



Examination of the effects of inbreeding on reproductive capacity in the Pacific oyster, *Crassostrea gigas*

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ABSTRACT

The Pacific oyster (*Crassostrea gigas*) has been widely cultured as a commercially important bivalve. Interbreeding of inbred strains is regarded as an efficient method to increase yield (heterosis), so the development of inbred lines is common in the breeding strategies of *C. gigas*. However, inbreeding usually reduces physiological efficiency or reproductive capacity. The effects of inbreeding on gonadal development and reproductive capacity remain to be explored in oysters. In the present study, the monthly gonadal development of an inbred and a control population was observed. Compared with the control population, the delayed gonadal development in the inbred population may require more time and cost for breeders in the artificial breeding process. We investigated how inbreeding influenced female and male gametic traits, including reproductive effort, egg diameter, fertilization rate, hatching rate, sperm motility, sperm longevity, and sperm curvilinear velocity, as well as sperm competitiveness based on a male's share of paternity. We found no inbreeding depression for egg diameter, fertilization rate, or sperm motility. However, maternal inbreeding decreased reproductive effort and hatching rate, and paternal inbreeding decreased sperm longevity, curvilinear velocity, and competitiveness. Our results show evidence of inbreeding depression in the gonadal development and reproductive capacity of *C. gigas*, which could help us in a better understanding and management of inbreeding in oyster aquaculture.

1. Introduction

Inbreeding refers to the mating or reproduction between close relatives that usually results in the reduction of the mean phenotypic value of various characteristics of offspring (inbreeding depression) such as growth, survival, and reproduction success rate (Leroy, 2015). Since Darwin's time, inbreeding depression has been extensively studied and documented in a variety of plant and animal species (Crnokrak and Roff, 1999; DeRose and Roff, 1999; Chapman et al., 2009; Coltman and Slate, 2003; Angeloni et al., 2011; Leroy, 2015). Numerous researches have reported the negative effects of inbreeding in aquatic animal species, including Echinodermata (Zhao et al., 2016; Feng et al., 2015), Arthropoda (Doyle, 2016), Chordata (Shikano and Taniguchi, 2003; Paul et al., 2022) and Mollusca (Zhang et al., 2020; Zhao et al., 2019).

The degree of inbreeding depression is known to exhibit variation across different traits. Numerous studies support the hypothesis that fitness traits, such as fertility and survival, tend to be more susceptible to inbreeding depression (DeRose and Roff, 1999). This increased

vulnerability can be attributed to factors like a higher prevalence of directional dominance in fitness traits (DeRose and Roff, 1999) or the fact that fitness traits are affected by a larger number of loci on average (Houle, 1992; Keller and Waller, 2002). Consequently, traits associated with gonads and gametes are expected to exhibit pronounced inbreeding depression. For example, testicular mass was found to decrease as the inbreeding coefficient increased in Alabama beach mice *Peromyscus polionotus* (Margulis and Walsh, 2002). A significant decrease in sperm activity and a significant increase in the proportion of abnormal spermatozoa in ejaculation were observed in inbred *Taeniopygia guttata* (Opatova et al., 2016). In *Poecilia reticulata*, inbred males showed a strong decrease in sperm quality due to the declining number of sperm bundles rather than to changes in the number of spermatozoa within sperm bundles (Zajitschek and Brooks, 2010). In females, inbreeding not only has the potential to reduce fertility by limiting sperm availability but may also directly impact female gametic traits such as clutch size and delay until laying (Ford et al., 2018). However, previous studies on the effects of maternal inbreeding have mainly focused on the offspring

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performance and little is known about the effects of maternal inbreeding on egg size and number (Paul et al., 2022; Ford et al., 2018), which often have profound existing effects on the offspring (Szulkin et al., 2007; Fox, 2013). In general, if inbreeding results in severe depression in gametic traits, it could reduce the reproductive fitness of individuals and populations, profoundly affecting mating systems and population dynamics (Pizzari and Parker, 2009; Snow and Spira, 1996). To date, the majority of studies concerning reproductive capacity have primarily concentrated on model organisms, birds, mammals, and insects (Losdat et al., 2018; Zajitschek and Brooks, 2010; Fitzpatrick and Evans, 2015; Fox, 2013). In mollusks, substantial inbreeding depression has been observed in gametogenesis (Feng et al., 2015), fertilized egg hatching, and larvae survival (Zheng et al., 2012). However, there is a notable absence of research specifically addressing the effects of inbreeding on the gametes themselves in males and females.

The Pacific oyster *Crassostrea gigas* is one of the most widely farmed aquaculture species around the world (Han and Li, 2020). Normally, cultured populations of *C. gigas* have limited initial population size, resulting in non-random mating between closely related individuals (inbreeding) (Langdon et al., 2003; Launey and Hedgecock, 2001). Inbreeding depression may become accentuated in selective breeding programs conducted in such cultured populations (Evans et al., 2004). Moreover, like most marine invertebrates and fishes, *C. gigas* exhibits high fecundity enabling stringent selection and rapid gain (Gjedrem, 2012). Nevertheless, great fertility is often accompanied by considerable variation in reproductive success (Boudry et al., 2002; Hedgecock et al., 2007). The effects of inbreeding on fecundity may further increase this variation, potentially leading to the loss of potentially beneficial alleles and net additive genetic variation (Han and Li, 2020), as inbred individuals may produce fewer offspring. The negative effects of inbreeding on yields, growth, and survival have been documented in *C. gigas* (Evans et al., 2004), while the effects of inbreeding on the reproductive capacity of *C. gigas* remain unclear.

In our breeding practice, a rare orange-shell strain of *C. gigas* was obtained based on four orange-shell mutant individuals. The orange-shell strain is a typical inbred strain with reduced allelic richness and expected heterozygosity due to the extremely small genetically-effective population size (Han et al., 2019). Additionally, the unique shell color, as a recessive trait, prevents it from being contaminated by wild oysters during culture (Han and Li, 2020). Therefore, the orange-shell strain provides an opportunity to assess the potential effects of inbreeding on the reproductive capacity of *C. gigas*.

In this study, the effects of inbreeding on reproductive capacity were detected by comparing the monthly gonadal development, sperm performance, and egg traits of the orange-shell strain with those of a control population established by wild oysters. In addition, artificial insemination was employed to determine whether inbreeding would reduce fertilization success in males under controlled conditions. The information gained in this study could contribute to further understanding of the effects of inbreeding on oysters.

2. Materials and methods

2.1. Experimental oysters

A rare orange-shell strain of *C. gigas* was obtained through three successive generations of family selection from 2011 to 2013 based on four orange-shell mutant individuals. To improve the growth performance of this strain, seven successive generations of mass selection were established from 2014 to 2020 (Han and Li, 2020). In May 2020, the 10th-generation orange shell strain (OS) and control group (CG) were cultivated based on the 9th-generation orange shell strain and wild population collected in Rongcheng, Shandong Province, China. Artificial fertilization and larval rearing management were conducted in the hatchery in Laizhou, Shandong Province. After settlement and metamorphosis, all spat were transferred and cultured in the same sea area in

Rongcheng.

To assess the inbreeding level of OS oysters, 36 oysters were randomly selected from the 10th-generation orange-shell strain, and the adductor muscle of each oyster was separated and immediately conserved in absolute ethyl alcohol at -30°C for microsatellite analysis. The genomic DNA was extracted according to the standard procedure of the phenol-chloroform method (Li et al., 2006). Six multiplex PCRs containing 18 microsatellite loci (Supplementary Table 1) were used to genotype according to Liu et al. (2017), and the inbreeding coefficient was calculated using GenAIEx 6.5 (Peakall and Smouse, 2012).

2.2. Annual variation of gonadal development

From November 2020 to October 2021, 30 oysters were collected monthly from each group. During sampling processing, the temperature and salinity of the surface seawater were measured in situ using a mercury thermometer and a portable refractometer. The oysters were immediately transported alive to the laboratory. Each specimen was examined histologically to determine sex and gametogenic stage. A 5-mm thick section of the gonad of each oyster was fixed in Bouin's fluid for 24 h and then transferred to 70% ethyl alcohol for replacement of Bouin's fluid. When Bouin's fluid was completely replaced, thick sections were dehydrated with serial dilutions of alcohol and embedded in paraffin wax. The wax blocks were sectioned at $5\ \mu\text{m}$ using a Leica RM microtome. Sections were stained with hematoxylin, counter-stained with eosin, and mounted on microscope slides. The prepared slides were examined to assess the sex and stage of gonadal development with a microscope (Olympus BX50).

2.3. Gametic traits

2.3.1. Sample collection

The oysters of both groups used in the subsequent experiment were transferred to the hatchery in Laizhou in June 2021. In each subsequent experiment, the sampled oysters were as similar as possible in size to avoid the influence of individual size on experimental results. In addition, about 200 wild oysters were collected from Rushan, Shandong Province.

2.3.2. Egg traits

The oysters were shucked to examine the size and swollenness of gonads and 60 female oysters (30 per group) with mature gonads were screened out. Eggs from each mature female were obtained as much as possible by gonad striping and transferred to a plastic beaker with 2 L filtered seawater. After completely mixing the seawater in the beaker, the number and diameter of eggs of 10 subsamples were quantified using a Sedgewick Rafter counting chamber and a light microscope ($10\times$) equipped with an ocular micrometer (Dudas and Dower, 2006). This method of determining fecundity should be considered a conservative estimate because of the underestimation of the number of eggs.

In the experiment on the effects of maternal inbreeding on the hatching rate, we always artificially mated females of two groups with wild males to ensure that the fertilized ovum was outbred to attribute the effects of experimental treatments to the inbreeding status of the experimental females. To be specific, 60 mature females (30 inbred females, 30 control females) were selected, and the eggs of each female were obtained by gonad striping, sifted through a $90\text{-}\mu\text{m}$ nylon screen, rinsed on a $25\text{-}\mu\text{m}$ screen, and finally immersed in filtered seawater. Then 30 mature wild males were selected and the gonad of each male was removed and dissolved in seawater. Then the sperm was sifted through a $90\text{-}\mu\text{m}$ nylon screen. For each female pair, microscopic examinations were performed to ensure that the number of eggs collected from both females was roughly the same, and then the eggs of both females were fertilized with an equal quantity of sperm from the same wild male. After fertilization, incubation was carried out at about 24°C for 24 h. Samples were taken at 2 h and 24 h after fertilization to

measure the fertilization and hatching rate. The fertilization rate was defined as the percentage of the number of fertilized eggs to the total number of eggs, and the hatching rate was defined as the percentage of the number of D-larvae to the number of fertilized eggs (Zhang et al., 2012).

2.3.3. Sperm traits

Thirty mature males were selected from each group according to the method described above. For each male, 10 µL sperm were pipetted and diluted with 100 mL filtered seawater. Before dilution with seawater, the sperm must be kept away from seawater to prevent premature activation. The diluted sperm was deposited on a slide and immediately transferred to a dark-field phase-contrast microscope, where sperm motion was video-recorded for 30 min. A computer-assisted sperm analysis (CASA) plug-in implemented in ImageJ software was used to analyze temporal dynamics of sperm motion after 0, 5, 10, 15, 20, 25, and 30 min of video recording from a video segment of 3 s at each time point (Wilson-Leedy and Ingermann, 2007). Sperm cells slower than 5 µm/s were considered immotile or moved by drift. Three standard sperm traits were quantified: (1) sperm motility, which estimates the percentage of motile sperm at the first focal time point; (2) sperm longevity defined as the rate of decrease in sperm motility across the 0–30 min video recording and (3) curvilinear velocity, the actual velocity along the trajectory.

2.3.4. Sperm competitiveness

To determine whether inbred males produce less competitive sperm, we artificially inseminated eggs of wild females with approximately equal numbers of sperm from control males and inbred males and then calculated their share of paternity. First, 90 mature oysters (30 control males, 30 inbred males, and 30 wild females) were dissected and eggs and sperm were obtained by gonad stripping. Before artificial insemination, the sperm from each male was diluted with filtered seawater. The diluted sperm was placed on a slide and the spermatozoa were counted using ImageJ with CASA plug-in. Five subsamples from each male were counted and the concentrations were adjusted to ensure that spermatozoa concentrations of both males of each male pair were approximately equal. Then diluted sperm of both males were fully mixed and the eggs of a wild female were inseminated with sperm mixture. Fertilized eggs of each female were hatched in separate plastic buckets. After 24 h of fertilization, D-larvae were collected and stored in 95% ethanol for genetic analysis. The adductor muscle of each male was stored in 95% ethanol until DNA extraction.

The genomic DNA of D-larvae was extracted using the Chelex®-100 method (Li and Kijima, 2005). The genomic DNA of the adductor muscle was extracted using the phenol-chloroform procedure (Li et al., 2006). Two multiplex PCRs containing 5 microsatellite loci (Table 1) (Liu et al., 2017) were employed for paternity analysis. The PCR condition was 3 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at the optimal annealing temperature, and 1 min at 72 °C, with a final extension of 5 min at 72 °C. Subsequent allele sizes were determined on the capillary sequencer, ABI 3130 genetic analyzer (Applied Biosystems), with

Table 1
Information of two multiplex PCRs used in the paternity test.

| Group | Locus | Primer sequence(5' ~ 3') | Product size (bp) |
|---------|------------|---|-------------------|
| Panel 1 | ucdCg-117 | F-TGTAACGACGGCCAGTCCAAGCTTGCACTCACTCAA R-GAGTGTCTGGTGTGCCAAAT | 281–355 |
| | ucdCg-120 | F-TGTAACGACGGCCAGTGGGTGAGATTTAGGGGAGA R-CTCCATCAAACCTGCCAAAC | 153–189 |
| | ucdCg-198 | F-TGTAACGACGGCCAGTGAAAGACACGACCGGAGAGA R-CTGATGATGCCACACCTG | 233–278 |
| Panel 2 | ucdCg-146 | F-TGTAACGACGGCCAGTCGCTCTGGTCTTTGTTCCAT R-ACCCCAACAGATCACAAATCC | 215–277 |
| | uscCgi-210 | F-TGTAACGACGGCCAGTTTCAACAATGAAGATGACAGTGC R-CCTCCTGCTGCCTCATATCA | 320–356 |

GeneScan LIZ 500 (Applied Biosystems) as internal size standard, and fragment lengths were assessed automatically with GeneMapper v4.0 (Applied Biosystems). The paternity assignment was performed with CERVUS 3.0 (Kalinowski et al., 2007).

2.4. Statistical analysis

All data were analyzed using SPSS 23.0. To investigate the effects of inbreeding on the fertility of oysters, the following eight traits were analyzed (four female traits and four male traits): (1) reproductive effort defined as the number of eggs divided by the weight of body mass without gonad, (2) egg diameter, (3) fertilization rate, (4) hatching rate, (5) sperm motility, (6) sperm longevity, (7) curvilinear velocity, (8) the equity of progeny from both sire assuming contributions from each sire to be equal respectively. A separate general linear model was carried out to test for the effects of inbreeding on each of the first seven traits above. The inbreeding status was used as the independent variable and the shell height was used as the covariate. By the independent sample *t*-test, the covariates and independent variables were confirmed to be mutually independent ($P > 0.05$), and the variance of the dependent variables in each group was also confirmed to be homogeneous ($P > 0.05$). The Shapiro-Wilk test was performed to verify that the dependent variables in each group conformed to a normal distribution ($P > 0.05$). In addition, there was a linear regression relationship between the covariates and the dependent variables in each group, and the regression line was proved to be parallel ($P > 0.05$, that is, the relationship between the covariates and dependent variables did not differ due to changes in the independent variables) by the interaction analysis of independent variables and covariates. These results indicated that all data were suitable for general linear models. For the eighth trait, a chi-squared test was performed to evaluate the equity of progeny from both sires, assuming contributions from each sire to be equal.

3. Results

3.1. Inbreeding level

The inbreeding coefficient of OS oysters was 0.31. In 2021, the inbreeding level of wild oysters in Rongcheng has been evaluated using 15 microsatellite markers, and the inbreeding coefficient was 0.07 (Zhang et al., 2020).

3.2. Environmental parameters

Monthly fluctuations in salinity and temperature were shown in Fig. 1. Salinity fluctuated between 29.4 and 32.0 psu. From November 2020 to February 2021, the surface seawater temperature decreased progressively, reaching a minimum of 3.3 °C in February 2021. From March onwards, the temperature increased gradually, reaching a maximum of 24.6 °C in August. The temperature began to decrease again in September 2021.

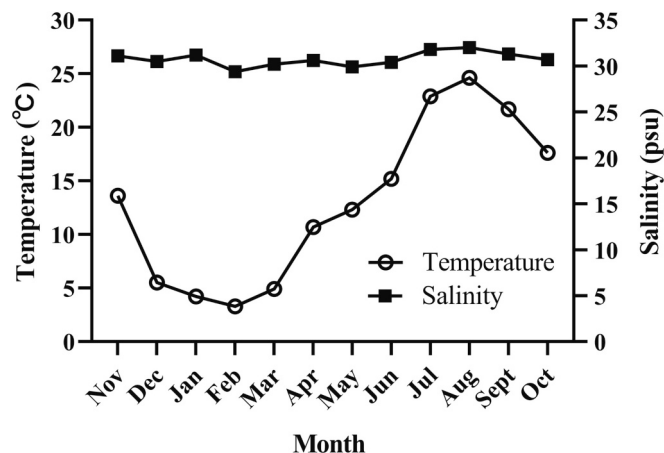


Fig. 1. Seasonal variation in surface seawater temperature and salinity in Sanggou Bay from November 2020 to October 2021.

3.3. Gametogenic activity

Gonadal development in both groups followed an annual cycle with peak activity over summer (May to July) and relative inactivity from August to April (Fig. 2). Due to the almost synchronous development of gonads between male and female oysters in the same group, the two are not displayed separately. However, gametogenesis of CG began in March with 40% at the early active stage, earlier than that of OS, which began in April. Similarly, the development stage was mainly observed in May in the control group, whereas 57% of the inbred oysters were at the development stage in June. In the control group, most oysters (60%) were fully developed in June, while any spawning activity was not observed. In the inbred group, most oysters (67%) were fully developed

in July. The spawning activity was observed in July and August in both groups. From September, the oysters returned to the resting stage again.

3.4. Gametic traits

Inbreeding had significant effects on reproductive effort and hatching rate, but egg diameter and fertilization rate were not affected by inbreeding (Table 2). Males of both groups had no significant difference in their sperm motility. The sperm of the control group had significantly higher curvilinear velocity and longevity (Table 2).

In the paternity assignment analysis, we randomly selected 30 offspring from each brood to determine the paternity at 95% confidence and conduct subsequent analysis. Totally, males of CG sired 72.89 ± 12.46% of offspring (n = 900), which was significantly different from

Table 2

Comparison of five traits between two groups and results of general linear models testing for effects inbreeding on seven traits.

| Traits | Inbreeding | Control | F | P |
|--|---------------|---------------|---------|--------------------------|
| Reproductive effort (*10 ⁶ ind/g) | 7.85 ± 1.30 | 10.57 ± 1.25 | 74.384 | 6.3703*10 ⁻¹² |
| Egg diameter (µm) | 53.84 ± 4.24 | 54.11 ± 6.98 | 0.019 | 8.9048*10 ⁻¹ |
| Fertilization rate (%) | 80.15 ± 2.73 | 80.40 ± 3.13 | 0.208 | 6.5002*10 ⁻¹ |
| Hatching rate (%) | 79.60 ± 2.76 | 87.95 ± 3.04 | 114.683 | 2.8762*10 ⁻¹⁵ |
| Curvilinear velocity (µm/s) | 143.08 ± 4.80 | 155.70 ± 3.81 | 124.115 | 6.1882*10 ⁻¹⁶ |
| Sperm motility (%) | 90.62 ± 8.77 | 92.73 ± 6.98 | 0.419 | 3.4631*10 ⁻¹ |
| Sperm longevity (%/min) | 1.57 ± 0.33 | 1.34 ± 0.32b | 6.892 | 1.1094*10 ⁻² |

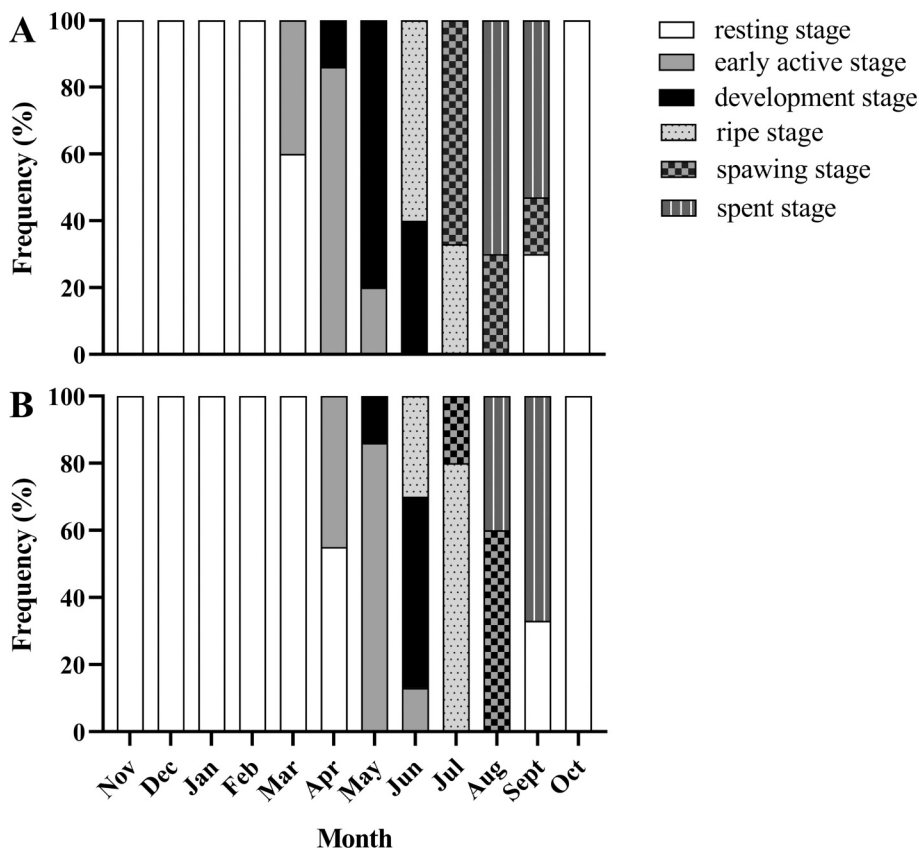


Fig. 2. Frequency distribution of gonadal development stages in CG (A) and OS (B) of *C. gigas*.

50% ($\chi^2 = 188.604, P < 0.05$). Among all 30 male pairs, 19 males of CG had significantly more offspring than corresponding inbred males (Table 3).

4. Discussion

It is generally accepted that inbreeding reduces the fitness of parents because they produce less-fit offspring who suffer from inbreeding depression due to increased homozygosity. However, the specific effects of inbreeding on different traits are not always clear (Marsh et al., 2017). In general, inbreeding is expected to have a more significant effect on traits that are closely related to fitness (Evans et al., 2004; Zheng et al., 2012; Huisman et al., 2016; Han and Li, 2018). Consequently, inbreeding depression often manifests in crucial reproductive traits within the range of inbreeding coefficients generated by natural mating systems (Losdat et al., 2018).

In this study, asynchronous gonadal development was observed between the two groups. The later initiation of gonadal development and shorter spawning time in the inbred oysters suggest that inbred oysters may require higher cumulative temperatures for gonadal development. In the current large-scale artificial breeding of the Pacific oyster, the parental oysters are usually cultured to sexual maturity in artificially heated seawater (Jennifer et al., 2021). As a result, inbred parental oysters may require more time or higher seawater temperatures to achieve sexual maturity (Leroy, 2015), which results in increased costs and reduced profits for breeders. Therefore, implementing reasonable control of inbreeding within commercial cultured populations becomes a necessary consideration. (Clark et al., 2013).

Our study provided evidence that maternal inbreeding negatively affects female reproductive success, corroborating previous findings of reduced reproductive success in inbred females across insects (Mattey et al., 2013; Ford et al., 2018), birds (Keller, 1998; Reid et al., 2003;

Jamieson et al., 2003; Richardson et al., 2004; Szulkin et al., 2007), and mammals (Huisman et al., 2016). We observed that female inbreeding status negatively affected reproductive effort, aligning with the hypothesis that the development and provision of eggs are energetically demanding and render these traits susceptible to inbreeding depression (Ebel and Phillips, 2016). From a mechanistic perspective, this decline in reproductive effort may stem from a decrease in primary oocyte numbers (Holmes et al., 2003), or general physiological deterioration (Lematre and Gaillard, 2017; Losdat et al., 2016). In addition, we discovered that inbred females had a significantly lower hatching rate of fertilized eggs than outbred females. Conversely, we found no evidence that maternal inbreeding influenced egg diameter or fertilization rate. Similarly, inbreeding had no significant effect on the fertilization rate in sea urchins *Strongylocentrotus intermedius* with an inbreeding coefficient of 0.375, indicating good fertilization robustness at this inbreeding level (Zhao et al., 2016). Thus, our results suggest that the diminished reproductive effort and hatching rate, rather than reductions in egg diameter or fertilization rate, shed light on potential mechanisms underlying the decrease in reproductive capacity due to maternal inbreeding.

The diploid genotype of males is more likely to affect multiple performances of sperm than the haploid genotype of sperm (Losdat et al., 2014; Pizzari and Parker, 2009), which may be associated with male reproductive success under directional selection (Birkhead et al., 1999; Hunter and Birkhead, 2002). The significant negative effects of inbreeding were reported in 48 cases of 99 sperm traits examined in 24 species, while no detectable effect was found in 50 cases, and significant positive effects were observed in 1 case (Losdat et al., 2014). Evidence of inbreeding depression for male sperm traits is widespread in closed populations such as domesticated and experimental animal species (Losdat et al., 2014). In contrast, the absence of inbreeding depression in some wild populations may result from immigration rates sufficient to maintain considerable genetic variation, thus preventing severe inbreeding despite limited population sizes (Keller et al., 2001; Gage et al., 2006; Terrell et al., 2016; Losdat et al., 2018). In this study, inbreeding had no significant effect on sperm motility but significant negative effects on curvilinear velocity and sperm longevity. In addition to these traits we measured, other sperm and ejaculate traits might be negatively influenced by inbreeding, including sperm count, size, and shape (Margulis and Walsh, 2002; Lawrence et al., 2017). In fact, regardless of whether sperm traits are negatively influenced by inbreeding, the more critical question is whether the fertilization success rate of inbred males is reduced in the context of sperm competition. Therefore, a formal test was conducted for the potential negative effects of inbreeding on sperm competitiveness.

Based on the result of paternity analysis, outbred males sired significantly more offspring than inbred males, suggesting that the sperm of inbred males exhibited reduced competitiveness. Although numerous studies have explored the effects of inbreeding on sperm traits (Roldan et al., 1998; Gomendio et al., 2000; Gage et al., 2006), only a few studies have focused on effects of inbreeding on sperm competition (Zajitschek et al., 2009). For example, in *Rhizoglyphus robini* and *Drosophila melanogaster*, sperm competitiveness was significantly lower in inbred females than in outbred males (Konior et al., 2005; Hughes, 1997). However, there was a nonlinear relationship between sperm competitiveness and inbreeding coefficient (F) in *P. reticulata* and *D. melanogaster*, where sperm competitiveness decreased at F = 0.5, but not at F = 0.25 (Ala-Honkola et al., 2013; Zajitschek et al., 2009). Moreover, the outcome of sperm competition may be affected by factors such as mating order, differences in sperm allocation, or female sperm selection. In our study, artificial insemination was employed to control for the potential variability of these factors, enhancing the reliability of results. Concurrently, our results suggest that the effects of inbreeding on paternity under sperm competition may be partially attributed to reduced sperm quality, as evidenced by the decrease in curvilinear velocity and longevity of sperm.

Table 3
The result of the chi-squared test.

| Male pair no. | Inbred offspring | Control offspring | Expected ratio | χ^2 |
|---------------|------------------|-------------------|----------------|----------------|
| 1 | 5 | 25 | 1:1 | 13.333 |
| 2 | 13 | 17 | 1:1 | 0.533 |
| 3 | 10 | 20 | 1:1 | 3.333 |
| 4 | 8 | 22 | 1:1 | 6.533 |
| 5 | 11 | 19 | 1:1 | 2.133 |
| 6 | 14 | 16 | 1:1 | 0.133 |
| 7 | 7 | 23 | 1:1 | 8.533 |
| 8 | 13 | 17 | 1:1 | 0.533 |
| 9 | 9 | 21 | 1:1 | 4.800 |
| 10 | 3 | 27 | 1:1 | 19.200 |
| 11 | 1 | 29 | 1:1 | 26.133 |
| 12 | 5 | 25 | 1:1 | 13.333 |
| 13 | 6 | 24 | 1:1 | 10.800 |
| 14 | 10 | 20 | 1:1 | 3.333 |
| 15 | 9 | 21 | 1:1 | 4.800 |
| 16 | 6 | 24 | 1:1 | 10.800 |
| 17 | 11 | 19 | 1:1 | 2.133 |
| 18 | 7 | 23 | 1:1 | 8.533 |
| 19 | 8 | 22 | 1:1 | 6.533 |
| 20 | 2 | 28 | 1:1 | 22.533 |
| 21 | 9 | 21 | 1:1 | 4.800 |
| 22 | 7 | 23 | 1:1 | 8.533 |
| 23 | 12 | 18 | 1:1 | 1.200 |
| 24 | 11 | 19 | 1:1 | 2.133 |
| 25 | 3 | 27 | 1:1 | 19.200 |
| 26 | 1 | 29 | 1:1 | 26.133 |
| 27 | 8 | 22 | 1:1 | 6.533 |
| 28 | 9 | 21 | 1:1 | 4.800 |
| 29 | 12 | 18 | 1:1 | 1.200 |
| 30 | 14 | 16 | 1:1 | 0.133 |
| Total | 244 | 656 | 1:1 | 188.604 |

Note: The numbers in bold mean observed ratios are significantly different from expected ratios.

5. Conclusion

In summary, we reported the first direct evidence that the gonadal development and gametic traits of *C. gigas* were negatively affected by inbreeding. The gonadal development of inbred oysters was later than that of control oysters by histological procedures, indicating that more time and cost were required in the artificial breeding process. Moreover, the negative effects of inbreeding on gametic traits may amplify the large variation in the reproductive success of oysters, leading to a higher risk of genetic drift and loss of potentially beneficial alleles (Xu et al., 2019; Boudry et al., 2002; In et al., 2016; Bentsen and Olesen, 2002). These results might indicate that the population under investigation has not purged the detrimental mutational load due to parental selection and artificial insemination that weakened the directional dominance of the related traits (DeRose and Roff, 1999). Various breeding programs should be considered in the future, including retention of adequately large population sizes, periodic introductions of unrelated parents, and systematic rotational line crossing to reduce the accumulation of inbreeding in the small breeding population of *C. gigas* (Evans et al., 2004). In this study, a single inbred strain was compared to one control group, which limits the scope of our findings. The study of multiple groups with different inbreeding levels would be needed to confirm and quantify the effects of inbreeding on reproductive capacity in *C. gigas* (Dégremont et al., 2022).

Author Statement

Jiafeng Fang: Performed the experiments and analyzed the data.

Qi Li: Convinced and designed the experiments, authored and revised the paper.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled, "Transcriptome analysis of inbreeding depression in the Pacific oyster *Crassostrea gigas*".

Data availability

Data will be made available on request.

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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.2023.739689>.

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