**Transcriptomic analysis of oyster *Crassostrea gigas* larvae
illustrates the response patterns regulated by
catecholaminergic system upon acute heat and bacterial
stress**

Zhaoqun Liu ^{a,b, c}, Lingling Wang^{d*}, Zhi Zhou ^e, Yu Liu ^d, Miren Dong ^d, Weilin Wang
^{a,b, c}, Xiaorui Song ^d, Mengqiang Wang ^a, Qiang Gao ^{a, d}, Linsheng Song ^{b, d}

Abstract

Bacterial infection and heat stress, as two major environmental threats of marine molluscs, could affect larval development and dramatically promote mortality of oysters. In the present study, next-generation sequencing, together with determinations of mRNA expression and measurements of enzyme activities, were employed to understand the response patterns of oyster larvae under acute heat and bacterial stress. After RNA-seq, a total of 9,472 differentially expressed genes including 4,895 significantly up-regulated ones and 4,577 significantly down-regulated ones were obtained from 12 transcriptome libraries. GO overrepresentation analysis of the up-regulated genes revealed that the neuroendocrine immunomodulation pathway was activated after acute heat and bacterial stimulation, in which the catecholaminergic regulation played an important role. GO overrepresentation analysis of the down-regulated genes suggested that the immune capacity of *Crassostrea gigas* larvae was suppressed under stress, which was further validated since superoxide dismutase (SOD) and phenoloxidase (PO) activities in the total protein extract of larvae decreased dramatically after stress. Moreover, the shell formation of trochophore was inhibited and severe mortality was caused after acute heat and bacterial stress. These results collectively indicated that acute heat and bacterial stress could significantly inhibit larval development and suppress immune response of oyster *C. gigas* larvae. And the neuroendocrine immunomodulation, especially the catecholaminergic regulation, played an indispensable role in the stress response of molluscan larvae.

Key words: *Crassostrea gigas* larvae; Transcriptomic analysis; Acute heat and bacterial stress; Catecholaminergic system; Immunomodulation

1. Introduction

As marine molluscs living in the intertidal and estuarine zones, oysters have to deal with tremendous harsh and extreme environments (Zhang et al., 2012). Abiotic factors such as waves, temperature, water acidity and density, could pose extreme stress on intertidal animals (Yang et al., 2014). In the past decades, severe mortalities of both juvenile and adult of pacific oysters *Crassostrea gigas* were reported during warm months throughout the year, named as summer mortality syndrome (Cotter et al., 2010; Samain et al., 2007). Evidences have proved that summer mortality is caused by combined effects of several factors, such as high water temperature, pathogen invasion, physiological challenge during maturation, etc (Bouchet et al., 2007; Dziejman et al., 2002; Le Roux et al., 2004; Soletchnik, 1997). For example, Lipovsky *et al* (Lipovsky, 1972) demonstrated that elevating temperature or high levels of pathogens dramatically promoted oyster mortality. In addition, summer mortality syndrome also happened during the development of some cultured marine bivalves, such as oyster *Crassostrea virginica*, on the eastern coast of America where the disease was named as JOD (Juvenile Oyster Disease) (Lee, 1996). The JOD affected only juvenile oysters and happened when the temperature of seawater went over 20°C, killing nearly 90% of the affected oysters within a few days (Ford and Borrero, 2001). Therefore, the summer mortality syndrome has become a huge threat for the bivalve aquaculture industry and needs to be well studied.

Based on our current knowledge, pathogen affection and heat stress are the two most important factors causing summer mortality (Lipovsky, 1972). In the past, most

of the research about heat stress and bacterial challenge focused only on adult molluscs. Recently, the influences of acute heat and bacterial threat on molluscan larvae have attracted a growing number of studies and some insights have been gained. For example, heat stress induces oxidative reaction (Abele et al., 2002; Vergote et al., 2005), and increases susceptibility to pathogen infections during reproduction (Li et al., 2007; Li et al., 2009). Heat stress can also affect directly on the immune system of molluscan larvae by inhibiting haemocyte phagocytic rate and killing capacity (Chen et al., 2007; Hegaret et al., 2003; Hegaret et al., 2004). Besides, when the temperature elevates, larvae need to up-regulate their metabolic rate to produce enough energy for survival, during which the organism might transit its aerobic respiration to anaerobic respiration due to insufficient oxygen supply, resulting in diminishment of thermal tolerance and severe mortality (Lannig et al., 2006; Li et al., 2007; Portner, 2002). Moreover, bacterial infection can also have serious consequences on oysters' survival, especially during the larvae stages (Genard et al., 2013). For instance, severe juvenile mortality was induced when they were treated with a *Vibrio* strain at the concentrations from 10^4 to 10^8 CFU individual⁻¹ (Lacoste et al., 2001a); while another *Vibrio* strain, named TNEMF6, was also demonstrated to be responsible for the unexplained summer mortalities of juvenile oyster *C. gigas* living in Bay of Morlaix (Le Roux et al., 2002). Although some progresses have been made in recent years, our understanding about the influences of acute heat stress and bacterial challenge on molluscan larvae is still far from comprehensive and further study on the underlying mechanism is urgently needed.

The resistances of oyster larvae to both heat stress and bacterial challenge can be seen as a kind of stress responses, which is a set of coordinated physiological

reactions enhancing host's capability for homeostasis maintenance (Ottaviani and Franceschi, 1996). Hormones and neurotransmitters, key molecules of the NEI (neuroendocrine-immune) regulatory network, are crucial for the regulation of responses to external and internal adverse stress (Chrousos, 2009). Among the first neurotransmitters responsive to stressors, catecholamines (CAs), also known as “stress hormones”, are most crucial in stimulating actions in response to a perceived threat (Ottaviani, 2011; Ottaviani and Franceschi, 1996). CAs were discovered at the beginning of the 20th century and consist of dopamine (DA), norepinephrine (NE) and epinephrine (E) (Kvetnansky et al., 2009; Sabban and Kvetnansky, 2001). In the past decades, CAs, together with their receptors and metabolic enzymes, have been identified in molluscs including bivalves, gastropods, etc (Liu et al., 2016a; Zhou et al., 2013; Zhou et al., 2011a; Zhou et al., 2012; Zhou et al., 2011b; Zhou et al., 2011c; Zhou et al., 2011d; Zhou et al., 2011f). Previous studies reported that stress could induce the synthesis and release of CAs in molluscs. For example, NE and E concentrations increased and reached the highest level at 72 h after salinity and heat stress stresses in scallop *Chlamys farreri* (Chen et al., 2008). The released CAs responsive to acute stress could interact with the immune system by influencing a vast array of immune indicators including haemocyte apoptosis index and phagocytic rate and the activities of enzymes associated with humoral immunity (Aladaileh et al., 2008; Zakharova, 2010). For example, after exposure to environmental stress, the NE and E contents in scallop serum elevated significantly (Chen et al., 2008), and both the superoxide dismutase (SOD) activity and heat shock protein (HSP) mRNA expression level were up-regulated (Wang et al., 2007). However, studies of the immunomodulation of catecholaminergic nervous system under stress conditions

during the ontogenesis of oyster are still limited.

Organisms living in the natural environment are often exposed to multiple dynamically-changing factors, and the quantification of such factors is usually carried out in laboratory conditions, in which just one parameter is varied (Dong et al., 2014). However, the combined effect of these factors may have a much stronger impacts. In recent years, the next-generation sequencing technology has been employed as a powerful tool for the obtaining of large amounts of data (Li et al., 2013; Mohd-Shamsudin et al., 2013). The new technology promises to explore the regulation mechanism of complex life process at the transcriptional level (Wang et al., 2014). In the current research, transcriptomes of *C. gigas* larvae after acute heat and bacterial stress were sequenced by Ion Torrent Proton sequencing platform, hoping to obtain high-coverage transcriptome data for the functional analysis. The primary goals of this study are to (1) identify the morphological changes and the transcriptional expression pattern of trochophore and D-hinged larvae of oyster *C. gigas*, (2) survey the differentially expressed genes of oyster larvae under acute heat and bacterial stresses, and (3) investigate the potential stress resistance patterns of oyster larvae based on both transcriptome analysis and biological validation.

2. Materials and methods

2.1. Oyster larvae and stress

Oysters *C. gigas* with the shell length of about 15cm, which was the normal size of 3-year old oysters, were sampled from the coastal area near Qingdao, China, and cultured in laboratory for over 10 days before subsequent experiments. Eggs and sperms were separately scratched from gonads of male and female oysters with small

toothpicks. The fertilization was performed by mixing eggs and sperms together, and then the successfully fertilized embryos were obtained and cultured at 20°C.

Trochophores collected at 18 h post-fertilization were equally divided into four groups, taking three parallels for each group. Larvae without any treatment were designated as the control group (Ctrl. group), while those in the heat stress group (Heat group) were bathed in 28°C seawater, which was near the maximum sea surface temperature during summer in near-shore regions of Shandong province, China (Zhang et al., 2014). In the bacterial stress group (Bacteria group), larvae were transferred to the tanks containing 10^8 CFU mL⁻¹ of live bacteria *Vibrio splendidus* since it was reported that *V. splendidus* was responsible for the juvenile mortality of oyster *C. gigas* (Lacoste et al., 2001b). Moreover, trochophore in the Bacteria+Heat group received synergistic stress of heat and bacteria. At 6 h after treatment, trochophore in all the four groups developed into D-hinged larvae, and they were identified microscopically and collected for subsequent experiments.

2.2. Mortality observation, sample collection and scanning electron microscopy

The mortality of oyster larvae was distinguished by microscopy and calculated in samples of 100-200 individuals. The collection of larvae was performed according to Liu *et al* (Liu et al., 2015b). Trochophore and D-hinged larvae were sampled at 18 and 24 h post-fertilization, respectively, and 1 mL of Trizol (10296010, Invitrogen, CA) was added to each tube containing larvae for RNA isolation. In addition, same amount of larvae were harvested and frozen immediately for total protein extraction. For each assay, six parallels were employed.

As for the samples in the scanning electron microscopy analysis, oyster larvae were

pre-fixed in 4% paraformaldehyde (PFA) before the addition of 5% glutaraldehyde and incubated for 4 hour at 24°C. Next, rinse the samples twice with 0.1 M cacodylate and dehydrate them with graded acetone. Then, coat the larvae with gold and a KYKY-2800B scanning electron microscope was employed for the sample observation.

2.3. The RNA-sequencing of transcriptome libraries

After the total RNA was isolated from larvae in each group, it was firstly quantified and qualified by Nanodrop 2000 (Thermo Scientific). Ribo-minus RNA was fragmented by RNase and then purified, while the rRNA was depleted. Ion Total RNA-Seq kit (Thermofisher) was used to convert mRNA into cDNA, and OneTouch Ion™ Template Kit (Thermofisher) was used to prepare template for RNA sequencing. Besides, cDNAs from each library were then sequenced using Ion Torrent Proton P1 chip according to the description of Chen et al (Chen et al., 2015). Three parallels were employed for each group. The data of sequencing is now available at NCBI in the SRA (Short Read Archive) database under the accession number of SRP085781.

2.4. Analysis of the differentially expressed transcripts

As for the bioinformatical analysis, LifeScope™ software was employed with little modification to align the reads, and “TopHat-Bowtie” was used for sequence mapping. The obtained transcripts were assembled by cufflinks (Trapnell et al., 2012) to acquire to differentially expressed genes between the control group and three challenge groups. Finally, the differential expression analysis was presented

graphically using the CummeRbund package included in the R/Bioconductor project.

2.5. Gene Ontology (GO) overrepresented analysis

Rstudio and Cytoscape ClueGo softwares were adopted to perform GO overrepresentation analysis of the identified significantly up- and down-expressed genes. The hypergeometric test with FDR value of 0.01 was used to do the GO enrichment, and the differentially expressed genes were selected as test set, while all identified genes were taken as the reference set. The significantly overrepresented GO terms were calculated from test set, and displayed as a network using BiNGO plug-in to Cytoscape (<http://cytoscape.org/>) (Maere et al., 2005).

2.6. The extraction of total protein

First of all, melt the sample and add 300 μL of PBS to each tube, followed by tissue homogenization to completely pulverize the larvae. Next, the shred larvae were centrifuged at 6,000 g at low temperature for 20 min to obtain the supernatant, which was regarded as total protein extract. Finally, quantify the protein concentration with a BCA Protein Quantification Kit (Beyotime) and adjusted the working concentration to 700 mg mL^{-1} .

2.7. The anti-bacterial assay and determination of PO and SOD activities

In the anti-bacterial assay, 50 μL of protein extract was mixed with 10 μL of live *Vibrio anguillarum* and added to a 96-well plate, incubating at 18°C for 3 h. Then add 200 μL of 2216E medium (HBO132-1, Hopebio, China) and incubate at 28°C. The OD value was detected for 24 h at intervals of 30 min and measured at 600 nm (Zhou

et al., 2013). The activity of SOD was tested by the kit (Jiancheng, A001-1, Nanjing), while phenoloxidase (PO) activity was measured according to the oxidation reaction of L-dopa (Asokan et al., 1997). The value of each parallel in Ctrl group was firstly normalized to 1.0 by being divided by the values of each parallel in the experimental groups, and the relative enzyme activities of SOD and PO are shown in Fig. 4.

2.8. Statistical analysis

Data statistical analyses were performed on SPSS using one-way ANOVA to test the effects of acute heat and bacterial stress on the development of oyster larvae. Values are given as Means $\pm$ Standard Error (SE), and a significance level of $p < 0.05$ was used for all tests.

3. Results

3.1. Morphological characteristics of oyster larvae after stress treatment

The performance of oyster larvae in the control and stress treatment groups was observed with scanning electron microscopy. Larvae in the control group successfully formed a uniform calcified shell, indicating the change of developmental stages (Fig. 1A). The larvae calcified shells in the Bacteria (Fig. 1B) and Heat (Fig. 1C) groups were less intact comparing with those in control group, while larvae in the Heat group seemed worse metamorphosized than those in the Bacteria group. In addition, larvae in the Bacteria+Heat group were hardly metamorphosized and the calcified shell was not observed at all (Fig. 1D).

The mortality rate in bacteria challenge group was 15.6% compared with 3.7% in the control group. The mortality rate in the heat stress group was 24.3%, which was

dramatically higher ($p < 0.05$) in comparison with that in control group. The highest mortality rate of 43.7% occurred in the synergistic Bacteria+Heat group, which was about 11.8-fold of the mortality rate in control group ($p < 0.05$).

3.2. The output of RNA sequencing

Twelve transcriptome libraries were constructed with RiboMinus RNA from oyster larvae in four groups, and three parallel libraries were included in each group. These libraries were sequenced using Ion Torrent Proton sequencing platform, and a total of 241,339,838 single end reads were obtained. The read numbers in each library were shown in Table 1.

After removing the non-coding RNA, the remaining clean reads in each library were mapped to oyster genome, and the successfully mapping rates ranged from 39.0% to 56.1% (Table 1). The uniquely mapping rates from 12 libraries fluctuated within the relatively small range (from 65.8% to 70.0%, Table 1).

3.3. Identification of the differentially expressed genes

The overall expression level of genes was estimated using the CummeRbund package, and the constant coefficients of variation of gene expression levels in four group samples were relatively small (Fig. 2A). The differences in gene expression level were determined with the cuffdiff module after acute heat and bacterial stress. In total, 9,472 differentially expressed genes were obtained, and the numbers of them were shown in Fig. 2B. There were two differentially expressed gene lists, including 4,895 significantly up-regulated and 4,577 down-regulated genes, respectively.

3.4. GO overrepresentation analysis of the significantly up-regulated genes

There were 534, 4,205 and 3,789 significantly up-regulated genes identified in the Bacteria, Heat and Bacteria+Heat groups compared with the Ctrl group, respectively. By comparing the gene lists of Bacteria and Ctrl group, a total of eight major GO terms relevant with stress adaptation were identified (Fig. S1, Table 2). The distributions of enriched GO terms between the gene lists of Heat and Ctrl groups were shown in Fig. S2. Meanwhile, there were seventeen major enriched GO terms between the gene lists of Bacteria+Heat and Ctrl groups (Fig. S3). The GO term overrepresentation analysis of those genes was completed at multiple GO levels (Fig. S7-S9).

3.5. GO overrepresentation analysis of the significantly down-regulated genes

About 280, 3,952 and 3,337 significantly down-regulated genes were identified in the Bacteria, Heat and Bacteria+Heat groups compared with the Ctrl group, respectively. Between the gene lists of Bacteria and Ctrl group, a total of three major GO terms related to responses to stimuli were revealed (Table 3, Fig. S4). There were nine major enriched GO terms in the gene lists between Heat and Ctrl groups (Fig. S5). Moreover, a total of six major GO terms were obtained from enriched gene lists of Bacteria+Heat and Ctrl groups (Fig. S6). The GO term overrepresentation analysis of those genes was completed at multiple GO levels (Fig. S10-S12).

3.6. The differentially expressed genes related to catecholaminergic nervous pathway

For the significantly up-regulated genes, two overrepresented GO term groups were related to catecholaminergic neuroendocrine regulation (Table 2 and Fig. 3), including

dopamine beta-monooxygenase activity (GO:0004500, FDR < 1.9E-3) and monooxygenase activity (GO:0004497, FDR < 12.0E-3). In particular, 3, 8 and 7 genes characterized by the overrepresented GO term of dopamine beta-monooxygenase activity were responsive to bacteria, heat and bacteria+heat treatments, respectively, while 8, 44 and 41 genes characterized by the overrepresented GO term of monooxygenase activity were in response to bacteria, heat and bacteria+heat treatments, respectively.

3.7. The SOD, PO and anti-bacterial activities of oyster larvae after acute heat and bacterial stress

To explore the influences of acute heat and bacterial challenge on the humoral innate immunity of oyster, the activities of SOD and PO were determined using commercially purchased kits. The relative SOD activities from Bacteria, Heat and Bacteria+Heat groups were significantly decreased by about 0.3-fold (Fig. 4A, $p < 0.05$), and the lowest activity was detected in Heat group. Relative PO activities were also down-regulated remarkably by about 0.4-fold at 6 h after heat treatment, while its activity decreased by about 0.3-fold post synergistic treatments of bacteria and heat (Fig. 4B, $p < 0.05$). No obvious alternation of PO activity was determined in Bacteria group ($p > 0.05$). Meanwhile, the anti-bacterial activities remained at basal level in all challenged groups (Fig. 5, $p > 0.05$).

4. Discussion

Accumulating evidences demonstrate that marine molluscs have evolved an ancient and effective adaptation mechanism to the changing environment. However, most of

the studies so far mainly focus on adult individuals, while the stress response processes in juveniles drew less attention. The developmental stages from trochophore to D-hinged larvae are critical for oysters and other bivalves since a calcified shell will be formed and the immune system becomes mature during this stage (Liu et al., 2015b; Mukherjee et al., 2013; Yue et al., 2013a). In this period, any slight disturbance on the metamorphosis can result in deathful consequences. In the present study, the expression of genes in the developmental larvae after acute bacterial and heat stress was investigated to illustrate the stress response patterns of marine molluscs during ontogenesis.

The metamorphosis of larvae is one of the most significant processes in molluscan life, during which many changes in morphology, physiology and habitat occur (Videla et al., 1998). The trochophore and D-hinged larvae stages are considered as the most critical stages for molluscs since tissue differentiation and organ formation are reported to happen and the innate immune system becomes mature during these stages (Lvoll et al., 2006; Tirape et al., 2007; Yue et al., 2013b). Thus, larvae at these two stages are vulnerable to a series of environmental stresses, such as temperature, pathogen infection, pollutants, ocean acidification, etc (Barros et al., 2013). In the current research, the metamorphosis of *C. gigas* larvae after acute heat and bacterial stress was observed by scanning electron microscopy. Both acute bacterial stress and heat stress could inhibit larvae metamorphosis by delaying the formation of calcified shell, even though most of the trochophore managed to metamorphosize into D-hinged larvae. A lot of trochophore stopped metamorphosing after the synergistic stimulation of bacteria and heat, while the rest showed extremely severe malformation during development (Fig. 1A). Moreover, the mortality rate of larvae significantly

increased to 15.6% and 24.3% after acute heat and bacterial treatments, respectively, comparing with only 3.7% in control group, while the mortality rate in Bacteria+Heat group dramatically increased to 43.7%. These results suggested that the acute heat and bacterial stress, especially synergistic stress, resulted in severe influences on the formation of calcified shell and significantly increased the mortality rate during the metamorphosis of molluscan larvae.

In the present study, 12 transcriptome libraries of *C. gigas* larvae were constructed and sequenced using Ion Torrent Proton sequencing platform to investigate the potential response pattern of molluscan larvae against acute heat and bacterial stress. Such high throughput RNA-Seq method intrigued us to identify a large number of differentially expressed genes under experimental stress. The overall statistical analysis revealed the relative dispersion and parallelism between three replicates in each group, suggesting that the transcriptome comparison was of good statistical confidence. The gene expression abundance analysis demonstrated that the transcriptome of oyster *C. gigas* larvae would be remodeled under stress, which might offer some clues for the delay of metamorphosis and severe mortality.

To further explore the response pattern of oyster larvae against acute heat and bacterial stress, the differentially expressed genes were sorted and the GO overrepresentation analysis was employed. Several components of the catecholaminergic nervous system were found in the up-regulated genes. For example, there were enriched GO terms of both monooxygenase activity and dopamine beta-monooxygenase activity in all the Bacteria, Heat and Bacterial+Heat groups. Previous reports have revealed that neuroendocrine regulation is crucial for the responses to external and internal adverse stress in molluscs (Liu et al., 2016a; Liu

et al., 2016b; Liu et al., 2015a; Liu et al., 2015b). And CAs are one of the very first appeared neurotransmitters during the ontogenesis of mollusc larvae, and act at the earliest developmental stages as signals influencing cell proliferation, differentiation, and neuritogenesis in different molluscs (Ottaviani, 2011; Ottaviani and Franceschi, 1996). For example, GO:0004500 and GO:0004497 IDs, related to monooxygenase activity and dopamine beta-monooxygenase activity, respectively, are among the overrepresented GO IDs in the upregulated genes lists outcoming from each challenged group with respect to the control one. According to previous study, the mRNA expression of monooxygenase was up-regulated at 12 h after bacterial challenge in scallop *C. farreri* (Zhou et al., 2011a). Meanwhile, the catecholaminergic neuroendocrine system in molluscs can be activated immediately and a higher level of CA is detected during acute stress. The contents of NE and DA in blood increased by about 2~2.5-fold in octopus *E. cirrhosa* after 5 min of air exposure (Chang et al., 2015), while NE and E concentrations increased and peaked at 72 h after acute temperature and salinity stresses in scallop *C. farreri* (Chen et al., 2008). Therefore, the results of transcriptomic analyses verified that catecholaminergic system could be activated under environmental threats. The CAs contents in oyster larvae might increase after acute heat and bacterial stress, resulting in the up-regulation of monooxygenase expression and the degradation of CAs.

Previous studies have demonstrated that CAs are released by a wide range of molluscs in response to acute stress, and widely disseminated to mediate various stress responses (Aladaileh et al., 2008; Lacoste et al., 2001a; Lacoste et al., 2001c). In the present study, several immune-related processes including neuropeptide Y receptor activity, steroid hormone mediated signaling pathway, N-acetyltransferase

activity, and neurotransmitter receptor activity were identified in the lists of down-regulated genes according to the GO enrichment analyses. In particular, heat and bacteria might affect the development of the immune system in oyster larvae via separate way. As shown in Fig. S7-S12, heat stress mainly suppressed the processes of steroid hormone receptor activity and steroid hormone mediated signaling pathway, while bacterial stress generally inhibited the processes of neuropeptide Y receptor activity, N-acetyltransferase activity and steroid hormone mediated signaling pathway. As mentioned before, the tissue differentiation and organ formation of oyster were reported to happen from gastrula larvae (Lvoll et al., 2006; Yue et al., 2013b), and haemocytes, the most important immune cells in oyster, were also firstly appeared at the gastrula-trochophore stages (Huan et al., 2012). Results of the transcriptomic analyses indicated that heat and bacterial stress could severely harm oyster larvae by disrupting the development and maturation of immune system through different ways, which might be the key reason causing severe larval mortality and malformation.

Moreover, functional validation further demonstrated that CAs released from the activated catecholaminergic system under stress could regulate the immune response of oyster larvae. We found that the relative SOD activities in Bacteria, Heat and Bacteria+Heat groups were significantly decreased, and the lowest activity was detected in Heat group. SOD is an important stress-response effector in bivalves, which is responsible for scavenging superoxide anions (Zhou et al., 2011e). In current results, challenged larvae had lower SOD activities, indicating the suppression of antioxidant defenses and impair on the innate immunity functions. Meanwhile, PO converts phenols to quinone and initiates melanogenic pathway during early phase of the immune process. The derived quinone compounds might be another source of

cytotoxic molecules (Cammarata et al., 1997). Similar to SOD, the PO activities also significantly declined after heat and bacteria+heat stress, further ascertaining that the immune response level was down-regulated under environmental stress. Finally, the anti-bacterial activity was measured since it is an ensemble accomplished by the multiple functions of oxidative toxicity and large number of immune effectors in the immune response of molluscs (Song et al., 2015; Wang et al., 2013). As shown in Fig. 5, the anti-bacterial activities were at the same level in all groups, suggesting that stress of bacteria and heat stress might neither enhance nor weaken oyster larvae's susceptibility to bacterial infection, although the overall immune response level was down-regulated. However, this point need to be further verified in the future research. A possible explanation to these results might be high concentrations of bacteria in the aquatic water is not the predominant factor that causes large-scale of larvae mortality of molluscs, while physical stressors such as heat stress, or multiple environmental factors working together, might play a more important role.

In conclusion, results of transcriptomic analysis suggested that the immune capacity of *C. gigas* larvae was suppressed under acute heat and bacterial stress, while functional validation revealed that the SOD and PO activities in larvae also decreased dramatically after challenge. Also, the shell formation of trochophore was inhibited and severe mortality was caused after acute heat and bacterial stress. These results indicated that acute heat and bacterial stress could significantly inhibit larval development and suppress immune response of oyster *C. gigas* larvae. The combined effects of multiple environmental factors could impose much stronger influence on organisms than single factor does. And, the neuroendocrine immunomodulation, especially the catecholaminergic regulation, plays indispensable roles in stress

response of molluscan larvae.

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Figure legends

Fig. 1. Morphological feature and mortality rate of oyster *C. gigas* larvae under acute heat and bacterial stress. (A) Morphological feature of larvae in the Ctrl group. Trochophore developed into D-hinged larvae and the calcified shell was successfully formed; (B) Morphological feature of larvae in the Bacterial group. Metamorphosis was delayed and the calcified shell was not uniform; (C) Morphological feature of larvae in the Heat group. Metamorphosis was delayed and the calcified shell was not uniform; (D) Morphological feature of larvae in the Bacterial+Heat group. Metamorphosis was almost stopped and the calcified shell was not observed.

Fig. 2. The squared coefficient of the expression level of all genes in four groups (A), and grouping of significantly up- (B1) and down-regulated (B2) genes in response to acute heat and bacterial stress. FPKM: Fragments Per Kilobase of exon per Million fragments mapped; CV²: the coefficient of variation to the power of two; vs: *Vibrio splendidus*.

Fig. 3. The overrepresented GO terms correlated with catecholaminergic regulation in the up-regulated genes identified in *C. gigas* larvae under acute heat and bacterial stress.

Fig. 4. SOD (A) and PO (B) activities in the total protein extract of oyster larvae after acute heat and bacterial challenge.

Fig. 5. The kinetic measurement of bacterial growth in the presence of total larval protein extract.