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Different responses between orange variant and cultured population of the Pacific oyster *Crassostrea gigas* at early life stage to temperature-salinity combinations

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Abstract

Although breeding of rare shell colour variants has drawn widespread attention from shellfish breeders, the potential disadvantages of their adaptive capacity have been ignored in practice. To explore the difference in adaptive capacity between orange shell variant (OSO) and commercially cultured population (CPO) of the Pacific oyster *Crassostrea gigas* at early life stage, the development to D-larvae and larval survival and growth (just 23 and 30°C for larval experiment) of them were compared under different temperature (16, 23 and 30°C) and salinity (17, 25 and 33 psu) combinations. In this study, at 23°C and 25 psu, for both OSO and CPO there was no difference in fertilization rates and survival ($p > .05$) (mean percentages of D-larvae after fertilized 40 hr $\geq$ 95.00%; mean larval survival rates on day 10 > 80.00%). However, the percentage of D-larvae of CPO at 40 hr was significantly ($p < .05$) higher than OSO at temperatures of 16 and 30°C and 25–33 psu and 17 psu at 23°C. Similarly, CPO has a better larval survival on day 10 and growth than OSO at salinities of 17 and 33 psu at 23°C. Overall, our results indicate that OSO can have an equally good performance like CPO at early life stage under optimal condition (23°C; 25 psu), but the potential disadvantages in adaptive capacity will be shown at suboptimal conditions. These findings can guide future hatchery breeding of OSO, and suggest the potential disadvantages in adaptive capacity in rare colour variants need more attention in further breeding.

KEYWORDS
adaptive capacity, *Crassostrea gigas*, early life stage, orange variant, temperature-salinity combination

1 | INTRODUCTION

The Pacific oyster (*Crassostrea gigas*) is one of the most widely farmed aquaculture species worldwide (FAO, 2014), and its shell colouration attracts constant attention of worldwide breeders (Brake, Evans & Langdon, 2004; Evans, Camara & Langdon, 2009; Ge, Li, Yu & Kong, 2015; Kang et al., 2013). In our selective breeding practice of the Pacific oyster, we obtained a number of orange variants (Figure 1) and obtained genetically stable strains through family selection. As orange shell colour is never been found in the wild populations of *C. gigas*, orange variant of *C. gigas* may become a valuable breeding line.

However, rare colour variants are commonly inferior in growth and/or environmental adaptive capacity than common colour shellfish in wild populations (Brake et al., 2004; Newkirk, 1980; Wolff & Garrido, 1991; Zheng, Xu, Zhang, Liu & Wang, 2008). This perhaps results from limited effective population size of the isolated colour populations, and small effective population can lead to inbreeding depression (Evans, Matson, Brake & Langdon, 2004; Hedgecock & Sly, 1990; Keller & Waller, 2002). Similarly, inbreeding and

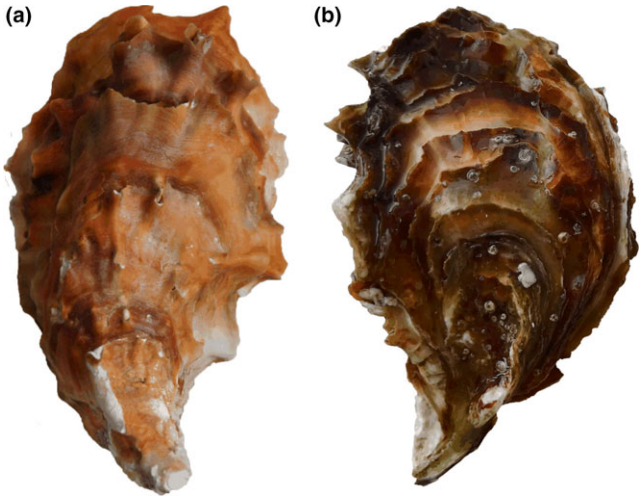

FIGURE 1 The left shell (a) and right shell (b) of orange shell variant of the Pacific oyster

inbreeding depression may be present in the orange shell variant obtained through artificial breeding from the cultured population of *C. gigas*. Inbreeding can make individuals more susceptible to adverse environmental conditions and increased mortality (Keller & Waller, 2002), and environmental stresses affect early-life stage of marine benthic invertebrates more than their adult counterparts (Ko et al., 2014). Although the effects of environmental factors on the early life stage of *C. gigas* have been intensively investigated (Helm & Millican, 1977; His, Robert & Dinet, 1989; Kheder, Moal & Robert, 2010; Ko et al., 2014; Nell & Holliday, 1988; Rico-Villa, Bernard, Robert & Pouvreau, 2010; Rico-Villa, Pouvreau & Robert, 2009; Robinson, 1992). Unfortunately, few studies have been undertaken to examine adaptive capacity of rare colour variants of *C. gigas* to environmental stresses.

This study was designed to compare the performance in embryonic and larval stages of orange shell variant and commercially cultured population of *C. gigas* under different temperature-salinity combinations. Those data will help elucidate the differences in adaptive capacity between orange shell variant and commercially cultured population of *C. gigas* at early life stage and facilitate the breeding of oyster with desirable colour traits.

2 | MATERIALS AND METHODS

2.1 | Broodstock collection

Orange shell individuals were found in the offspring of purple-black shell colour individuals, which were produced by crossing females with black shell colouration and males with purple shell colouration selected from the cultured population of *C. gigas*. Using those orange shell individuals as parents, we had bred genetically stable strains of *C. gigas* with orange left and right shell colour (OSO), combining four generations successive family selection for fixing orange shell colour from 2009 to 2013, and three generations successive mass selection to promote growth from 2014 to 2016. In May 2016, 1-year-old OSO were collected from Rushan, Shandong province, China (36.4°N, 121.3°E). During the same period, random samples of equivalent aged commercially cultured *C. gigas* (CPO) with common shell colour were obtained from local oyster farms. Commercially cultured oysters were hatchery produced by using cultured populations as parents and no selected strains or lines have been used. Both OSO and CPO were transferred to Laizhou Haiyi hatchery, Yantai, Shandong province, and conditioned at ambient conditions (temperature: 23°C; salinity: 25 psu) prior to experiment.

2.2 | Fertilization and larval rearing

Two hundred adult individuals of OSO (shell height, 10.38 ± 2.88 cm) and CPO (shell height,10.54 ± 4.06 cm) were dissected and gametes were obtained by gonad striping (50 males and 50 females respectively). Fertilized eggs were divided into two parts, one was transferred immediately into 2 L tanks under various temperature-salinity combinations for the embryonic experiment at a concentration of 50 eggs/ml and the other was reared at ambient conditions (temperature: 23°C; salinity: 25 psu), for the subsequent larval experiment.

After 24 hr incubation, D-larvae (shell length: OSO, 75.41 ± 3.66 μm; CPO, 75.71 ± 3.81 μm; $n = 30$, $p > .05$) were collected and transferred into 25 L tanks at an initial stocking density of 2 larvae/ml, and treated with various salinity-temperature combinations, acclimated for 1 day by adjusting 0.5°C/hr and 1 psu/hr (Rico-Villa et al., 2009). Veliger were fed with *Isochrysis galbana* at early stage (shell length < 120 μm) and mixture of *I. galbana* and *Platymonas subcordigoramis* at later stage, and feed ration was increased with larval growth. During the initial period, culture water was exchanged 1/3 daily, and gradually increasing exchanged water volume to 1/2 twice everyday as larvae grew. Prior to each water change, replacement water was filtered through sand filters and non-wovens polypropylene fabric and adjusted to the test conditions. The larval experiment ended when 20%–30% larvae were competent (larvae with active foot and eyes).

2.3 | Experimental design

A two-factor experimental design was used to examine the effect of temperature and salinity on the development to D-larvae, and larval survival and growth of OSO compared with CPO. For the experiment of the development of embryos to D-larvae, there were three levels of temperature (16, 23 and 30°C) and three levels of salinity (17, 25 and 33 psu) at each temperature, and each tested group included three replicates. Similarly, for the experiment of the larval growth and survival, there were two temperatures (23 and 30°C) and three salinities (17, 25 and 33 psu) at each temperature, and each test group included three replicates. Water temperature was maintained by water bath with immersed heaters or water chiller (HC-150A, 33ILEA, China) and a temperature regulator, while water salinity adjusted by diluting natural seawater with filtered freshwater or adding aquarium salt and measured by a refractometer (ATAGO).

The levels for each temperature and salinity were based on the previous studies about optimal larval rearing conditions (Helm & Millican, 1977; Ko et al., 2014; Robinson, 1992) and the range of environmental conditions of aquaculture area in the Yellow Sea (Chu, Yuchun & Kuninaka, 2005; Liu, Hu & Tang, 2008). The temperature-salinity combination of 23°C and 25 psu was optimal larval rearing environment in this study and consistent with other studies (Dineshram et al., 2016; Ko et al., 2014).

2.4 | Measurement

In the embryonic experiment, replicates were sampled at 24 and 40 hr post-fertilization. Before sampled, the water in each tank was completely mixed. Then, a 5 ml sample was collected randomly and fixed by the addition of 1% Lugol's solution. The percentage of normal D-larvae in each replicate sample was recorded under a light microscope (100×).

In the larval experiment, replicates were sampled every 3 days starting on day 4 until the day of 20%–30% larvae were competent. A 50 ml sample was collected randomly after the water was mixed as above and fixed by the addition of 1% Lugol's solution to determine the mean shell length and survival. The total number of filled larval shells (distinguishing from empty larval shells) and the shell length of 30 larvae randomly selected in each replicate sample were quantified using a light microscope (100×) fitted with an ocular micrometer. Mean larval growth rates were determined from the initial measurement to the day of 20%–30% larvae were competent. Larval survival was based on an initial density.

2.5 | Statistical analysis

Percentage data were given as mean ± *SD* ($n = 3$) in the tables and figures. The data of the percentage development of embryos to D-larvae stage and the larval survival rates were analysed by four-way ANOVA, with populations (OSO and CPO), temperature, salinity and exposure time (hr or days) as fixed factors. If there was an interaction among the factors, the effects of each factor were analysed separately at each level of the other factors by one-way ANOVA followed by multiple comparison Tukey test. As the larvae reared at 30°C and salinities 17 and 33 psu were stopped ahead because of the mass mortality, the mean larvae growth rates under the rest temperature-salinity combinations were analysed using one-way ANOVA followed by multiple comparison Tukey test. Prior to analysis, the data were checked for homogeneity of variances using Levene's test respectively. All statistical analyses were performed using SPSS (Statistical Package for Social Science) 20.0 software. Differences were considered statistically significant if $p < .05$.

3 | RESULTS

3.1 | The development to D-larvae

All four factors, exposure time, temperature, salinity and population, interacted with each other to influence the development to D-larvae (Table 1a). Overall, at all other test conditions, percentage development increased between 24 hr and 40 hr post-fertilization, except the CPO that had approached 100% within 24 hr at temperature 23°C and salinities of 25 and 33 psu and neither CPO nor OSO developed normal D-larvae at temperatures of 17 and 30°C and 17 psu (Figure 2). Extending the exposure time from 24 to 40 hr narrowed the gap between populations at 23°C and salinities of 25 and 33 psu, but increased the gap between populations at other test conditions. Within fertilization 24 hr (Figure 2a), over 75% embryos of both OSO and CPO developed to D-larvae at 23°C and salinities of 25 and 33 psu, and the percentage of OSO development was significantly ($p < .05$) lower than CPO, which were 88.33 ± 3.51% and 97.33 ± 0.58% (25 psu), 79.00 ± 4.00% and 92.33 ± 2.52% (33 psu) respectively. After 40 hr, percentage of OSO development increased to the similar ($p > .05$) level as CPO at 23°C and salinities of 25 and 33 psu. However, at temperatures of 16 and 30°C and 33 psu, CPO developed significantly ($p < .05$) faster than OSO from 24 to 40 hr, especially the percentage development of CPO was about twice as high as OSO at 16°C and 33 psu, although no significant ($p > .05$) difference between them within 24 hr. In addition, at temperatures 16 and 30°C and 25 psu and 23°C at 17 psu, the percentage development of CPO has been significantly ($p < .05$) higher than OSO at both 24 and 40 hr post-fertilization, and the gap between them increased from 6%–24% at 24 hr to 12%–35% at 40 hr.

3.2 | The growth and survival rate of larvae

The larvae reared at 30°C were stopped on day 10, as 20%–30% larvae of both populations were competent at 30°C and 25 psu and both populations at salinities of 17 and 33 psu occurred the mass mortality (survival rate at 17 psu: OSO 4.07 ± 3.73%, CPO 7.32 ± 2.44%; survival rate at 33 psu: OSO 8.13 ± 3.73%, CPO 10.57 ± 5.08% respectively). Moreover, the survival rate of OSO and CPO at the end of the larval rearing experiment (16 days for 25 psu, 19 days for salinities 17 and 33 psu; data not shown) were similar ($p > .05$) to each of that on day 10 at 23°C and all salinities. Consequently, the survival rates of OSO and CPO under different temperature-salinity combinations were analysed on day 4, 7 and 10 only (Figure 3a–c).

All four factors, exposure time, temperature, salinity and population, interacted with each other to affect the larval survival rate (Table 1b). At 23°C, the population had no effect on the larval survival rate at 25 psu with extending exposure time, but the effect of population was gradually enhanced at salinities of 17 and 33 psu. At 23°C and 25 psu, the survival rates of CPO and OSO were above 75% within 10 days and no significant ($p > .05$) difference between them. However, at 23°C and salinities of 17 and 33 psu, the CPO survival rate was similar ($p > .05$) to OSO on day 4 (Figure 3a), but significantly ($p < .05$) higher than OSO on day 10 (Figure 3c), especially at 17 psu, the survival rate of CPO was about threefold higher ($p < .05$) than that of OSO on day 10 (79.33 ± 19.01% and 26.67 ± 6.11%). In addition, high temperature (30°C) significantly

TABLE 1 Analysis of variance (ANOVA) on (a) the mean percentage of embryos to reach the D-larvae stage (excluding abnormal D-larvae) and (b) the mean larval survival rate

		(a) % Normal D-larvae			(b) Larval survival rate		
		$F = 1.468$ $p = .113 > .05$			$F = 1.321$ $p = .159 > .05$		
Source of variation	*Df*	MS	*F*	*p*	MS	*F*	*p*
Temperature (Te)	2 (1)	2.318	832.507	<.001	5.782	950.758	<.001
Salinity (Sa)	2	1.174	421.652	<.001	0.541	88.966	<.001
Populations (Po)	1	0.573	205.919	<.001	0.224	36.796	<.001
Exposure Time (Et)	1 (2)	0.285	102.365	<.001	1.667	274.088	<.001
Te × Sa	2	0.537	193.015	<.001	0.042	6.900	.002
Te × Po	2 (1)	0.052	18.744	<.001	0.178	29.267	<.001
Te × Et	2	0.033	11.831	<.001	0.540	88.770	<.001
Sa × Po	2	0.017	6.005	.004	0.075	12.401	<.001
Sa × Et	2 (4)	0.032	11.370	<.001	0.012	1.922	.116
Po × Et	1 (2)	0.045	16.259	<.001	0.029	4.796	.011
Te × Sa × Po	2	0.038	13.534	<.001	0.131	21.469	<.001
Te × Sa × Et	2 (4)	0.027	9.815	<.001	0.057	9.452	<.001
Te × Po × Et	2	0.047	16.836	<.001	0.012	1.964	.148
Sa × Po × Et	2 (4)	0.012	4.346	.018	0.010	1.568	.192
Te × Sa × Po × Et	2 (4)	0.021	7.435	.001	0.023	3.713	.008
Residual	56 (72)	0.003			0.006		
Total	84 (108)						

OSO, orange shell variant of the Pacific oyster; CPO, commercially cultured population of the Pacific oyster.
This was a four-way analysis with populations (OSO and CPO), temperature (16, 23, and 30°C for embryonic experiment, 23 and 30°C for the larval experiment), salinity (17, 25, and 33 psu) and exposure time (24 and 40 hr for embryonic experiment, 4, 7 and 10 days for larval experiment) as fixed factors, $n = 3$.
Since there was no normal D-larvae developed at 17 psu and 16/30°C for both OSO and the CPO, these tested groups were excluded from the analysis of % normal D-larvae.
Prior to analysis, data were tested for homogeneity of variances, levene's test.

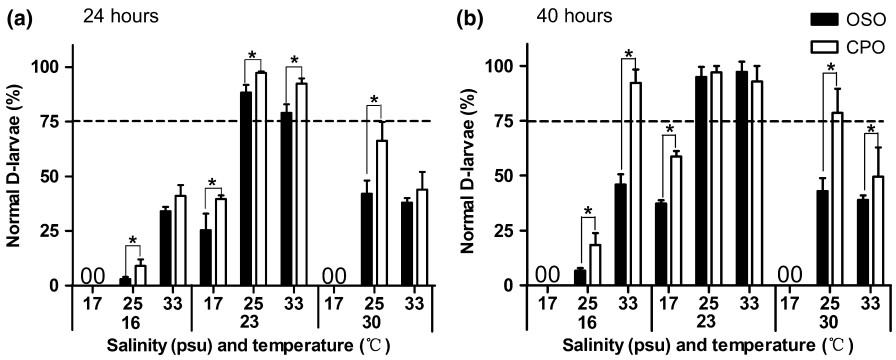

FIGURE 2 The percentage (mean ± *SD*, $n = 3$ replicates) of OSO and CPO embryos to reach the D-larvae stage (excluding abnormal D-larvae) after exposed to different salinity (17, 25, and 33 psu) and temperature (16, 23, and 30°C) combinations for 24 hr (a) and 40 hr (b) post-fertilization, * $p < .05$. OSO, orange shell variant of the Pacific oyster; CPO, commercially cultured population of the Pacific oyster

($p < .05$) reduced the larval survival rates with extending exposure time, and no significant ($p > .05$) difference between OSO and CPO in survival rate on day 10 at each test condition at 30°C (Figure 3c). On day 4, survival rates at 30°C and 17 psu were below 75% while all other test conditions over 75% (Figure 3a). The larval survival rates at 30°C and all salinities had a reduction on day 7 (Figure 3b), and on day 10, only about 25% of the larvae survived at 25 psu and less than 11% at other test conditions (Figure 3c).

The mean larval growth rates of CPO and OSO at each test condition had no significant ($p > .05$) difference, and CPO and OSO larvae grew fastest at 30°C and 25 psu (Figure 3d).

4 | DISCUSSION

In this study, OSO and CPO displayed different responses to various temperature-salinity combinations in the development to D-larvae,

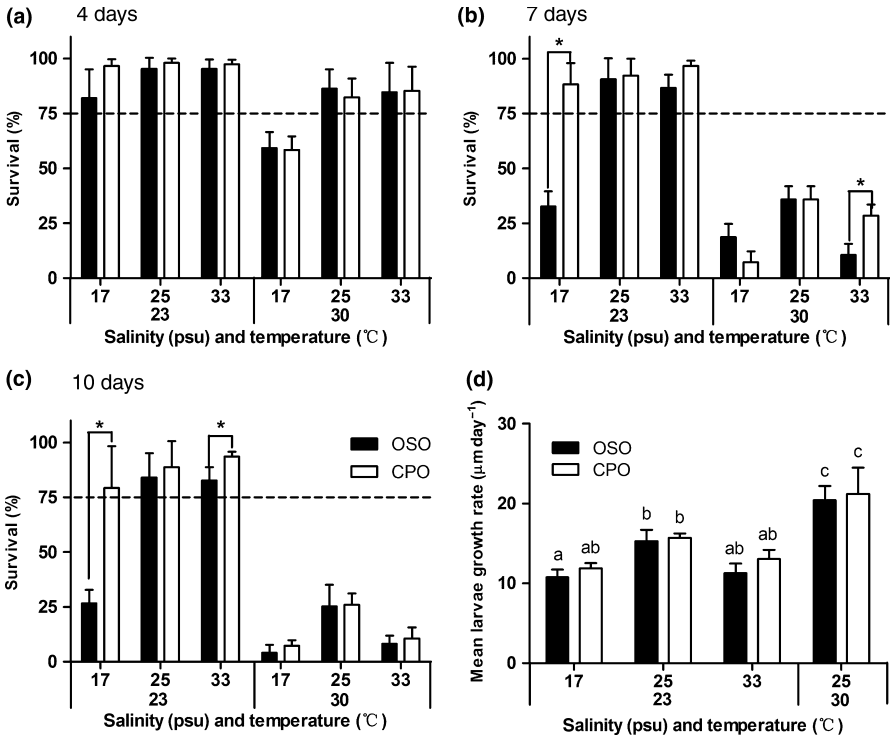

FIGURE 3 The percentage (mean ± *SD*, *n* = 3 replicates) of OSO and CPO larval survival after exposure to salinity (17, 25, and 33 psu) and temperature (23°C and 30°C) combinations for (a) 4 day, (b) 7 day and (c) 10 day, and (d) mean larval growth rate (mean ± *SD*, *n* = 3 replicates) of OSO and CPO from the initial measurement (shell length: OSO 75.41 ± 3.66 μm, CPO 75.71 ± 3.81 μm; *n* = 30, $p > .05$) to the day of 20%–30% larvae completed exposure to above temperature-salinity combinations, excluding larvae under 30°C and salinities of 17 and 33 psu as mass mortality (see Figure 2). * $p < .05$. OSO, orange shell variant of the Pacific oyster; CPO, commercially cultured population of the Pacific oyster. Mean larval growth rates were analysed by one-way ANOVA followed by multiple comparison Tukey test and share the same superscript letters are not significantly different ($p > .05$)

larval survival and growth. For the percentage of D-larvae development and larval survival, there were two major observations on comparing OSO with CPO. Firstly, OSO were vulnerable to the environmental stresses. At optimal conditions (temperature:23°C; salinity: 25 psu), for both OSO and CPO there was no difference in early stage development. At 23°C and 33 psu, the number of D-larvae of OSO at 40 hr was similar ($p > .05$) to CPO and had a high level (>90%), but the survival rate of OSO on day 10 was significantly ($p < .05$) lower than that of CPO. Moreover, embryos of OSO developed slower than CPO at 23°C and 17 psu and temperatures of 16 and 30°C at salinities of 25 or 33 psu, and the larvae survived on day 10 of OSO were almost 1/3 of that of CPO at 23°C and 17 psu. Different responses between OSO and CPO to optimal and suboptimal conditions here are consistent with the results of previous studies conducted with colour variants of other bivalve molluscs. As the larvae reared at optimal conditions, no difference in growth and survival among orange, purple and white shell colour variants of *Argopecten irradians irradians* was observed (Zheng, Zhang & Liu, 2005). Comparing with the experimental conditions, the wild conditions are more variable. Two species of scallops, *Argopecten purpuratus* (Wolff & Garrido, 1991) and *A. irradians irradians* (Zheng et al., 2008) were found in the wild that the growth and/or survival of rare lighter shell colour variants is lower than common darker shell colour individuals. Similar results have been reported for *Mytilus edulis* (Newkirk, 1980) and *C. gigas* (Brake et al., 2004). All these results suggest that the disadvantage in adaptive capacity of rare colour variants to environmental stresses may be not shown in optimal conditions, but be observed in suboptimal conditions. Several studies have demonstrated that shell colours are genetically controlled by a relatively simple genetic basis (Ge et al., 2015; Ky, Nakasai, Pommier, Sham Koua & Devaux, 2016). Furthermore, isolation of rare colour variants may have limited effective population sizes, small effective population sizes lead to inbreeding and inbreeding depression, which can strongly affect individual and population viability (Evans et al., 2004; Hedgecock & Sly, 1990; Keller & Waller, 2002).

Secondly, at severe environmental stress, both OSO and CPO had a bad performance at early life stage and no significant ($p > .05$) difference between them. All the embryos failed to develop to normal D-larvae at temperatures of 16 and 30°C and 17 psu (Figure 2a–b). This perhaps results from low salinity can dramatically decrease the development of embryos to D-larvae (Gamain et al., 2016), especially with the interactive effects of suboptimal temperature (Dove & Connor, 2007; O'Connor & Lawler, 2004). Moreover, all the larvae had a mass mortality at high temperature, and the survival rates at high temperature (30°C) are much lower than the results of the previous studies about *C. gigas* larvae. Helm and Millican (1977) found the percentage of 6-day survival reduced to 60% under 32°C and 30–34 psu. Rico-Villa et al. (2009) and Kheder et al. (2010) found larvae reared at optimal salinity survived over 17–32°C with high survival (>90%). The results may be explained by geographic factors, as the adaptive capacity to climatic conditions is different among distinct geographic population of species (e.g., *C. gigas*) that had a wide distribution (Duarte et al., 2014; Eierman & Hare, 2013; Robinson, 1992). Consequently, it is necessary to study the adaptive capacity of OSO compared with CPO, taking into account the intraspecific variability in the responses of *C. gigas* to different environmental stressors. In addition, it is pretty sure that these temperature-salinity combinations should be avoided in the OSO breeding, as high mortality occurred at the early life stage will exacerbate inbreeding

in the breeding program without properly structured, which is harmful for the further breeding (Hedgecock et al., 2007).

It should be noted that larval density was not examined as a factor in experiment on larval growth rate conditions and therefore larval density may still be a problem (Liu, Dong, Tang, Zhang & Xiang, 2006; Taylor, Southgate, Rose & Keegan, 1998; Yund & McCartney, 2016). However, no significant ($p > .05$) difference in larval growth and survival rates between CPO and OSO at 23 and 25 psu, consistent with the results of D-larvae development. Therefore, those results suggest that the OSO can have an equally good performance like CPO at early life stage under optimal condition. However, the survival rates of OSO on day 10 were significantly ($p < .05$) lower than that of CPO at 23°C and salinities of 17 and 33 psu, which means that the test condition effects on the larval growth rates of OSO may have been soothed by low larval density. Subsequently, although the larval growth rates of OSO at 23°C and salinities of 17 and 33 psu were just slightly lower than CPO here, in fact the disadvantage in growth of OSO might be more obvious than recorded. In addition, the fastest growth rate at high temperature may be due to the combination of temperature and larval density.

In conclusion, the OSO can have an equally good performance like CPO at early life stage under optimal condition for this experiment, but the potential disadvantages in adaptive capacity to environmental stresses will be shown at suboptimal conditions. Suboptimal temperatures (16 and 30°C) at all salinities and low salinity (17 psu) at optimal temperature (23°C) can significantly ($p < .05$) reduce the development of OSO D-larvae. Similarly, low salinity (17 psu) at optimal temperature (23°C) can reduce the larval survival and growth of OSO. In addition, there are a few suggestions on the breeding of OSO. (i) During the development of embryos and rearing of larvae, the water should be maintained at around 23°C and 25 psu. (ii) At optimal temperature (23°C) and salinity (25–33 psu), prolonging the embryonic incubation from 24 to 40 hr appropriately can increase the development to D-larvae by 7%–23%. Finally, these findings suggest the potential disadvantages in adaptive capacity in rare colour variants needs more attention in further breeding.

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