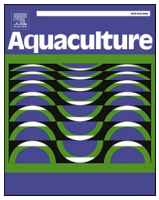
Aquaculture 557 (2022) 738314

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Transcriptome analysis of inbreeding depression in the Pacific oyster *Crassostrea gigas*

Jiafeng Fang [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

ARTICLE INFO

Keywords:
Inbreeding depression
Pacific oyster
RNA-seq
Differential expression analysis
Enrichment analysis

ABSTRACT

The Pacific oyster (*Crassostrea gigas*) is widely cultured worldwide. Because intercrossing inbred strains improves yield (heterosis), development of inbred lines is needed to implement this breeding strategy. However, inbreeding usually reduces the mean phenotypic value shown by characters connected with physiological efficiency or reproductive capacity. The effects of inbreeding on growth and survival of *C. gigas* have been reported by numerous studies. However, effects of inbreeding on gene expression still remains to be explored in oysters. In this study, the relationship between gene expression and inbreeding depression was investigated in an inbred line of the Pacific oyster. Two inbred groups, an F1 group containing 15 inbreeding families and an F2 group containing 15 families with a presumed relative higher inbreeding level performing worse in growth and survival rate compared to the F1 group, were constituted by using this inbred line. The shell height and survival rate of two inbred groups were compared with those of the wild group including 15 families (CF). A total of 9 RNA-seq libraries were constructed using oyster gills. Based on differential gene analysis, 2430 differentially expressed genes (DEGs) in the F1 group and 3741 in the F2 group were identified respectively compared to the CF group. A total of 1746 common DEGs were counted shared in two inbred groups, of which 911 were up-regulated and 835 were down-regulated. GO and KEGG analysis showed that common DEGs were significantly enriched in the immune response and actin nucleation pathways. Additionally, 695 DEGs were identified between two inbred groups, and GO analysis revealed that these DEGs were significantly in the pathways related to immune and stress response. Our results show the preliminary exploration of the effect of inbreeding on gene expression of *C. gigas*, which is helpful for us to better understand and manage inbreeding depression of bivalves, and may potentially benefit the breeding of oyster in aquaculture.

1. Introduction

Inbreeding, characterized as mating among closely related individuals, normally results in inbreeding depression, the reduction of the mean phenotypic value connected with a given trait, such as fecundity and survival (Leroy, 2015). The effects of inbreeding depression have been observed for well over a century in domesticated as well as natural populations of plants and animals (Charlesworth and Wills, 2009). There are cumulative lines of studies that described the deleterious effects of inbreeding depression in aquatic animal species, including Nemertea (Caplins and Turbeville, 2015), Mollusk (Zheng et al., 2012; Janicke et al., 2014), Echinodermata (Zhao et al., 2016) and Chordate (Zajitschek and Brooks, 2010; Phillippi and Yund, 2017). However, only a few of them have explored the genome-wide analysis on inbreeding depression (Plough and Hedgecock, 2011; Plough, 2012; Venney et al., 2016; Fellous et al., 2018).

Studying the relationship between inbreeding and gene expression could help to understand the importance of specific genes, genetic networks and physiological pathways for inbreeding depression (Mandy et al., 2015; Zhao et al., 2019), which allows us to better understand, estimate and manage inbreeding depression (Hansson et al., 2014). However, the research of inbreeding depression was stagnating at the phase of symptom description for a long time and interrogations about the effect of inbreeding on gene expression still remain inconclusive. (Zhao et al., 2019; Leroy, 2015). Recently, the development of molecular tools made it possible to perform studies on variation in inbreeding-induces gene expression at the level of the whole-genome (Huisman et al., 2016). Relevant researches performed in several model organisms

* 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).

https://doi.org/10.1016/j.aquaculture.2022.738314
Received 27 November 2021; Received in revised form 26 April 2022; Accepted 28 April 2022
Available online 5 May 2022

showed different expression patterns (Mandy et al., 2015; Hansson et al., 2014; Bos et al., 2016; Kristensen, 2005; Ayroles et al., 2010). For commercially important mollusk bivalves, the research on transcriptomes under inbreeding circumstances was only reported in yesso scallop *Patinopecten yessoensis*. Zhao et al. (2019) identified 9 genes involved in inbreeding depression in *Pseudoceros yessoensis*, which were related to a perturbing lipid metabolism, a severe inflammatory reaction or immune response, and a stress-responsive behavior.

The Pacific oyster *Crassostrea gigas* is the most widely cultured shellfish species around the world. Normally, cultured populations of *C. gigas* are established with few breeders and the culture of *C. gigas* depends on hatchery-produced seed, so inbreeding depression of performance characters has become pervasive (Langdon et al., 2003; Launey and Hedgecock, 2001). The effects of inbreeding have become more apparent as breeders attempt to establish selected lines from hatcheries with small effective population size (Evans et al., 2004). Meanwhile the great fecundity and very high larval mortality increase the risk of inbreeding (Hedgecock and Davis, 2007). The effects of inbreeding on growth and survival traits during larval and adult stages have been revealed by an abundance of studies (Dégremont et al., 2007). To date, some researches focused on the effects of inbreeding on yield, individual growth rate and survival (Evans et al., 2004). Plough and Hedgecock (2011) reported that deleterious mutations were predominantly expressed before the juvenile stage (90%), mostly at metamorphosis (50%) by quantitative trait locus analysis of stage-specific inbreeding depression in *C. gigas*. However, the effect of inbreeding on gene expression has not been reported, which is essential to understand the genetic basis of inbreeding depression in *C. gigas*.

In our previous breeding practice of *C. gigas*, four individuals with orange shell color were unexpectedly identified (Han and Li, 2018, 2020). Based on these four individuals (two males and two females), two full-sib families were produced in 2011 to serve as the first generation in this study. Subsequently, two successive generations of family selection were established from 2012 to 2013 in order to remedy shell color. For purposes of improving growth performance, five generations of mass selection were performed from 2014 to 2018 (Supplementary Fig.1) allowing a genetically stable orange-shell line to be obtained. The number of parents in each generation of mass selection was about 100, and the selection intensity was about 1.9 (Han et al., 2019). The orange-shell line has obvious characteristics of an inbred strain. For example, compared to wild populations, significant reduction in allelic richness (Ar: 68.17–74.91%) and expected heterozygosity (He: 34.21–39.24%) were observed due to the extra small genetically-effective population size (Han et al., 2019). Additionally, the special shell color, as a recessive trait, keeps it from being contaminated by wild germplasm during cultivation (Han and Li, 2020). Therefore, the orange shell line is suitable for exploring the effects of inbreeding-induced gene expression in *C. gigas*.

In the present study, we investigate the effects of inbreeding on gene expression through comparing transcriptome-wide gene profilings of *C. gigas* with three different inbreeding levels, and also discuss the genetic basis of inbreeding depression in *C. gigas*. This study would contribute to further understanding of oyster inbreeding depression, which may potentially benefit the genetic breeding for oyster aquaculture.

2. Materials and methods

2.1. Experimental animals

In this study, all the 45 families were established in June 2019. The parents of 15 high inbreeding families (F2) were selected from the progeny of 15 full-sib families, which were established based on the 8th generation orange-shell line by crossing one female to one male in June 2018. One male and one female were selected randomly from each family constructed in 2018 to establish 15 full-sib F2 families in June 2019. The parents of lower inbreeding families (F1) were selected from the 8th generation orange-shell line randomly, and 15 families were produced by crossing one female to one male (Supplementary Fig. 1). The 15 families of non-inbred control (CF) group were established by crossing one female to one male, using the wild individuals collected from Laizhou Bay as parents. All spat were cultured in Sanggou Bay, Rongcheng, Shandong province. They were placed on nylon ropes and cultured on suspended longlines. All spat were cultured by the long-line method by 30 days, and then 200 oysters from each family were placed in 10-layer lantern nets with 20 individuals per layer.

2.2. Growth and survival perfomances

During grow-out stage, 30 oysters were randomly selected from each lantern for 60-day-old, 150-day-old and 300-day-old age classes. The shell height of each oyster was measured with an electronic vernier caliper (0.01 mm). The dead oysters were removed at each sampling, and the survival rate of each family was calculated based on the total number of living oysters from the corresponding lantern.

Differences in shell height and survival rate among three groups were analyzed with one-way analysis of variance followed by multiple comparison Tukey test using SPSS (Statistical Package for Social Science) 22.0 software. Differences were considered significant if $p < 0.05$.

2.3. RNA isolation, library construction and Illumina sequencing

At the last sampling (300-day-old age), experimental samples were collected. For each group, the average individual size of these collected samples was approximately equal to that of the family, while the average individual size of the family was approximately equal to that of the corresponding population. In this way, the transcriptome data can represent the overall situation of the corresponding group as much as possible, to reduce the effects of different oyster growth rates on gene expression among the three groups. But from another point of view, the difference in growth may also be the result of inbreeding. Gills of three individuals from each family were dissected and flash-frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction.

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina NovaSeq 6000 platform and 150 bp paired-end reads were generated.

2.4. Quality control and reads count

Raw data (raw reads) were firstly processed through in-house perl scripts to get rid of adapter sequences. Then reads containing more than 5 ambiguous bases (N) were removed and only the high-quality reads (clean reads) which more than 80% bases quality scored over 20 could be kept for further analysis.

Then Hisat2 (v2.0.4) was used to align clean reads to the indexed reference genome (Li et al., 2009), which can generate a database of splicing junctions based on the gene model annotation file and provide better mapping results (Kim et al., 2015). For each gene, raw counts were obtained using featureCounts, a read summarization tool of Subread (v2.0.0) (Yang et al., 2014).

2.5. Differential expression analysis

Differential expression analysis of three groups was performed with the R package DESeq2 (v4.0.3) (Love et al., 2014), using a model based

on the negative binomial distribution to calculate the *P*-value. *P*-value of each gene were revised by the Benjamini and Hochberg approach to control the false discovery rate (FDR). Genes with FDR < 0.05 and fold-change >2 were regarded as differentially expressed genes (DEGs).

2.6. Functional annotation

Gene ontology (GO) enrichment analysis of DEGs was conducted using the R package clusterProfiler, in which gene length was corrected. GO terms with corrected P-value <0.05 were considered significantly enriched by DEGs. As well, clusterProfiler R package was used to test the statistical enrichment of DEGs in KEGG pathways to understand high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem from molecular-level information (Kanehisa et al., 2007), and P-value <0.05 was regarded as significantly enriched in the pathway.

2.7. Quantitative real-time PCR validation

To verify the results of RNA-seq, 12 DEGs were chosen for quantitative real-time PCR (qRT-PCR) analysis. The samples used for the qRT-PCR and RNA-seq analysis were the same. The PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) was used for synthesizing the total cDNA for qRT-PCR. Gene-specific primers were designed using Primer Premier 5.0 (Supplementary Table 1). Eukaryotic elongation factor 1 (eEF-1) gene was used a control to normalize gene expression by qRT-PCR (Renault et al., 2011). Cycling parameters were 94 °C for 5 min and then 40 cycles of 94 °C for 30s, 56 °C for 30s and 72 °C for 15 s. Relative gene expression levels were calculated by the $2^{-\triangle\triangle Ct}$ method (Schmittgen and Livak, 2008).

3. Results

3.1. Comparison of growth and survival rate

The shell height and survival rate of three groups were shown in Fig. 1. Apparently, shell height and survival rate of two inbred groups were significantly lower than those of the CF group, and were lowest in the F2 group ($P < 0.05$). In addition, the survival rate of inbreeding groups was more impacted by inbreeding than the shell height when compared with the CF group.

3.2. Sequence analysis

A total of 181.4 million clean reads were obtained after quality filter of 193.7 million 150 PE raw reads, with Q20 varying from 98.09% to 98.48%. The clean bases of each sample ranged from 5.44 to 6.84 Gb, which is about 12× of the oyster genome size. Totally, 75.52–79.37% of clean reads were aligned to *C.gigas* reference genome, of which 67.85–70.83% were uniquely aligned and 7.44–8.55% had multiple alignment positions on the reference genome (Table 1).

3.3. Differential expression analysis

Two thousand four hundred and thirty genes were significantly differentially expressed (FDR < 0.05) between the CF and the F1 groups. Among these DEGs, 1207 of them were expressed at lower levels and 1223 genes were expressed at higher levels. In the F2 group, 3741 genes were significantly differentially expressed compared to the CF group, of which 1614 genes were down-regulated and 2127 genes were up-regulated. When all DEGs from two inbred groups were further analyzed, 1746 common DEGs were found, and 911 and 835 were up- and down-regulated respectively (Fig. 2). In each inbreeding group, top 30 DEGs were ranked and 14 common DEGs were found (Table 2), including peroxidase-like protein, fucolectin, deoxynucleoside triphosphate triphosphohydrolase SAMHD1-like, bcl-2 homologous antagonist/killer-like, etc.

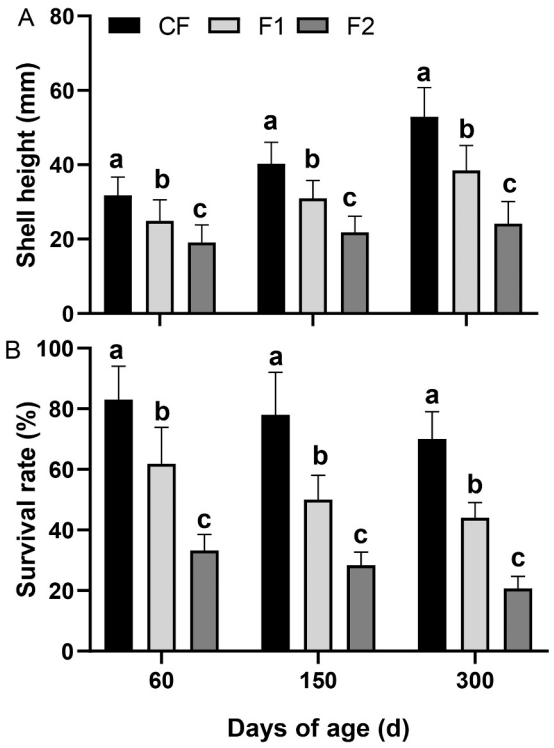


Fig. 1. Comparison of shell height (A) and survival rate (B) among three experimental groups. Different letters of the same days of age indicated significant differences among three groups ($P < 0.05$). The survival rate is cumulative over the whole experimental time (i.e. 300 days).

A total of 695 DEGs were identified between groups F1 and F2, of which 315 genes were expressed at higher levels in the F2 group, while 380 genes were expressed at higher levels in the F1 group (Fig. 3 and Supplementary Table 2).

The results of qRT-PCR verification of 12 common DEGs were compared with those from RNA-seq analysis. In general, the expression levels of 12 genes detected by qRT-PCR were consistent with the results of RNA-seq analysis (Supplementary Fig. 2).

3.4. GO and KEGG enrichment analysis of DEGs

GO term enrichment analysis was performed to further understand the biological functions of 1746 common DEGs. The common up-regulated genes were found mainly enriched in “negative regulation of fibroblast proliferation”, “cellular response to interleukin-6” and “response to interleukin-6” (Fig. 4 and Supplementary Table 3), which are associated with wound healing and immunoreaction. In a total of 76 terms, almost half of significantly enriched GO terms were related to immune response. The remainder of the GO terms were associated with synthesis and metabolism of lipids, proteins and glycogen and disease resistance. The common 835 down-regulated genes were found mainly enriched in GO terms involved with virus releasing from host cell, calcium ion-dependent exocytosis and actin nucleation (Fig. 5 and Supplementary Table 4).

To further understand the metabolism process and signal transduction pathways involved in these common DEGs, KEGG enrichment analysis was performed. The results revealed that common up-regulated DEGs were significantly enriched in 17 pathways (Fig. 6 and Supplementary Table 5). More than half of pathways were related to the immune reaction and apoptosis, including “NF-kappa B signaling pathway”, “NOD-like receptor signaling pathway”, “Apoptosis”, etc.

GO enrichment analysis was also performed for the DEGs between

Table 1
Summary of RNAseq sequencing data in gills of *C.gigas*.

Sanple name	Raw reads	Clean reads	Q20(%)	Clean bases(G)	Total mapped	Uniquely mapped	Multiple mapped
CF-1	21,840,187	21,079,810	98.43	6.32	16,731,045 (79.37%)	14,931,050 (70.83%)	1,799,995 (8.54%)
CF-2	23,311,950	22,404,700	98.25	6.7267.857	17,486,868 (78.05%)	15,571,008 (69.50%)	1,915,860 (8.55%)
CF-3	24,379,220	22,811,249	98.28	6.84	17,455,168 (76.52%)	15,588,863 (68.34%)	1866′305 (8.18%)
F1–1	19,666,826	18,429,800	98.36	5.53	14,323,641 (77.72%)	12,814,528 (69.35%)	1,509,113 (8.37%)
F1–2	22,105,079	20,820,519	98.28	6.25	16,173,379 (77.68%)	14,467,376 (69.49%)	1,706,003 (8.19%)
F1–3	21,124,596	19,800,591	98.27	5.94	15,278,136 (77.16%)	13,675,754 (69.07%)	1,602,382 (8.09%)
F2–1	20,445,757	18,580,019	98.09	5.57	14,031,630 (75.52%)	12,606,928 (67.85%)	1,424,702 (7.67%)
F2–2	21,040,801	19,375,630	98.48	5.81	14,926,985 (77.04%)	13,437,038 (69.35%)	1,489,947 (7.69%)
F2–3	19,779,370	18,129,753	98.39	5.44	14,322,504 (79.00%)	12,973,033 (71.56%)	1,349,471 (7.44%)

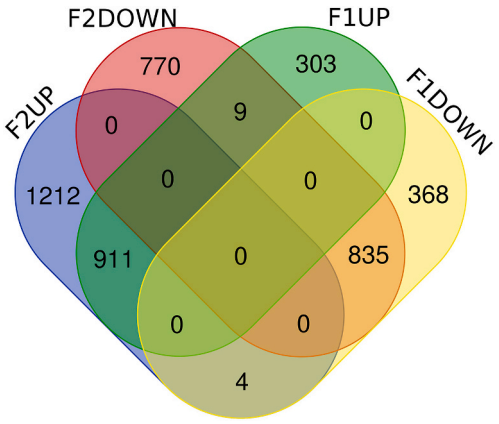


Fig. 2. Venn diagrams showing the overlap of DEGs between F1 and F2. All DEGs were obtained by comparing the transcriptome data of F1 or F2 with CF respectively.

Table 2
Top fourteen common differentially expressed genes in F1 and F2 compared to CF.

Gene symbol	Gene description
Up-regulated	
LOC105323064	shematrin-like protein 2
LOC105324729	peroxidase-like protein
LOC105344449	uncharacterized
LOC117687744	uncharacterized
LOC105322773	uncharacterized
LOC105340266	uncharacterized
LOC117682969	fucolectin-7-like
LOC117684920	nectin-4-like
LOC117686615	complement C1q tumor necrosis factor-related protein 3-like
LOC105329996	uncharacterized
LOC117691357	coadhesin-like
LOC105344934	uncharacterized
Down-regulated	
LOC117687819	deoxynucleoside triphosphate triphosphohydrolase SAMHD1-like
LOC117692211	bcl-2 homologous antagonist/killer-like

the F1 and F2 group. For the 315 up-regulated genes in the F2 group, enriched GO terms involve many aspects, including ion homeostasis, transmembrane transport, oxidative stress, biomineralization, etc. (Fig. 7 and Supplementary Table 6). The 380 down-regulated genes in the F1 group were significantly enriched in the terms related to autophagy, replication and release of viruses, clathrin, immune cell, etc. (Fig. 8 and Supplementary Table 7). DEGs between two inbred groups were not enriched in any pathways in the KEGG enrichment analysis.

4. Discussion

In this study, inbreeding caused reductions of survival rate and growth in *C. gigas* and inbreeding depression was stronger in the survival rate than growth. These results support the viewpoint that inbreeding usually causes a decline in multiple traits, and inbreeding depression is expected to be more serious in traits more closely associated with fitness (Evans et al., 2004; Zheng et al., 2012; Huisman et al., 2016; Han and Li, 2018). At the same time, the higher inbreeding level led to a further decline in these two traits.

Although the fitness consequences of inbreeding have been studied in *C. gigas* (Camara et al., 2007), the effect of inbreeding on gene expression has not been reported.

We performed transcriptome comparative analysis of two groups of inbred oysters with wild oysters. A total of 2430 and 3741 genes were identified to be differentially expressed in F1 and F2 groups respectively compared with the wild control group, and 14 genes with the highest degree of differential expression were shared in two inbred groups. Functional analysis showed that several genes of these were related to antioxidation and immunoreaction. Peroxidase-like protein was found to play a significant role in defense mechanisms (Hiraga et al., 2001). The previous study of peroxidase-like protein in the clam *Meretrix meretrix* showed that its genetic regulation is to prevent an increase in ROS in organisms when clams were exposed to polychlorinated biphenyls (Li et al., 2021). Thus, we inferred that the up-regulated of peroxidase-like protein in inbred oysters may reflect elevated levels of ROS in cells which can disrupt the structure of carbohydrates, nucleic acids, lipids and proteins, and contribute to the reduced adaptability and lower survival. Lectins play key roles in the innate immunity as a group of carbohydrate-binding proteins (Gorbushin and Borisova, 2015). F-type lectin (fucolectin) is fucose-binding proteins which mediate molecular recognition (Vasta et al., 2017). PmF-lectin from *Pinctada martensii* was highly expressed in gill and hemocytes after being exposed to *Vibrio alginolyticus*, which supported its important roles against microbial invasion (Chen et al., 2011). In addition, bcl-2 homologous antagonist/killer-like is an important regulator of apoptosis which is required for the maintence of tissue homeostasis by purging superfluous or potentially harmful cells (Zohny and El-Shinawi, 2011; Lin et al., 2019), and deoxynucleoside triphosphate triphosphohydrolase SAMHD1-like participates in the regulation of innate immune responses or has the function as a potent cellular restriction factor against retroviruses. Differential expression of genes indicated that inbreeding may

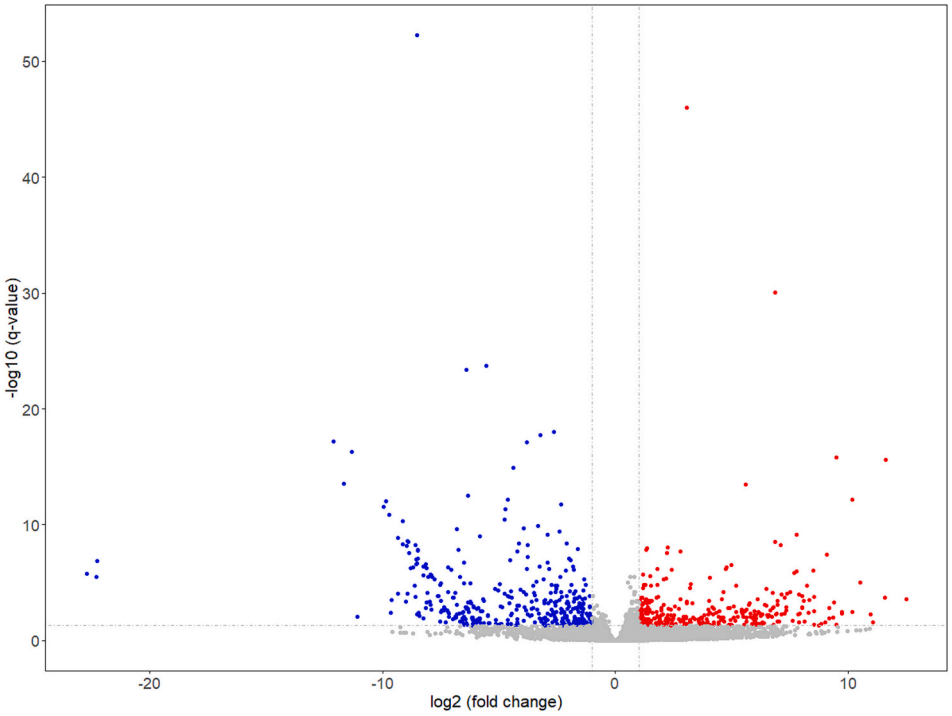


Fig. 3. Volcano plot based on comparison of gene expression levels between F1 and F2. DEGs were obtained by comparing the transcriptome data of F1 or F2. The red dots represent the DEGs expressing at a higher level in F2 and the blue dots represent the DEGs expressing at a higher level in F1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

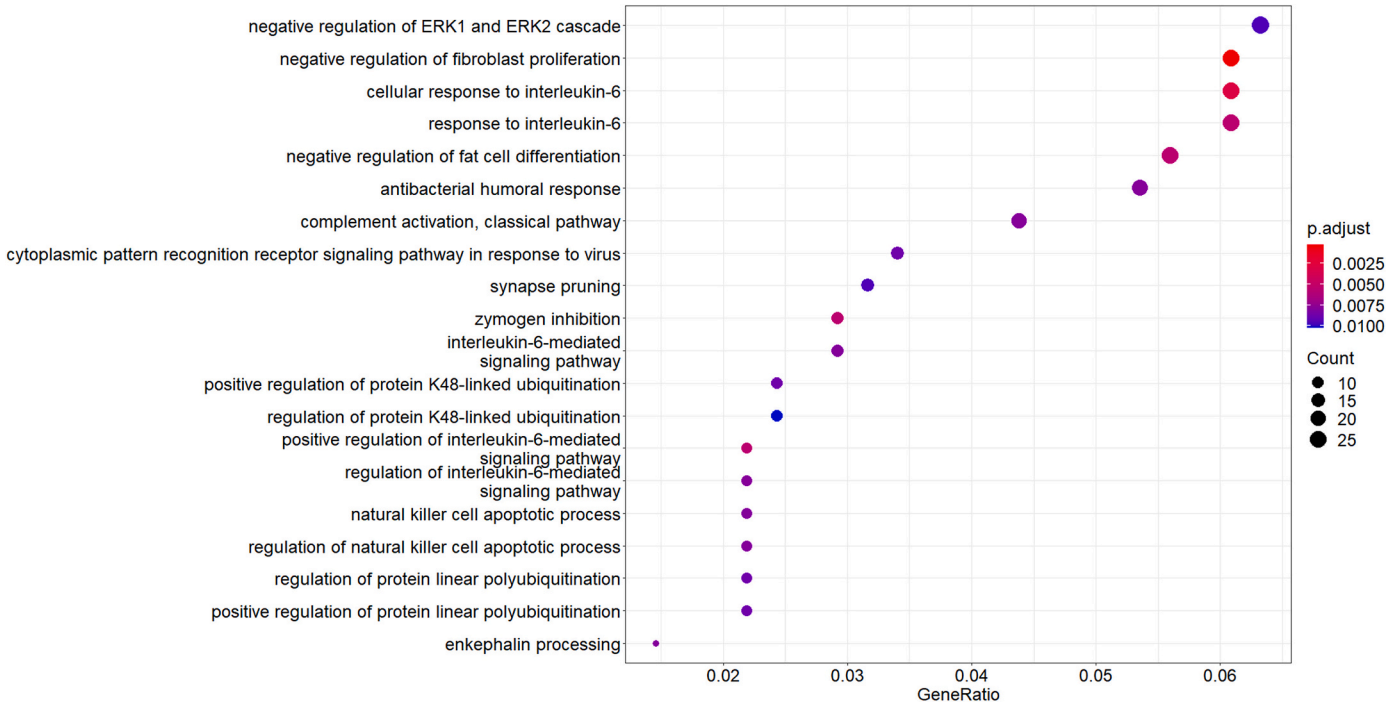


Fig. 4. GO enrichment analysis of common differentially expression genes that were expressed at higher levels in F1 and F2 compared with the CF group.

contribute to impaired immunologic homeostasis, which could partly explain the low survival rate of inbred oysters.

A total of 1746 DEGs were shared in two inbred groups. Based on these common DEGs, GO and KEGG analysis were conducted. Gene functional annotation analysis showed that DEGs were significantly enriched in immune responses, stress responses and actin nucleation pathways.

Bivalves have a comprehensive repertoire of immune cells, genes and proteins like many invertebrates. Immune cells are highly responsive to the recognition of microbe-associated molecular patterns because of a range of pattern recognition receptors, which usually results in a series of responses to infection, including chemotaxis, complex intracellular

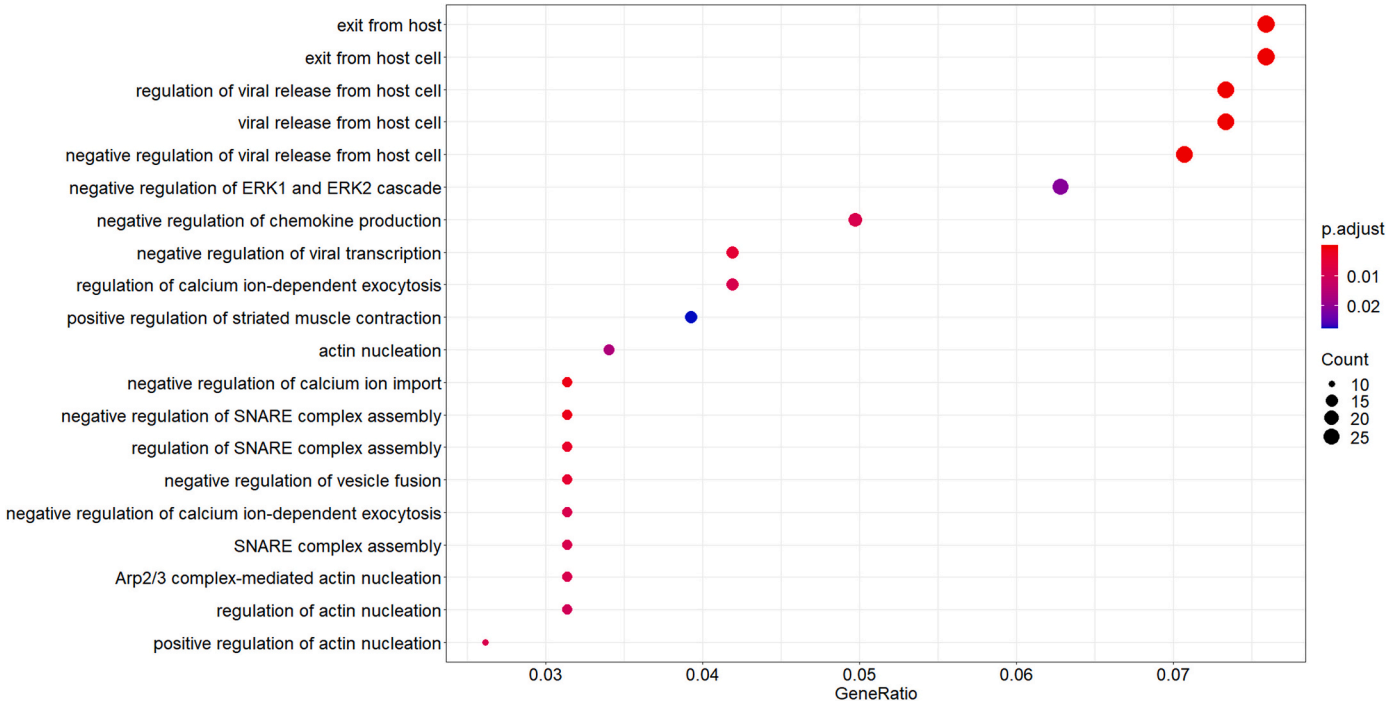


Fig. 5. GO enrichment analysis of common differentially expression genes that were expressed at higher levels in CF compared with both F1 and F2 groups.

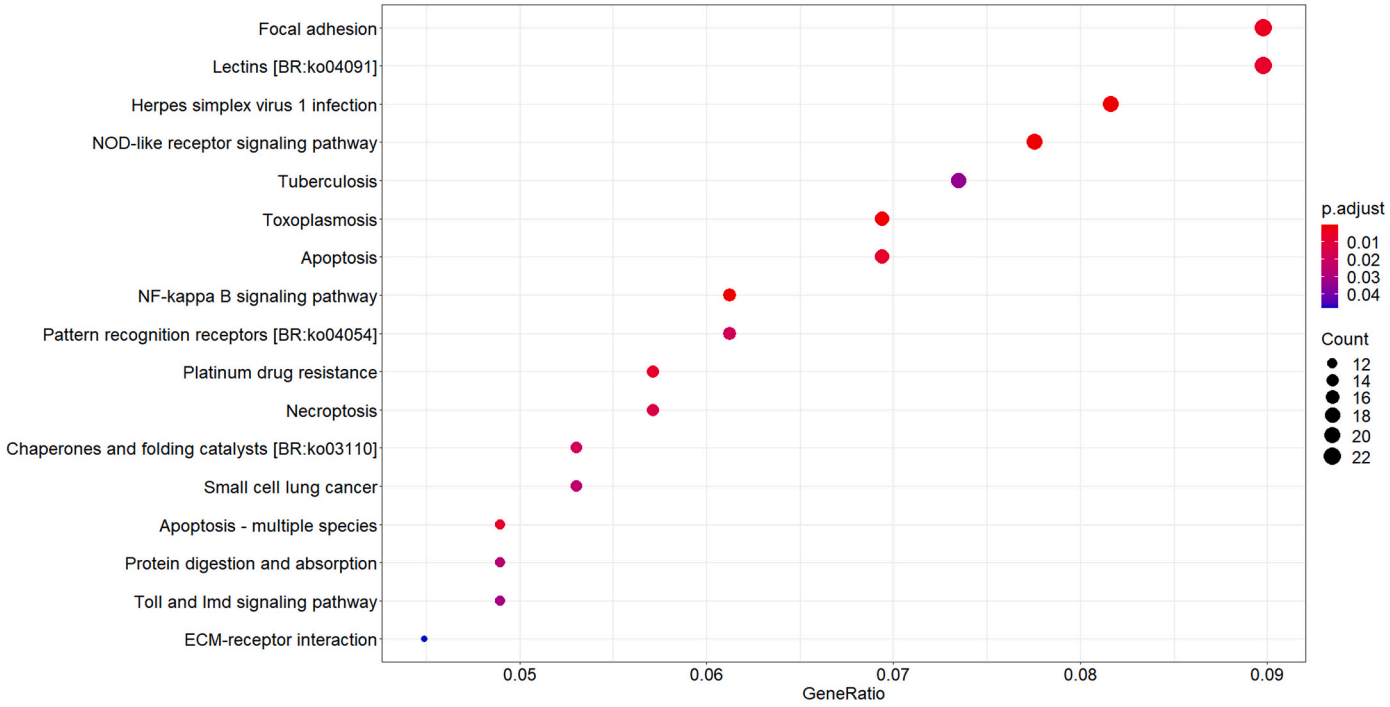


Fig. 6. KEGG enrichment analysis of common differentially expression genes that were expressed at higher levels in F1 and F2 compared with the CF group.

signaling, apoptosis and the induction of anti-viral states (Bassem and David, 2015). As revealed in this study, up-regulated common DEGs were found mainly enriched in pathways related to interleukin-6 (IL-6), natural killer (NK) cell apoptotic process, cytoplasmic pattern recognition receptor signaling pathways in response to virus and monocyte chemotactic protein-1 (MCP-1). IL-6 is a soluble mediator with a pleiotropic effect on inflammation, immune response, and hematopoiesis (Toshio et al., 2014). For example, it is possible to induce differentiation of activated B cells into antibody-producing cells in an infection lesion (Kishimoto, 1985). NK cells are essential to defend against a variety of pathogens. NK cells are activated by pattern recognition receptors and elicit the production of tumor necrosis factor (TNF) and interferon-gamma (IFN-γ), contributing to antibacterial defense (Souza-Fonseca-Guimaraes et al., 2011; Dillon et al., 2014). MCP-1 is an important chemokine secreted in response to stimulation by TNF and IFN-γ (Bilgic et al., 2009), which is of particular importance in the homeostatic response (Gerszten et al., 1999). Down-regulated DEGs were enriched in the pathways related to the negative regulation of viral

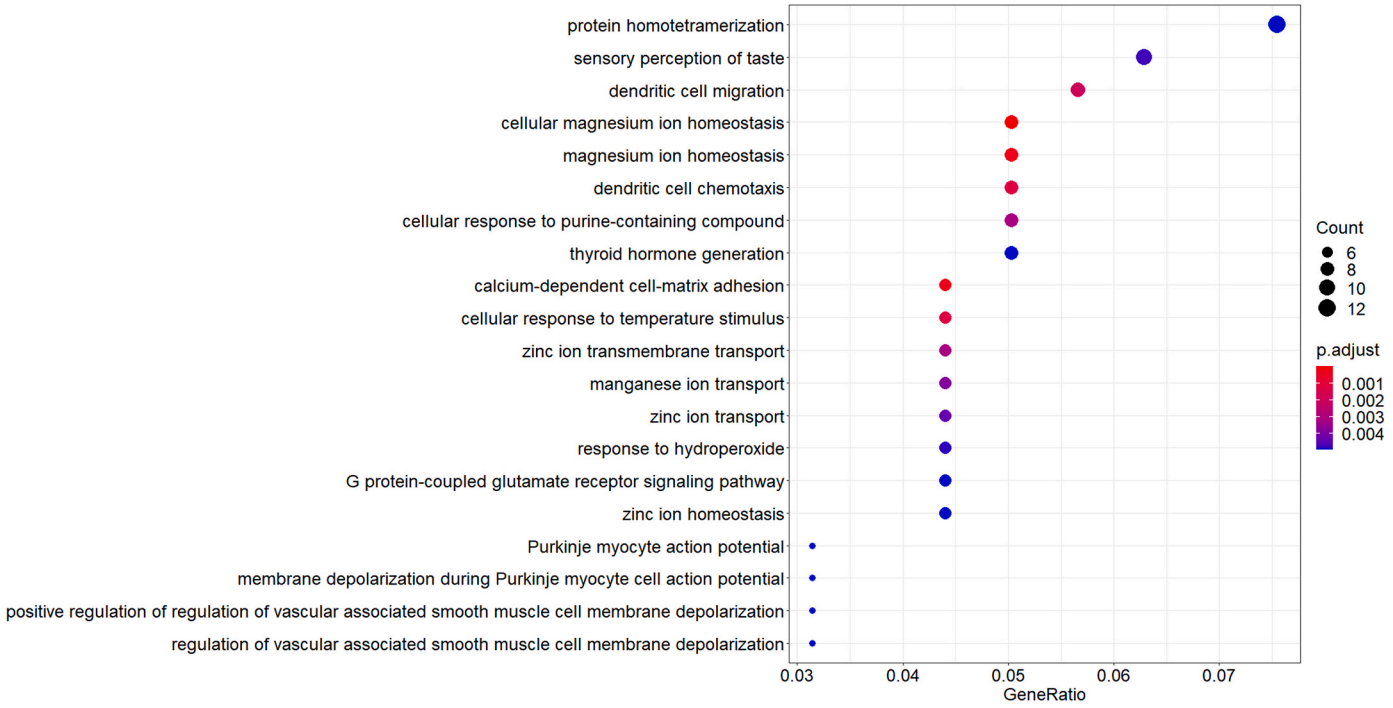


Fig. 7. GO enrichment analysis of differentially expression genes betweenF1 and F2 that were expressed at higher levels in F2.

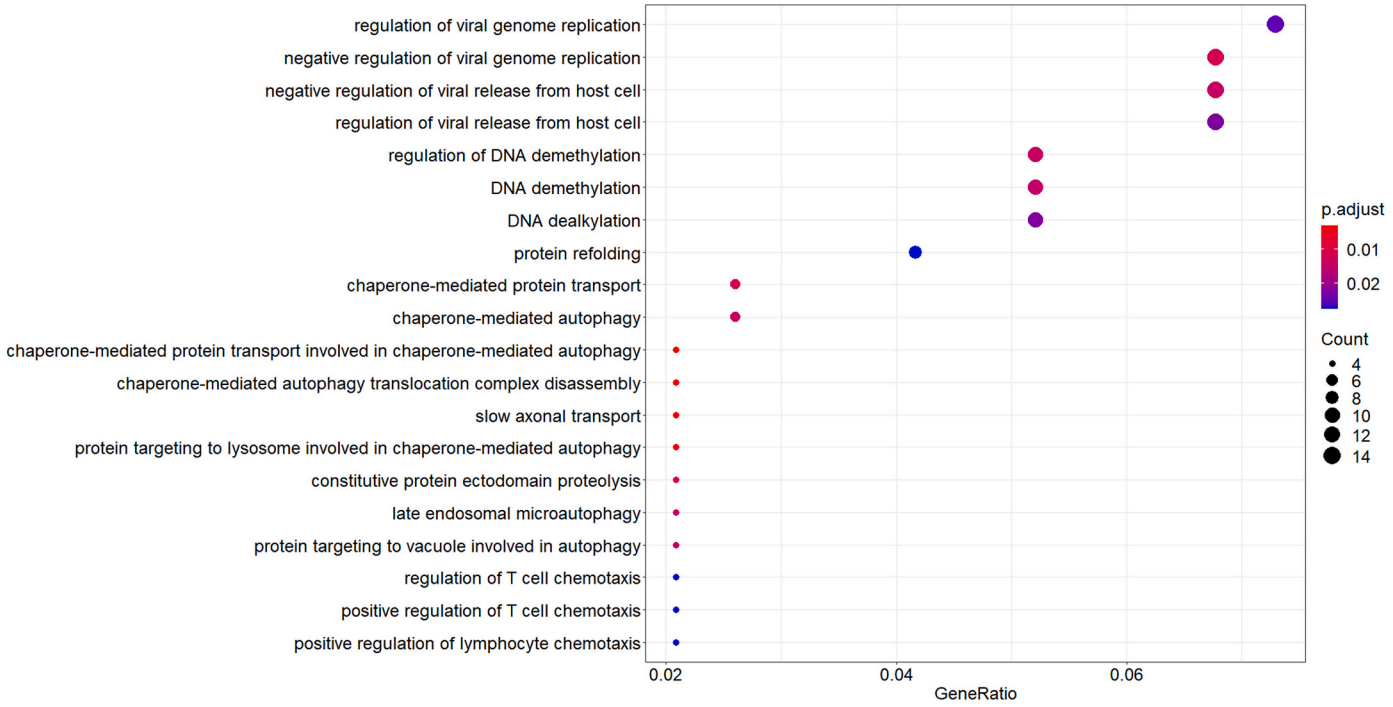


Fig. 8. GO enrichment analysis of differentially expression genes between F1 and F2 that were expressed at higher levels in F1.

transcription and release, that could mean faster virus multiplication in the inbreed oysters. Overall, it is considerably reasonable that DEGs in this study would contribute to low stress tolerance and low survival rate in inbred individuals through impaired immunologic homeostasis.

In addition, some of common DEGs were found to be involved in many pathways related to stress-response related pathways, including protein ubiquitination and regulation of lipid, glycogen and protein. In bivalves, the function of ubiquitination under temperature, season and oxidative stress has been wildly studied (Hofmann and Somero, 1995; Buckley et al., 2001; Mcdonagh and Sheehan, 2006). Damaged proteins are removed from cells by the ubiquitin-proteasome pathway (UPP), which selectively degrade abnormal cytosolic and nuclear proteins or short-lived intracellular regulatory proteins, and is important for normal cell growth and viability (Mcdonagh and Sheehan, 2006). When bivalves cope with stress, protein, lipid and glycogen have been shown to influence fitness related detoxification systems, defense, and physiology of organisms under stress (Bartlett et al., 2020). It is likely that abnormal protein ubiquitination and energy metabolism lead to low tolerance and

low survival phenotypes in inbred offspring in response to biological or abiotic stress.

Actin filaments are important parts of the cytoskeleton and participate in many cellular functions, including structural integrity, shape, cell division, organelle transport and cell motility. Cell motility plays an important role in a variety of biological processes such as the wound healing, development of organisms, growth and immune response (Ananthakrishnan and Ehrlicher, 2007). As revealed in this study, in inbred oysters, down-regulation of genes related to actin nucleation may indicate that negative effect of inbreeding on cell division and movement, which could be probably associated with the low growth of inbred oysters.

It was observed that oysters with a higher inbreeding level had more DEGs, which means that more recessive deleterious alleles were expressed in F2 than in F1, as lower survival rates and slower growth were observed in F2.

According to results of our GO analysis, a considerable part of the up-regulated DEGs in the F2 group were related to the homeostasis and transmembrane transport of metal ions and biomineralization. Metal ions are essential for numerous metabolic processes and their homeostasis is important for life. They always play an essential role in multiple bodily functions including immunity, regulation of cellular energy, reproduction and digestion in various forms such as free state, metalloenzymes or metalloprotein, and an aberration in metal ion homeostasis may lead to cell death and severe diseases (Nelson, 2014; Romani, 2011). For example, magnesium is necessary to regulate numerous cellular functions and enzymes, including signaling progress, ion channels and metabolic cycles (Romani, 2011), and the regulatory function of zinc ion impacts virtually all aspects of cell biology, including the transmission of information within and between cells (Maret, 2017). Up regulation of the genes related to the homeostasis and transmembrane transport of metal ions may mean the oysters with higher inbreeding level have lower metabolic efficiency, which leads to slower growth and higher mortality.

Biomineralization is essential for the formation of shell which is a complex structure made of organic and mineral components (Yarra et al., 2016). Up-regulation of genes negatively regulating biomineralization and biomineral tissue development leads to the decreased rate of shell growth. Negative regulation of mineralization and osteoblast differentiation may also contribute to the slow growth of inbred oysters.

The down-regulated DEGs observed in the F2 group seems to be more related to chaperone-mediated autophagy (CMA), clathrin, genome replication and release of virus and chemotaxis of immune cells. CMA contributes to the control of cellular quality and maintains cellular energetic balance and eliminate the damage of oxidative stress by selectively degrading soluble proteins in lysosomes and promoting the degradation of damaged proteins (Tasset and Cuervo, 2016; Kiffin, 2004). For oysters with a higher inbreeding level, CMA activity may decline with down-regulation of related genes, thus oxidative damage in cells increases. The effects of clathrin in cells are diverse, including tissue development, tissue structure, metabolic pathways and disease (Kit et al., 2020). Down-regulation of genes related to clathrin may lead to more severe inbreeding depression in growth and survival rate. Additionally, down-regulation of genes regulating the genome replication and release of virus and chemotaxis of immune cells may result in lower resistance to bacterial and viral infections, which may be another reason for lower survival rate in oysters with a higher inbreeding level.

5. Conclusion

The results of gene differential expression analysis showed that the influence of inbreeding on oyster gene expression was very complex and involved in many aspects of biological processes. The genetic functional analysis, suggested that reduced immune and stress responses and a perturbing energy metabolism may induce the abnormal cellular physiological situation, lead to inbreeding depression: reduced growth and survival rates in *C. gigas*. Our results only show the effect of inbreeding on gene expression during the studied stage, and due to selection favoring heterozygotes, the inbreeding degree of surviving individuals may be lower than expected. In the future, the stage-specific transcriptome analyses need to be explored so that we can have a more comprehensive understanding of the influence of inbreeding on all stages of oyster life history.

Author statement

Jiafeng Fang: Performed the experiments and analyzed the data.
Chengxun Xu: Performed the experiments.
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 *C. gigas*".

Acknowledgements

This work was supported by the grants from National Natural Science Foundation of China (31972789), Earmarked Fund for Agriculture Seed Improvement Project of Shandong Province (2020LZGC016), Science and Technology Development Project of Weihai City (2018NS01), and Industrial Development Project of Qingdao City (20-3-4-16-nsh).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738314.

References

Ananthakrishnan, R., Ehrlicher, A., 2007. The forces behind cell movement. Int. J. Biol. Sci. 3, 303–317. https://doi.org/10.7150/ijbs.3.303.

Ayroles, J.F., Hughes, K.A., Rowe, K.C., Reedy, M.M., Rodriguez-Zas, S.L., Drnevich, J. M., Paige, K.N., 2010. A genomewide assessment of inbreeding depression: gene number, function, and mode of action. Conserv. Biol. 23, 920–930. https://doi.org/10.1111/j.1523-1739.2009.01186.x.

Bartlett, J., Maher, W., Ubrihien, R., Krikowa, F., Edge, K., Pott, J., Taylor, A., 2020. Fitness of two bivalves Saccostrea glomerata and Ostrea angasi exposed to a metal contamination gradient in Lake Macquarie, NSW Australia: integrating subcellular, energy metabolism and embryo development responses. Ecol. Indic. 110, 105869 https://doi.org/10.1016/j.ecolind.2019.105869.

Bassem, A., David, R., 2015. Immune responses to infectious diseases in bivalves. J. Invertebr. Pathol. 131, 121–136. https://doi.org/10.1016/j.jip.2015.05.005.

Bilgic, H., Ytterberg, S.R., Amin, S., McNallan, K.T., Wilson, J.C., Koeuth, T., Reed, A.M., 2009. Interleukin-6 and type I interferon–regulated genes and chemokines mark disease activity in dermatomyositis. Arthritis Rheum-US 60, 3436–3446. https://doi.org/10.1002/art.24936.

Bos, N., Pulliainen, U., Sundstrom, L., Freitak, D., 2016. Starvation resistance and tissue-specific gene expression of stress-related genes in a naturally inbred ant population. R. Soc. Open Sci. 3, 160062 https://doi.org/10.1098/rsos.160062.

Buckley, B.A., Owen, M.E., Hofmann, G.E., 2001. Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (*genus Mytilus*) changes as a function of thermal history. J. Exp. Biol. 204, 3571–3579. https://doi.org/10.1002/0471142905.hg0118s57.

Camara, M.D., Evans, S., Langdon, C., 2007. Inbreeding effects on growth and survival in a naturalized population of the Pacific oyster (*Crassostrea gigas*) revealed using molecular marker-based estimates of parental relatedness. Aquaculture 272. https://doi.org/10.1016/j.aquaculture.2007.07.038. S247-S247.

Caplins, S.A., Turbeville, J.M., 2015. High rates of self-fertilization in a marine ribbon worm (Nemertea). Biol. Bull. 229, 255–264. https://doi.org/10.1086/BBLv229n3p255.

Charlesworth, D., Wills, J.H., 2009. The genetics of inbreeding depression. Nat. Rev. Genet. 10, 783–796. https://doi.org/10.1038/nrg2664.

Chen, J., Xiao, S., Yu, Z., 2011. F-type lectin involved in defense against bacterial infection in the pearl oyster (*Pinctada martensii*). Fish Shellfish Immun. 30, 750–754. https://doi.org/10.1016/j.fsi.2010.12.025.

Dégremont, L., Ernande, B., Bedier, E., Boudry, P., 2007. Summer mortality of hatchery-produced Pacific oyster spat (*Crassostrea gigas*). I. Estimation of genetic parameters for survival and growth. Aquaculture 262, 41–53. https://doi.org/10.1016/j.aquaculture.2006.10.025.

Dillon, S.M., Lee, E.J., Bramante, J.M., Barker, E., Wilson, C.C., 2014. The natural killer cell interferon-gamma response to bacteria is diminished in untreated HIV-1 infection and defects persist despite viral suppression. J. Acquir. Immune Defic. 65, 259–267. https://doi.org/10.1097/01.qai.0000435603.50598.2b.

Evans, F., Matson, S., Brake, J., Langdon, C., 2004. The effects of inbreeding on performance traits of adult Pacific oysters (*Crassostrea gigas*). Aquaculture 230, 89–98. https://doi.org/10.1016/j.aquaculture.2003.09.023.

Fellous, A., Labed-Veydert, T., Locrel, M., Voisin, A.S., Earley, R.L., Silvestre, F., 2018. DNA methylation in adults and during development of the self-fertilizing mangrove rivulus, *Kryptolebias marmoratus*. Ecol. Evol. 8, 6016–6033. https://doi.org/10.1002/ece3.4141.

Gerszten, R.E., Garcia-Zepeda, E.A., Lim, Y.C., Yoshida, M., Rosenzweig, A., 1999. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 398, 718–723. https://doi.org/10.1038/19546.

Gorbushin, A.M., Borisova, E.A., 2015. Lectin-like molecules in transcriptome of *littorina littorea* hemocytes. Dev. Comp. Immunol. 48, 210–220. https://doi.org/10.1016/j.dci.2014.10.007.

Han, Z.Q., Li, Q., 2018. Different responses between orange variant and cultured population of the Pacific oyster *Crassostrea gigas* at early life stage to temperature-salinity combinations. Aquac. Res. 49, 2233–2239. https://doi.org/10.1111/are.13680.

Han, Z.Q., Li, Q., 2020. Mendelian inheritance of orange shell color in the Pacific oyster *Crassostrea gigas*. Aquaculture 516, 734616. https://doi.org/10.1016/j.aquaculture.2019.734616.

Han, Z., Li, Q., Liu, S.K., Kong, L.F., 2019. Genetic variability of an orange-shell line of the Pacific oyster Crassostrea gigas during artificial selection inferred from microsatellites and mitochondrial COI sequences. Aquaculture 208, 159–166. https://doi.org/10.1016/j.aquaculture.2019.04.074.

Hansson, B., Naurin, S., Hasselquist, D., 2014. Does inbreeding affect gene expression in birds? Biol. Lett. 10, 20140648. https://doi.org/10.1098/rsbl.2014.0648.

Hedgecock, D., Davis, J.P., 2007. Heterosis for yield and crossbreeding of the Pacific oyster *Crassostrea gigas*. Aquaculture 272, S17–S29. https://doi.org/10.1016/j.aquaculture.2007.07.226.

Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., Matsui, H., 2001. A large family of class iii plant peroxidases. Plant Cell Physiol. 42, 462–468. https://doi.org/10.1093/pcp/pce061.

Hofmann, G.E., Somero, G.N., 1995. Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel Mytilus trossulus. J. Exp. Biol. 198, 1509–1518.

Huisman, J., Kruuk, L.E.B., Ellis, P.A., Clutton-Brock, T., Pemberton, J.M., 2016. Inbreeding depression across the lifespan in a wild mammal population. P. Natl. Acad. Sci. U.S.A. 113, 3585–3590. https://doi.org/10.1073/pnas.1518046113.

Janicke, T., Vellnow, N., Lamy, T., Chapuis, E., David, P., 2014. Inbreeding depression of mating behavior and its reproductive consequences in a freshwater snail. Behav. Ecol. 25, 288–299. https://doi.org/10.1093/beheco/art122.

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Yamanishi, Y., 2007. KEGG for linking genomes to life and the environment. Nucleic Acids Res. 36, D480–D484. https://doi.org/10.1093/nar.gkm882.

Kiffin, R., 2004. Activation of chaperone-mediated autophagy during oxidative stress. Mol. Biol. Cell 15, 4829–4840. https://doi.org/10.1091/mbc.E04-06-0477.

Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360. https://doi.org/10.1038/nmeth.3317.

Kishimoto, T., 1985. Factors affecting B-cell growth and differentiation. Annu. Rev. Immunol. 3, 133–157. https://doi.org/10.1146/annurev.iy.03.040185.001025.

Kit, B., Lisa, R., Frances, M.B., 2020. Clathrin's life beyond 40: connecting biochemistry with physiology and disease. Curr. Opin. Cell Biol. 65, 141–149. https://doi.org/10.1016/j.ceb.2020.06.004.

Kristensen, T.N., 2005. Genome-wide analysis on inbreeding effects on gene expression in Drosophila melanogaster. Genetics 171, 157–167. https://doi.org/10.1534/genetics.104.039610.

Langdon, C., Evans, F., Jacobson, D., Blouin, M., 2003. Yields of cultured Pacific oysters Crassostrea gigas Thunberg improved after one generation of selection. Aquaculture 220, 227–244. https://doi.org/10.1016/S0044-8486(02)00621-X.

Launey, S., Hedgecock, D., 2001. High genetic load in the pacific oyster crassostrea gigas. Genetics 159, 255–265. https://doi.org/10.1093/genetics/159.1.255.

Leroy, G., 2015. Inbreeding depression in livestock species: review and meta-analysis. Anim. Genet. 45, 618–628. https://doi.org/10.1534/genetics.104.039610.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Durbin, R., 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352.

Li, Y.Q., Liu, S.S., Cheng, H.L., Chen, X.N., Shen, X., Cai, Y.F., 2021. Dynamic transcriptome response in meretrix meretrix to aroclor 1254 exposure - sciencedirect. Ecotox. Environ. Safe. 207, 111485 https://doi.org/10.1016/j.ecoenv.2020.111485.

Lin, S.M., He, Y.Y., Gong, Y., Zhang, Y.L., Ma, H.Y., Zheng, H.P., Li, S.K., 2019. *Sp*BOK inhibits WSSV infection by regulating the apoptotic pathway in mud crab (*scylla paramamosain*). Dev. Comp. Immunol. 106, 103603 https://doi.org/10.1016/j.dci.2019.103603.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8.

Mandy, M., Nina, S., Jon, A., Bengt, H., 2015. Inbreeding affects gene expression differently in two self-incompatible *Arabidopsis lyrata* populations with similar levels of inbreeding depression. Mol. Biol. Evol. 32, 2036–2047. https://doi.org/10.1111/age.12178.

Maret, W., 2017. Zinc in cellular regulation: the nature and significance of "zinc signals". Int. J. Mol. Sci. 18, 2285. https://doi.org/10.3390/ijms18112285.

Mcdonagh, B., Sheehan, D., 2006. Redox proteomics in the blue mussel Mytilus edulis: carbonylation is not a pre-requisite for ubiquitination in acute free radical-mediated oxidative stress. Aquat. Toxicol. 79, 325–333. https://doi.org/10.1016/j.aquatox.2006.06.020.

Nelson, N., 2014. Metal ion transporters and homeostasis. EMBO J. 18, 4361–4371. https://doi.org/10.1093/emboj/18.16.4361.

Phillippi, A.L., Yund, P.O., 2017. Self-fertilization and inbreeding depression in three ascidian species that differ in genetic dispersal potential. Mar. Biol. 164, 179. https://doi.org/10.1007/s00227-017-3214-x.

Plough, L.V., 2012. Environmental stress increases selection against and dominance of deleterious mutations in inbred families of the Pacific oyster *Crassostrea gigas*. Mol. Ecol. 21, 3974–3987. https://doi.org/10.1111/j.1365-294x.2012.05688.x.

Plough, L.V., Hedgecock, D., 2011. Quantitative trait locus analysis of stage-specific inbreeding depression in the Pacific oyster *Crassostrea gigas*. Genetics 189, 1473–1486. https://doi.org/10.1534/genetics.111.131854.

Renault, T., Faury, N., Barbosa-Solomieu, V., Moreau, K., 2011. Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, *Crassostrea gigas*, challenged with Ostreid herpesvirus 1. Dev. Comp. Immunol. 35, 725–735. https://doi.org/10.1016/j.dci.2011.02.004.

Romani, A., 2011. Cellular magnesium homeostasis. Arch. Biochem. Biophys. 512, 1–23. https://doi.org/10.1016/j.abb.2011.05.010.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3 (6), 1101–1108. https://doi.org/10.1038/nprot.2008.73.

Souza-Fonseca-Guimaraes, F., Adib-Conquy, M., Cavaillon, J.M., 2011. Natural killer (NK) cells in antibacterial innate immunity: angels or devils? Mol. Med. 18, 270–285. https://doi.org/10.2119/molmed.2011.00201.

Tasset, I., Cuervo, A.M., 2016. Role of chaperone-mediated autophagy in metabolism. FEBS J. 283, 2403–2413. https://doi.org/10.1111/febs.13677.

Toshio, T., Masashi, N., Tadamitsu, K., 2014. IL-6 in inflammation, immunity, and disease. CSH Perspect. Biol. 6, a016295 https://doi.org/10.1101/cshperspect.a016295.

Vasta, G.R., Mario, A.L., Bianchet, M.A., Matteo, C., Feng, C., Keiko, S., 2017. F-type lectins: a highly diversified family of fucose-binding proteins with a unique sequence motif and structural fold, involved in self/non-self-recognition. Front. Immunol. 8, 1648. https://doi.org/10.3389/fimmu.2017.01648.

Venney, C.J., Johansson, M.L., Heath, D.D., 2016. Inbreeding effects on gene-specific DNA methylation among tissues of Chinook salmon. Mol. Ecol. 25, 4521–4533. https://doi.org/10.1111/mec.13777.

Yang, L., Smyth, G.K., Wei, S., 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930. https://doi.org/10.1093/bioinformatics/btt656.

Yarra, T., Gharbi, K., Blaxter, M., Peck, L.S., Clark, M.S., 2016. Characterization of the mantle transcriptome in bivalves: Pecten maximus, Mytilus edulis and Crassostrea gigas. Mar. Genomics 27, 9–15. https://doi.org/10.1016/j.margen.2016.04.003.

Zhao, C., Sun, P., Wei, J., Zhang, L.S., Zhang, W.J., Song, J., et al., 2016. Larval size and metamorphosis are significantly reduced in second generation of inbred sea urchins *Strongylocentrotus intermedius*. Aquaculture 452, 402–406. https://doi.org/10.1016/j.aquaculture.2015.11.024.

Zajitschek, S.R.K., Brooks, R.C., 2010. Inbreeding depression in male traits and preference for outbred males in *Poecilia reticulata*. Behav. Ecol. 21 (4), 884–891. https://doi.org/10.1093/beheco/arq077.

Zhao, L., Li, Y.P., Lou, J.R., Yang, Z.H., Liao, H., Fu, Q., Bao, Z.M., 2019. Transcriptomic profiling provides insights into inbreeding depression in yesso scallop *Patinopecten yessoensis*. Mar. Biotechnol. 21, 623–633. https://doi.org/10.1007/s10126-019-09907-9.

Zheng, H.P., Li, L., Zhang, G.F., 2012. Inbreeding depression for fitnessrelated traits and purging the genetic load in the hermaphroditic bay scallop *Argopecten irradians irradians* (Mollusca: Bivalvia). Aquaculture 366, 27–33. https://doi.org/10.1016/j.aquaculture.2012.08.029.

Zohny, S.F., El-Shinawi, M., 2011. Significance of survivin and Bcl-2 homologous antagonist/killer mRNA in detection of bone metastasis in patients with breast cancer. Med. Oncol. 28, 108–114. https://doi.org/10.1007/s12032-010-9724-8.