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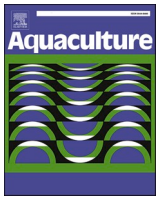

Determination of optimal temperature, salinity, and stocking density combinations for larval growth and survival of diploid, triploid, and tetraploid Fujian oyster (*Crassostrea angulata*)

Yuanxin Liang [a], Xianchao Bai [a], Chengxun Xu [a], Qi Li [a,b,*]

[a] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China,

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ABSTRACT

For nurseries, thriving larvae mean higher incomes. Three factors - temperature, salinity and stocking density - are critical to the success of larval rearing. However, the effects of these three factors on the performance of diploid, triploid and tetraploid *Crassostrea angulata* larvae have not yet been comprehensively investigated. In this study, the combined effects of temperature (T, 12.0–35.0 °C), salinity (S, 10.0–38.0 psu) and stocking density (D, 0.5–10.0 ind. ml^{-1}) on the accumulated growth rate (AGR) and survival rate (SR) of diploids (DD), triploids (DT) and tetraploids (TT) *C. angulata* larvae were comprehensively assessed using a central composite design and response surface methodology. The results revealed that suitable temperature-salinity-stocking density combinations significantly increased the AGR and SR of larvae from all three groups, while unsuitable environments (e.g., ultra-high temperature - extra-high salinity -high stocking density combinations or ultra-high temperature - extra-low salinity - high stocking density combinations) significantly reduced the AGR and SR of the larvae, albeit to different magnitudes. The optimal temperature-salinity-stocking density combinations were obtained by model optimization. The maximum AGR and SR of DT reached at 23.6 °C, 26.5 psu and 0.5 ind. ml^{-1} combinations with values of 17.13 μm day^{-1} and 64.81%, respectively. The combinations of 24.0 °C - 25.89 psu - 1.99 ind. ml^{-1} and 25.9 °C - 25.96 psu - 0.5 ind. ml^{-1} were the optimal breeding conditions for DD and TT (DD: AGR = 13.10 μm day^{-1}, SR = 63.55%; TT: AGR = 10.11 μm day^{-1}, SR = 25.89%). In addition, validation experiments were carried out under the obtained optimum conditions. The AGR and SR of DD, DT and TT obtained from the validation experiments were basically in conformity with the maximum AGR and SR obtained under the theoretically predicted conditions. Moreover, the growth and survival of tetraploids remained lower than those of diploid and triploid counterparts, even under optimal breeding conditions. This means that more attention should be paid to the tetraploids so as to safeguard the succession of this fragile population. The application of the findings of this study will help to improve the breeding efficiency of *C. angulata* with different ploidies in larval culture.

1. Introduction

Oyster farming is of vital importance to a number of countries around the world. Many scholars have brainstormed ways to improve the yield and quality characteristics of oysters. The resistance of the Pacific oyster *Crassostrea gigas* to pathogens such as *Vibrio aestuarianus* or *V. alginolyticus* has been significantly improved through selective breeding, leading to a substantial increase in their survival rate (Dégremont et al., 2020; Azéma et al., 2017; Yang et al., 2021). Four lines of *C. gigas* with noble, special colors have been bred through generations of mass selection and were known as the Black-shell line, White-shell line, Golden-shell line and Orange-shell line (Han et al., 2020; Wan et al., 2017). Moreover, the aquatic traits (shell height, wet weight, survival rate, nutritional quality, etc.) of oysters have been significantly improved by crossbreeding (Bartley et al., 2001; Tan et al., 2020; Hedgecock et al., 1995; Hedgecock and Davis, 2007; Rawson and Feindel, 2012; Liang et al., 2022a, 2022b). However, the disadvantages of summer spawning in diploids make them less palatable in summer and difficult to supply to the market (Normand et al., 2009). In addition, the high reproductive effort has also been reported to be correlated with

* Corresponding author at: Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, 266003, China.
E-mail address: qili66@ouc.edu.cn (Q. Li).

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Table 1
The combined effects of temperature (T), salinity (S) and stocking density (D) on the accumulated growth rate (AGR) and survival rate (SR) of diploids (DD), triploids (DT) and tetraploids (TT) *Crassostrea angulata* larvae. Notice that coded and actual levels of the two factors are given.

Run	Code			Actual			AGR (μm day^{-1})			SR (%)		
	Temperature	Salinity	Stocking density	Temperature (°C)	Salinity (psu)	Stocking density (ind. ml^{-1})	DD	DT	TT	DD	DT	TT
1	0	α	0	23.5	38	5.25	8.14 ± 0.42^{b}	11.14 ± 0.32^{a}	4.59 ± 0.82^{c}	16.35 ± 1.7^{a}	17.35 ± 1.44^{a}	10.35 ± 0.86^{b}
2	0	0	α	23.5	24	10	8.21 ± 0.73^{b}	11.68 ± 0.58^{a}	4.99 ± 0.8^{c}	38.66 ± 0.17^{a}	39.66 ± 4.4^{a}	11.23 ± 1.74^{b}
3	0	0	0	23.5	24	5.25	12.2 ± 2.45^{a}	14.88 ± 1.94^{a}	7.42 ± 0.88^{b}	59.65 ± 0.61^{a}	61.23 ± 2.29^{a}	18.65 ± 1.43^{b}
4	-1	1	-1	16.66	32.32	2.43	9.71 ± 0.03^{b}	13.88 ± 1.61^{a}	7.05 ± 0.76^{c}	39.86 ± 0.4^{a}	40.86 ± 2.37^{a}	16.86 ± 1.65^{b}
5	0	-α	0	23.5	10	5.25	4.91 ± 0.58^{b}	7.4 ± 0.55^{a}	1.36 ± 0.34^{c}	3.55 ± 0.33^{a}	3.22 ± 0.96^{a}	4.35 ± 0.77^{a}
6	0	0	0	23.5	24	5.25	12.41 ± 1.15^{a}	14.62 ± 1.02^{a}	7.06 ± 0.76^{b}	59.86 ± 0.3^{b}	61.56 ± 0.27^{a}	20.56 ± 0.45^{c}
7	0	0	0	23.5	24	5.25	12.23 ± 1.67^{a}	14.58 ± 0.37^{a}	7.54 ± 1.06^{b}	59.66 ± 0.53^{a}	61.35 ± 3.44^{a}	19.86 ± 2.21^{b}
8	0	0	-α	23.5	24	0.5	12.9 ± 1.41^{b}	17.18 ± 0.77^{a}	9.86 ± 1.6^{b}	63.44 ± 3.18^{a}	64.44 ± 4.18^{a}	25.35 ± 4.09^{b}
9	0	0	0	23.5	24	5.25	12.18 ± 3.46^{ab}	15.12 ± 3.82^{a}	7.13 ± 0.81^{b}	58.68 ± 3.22^{a}	60.89 ± 1.95^{a}	21.66 ± 0.82^{b}
10	-α	0	0	12	24	5.25	7.69 ± 0.47^{a}	10.69 ± 2.2^{a}	4.14 ± 0.16^{b}	19.67 ± 0.49^{a}	20.67 ± 1.73^{a}	13.56 ± 2.3^{b}
11	-1	-1	-1	16.66	15.68	2.43	6.88 ± 1.03^{b}	9.34 ± 0.02^{a}	3.53 ± 1.17^{c}	24.56 ± 0.91^{a}	25.56 ± 2.1^{a}	17.86 ± 2.24^{b}
12	0	0	0	23.5	24	5.25	12.22 ± 0.96^{b}	15.03 ± 0.26^{a}	7.22 ± 1.13^{c}	58.53 ± 2.62^{a}	60.29 ± 1.3^{a}	21.35 ± 2.71^{b}
13	1	1	1	30.34	32.32	8.07	8.47 ± 0.59^{a}	11.47 ± 1.98^{a}	4.92 ± 0.87^{b}	12.55 ± 0.74^{ab}	13.55 ± 2.29^{a}	9.2 ± 0.26^{b}
14	0	0	0	23.5	24	5.25	11.41 ± 0.56^{ab}	14.79 ± 2.6^{a}	7.73 ± 0.85^{b}	57.86 ± 5.43^{a}	60.36 ± 1.42^{a}	21.56 ± 3.29^{b}
15	1	-1	-1	30.34	15.68	2.43	9.9 ± 0.78^{a}	13.23 ± 0.67^{a}	5.86 ± 2.57^{b}	18.53 ± 0.93^{a}	19.53 ± 1.58^{a}	15.18 ± 0.53^{b}
16	α	0	0	35	24	5.25	10.74 ± 0.48^{b}	13.74 ± 0.2^{a}	7.19 ± 1.25^{c}	5.35 ± 1.14^{a}	5.56 ± 2.27^{a}	4.88 ± 1.63^{a}
17	-1	-1	1	16.67	15.68	8.07	5.29 ± 2.3^{a}	8.29 ± 0.3^{a}	1.74 ± 0.45^{b}	14.56 ± 1.3^{a}	15.56 ± 1.72^{a}	11.21 ± 0.95^{b}
18	-1	1	1	16.67	32.32	8.07	7.54 ± 2.02^{a}	10.54 ± 0.25^{a}	3.99 ± 0.88^{b}	22.33 ± 0.71^{a}	23.33 ± 3.14^{a}	15.65 ± 2.43^{b}
19	1	1	-1	30.34	32.32	2.43	12.09 ± 0.5^{b}	15.09 ± 0.44^{a}	8.54 ± 1.38^{c}	26.53 ± 1.45^{a}	27.53 ± 2.38^{a}	18.55 ± 2.94^{b}
20	1	-1	1	30.34	15.68	8.07	7.49 ± 1.16^{b}	10.49 ± 0.2^{a}	3.94 ± 1.29^{c}	13.56 ± 2.3^{a}	14.56 ± 2.4^{a}	0.35 ± 0.14^{b}

The star arm α = |1.682| for this experimental design.

high summer mortality in oysters (Cowan et al., 2023; Cotter et al., 2010; Samain et al., 2007). Fortunately, the emergence of triploids has turned this nightmare around. Due to their sterility, triploids can maintain favorable tastes during the reproductive period. Moreover, triploids also exhibited significant advantages over diploids in terms of growth, survival or disease resistance (Walton et al., 2013; Allen and Downing, 1986; Nell, 2002; Dégremont et al., 2015; Wadsworth et al., 2019; Guo et al., 1996; Piferrer et al., 2009).

The thriving of larvae is crucial for the nursery, and environmental factors, especially temperature, salinity, and stocking density, exert significant impacts on their growth. The accumulated growth rate and survival rate for the larvae of the black shell strain of *C. gigas* were significantly higher under optimum conditions than under other stress conditions (Xu et al., 2020). Rico-Villa et al. (2009) and Kheder et al. (2010) found that larvae reared under optimum salinity survived above 17–32 °C with high survival rates (> 90%). Furthermore, significant effects of temperature or salinity on larval performance have also been reported in the Sydney rock oyster *Saccostrea glomerata* (Dove and O'Connor, 2007), *C. sikamea*, *C. ariakensis* and their hybrids (Xu et al., 2011). In addition, environmental factors also had significant effects on oyster larvae with polyploids. The growth rate of tetraploid *C. gigas* larvae was significantly lower at 18 °C than that at 28 °C, and the optimum conditions for breeding tetraploid larvae were a temperature of 23–28 °C and a salinity of 25–30 psu (Li et al., 2022a, 2022b). Apart from temperature and salinity, stocking density also played a vital role in the growth of shellfish larvae. It has been widely recognized as a key factor in intensive aquaculture and a potential source of chromium stress, affecting the physiology and behavior of cultured species (Velasco and Barros, 2008; Xu et al., 2020). Studies have reported synergistic effects of temperature, salinity and stocking density on the performance of wild populations of *C. gigas* (Xu et al., 2020). However, detailed information on the combined effects of temperature, salinity and stocking density on the successful breeding of *C. angulata* larvae, especially triploids and tetraploids, and the identification of the best combination of factors of practical relevance is still unclear to date. There is no doubt that an in-depth study of these issues will be beneficial in maintaining optimum breeding conditions for *C. angulata*, ensuring larval growth and survival and increasing production.

Oyster farming is of vital importance to China. >6 million tonnes of oysters were farmed in China in 2022, accounting for 39.50% of the total mariculture production (BOF (Bureau of Fisheries), 2022). Four economically valuable farmed oysters in China belong to the genus *Crassostrea*: *C. gigas*, *C. angulata*, *C. ariakensis*, and *C. hongkongensis* (Li et al., 2022a, 2022b). Of these, the most productive economic species, *C. angulata*, reached an annual production of 2.07 million tonnes in 2022, accounting for 38% of the total production of farmed oysters in

China (BOF (Bureau of Fisheries), 2022).

Recently, the successful breeding of tetraploid *C. angulata* has made large-scale production of triploids a reality (Liang et al., 2023). However, the optimal breeding conditions for triploid and tetraploid larvae have not been well studied. In this study, the central composite design and response surface methodology were used to comprehensively assess the performance of diploid, triploid and tetraploid larvae of *C. angulata* under different temperature-salinity-stocking density combinations. The aims of this study were (1) to investigate the combinatorial effects of temperature, salinity and stocking density on the characteristics (growth and survival) of diploid, triploid and tetraploid *C. angulata* larvae; (2) to model the relationship between characteristics (growth or survival) and temperature-salinity-stocking density combinations in *C. angulata* with different ploidies; and (3) to determine the optimal temperature-salinity-stocking density combinations for diploid, triploid and tetraploid *C. angulata* using statistical optimization techniques.

2. Materials and methods

2.1. Experimental oysters

One-year-old diploid *C. angulata* were collected from Putian, Fujian Province (25.43°N, 119.01°E) in May 2022 and transferred to Litao Seedlings Ltd. in Laizhou, Shandong Province (37.3°N, 119.9°E), and the first generation was successfully obtained through mass selection. The tetraploids *C. angulata* were produced by inhibiting the emission of the first polar body of the fertilized eggs from triploid eggs and diploid sperm (Liang et al., 2023). The diploid and tetraploid populations were transferred to Jiaonan, Shandong Province (36.00°N, 119.37°E) for rearing in October 2022.

One-year-old diploids and tetraploids *C. angulata* were collected from Jiaonan, Shandong Province in March 2023. These parental oysters were then transported to Litao Seedlings Ltd. in Yantai, Shandong Province, China. All groups temporarily remained in conditioned water (temperature: 24.0 ± 2.0 °C; salinity: 28.0 ± 2.0 psu) until gonadal maturity.

2.2. Artificial insemination

Gonadally mature oysters were selected according to their shell height (SH) and living weight (LW) (DD: SH = 66.3 ± 7.68 mm, LW = 48.78 ± 5.87 g; TT: SH = 83.19 ± 11.73 mm, LW = 75.12 ± 10.27 g). Diploids and tetraploids were opened and the sexes were identified under a light microscope. The hemolymph of each putative tetraploid oyster was then extracted and a portion of the gill filament was also taken for double testing of ploidy by flow cytometry. The sperm or eggs from three oysters were collected separately and divided equally into two for artificial fertilization. The insemination was carried out by (1) mixing the eggs of diploids with sperm from diploids or tetraploids to produce the DD (diploids ♀ × diploids ♂) and DT (diploids ♀ × tetraploids ♂) groups, respectively; and (2) mixing the eggs of tetraploids with sperm from tetraploids to produce the TT (tetraploids ♀ × tetraploids ♂) group. Large-scale production of triploids was produced by crossbreeding between male tetraploids and female diploids. Thus, diploids and tetraploids were crossed in only one direction in this study, that is, female diploids were crossed with male tetraploids to produce triploids (diploids ♀ × tetraploids ♂). During artificial fertilization, the presence of 8 to 10 sperm around each egg was ensured.

Once fertilization was completed, the fertilized eggs of each group were transferred to 100 L polyethylene plastic buckets for incubation. Later, the corresponding number of vigorous D-larvae were selected into 25 L plastic buckets according to the pre-calculated density and acclimated to the experimental temperature and salinity combinations for 1 day by adjusting 0.5 °C/h and 0.5 psu/h, respectively. The temperature, salinity and stocking density in each tank were set according to the parameters set by the central composite design (Table 1). The high temperature was maintained by electric heaters (accuracy 0.1 °C), the low temperature was regulated by a low-temperature refrigerator (accuracy 0.1 °C), and the temperature was controlled using an electric thermometer (accuracy 0.1 °C). The low salinity seawater was obtained by adding dechlorinated fresh water to the seawater, while high salinity seawater was obtained by adding sea salt, and the salinity was measured using a refractometer (ATAGO) (accuracy 0.1%). *Isochrysis galbana* was fed to the larvae at the early veliger stage, and appropriate *Platymonas sp* were supplied as larval growth. During the experimental period, 1/3 of the water was changed every 2 days. Prior to each water change, the replacement water was filtered through a sand filter and non-woven polypropylene fabric and adjusted to the experimental conditions (Li et al., 2011). The larval experiment was completed when 20–30% of the larvae have developed eye spots (25 days after fertilization).

2.3. Experimental design

Larval performance (accumulated growth rate, AGR and survival rate, SR) was assessed using a central composite design (CCD) engineered through Design Expert software version 13 (Minneapolis, USA) with three factors, temperature (T, °C), salinity (S, psu) and stocking density (D, ind. ml^{-1}). The ranges of the three factors were determined based on the results of previous experiments and seasonal variations in seawater temperature and salinity in Shandong and Fujian Provinces. Throughout the experiment, the maximum temperature was set at 35.0 °C and the minimum at 12.0 °C, the salinity ranged from 10.0 to 38.0 psu and the stocking density was from 0.5 to 10.0 ind. ml^{-1}. Five levels were set for each factor in this experiment, coded as -α, −1, 0, 1 and + α, with α being the star arm. The actual level corresponding to each coded level for each factor was displayed in Table 1. Due to the rotatable nature of the CCD design used in the experiments, the number of factorial points, axial points and centroid points was 8, 6 and 6, respectively. Thus, the whole design consisted of 20 experimental points or runs, in which case $\alpha = \pm |1.682|$. Six replications were performed for each run to ensure reliability (Table 1). The order of runs was randomized to avoid a systematic trend of errors.

2.4. Measurements

The accumulated growth rate (AGR) and survival rate (SR) of each group were measured during the experiment. Thirty larvae were randomly sampled from each group and their shell heights were measured using image analysis software (image-pro plus 6.0). The accumulated growth rate and survival rate were calculated with the following equations:

$$\mathrm{AGR}\ \left(\mu\mathrm{m\ day}^{-1}\right) = \left(\mathrm{SH_t}–\mathrm{SH_{t0}}\right)/\mathrm{T}.$$

$$\mathrm{SR}\ (\%) = \mathrm{N_t} \times 100/\mathrm{N_{t0}}.$$

Where SH_t is the mean shell height of each group at the end of the experiment; SH_{t0} is the initial shell height of each group; T is the total number of days of the experiment; N_t is the total number of living larvae in 100 ml taken randomly at the end of the experiment; N_{t0} is the total number of D - larvae in 100 ml taken at random.

2.5. Statistical analyses

Data are presented as mean ± standard deviation (SD). The accumulated growth rates were log-transformed on a base of 10, and the survival rate for each group was arcsine converted to improve normality and homoscedasticity. Differences in growth or survival between the three groups under the same condition were analyzed by one-way ANOVA and Tukey's test for multiple comparisons in SPSS 26.0. Significance was set at $P < 0.05$.

A central composite design will be most appropriate when examining

Table 2
ANOVA for reduced cubic models for diploids, triploids and tetraploids *Crassostrea angulata*.

Source	Sum of squares		d. f.		Mean square		*F* value		*P* value	
	AGR	SR	AGR	SR	AGR	SR	AGR	SR	AGR	SR
DD										
Model	121.63	8720.85	11	11	11.06	792.80	102.43	1004.79	< 0.0001	< 0.0001
T	13.66	215.29	1	1	13.66	215.29	126.55	272.85	< 0.0001	< 0.0001
S	5.22	194.86	1	1	5.22	194.86	48.32	246.97	0.0001	< 0.0001
D	22.88	307.02	1	1	22.88	307.02	211.96	389.12	< 0.0001	< 0.0001
TS	0.46	32.32	1	1	0.46	32.32	4.22	40.96	0.0739 ^{NS}	0.0002
TD	0.64	9.20	1	1	0.64	9.20	5.97	11.66	0.0404	0.0092
SD	0.40	34.20	1	1	0.40	34.20	3.71	43.34	0.0903 ^{NS}	0.0002
T^2	15.97	3985.47	1	1	15.97	3985.47	147.93	5051.12	< 0.0001	< 0.0001
S^2	57.86	4431.11	1	1	57.86	4431.11	535.97	5615.90	< 0.0001	< 0.0001
D^2	4.83	130.04	1	1	4.83	130.04	44.74	164.81	0.0002	< 0.0001
TSD	0.05	0.27	1	1	0.05	0.27	0.46	0.35	0.517 ^{NS}	0.5721 ^{NS}
T^2S	0.02	8.03	1	1	0.02	8.03	0.15	10.18	0.7045 ^{NS}	0.0128
T^2D	/	/	/	/	/	/	/	/	/	/
TS^2	/	/	/	/	/	/	/	/	/	/
Residual	0.86	6.31	8	3	0.11	0.79				
Lack of Fit	0.24	3.10	3	5	0.08	1.03	0.66	1.61	0.6127 ^{NS}	0.299 ^{NS}
Pure Error	0.62	3.21	5	19	0.12	0.64				
Cor Total	122.50	8727.16	19							
DT										
Model	132.73	9130.94	10	13	13.27	702.38	148.67	2153.14	< 0.0001	< 0.0001
T	13.07	114.16	1	1	13.07	114.16	146.38	349.94	< 0.0001	< 0.0001
S	18.56	99.83	1	1	18.56	99.83	207.86	306.02	< 0.0001	< 0.0001
D	29.29	307.02	1	1	29.29	307.02	328.06	941.18	< 0.0001	< 0.0001
TS	1.95	32.32	1	1	1.95	32.32	21.85	99.08	0.0012	< 0.0001
TD	0.49	9.20	1	1	0.49	9.20	5.43	28.21	0.0447	0.0018
SD	1.26	34.20	1	1	1.26	34.20	14.07	104.83	0.0045	< 0.0001
T^2	13.87	4164.56	1	1	13.87	4164.56	155.36	12,766.41	< 0.0001	< 0.0001
S^2	58.94	4669.22	1	1	58.94	4669.22	660.13	14,313.46	< 0.0001	< 0.0001
D^2	0.56	150.70	1	1	0.56	150.70	6.32	461.98	0.0331	< 0.0001
TSD	0.25	0.27	1	1	0.25	0.27	2.78	0.84	0.1296 ^{NS}	0.3949 ^{NS}
T^2S	/	0.65	/	1	/	0.65	/	2.00	/	0.2073 ^{NS}
T^2D	/	8.03	/	1	/	8.03	/	24.63	/	0.0025
TS^2	/	1.74	/	1	/	1.74	/	5.34	/	0.0603 ^{NS}
Residual	0.80	1.96	9	6	0.09	0.33				
Lack of Fit	0.57	0.56	4	1	0.14	0.56	3.03	2	0.1275 ^{NS}	0.2163 ^{NS}
Pure Error	0.23	1.40	5	5	0.05	0.28				
Cor Total	133.53	9132.90	19	19						
TT										
Model	95.30	822.94	11	9	8.66	91.44	102.27	44.25	< 0.0001	< 0.0001
T	10.68	79.25	1	1	10.68	79.25	126.13	38.35	< 0.0001	0.0001
S	5.22	48.55	1	1	5.22	48.55	61.58	23.50	< 0.0001	0.0007
D	25.28	227.88	1	1	25.28	227.88	298.42	110.29	< 0.0001	< 0.0001
TS	0.56	9.64	1	1	0.56	9.64	6.57	4.66	0.0335	0.0562 ^{NS}
TD	0.06	33.29	1	1	0.06	33.29	0.70	16.11	0.4263 ^{NS}	0.0025
SD	1.10	14.91	1	1	1.10	14.91	13.02	7.21	0.0069	0.0229
T^2	6.21	186.44	1	1	6.21	186.44	73.31	90.23	< 0.0001	< 0.0001
S^2	37.24	261.29	1	1	37.24	261.29	439.62	126.45	< 0.0001	< 0.0001
D^2	0.02	2.19	1	1	0.02	2.19	0.20	1.06	0.6673 ^{NS}	0.3271 ^{NS}
TSD	0.02	/	1	/	0.02	/	0.27	/	0.6156 ^{NS}	/
T^2S	0.16	/	1	/	0.16	/	1.87	/	0.209 ^{NS}	/
T^2D	/	/	/	/	/	/	/	/	/	/
TS^2	/	/	/	/	/	/	/	/	/	/
Residual	0.68	20.66	8	10	0.08	2.07				
Lack of Fit	0.34	13.70	3	5	0.11	2.74	1.71	1.97	0.2803 ^{NS}	0.2375 ^{NS}
Pure Error	0.33	6.96	5	5	0.07	1.39				
Cor Total	95.97	843.60	19	19						

NS letter superscripts indicate $P > 0.05$, which means no significant difference. / stands for the factors are not contained in the model.

the combined effects of several continuously changing variables or factors on a particular response (Montgomery, 2005). By fitting a response surface to our experimental data, we can greatly facilitate the quantification of the relationship between factors and response. Therefore, three-dimensional response surfaces were plotted by the effects of different combinations on the responses. The data were analyzed by Design Expert 13 and response surface models for temperature, salinity and stocking density were selected and the hierarchy was automatically corrected using reverse stepwise regression (Alpha out of 0.1). The general formula for the model is:

$$Y = \beta_0 + \beta_1 T + \beta_2 S + \beta_3 D + \beta_{12} T \times S + \beta_{13} T \times D + \beta_{23} S \times D + \beta_{11} T^2 + \beta_{22} S^2 + \beta_{33} D^2 + \beta_{123} T \times S \times D + \beta_{112} T^2 \times S + \beta_{113} T^2 \times D + \beta_{122} T \times S^2 + e.$$

Here, Y indicates the responses (AGR and SR). β_0 represent the intercept of the regression equation. β_1, β_2 and β_3 are the linear effects of temperature, salinity and stocking density, respectively; β_{12}, β_{13}, β_{23} are the interactive effects of temperature and salinity, temperature and stocking density, and salinity and stocking density, respectively, β_{11}, β_{22}, β_{33} are the quadratic effects of temperature, salinity and stocking density, respectively; β_{123} are the cubic effects of temperature, salinity and

Table 3
Regression coefficients, standard errors, and 95% confidence intervals for the predicted models of accumulated growth rate and survival rate of diploids, triploids and tetraploids *Crassostrea angulata* larvae.

Factors	Coefficient estimate		d. f.		Standard error		95% CI			
							Low		High	
	AGR	SR	AGR	SR	AGR	SR	AGR	SR	AGR	SR
DD										
Intercept	12.11	59.07	1	1	0.13	0.36	11.80	58.23	12.42	59.90
T	1.00	−3.97	1	1	0.09	0.24	0.80	−4.52	1.21	−3.42
S	0.96	3.78	1	1	0.14	0.24	0.64	3.22	1.28	4.33
D	−1.29	−7.37	1	1	0.09	0.37	−1.50	−8.23	−1.09	−6.51
TS	−0.24	−2.01	1	1	0.12	0.31	−0.51	−2.73	0.03	−1.29
TD	−0.28	1.07	1	1	0.12	0.31	−0.55	0.35	−0.02	1.80
SD	−0.22	−2.07	1	1	0.12	0.31	−0.49	−2.79	0.04	−1.34
T^2	−1.05	−16.63	1	1	0.09	0.23	−1.25	−17.17	−0.85	−16.09
S^2	−2.00	−17.53	1	1	0.09	0.23	−2.20	−18.07	−1.80	−17.00
D^2	−0.58	−3.00	1	1	0.09	0.23	−0.78	−3.54	−0.38	−2.46
TSD	−0.08	−0.19	1	1	0.12	0.31	−0.35	−0.91	0.19	0.54
T^2S	0.07	/	1	/	0.18	/	−0.35	/	0.49	/
T^2D	/	1.56	/	1	/	0.49	/	0.43	/	2.68
TS^2	/	/	/	/	/	/	/	/	/	/
DT										
Intercept	14.84	60.96	1	1	0.12	0.23	14.57	60.39	15.12	61.53
T	0.98	−4.49	1	1	0.08	0.24	0.80	−5.08	1.16	−3.90
S	1.17	4.20	1	1	0.08	0.24	0.98	3.61	1.35	4.79
D	−1.46	−7.37	1	1	0.08	0.24	−1.65	−7.95	−1.28	−6.78
TS	−0.49	−2.01	1	1	0.11	0.20	−0.73	−2.50	−0.25	−1.52
TD	−0.25	1.07	1	1	0.11	0.20	−0.49	0.58	−0.01	1.57
SD	−0.40	−2.07	1	1	0.11	0.20	−0.64	−2.56	−0.16	−1.57
T^2	−0.98	−17.00	1	1	0.08	0.15	−1.16	−17.37	−0.80	−16.63
S^2	−2.02	−18.00	1	1	0.08	0.15	−2.20	−18.37	−1.84	−17.63
D^2	−0.20	−3.23	1	1	0.08	0.15	−0.38	−3.60	−0.02	−2.87
TSD	0.18	−0.19	1	1	0.11	0.20	−0.06	−0.68	0.42	0.31
T^2S	/	−0.44	/	1	/	0.31	/	−1.21	/	0.32
T^2D	/	1.56	/	1	/	0.31	/	0.79	/	2.32
TS^2	/	0.72	/	1	/	0.31	/	−0.04	/	1.49
TT										
Intercept	7.36	20.54	1	1	0.12	0.59	7.09	19.23	7.63	21.85
T	0.88	−2.41	1	1	0.08	0.39	0.70	−3.28	1.07	−1.54
S	0.96	1.89	1	1	0.12	0.39	0.68	1.02	1.24	2.75
D	−1.36	−4.08	1	1	0.08	0.39	−1.54	−4.95	−1.18	−3.22
TS	−0.26	1.10	1	1	0.10	0.51	−0.50	−0.03	−0.03	2.23
TD	−0.09	−2.04	1	1	0.10	0.51	−0.32	−3.17	0.15	−0.91
SD	−0.37	1.36	1	1	0.10	0.51	−0.61	0.23	−0.13	2.50
T^2	−0.66	−3.60	1	1	0.08	0.38	−0.83	−4.44	−0.48	−2.75
S^2	−1.61	−4.26	1	1	0.08	0.38	−1.78	−5.10	−1.43	−3.41
D^2	−0.03	−0.39	1	1	0.08	0.38	−0.21	−1.23	0.14	0.45
TSD	−0.05	/	1	/	0.10	/	−0.29	/	0.18	/
T^2S	0.22	/	1	/	0.16	/	−0.15	/	0.59	/
T^2D	/	/	/	/	/	/	/	/	/	/
TS^2	/	/	/	/	/	/	/	/	/	/

AGR and SR represent accumulated growth rate and survival rate, respectively. T, S and D are temperature, salinity and stocking density, respectively. DD, DT and TT indicate diploids, triploids and tetraploids *Crassostrea angulata* larvae, respectively. The values in the table are all coded values, and the coefficients are estimated according to the coded values. / stands for the factors are not contained in the models.

stocking density. β_{112}, β_{113}, β_{122} are the cubic effects of temperature and salinity, temperature and stocking density, respectively; *e*, random error, is assumed to conform to the normal distribution with a mean of zero.

The significance of each model was tested using analysis of variance (ANOVA) and the adequacy of the model was determined using the lack-of-fit test. Once the models were built, we used the coefficient of determination (R^2), adjusted coefficient ($Adj\text{-}R^2$), predicted coefficient ($Pred\text{-}R^2$) and adeq precision to indicate the goodness of fit and predictive ability of these models. Three-dimensional response surface plots and corresponding contour maps were generated with Origin 22 (Northampton, USA) and applied to assess the combined effects of temperature, salinity and stocking density on larval growth and survival. $P < 0.05$ was considered statistically significant in this study.

3. Results

3.1. The significance, adequacy and fit of the models

ANOVA results demonstrated the resultant model equations for both accumulated growth rate (AGR) and survival rate (SR) of the diploids (DD), triploids (DT) and tetraploids (TT) of *C. angualta* can represent the experimental data ($P < 0.0001$) (Table 2). For AGR of the three groups, the pure error mean squares were all very small (0.1239 for DD, 0.0469 for DT and 0.067 for TT), indicating the experimental data could be reproduced very well. And the F-value of the lack-of-fit for AGR of DD, DT and TT were 0.6569, 3.03 and 1.71 ($P > 0.05$), respectively, meaning the resulting models are very appropriate. Moreover, the lack-of-fit tests for SR of the three groups also imply the lack-of-fit is not significant relative to the pure error ($P > 0.05$) (Table 2). Thus, the models suggested here both for AGR and SR of the three groups are of great adequacy.

Table 4
Fitting statistics for models with different responses.

Item	DD		DT		TT	
	AGR	SR	AGR	SR	AGR	SR
R^2	0.9929	0.9993	0.994	0.9998	0.9929	0.9755
Adj-R^2	0.9833	0.9983	0.9873	0.9993	0.9832	0.9535
Pred-R^2	0.9407	0.9871	0.9398	0.9863	0.8684	0.8655
Adeq precision	30.7338	86.974	43.2476	128.1133	37.0515	26.4587
C.V. %	3.41	2.64	2.36	1.64	5.03	9.64

R^2 represents determination coefficient. *Adj-*R^2 is the adjusted R^2. *Pred-*R^2 stands for the predicted R^2. The two values, *Adj-*R^2 and *Pred-*R^2, are high and close (difference < 0.2), indicating that the regression model adequately describes the experimental process. Adeq precision measures the signal to noise ratio. A ratio >4 is desirable. C.V. $< 10\%$ indicates a high degree of confidence and precision in the experiment.

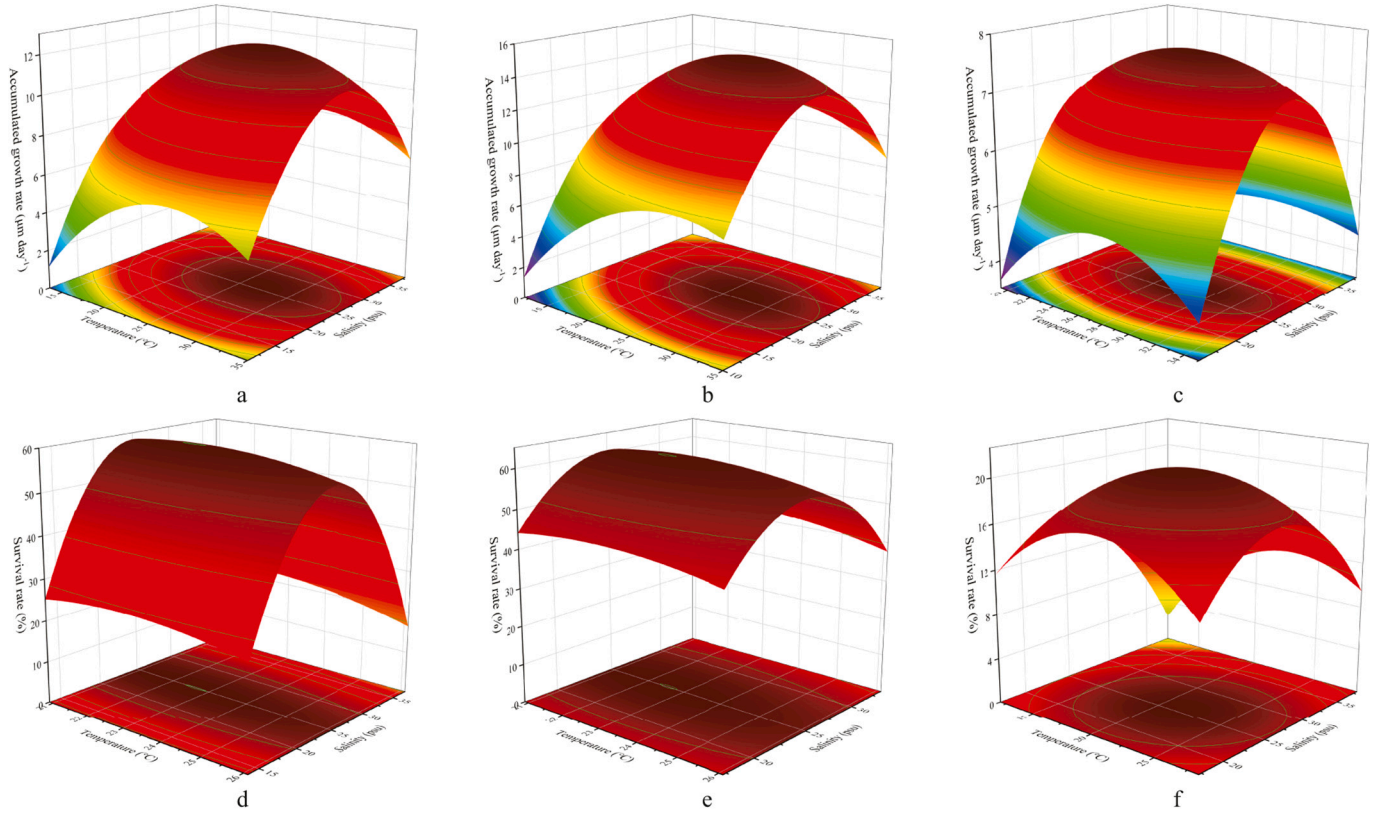

Fig. 1. Response surface plots of the combined effects of temperature and salinity on the accumulated growth rate (AGR) and survival rate (SR) of diploids (DD), triploids (DT) and tetraploids (TT) Fujian oyster *Crassostrea angulata* (stocking density = 5.25 ind. ml^{-1}). (1) a and d represent AGR and SR for DD, respectively. (2) b and e are AGR and SR for DT, respectively. (3) c and f indicate AGR and SR for TT, respectively.

By means of statistical analysis, the coefficient was estimated (Table 3). Besides, the ANOVA analysis revealed that the effects of temperature, salinity and stocking density were significantly affecting the AGR and SR among the all three groups ($P < 0.05$) (Table 2). Whereas, the cubic effects of the three factors are nonsignificant for both AGR and SR ($P > 0.05$). The linear effects of the temperature-salinity interaction and salinity-stocking density interaction are nonsignificant for AGR of DD ($P > 0.05$). And the linear effect of the temperature-salinity interaction is also nonsignificant for SR of TT ($P > 0.05$). Moreover, we computationally modeled the AGR and SR of DD, DT, and TT as a function of temperature (T), salinity (S), and stocking density (D) (Supplementary file 1).

The goodness of fit for the six model equations was assessed using determination coefficients (R^2), resulting in R^2 values of 0.9929 for AGR and 0.9993 for SR of DD, respectively (Table 4). Moreover, all the values of R^2 were ≥ 0.97 for both AGR and SR of DT and TT. The adjusted R^2 (*Adj-*R^2) were all in reasonable agreement with the predicted R^2 (*Pred-*R^2), in which the differences between the two factors were all <0.2. Besides, all the values of adeq precision were much >4 for the six model equations. Such a high ratio indicates an adequate signal. Furthermore, all the values of *Pred-*R^2 were ≥ 0.86 for both AGR and SR of DD, DT and TT. Based on these values, these models were of greater predictability. Therefore, these six model equations can be used to navigate the design space.

3.2. Variations in AGR and SR for three groups under the combined effects of temperature, salinity and stocking density

By looking at these contours or response surfaces, they are all single peaked (Figs. 1, 2, 3). All the AGR and SR decreases with decreasing or increasing temperature and salinity. In addition, except for DD, the AGR and SR of the other two groups decreased with increasing stocking density. In other words, AGR and SR varied curvilinearly when temperature and salinity varied linearly, while AGR and SR varied linearly when stocking density varied linearly. It shows that there is an optimum value on the response surface, and deviation from this optimum value

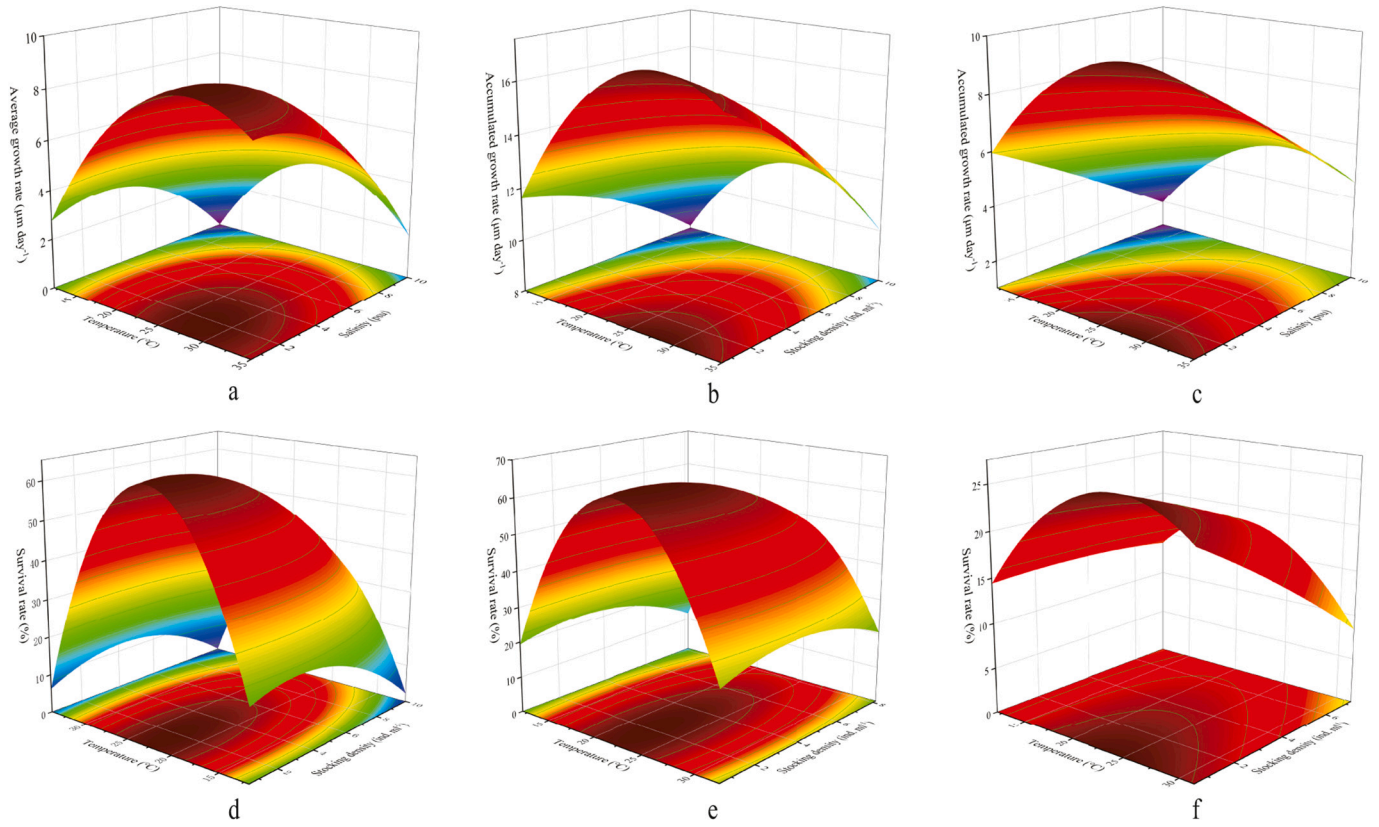

Fig. 2. Response surface plots of the combined effects of temperature and stocking density on the accumulated growth rate (AGR) and survival rate (SR) of diploids (DD), triploids (DT) and tetraploids (TT) Fujian oyster *Crassostrea angulata* (salinity = 24.0 psu). (1) a and d represent AGR and SR for DD, respectively. (2) b and e are AGR and SR for DT, respectively. (3) c and f indicate AGR and SR for TT, respectively.

leads to a significant decrease in AGR and SR. For DT, the highest AGR and SR were achieved at 22.0–24.0 °C – 25.0-27.0 psu – 0.5 – 1.0 ind. ml^{-1} combinations, with up to 16.0 μm day^{-1} or more for AGR and ca. 60.0% for SR. The AGR and SR of DD and TT also reached optimal levels under this temperature-salinity-stocking density combination, but differences existed between their optimal values. In addition, the response surface plots showed that under extreme conditions (temperatures below 3.0 °C or above 35.0 °C, salinities below 5.0 psu or above 38.0 psu, and stocking densities in high levels), the AGR and SR of the three groups were very low or even below 0. This suggested that the growth and survival of larvae of the three groups were significantly compromised by the extreme environmental factors.

3.3. Optimization and verification

For larval rearing, it is desirable that both AGR and SR of the larvae are simultaneously maximized. The optimal temperature-salinity-stocking density combinations of AGR and SR for DD, DT and TT can be obtained by maximizing these two corresponding desirability functions. Simultaneous optimization of the fitted response equations for temperature, salinity and stocking density showed that the AGR and SR of DD reached maximum values of 13.10 μm day^{-1} and 63.55% at the combination of 24.0 °C, 25.89 psu and 1.99 ind. ml^{-1}, respectively. The maximum values of AGR and SR of DT (17.13 μm day^{-1} for AGR and 64.81% for SR) were achieved under the combined conditions of temperature at 23.6 °C, salinity at 26.5 psu and stocking density at 0.5 ind. ml^{-1}. For TT, the optimal cultivation condition was a combination of 25.9 °C - 25.96 psu - 0.5 ind. ml^{-1}, under which AGR and SR reached their maximum values of 10.11 μm day^{-1} and 25.98%, respectively.

In order to further verify the reliability of the response surface optimization conditions, validation experiments were carried out under the resulting optimal conditions. That is, AGR and SR were evaluated for DD, DT and TT at the combinations of 24.0 °C - 25.89 psu - 1.99 ind. ml^{-1}, 23.6 °C - 26.5 psu - 0.5 ind. ml^{-1} and 25.9 °C - 25.96 psu - 0.5 ind. ml^{-1}, respectively. The obtained AGR for DD, DT and TT are 16.99 μm day^{-1}, 13.21 μm day^{-1} and 10.56 μm day^{-1}, respectively. The values of SR are 63.11%, 62.00% and 24.12% for DD, DT and TT, respectively. This is basically in line with the maximum AGR and SR obtained under the theoretically predicted conditions, which indicates that the model optimization conditions are reasonable and effective.

4. Discussion

The growth and survival of mollusks were not only affected by one single environmental factor alone. A central composite design is most appropriate when studying the integrated effects of several continuously varying variables or factors on a given response (Montgomery, 2005). In this study, significant effects of temperature, salinity, stocking density and their interactions were exhibited in the growth and survival of *C. angulata* in all three ploidies.

4.1. Effects of temperature, salinity, stocking density and their interactions on characteristics of C. angulata

In this study, significant effects of temperature on the growth and survival of *C. angulata* were observed regardless of its ploidy. Temperature was considered to be the most essential modifier for energy flow and growth of the organism (Xu et al., 2020). Studies on *C. gigas* (Rico-Villa et al., 2009; Han and Li, 2018) and *Ostrea angasi* (O'Connor et al., 2015) also indicated that the larval growth was proportional to temperature within a certain range. This may be related to an increase in larval feeding rate and digestibility to meet the metabolic demands of

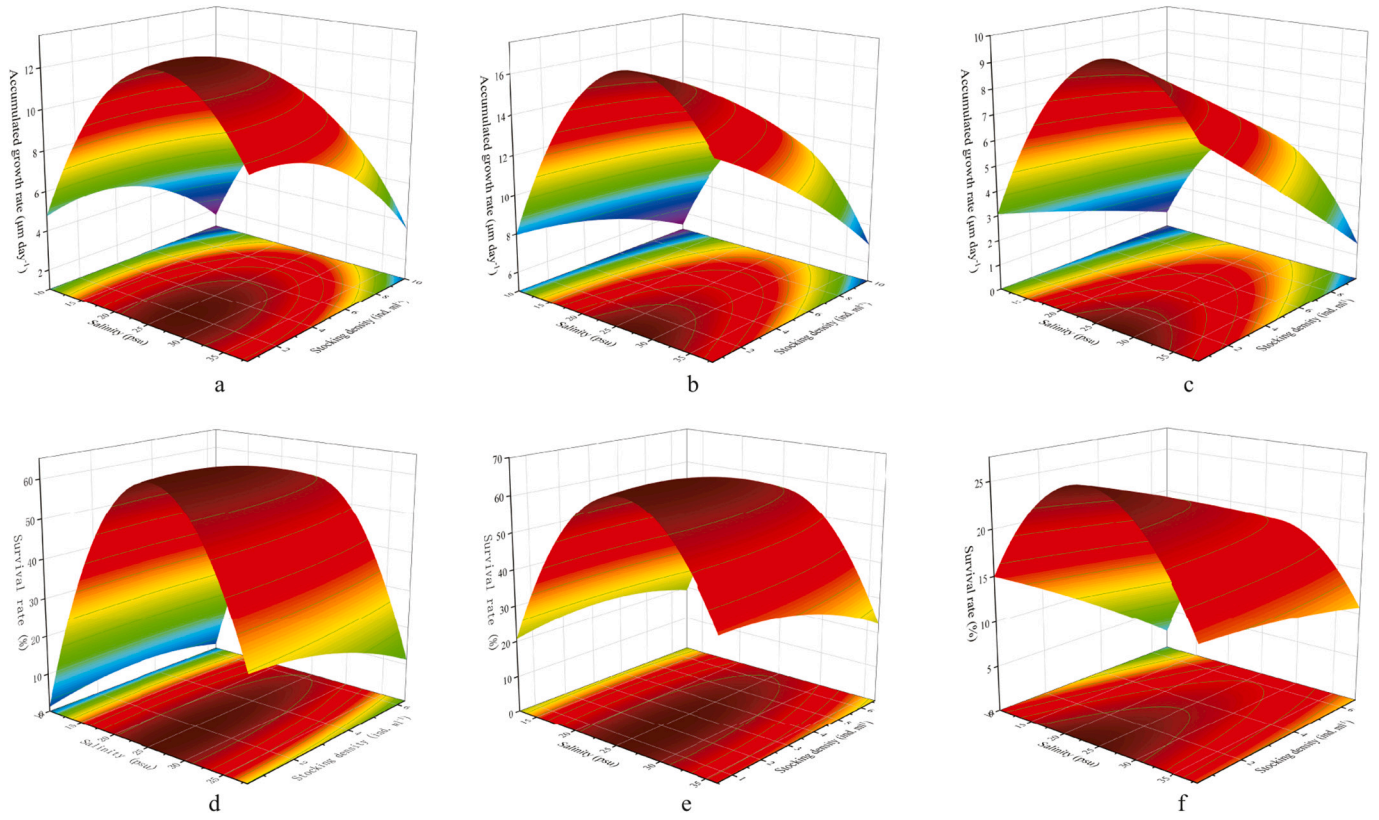

Fig. 3. Response surface plots of the combined effects of salinity and stocking density on the accumulated growth rate (AGR) and survival rate (SR) of diploids (DD), triploids (DT) and tetraploids (TT) Fujian oyster *Crassostrea angulata* (temperature = 23.5 °C). (1) a and d represent AGR and SR for DD, respectively. (2) b and e are AGR and SR for DT, respectively. (3) c and f indicate AGR and SR for TT, respectively.

rapid growth (Rico-Villa et al., 2009). In contrast, when the temperature exceeds the normal range, the growth rate is negatively correlated with the increase in temperature. This was also confirmed in this study. Mollusks have the ability to adjust their physiological activities to environmental temperature changes within an upper and lower margin of non-lethal temperatures (Rico-Villa et al., 2009). However, when the temperature rises to a certain point, it would accelerate the development of pathogenic microorganisms in the water, increase oxygen consumption, and weaken the feeding activity of larvae. The growth and survival of the larvae will be reduced (Xu et al., 2020; Gruffydd and Beaumont, 1972). Additionally, temperature stress can reduce the activity of blood cells and enzymes of shellfish, weaken the immune defense ability of the organism, and adversely affect respiratory feeding, growth and survival (Meng et al., 2021; Rahman et al., 2019).

Salinity has a great influence on the immune resistance of mollusks. A high larval mortality was observed in extreme cases of too-high or too-low salinity concentrations in this study, mainly due to changes in the osmotic pressure of seawater that exceeded its regulatory capacity (Xu et al., 2017). Zhao et al. (2012) proposed that cell adhesion and communication, signaling receptors and cytoskeleton-related genes might play important roles in salt shock. These genes may contribute to oyster recovery adaptation to hypotonic shock and osmotic stress signals. And the expression of these genes may be suppressed in extreme environments.

Several studies have demonstrated that high-density culturing could lead to inhibitory effects on the growth and/or survival of shellfish larvae in both recirculation aquaculture systems and static systems (Ramos et al., 2021; Andersen et al., 2000; Rico-Villa et al., 2009; Xu et al., 2020). This reduction in the growth and survival of larvae may be related to the deterioration of water quality (Sarkis et al., 2006). Excretion products from high densities of larvae, which are mainly composed of nitrogen compounds, with ammonia dominating, can deteriorate water quality, especially under limited rearing conditions (Xu et al., 2020). The deterioration of water quality caused by high-density breeding cannot be completely eliminated, even in recirculating aquaculture systems (Ramos et al., 2021). In addition, as high-density breeding forces more physical contact among larvae, resulting in increased shell and tissue damage, this may be an additional reason for the low survival rate due to high density in this study (Loosanoff and Davis, 1963; Sprung, 1984; Avila et al., 1997).

The results of stepwise regression and ANOVA of the six model equations showed that the two-by-two linear interaction effects between the three factors of temperature, salinity and stocking density were significant for the responses, suggesting that the breeding process of larvae is not affected by one single factor, but rather a combination of multiple factors that are mutually constraining and act in concert. However, their cubic effects were not significant for all responses in this study, which has also been reported in the study of the black shell strain of *C. gigas* (Xu et al., 2020).

4.2. Effects of ploidy on characteristics of C. angulata

Significant effect of ploidy on the growth and/or survival of *C. angulata* was observed even under optimal breeding conditions in this study. Tetraploids performed the poorest among the three groups. A study on tetraploid *C. gigas* also pointed out that tetraploid larvae were less adaptable to the environment than diploid larvae (Li et al., 2022a, 2022b). The low adaptive capacity of tetraploids may be related to the higher energy required to segregate four sets of chromosomes and develop giant cells during meiosis and mitosis (McCombie et al., 2005). Moreover, this difference may be also closely related to the vulnerability of tetraploids to adapt to genomic changes (Comai, 2005). The growth rates of triploids of *C. angulata* were significantly greater than these of diploids and tetraploids under optimal conditions in this study. Superior

traits of triploids over diploids and tetraploids were also reported in *C. gigas* (Allen and Downing, 1986; Guo et al., 1996; Nell, 2002), *C. sikamea* (Wu et al., 2019) and *C. hongkongensis* (Qin et al., 2019). The gigantic cell sizes of triploids and the hybrid vigor generated by the increased heterozygosity of the triploid genome may be the possible reasons for the outstanding performance of triploids at the early larval stage (Guo and Allen, 1994; Wang et al., 2002; Piferrer et al., 2009). In addition, we speculate that the ingestion rates and food utilization of triploids were higher than those of diploids and tetraploids, leading to superior performance of triploids.

Just minor differences existed in the optimal temperature - salinity - stocking density combinations among diploids, triploids and tetraploids of *C. angulata* in this study. However, the traits of oysters varied with ploidy even under optimal conditions. Furthermore, a study on *C. gigas* also showed that optimal breeding conditions for tetraploid and diploid larvae were similar, but diploids showed growth and survival advantages compared to tetraploids (Li et al., 2022a, 2022b). Therefore, we conjectured that the differences between traits of oysters with different ploidy were most likely attributed to their nature rather than to environmental factors.

5. Conclusions

Significant effects of temperature, salinity and stocking density combinations on the larval of *C. angulata* with different ploidies were found in this study. The best performance in the three groups were obtained at temperatures of 23.0–26.0 °C, salinities of 25.0–27.0 psu and stocking densities of 0.5–2.0 ind. ml^{-1}. Moreover, the optimum combinations for AGR and SR for the three groups were obtained by optimal modeling equations. The growth and survival of tetraploids were at a lower level even under optimal conditions, and in particular the survival rate was significantly lower than that of diploids and triploids. This indicates considerable attention needed to be drawn to the performance of this vulnerable group in actual production. Validation experiments showed that the optimal combinations obtained from the modeling could be applied to the breeding of *C. angulata*. Consequently, optimum conditions obtained by optimizing the models can be used in the practical production. Applying these optimization factors will help to improve the nursery quality of *C. angulata*.

CRediT authorship contribution statement

Yuanxin Liang: Conceptualization, Investigation, Writing – original draft. **Xianchao Bai:** Data curation. **Chengxun Xu:** Resources. **Qi Li:** Conceptualization, Funding acquisition, Resources, 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.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.740868.

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