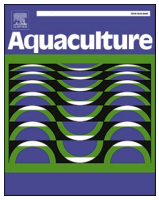
Aquaculture 555 (2022) 738219

Contents lists available at ScienceDirect

Aquaculture

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



Comparative transcriptomic analyses reveal differences in the responses of diploid and triploid Pacific oysters (*Crassostrea gigas*) to thermal stress

Yongguo Li [a,1], Kunyin Jiang [a,1], 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:
Crassostrea gigas
Diploid, triploid
Heat stress
Survival rate
Transcriptomic analyses

ABSTRACT

Triploid oysters often have higher growth rates and survival rates than diploid oysters. However, some studies have observed the opposite patterns. Triploid oysters have also been observed to show reduced resistance to stress and disease compared with diploid oysters. We compared the transcriptional patterns of diploid and triploid Pacific Oysters (*Crassostrea gigas*) in response to acute and prolonged thermal stress. The survival rate of triploid *C. gigas* was lower than that of diploid oysters after prolonged heat stress at 28 °C. A total of 779 and 396 differentially expressed genes (DEGs) were detected in diploid and triploid oysters exposed to acute heat stress, respectively, and the number of DEGs was 418 in diploids and 483 in triploids after prolonged heat stress. Functional enrichment analysis revealed that the DEGs were significantly enriched in protein processing in the endoplasmic reticulum pathway, NF-kappa B signaling pathway, NOD-like receptor signaling pathway, and TNF signaling pathway in both diploid and triploid *C. gigas* under heat stress. Genes encoding translation initiation factors, including *MKNK1, eIF4G3, eIF2a kinase* 1, and *eIF2a kinase* 3, were differentially expressed under acute heat stress in diploid oysters. The number of differentially expressed *HSP* and *IAP* genes was greater in diploids than in triploids under acute heat stress, and the expression levels of most of these *HSP* and *IAP* genes were higher in diploid oysters than in triploid oysters. Overall, our findings revealed differences in the response of diploid and triploid *C. gigas* to thermal stress. Triploid *C. gigas* might be less capable of rapidly modifying the inflammatory response and apoptosis in response to heat stress compared with diploid *C. gigas*. Different genetic lines of diploid and tetraploid oysters should be used to produce triploid oysters with increased stress resistance.

1. Introduction

The Pacific Oyster, *C. gigas*, is one of the most widely cultured shellfish with an important commercial and ecological value (Xing et al., 2018; Zhu et al., 2016). Triploid oysters were developed approximately 40 years ago to increase oyster production (Stanley et al., 1981), and they are now widely cultured in Asia, North America, Australia, and Europe (Nell, 2002; Wadsworth, 2018). Triploid oysters have higher growth performance, survival rates, and meat quality compared with diploid oysters (Hand et al., 1998; Nell et al., 1994; Vialova, 2020; Wadsworth et al., 2019a). Triploid oysters are considered reproductively sterile, and their meat weight is maintained throughout the spawning season; this greatly enhances their marketability compared with diploid oysters, which tend to decrease in meat weight during the spawning season (Matt et al., 2020). Triploid oyster production has increased substantially to meet the continually growing demand for oysters by consumers (Haure et al., 2021). There are currently two main approaches for producing triploid oysters: chemical induction or crosses of diploid females with tetraploid males (Gérard et al., 1999; Guo et al., 1996). Only the latter method is capable of generating a fully triploid population (Guo et al., 1996). Triploid oysters have faster growth rates than diploids, especially at sites with high trophic richness (Normand et al., 2008). The faster growth of triploid oysters likely stems from their sterility, increased heterozygosity, and cell size (Wang et al., 2002). Sterility allows the energy normally required for spawning to be reallocated to somatic growth (Guo and Allen, 1994).

Several studies of the survival of diploid and triploid oysters and their resistance to various pathogens have been conducted, and the results of these studies have varied (De Decker et al., 2011; Hand et al., 2004). Triploid *C. gigas* might be more resistant to summer mortality

* Corresponding author at: Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qindao 266003, China.
E-mail address: qili66@ouc.edu.cn (Q. Li).
[1] Yongguo Li and Kunyin Jiang contributed equally to this work.

https://doi.org/10.1016/j.aquaculture.2022.738219
Received 1 November 2021; Received in revised form 28 March 2022; Accepted 1 April 2022
Available online 4 April 2022

than diploids and possess other advantages for surviving in warm climates (Shpigel et al., 1992). Some studies have shown that triploid oysters have higher survival rates following pathogen exposure compared with diploid oysters (Hand et al., 1998; Pernet et al., 2012), whereas other studies have found the opposite pattern (Cheney et al., 2000; De Decker et al., 2011; Garnier-Gere et al., 2002). Garnier-Gere et al. (2002) found that growth rates of triploid *C. gigas* were higher than those of diploid oysters at both oligotrophic and eutrophic sites; however, the survival rates of triploid and diploid oysters were similar at both sites. Duchemin et al. (2007) showed that variation in immune parameters among seasons is lower in triploid oysters than in diploid oysters, which indicates that triploids are less sensitive to environmental cues. The cumulative mortality of triploid Eastern Oyster (*Crassostrea virginica*) in the northern Gulf of Mexico is higher than that of diploid oysters (Wadsworth et al., 2019b). Triploid oysters are physiologically weak and thus more vulnerable to infection by pathogens than diploid oysters during the winter months, which is a period when diploid individuals do not incur the costs of reproductive activity (De Decker et al., 2011).

High mortality of both diploid and triploid oysters has been reported during the summer months over the past several decades (Dégremont et al., 2012), and this has motivated increased interest in the effect of heat stress on oysters (Lang et al., 2009; Li et al., 2007; Zhang et al., 2019). Oysters have evolved various mechanisms, as well as a diverse set of genes, to facilitate adaptation to temperature extremes (Zhang et al., 2012a). Under heat stress, the basic life activities of *C. gigas*, including energy metabolism, the unfolded protein response, and anti-apoptotic system, are affected, and several genes involved in the refolding and stabilization of protein structure are activated to repair stress-related injuries (Yang et al., 2017). If heat stress persists, stress-related injuries become irreparable, which eventually leads to the activation of apoptotic genes (Yang et al., 2017). Analysis of the oyster genome and transcriptome data has suggested that the expansion of heat shock protein 70 protein (HSP70s) and inhibitor of apoptosis (IAPs) families has likely played a key role in mediating the adaptation of oysters to sessile life in the intertidal zone, which is a physiologically stressful environment (Zhang et al., 2012a).

High temperatures have a substantial effect on the energy metabolism and immune response of oysters and thus increase their susceptibility to pathogens (Delisle et al., 2018; Gagnaire et al., 2006). Many studies have examined the response of *C. gigas* to high temperature (Lim et al., 2016; Liu et al., 2019; Nash et al., 2019; Yang et al., 2017). Few comparative transcriptomic analyses of triploid and diploid oysters have been conducted to date. Here, we used high-throughput RNA sequencing (RNA-seq) to characterize differences in expression patterns between triploid and diploid *C. gigas* under acute and prolonged thermal stress. Overall, our aim was to reveal the responses of diploid and triploid *C. gigas* to thermal stress.

2. Materials and methods

2.1. Oysters and thermal stress

Hatchery-produced triploid and diploid *C. gigas* (two years in age) were used in this experiment. Triploid oysters were produced by crossing 150 diploid females with 30 tetraploid males, and diploid oysters were produced by crossing 50 diploid females with 50 diploid males. All oysters were cultured in a farm in Sanggou Bay, Shandong Province, China. Prior to experiments, the ploidy level of oysters was measured using a flow cytometer (Beckman Coulter, Brea, CA, USA). Diploid and triploid *C. gigas* were acclimatized to the experimental environment in 500-L plastic tanks at 18 °C for 10 d. Oysters were fed *Chlorella vulgaris*, and approximately one-third of the seawater was exchanged daily. After acclimatization, triploid and diploid oysters were transferred to 28 °C seawater. There were three replicates for both triploid and diploid oysters, and there were 90 oysters in each replicate. Dead oysters were removed from the tank as soon as they were detected. Surviving oysters were cultured at 28 °C until no deaths were observed for two consecutive days (Fig. 1). Three surviving individuals were randomly selected for sampling from each replicate at 0 h, 12 h, and 9 d; samples from each of these sampling events were referred to as A0d and A0t, A12d and A12t, and Afd and Aft, respectively. The characters "0," "12," and "f" refer to the timing of sampling (0 h, 12 h, and the final sampling event after no deaths were observed for two consecutive days, respectively), and "d" and "t" stand for diploid and triploid, respectively. Whole gill tissues were collected and frozen immediately in liquid nitrogen; they were then stored at −80 °C until RNA extraction. The final survival rate of triploid and diploid oysters was calculated.

2.2. RNA extraction and library construction for transcriptome sequencing

Total RNA was extracted from gill tissues using the TRIzol Reagent (Ambion, USA) per the manufacturer's protocol. The quality and integrity of total RNA were detected using a 1% agarose gel as well as an RNA Nano 6000 Assay Kit and Bioanalyzer 2100 system (Agilent Technologies, CA, USA), respectively. The total RNA of each of the three diploid and triploid samples from each time point was pooled into single samples with the same concentration. The mixed total RNA samples were referred to as A0d_1, A0d_2, A0d_3, A0t_1, A0t_2, A0t_3, A12d_1, A12d_2, A12d_3, A12t_1, A12t_2, A12t_3, Afd_1, Afd_2, Afd_3, Aft_1, Aft_2, and Aft_3. One μg of total RNA per sample was used for the RNA sample preparations. Poly-T oligo-attached magnetic beads were used to purify total RNA. Random hexamer primers and M-MuLV Reverse Transcriptase (RNase H) were used to synthesize first-strand cDNA. DNA Polymerase I and RNase H were used to synthesize second-strand cDNA. An AMPure XP system (Beckman Coulter, Beverly, USA) was used to purify the library fragments and obtain cDNA fragments with a length of 370–420 bp. Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer PCR were used to perform PCR. After purification of the PCR products (AMPure XP system), an Agilent Bioanalyzer 2100 system was used to assess the quality of the library. The NEBNext mRNA Library Prep Master Mix Set and Illumina HiSeq X sequencing were used to construct RNA-seq libraries.

2.3. Sequence filtering, mapping, and differential expression analysis

The quality of the raw reads was assessed using FastQC v0.11.8, and the Q20, Q30, and GC content of the clean data were determined. The reference *C. gigas* genome (cgigas_uk_roslin_v1) was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/genome). The index of the reference genome was constructed using Hisat2 v2.2.0, and paired-end clean reads were aligned to the reference genome. All downstream analyses were conducted using the clean reads. The number of reads mapped to the reference genome gene was counted using featureCounts software. The fragments per kilobase per million fragments mapped values of each gene were calculated based on the length of the gene and the number of reads mapped to this gene. The abundances of each gene in read count values were normalized using the transcripts per million (TPM) method. Principal component analysis (PCA) was used to examine variation in the gene expression patterns of samples among the different time points. Correlations among all samples were analyzed using RStudio v1.4.1717 software. The DESeq2 R package was used to analyze differential expression patterns (Love et al., 2014). Differentially expressed genes (DEGs) were identified using the following criteria: adjusted $P < 0.05$ and $|\log_2(\text{fold change})| > 1$.

2.4. Functional annotation and enrichment analysis

The functional annotations of proteins were predicted using eggNOG v5.0 (Huerta-Cepas et al., 2019), and this information was associated with the genome annotation file. Gene Ontology (GO) analysis of DEGs

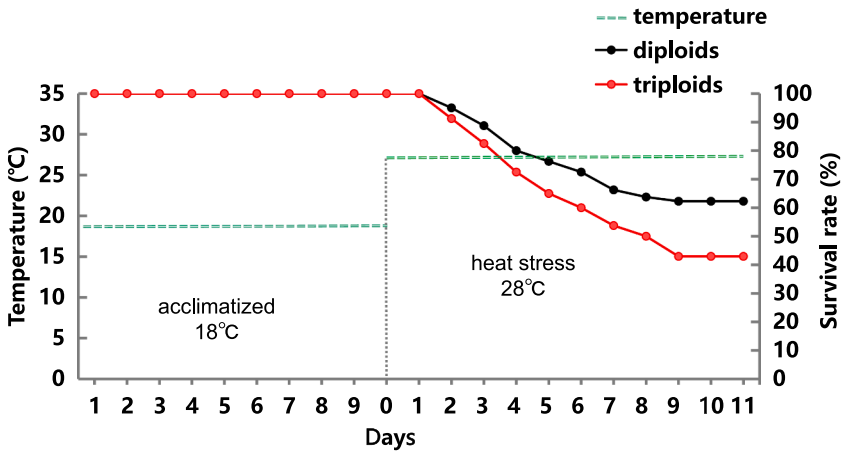


Fig. 1. The experimental temperature and survival rate of triploid and diploid *Crassostrea gigas*. The red and black solid lines indicate the survival rate of triploids and diploids, respectively. The green dotted line indicates the experimental temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was conducted using the ClusterProfiler v4.0.2 R package (Ashburner et al., 2000). DEGs were considered significantly enriched in GO terms when *P* was less than 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis can provide key insights into the high-level functions and utilities of biological systems (Ogata et al., 1999). The KEGG pathways in which DEGs were enriched were determined using the ClusterProfiler v4.0.2R package.

2.5. Validation and expression analysis of selected DEGs

The expression levels 15 DEGs were validated using quantitative real-time PCR (qPCR). Primers for qPCR were designed using Primer Premier 5 software. The elongation factor 1-α (*EF1-α*) gene was used as the reference gene for qPCR analysis because a previous study has shown that it is stably expressed under stress conditions (Du et al., 2013). A series of five 10-fold dilutions of cDNA was conducted, and the efficiency of PCR amplification was evaluated using the following equation: Efficiency $= 10^{(-1/\text{slope})}$. For qPCR, the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China) were used to reverse-transcribe total RNA to cDNA. qPCR reactions were performed using TB Green® Premix Ex Taq™ II (Takara, China) in a total volume of 20 μL containing 2 μL of diluted cDNA, 1 μL of each primer, 10 μL of TB Green PCR Master Mix, and 6 μL of H_2O with the following thermal cycling conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 65 °C for 1 min. Triplicate reactions were conducted for each sample in the Roche LightCycler 480 Real-Time PCR System (Roche, Switzerland). Expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

3. Results

3.1. Survival statistics

The resistance of triploid and diploid oysters to thermal stress was evaluated based on their survival rates. The survival of diploid and triploid oysters was 100% during the acclimatization period (Fig. 1). Acute heat stress for 12 h did not result in the death of any oysters. The first deaths of both diploid and triploid oysters were observed after 2 d of heat stress. The number of deaths of triploid oysters was greater than that of diploid oysters during prolonged heat stress, especially at 8 and 9 d. The survival of diploid and triploid oysters gradually stabilized after 9 d of heat stress, as no dead oysters were observed thereafter. The survival rate of triploid oysters (42.95 ± 10.32%) was lower than that of diploid oysters (62.27 ± 8.81%) by the end of the experiment.

3.2. Transcriptome assemblies

A total of 867,834,454 raw paired-end reads were obtained (Table S1). After filtering, 831,151,096 clean reads (Q30: 93.23–94.70%) were retained and used for transcriptome assembly. The clean reads were submitted to the NCBI SRA database under the accession number PRJNA762441. The average alignment rate at which the clean data for each sample were mapped to the *C. gigas* reference genome was 78.12% (Table S1). After alignment, the abundances of whole genes were estimated to read count values and normalized using the TPM method. The correlations among all samples ranged from 0.83 to 0.99 (Fig. S1A). Samples from the same time point were clustered together according to PCA. PC1 explained 52.06% of the variation in the data and was highly correlated with the differential gene expression patterns of *C. gigas* and thermal stress (Fig. S1B). The Aft and Afd groups were clustered into a single branch, and the A0t, A0d, A12t, and A12d groups were clustered into another branch according to hierarchical clustering analysis (Fig. S1C).

3.3. DEGs

The expression distribution of all transcripts is shown in Fig. 2. We detected a total of 779 DEGs in the A12d vs. A0d comparison, including 218 up-regulated genes and 561 down-regulated genes (Fig. 2A); 396 DEGs were detected in the A12t vs. A0t comparison, including 137 up-regulated genes and 259 down-regulated genes (Fig. 2B); 418 DEGs were detected in the Afd vs. A0d comparison, including 181 up-regulated genes and 237 down-regulated genes (Fig. 2C); and 483 DEGs were detected in the Aft vs. A0t comparison, including 290 up-regulated genes and 193 down-regulated genes (Fig. 2D). The number of DEGs was higher in diploid oysters than in triploid oysters under acute heat stress for 12 h (Fig. 2E). After prolonged heat stress, the number of DEGs in diploid oysters was 418, and the number of DEGs in triploid oysters was 483. A Venn diagram revealed the shared and unique DEGs among the A12d vs. A0d, A12t vs. A0t, Afd vs. A0d, and Aft vs. A0t comparisons (Fig. 2F).

The expression of several genes was significantly altered in diploid and triploid *C. gigas* under acute heat stress, including those encoding baculoviral IAP repeat-containing proteins (*BIRCs*), calnexin (*CANX*), ATP-dependent RNA helicase (*DDX58*), DnaJ homolog subfamily C members (*DnaJs*), heat shock proteins (*HSP20*, *HSP70*, *HSPA5*, and *HSPB1*), and transient receptor potential cation channel subfamily M member (*TRPM*) (Table S2). Unique DEGs detected in the A12d group included MAP kinase-interacting serine/threonine-protein kinase 1 (*MKNK*1), eukaryotic translation initiation factor 4 gamma 3 isoform X5

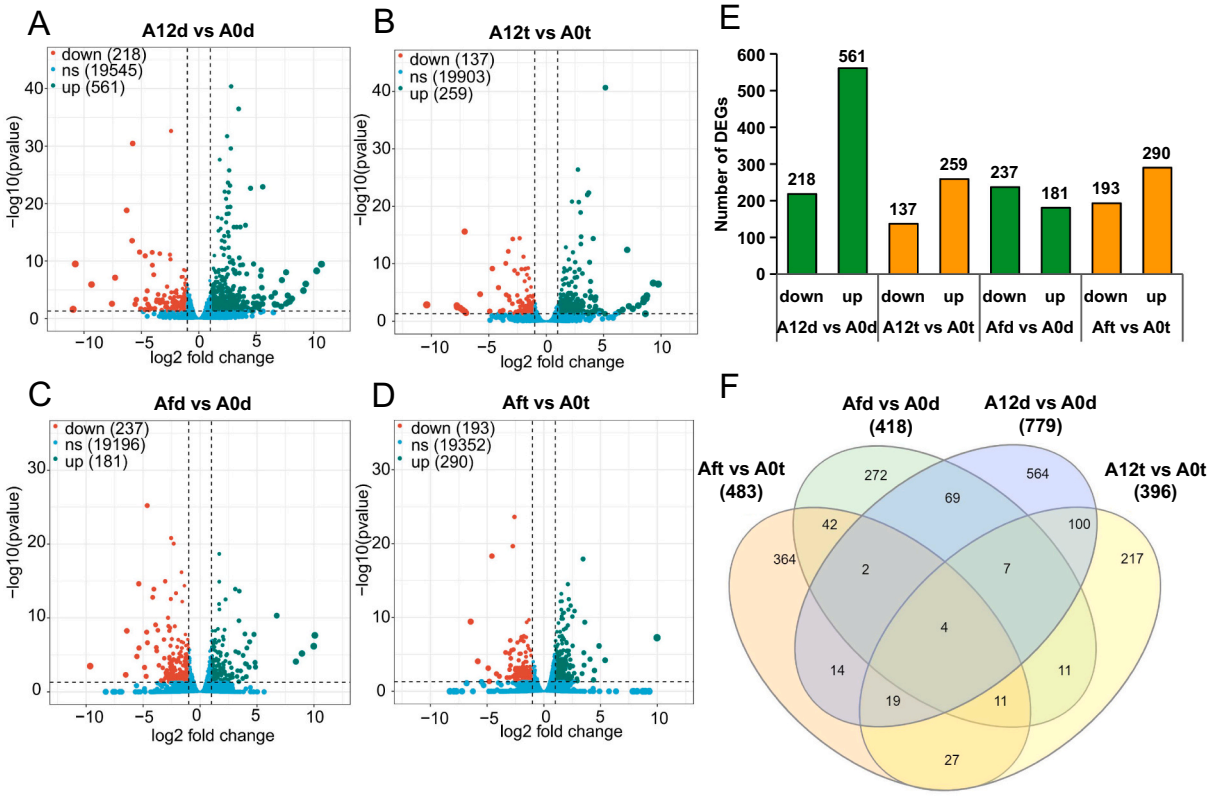


Fig. 2. Comparison of differentially expressed genes (DEGs). Volcano plot of DEGs in the A12d (A), A12t (B), Afd (C), and Aft (D) groups. Number of up-regulated and down-regulated DEGs in each group (E) and Venn diagram of overlapping DEGs among these four groups (F).

(*eIF4G3*), eIF2-alpha kinases (*eIF2α kinase1* and *eIF2α kinase3*), caspase 2 (*CASP*2), TNF receptor superfamily member 19 (*FADD*), TNF receptor-associated factor 2 (*TRAF*2), DNA damage-regulated autophagy modulator 2 (*DRAM*2), and nitric oxide synthase (*NOS*). After prolonged heat stress, the expression of the genes NADPH oxidase 5 (*NOX*5), caspase-8 (*CASP*8), cytochrome *b*5 (CYB5), complement c1q-like 4 (*C1qL*4), and complement c1q tumor necrosis factor-related protein 3 (*C1qTNF*3) was highly up-regulated in both diploid and triploid oysters. Some unique DEGs were also detected in the Aft group, including DNA replication licensing factors (*MCM*s), cytochrome P450 3A9 isoform X2 (*CYP3A9*), and *TRAF*3. We detected four DEGs in both diploids and triploids during exposure to heat stress, including serine/arginine-rich splicing factor 4 (*SRSF*4), protein disulfide-isomerase A6 (*PDIA*6), heat shock protein 90-kDa beta (*HSP90B*1), and peptidyl-prolyl *cis-trans* isomerase B isoform X2 (*PPIase*) (Table S2).

3.4. GO analysis

A total of 829, 673, 458, and 685 GO terms were enriched in the A12d, A12t, Afd, and Aft groups, respectively (Fig. 3A & C). The proportions of DEGs in the GO categories biological process, molecular function, and cell component in these four groups were similar (Fig. 3A). The number of overlapping and unique GO terms in these four groups is shown in Fig. 3C. The overlapping terms among all four groups from the top 30 GO terms were positive regulation of cellular process (GO:0048522) and catalytic activity (GO:0003824). Other overlapping or unique GO terms related to the stress response included cellular response to stimulus (GO:0051716), response to stress (GO:0006950), regulation of response to stimulus (GO:0048583), and response to stimulus (GO:0050896) (Fig. 4A–D). We found that the magnitude of the changes in the expression of DEGs involved in stress responses, cell process, and metabolism differed in diploids and triploids (Fig. 4E & F). The expression of DEGs in these GO terms was slightly lower in triploid oysters than in diploid oysters at 12 h and slightly higher in triploid oysters than in diploid oysters at 9 d. The number of DEGs enriched in these GO terms decreased in diploids and increased in triploids from 12 h to 9 d under heat stress.

3.5. KEGG pathway enrichment analysis

DEGs in the A12d, A12t, Afd, and Aft groups were significantly enriched in 14, 19, 5, and 15 KEGG pathways, respectively (Fig. 3B & D). Under acute heat stress, the significantly enriched signaling pathways shared by diploids and triploids were TNF signaling pathway, NF-kappa B signaling pathway, NOD-like receptor signaling pathway, thyroid hormone synthesis, toxoplasmosis, and cytosolic DNA-sensing pathway (Fig. 5A & B). Most DEGs were significantly enriched in the "protein processing in the endoplasmic reticulum" pathway in the A12d (25/128 gene ratio) and A12t (21/128 gene ratio) groups. After prolonged thermal stress, the number of significantly enriched KEGG pathways was lowest in the Afd group (five enriched pathways), and no overlap was observed in these pathways with other groups (Fig. 3D); by contrast, a total of 15 significantly enriched KEGG signaling pathways were detected in the Aft group. The IL-17 signaling pathway was specifically enriched in triploids (in both the A12t and Aft groups, Fig. 5B & D). The co-expression network of the top KEGG pathways revealed the relationships among DEGs in the different pathways (Fig. S2A–C). The key DEGs in these pathways are shown in Fig. S2D–F.

3.6. Analysis of DEG families

A heatmap of the DEGs in the A12d, A12t, Afd, and Aft groups was constructed to characterize the global expression profiles of DEGs in each group (Fig. 6A). The expression of expanded gene family members (*HSP*s and *IAP*s) was significantly altered under heat stress. The expression of these differentially expressed *HSP* and *IAP* members

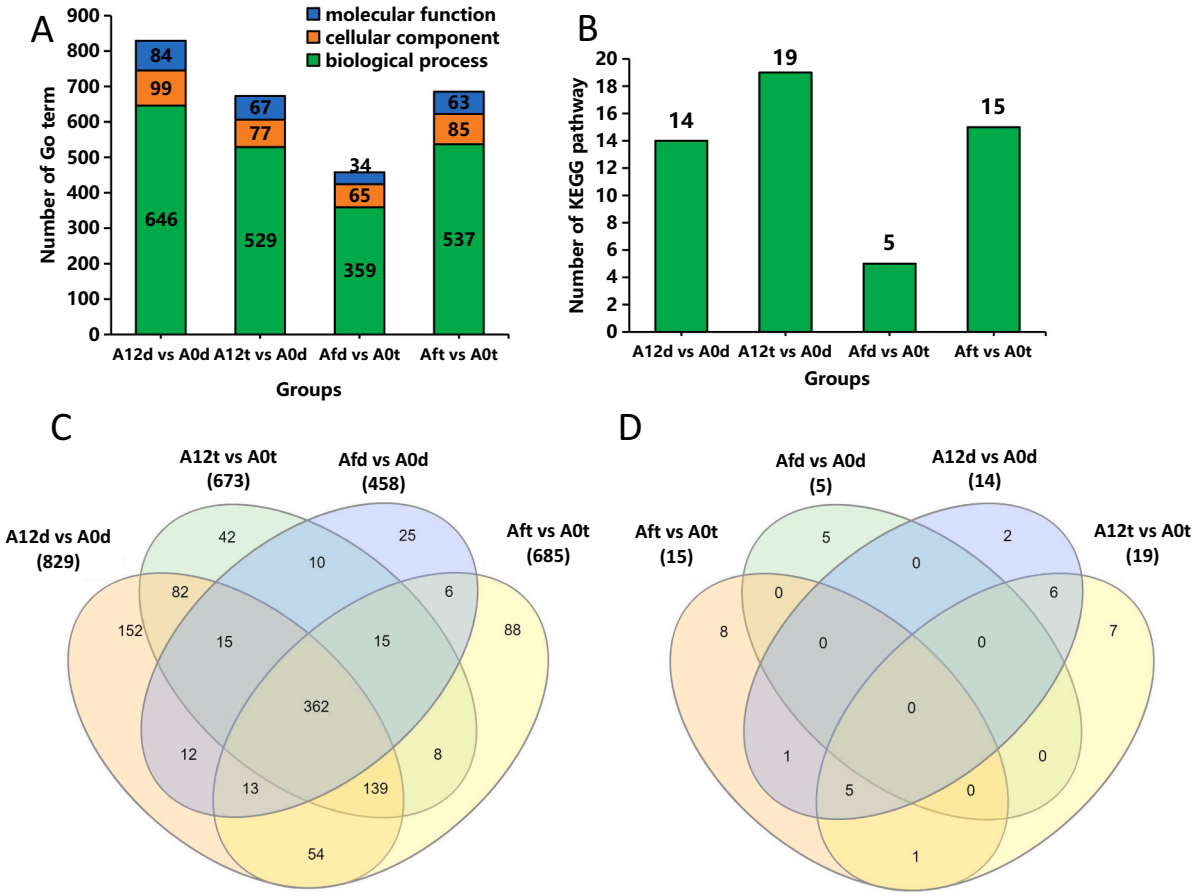


Fig. 3. Distribution of Gene Ontology (GO) terms assigned to biological process, molecular function, and cellular component (A). The number of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the different groups ($P < 0.05$) (B). Venn diagram of overlapping GO terms in the different groups (C). Venn diagram of overlapping KEGG pathways in the different groups (D).

differed in diploids and triploids. A total of 30 *HSP* DEGs were detected, and their expression patterns in each group are shown in a line chart (Fig. 6B). In diploids, the expression of 23 *HSP*s was significantly up-regulated in the A12d group, and only two significantly up-regulated *HSP*s were detected in the Afd group. However, in triploids, the expression of 17 and 10 *HSP*s was significantly up-regulated in the A12t and Aft groups, respectively. A total of 27 of the 30 *HSP*s showed higher expression in the A12d group than in the A12t group (Fig. 6D). Under heat stress, a total of 11 significantly modulated *IAP*s (*BIRC* and *IAP*) were detected; 8 of the 11 *IAP*s were significantly up-regulated in the A12d group, and 0 *IAP*s were significantly up-regulated in the Afd group (Fig. 6C). The expression levels of most of these *IAP*s were higher in the A12d group than in the A12t group (Fig. 6E).

3.7. qPCR validation

The accuracy of the RNA-seq results was evaluated using qPCR. We used 15 DEGs involved in the response to acute and prolonged thermal stress. The specific primers for these genes are listed in Table S3. The amplification efficiencies of these primers ranged from 0.90 to 1.10. The fold change of the selected DEGs detected by qPCR was compared with that determined by RNA-seq expression analysis (Table 1). Fold-change values for most of these DEGs were similar, indicating that the results of the RNA-seq expression analysis are accurate.

4. Discussion

Temperature has a significant effect on the growth and metabolism of oysters (Lim et al., 2016). Some studies have shown that the survival rate of triploid oysters is lower than that of diploid oysters. Other studies have found that the survival rate of triploid oysters is higher than that of diploid oysters and that the difference in the survival of triploid and diploid oysters is particularly pronounced in the summer. We compared the survival rate and the transcriptomic response of diploid and triploid *C. gigas* to acute and prolonged thermal stress. All oysters survived exposure to acute heat stress. However, the survival rate of triploid *C. gigas* was lower (42.95 ± 10.32%) than that of diploid *C. gigas* (62.27 ± 8.81%) after prolonged heat stress. Prolonged high temperature substantially reduced the survival of both diploid and triploid oysters, and the survival rate of triploid oysters was lower than that of diploid oysters. Overall, our findings indicated that the high-temperature tolerance of triploid oysters is weaker than that of diploid oysters during the winter months when diploids do not incur the costs of reproductive activity.

4.1. Responses of diploid and triploid C. gigas to heat stress

The response of oysters to thermal stress is a complex process involving various regulatory pathways and genes (Zhang et al., 2012b). Our comparative transcriptome analysis identified 100 DEGs in diploid and triploid *C. gigas* under acute heat stress. Most of the shared DEGs were enriched in the following pathways: protein processing in the endoplasmic reticulum pathway, NF-kappa B signaling pathway, NOD-like receptor signaling pathway, and TNF signaling pathway. Under heat stress, misfolded and unfolded proteins accumulate in the endoplasmic reticulum, which results in persistent endoplasmic reticulum

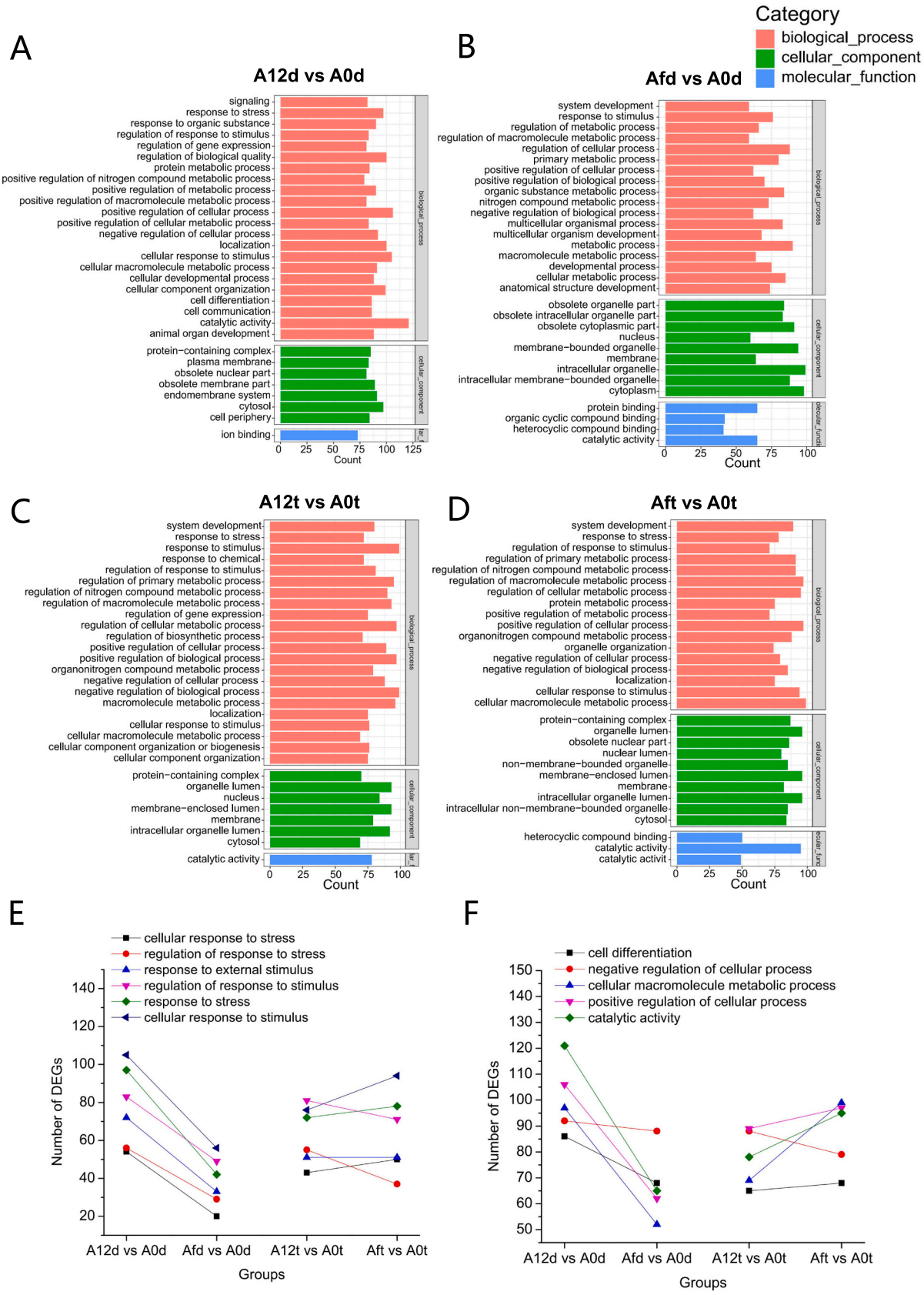


Fig. 4. The top 30 enriched GO terms derived from the GO enrichment analysis of the A12d (A), Afd (B), A12t (C), and Aft (D) groups. The number of DEGs significantly enriched in stress response, cell process, and metabolism-related GO terms in the A12d, Afd, A12t, and Aft groups (E & F).

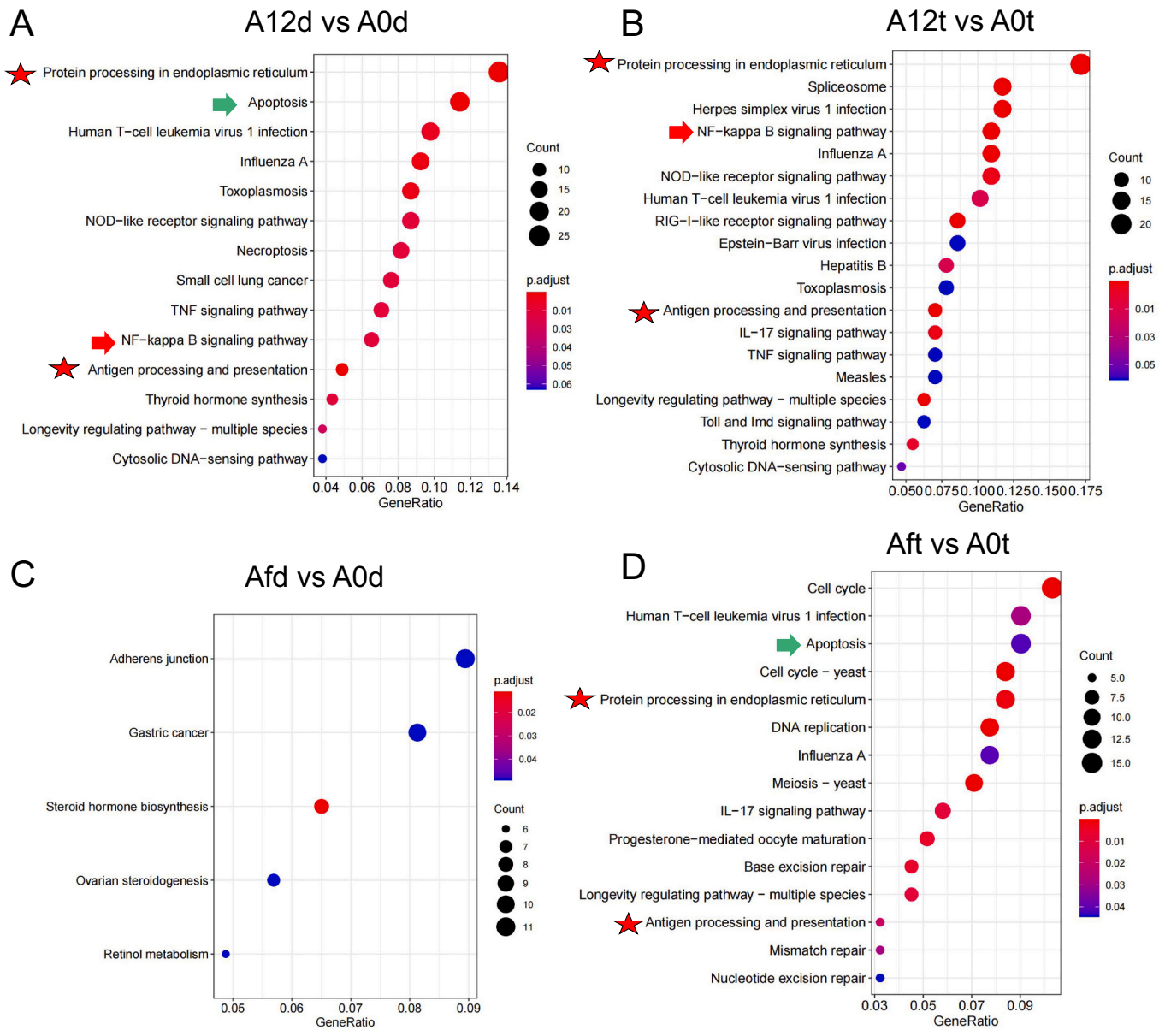


Fig. 5. KEGG enrichment analysis of DEGs in the A12d (A), A12t (B), Afd (C), and Aft (D) groups. The arrows and pentagrams indicate key pathways.

stress and cell death (Haynes et al., 2004; Li et al., 2021). Protein processing in the endoplasmic reticulum pathway can alleviate endoplasmic reticulum stress and improve cell survival by helping misfolded proteins refold into their correct molecular conformation or promoting the degradation of misfolded polypeptides (Haynes et al., 2004; Huang et al., 2018; Xiao et al., 2021). The NF-kappa B signaling pathway is a well-known intracellular signaling cascade that plays a key role in the transcription factor NF-kappa B family (Huang et al., 2012). The activation of NF-kappa B regulates several genes involved in various processes (Barnes, 1997; Nelson et al., 2004). NF-kappa B plays a key role in intracellular transduction, stress signal sensing, the endocrine system, and the immune response of bivalves in response to stress (Nie et al., 2020a). The TNF signaling pathway and NOD-like receptor signaling pathway are involved in the activation of NF-kappa B, cytokine production, and apoptosis (Caruso et al., 2014; Hsu et al., 1995). Thus, the NF-kappa B pathway might contribute to the regulation of the response to heat stress in *C. gigas*.

We also detected differences in the transcriptional responses of diploids and triploids to acute heat stress. The expression of some translation initiation factor-related genes, including *MKNK1, eIF4G3, eIF2a kinase* 1, and *eIF2a kinase* 3, was significantly up-regulated in the A12d group; however, no significant changes in the expression of these genes were observed in the A12t group. Phosphorylation of eIF2 (EIF2AK) by protein kinases can alleviate cellular injury by inducing apoptosis in response to high temperatures (Wek et al., 2006). Phosphorylation of eIF2 results in a rapid reduction in translational initiation and the repression of global protein synthesis, which allows cells to conserve resources and alters the expression of genes to mediate the response to stress (Wek et al., 2006). Apoptosis is a genetically programmed process involved in the elimination of damaged or redundant cells without triggering inflammation (Sokolova, 2009). In our study, the apoptosis pathway was enriched in diploid oysters following acute heat stress. Most of the DEGs enriched in the apoptosis pathway were IAPs, and the expression of these IAPs was significantly up-regulated. X-linked IAPs can inhibit caspases by directly binding to them, and cIAPs bind to caspase-3/7 to mark them for proteasomal degradation (Shiozaki et al., 2003). cIAPs also bind to TRAFs, which block TRAF-induced cell death and activate signaling pathways that induce the expression of pro-

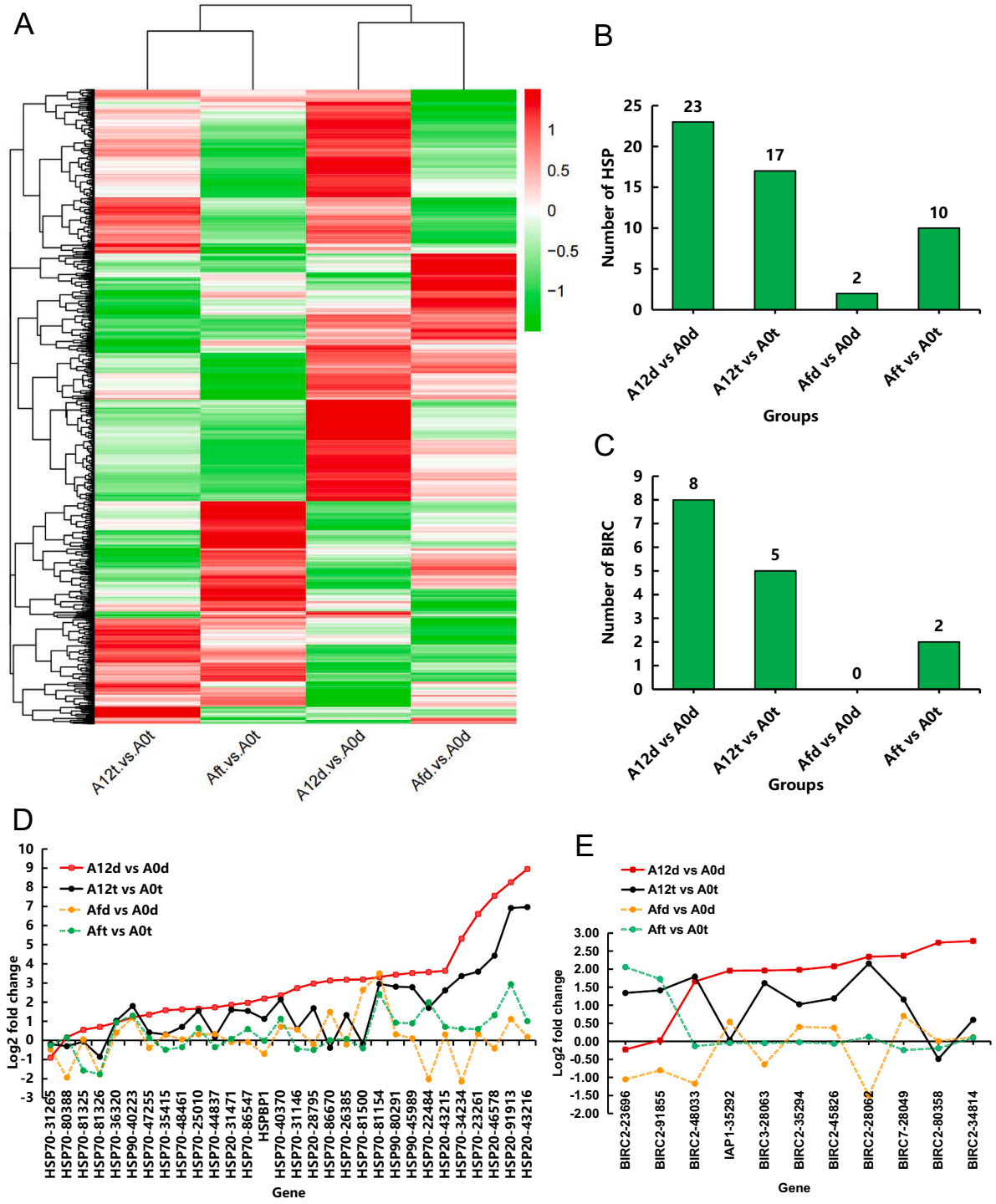


Fig. 6. The expression patterns of all DEGs in the A12d, A12t, Afd, and Aft groups (A). The number of differentially expressed *HSP* (B) and *BIRC* genes (C). Fold change of differentially expressed *HSP* genes (D) and *BIRC* genes (E).

survival proteins (Rothe et al., 1995). These findings suggest that the response of diploid *C. gigas* to acute heat stress was stronger and more rapid than that of triploids and that the heat stress response of both diploid and triploid *C. gigas* involved a reduction in translational initiation and the induction of apoptosis.

The response to thermal stress in *C. gigas* is a complex process. Study of *C. gigas* under prolonged heat stress can provide more detailed information on the response of diploid and triploid oysters to heat stress. No deaths of triploid and diploid oysters were observed for two consecutive days following exposure to prolonged heat stress, which indicates that both triploid and diploid oysters became adapted to heat stress. The number of DEGs was lower in diploid oysters than in triploid oysters during this period of adaptation to prolonged heat stress. DEGs in the Afd group were mainly involved in biological adhesion, calcium ion binding, cytochrome P450 family, and RNA-mediated transposition, whereas DEGs in the Aft group were mainly involved in the inflammatory response, apoptosis, transcription regulation, and cell cycle. Triploid oysters appear to be less sensitive to environmental cues than diploid oysters (Duchemin et al., 2007). These findings indicate that changes in the inflammatory response and apoptosis occurred more

Table 1
Validation of RNA-Seq results using qPCR.

Group	ID	Gene abbreviation	Log2 fold change	
			RNA-seq	qPCR
A12d vs A0d	LOC105343216	*HSP20*	8.95	8.90
	LOC105334234	*HSP70*	5.31	3.05
	LOC105347317	*HSPBP1*	2.05	2.65
	LOC105323151	*DNAJB5*	1.79	1.86
	LOC105317959	*CDC37*	1.66	2.03
	LOC105330898	*DNAJC3*	1.49	1.72
	LOC105344505	*CANX*	1.30	1.19
	LOC105333990	*DDX58*	1.29	3.04
	LOC105343206	*DRAM2*	1.22	1.51
	LOC105340223	*HSP90B1*	1.21	1.03
	LOC105326897	*TRPM2*	−1.12	−0.86
A12t vs A0t	LOC105343216	*HSP20*	6.97	6.87
	LOC105334234	*HSP70*	3.36	5.17
	LOC105340223	*HSP90B1*	1.80	3.37
	LOC105333990	*DDX58*	1.50	1.23
	LOC105330898	*DNAJC3*	1.37	1.37
	LOC105344505	*CANX*	1.25	2.45
	LOC105341798	*DDX58*	1.23	1.49
	LOC105317959	*CDC37*	1.03	1.11
	LOC105326897	*TRPM2*	−1.34	−1.02
Afd vs A0d	LOC117688268	*NOX5*	1.28	4.06
	LOC105340223	*HSP90B1*	1.20	1.37
	LOC105336572	*CYP2J2*	−1.24	−2.42
Aft vs A0t	LOC105340223	*HSP90B1*	1.30	1.34
	LOC105348057	*NOX5*	1.18	1.83
	LOC117688268	*NOX5*	1.09	3.19
	LOC105336572	*CYP2J2*	−1.26	−3.36

slowly in triploid oysters than in diploid oysters. Thus, prolonged heat stress had a more pronounced effect on the inflammatory response and induced more cell damage in triploid oysters than in diploid oysters.

4.2. Evidence for the susceptibility of triploid oysters to heat damage

In our study, the number of DEGs was lower in triploid oysters than in diploid oysters during exposure to acute thermal stress. The survival rate of triploids was lower than that of diploids following exposure to prolonged thermal stress. Consistent with this finding, the number of enriched KEGG pathways and GO terms, especially stress response-related GO terms, was lower in diploids in the Afd group than in triploids in the Aft group. Only five enriched KEGG pathways were detected in the Afd group, and none of these were stress response-related pathways; by contrast, in the Aft group, 15 enriched KEGG pathways related to the immune response, apoptosis, and cell cycle were enriched in the Aft group. The number of DEGs involved in stress responses, cell process, and metabolism-related GO terms was lower in the Afd group than in the Aft group during prolonged heat stress. These findings indicate the gradual adaptation of diploids to heat stress, followed by recovery to a physiologically stable state; the adaptation of triploids to heat stress and the recovery process took much longer by comparison.

A greater number of *HSP*s and *IAP*s were differentially expressed in diploid oysters than in triploid oysters following acute heat stress. The expression of most *HSPs* and *IAPs* was higher in diploid oysters than in triploid oysters under acute heat stress. *HSP*s are a diverse group of heat-induced molecular chaperones that are conserved in eukaryotes and involved in the response to high-temperature stress. *HSP*70s are thought to be expanding in *C. gigas* (Zhang et al., 2012a), and the induced expression of *HSP*70 genes can mediate the adaptation of *C. gigas* to high-temperature stress (Ding et al., 2020). Recent studies have also demonstrated that increases in the concentration of molecular chaperones can mediate the repair of heat-induced cellular damage (Dong et al., 2010; Meistertzheim et al., 2007; Villamil et al., 2007). In our study, a total of 30 *HSP* genes were differentially expressed in at least one group. Among them, 27 of the 30 *HSP* genes were differentially expressed in the A12d group compared with 17 of the 30 *HSP* genes in the A12t group. The expression of 26 of the 30 *HSP* genes was higher in diploid oysters than in triploid oysters. Previous studies have shown that the high inducibility of HSPs in oysters allows them to cope with acute thermal stress (Clegg et al., 1998; Ivanina et al., 2009). The expression of HSP70 gene family members is significantly up-regulated in thermotolerant oysters but down-regulated in thermosusceptible oysters (Kim et al., 2017). The number of differentially expressed *BIRCs* was higher in diploid oysters than in triploid oysters following acute heat stress. *BIRC* genes belong to the IAP gene family and encode negative regulatory proteins that suppress caspases to prevent apoptotic cell death (Nie et al., 2020b; Wu et al., 2019). In bivalves, the expression of some IAPs is up-regulated in response to different types of stress (Song et al., 2021; Zhang et al., 2012a). In *C. gigas*, the anti-apoptosis system, especially the expansion of the IAP family, plays a key role in their ability to withstand various biotic and abiotic stresses (Zhang et al., 2012a). In our study, the expression of 8 of the 11 differentially expressed *BIRC* genes was higher in diploid oysters than in triploid oysters following acute heat stress. Overall, these findings indicate that the response of triploid oysters to heat stress might be weaker and slower compared with that of diploid oysters.

Consistent with our results, previous studies have indicated that triploidy might negatively affect survival. Duchemin et al. (2007) found that triploid *C. gigas* is less sensitive to environmental cues than diploid *C. gigas* because there is less variation in the total hemocyte count among seasons in the former than in the latter. Triploid *C. gigas* in Puget Sound, Washington, and Tomales Bay, California show consistently higher mortality rates than diploid oysters (Cheney et al., 2000). Wadsworth (2018) found that the mortality of triploid *C. virginica* was significantly higher than that of diploid oysters in the northern Gulf of Mexico. Our experiment was conducted in the winter, a period when diploid oysters do not incur the costs of reproductive activity. The low survival rate and the weaker response of triploids to heat stress might stem from an intrinsic depression in their performance during the winter months (De Decker et al., 2011).

The high meat weight of triploid oysters is maintained throughout the year, indicating that triploid oysters require more costs to sustain their homeostasis in response to environmental changes (De Decker et al., 2011). Although triploid oysters have larger cells, increases in genomic content (three sets of chromosomes) might alter the cell nucleus: cell surface area ratio, which might negatively affect the ability of triploids to withstand thermal stress. Higher temperatures have also been shown to negatively affect the survival of triploid fish to a greater degree than diploid fish (Hyndman et al., 2003). The adverse effects of high temperatures might be particularly strong on triploid oysters during the winter when the costs of the reproductive activity of diploids are low.

Previous studies have shown that the resistance of diploid oysters to heat stress can be transmitted to their triploid progeny (Hand et al., 2004). Additional research is needed to develop diploid oysters resistant to thermal stress through mass or family selection and evaluate the degree to which thermal tolerance can be passed on to triploid progeny.

5. Conclusions

We conducted a comparative transcriptomic analysis of the gills of diploid and triploid *C. gigas* following exposure to acute and prolonged heat stress. Analysis of the DEGs revealed differences in the response of diploid and triploid *C. gigas* to acute and prolonged thermal stress. The response of diploid *C. gigas* to acute heat stress, which involved reducing translational initiation and inducing apoptosis, was more effective and rapid compared with that of triploid oysters, and this likely explains the lower thermal tolerance and thermal adaptive capacity of triploid oysters compared with diploid oysters. *C. gigas* lines resistant to thermal stress need to be developed to produce triploids with high thermal resistance.

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.aquaculture.2022.738219.

Credit author statement

Yongguo Li: Investigation, Methodology, Resources, Data curation.
Kunyin Jiang: Performed the experiments, analyzed the data, authored or revised the paper.Formal analysis, Writing - original draft, Writing - review & editing.
Qi Li: Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (31972789), Earmarked Fund for Agriculture Seed Improvement Project of Shandong Province (2020LZGC016), China Agriculture Research System Project (CARS-49), Science and Technology Development Project of Weihai City (2018NS01), and Industrial Development Project of Qingdao City (20-3-4-16-nsh).

References

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Sherlock, G., 2000. Gene ontology: tool for the unification of biology. Gene Ontol. Consort. Nat. Genet. 25, 25–29. https://doi.org/10.1038/75556.

Barnes, P.J., 1997. Nuclear factor-κB. Int. J. Biochem. Cell Biol. 29, 867–870. https://doi.org/10.1016/S1357-2725(96)00159-8.

Caruso, R., Warner, N., Inohara, N., Nunez, G., 2014. NOD1 and NOD2: signaling, host defense, and inflammatory disease. Immunity. 41, 898–908. https://doi.org/10.1016/j.immuni.2014.12.010.

Cheney, D.P., Macdonald, B.F., Elston, R.A., 2000. Summer mortality of Pacific oysters, *Crassostrea gigas* (Thunberg): initial findings on multiple environmental stressors in Puget Sound, Washington, 1998. J. Shellfish Res. 19, 353–359.

Clegg, J.S., Uhlinger, K.R., Jackson, S.A., Cherr, G.N., Friedman, C.S., 1998. Induced thermotolerance and the heat shock protein-70 family in the Pacific oyster *Crassostrea gigas*. Mol. Mar. Biol. Biotechnol. 7, 21–30. https://doi.org/10.1007/s002489900076.

De Decker, S., Normand, J., Saulnier, D., Pernet, F., Castagnet, S., Boudry, P., 2011. Responses of diploid and triploid Pacific oysters *Crassostrea gigas* to Vibrio infection in relation to their reproductive status. J. Invertebr. Pathol. 106, 179–191. https://doi.org/10.1016/j.jip.2010.09.003.

Dégremont, L., Garcia, C., Frank-Lawale, A., Allen, S.K., 2012. Triploid oysters in the Chesapeake Bay: comparison of diploid and triploid *Crassostrea virginica*. J. Shellfish Res. 31, 21–31. https://doi.org/10.2983/035.031.0103.

Delisle, L., Petton, B., Burguin, J.F., Morga, B., Corporeau, C., Pernet, F., 2018. Temperature modulate disease susceptibility of the Pacific oyster *Crassostrea gigas* and virulence of the Ostreid herpesvirus type 1. Fish Shellfish Immunol. 80, 71–79. https://doi.org/10.1016/j.fsi.2018.05.056.

Ding, F.F., Li, A., Cong, R.H., Wang, X.X., Wang, W., Que, H.Y., Li, L., 2020. The phenotypic and the genetic response to the extreme high temperature provides new insight into thermal tolerance for the Pacific oyster *Crassostrea gigas*. Front. Mar. Sci. 7, 399. https://doi.org/10.3389/fmars.2020.00399.

Dong, Y.W., Ji, T.T., Meng, X.L., Dong, S.L., Sun, W.M., 2010. Difference in thermotolerance between green and red color variants of the Japanese sea cucumber, *Apostichopus japonicus* Selenka: Hsp70 and heat-hardening effect. Biol. Bull. 218, 87–94. https://doi.org/10.1086/BBLv218n1p87.

Du, Y., Zhang, L., Xu, F., Huang, B., Zhang, G., Li, L., 2013. Validation of housekeeping genes as internal controls for studying gene expression during Pacific oyster (*Crassostrea gigas*) development by quantitative real-time PCR. Fish Shellfish Immunol. 34, 939–945. https://doi.org/10.1016/j.fsi.2012.12.007.

Duchemin, M.B., Fournier, M., Auffret, M., 2007. Seasonal variations of immune parameters in diploid and triploid Pacific oysters, *Crassostrea gigas* (Thunberg). Aquaculture. 264, 73–81. https://doi.org/10.1016/j.aquaculture.2006.12.030.

Gagnaire, B., Frouin, H., Moreau, K., Thomas-Guyon, H., Renault, T., 2006. Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish Shellfish Immunol. 20, 536–547. https://doi.org/10.1016/j.fsi.2005.07.003.

Garnier-Gere, P.H., Naciri-Graven, Y., Bougrier, S., Magoulas, A., Heral, M., Kotoulas, G., Gerard, A., 2002. Influences of triploidy, parentage and genetic diversity on growth of the Pacific oyster *Crassostrea gigas* reared in contrasting natural environments. Mol. Ecol. 11, 1499–1514. https://doi.org/10.1046/j.1365-294x.2002.01531.x.

Gérard, A., Ledu, C., Phélipot, P., Naciri-Graven, Y., 1999. The induction of MI and MII triploids in the Pacific oyster *Crassostrea gigas* with 6-DMAP or CB. Aquaculture. 174, 229–242. https://doi.org/10.1016/S0044-8486(99)00032-0.

Guo, X., Allen, S.K., 1994. Viable tetraploid Pacific oyster (*Crassostrea gigas* Thunburg) produced by inhibiting polar body I in eggs of triploids. Mol. Mar. Biol. Biotechnol. 3, 42–50.

Guo, X., DeBrosse, G.A., Allen, S.K., 1996. All-triploid Pacific oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. Aquaculture. 142, 149–161. https://doi.org/10.1016/0044-8486(95)01243-5.

Hand, R.E., Nell, J.A., Maguire, G.B., 1998. Studies on triploid oysters in Australia. X. Growth and mortality of diploid and triploid Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley). J. Shellfish Res. 17, 1115–1127. https://doi.org/10.1016/0044-8486(94)90040-X.

Hand, R.E., Nell, J.A., Thompson, P.A., 2004. Studies on triploid oysters in Australia XIII. Performance of diploid and triploid Sydney rock oyster, *Saccostrea glomerata* (Gould, 1850), progeny from a third generation breeding line. Aquaculture. 233, 93–107. https://doi.org/10.1016/j.aquaculture.2003.09.017.

Haure, J., François, C., Dégremont, L., Ledu, C., Maurouard, É., Girardin, F., Benabdelmouna, A., 2021. Physiological comparisons of Pacific cupped oysters at different levels of ploidy and selection to OsHV-1 tolerance. Aquaculture. 544, 737111 https://doi.org/10.1016/j.aquaculture.2021.737111.

Haynes, C.M., Titus, E.A., Cooper, A.A., 2004. Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. Mol. Cell 15, 767–776. https://doi.org/10.1016/j.molcel.2004.08.025.

Hsu, H., Xiong, J., Goeddel, D.V., 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell. 81, 495–504. https://doi.org/10.1016/0092-8674(95)90070-5.

Huang, X.D., Liu, W.G., Guan, Y.Y., Shi, Y., Wang, Q., Zhao, M., He, M.X., 2012. Molecular cloning and characterization of class I NF-kappaB transcription factor from pearl oyster (*Pinctada fucata*). Fish Shellfish Immunol. 33, 659–666. https://doi.org/10.1016/j.fsi.2012.06.029.

Huang, J., Li, Y., Liu, Z., Kang, Y., Wang, J., 2018. Transcriptomic responses to heat stress in rainbow trout *Oncorhynchus mykiss* head kidney. Fish Shellfish Immunol. 82, 32–40. https://doi.org/10.1016/j.fsi.2018.08.002.

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernandez-Plaza, A., Forslund, S.K., Cook, H., Bork, P., 2019. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res. 47, D309–D314. https://doi.org/10.1093/nar/gky1085.

Hyndman, C.A., Kieffer, J.D., Benfey, T.J., 2003. Physiology and survival of triploid brook trout following exhaustive exercise in warm water. Aquaculture. 221, 629–643. https://doi.org/10.1016/S0044-8486(03)00119-4.

Ivanina, A.V., Taylor, C., Sokolova, I.M., 2009. Effects of elevated temperature and cadmium exposure on stress protein response in eastern oysters *Crassostrea virginica* (Gmelin). Aquat. Toxicol. 91, 245–254. https://doi.org/10.1016/j.aquatox.2008.11.016.

Kim, B.M., Kim, K., Choi, I.Y., Rhee, J.S., 2017. Transcriptome response of the Pacific oyster, *Crassostrea gigas* susceptible to thermal stress: a comparison with the response of tolerant oyster. Mol. Cell. Toxicol. 13, 105–113. https://doi.org/10.1007/s13273-017-0011-z.

Lang, R.P., Bayne, C.J., Camara, M.D., Cunningham, C., Jenny, M.J., Langdon, C.J., 2009. Transcriptome profiling of selectively bred Pacific oyster *Crassostrea gigas* families that differ in tolerance of heat shock. Mar. Biotechnol. (NY). 11, 650–668. https://doi.org/10.1007/s10126-009-9181-6.

Li, Y., Qin, J.G., Abbott, C.A., Li, X., Benkendorff, K., 2007. Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas*: an explanation for summer mortality in Pacific oysters. Am. J. Phys. Regul. Integr. Comp. Phys. 293, R2353–R2362. https://doi.org/10.1152/ajpregu.00463.2007.

Li, C., Zhao, W., Qin, C., Yu, G., Ma, Z., Guo, Y., Chen, J., 2021. Comparative transcriptome analysis reveals changes in gene expression in sea cucumber (*Holothuria leucospilota*) in response to acute temperature stress. Comp. Biochem. Physiol. Part D Genom. Proteom. 40, 100883 https://doi.org/10.1016/j.cbd.2021.100883.

Lim, H.J., Kim, B.M., Hwang, I.J., Lee, J.S., Choi, I.Y., Kim, Y.J., Rhee, J.S., 2016. Thermal stress induces a distinct transcriptome profile in the Pacific oyster *Crassostrea gigas*. Comp. Biochem. Physiol. Part D Genom. Proteom. 19, 62–70. https://doi.org/10.1016/j.cbd.2016.06.006.

Liu, Y., Li, L., Huang, B., Wang, W., Zhang, G., 2019. RNAi based transcriptome suggests genes potentially regulated by HSF1 in the Pacific oyster *Crassostrea gigas* under thermal stress. BMC Genomics 20, 639. https://doi.org/10.1186/s12864-019-6003-8.

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.

Matt, J.L., Guévélou, E., Small, J.M., Allen, S.K., 2020. A field test investigating the influence of brood stock origin and ploidy on the susceptibility of *Crassostrea virginica* to "triploid mortality" in the Chesapeake Bay. Aquaculture. 526, 735375 https://doi.org/10.1016/j.aquaculture.2020.735375.

Meistertzheim, A.L., Tanguy, A., Moraga, D., Thebault, M.T., 2007. Identification of differentially expressed genes of the Pacific oyster *Crassostrea gigas* exposed to prolonged thermal stress. FEBS J. 274, 6392–6402. https://doi.org/10.1111/j.1742-4658.2007.06156.x.

Nash, S., Johnstone, J., Rahman, M.S., 2019. Elevated temperature attenuates ovarian functions and induces apoptosis and oxidative stress in the American oyster, *Crassostrea virginica*: potential mechanisms and signaling pathways. Cell Stress Chaperones 24, 957–967. https://doi.org/10.1007/s12192-019-01023-w.

Nell, J.A., 2002. Farming triploid oysters. Aquaculture. 210, 69–88. https://doi.org/10.1016/s0044-8486(01)00861-4.

Nell, J.A., Cox, E., Smith, I.R., Maguire, G.B., 1994. Studies on triploid oysters in Australia. I. The farming potential of triploid Sydney rock oysters *Saccostrea*

commercialis (Iredale and Roughley). Aquaculture. 126, 243–255. https://doi.org/10.1016/0044-8486(94)90040-x.

Nelson, D.E., Ihekwaba, A., Elliott, M., Johnson, J.R., Gibney, C.A., Foreman, B.E., Spiller, D.G., 2004. Oscillations in NF-κB signaling control the dynamics of gene expression. Science. 306, 704–708. https://doi.org/10.1126/science.1099962.

Nie, H., Wang, H., Jiang, K., Yan, X., 2020a. Transcriptome analysis reveals differential immune related genes expression in *Ruditapes philippinarum* under hypoxia stress: potential HIF and NF-kappaB crosstalk in immune responses in clam. BMC Genomics 21, 318. https://doi.org/10.1186/s12864-020-6734-6.

Nie, H., Jiang, K., Li, N., Li, D., Yan, X., 2020b. Transcriptomic analysis of *Ruditapes philippinarum* under aerial exposure and reimmersion reveals genes involved in stress response and recovery capacity of the Manila clam. Aquaculture. 524, 735271 https://doi.org/10.1016/j.aquaculture.2020.735271.

Normand, J., Le Pennec, M., Boudry, P., 2008. Comparative histological study of gametogenesis in diploid and triploid Pacific oysters (*Crassostrea gigas*) reared in an estuarine farming site in France during the 2003 heatwave. Aquaculture. 282, 124–129. https://doi.org/10.1016/j.aquaculture.2008.06.026.

Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., Kanehisa, M., 1999. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 27, 29–34. https://doi.org/10.1093/nar/27.1.29.

Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., Keck, N., 2012. Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquac. Environ. Interact. 2, 215–237. https://doi.org/10.3354/aei00041.

Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M., Goeddel, D.V., 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell. 83, 1243–1252. https://doi.org/10.1016/0092-8674(95)90149-3.

Shiozaki, E.N., Chai, J., Rigotti, D.J., Riedl, S.J., Li, P., Srinivasula, S.M., Shi, Y., 2003. Mechanism of XIAP-mediated inhibition of caspase-9. Mol. Cell 11, 519–527. https://doi.org/10.1016/s1097-2765(03)00054-6.

Shpigel, M., Barber, B.J., Mann, R., 1992. Effects of elevated-temperature on growth, gametogenesis, physiology, and biochemical-composition in diploid and triploid Pacific oysters, *Crassostrea-gigas* Thunberg. J. Exp. Mar. Biol. Ecol. 161, 15–25. https://doi.org/10.1016/0022-0981(92)90186-E.

Sokolova, I.M., 2009. Apoptosis in molluscan immune defense. Isj-Invertebr. Surv. J. 6, 49–58.

Song, H., Guo, X., Sun, L., Wang, Q., Han, F., Wang, H., Zhang, T., 2021. The hard clam genome reveals massive expansion and diversification of inhibitors of apoptosis in Bivalvia. BMC Biol. 19, 15. https://doi.org/10.1186/s12915-020-00943-9.

Stanley, J.G., Allen, S.K., Hidu, H., 1981. Polyploidy induced in the American oyster, *Crassostrea virginica*, with cytochalasin B. Aquaculture. 23, 1–10. https://doi.org/10.1016/0044-8486(81)90002-8.

Vialova, O.Y., 2020. Comparative morphological analysis of diploid and triploid oysters, *Crassostrea gigas*, farmed in the Black Sea. Turk. J. Vet. Anim. Sci. 44, 740–746. https://doi.org/10.3906/vet-1907-50.

Villamil, L., Gomez-Leon, J., Gomez-Chiarri, M., 2007. Role of nitric oxide in the defenses of *Crassostrea virginica* to experimental infection with the protozoan parasite Perkinsus marinus. Dev. Comp. Immunol. 31, 968–977. https://doi.org/10.1016/j.dci.2007.01.006.

Wadsworth, P.C., 2018. Comparing Triploid and Diploid Growth and Mortality in Farmed Oysters, *Crassostrea virginica*, in the Northern Gulf of Mexico. Master thesis. Auburn University, Auburn, p. 2018.

Wadsworth, P., Wilson, A.E., Walton, W.C., 2019a. A meta-analysis of growth rate in diploid and triploid oysters. Aquaculture. 499, 9–16. https://doi.org/10.1016/j.aquaculture.2018.09.018.

Wadsworth, P., Casas, S., La Peyre, J., Walton, W., 2019b. Elevated mortalities of triploid eastern oysters cultured off-bottom in northern Gulf of Mexico. Aquaculture. 505, 363–373. https://doi.org/10.1016/j.aquaculture.2019.02.068.

Wang, Z.P., Guo, X.M., Allen, S.K., Wang, R.C., 2002. Heterozygosity and body size in triploid Pacific oysters, *Crassostrea gigas* Thunberg, produced from meiosis II inhibition and tetraploids. Aquaculture. 204, 337–348. https://doi.org/10.1016/S0044-8486(01)00845-6.

Wek, R.C., Jiang, H.Y., Anthony, T.G., 2006. Coping with stress: eIF2 kinases and translational control. Biochem. Soc. Trans. 34, 7–11. https://doi.org/10.1042/BST20060007.

Wu, D., Wang, C., Zhang, W., Peng, K., Sheng, J., Wang, J., Hong, Y., 2019. Molecular characterization of an inhibitor of apoptosis protein (IAPs) in freshwater pearl mussel, *Hyriopsis schlegelii*. Bioengineered. 10, 365–373. https://doi.org/10.1080/21655979.2019.1653738.

Xiao, Q., Huang, Z., Shen, Y., Gan, Y., Wang, Y., Gong, S., Ke, C., 2021. Transcriptome analysis reveals the molecular mechanisms of heterosis on thermal resistance in hybrid abalone. BMC Genomics 22, 650. https://doi.org/10.1186/s12864-021-07954-y.

Xing, D., Li, Q., Kong, L., Yu, H., 2018. Heritability estimate for mantle edge pigmentation and correlation with shell pigmentation in the white-shell strain of Pacific oyster, *Crassostrea gigas*. Aquaculture. 482, 73–77. https://doi.org/10.1016/j.aquaculture.2017.09.026.

Yang, C., Gao, Q., Liu, C., Wang, L., Zhou, Z., Gong, C., Song, L., 2017. The transcriptional response of the Pacific oyster *Crassostrea gigas* against acute heat stress. Fish Shellfish Immunol. 68, 132–143. https://doi.org/10.1016/j.fsi.2017.07.016.

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Wang, J., 2012a. The oyster genome reveals stress adaptation and complexity of shell formation. Nature. 490, 49–54. https://doi.org/10.1038/nature11413.

Zhang, L., Hou, R., Su, H., Hu, X., Wang, S., Bao, Z., 2012b. Network analysis of oyster transcriptome revealed a cascade of cellular responses during recovery after heat shock. PLoS One 7, e35484. https://doi.org/10.1371/journal.pone.0035484.

Zhang, H., Wang, H., Chen, H., Wang, M., Zhou, Z., Qiu, L., Song, L., 2019. The transcriptional response of the Pacific oyster *Crassostrea gigas* under simultaneous bacterial and heat stresses. Dev. Comp. Immunol. 94, 1–10. https://doi.org/10.1016/j.dci.2019.01.006.

Zhu, Q., Zhang, L., Li, L., Que, H., Zhang, G., 2016. Expression characterization of stress genes under high and low temperature stresses in the Pacific oyster, *Crassostrea gigas*. Mar. Biotechnol. (NY). 18, 176–188. https://doi.org/10.1007/s10126-015-9678-0.