI, Tubule Area Index.

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The Pacific cupped oyster, *Crassostrea gigas* (Thunberg, 1793), is a successive and irregular hermaphrodite, predominantly of male gender during the first year and then able to change sex at each reproductive season [1]. Its protandry has recently been questioned [2]. Its gonad corresponds to a network of gonadal tubules imbricated in a connective tissue including the storage tissue. Its breeding cycle is annual and seasonal with four different stages of gametogenesis, which were described by Berthelin et al. [4]; Franco et al. [5]. During the sexual resting stage (stage 0), only some undifferentiated germ cells with no mitotic activity are present in the gonadal tubules, which do not allow sex identification. Stage 1 is characterized by gonial proliferation, and the sex of individuals can be determined by histological methods at the end of the stage. During stage 2, successive differentiations (active spermatogenesis or oogenesis) conduct the gonadal cells to stage 3, corresponding to the mature reproductive stage [4–6].

Triploid (3n) oysters were first introduced in the 1980s [7] for purposes of shellfish aquaculture. Now, triploid oysters have become an important part of aquaculture, thanks to their ability to grow faster than diploid (2n) animals, their supposed lack of gametogenesis [8–10], and their superior taste quality [11,12]. Their great performance in terms of growth is due to the reallocation of energy to the growth of somatic tissues at the expense of the growth and differentiation of the gonad [8–10,13]. Nevertheless, it has been proven that many triploid oysters are not totally sterile even if their reproductive capacities are significantly reduced compared to diploid animals [14,15] Among them, 3n alpha shows an active gametogenesis-like diploid animals but low gametes at sexual maturity stage while 3n beta showed a locked gametogenesis with an accumulation of abnormal gonia at stage 1 and only few mature gametes at sexual maturity [14]. The sterility triploid oysters are therefore partial and very heterogeneous depending on the animals. However, this heterogeneity is poorly documented in triploid oysters as is their gametogenesis. Triploid gametogenesis is insubstantially documented as well as its reproduction. This heterogeneity may be due to environmental factors such as temperature, photoperiod, and food availability [15–17]. Another explanation for this diversity could come from the different methods of inducing triploidy and from the selection of the genitors [1,18].

The first method used to induce the triploids consists of blocking the expulsion of polar bodies with the cytochalasin B or with other cell cycle inhibitors [19]. The animals obtained as a result of this technique of induction are called 'chemical' triploid oysters [20,21]. This chemical method has a number of disadvantages including its incomplete effectiveness and the significant toxicity of the chemical used. It was therefore abandoned to be replaced by a more natural method, that is, the cross-breeding between tetraploid and diploid animals. The resulting offspring are named 'natural' triploid oysters [19,11]. For this purpose, tetraploid (4n) oysters are produced by chemical induction techniques by inhibiting the expulsion of polar body I in oocytes of triploid fertilized oysters and then fertilizing these oocytes with haploid sperm. Although this method triggers the production of viable 4n oysters that reach sexual maturity [9,22], in the end it only produces 80–90% triploid embryos, when it should theoretically produce 100% [9,11]. In order to increase the induction rate, a new method was developed. It consist in producing tetraploid oysters by inhibiting the expulsion of polar body II with cytochalasin B in diploid oocytes and then fertilizing these oocytes with diploid sperm from tetraploid males [10,23]. This method is still currently used to produce triploid oysters [10].

However, the induction of polyploidy can cause many changes at the molecular, chromosomal, and cellular levels, resulting in phenotypic variations [24]. Indeed, the presence of an additional set of chromosomes in triploid animals may be critical during biological processes such as mitosis and meiosis that undergo cell divisions [25]. It may then impact the levels of transcripts, especially during the regulatory mechanisms of gene dosage compensation [26,27]. During the mitosis of polyploid cells, impaired mitotic spindle assembly can cause a chaotic segregation of chromatids and the production of aneuploid cells [27,28]. The transcriptomic profiling of gametogenesis has shown that such disruptions of the mitosis can be responsible for the impaired gametogenesis observed in triploid oyster [29]. All triploids displayed a downregulation of genes associated with cell division, and the comparison of 3n alpha and 3n beta transcriptomes with 2n revealed the involvement of a cell cycle checkpoint during mitosis in the successful but delayed development of gonads in 3n alpha oysters [29].

The aim of this study is to understand the molecular and cellular regulations that take place at certain checkpoints of the cell cycle, in mitosis and meiosis during gametogenesis, in both groups of triploid oysters (α and β), focusing only on male oysters. This study also aims to reach a finer understanding of the differences in the structure of the gonad which are at the origin of their differences in terms of reproductive effort. In order to do this, we compared α and β triploid oysters with diploid ones. First, we assessed the

reproductive effort by means of a histological quantitative approach focusing on the number and area of gonadic tubules. We also highlighted molecular expression profiles by means of a transcriptomic quantification method in order to explore the regulation of the spermatogenesis. Finally, the dynamic and remodeling of the chromatin was assessed during mitosis and meiosis occurring during the gametogenetic cycle.

Materials and methods

Animals and rearing conditions

Diploid and triploid juveniles (*Crassostrea gigas*) in their 1st year of life were provided by a commercial farm (Calvados, France). Triploid oysters were produced by crossing tetraploid males with diploid females. Oysters were maintained on field at Cricqueville en Bessin (Calvados) between December 2017 and May 2018. The animals were sampled every 15 days until May, and after that monthly until September 2018. For each individual, pieces of gonad and gill tissues were sampled, frozen in liquid nitrogen, and stored at −80 °C for total RNA extraction and qPCR. A transverse section of gonad was made and fixed in Davidson's solution for histology and immunocytochemistry.

Ploidy determination

Ploidy was determined individually for both diploid and triploid oysters by using a statistically representative group of 25 animals for each ploidy. From each animal, a piece of gills was dissected and the ploidy was certified by flow cytometry. To do this, a Muse Cell Cycle Kit with a Guava Muse Cell Analyzer (Luminex, Austin, TX, USA) was used following the manufacturer's instructions. The DNA content was determined by propidium iodide labeling from a cell suspension provided to centrifugate shredded gills in the final volume of cold 70% ethanol.

Histological analysis

Transverse sections of gonad were fixed in Davidson's solution (10% glycerol, 20% formaldehyde, 30% ethanol (95%), 30% sterile seawater, 10% acetic acid). They were then dehydrated in ethanol and embedded in paraffin wax. Three-micrometer-thick sections were stained with Prenant-Gabe trichrome [30]. The gametogenetic stages and sex were determined *a posteriori*. For diploid oysters, it was based on the criteria described by Refs [4] and [5]: stage 0 (sexual rest, undifferentiated cells), stage 1 (gonia proliferation), stage 2 (maturation; spermatogenesis or oogenesis), and stage 3 (ripe gonads before spawning). For 3n oysters, α and β patterns were determined according to Ref. [14]: stage 0 (sexual rest as in 2n), stage 1 (α-pattern: gonia proliferations as in 2n and β-pattern with highly disturbed gametogenesis, with numerous germ cells locked in prophase of mitosis) (Appendix S1), stage 2 (α-pattern: maturation as in 2n and β-pattern still exhibiting locking events), stage 3 (α-pattern: mature animals closely resembling 2n and β-pattern nearly completely sterile, with only a few gametes) (Appendix S2).

Quantitative analysis of reproductive effort

To evaluate the reproductive effort, each of the histological sections was scanned at 20× (0.25 µm·pixel^{-1}) using a ScanScope CS microscope slide scanner (Leica Biosystems, Nanterre, France). For each image, a downsampling by 8 was applied to allow an analysis with Python algorithms [31] and using the ILASTIK software [32]. A color normalization [33] of all images was first carried out to enhance the performance of image analysis algorithms. Then, three different tissues were detected on the images: the whole tissue section without the gills that had previously been removed, the gonadal tubules (GT) and the storage tissue (ST). The whole tissue area was extracted, thanks to a threshold intensity component of the color space 'Hue, Saturation and Intensity'. GT areas were obtained with a threshold on components L* and a* of color space 'CIE L*a*b*', where L* represents the lightness from black to white, a* represents colors ranging from green to red, and b*represents the colors ranging from blue to yellow. ST areas were obtained with a pixel classification method using the ILASTIK software [32]. The areas of these three different tissues were computed in pixels so as to access the reproductive effort of diploid and triploid oysters, using gonadal area (GA) measurement. This area, located around the digestive gland, contains GT and ST and is known to vary in proportions depending on the gametogenetic stage, in diploid oysters as in triploid oysters. Here, to be even more precise, the Gonadal Tubule Index (GTI) and the Tubule Area Index (TAI) were specifically measured. The GTI represents the percentage of GA in relation to the whole animal cross section [GTI = (GA/surface of the whole cross section) * 100] and TAI is the percentage of GT in relation to the whole animal cross section [TAI = (GT/surface of the whole cross section) * 100]. The average number of tubules per cross section and the mean area per tubule was also measured in group of animals at the beginning of the gametogenetic stage (stages 0 and 1). Area (A), circularity, perimeter (P), major and minor axes, and compacity of each tubule were obtained. The sorting of the tubules was carried out using the Gravelius compactness coefficient: $K = P\frac{P}{2\sqrt{\pi A}} A = 0.28\frac{P}{A}$ with the threshold of $K < 1.7$ to keep only the tubules with circular cross sections [34,35]. To ensure that the number of these tubules was representative of the totality of the tubules, we have compared their proportion to that of all tubules for each animal and no significant difference was found.

RNA Extraction

Ten individual gonad samples and three gill samples for gametogenetic stage of diploid and triploid oysters were used for the quantitative expression analysis. The tissues were grounded in 500 µL of TriReagent (Sigma-Aldrich, Saint Quentin Fallavier, France). One hundred microliters of bromo-3-chloropropane (Sigma-Aldrich) was then added, and the samples were vortexed before centrifugation (12 000 ***g***, 10 min, 4 °C). The aqueous phase was retrieved and the total RNA extraction was performed using a NucleoSpin RNA Clean-up Kit (Macherey-Nagel, Hoerdt, France), following the manufacturer's instructions. RNA concentration, purity, and integrity were checked by spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Waltham, MA, USA). To prevent genomic DNA contamination, RNA samples were treated with DNAse I (1 $U \cdot \mu L^{-1}$ of total RNA, Sigma). Then, the total RNA was reverse-transcribed to prepare for the cDNA. Two hundred and fifty nanograms of total RNA from each sample was reverse-transcribed using 200 U of MMuLV-RT (Moloney Murine Leukemia Virus Reverse transcriptase, Promega, Charbonnières les bains, France) in the presence of 12 U of RNase inhibitor (RNasin, Promega), 5 mM of RNAse free dNTPs, and 100 ng of random primers in the appropriate buffer (Promega) at 37 °C during 90 min. cDNA was stored at −20 °C until further usage.

Quantitative real-time PCR (qPCR)

The expression of mRNA in oysters during the gametogenetic cycle was investigated using a quantitative real-time PCR analysis. The primer sequences were designed using the Primer 3 software (Table 1) and synthesized by Eurogentec (Seraing, Belgium). The reference gene, elongation factor 1α (EF1α), was selected among two housekeeping genes [EF1α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. The high stability of this housekeeping gene was previously identified by Refs [36,37] and was further confirmed in the present study. The qPCR mix contained 1X *GoTaq SYBR Green Mix* (Promega), 5 ng cDNA, and 15 µM of forward and reverse primers in a final volume of 15 µL. The amplification conditions consisted of one cycle at 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 45 s. The specific amplification of the target sequence was estimated from the melting temperature curves. The relative expression level of each target gene was calculated based on the threshold value (C_t) deviation of this target gene from the housekeeping gene (EF1α).

Statistical analysis

The gene expression levels and the measures assessed by quantitative histology between diploid and triploid oysters at each gametogenetic stage were illustrated on graphs by means and SEM. The results were analyzed using a Student's parametric *t*-test with a *P*-value < 0.01. All analyses were performed with the PRISM.V6 (GraphPad) software.

Immunohistochemistry

Five-micrometer sections were deparaffinized, rehydrated, and washed in 1X phosphate-buffered saline (PBS) during 10 min. The sections were permeabilized with 200 µL of 0.1% Triton X-100 in 1X PBS for 10 min at room temperature. Antigen retrieval was performed with 200 µL of freshly made 2N hydrochloric acid in 1X PBS in a 37 °C incubator during 30 min. After denaturation, nonspecific sites were blocked with an incubation in 100 µL of 0.1 M Tris/HCl (pH8.3) for 10 min followed by one in 500 µL of blocking solution [0.25% bovine serum albumin (BSA),

Table 1. List of names, sequences, and corresponding gene names and NCBI ID numbers (*: reference gene) of the primers used for quantitative real-time PCR analysis in *Crassostrea gigas*. 'F' and 'R' indicate, respectively, forward and reverse primers.

Primer	Nucleotide sequences (5′-3′)	Gene name	NCBI Gene ID
EF1α-F	ACGACGATCGCATTTCTCTT	*Elongation factor 1-alpha**	105338957
EF1α-R	ACCACCCTGGTGAGATCAAG		
GAPDH-F	TTGTCTTGCCCTCTTGC	*Glyceraldehyde-3-phosphate dehydrogenase**	105340512
GAPDH-R	CGCCAATCCTTGTTGCTT		
CDC20-F	TACCAAAGGACCAGGCATGC	*Cell division cycle 20*	105332569
CDC20-R	GTGTTTTCGCCGTTGTACTGA		
MAD2L1-F	ACCACGTCAGAAGTCAGAGA	*Mitotic arrest-deficient 2-like 1*	105340350
MAD2L1-R	CAGCAGGGGTAGGAATGTGA		
MIS12-F	GGACAGGGTACAGATGACACA	*MIS12 Kinetochore Complex Component*	105330856
MIS12-R	GCTATGATGTGTTTACGGAGCT		
RAD21-F	TGCCATCAACACTTTCCTTCAG	*RAD21 Cohesin Complex Component*	105337371
RAD21-R	ACCCGTGCAATCTTTTCCAC		
BUB3-F	GTTGTGTCGAGTACTGCCCA	*BUB3 Budding uninhibited by benomyl 3*	105332696
BUB3-R	CTTGTCCGGTTGAGTGAAGGA		

0.5% Triton X-100 in 1X PBS] for 1 h at room temperature in a humid chamber. After that, the slides were incubated overnight with a rabbit polyclonal phospho-histone H3 (Ser10) antibody (Millipore 06–570, Millipore Corporation, Guyancourt, France) diluted (1 : 500 previously validated by Cavelier et al. [38]) in a blocking solution at 4 °C in a humid chamber. The next day, the sections were washed three times (15 min each) with 0.1% PBS Tween-20 and then incubated during 1 h with a secondary antibody, Donkey Anti-Rabbit IgG H&L (1 : 1000) (Alexa Fluor 594) (ab150080) and with Hoechst 20 mM (1 : 1000) in a blocking solution at room temperature. Three further washes with 0.1% PBS Tween-20 were then performed, and the slides were mounted in mounting medium (Ibidi, Sigma-Aldrich). The image analysis was performed with a FV1000 confocal laser scanning biological microscope (Olympus, Rungis, France).

Results

Reproductive effort during spermatogenesis

The reproductive effort during the gametogenetic cycle was studied by quantitative histology analysis on transversal sections of male diploid and triploid oysters. It was first measured as the percentage of gonadal area occupying the whole cross section (GTI) (Fig. 1A). During the quiescent stage (stage 0), while alpha and beta triploids cannot be distinguished, no significant difference was observed between 2n and 3n animals, with around 10–15% of the gonadal area. At stage 1, GTI was 20% for diploid, 17% for alpha triploid, and 10% for beta triploid oysters. At the end of the gametogenetic cycle (stage 3), it reached 85% in diploid, 71% in alpha triploid, and 23% in beta males triploid. A pairwise comparison within each gametogenic stage found significant differences between diploid and alpha and beta triploid oysters from stage 1 to stage 3. Whatever the stage, the GTI of 3n beta was always significantly lower in comparison with diploid and alpha triploid males. The GTI of alpha triploids was also significantly lower compared with diploid oyster, but only at stage 3. A fine analysis was also made to quantify more precisely the occupation of the tubules in the whole animal section through the Tubule Area Index (TAI) (Fig. 1B). In comparison with diploid oysters, the TAI was significantly reduced in triploid animals at stages 2 and 3. At stage 3, it was significantly reduced by half in alpha triploid oysters and by eight in beta triploid male oysters. The TAI not only takes into account the number of tubules but also their surface, which increases during gametogenesis, according to the filling rate of the tubules by germ and somatic cells. Thus, a complementary analysis focusing on the number of tubules and on the mean area per tubule was performed in order to find out whether the decrease in TAI in triploids could be due to a decrease in the number of tubules and/or in their surface. This analysis was only conducted at stages 0 and 1, when the tubules are clearly individualized on the section (Fig. 2). These stages are also relevant because (a) stage 0 corresponds to the very beginning of the gametogenesis, illustrated by scarce tubules filled with very few cells, and (b) stage 1 of gonia proliferation was described as a crucial stage to understand gametogenesis in triploids [14]. This analysis showed that at stage 0, diploid animals have a significantly higher number of tubules per section than triploid males (Fig. 2A), although the mean tubule area was similar for both animals (Fig. 2B). At stage 1, diploid and alpha triploid oysters presented the same mean values of tubules areas, while beta triploid oysters highlighted a significantly reduced mean area per tubule compared with both diploid and alpha triploid oysters (Fig. 2B), in line with the results of the GTI (Fig. 1A).

Molecular regulations during spermatogenesis

Here, the objective was to gain more insights into the regulatory gene network involved in the gametogenetic disruption in alpha and beta triploid oysters. For this purpose, genes were selected among those which appeared dysregulated between diploid and triploid oysters in a previous study carried out by Refs [29,36] and using large-scale approaches. In order to gain insight into the genes involved in gametogenetic disruption beta oysters, several ones have been selected from the previous studies from Refs [29,36], appearing dysregulated between the 2n and 3n animals. We especially focused on those involved in the kinetochore structure and its regulation during mitotic and meiotic progression, namely mad2l1 (mitotic arrest-deficient 2-like 1), mis12 (kinetochore complex component), cdc20 (cell division cycle 20), bub3 (budding uninhibited by benomyl 3), and rad21 (a component of the cohesin complex). This meant performing an expression analysis by quantitative PCR on diploid and triploid male gonads at stage 0 of sexual rest and at stages 1 and 2 when mitosis and meiosis take place, respectively (Fig. 3). In diploid and alpha triploid oysters, the trend for all transcripts corresponded to a progressive increase through the gametogenesis, from stage 0 to stage 2. In contrast, in beta triploid oysters, despite an increase in all gene expressions between stages 0 and 1,

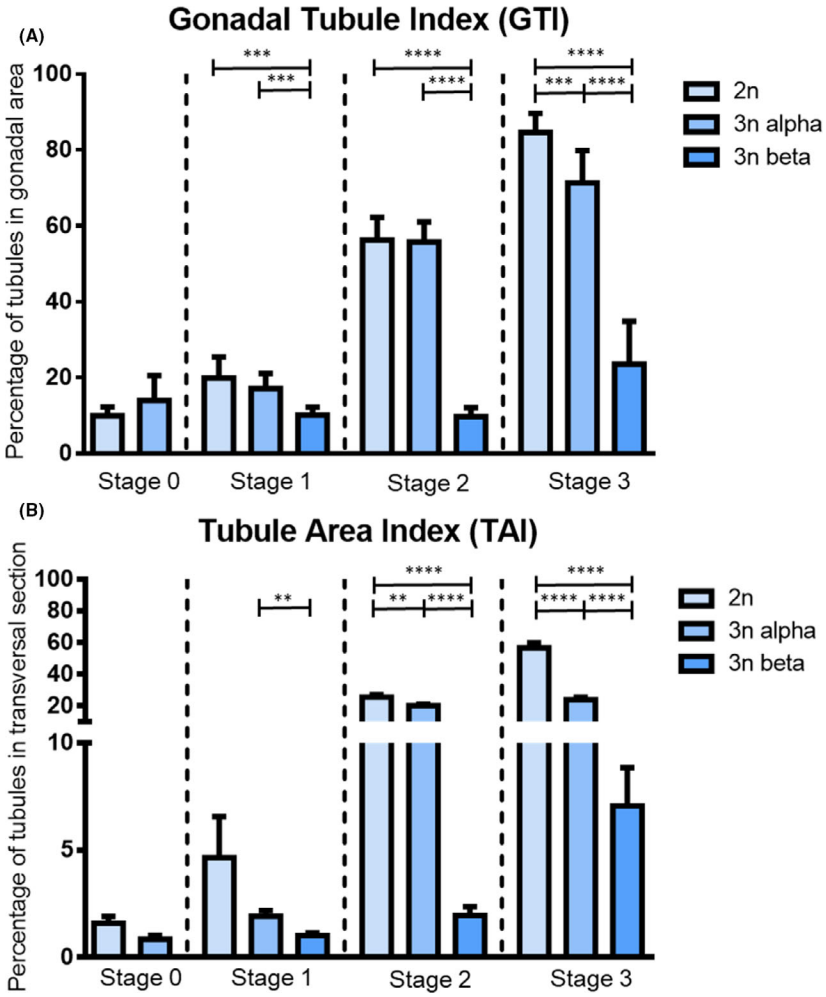

Fig. 1. Quantification of reproductive effort in male *Crassostrea gigas* oysters. Assessed through the Gonadal Tubule Index(GTI) (A) and the Tubule Area Index (TAI) (B) in a transverse sections of diploid ($n = 36$) and triploid ($n = 64$) oysters during the gametogenic cycle (stage 0: resting period, stage 1: gonia proliferations, stage 2: maturation, and stage 3: mature animals). By means of a statistical analysis (Student's test) comparing triploid and diploid animals during the gametogenetic cycle, the significant differences are indicated by asterisks (****$P < 0.0001$, ***$P < 0.0005$ and **$P < 0.001$). Bars represent standard error of mean.

a significantly lower expression was highlighted at stage 2 compared with diploid and alpha triploid oysters. Such significantly lower mRNA expression in triploids compared with diploids was also observed for MIS12 in stage 0.

Cell divisions and chromatin remodeling along the cell cycle during early spermatogenesis

To follow the cell divisions at the origin of male gonial proliferations in diploid and triploid oysters, we traced the dynamic distribution of the chromatin at stage 1, when mitoses are most frequent, but also just before at stage 0 and then at stage 2 when meiosis occurs. For that purpose, the histone H3 phosphorylated on the Serine 10 residue was detected by fluorescent immunohistochemistry (IHC), using a H3-phosphoS10 antibody (H3S10p) in association with a DNA staining with Hoechst dye. H3S10p-specific signal was certified by the lack or low labeling in Hoechst-stained nuclei in the storage tissue (tissue with low or reduced mitotic activity) and on the negative controls using no primary antibody. Confocal images revealed an intense signal associated with the stained chromatin in the gonadal tubules, with special and temporal variations depending on the mitotic stages (Fig. 4). Thus, a staining was observed starting from the G2 phase until the telophase (Fig. 4A). At the G2 phase, the Hoeschst staining (blue) and the H3S10p staining (red) were diffuse and they overlapped each other through the whole nucleus. Then, they progressively reached the periphery of the nucleus to finally become perinuclear in the late prophase. At the prometaphase, when the chromosomes start to condense and at the metaphase when all chromosomes compact on the equatorial plate, the H3S10p signal overlapped with condensed chromosomes but this pattern could also be observed outside the chromosome localization area. At the anaphase, when sister chromosomes are getting apart after centromeric fission, both signals were overlapping again.

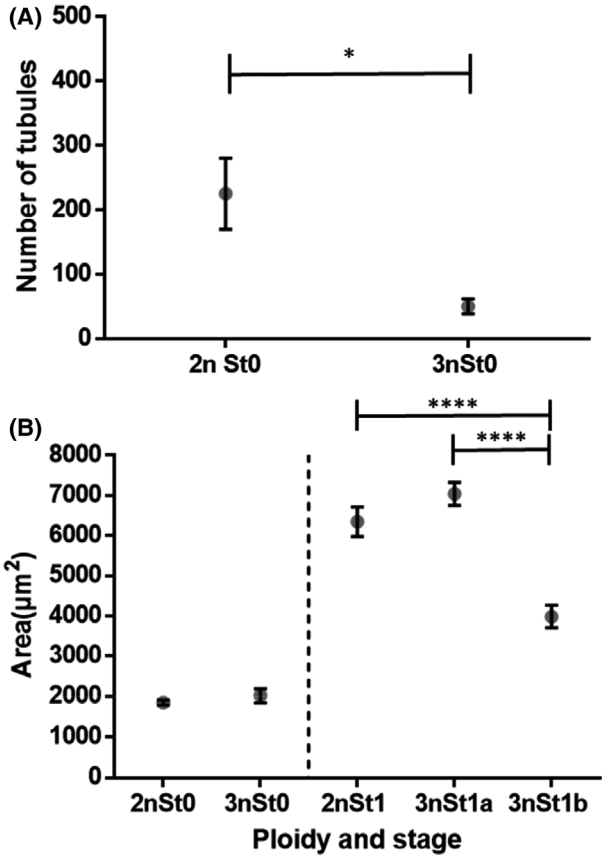

Fig. 2. Quantification of the average number of gonadal tubules (A) and the mean area per tubule (B) in a transverse section of male diploid (2n) ($n = 12$) and triploid (3n) ($n = 27$) oysters during the gametogenic cycle (stage 0: resting period, stage 1: gonia proliferations). By means of a statistical analysis (Student's test) comparing triploid and diploid animals at each gametogenetic cycle, the significant differences are indicated by asterisks (****$P < 0.0001$ and *$P < 0.05$). Bars represent standard error of mean.

At the telophase, when the separated chromosomes started to uncoil and to become less condensed, the immunofluorescence appeared as aligned patches, localized on the chromosomal areas. When the relative frequency of each mitosis stage was counted at stages 0 and 1 and compared between diploid and triploid oysters (Fig. 4B), it appeared that at stage 0 of gametogenesis, most of the cells in diploid and triploid oysters were in the late prophase (63% for diploids and 30% for triploids) and in the prometaphase/metaphase (30% for diploids and 55% for triploids). However, diploids were rather in the late prophase whereas triploids were in the prometaphase/metaphase. Nonetheless, at this stage, the number of mitosis figures is low. In stage 1 when cells proliferate, when alpha and beta triploids can be distinguished, the cells in mitosis were still predominantly in the late prophase and prometaphase/metaphase. Interestingly, diploids and alpha triploids were rather in the metaphase whereas beta triploid oysters were in the late prophase. Looking specifically at the signal distribution in prophase (Fig. 5), at stage 0, the same staining was observed in diploids and in triploids, with heterochromatin localized at the center of the nucleus while H3S10p staining was observed at its periphery. In contrast, at stage 1, the staining was different in beta triploid animals compared with diploid and alpha triploid oysters. In these last two cases, heterochromatin was observed at the periphery of the nucleus and was overlapping with the H3S10p staining while in beta triploid heterochromatin was localized at the center of the nucleus while H3S10p staining was observed at its periphery. When looking more broadly within the tubules and later on during the gametogenetic cycle (Fig. 6), it appeared that the cells labeled H3S10p were (a) less numerous in triploid oysters (especially beta triploid) than in diploid ones whatever the gametogenetic stage and (b) still present at stage 2, which encounters meiosis.

Discussion

Could disturbances in the establishment of the gonadal tubules and in the gametogenesis during stages 0 and 1 of spermatogenesis explain the lower reproductive effort of beta triploid oysters?

Two strategies of gametogenesis were previously described, in triploid oysters by Jouaux et al. [14], a α-pattern, corresponding to animals displaying numerous proliferating gonia at stage 1, resulting in abundant gametes at stage 3 and a β-pattern, which was associated with a locked gametogenesis and only few mature gametes at sexual maturity. The authors supported these results by demonstrating, at stage 3, a lower reproductive effort in triploid oysters compared to diploid, using the Gonadal Area Index (GAi) and the Gonadal Tubule Index (GTi). In the present work that specifically focuses on triploid males, the same both patterns were observed (results not shown). In contrast, in Jouaux et al. [14], the reproductive effort was assessed from stage 0, at various stages of the gametogenetic cycle, and not only with GAi and GTi but also by estimating the average number of tubules per cross section and the mean area per tubule. Thus, in this study, at stage 3 of sexual maturity, as stated by Jouaux et al. [14], the GTI and TAI were lower in alpha triploids and beta triploids compared with diploid oysters, thus confirming the idea of a lower

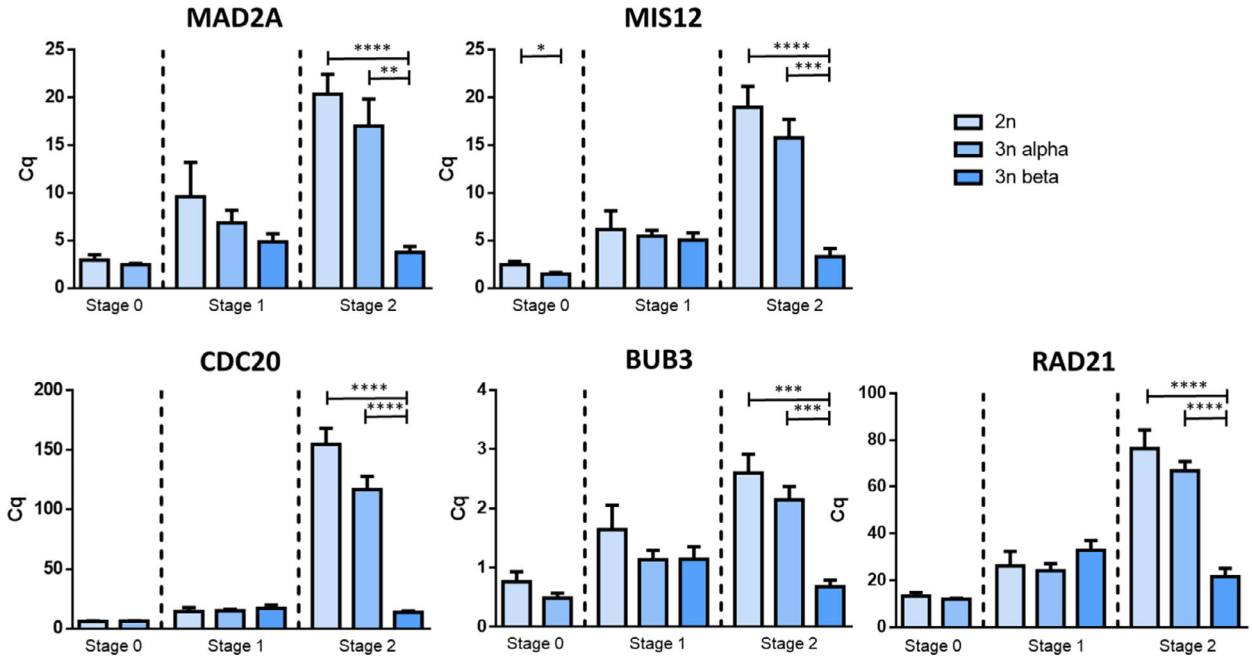

Fig. 3. Quantitative PCR analysis of relative mRNA expressions of MAD2L1, MIS12, CDC20, BUB3, and RAD21 in gonad of male diploid and triploid *Crassostrea gigas* during the gametogenetic cycle (stage 0: resting period, stage 1: gonia proliferations, stage 2: maturation). Each bar represents the mean of expression level of the target transcript replicates ($n = 10$) related to the housekeeping gene EF1α. By means of a statistical analysis (Student's test) comparing triploid and diploid animals at each gametogenetic cycle, the significant differences are indicated by asterisks (****$P < 0.0001$, ***$P < 0.0005$, **$P < 0.001$ and *$P < 0.05$). Bars represent standard error of mean.

reproductive effort at this stage of sexual maturity. In alpha triploids, such differences were explained by Guo and Allen [9] and Gong et al. [18] by differential fecundities between diploid sand triploids, although they made a distinction between alpha and beta. Jouaux et al. [14] disagreed, since they observed equivalent fecundities between diploids and alpha triploids. In beta triploids, our results also demonstrated that the lower reproductive effort occurred very early, as soon as stages 0 or 1. According to our results, this could be explained by a lower number of tubules as observed at stage 0 in triploid oysters compared with diploid, but also at stage 1 by a significantly lower mean area per tubule (knowing that the number of tubules is not lower; results not shown). At this stage, and knowing that tubules are filled with proliferating gonia, a lower mean area per tubule may suggest an impaired gametogenesis with the presence of fewer germ cells within the tubules. Indeed, this latter idea is supported by previous work done by Jouaux et al. [14] who mentioned the presence of numerous germ cells locked in the mitosis prophase at stage 1 in beta triploid oysters, thus suggesting a stop in the gametogenesis at this point. Taken all together, our results suggest that the lower reproductive effort observed in beta triploid oysters may be due to disturbances occurring as soon as stage 0 or 1 of the reproductive cycle. These disturbances would occur during the establishment of the gonadal tubules and during germ cells mitosis occurring during spermatogenesis.

Could chromatin remodeling and dysregulations of genes involved in epigenetic modifications impair mitosis in stage 1 of spermatogenesis in 3n β oysters?

In order to deepen the understanding of the cellular impairments of mitosis occurring in beta triploid oysters early during spermatogenesis, we performed an immunofluorescence labeling of the phosphorylated histone H3 on the serine 10, using a H3S10p antibody. This marker is associated with each step of mitosis and meiosis. The expression pattern of H3 is exposed to dynamic changes during spermatogenesis [39]. In our study, the frequencies of each mitosis phase measured at stages 0 and 1 of the reproductive cycle showed that beta triploid males were locked between the end of the prophase and the start of the prometaphase. These results are in line with those observed by Jouaux et al. [14]; that is, the gonia is locked in the prophase in beta triploid oysters. Previous studies have demonstrated a link between the histone H3

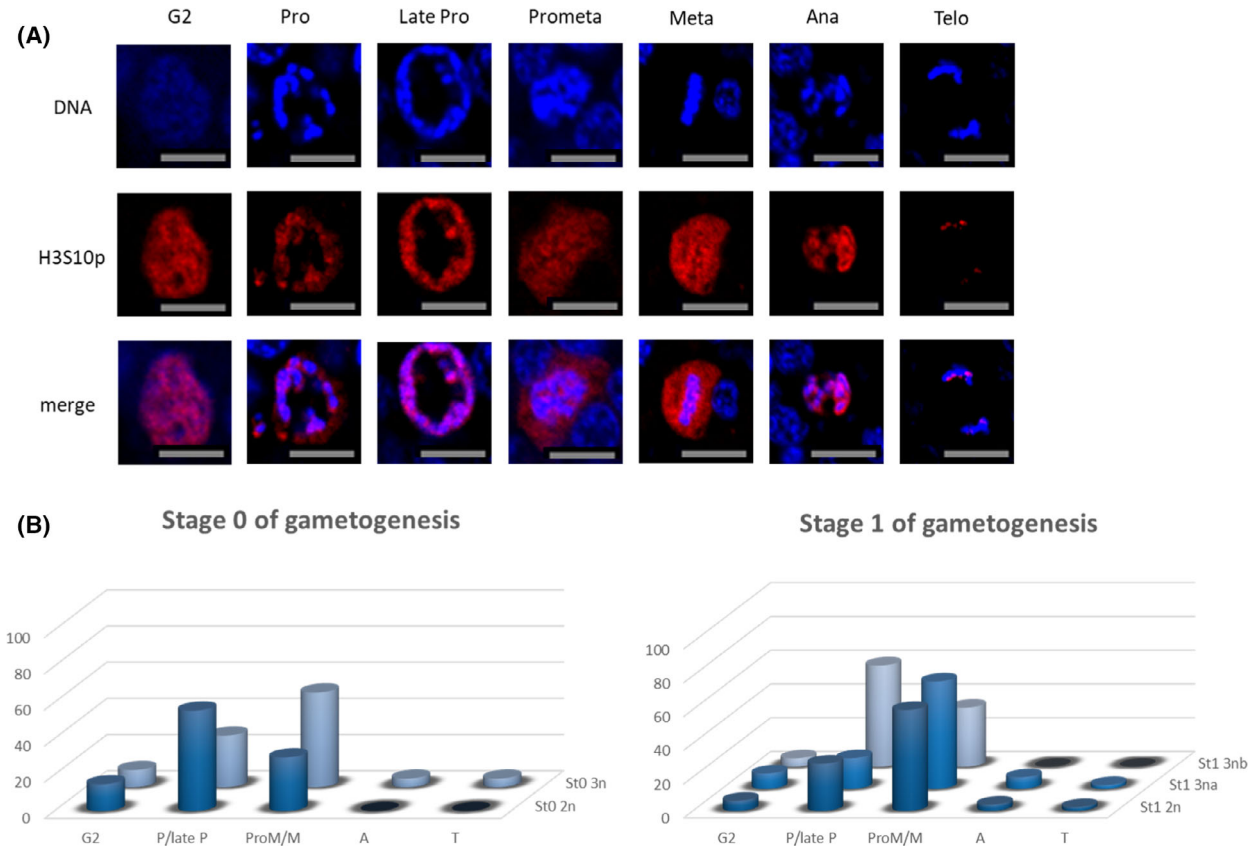

Fig. 4. Immunofluorescence labeling of H3S10p (red) at different stages of mitosis in cross sections of gonadal tubules of male diploid oysters during stage 1 of the gametogenetic cycle. DNA was stained with Hoechst (blue) (A). Relative frequency of each figure of mitosis during the early stages of gametogenesis (stages 0 and 1) of diploid and triploid oysters (B). Scale bar: 5 μm.

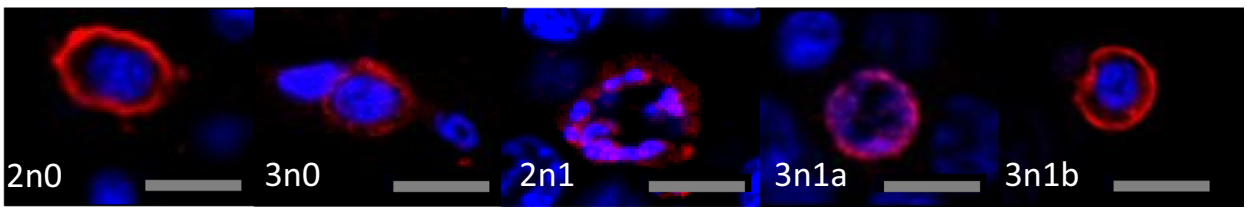

Fig. 5. Germ cells at prophase in male diploid (2n) and triploid (3na: 3n alpha and 3nb: beta) oysters during early stages of gametogenetic cycle (0: stage 0 and 1: stage 1). DNA was stained with Hoechst (blue). Scale bar: 5 μm.

phosphorylation and chromosome segregation during the cell division process. The histone H3 is phosphorylated at Ser 10 during the prophase, with a peak level at the metaphase, and it is dephosphorylated at the anaphase to get out for of the division cycle [40,41]. A mutation of this phosphorylation in the ciliated protozoan *Tetrahymena thermophile* causes condensation and segregation defects, suggesting that the H3 phosphorylation is correlated with chromosome condensation during mitosis and meiosis [42]. Moreover, underphosphorylation of histone H3 has also been connected to aberrant chromosome behavior during mitotic and meiotic division in insects [43]. In our study, in diploid oysters, during mitosis, chromatin staining was usually colocalized with H3S10p staining, except at the prometaphase and metaphase, when chromatin was highly condensed. These results also highlight a link between the histone H3 phosphorylation and chromatin remodeling during mitosis. Besides, this remodeling could be explained, as suggested by some authors, [44] by an increase in negative charge due to the phosphorylated histone H3 that makes it dissociate from chromosomal DNA, therefore causing chromatin condensation. In the same way, in

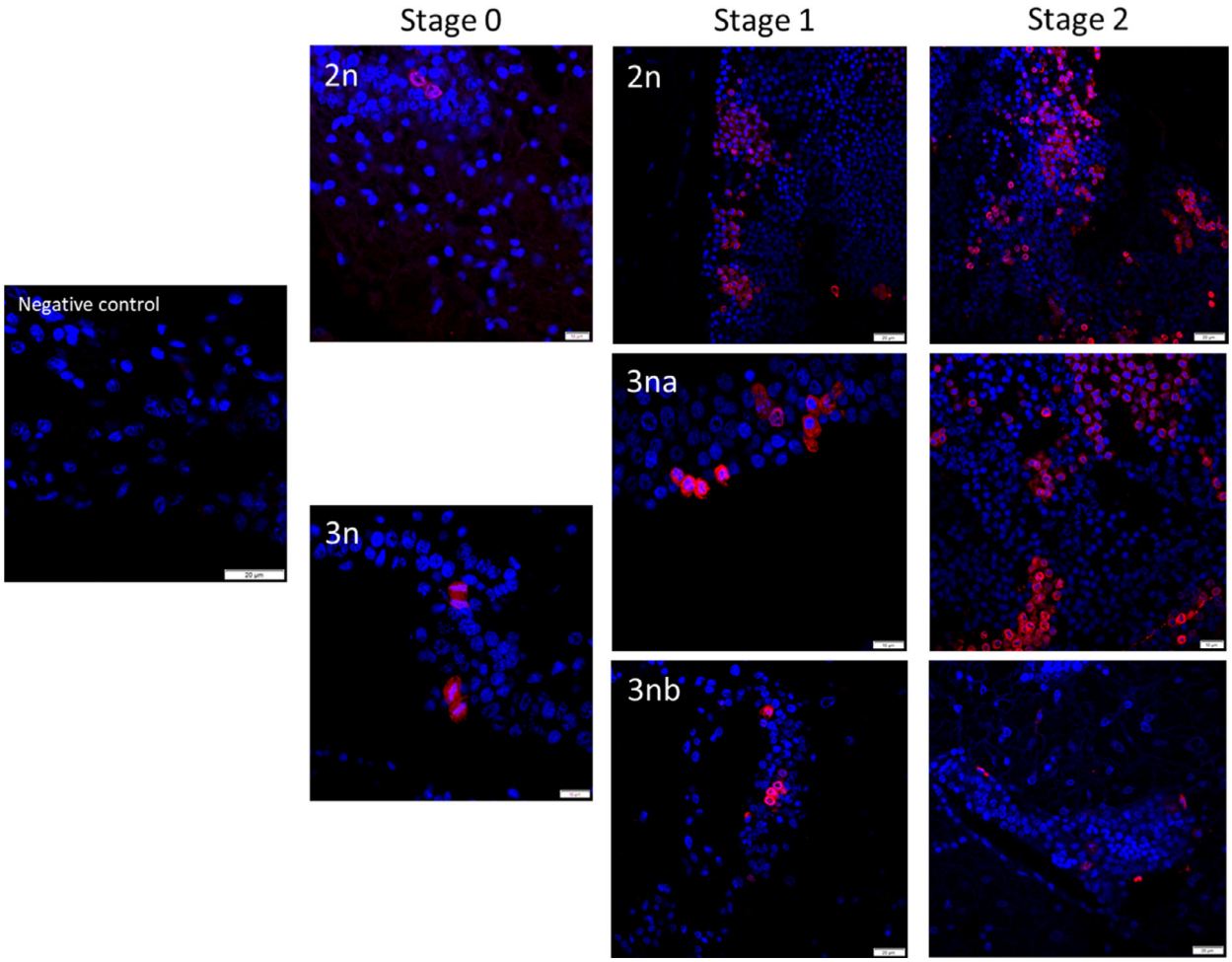

Fig. 6. Immunofluorescence labeling of H3S10p (red) in cross sections of gonadal tubules in male diploid (2n) and triploid (3na: 3n alpha and 3nb: beta) oysters during the gametogenetic cycle (stage 0: resting period, stage 1: gonia proliferations and stage 2: maturation). DNA was stained with Hoechst (blue). Scale bar: 20 μm.

beta triploid male oysters, the particular pattern of germ cells locked between the late prophase and the beginning of the metaphase could be explained by a dysregulation of the histone H3 phosphorylation followed by an abnormal condensation of the germ cell nuclei. This hypothesis is also supported by our proteomic results (not shown) which highlighted a significant downregulation of the expression level of protein histone H3 in beta triploid oysters and suggests a dysregulation at the post-translational level. Further studies examining post-translational modifications of histones are therefore necessary in order to maintain this hypothesis. Our results of H3S10p staining at stage 2 of the gametogenesis (meiotic stage) also suggest that the blocking of the germ cells persists throughout the gametogenetic cycle, in contrast with the results of Ref. [8] who only suggested a delay. Our results also showed a particular pattern of chromatin localization in prophase in beta triploid males only, at the center of the nucleus while the H3S10p staining was localized at its periphery. Indeed, the frequent organization of the nucleus has two types of heterochromatin: constitutive and facultative at the periphery of the nucleoplasm, near or associated with lamina and euchromatin at central position. A unique exception to chromatin organization in eukaryotes is found in rod photoreceptor cells of nocturnal mammals [45]. In these cells as in the germ cells of beta triploid males, the positions of eu- and heterochromatin are inverted in the nuclei. This peculiar chromatin remodeling happens during the development and the cellular differentiation. In beta triploid male oysters, this peculiar chromatin localization during mitosis could be associated with the peripheral labeling of H3S10p.

Indeed, in rod cells it is associated with major post-translational modifications of the histones, mainly the epigenetic marks H3K9me3 and H4K20me3 and with nuclear tether proteins, Lamin-A/C and LBR (Lamin B Receptor) [46,47]. These latter two proteins are involved in the tethering of LAD domains in association with HP1 (heteroprotein 1) and H3K9me2/3. LBR is used to attach the nuclear lamina to the inner nuclear membrane and to bind HP1-associated heterochromatin. It acts in association with H4K20me2PRR14, which requires Lamin-A/C for localization at the inner nuclear periphery [48]. The loss of tethering of the nuclear lamina induces alterations of the genome architecture [49]. Surprisingly, in 2n oysters, a similar central localization of chromatin and peripheral localization of the Histone H3p were also observed in germ cells at stage 0 (resting gametogenetic stage). This startling localization requires further studies. However, in mice, a role of epigenetic modifications during very early spermatogenesis was recently mentioned, when prospermatogonia differentiate into differentiating or undifferentiated spermatogonia after birth [50]. A demethylation of H3K9me2 by the JMJD1A and B demethylases would be at the origin of this differentiation of spermatogonia [50]. Taken all together, our results therefore suggest (a) that epigenetic modifications appear essential to the progression of spermatogenesis in diploid oysters and (b) that their probable deregulation might be involved in the blocking observed in beta triploid oysters.

Could dysregulations in genes involved in the spindle assembly checkpoint (or SAC) be a sign of impaired meiosis at stage 2 of spermatogenesis in 3n β oysters?

The frequent infertility of triploid individuals is a general pattern directly related to their ploidy, as mentioned for numerous species. It is frequently due to irregular chromosomal pairing and perturbed

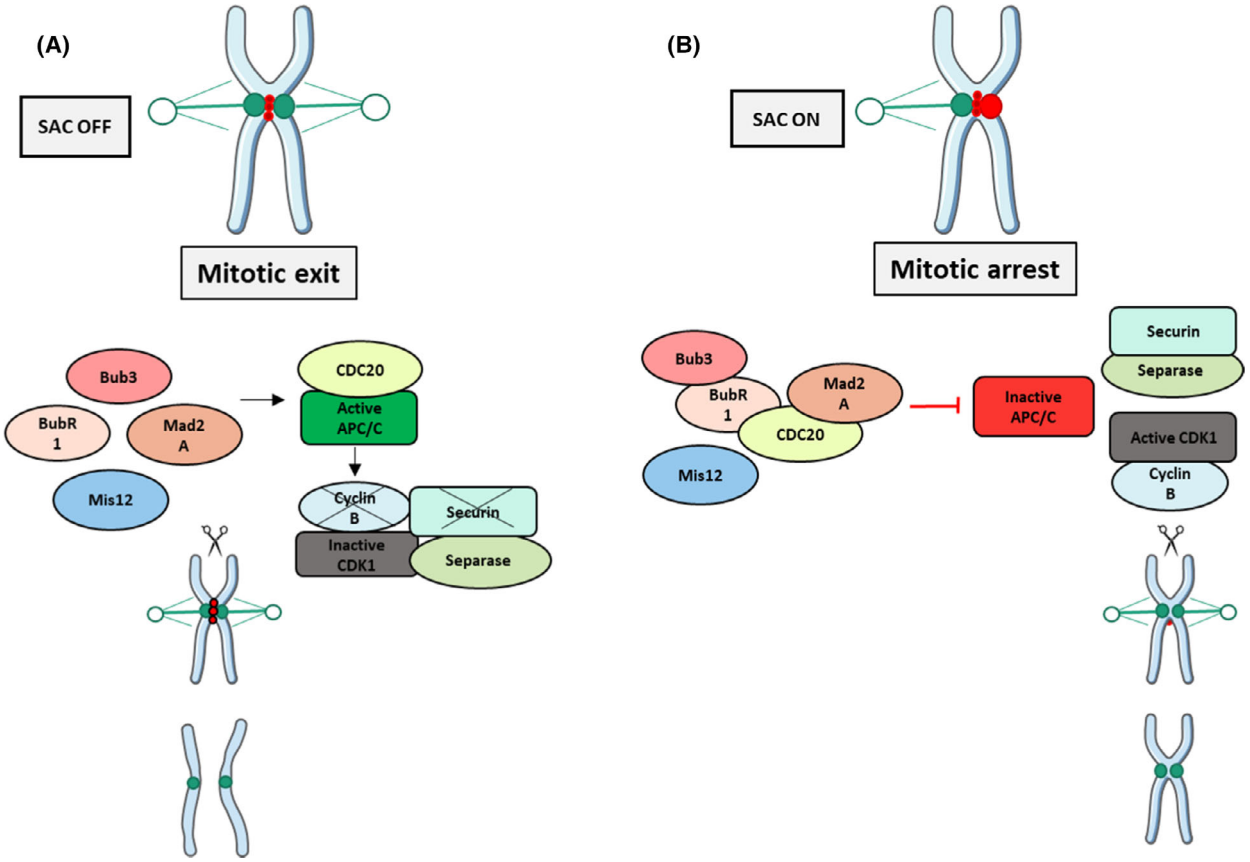

Fig. 7. Speculative model for the coordination of the metaphase–anaphase transition. Mitotic and meiotic chromosome segregation is controlled by spindle assembly checkpoint (SAC). (A) The unpaired and mismatched kinetochores activate the SAC signals and block the cell cycle by keeping the anaphase-promoting complex (APC/C) inactive. (B) A correct and stable microtubule's attachment is sufficient for SAC silencing and mitotic exit via the activation of the APC/C.

segregation during mitotic and meiotic events [20,51–53] In our study, the mRNA expression of genes establishing the normal spindle-kinetochore interaction during cell divisions was assessed by qPCR. A significantly lower expression of these genes was observed at stage 2 in beta triploid male oysters, suggesting their downregulation during mitosis and meiosis.

The segregation of the chromosomes during the mitosis or meiosis requires their attachment to the kinetochore, a large protein complex which ensures the assembly of the centromere of each chromosome to the spindle of microtubules. This complex is controlled by a molecular surveillance mechanism that checks the correct microtubule attachment, named spindle assembly checkpoint (or SAC) (Fig. 7). During equational meiosis (as during mitosis), and once all centromeres are associated with the microtubules, the SAC is silenced, therefore allowing the mitotic exit via the activation of the APC/C (Fig. 7A). More precisely, when all kinetochores are correctly associated with the microtubules at the prometaphase, the activation of APC/C^{CDC20} promotes Cyclin B and Securin proteolysis by the proteasome. Thus, by inhibiting the APC/C, the MCC stabilizes these substrates and their destruction induces a mitotic exit [54,55]. Then, the separation of sister chromatids takes place under the control of a cohesion protease separase such as Rad21 [56,57]. During the unpaired attachment of the microtubules to the kinetochore, the SAC signal is activated and locks the cell cycle by inactivating the anaphase-promoting complex (APC/C) (Fig. 7B). Then, the SAC acts through numerous effectors forming the mitotic checkpoint complex (MCC). From their effectors of the MCC, the three SAC proteins Mad2l1, BubR1, and Bub3, in interaction with CDC20, participate to the formation of the outer kinetochores, that is, protein complexes formed by numerous proteins including Mis12. They bind and then inhibit the APC/C^{CDC20} complex required to prevent the entry into the anaphase. Based on our results, we can posit the hypothesis of a potential impairment during meiosis in beta triploid male oysters. During the first division, homologous chromosomes may be held together through chiasmata that are supported by cohesion along chromosome arms. Sister kinetochores would be associated with each other and thus co-oriented. Homologous kinetochores would be pulled by spindle microtubule into the opposite pole (homologous biorientation). The homologous recombination of chromosomes during meiosis would make the kinetochore's attachment to the chromosome more difficult [58]. In male oysters, this step may occur at stage 2 of the gametogenesis, when germ cells differentiate from gonia to spermatozoa. In beta triploid oysters, the downregulation of SAC actors at stage 2 could reduce the chances of spindle chromosome attachment. In addition, the decrease in cdc20 expression level would lead to a decreased activation of APC. Maintaining SAC activation could result in a cell cycle arrest and lock the entry of the cell into the anaphase during the meiosis. Therefore, a deregulation of the meiotic checkpoint may induce an impairment in the chromatids segregation in beta triploid oysters. Furthermore, a downregulation of the cohesion rad21 may induce chromosome instability, thus amplifying the locking of germ cell in beta triploid oysters. All this would therefore prevent the germ cells from completing their meiosis. In contrast, in alpha triploid oysters, the expression profile of the kinetochore actors was similar to that of diploid oysters, which would suggest an activation of the APC, and therefore, a normal segregation and a meiosis exit.

Then, our work allowed us to assume that the lower reproductive effort of beta triploid male oysters may be (a) first due to disturbances in the establishment of the gonadal tubules and to a locking of germ cells due to a misregulation of the chromatin remodeling by modifications of epigenetic marks, during quiescent and mitotic stages of spermatogenesis (stages 0 and 1) and (b) in a second time, to an aberrant segregation of chromosomes during the meiosis due to the perturbation of the SAC mechanism. Hence, these results lead to a more detailed study of the actors of the chromatin structure, through the analysis of the post-translational modifications of histones (methylation, acetylation essentially) and associated proteins (as isoforms of HP1, LBR, Lamins) and their expression during the complete spermatogenesis. It is also important to follow the proteomic expression of the kinetochore actors.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

CL and FM designed the experiment. FM, NE, NVN, ML, and CL performed the experiments. FM, ASM, and CL analyzed the data. FM, CL, and ASM wrote and edited the manuscript, and ASM and CL supervised the project.

Data accessibility

The data presented in this study will be made available upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Histological cross sections of *Crassostrea gigas* male gonadal area at stage 1 of gametogenesis (gonial proliferation). The gonadal area is composed of the gonadal tubule (GT) and of the storage tissue (ST). Diploid (A) and alpha triploid male oysters (B) present a proliferation of germinal lineage in the gonadal tubule with the figures of mitosis (M) especially for diploid oyster whereas beta triploid oyster (C) exhibit locking events (clear cytoplasmic area: asterisk and condensed nuclei: arrow).

Appendix S2. Gametogenetic stages in male diploid (2n) and triploid (3nα: 3n alpha and 3nβ: beta) oysters. (stage 0: sexual resting period, stage 1: gonial proliferation, stage 2: maturation, and stage 3: sexual maturity).

ARTICLES FOR FACULTY MEMBERS

PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

Title/Author	**Refinement of a classification system for gonad development in the triploid oyster Crassostrea gigas / Yang, Q., Yu, H., & Li, Q.**
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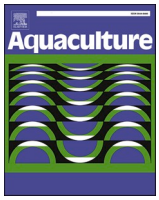

Refinement of a classification system for gonad development in the triploid oyster *Crassostrea gigas*

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ABSTRACT

Breakthroughs in polyploidy technologies have enabled the scaling-up of the commercial utilization of phenotypically-sterile triploid *Crassostrea gigas*. Compared with diploid oysters, gonad development in triploids is retarded, but not absent. Although histological methods of characterizing gonadal development in triploid *C. gigas* has been reported, the selection of sampling sites and densities might lead to bias when taking seasonal variations into consideration. Moreover, previously-employed classification systems were found to be incompatible with our long-term observations. In this study, by sampling Pacific oysters at different seasons, over several years and from different locations, we were able to establish a new classification system for gonadal development in triploid Pacific oyster based on histological analysis. Based on the types of germ cells, triploids were grouped into female (divided into female α and female β), male and hermaphrodites during the reproductive season. Surprisingly, female α was partially sterile, showing active gametogenesis and few abnormal germ cells, which were defined as β gonia. Unlike female α, triploid female β had the most severely retarded gonad along with abnormal morphological features in the gonia. We inferred that β gonia were correlated with female triploid sterility. Despite the presence of numerous gametes in triploid male gonads, the fecundity of triploid males was still severely reduced compared with that of diploids. In addition, maturation arrest of spermatogenesis in triploid males was detected histologically. Based on the types of germ cells and the presence of β gonia, hermaphrodites were further divided into hermaphrodite I and hermaphrodite II. An almost male-like triploid was redefined as a hermaphrodite triploid. The gonadal development of triploids from different sites was mostly similar. As shown via annual histological analysis, the increase in the percentage of female α and the decrease in the percentage of female β matched the seasonal development of the oyster gonad. Hence, we speculated that gonad development in female triploid oysters was delayed.

1. Introduction

Triploid aquatic species are widely used in aquaculture since their growth, survival rates and flesh quality are significantly enhanced compared to diploids (Rasmussen and Morrissey, 2007; Maxime, 2010). Optimization of polyploidy technologies has succeeded in producing triploids in many species, such as Atlantic salmon (*Salmo salar*) (Murray et al., 2018), rainbow trout (*Oncorhynchus mykiss*) (Weber et al., 2014), shrimp (Manan and Ikhwanuddin, 2021) and oysters (Nell, 2002). In addition, potential genetic contamination with gene-editing technology has driven researchers to find an alternative method for reproductively sterile aquatic animals. The polyploidy approach is a viable strategy to induce sterility.

In general, triploids have an extra set of chromosomes, which usually prevents meiotic sister chromatids from separating and makes it difficult to produce functional gametes (Zhang et al., 2021). Theoretically, triploids are sterile (Benfey, 1999). However, some triploid aquatic animals are not completely sterile. For example, triploid females of *Oncorhynchus masou* exhibited abnormal gonadal development without germ cells, whereas triploid males showed active gametogenesis (Gray et al., 1993; Tiwary et al., 2001). A similar phenomenon has been reported in several triploid fish (Krisfalusi and Cloud, 1996; Carrasco et al., 1998). In triploid shellfish, the potential to produce gametes and viable offsprings was limited, particularly in triploid oysters (Meng et al., 2012; Suquet et al., 2016; Matt and Allen Jr, 2021). The reproductive capabilities of triploid oysters vary between individuals.

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Relative to diploid oysters, the gonads in triploids were retarded but not absent in Pacific oysters (*C. gigas*) (Guo and Allen, 1994a; Beaumont and Fairbrother, 1991; Gong et al., 2004 ; Suquet et al., 2016). Jouaux et al. (2010) reported that 25% of triploid *C. gigas* could produce numerous gametes, and Hermabessiere et al. (2016) found that 46% of triploids produced numerous gametes. In *Crassostrea virginica*, 19% of triploids were observed with substantial numbers of gametes (Matt and Allen Jr, 2021). Moreover, fertile triploid oysters could be found in both sexes, and mating experiments using triploid parents could produce viable gametes (Guo and Allen, 1994a; Gong et al., 2004; Suquet et al., 2016).

Triploid oysters can be applied as an interesting model to elucidate the effect of triploidy on gametogenesis. Therefore, characterization of gametogenesis and a reasonable and scientific classification system developed for gonadal development in triploid oysters are necessary for further studies. Jouaux et al. (2010) previously established a classification method for gametogenesis in triploid Pacific oysters. Two types of gametogenic patterns were defined: α-patterns, which had a great number of gametes, and β-patterns, showing abnormal, disturbed gametogenesis. The appearance of the β-pattern was due to the presence of abnormal gonia. However, this classification system in triploid Pacific oysters seemed inapt for triploid *C. virginica* because gonad development in triploid *C. virginica* was quite different from that described by Jouaux et al. (2010) for triploid *C. gigas*. The fecundity described in triploid *C. gigas* was not observed in triploid *C. virginica* (Matt and Allen Jr, 2021). Based on the features of gonia and the presence of mature gametes, a classification system was developed to assess gonad development in triploid *C. virginica* (Matt and Allen Jr, 2021). Matt and Allen Jr (2021) hypothesized that the nature of gonia indicated sex, of which 'normal' gonia were associated with males, while irregular gonia were associated with females. In addition, they proposed that it was difficult to determine the stage of gametogenesis in triploids because of the lack of synchrony between follicle development and gamete production. Therefore, it is of great interest to note the stark difference in gonad development between the two *Crassostrea* oysters. In addition, different sampling sites and densities might cause bias when considering seasonal variation. Attention has been given to specific gonad development in triploid *C. gigas* over recent years. Jouaux et al. (2010) documented similar phenomena in which abnormal gonia and a small portion of triploid oysters contained numerous gametes. Nonetheless, the classification criteria proposed by Jouaux et al. (2010) did not fully explain our results based on long-term observations of triploid *C. gigas*. Some findings bear a marked resemblance to those described in triploid *C. virginica* (Matt and Allen Jr, 2021).

In this study, we aimed to characterize the gametogenesis of triploid Pacific oysters from different sites and compare them with diploids through fine histological analysis. The extent of gonad development in triploid Pacific oysters during an annual reproductive cycle was analyzed to obtain a better understanding of the fecundity of triploids. We developed a method to distinguish the sex of triploid *C. gigas* in early development using the nature of the type of germ cells and proposed a new classification system for gonad development in triploid Pacific oysters based on histological observations of a great number of samples. This study provided new insights into the gonad development of triploid oysters and valuable information to the aquaculture industry. Since triploid eggs have been successfully used to induce tetraploid oysters (Guo and Allen, 1994b), understanding gonad development in female triploids is of great significance for the induction of tetraploid oysters.

2. Materials and methods

2.1. Sample collection

All diploid and triploid Pacific oysters in this study were located in Shandong Province in China. The triploids were bred by crossing tetraploids and diploids. Two hundred triploids and 100 diploids were cultured in Rushan (Rs), Shandong (36.4 N, 121.3 E) and were sampled in June 2018. To assess the annual development cycle of gonads in triploid Pacific oysters, a total of 1000 one-year-old crossed triploids and 700 one-year-old diploids were cultivated in Qingdao (Qd), Shandong (36.2 N, 120.6 E), in 2018. The diploid and triploid oysters in Qingdao (Qd) were collected each month from April 2019 to March 2020. In April, 100 diploid and 100 triploid *C. gigas* were collected. From May to July, 100 diploids and 200 triploids were collected, while 40 diploids and 40 triploids were collected from other months. To compare gonad development of triploids in different locations in the same year, 300 crossed triploids and 100 diploids in Rongcheng (Rc), Shandong (37.1 N, 122.4 E) were collected in June 2019.

2.2. Ploidy verification

Before sampling, ploidy was evaluated in all triploid Pacific oysters. After dissection, approximately 1 mm^3 of gill tissue was dissected in 1 ml PBS (phosphate buffer saline) and diluted in 3 ml 100% ethanol. After a short centrifugation (300 *g*, 25 °C, 5 min) and removal of the supernatant, cell pellets were resuspended in 1 ml PBS. Thirty microliters of the solution containing propidium iodide was added. After a 20 min incubation, ploidy was analyzed using flow cytometry (CytoFlex Beckman Coulter, US).

2.3. Histology

Gonad tissue from each sample was fixed in Bouin's fluid for 24 h and then stored in 70% ethanol. Subsequently, sections were dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin. The sections were observed and photographed using an Olympus BX53 microscope (Olympus, Japan).

2.4. Gonad development

Histological assessment covered each triploid and diploid. Stages of gametogenic development of diploids were assigned according to Franco et al. (2008) and Normand et al. (2008). Diploids were divided into six stages: stage 0: inactive stage; stage I: proliferative stage; stage II: growth stage; stage III: mature stage; IV, spawning stage; and V, reabsorption stage, based on the morphology of the follicles, follicle contents, and follicle coverage.

As gonad development in triploids deviated considerably from that in diploids, we did not adopt the standard of diploids to judge the stage of triploid gonad development. The observation of triploid gonads was mainly through the type of germ cells, the number and location of gametes, and the morphology of the follicles. Different gonads of triploids from the same time and location were compared and classified. To further analyze the differences in triploid gonad development, diploid and triploid gonads at the same time and location were compared.

3. Results

3.1. Classifying gonad development in triploids during the reproductive season

The gonad development of triploids was abnormal, which was quite different from that of diploids. One of the most striking differences was the abnormal gonadal cells in triploids, as shown in Figs. 1, 2, and 3 (arrowhead). Abnormal gonadal cells always presented with condensed and rod-shaped chromosomes that were acidophilic. This kind of gonadal cell was along the inner side of the gonadal tubule with a changeable number and was named β gonia according to Matt and Allen Jr (2021). We found that β gonia were present in most triploid gonads and throughout the course of gametogenesis. The presence of abnormal cells (β gonia) and the relative abundance of gametes and triploids were classified according to the types of germ cells. Because germ cells could be observed during the proliferative stage, the categories of triploids

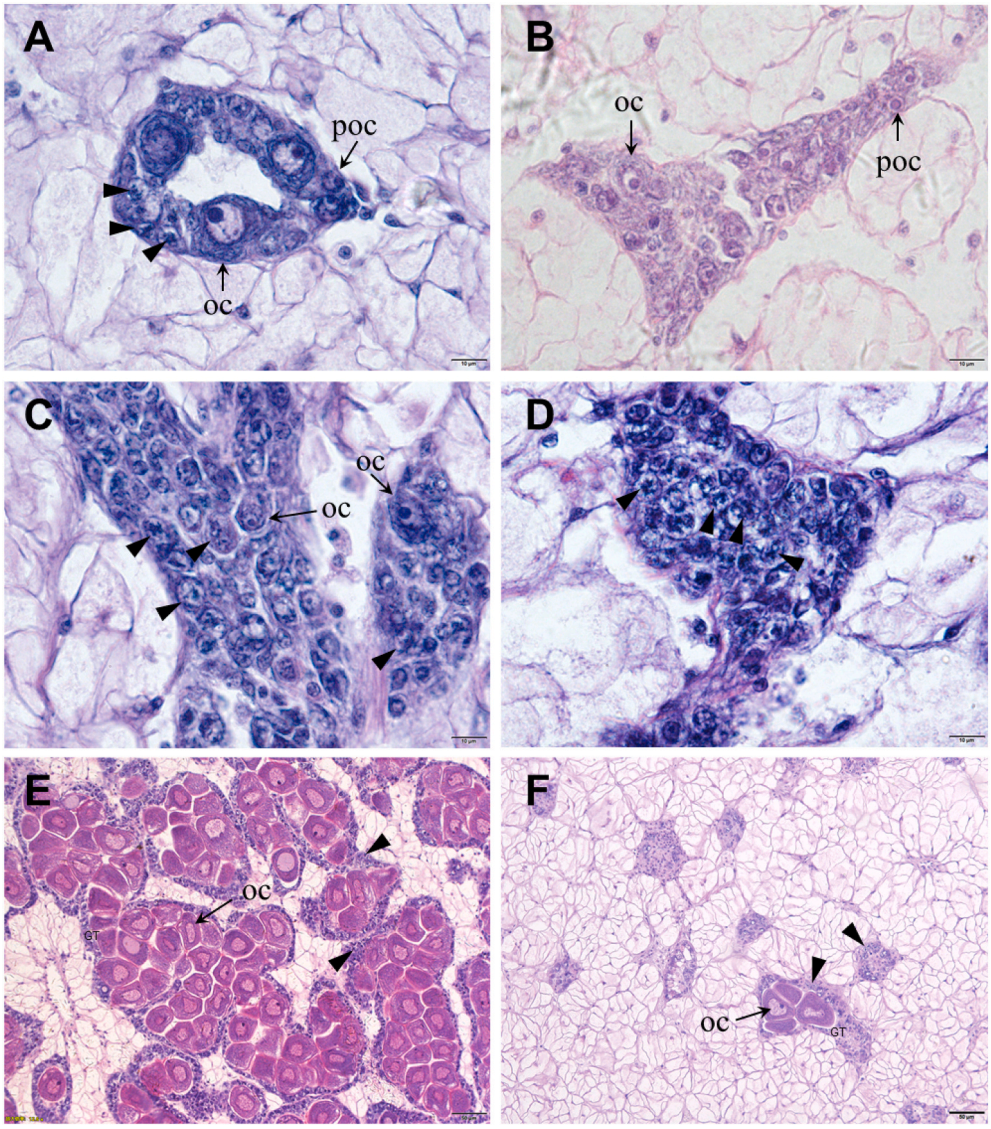

Fig. 1. Gonadal follicles of female diploid and triploid *C. gigas*. A, C&D. Female triploid at early stage; B. Female diploid at early stage; E. Female α triploid; F. Female β triploid. GT: gonadal tubule; poc: previtollogenic oocyte; oc: oocyte; β gonia (arrowhead). A–D, Scale bar = 10 μm; E–F, Scale bar = 50 μm.

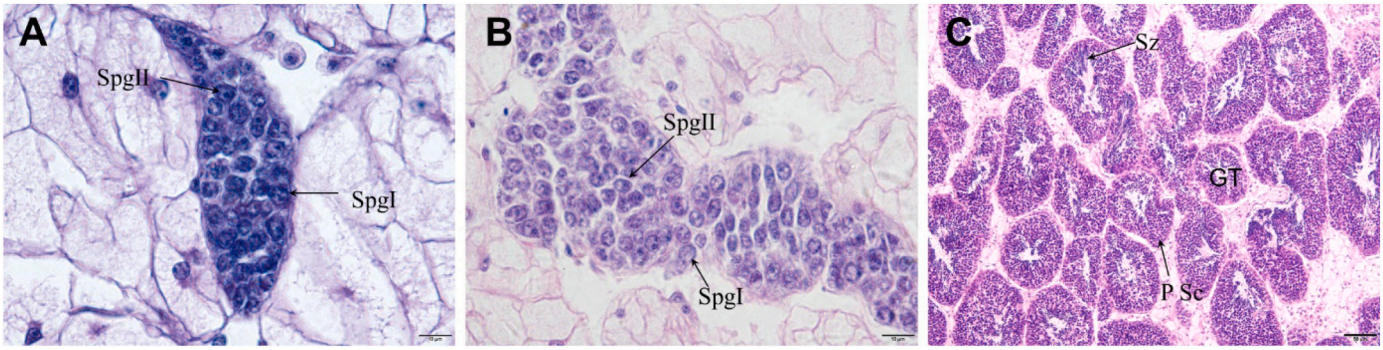

Fig. 2. Gonadal follicles of male diploid and triploid *C. gigas*. A. Male triploid at early stage; B. Male diploid at early stage; C. Male triploid. GT: gonadal tubule; SpgI: Type I spermatogonium; SpgII: type II spermatogonium; P Sc: primary spermatocyte. Sz: spermatozoa. β gonia (arrowhead). A–B, Scale bar = 10 μm; C, Scale bar = 50 μm.

started from the early development of triploid gonads.

3.1.1. Female

The sex of triploids can be distinguished in the early stage of gonad development. At the early development of triploids, previtellogenic

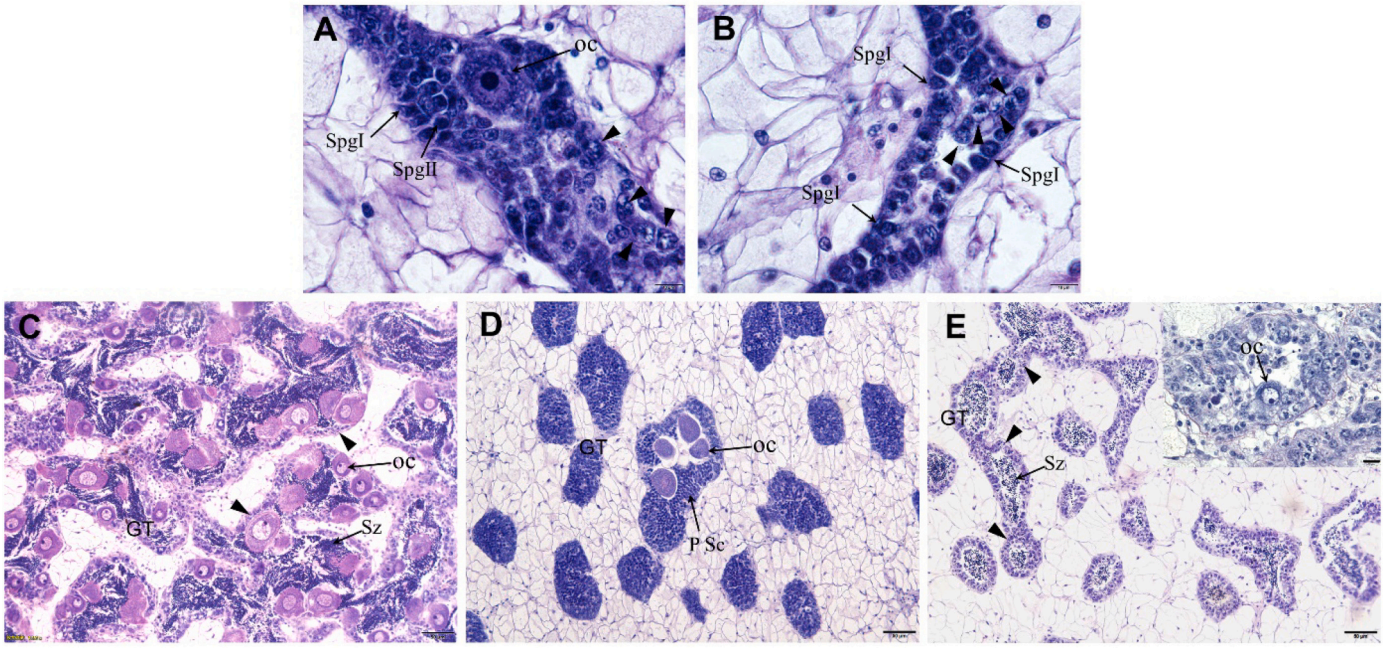

Fig. 3. Gonadal follicles of hermaphrodite triploid *C. gigas*. A& B. Hermaphrodite at early stage; C&F. Hermaphrodite I; E. Hermaphrodite II. GT: gonadal tubule; SpgI: Type I spermatogonium; SpgII: type II spermatogonium; P Sc: primary spermatocyte. Sz: spermatozoa; oc: oocyte; β gonia (arrowhead). A–B, Scale bar = 10 μm; C–E, Scale bar = 50 μm.

oocytes, oocytes and β gonia could be observed (Fig. 1A). There was an obvious nucleolus in the center of the previtellogenic oocyte, which accounted for a great proportion of the cells. The volume of the oocytes was larger than that of previtellogenic oocytes. In comparison, female diploids at early gametogenesis consisted of primary oocytes of homogeneous size (Fig. 1B), while the size of oocytes in female triploids was inhomogeneous (Fig. 1A). Sometimes β gonia occupied a large proportion of the follicle. As shown in Fig. 1C, two oocyte were detected, while the others were β gonia. Some follicles were even filled with β gonia (Fig. 1D).

With the development of gonads in female triploids, fecundity varies from individual to individual. The number of oocytes in triploid was varied. Some produced plenty of oocytes, while some produced few oocytes. To distinguish these two kinds of female triploids, female α and female β were used.

3.1.1.1. Female α. The female α triploid produced many oocytes (Fig. 1E). Follicles were filled with mature oocytes, and β gonia were distributed at the inner side of follicles. The gonads of female triploids were generally inhibited, and the number of oocytes in triploids was generally lower than that in diploids. However, a few female α were similar to diploids in the mature stage per histological sections, in which oocytes filled the gonad without connective tissue. In our study, only seven female triploids from 456 triploids in June were close to diploids.

3.1.1.2. Female β. Most triploid females were classified as female β, which had no or a few oocytes (Fig. 1F). The follicles of female β always consisted of β gonia.

3.1.2. Males

The follicles of male triploids contained closely arranged and orderly spermatogenic cells (Fig. 2). The gonads of male triploids (Fig. 2A) were similar to those of diploids (Fig. 2B) during early gametogenesis. However, with the development of gonad, the gonads of male triploids did not change substantially, and exhibited numerous primary spermatocytes, spermatids and few sperm in the follicles of male triploids, similar to the diploids of the same growth stage (Fig. 2C). Primary spermatocytes or spermatogonia were present along the follicle wall of male triploids, while a few spermatids were at the center of the follicle. No male triploid that produced as many spermatozoa as the mature diploids was observed. In addition, β gonia were absent in male triploids.

3.1.3. Hermaphrodites

Hermaphrodites could be identified according to gametogenesis cells and β gonia at early gametogenesis (Fig. 3). As shown in Fig. 3A, oocytes, primary spermatocytes and β gonia were observed at the same follicle. Primary spermatocytes and β gonia without oocytes were found in some hermaphrodites (Fig. 3B). Through histological observation, two types of hermaphrodites could be identified based on germ cells: hermaphrodite I (Fig. 3C) and hermaphrodite II (Fig. 3D). The major difference between the two types of hermaphrodites was the presence of β gonia.

3.1.3.1. Hermaphrodite I. Most triploid hermaphrodites were classified as hermaphrodite I, which contained spermatogenic cells, oocytes and β gonia. β gonia were present along the follicle wall of hermaphrodite I. Spermatogenic cells in hermaphrodite I were apparent, including primary spermatocytes, spermatids and sperm. In most cases, mature spermatids were more abundant than other spermatogenic cells in follicles. Many sperms detached from the germinal lining of the wall. The number of oocytes was variable. Sometimes it was difficult to observe oocytes in histological sections. As shown in Fig. 3E, the triploid contained spermatozoa and β gonia without oocytes, but when more sections of this sample were observed, the oocytes could be found (shown in the small picture). Thus, we also classified this type of triploid as hermaphroditic I.

3.1.3.2. Hermaphrodite II. Hermaphrodite II was rarely observed. Gonadal tubules of hermaphrodite II were filled with a number of spermatocytes surrounded by a few oocytes, and no β gonia were observed.

3.2. Categories of triploids in different locations

To assess gonad development in triploids from different locations,

triploids from Rushan (Rs), Rongcheng (Rc) and Qingdao (Qd) were classified according to the system standard mentioned above (Table. 1). The diploids, collected at the same time, were already in the mature stage (Supplement Fig. S1). The ratios of triploids assigned to five categories were similar at the three sites. The percentage of female β was the largest, and ranged from 44.44% to 52.20%. Hermaphrodite I ranged from 26.42–33.33%. Hermaphrodite II accounted for a very small proportion, with only six individuals found. The percentages of females α and males were similar, and were 5.56%–13.21% and 7.55%–12.59%, respectively.

3.3. Annual analysis of gonad development in triploids

Annual histological analysis of gonad development in 1000 triploid Pacific oysters began in April 2019 and lasted over a period of one year at Qd. The gonad development of triploid *C. gigas* could be divided into two phases: the gametogenesis phase from April to August and the reproductive inactivation phase from September 2019 to March 2020. Males and females were indistinguishable at the reproductive inactivation phase. Thus, only the triploid oysters of the gametogenesis phase were classified (Fig. 4); the gonadal development of triploids of the other phase were presented in Supplement Fig. S2-S4. From April to August 2019, the percentage of female α increased from 2.41% to 35.14%. From April to June 2019, the percentage of female β increased from 32.53% to 52.20% and then decreased to 16.22% in August. The percentage of males changed slightly in April (34.95%) and May (30.30%) and then decreased in July and August (22.22% and 24.32%) but reached its lowest value in June (7.55%). The ratio of hermaphrodite I decreased from April (30.12%) to July (13.89%). Hermaphrodite II made up a small, relatively unchanging percentage (0–4.05%).

In September, the diploids were in the reabsorption stage, while most triploids were in reabsorption except that some triploids spawned (Supplement Fig. S2). From October 2019 to February 2020, gonads of diploids and triploids were at resting (Supplement Fig. S3). In March 2020, the diploid and triploid gonads began to redevelop (Supplement Fig. S4).

3.4. Comparison of gonad development between diploid and triploid males

We compared diploid and triploid males in the same culture area from April to September 2019 (Fig. 5 and Fig. 6). The gonad tubules of diploid males in April contained a number of primary spermatocytes (Fig. 5A). Triploid males were similar to diploid males (Fig. 6A). In May, the diploid male gonads were filled with spermatogenic cells without connective tissue, and spermatocytes, spermatocytes, or spermatozoa appeared at the same time (Fig. 5B). For triploids, there was space between follicles, and spermatocytes and spermatocytes appeared (Fig. 6B). In June, diploids were in the mature stage, and the follicles were full of mature sperms. Follicles became loose, and sperms began spawning in July. During August, connective tissue appeared again, and sperms became less abundant. However, with the growth of gonads from June to August, the follicles in male triploids seemed to stop spermatogenesis, and the gonads looked similar, containing many spermatocytes and spermatocytes and few spermatozoa. In September, gonadal tubules of diploids were empty, while the composition of triploid follicles did not change.

Table 1
Percentage of individuals classified in each category of gonad development.

	Female α	Female β	Male	Hermaphrodite I	Hermaphrodite II
2018 Rs	10.37%	44.44%	12.59%	30.37%	2.22%
2019 Qd	13.21%	52.20%	7.55%	26.42%	0.63%
2019 Rc	5.56%	48.77%	11.11%	33.33%	1.23%

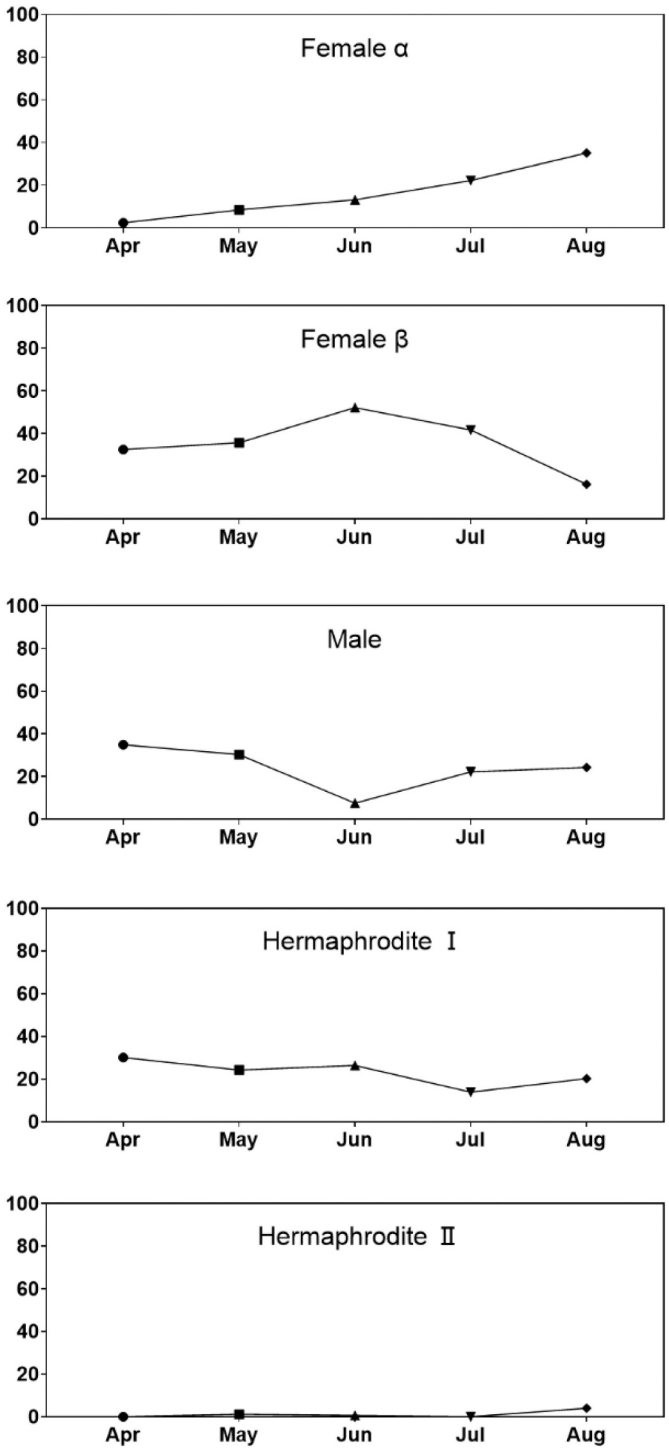

Fig. 4. Percentage of triploid *C. gigas* individuals of each category of gonad development sampled from Qingdao, Shandong.

4. Discussion

4.1. Characteristics of triploid gonad development

Our findings indicated that triploid oysters were characterized by abnormal gonad cells and β gonia. In keeping with previous reports, β gonia had rod-like condensed chromosomes that were basophilia

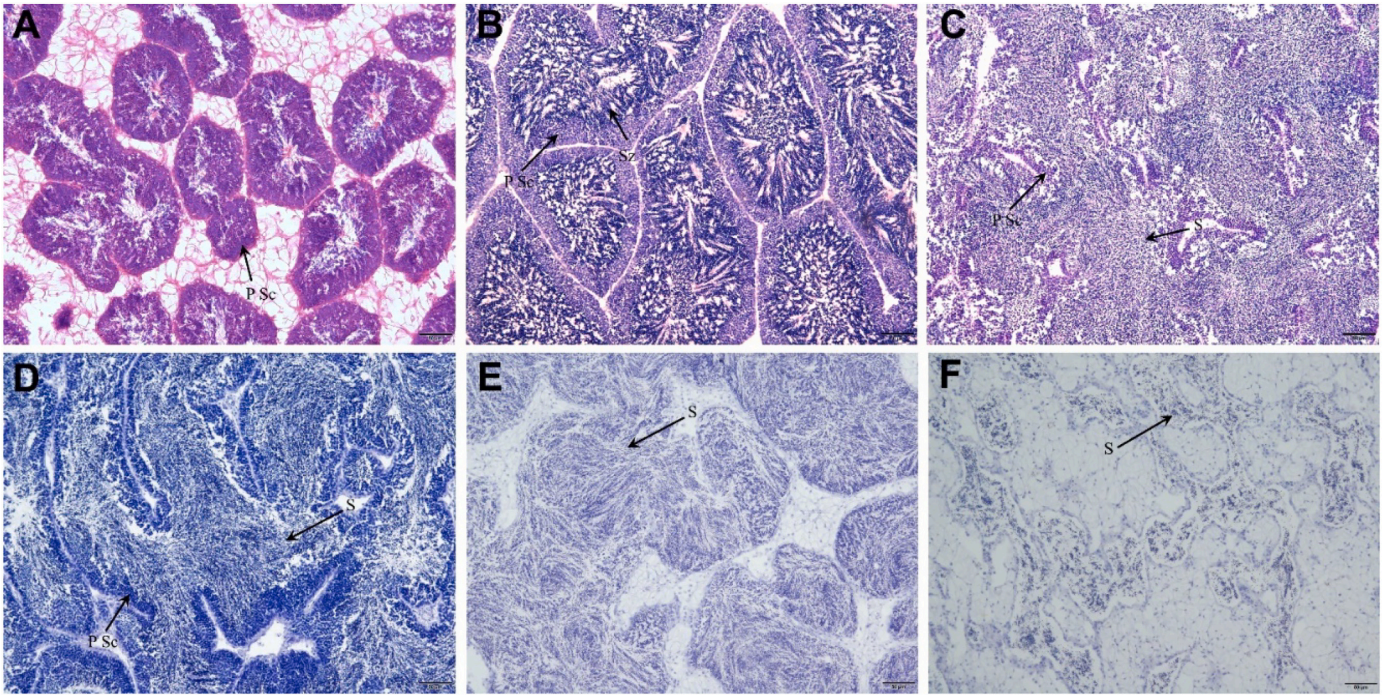

Fig. 5. Male diploids from April to September sampled from Qingdao, Shandong. A. April; B. May; C. June; D. July; E. August; F. September. Scale bar = 50 μm.

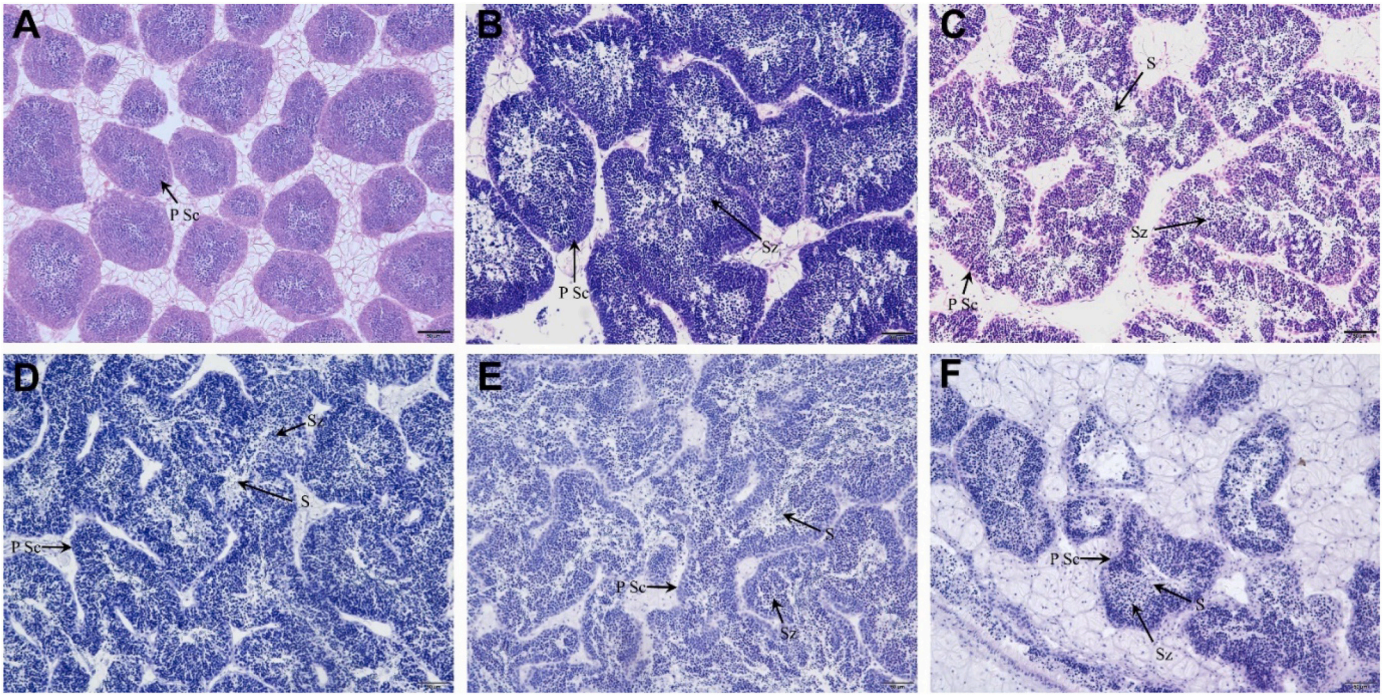

Fig. 6. Male triploids from April to September sampled from Qingdao, Shandong. A. April; B. May; C. June; D. July; E. August; F. September. Scale bar = 50 μm.

(Jouaux et al., 2010; Matt and Allen Jr, 2021). There were different explanations for the appearance of abnormal gonad cells. Jouaux et al. (2010) found abnormal cells present in sterile triploids that exhibited locking of gonial mitosis events in both males and females. However, Matt and Allen Jr (2021) observed abnormal gonad cells in all triploid *C. virginica* females and referred to them as abnormal oogonia. Consistent with the latter observation, the appearance of β gonia was associated with the oogenesis of triploid *C. gigas*.

β gonia were observed in the early stage of gonad development in triploid *C. gigas*, in accordance with the previous observation by Jouaux et al. (2010). Unlike diploid oysters, males and females had obvious phenotypic differences at stage I. Jouaux et al. (2010) considered that sex identification was never possible for triploid *C. gigas* at that stage due to the abnormal aspect of the gonial proliferating fold in the inner part of the gonadal tubule. Conversely, with fine microscopic observation, apparent oogonia and spermatogonia made it easy to distinguish the sex of triploids. Additionally, we identified β gonia from normal germ cells at the early stage of gametogenesis. In addition, β gonia were present in

triploids throughout the reproductive season, especially in triploids with oocytes. Therefore, β gonia served as an important criterion in our classification system for triploid Pacific oysters. In addition, the number of gametes varied greatly among triploid *C. gigas*. We used female α and female β to define triploid females with more oocytes and fewer oocytes, respectively.

The types of gonadal cells at the early stage largely indicated subsequent gonadal development. At the early stage of triploid gonad development, we observed different patterns of germline cells arrangement. Some gonads exhibited a compact and homogeneous germ cell arrangement in appearance, with deep alkaline staining, indicating an association with male fate. We also found that some male germ cells were interspersed with β gonia at early stage, indicating that these individuals would become Hermaphrodite I. Previtellogenic oocytes and oocytes were found in the early development of triploid female gonads. β gonia were present in all triploid females at the early growing stage, suggesting that β gonia were associated with triploid oogenesis. Based on the number of oocytes and β gonia, the females could be portioned into two types. The individuals with rare oocytes or previtellogenic oocytes but many β gonia in the gonads were female β, and the others were female α. The triploids with oocytes and spermatocytes would undoubtedly be hermaphrodites. The histologic types could be identified at early stage.

High variation in reproductive capacity exists among triploid oysters. The number of gametes in some triploids was comparable to that in normal diploid organisms, while others were sterile (Guo and Allen, 1994a; Eudeline et al., 2000; Normand et al., 2008; Jouaux et al., 2010). It was quite difficult to define the stage of gonad development in triploids. In our study, we found that the beginning and end of the reproductive period in triploids were similar to those observed in diploid gonads. Thus, to facilitate the study of triploid gonads, we suggested that judging the stage of triploid gonad development could refer to diploids raised at the same time and same area, which could be classified as stage 0, inactive stage; stage I: proliferative stage; stage II: growth stage; stage III: mature stage; IV, spawning stage; and V, reabsorption stage.

4.2. The sterility of triploid females

Sterility is common in triploid organisms. The meiosis process was obstructed in triploids because the extra haploid set of chromosomes made it difficult to develop functional gametes (Zhang et al., 2021). In practice, fecundity varies in triploids from different species. In *C. gigas*, oocyte maturation was arrested in the metaphase of the first meiosis (Li et al., 2000). Given the high error rate seen in meiotic products (Martin, 2008), the level of sterility in females was supposed to be lower. In fact, it was generally high in females, with 25% of triploids producing plenty of eggs (Jouaux et al., 2010). In our study, 5.56% ~ 13.21% of triploids produced numerous oocytes.

According to histological analysis, the percentage of female α increased gradually with gonad development, while the percentage of female β decreased in July and August, which matched the seasonal development of the oyster gonad. Therefore, we speculated that gonad development in female triploid oysters may be delayed. As previously reported, the diameter of mature eggs in triploid oysters was 11% larger than that of diploids (Jeung et al., 2016). The growth of triploid eggs requires more energy material than diploid eggs, which may result in a delay in gonad development in female triploids. Abnormal cells, β gonia, associated with undeveloped oocytes have already been mentioned above. We hypothesized that β gonia were directly related to female triploid sterility. However, studies on β gonia were quite limited.

4.3. The fecundity of triploid males

To reveal fecundity in triploid males of *C. gigas*, we specifically compared gonad development between diploid and triploid males. The results showed that normal diploids underwent the normal maturation of spermatogenesis during the reproductive season. However, when oysters were in the growth stage, the gonads of male triploids were similar to those of diploids only in April (Fig. 4A and Fig. 5A). After that, the gonad development of the triploid males seemed to be arrested, along with the maintenance of the dominance of spermatocytes and spermatids and the minority of sperms in gonad tubules. The same phenomenon was also reported by Allen and Downing (1990). Similarly, Xiang et al. (2006) claimed that even though sperms were observed in the vas deferens of triploid shrimp, ultrastructural observation indicated that they were not mature sperm but spermatids. In addition, comparative analysis of testis transcriptomes in cyprinid fish revealed that genes associated with sperm flagellar assembly and motility were downregulated in triploid fish, suggesting that the variational expression of these genes may be a critical factor for sperm formation and lead to sterility of triploid male fish (Xu et al., 2015; Li et al., 2019). Previous studies speculated that male triploid *C. gigas* could proceed with early meiosis normally and produce spermatocytes, but problems occurred in spermatocytes and spermatids in later meiosis (Lee, 1988; Allen and Downing, 1990; Barber and Mann, 1991; Guévélou et al., 2019). Thus, we speculated that unusual meiosis and obstacles of spermatozoa assembly may lead to the reduced fecundity of triploid males.

Additionally, the diploid and triploid oysters entered the resting stage simultaneously at the end of the reproductive season. We did not find any evidence that gonad development of male triploids was retarded. Although the gonads of triploid males contained numerous gametes, most of them might not be able to develop into mature sperm. The fecundity of male triploid *C. gigas* was still severely reduced, which was overestimated in the previous study (Jouaux et al., 2010). Moreover, the male-like triploid, which was regarded as β male triploid and a kind of sterile triploid by Jouaux et al. (2010), was confirmed to be hermaphrodite triploid in the present study.

Credit author statement

Qiong Yang: Writing - original draft. **Hong Yu**: Conceptualization, Writing - review & editing. **Qi Li**: Writing - review & editing.

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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ARTICLES FOR FACULTY MEMBERS

PRODUCTION OF TRIPLOID OYSTER TO ENHANCE OYSTER CULTURE PRODUCTION AND MARKETABILITY

Title/Author	**Transcriptomic and metabolomic analyses provide insights into the growth and development advantages of triploid Apostichopus japonicus / Xie, J., Sun, Y., Cao, Y., Han, L., Li, Y., Ding, B., Gao, C., Hao, P., Jin, X., Chang, Y., Song, J., Yin, D., & Ding, J.**
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ORIGINAL ARTICLE

Transcriptomic and Metabolomic Analyses Provide Insights into the Growth and Development Advantages of Triploid *Apostichopus japonicus*

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Abstract

Polyploid breeding is widely used in aquaculture as an important area of new research. We have previously grown *Apostichopus japonicus* triploids with a growth advantage. The body length, body weight, and aestivation time of triploid and diploid *A. japonicus* were measured in this study, and the transcriptome and metabolome were used to examine the growth advantage of triploids *A. japonicus*. The results showed that the proportion of triploid *A. japonicus* with a body length of 6–12 cm and 12–18 cm was significantly higher than that of diploid *A. japonicus*, and triploid *A. japonicus* had a shorter aestivation time (39 days) than diploid (63 days). We discovered 3296 differentially expressed genes (DEGs); 13 DEGs (for example, *cyclin-dependent kinase 2*) related to growth advantage, immune regulation, and energy storage were screened as potential candidates. According to Gene Ontology (GO) enrichment analysis, DEGs were significantly enriched in the cytoplasm (cellular component), ATP binding process (molecular function), oxidation–reduction process (biological process), and other pathways. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment data, DEGs were significantly enriched in ribosome production and other areas. We discovered 414 significant differential metabolites (SDMs), with 11 important SDMs (for example, nocodazole) linked to a growth advantage. SDMs are significantly enriched in metabolic pathways, as well as other pathways, according to the KEGG enrichment results. According to a combined transcriptome and metabolome analysis, 6 DEGs have regulatory relationships with 11 SDMs, which act on 11 metabolic pathways together. Our results further enrich the biological data of triploid *A. japonicus* and provide useful resources for genetic improvement of this species.

Keywords Triploid *Apostichopus japonicus* · Transcriptome · Metabolome · Growth · Aestivation

Introduction

Polyploidy, which was first proposed by Winkler (1916), refers to the occurrence of three or more genomes in each somatic cell (Joana et al. 2018). Most polyploids naturally exist in fish but, at present, artificial polyploidy is frequently used. Polyploid breeding first appeared in the artificial induction of triploid carp by Makino and Ojima (Ren et al. 2018). Since then, polyploid breeding of aquatic animals has been widely used in fish and shellfish. Polyploid breeding has good market value in shellfish. Guo invented the production method for tetraploid mollusks (oysters, scallops, clams, mussels, and abalones) in 1995, and proposed the method of producing triploid by mating tetraploid and diploid (Guo and Allen 1994; Guo et al. 1996). Compared with diploid oysters, triploid oysters have the advantages of large size, fast growth (Guo et al. 2009; Guo 2021), and high nutritional value (Qin et al. 2018). At present, polyploid breeding has been applied or studied in more than 40 types of fish and more than 20 types of economic shellfish and crustaceans (Song et al. 2004).

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Apostichopus japonicus (Echinodermata: Holothuroidea: Aspidochirotida: Stichopodidae) is an important economic echinoderm, and is a species known to be of the best quality among the more than 20 types of edible sea cucumbers. However there have been few studies on the polyploid breeding of echinoderms, except that Chang successfully induced triploid and tetraploid *A. japonicus* using cytochalasin B and 6-dimethylaminopurine (6-DMAP), and examined the inducing drug concentration, treatment time, treatment start time, and the survival rate of larvae (Chang and Xiang 2002). Ding then proposed the method of inducing triploid *A. japonicus* by hydrostatic pressure (Ding et al. 2007). Han et al (2021) used transcriptome and methylation to study the role of methylation changes of different genes in triploid *A. japonicus*. However, the molecular mechanism of controlling the dominant traits such as fast growth and short aestivation time of triploid *A. japonicus* is not clear. Therefore, we expect to determine the significantly different genes, metabolites, and metabolic pathways related to triploid *A. japonicus* which participate in growth immunity, and understand the relationship between different genes and different metabolites.

In recent years, exploring the growth and development (Sun et al. 2017; Xing et al. 2021), immune function (Li et al. 2018; Shi et al. 2020), and phylogeny (Zhao et al. 2020; Carmona et al. 2017) of aquaculture animals using various omic techniques have gradually become a research hotspot (Chen et al. 2020). The combined analysis of transcriptome and metabolome is widely used to reveal the molecular mechanism in organisms (Wang et al. 2020; Liu et al. 2019; Kong et al. 2020). Therefore, we used the combined transcriptome and metabolome sequencing technology to analyze triploid *A. japonicus*. The different gene expression patterns of triploid and diploid *A. japonicus* have been identified. Some differential genes and metabolites in triploid *A. japonicus* that stimulate growth, metabolism, immune regulation, and protein synthesis have been found and verified, and differential genes and differential metabolites were jointly analyzed. The results in the present study will enrich the basic biological data on triploid *A. japonicus*, and provide resources for the future breeding of triploid *A. japonicus*.

Materials and Methods

Culture and Detection of *A. japonicus*

The triploid *A. japonicus* induced by hydrostatic pressure came from the same batch as the diploid control group produced at the Ministry of Agriculture and Rural Affairs' North Key Laboratory of Marine Aquaculture at Dalian Ocean University, and all were 1.5-year-old *A. japonicus*. During the experiment, the breeding conditions were as follows: water temperature 14 ± 1.5 °C, salinity 30 ± 1, and pH 7.0. During the breeding process, the water was changed every 2 days, and feeding was done once a day (feed formula: sea mud, compound feed, spirulina powder, purslane powder). We used flow cytometry (Sysmex, Japan) to determine the ploidy of *A. japonicus*, and the procedure was the same as Han's (Han et al. 2021).

Analysis of Growth Characters

To determine the ploidy of *A. japonicus*, a tiny amount of tube feet was cut and dissolved in 1.2-ml cell lysate (cystatin UV precise P/05–5002, Japan) in a 1.5-ml centrifuge tube. Flow cytometry was used to detect *A. japonicus* after the cell components had been destroyed (Han et al. 2021). Flow cytometry detected 2682 *A. japonicus*, including 510 triploid *A. japonicus* and 2172 diploid *A. japonicus*. The body lengths of all *A. japonicus* were then measured. To make comparisons easier, we divided the body length of *A. japonicus* into three groups (0–6 cm, 6–12 cm, and 12–18 cm) and compared its proportion in each group (Fig. 1) (Table S1). We compared the aestivation times of triploid and diploid *A. japonicus* which is defined as the time starting with the cessation of feeding, body contraction, and inactivity and ending with relaxation, activity, and feeding (Table 1).

Sample Preparation

In 2020, three *A. japonicus* were dissected in each experimental group, yielding a total of 22 groups of samples, 6 of which were subjected to transcriptome sequencing (3 triploid *A. japonicus*, 3 diploid *A. japonicus*) and 16 of which were subjected to metabolomic analysis (8 triploid *A. japonicus*, 8 diploid *A. japonicus*). The body wall tissues were quickly frozen in liquid nitrogen and kept at −80 °C in a refrigerator.

RNA Extraction and Quality Control

Total RNA was extracted from the body wall tissue of the *A. japonicus* using a Mirvana™ miRNA Isolation Kit, Ambion (USA), and the purity and concentration of total RNA were detected using the Agilent 2100 and Nanodrop (Severino et al. 2013). RNA detected with a purity between 2.0 and 2.1 was stored at − 80 ℃. Gene expression was measured by the FPKM value (Li et al. 2021; Han et al. 2021).

RNA-Seq and Screening for Key Differential Genes and Data Analysis

The Cutadapt program (https://cutadapt.readthedocs.io/en/stable/,version:cutadapt-1.9) was used to eliminate

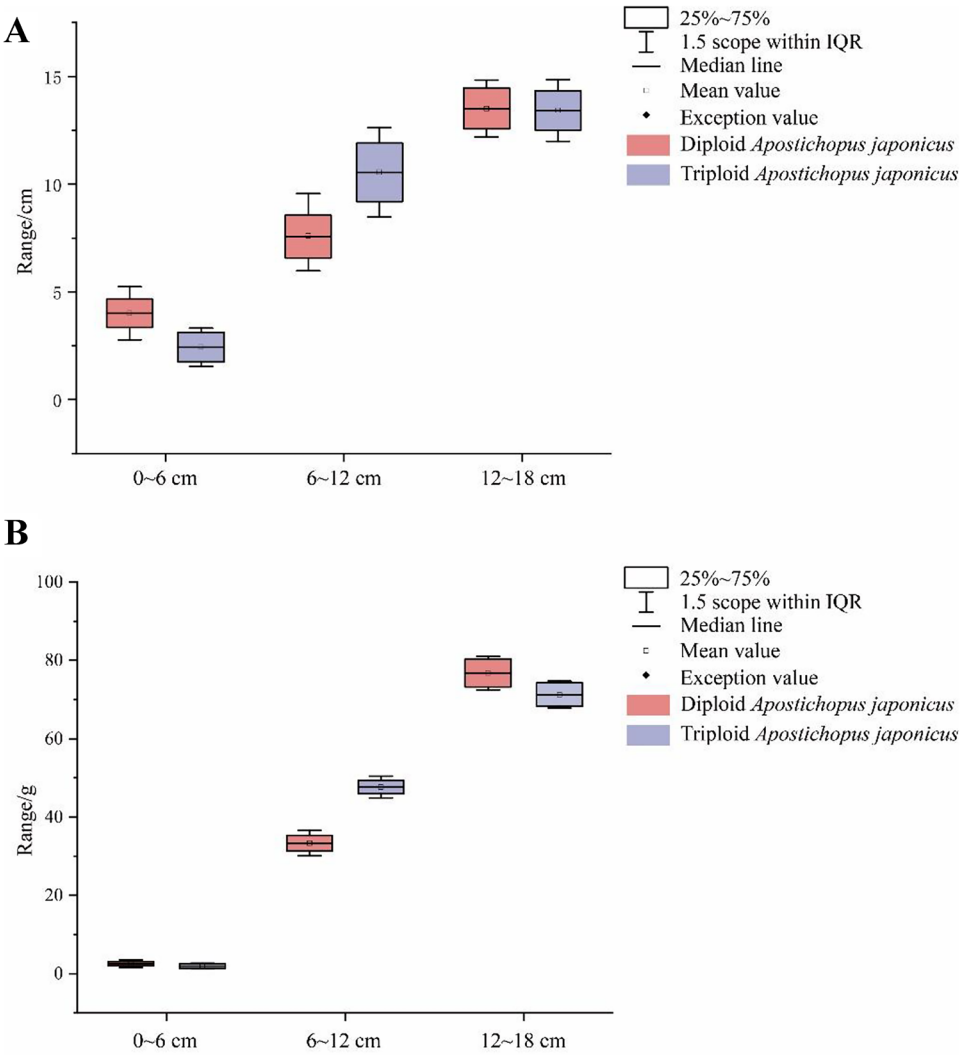

Fig. 1 Box diagram of body length (**A**) and body weight (**B**) of triploid and diploid *Apostichopus japonicus*

adaptor contamination from reads (command line: cutadapt -a ADAPT1 -A ADAPT2 -o out1.fastq -p out2.fastq in1.fastq in2.fastq -O 5 -m 100). After removing the low-quality bases and uncertain bases, we utilized the HISAT2 software (https://daehwankimlab.github.io/hisat2/,version:hisat2-2.0.4) to map reads to the genome. StringTie (http://ccb.jhu.edu/software/stringtie/,version:stringtie-1.3.4d.Linux × 86 64) with default parameters was used to build the mapped readings of each sample. Then, using the gffcompare program (http://ccb.jhu.edu/software/stringtie/gffcompare.shtml,version:gffcompare-0.9.8.Linux × 86 64), all transcriptomes from all samples were combined to rebuild a full transcriptome. StringTie and ballgown (http://www.bioconductor.org/packages/release/bioc/html/ballgown.html) were used to estimate the expression levels of all transcripts and perform expression level for mRNAs by calculating FPKM once the final transcriptome was created. The differentially expressed mRNAs were selected with Fold Change > 2 or Fold Change < − 2 and p-value < 0.05 by R package edgeR (Robinson et al. 2010)

Table 1 Comparison of aestivation time between triploid *Apostichopus japonicus* and diploid *Apostichopus japonicus*

Group	Observation time	Aestivation date	End of aestivation	Aestivation duration
Triploid group	2019	July 27	September 3	38 days
Diploid group	2019	July 12	September 5	55 days
Triploid group	2020	July 24	September 1	39 days
Diploid group	2020	July 9	September 10	63 days

(https://bioconductor.org/packages/release/bioc/html/edgeR.html) or DESeq2 (Pertea et al. 2015) (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html). We improved our understanding of the biological functions of the genes by using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases; enrichment analysis of GO and KEGG was performed using GSEA (gene set enrichment analysis) (https://www.omicstudio.cn/login). All RNA clean data were submitted to the Short Read Archive (SRA) Sequence Database at the National Center for Biotechnology Information (NCBI) (Accession No. PRJNA760261).

qRT-PCR Validation and Analysis of DEGs

Thirteen DEGs were validated by qRT-PCR to further validate the RNA-Seq results (Ge et al. 2020). Primer Premier 6.0 was used to create primer syntheses for the differential genes (Table 2). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of DEGs (Arocho et al. 2006).

Analysis of the Metabolicome, Liquid Phase Analysis, and Mass Spectrometry

For each sample, 100 mg of *A. japonicus* body wall was weighed, ground with liquid nitrogen, and treated before loading the metabolome according to Yu et al. (2018). Before loading, all metabolic samples were kept at − 80 °C. Ultra-high pressure liquid phase and a TripleTOF5600plus (SCIEX, UK) high-resolution mass spectrometer are used for analysis during liquid phase analysis and mass spectrometry. Its model and parameter settings are similar to those of Wang et al. (2019). Quality control (QC) samples were added before, during, and after the test. Prepare QC samples by combining the same number of samples to determine the instrument status and evaluate the stability of liquid chromatography-mass spectrometry (LC–MS/MS) (Li et al. 2019).

Screening of Key Metabolites

The *q*-value by BH correction was calculated using univariate analysis (Fold Change) and the *t* test. PLS-DA VIP values were combined with multivariate statistical analysis to identify metabolites that were differentially expressed. Differential ions fulfilled the following criteria: ratio ≥ 2 or ratio ≤ 1/2, *q*-value ≤ 0.05, and VIP = 1 or higher. The KEGG enrichment pathway was used to investigate the importance of enrichment (Zheng et al. 2019).

Table 2 Primer sequences of the tested genes used in the quantitative RT-PCR analysis

Gene name	Primer (5′ → 3′)
CYTB	F TGACAGGACCGCTACGAAAGAGG R AAAGTTTTCTTGGGGCCGGAAGG
CDK2	F CACTGCTGAAGGAGTTGGACCATG R ATCGGCTGGAGACCTTGACTGG
CDC45	F AACAGACGAAGATCACGCAACCTC R CAAGTTCAGGAAGTGGCGGGATTC
ORC1	F CAGTGACGATGAGGAGGAGGAGAG R GGAGTTGCTGCTTTAGCGGAGAC
GAMT	F GGAAGGGGAAGACTGTAAGAGC R AATAATACGCACAAGAGGCAGG
UGT	F GGCAGTGTTGGATCCGTTGATGG R CGGTGGTGAAGTCGGCATTGG
PGM	F GCAGCAGCCAATCAGGTGAGG R GTGAGTATAATGCCGCCGGTAGC
MRPs	F TCTTAGACAACGGGTGGCAAT R AAGAATGTCGGGTGGTCCTG
GP96	F CAAGTCGAGGAGGATGGTGAAAGC R CCAGCAAAGGCAGCGGAGTC
HSC70	F GCCTACCAGAGAATTGCCACATCC R ACATCGGGCACTCTTTGTTCTACC
HK	F CGGGGAAGTAATTTCAGAGTCC R ACGGCAGCGATCAATGCT
CBRs	F TCACAGGTTCCAACAAGGGC R CTGAGTACATCTCCCCTCTGCC
Histidine methyltransferase	F TCAATACGAAAGCCACCAAATG R CTTTACCGTCCTCTTTCGTCG
DPD	F TCCAGATGCCTCAAGTGCG R CAATCCACTGTCCGTCATCGT

Combined RNA-Seq and Metabolome Analysis

The process of discovering transcriptome and metabolome associations can be broken down into three steps: (1) the KEGG metabolic pathway connects the transcriptome and metabolome, (2) data screening for statistically significant differences in data and regulatory relationships, and (3) GO and KEGG enrichment analysis.

Results and Analysis

Growth Characteristics of Triploid and Diploid *A. japonicus*

The flow cytometer results showed that the peak value of diploid *A. japonicus* was around 200 and that of triploid *A. japonicus* was around 300 (Fig. S6). This outcome is consistent with that of Han (Han et al. 2021). The measurement results revealed that the proportions of triploid *A. japonicus* in the 12–18- and 6–12-cm groups were higher than those in the diploid control group (Fig. 1 and Table S1). Aestivation

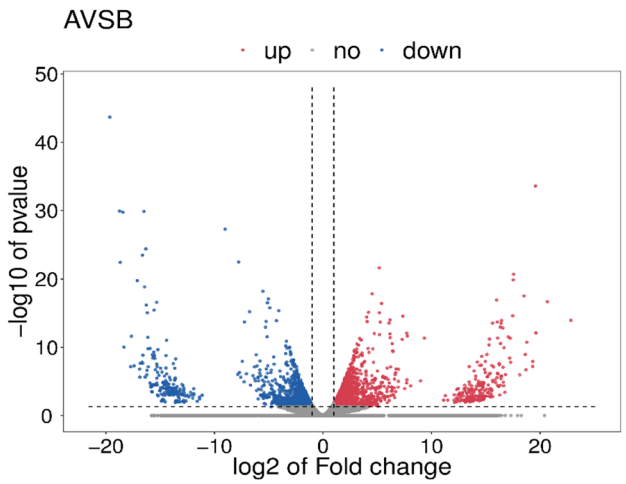

Fig. 2 Volcano plot of differentially abundant genes. Note that the red dots represent up-regulation and the blue dots represent down-regulation. **A** Triploid *Apostichopus japonicus* and **B** diploid *Apostichopus japonicus*

of triploid *A. japonicus* cultured in 2016 was observed. The results showed that aestivation duration of triploid *A. japonicus* in 2019 was 38 days, while that of diploid *A. japonicus* in the control group was 55 days. The triploid *A. japonicus* aestivation duration in 2020 was 39 days, while that of diploid *A. japonicus* in the control group was 63 days (Table 1). According to these data, triploid *A. japonicus* had a shorter aestivation time and greater growth advantage.

Overview of Transcriptome Sequencing

The transcriptome data obtained were analyzed. All samples were sequenced independently. In diploid *A. japonicus*, 117.5-M raw reads were obtained, while in triploid *A. japonicus*, 141.83-M raw reads were obtained. The diploid obtained 106.79-M clean reads after removing the redundant data, while the triploid obtained 125.24-M clean reads. Diploid *A. japonicus* transcriptome Q30 was 98.01%, and

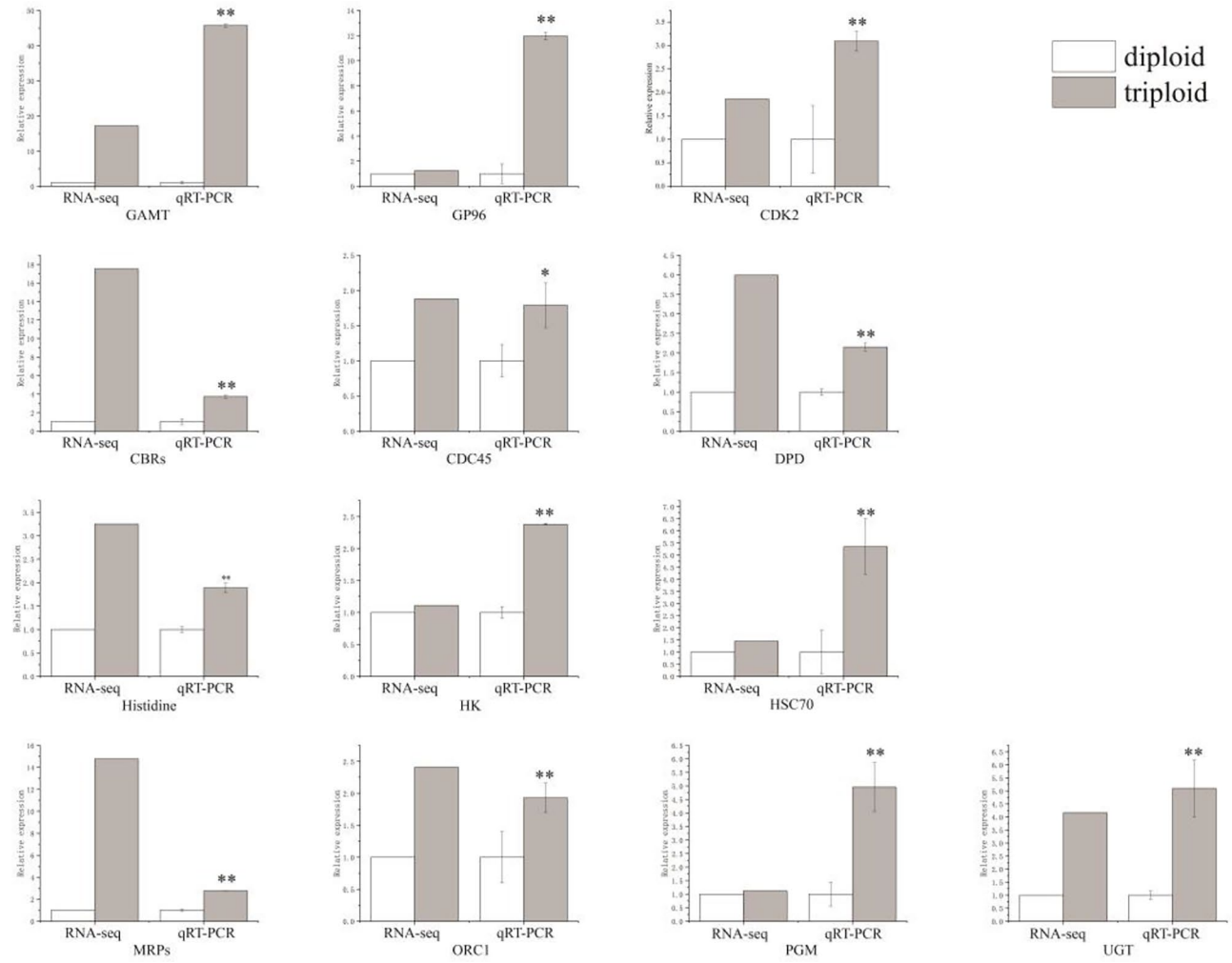

Fig. 3 Quantitative RT-PCR validation of differentially expressed genes. *Significant differences at $p < 0.05$ vs. diploid (triploid). **Highly significant differences at $p < 0.01$ vs. diploid (triploid). The results are expressed as mean ± SEM and one-way ANOVA

triploid *A. japonicus* transcriptome Q30 was 98.16%, demonstrating the reliability of transcriptome data. When the GC content of diploid and triploid was compared, it was discovered that diploid had a GC content of 41–43% and triploid had a GC content of 42% (Table S2). A total of 3296 differential genes were identified, with 1856 being up-regulated and 1440 being down-regulated (Fig. 2).

DEG Enrichment Results in GO and KEGG

The top 50 GO terms with the highest enrichment of differential genes were enriched and analyzed (Fig. S1). The results revealed that the most significantly enriched GO term in the cellular component was cytoplasmic, with 348 DEGs (up: 244, down: 104). The nucleus was the second most important location, with 314 DEGs (up: 233, down: 81). Among the molecular functions, the ATP binding process had the most differential genes (172 DEGs) (up: 126, down: 46). The second process was metal ion binding, which had 144 DEGs (up: 79, down: 65). The oxidation–reduction process was the most important item in the biological process classification, with 96 DEGs (up: 55, down: 41). Proteolysis was the second most important process, accounting for 90 DEGs in total (up: 36, down: 54).

Pathway items with more than 21 differential genes were screened, and the top 20 were chosen based on the − log10 *p*-value corresponding to each item in descending order. Through differential gene screening and enrichment analysis, 1114 differential genes were discovered to be enriched in 224 KEGG signaling pathways, 32 of which were significant ($p < 0.05$). DEGs were significantly enriched in pathways related to ribosome biogenesis in eukaryotes (62 DEGs; up: 57, down: 5), protein processing in the endoplasmic reticulum (52 DEGs; up: 45, down: 7), ECM-receptor interaction (50 DEGs; up: 21, down: 29), RNA transport (44 DEGs; up: 41, down: 3), and lysosomes (43 DEGs) (Fig. S2).

Screening for Key Differential Genes

According to the gene functions annotated by GO and KEGG, we discovered 13 key significantly different genes that may participate in the mechanism of growth advantage in triploid *A. japonicus*, and 13 genes were up-regulated, namely, *cyclin-dependent kinase 2* (*CDK2*), *cell division cycle 45* (*CDC45*), *origin recognition complex subunit 1* (*ORC1*), *multi-resistance associated proteins* (*MRPs*), *hexokinase* (*HK*), *UDP glucuronosyltransferase* (*UGT*), *phosphoglucomutase* (*PGM*), *carbonyl reductase* (*CBRs*), *guanidinoacetate methyltransferase* (*GAMT*), *glycoprotein 96* (*GP96*), *heat-shock cognate protein 70* (*HSC70*), *histidine methyltransferase*, and *dihydropyrimidine dehydrogenase* (*DPD*) (Figs. 3 and 7; Table 2).

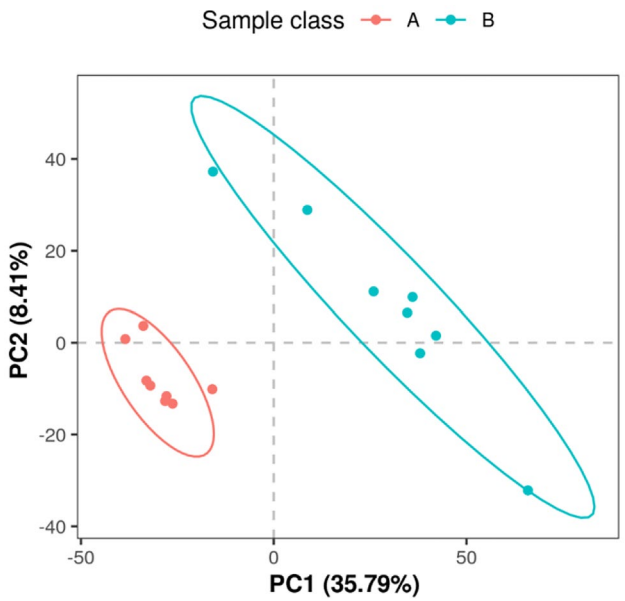

Fig. 4 Comparison group PLS-DA analysis. Note that each point in the figure represents a sample, and the similarities and differences among all samples are reflected in the tendency of separation and aggregation of samples in the figure. The aggregation of points indicates that the observed variables have a high degree of similarity, while the dispersion of points indicates that the observed variables have obvious differences. **A** Triploid *Apostichopus japonicus* and **B** diploid *Apostichopus japonicus*

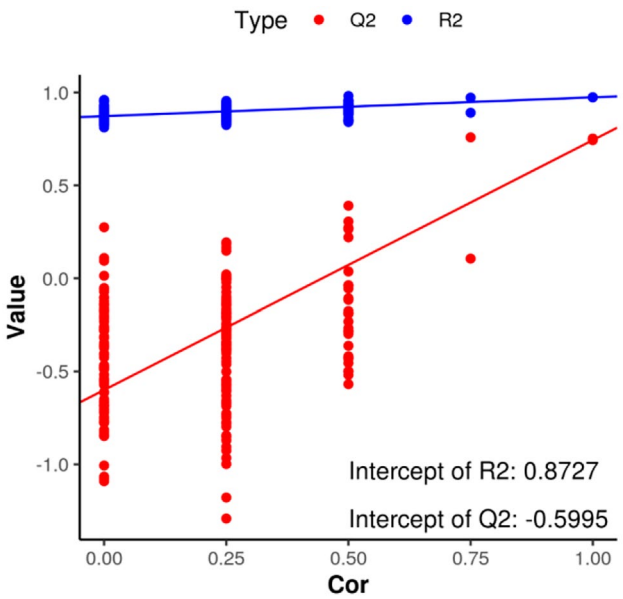

Fig. 5 Triploid and diploid *Apostichopus japonicus* replacement test. Note that Q_2 represents the prediction rate of the model and R_2 represents the interpretation rate of the model. $Q_2 = -0.56510 < 0$, $R_2 = 0.8762$

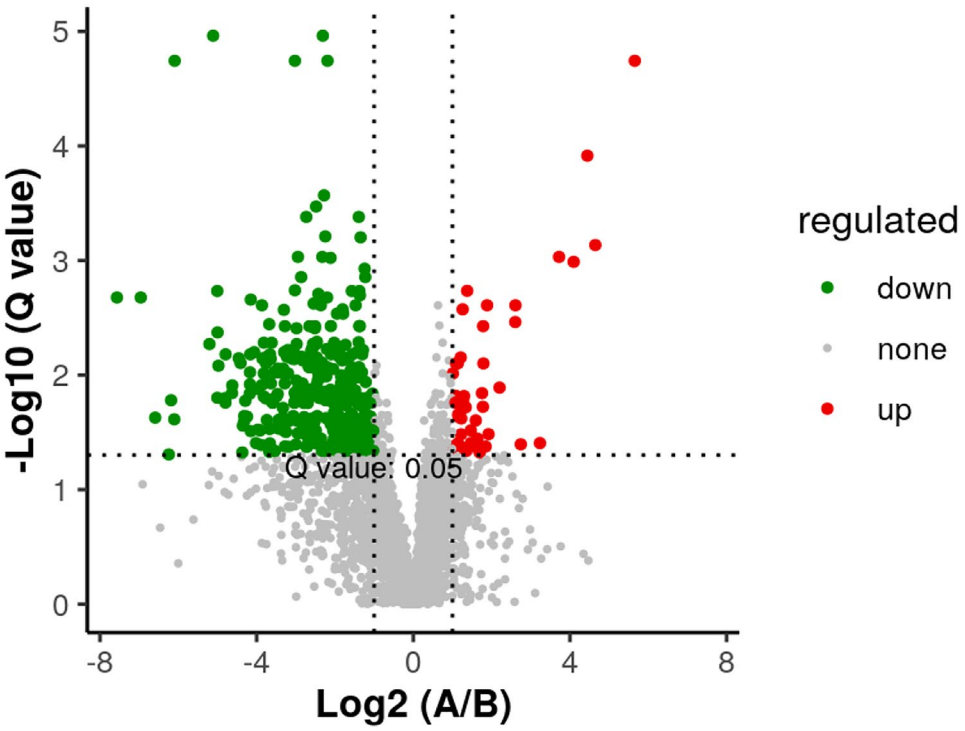

Fig. 6 Volcano plot of differentially abundant metabolites. Note that the red dots represent up-regulation and the green dots represent down-regulation. **A** Triploid *Apostichopus japonicus* and **B** diploid *Apostichopus japonicus*

qRT-PCR Validation

The 13 key DEGs identified by transcriptome analysis were validated using qRT-PCR (Table 2). The verification results agreed with the RNA-Seq results (Fig. 3), demonstrating the reliability of the experimental results.

Partial Least Squares Discriminant Analysis (PLS-DA) of Comparative Groups

A PLS-DA model was established between each pair of groups using VIP ≥ 1.0 as the screening condition in order to perform principal component analysis on the identified metabolic ions (Fig. 4). In this study, samples from the triploid and control groups were found to have a high degree of dispersion, with no overlap between the two groups of samples. The results were reliable, and the samples were analyzed further by metabolome.

During model validation, linear regression was performed between the original classification Y matrix and the Y matrix of N different permutations with R_2Y and Q_2Y, and the obtained regression line and Y-axis intercept values were R_2 and Q_2, respectively. They were used to determine whether the model was over-fitting. $Q_2 = -0.56510 < 0$, $R_2 = 0.8762$. This demonstrated that the model did not involve over-fitting and that the differential metabolite analysis was sufficiently accurate (Fig. 5).

Differential Metabolite Statistical Analysis and Screening

A total of 414 metabolites were identified, including 306 positive metabolites (up: 44, down: 262) and 108 negative metabolites (up: 6, down: 102).

We discovered 11 key significantly different metabolites (SDMs). Nocodazole, lactose, lactulose, gentiobiose, hypoxanthine (HX), 2-oxoglutarate, rhododendrin, arginine, uridine, spongouridine, and aspartame were among the SDMs (Figs. 6 and 7).

KEGG Pathway Enrichment Results

According to the KEGG results, a total of 97 metabolic pathways were enriched. Significantly enriched in metabolic pathways (SDMs: 578, POS: 505, NEG: 73), porphyrin and chlorophyll metabolism (SDMs: 6, POS: 6, NEG: 0), fatty acid metabolism (SDMs: 9, POS: 8, NEG: 1), amino sugar and nucleotide sugar metabolism (SDMs: 14, POS: 14, NEG: 0), and fructose and mannose metabolism (SDMs: 6, POS: 4, NEG: 2) (Fig. S3).

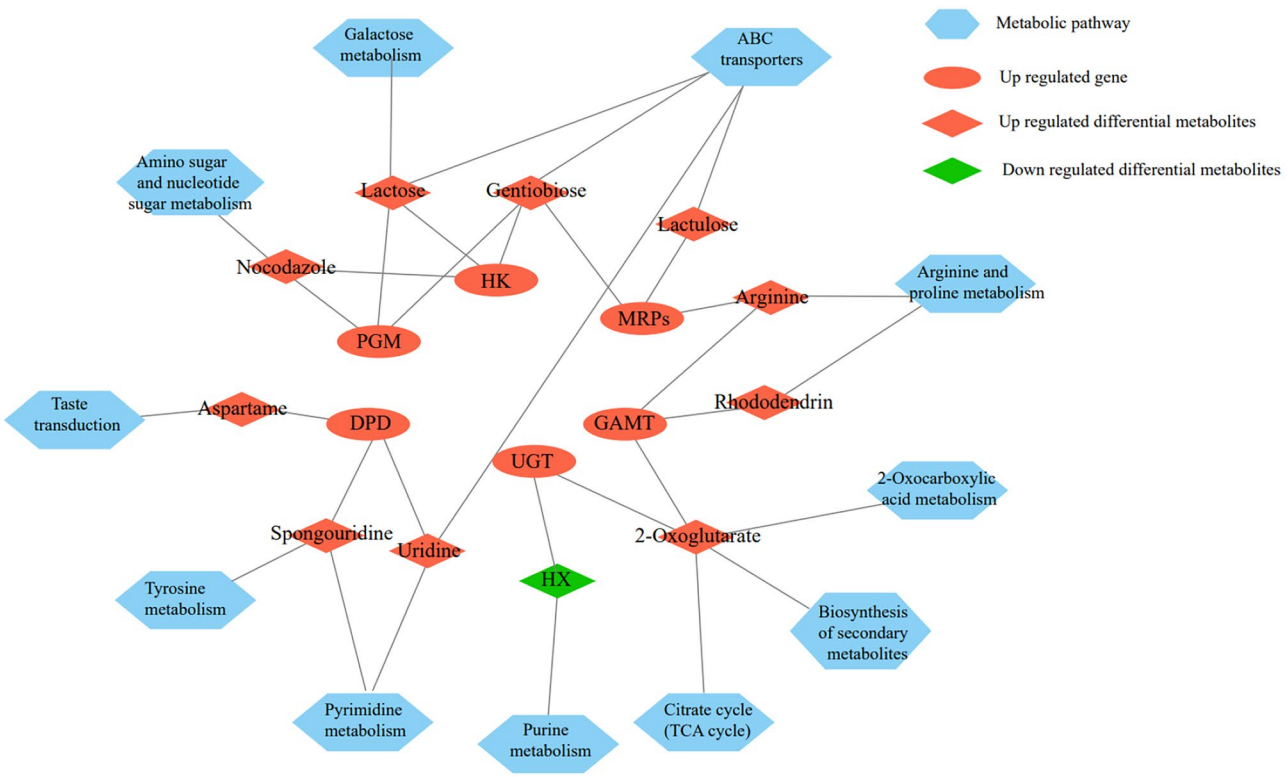

Fig. 7 Results of combined transcriptomic and metabolomic analyses

Combined RNA-Seq and Metabolome Analysis Results

We discovered that 6 DEGs may have regulatory relationships with 11 SDMs using a combined RNA-Seq and metabolome analysis, with 1 pair having negative regulation and 10 pairs having positive regulation (Fig. 7). We used GO enrichment analysis to look at the target genes of different metabolites. A total of 404 target genes (POS: 235, NEG: 165) were found to be enriched in 1151 items (POS: 666, NEG: 485). There were 572 items in terms of biological processes, mostly oxidation reduction processes (56 DEGs, POS: 28, NEG: 28) and metabolic processes (38 DEGs, POS: 20, NEG: 18), among others. There were 459 items in terms of molecular functions, mostly oxidative enzyme activity (40 DEGs, POS: 20, NEG: 20) and catalytic activity (44 DEGs, POS: 25, NEG: 19), among others. There were 120 cellular components, mostly cytosol (56 DEGs, POS: 30, NEG: 26) and cytoplasm (70 DEGs, POS: 50, NEG: 20), among others (Fig. S4); KEGG enrichment analysis of differential metabolite target genes revealed that 417 (POS: 246, NEG: 171) genes were enriched in 181 (POS: 92, NEG: 89) pathways, with purine metabolism (82 DEGs, POS: 41, NEG: 41) and pyrimidine metabolism (58 DEGs, POS: 33, NEG: 25) being the most enriched (Fig. S5).

Discussion

The length of *A. japonicus* is an important economic characteristic. In this study, the length of diploid and triploid *A. japonicus* was significantly different under the same culture conditions. Among *A. japonicus* with a body length of 6–12 cm, the percentage of triploid *A. japonicus* was 4.46% higher than that of diploid *A. japonicus*. Among *A. japonicus* with a body length of 12–18 cm, the percentage of triploid *A. japonicus* was 1.37% higher than that of diploid *A. japonicus*. This was consistent with the triploid breeding results of other aquatic species (Peruzzi et al. 2018; Liu et al. 2018; Garnier-Géré et al. 2002). Following a comparative analysis, the aestivation time of triploid *A. japonicus* was shorter. This study proved that triploid *A. japonicus* had a better growth advantage.

Following sequencing analysis, we found that 6 DEGs had regulatory relationships with 11 SDMs, which jointly acted on 11 metabolic pathways (Fig. 7). The observed potential candidate genes mainly involved functions such as promoting growth and development, immune regulation, accumulation of carbohydrate, energy storage, and synthesis of beneficial metabolites; the differential metabolites were mainly concentrated in functions such as growth, immunity, carbohydrate synthesis, and taste improvement.

The results showed that triploid *A. japonicus* had certain growth advantages. We investigated whether the significant growth advantage of triploid *A. japonicus* was related to the enhancement of cell division, as the expressions of *CDK2*, *CDC45*, *ORC1*, and *histidine methyltransferase* were significantly higher in triploids. *CDK2* plays a key role in cell cycle regulation and participates in a series of biological processes. Existing research shows that *CDK2* may be involved in DNA damage and phosphorylation of protein interactions and antitumor activity (Tadesse et al. 2020; Spencer et al. 2013). *CDC45* can participate in the formation of the eukaryotic replication helicase and play an important role in the initial stage of DNA replication; thus, it can promote the cell cycle (Rios-Morales et al. 2019). Research has shown that *DNAJA1* after being activated by stable *CDC45* promotes the cell cycle (Yang et al. 2020). *ORC1* can participate in eukaryotic replication and maintain genome stability (De et al. 2019). The studies by Okano have proved that *ORC1* is essential for cell mitosis (Okano-Uchida et al. 2018). *Histidine methyltransferase* can regulate the differentiation of muscle cells and promote the growth and development of *A. japonicus* (Shu and Du 2021). The participation of genes in immune regulation contributes to the growth of triploid *A. japonicus*; the expressions of *UGT* , *GP96*, and *HSC70* are much higher in triploids. *UGT* is a glycoprotein attached to the endoplasmic reticulum cavity of microsomes. Bigo et al. (2013) indicated that *UGT* acted as a key metabolic protein in organisms to hinder the accumulation of toxic hydrophilic compounds. Primarily, it catalyzes the combination of endogenous or exogenous compounds with the cofactor uridine diphosphate glucuronic acid, thereby enhancing the polarity of lipophilic substrates and facilitating their excretion via urine or bile. For this reason, it has been proposed that triploid *A. japonicus* has a higher immunity and stronger stress resistance. The two heat shock proteins, *GP96* and *HSC70*, are generated at abnormal temperatures in an environment with extremely low oxygen content and oxidized free radicals during the bacterial infection process. They are able to repair denatured proteins, accelerate the recovery of normal proteins, and strongly protect cells when exposed to stress (Zininga et al. 2018). High temperature can induce the high expression of *HSC70*. The up-regulated expression of *HSC70* gene can protect against injury due to high temperature (Sun et al. 2016). *GP96* is highly expressed following increased temperature; therefore, it can resist high temperature (Tang et al. 2009). This may be the reason why the aestivation time of triploid *A. japonicus* is shorter. Also, they are important players in the processing of proteins in the endoplasmic reticulum (Cosin-Roger et al. 2018; Stricher et al. 2013). *HSC70* promote the processing and synthesis of proteins and benefits the growth of organisms (Liu et al. 2012); thus, it could be speculated that triploid *A. japonicus* has potential immune regulation ability than diploid *A. japonicus*. *DPD* is an enzyme mainly involved in the metabolism of pyrimidines in organisms. It contains the ability to metabolize the toxic pyrimidine analogue fluorouracil (5-FU). *DPD* is the rate limiting enzyme of fluoropyrimidine metabolism and can convert 5-FU and its metabolites into non-cytotoxic products and excrete them out of the body (Sharma et al. 2019). *DPD* expression was increased in triploid *A. japonicus*. Therefore, we speculate that triploid *A. japonicus* has a better ability to excrete toxins.

Carbohydrate accumulation can provide energy for triploid *A. japonicus*, and its related genes are *HK* and *PGM*, which are significantly up-regulated. *HK* is a pivotal rate-limiting enzyme in glycolysis; it is able to stimulate the utilization of glucose by cells and performs an extremely important function in energy metabolism (Patra et al. 2013). The energy provided by *HK* up-regulation may be used to resist the adverse effects at high temperature (Scaraffia and Gerez 2000). Variations in *HK* activity will give rise to changes in glycolysis and the formation of pentose phosphates, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), and glycogens (Anderson et al. 1971). *HK* with high activity is therefore beneficial to allow it to tackle hypoxia and high temperature stress. *PGM* participates in glycolysis and gluconeogenesis in vivo. Fragmentary or missing *PGM* will affect the synthesis of some elements of the cell wall (Morava 2014), and *PGM* deficiency greatly influences the glycosylation of proteins (Beamer 2015). Hence, high expression levels of related genes are speculated to be a vital influencing factor on the rapid growth and development of triploid *A. japonicus*. These results showed that the genes related to energy metabolism were up-regulated. *MRPs* and *GAMT* were significantly up-regulated in triploid *A. japonicus*. *MRPs* and *GAMT* are all related to the ATP energy supply. *MRPs* are members of the subfamily C of the ABC transporter superfamily and serve as efflux pumps for ATP (Zhang et al. 2015). They can pump antitumor drugs conjugated to glutathione, glucuronate, or sulfate out of cells. Arginine can generate creatine and glycocyamine under the action of *GAMT*. Studies have shown that creatine plays an important role in the conversion of ATP and participates in stabilizing a form of ATP (Joncquel-Chevalier et al. 2015). It is speculated that the up-regulation of *MRPs* and *GAMT* promotes the energy supply of triploid *A. japonicus*. Genes related to the synthesis of beneficial metabolites were up-regulated. *CBRs* is a type of oxidoreductase protein, which is widely found in bacteria, fungi, yeasts, animals, and plants. With coenzyme NAD(P) + and NAD(P)H as the electron acceptor and donor, respectively, *CBRs* can specifically catalyze the interconversion between ketone (aldehyde) and alcohol, and contribute to the synthesis of valuable hydroxy compounds and metabolites (Forrest and Gonzalez 2000).

According to the combined analysis of transcriptome and metabolome, we screened 11 key SDMs which are related to growth and development (SDMs: nocodazole, rhododendrin, 2-oxoglutarate, arginine, lactulose, and HX), immunity (SDMs: uridine and spongouridine), and taste (SDMs: lactose, gentiobiose, and aspartame), respectively. Nocodazole, rhododendrin, 2-oxoglutarate (AKG), arginine and lactulose are positively regulated by the DEGs *HK* (SDM: nocodazole), *PGM* (SDM: nocodazole), *GAMT* (SDMs: rhododendrin and arginine), and *MRPs* (SDMs: arginine and lactulose). HX is negatively regulated by *UGT* . They are involved in the metabolic pathways of amino sugar and nucleotide sugar metabolism, arginine and proline metabolism, TCA cycle, 2-oxocarboxylic acid metabolism, biosynthesis of secondary metabolites, ABC transporters, and purine metabolism. Nocodazole is a mitotic blocker in the same class as colchicine, and it can induce the arrest of cell division in the *M* phase and lead to cell synchronization (Cooper et al. 2006). Attia found that nocodazole was a germ cell mutagen, which induced mutation lethality in male germ cells (Attia et al. 2015). Based on its ability to kill germ cells, it was inferred that the up-regulated expression of such metabolites is related to the sterility of triploid *A. japonicus*. According to research carried out by Kim (Kim et al. 2019), rhododendrin significantly enhanced the activity of dopaminergic neurons. The metabolite AKG can promote growth. Studies have shown that AKG can promote the synthesis of muscle protein (Pierzynowski and Sjodin 1998). In piglets with slow perinatal growth, AKG as an energy donor can promote bone metabolism (Tomaszewska et al. 2021). Arginine is a basic component of various proteins, and its precursor can regulate cell proliferation, differentiation, and homeostasis (Bulau et al. 2006). Research carried out by Deng indicated that lactulose can promote enterocinesia (Deng et al. 2021). It may accelerate the intestinal absorption of nutrients and provide more energy for the body. HX is an important purine alkaloid that degrades fats. The down-regulation of HX benefits the development of triploid *A. japonicus*. Therefore, these metabolites may lead to the rapid growth and development of triploid *A. japonicus*. Uridine and spongouridine are positively regulated by *DPD*, and they are jointly involved in the metabolic pathways of ABC transporters, pyrimidine metabolism, and tyrosine metabolism. Uridine is a ribose extracted from nucleic acids. Walker revealed that uridine reduced cell apoptosis, and prevented mitochondrial DNA deletion and mitochondrial depolarization (Walker and Venhoff 2005). It has been found that spongouridine, a metabolite with antibacterial ability, inhibited bacteria and fungi to some extent (Hamoda et al. 2021). It is speculated that the up-regulation of these metabolites can enhance the antibacterial immunity of triploid *A. japonicus*. Lactose, gentiobiose, and aspartame are positively regulated by the DEGs *HK* (SDMs: lactose and gentiobiose), *PGM* (SDMs: lactose and gentiobiose), and *DPD* (SDM: aspartame). They are involved in the metabolic pathways of galactose metabolism, ABC transporters, and taste transduction. Lactose, one of the sources of dietary energy, can effectively improve the quality of products, maintain the color of products, and increase the overall sugar content without making the products too sweet and greasy. Luo and Jiang (2006) proved that lactose improved the flavor of shredded squid and reduced water activity. As a type of functional oligosaccharide, gentiobiose can effectively improve food flavors, and function as an immune regulator in vitro (İspirli et al. 2019). Aspartame is an artificial dipeptide sweetener that is able to affect a variety of cells and tissues (Choudhary and Pretorius 2017). It is presumed that the up-regulated expression of these metabolites can enhance the nutritional value of triploid *A. japonicus*, and optimize flavor and taste.

Compared with diploid *A. japonicus*, triploid *A. japonicus* had more advantages in terms of gene regulation ability, error-tolerant rate of gene expression, immunity, growth and development, stress resistance, energy conversion rate, adaptability to a harsh living environment, edible value, and taste. Our results further enrich the biological data of triploid *A. japonicus* and provide useful resources for genetic improvement of this species.

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Author Contribution YQC and JD designed and conceived the whole experiments. JHX and YS collected important background information. JHX, YS, YC, LSH, BCD, YXL, CG, PFH, XJ, JS, and DHY performed the experiments. JHX and YS carried out literature retrieval, data collection, and data analysis. YC and LSH provided help for data analysis. JHX, YS, and JD wrote the paper. YQC ang JD checked the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of Interest The authors declare no competing interests.

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