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EDITED BY

Bin Xia,
Qingdao Agricultural University, China

REVIEWED BY

Maocang Yan,
Zhejiang Mariculture Research Institute,
China
Zhiguo Dong,
Jiangsu Ocean University, China

*CORRESPONDENCE

Xiumei Zhang
✉ zhangxiumei@zjou.edu.cn

[†]These authors have contributed equally to this work

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Physiological and transcriptomic analyses reveal critical immune responses to hypoxia and sulfide in the haemolymph of clam *Tegillarca granosa*

Zhengkuan Yang^{1†}, Yihang Wang^{1†}, Puyuan Jiang¹, Feiyu Xia¹, Ying Xu¹, Xiaofei Tian¹ and Xiumei Zhang^{1,2*}

¹Fishery College, Zhejiang Ocean University, Zhoushan, China, ²Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

Hypoxia and sulfide are inducing potential damage to aquatic organisms. However, the effects of hypoxia and sulfide on their immune systems and molecular mechanisms are not fully understood. In the present study, the clam *Tegillarca granosa* was exposed to hypoxia alone or in combination with sulfide (0.1, 0.5 mM) to investigate the physiological and transcriptomic responses in haemolymph. The *IBR* analysis revealed that moderate sulfide stimulated immune responses *via* increasing the total hemocyte counts, phagocytic activity, antibacterial activity, and antioxidant activity. The transcriptomic analysis revealed many critical signaling pathways (Toll and Imd, FoxO, NLR) and biological processes (antimicrobial/antibacterial peptide, interferon, interleukin, leukocyte, lymphocyte, mitophagy) involved in the immunostimulation. Our results would offer insights into the sulfide-tolerant molecular mechanisms in this species and provide a useful tool for assessing the integrated biological impacts of hypoxia and sulfide on shellfish.

KEYWORDS

hypoxia, sulfide, *Tegillarca granosa*, haemolymph, Immunomodulatory

1 Introduction

Since the mid to 20 th century, aquatic hypoxia caused by water eutrophication has expanded more and more quickly in worldwide ocean, especially in gulfs, lakes, estuaries, and aquaculture farming zones (Diaz, 2001; Bocaniov and Scavia, 2016; Saleh et al., 2021; Whitney and Vlahos, 2021). Recently, aquatic hypoxia has become a worldwide concern for environmental issues as it can reduce biodiversity and change community structures in aquatic ecosystem (Gobler and Baumann, 2016; Zhai et al., 2019). It also has direct impacts on the behavior, physiology, and immunity of aquatic animals (Kvamme et al., 2013;

Abdel-Tawwab et al., 2019; Zheng et al., 2022). Sulfide, which is produced under anaerobic conditions through decomposition of organic matter and reduction of sulfate, is commonly found in the bottom layer and sediment of aquatic environments (Cutter and Krahforst, 1988; Asaoka et al., 2018). According to previous studies, the content of sulfide could reach to high levels and retained for a long time under hypoxic condition, which means benthic fauna is easily co-exposed to both hypoxia and sulfide conditions (Jørgensen, 1990; Wang and Zhang, 2021). Most commercial marine bivalves are bred in the coastal areas where sulfide and hypoxia frequently happen (Bagarinao and Lantin-Olaguer, 1998; Chang et al., 2013; Kodama et al., 2018; Wang et al., 2021). For instance, the content of sulfide in the sediment of Gamo Lagoon could stay at 6.6 mmol/L during warmer months (Kanaya et al., 2016; Asaoka et al., 2018). Evidences have indicated that high content of sulfide (1 mmol/L) was likely to become lethal level to most aquatic fauna including benthic bivalves, nematodes and polychaete species (Affonso and Rantin, 2005; Hargrave et al., 2008). Due to limited mobility to escape, these species easily suffer from the stressors (Aelion and Warttinger, 2009; Asaoka et al., 2018; Losyuk et al., 2021). Hence, prolonged exposure of hypoxia and sulfide has become a great threat for coastal farming organisms (Christensen et al., 2003; Srithongouthai and Tada, 2017; Meng et al., 2019).

Many studies on marine invertebrates suggested that sulfide exposure could be related to immunosuppression. For instance, 48 h exposure to 0.4–4 mg/L sulfide decreased the total haemocyte count (THC) and phagocytic activity of the haemocytes in the shrimp *Macrobrachium nipponense*, and higher level of sulfide (1.2–4.4 mg/L) remarkably restrained the total antioxidant capacity (Guan et al., 2011). Concentrations of sulfide higher than 528 µg/L increased the susceptibility of kuruma shrimp *Marsupenaeus japonicus* against *Vibrio alginolyticus* infection by a depression in immune responses including THC, phenoloxidase activity, phagocytic activity and bacterial clearance efficiency (Cheng et al., 2007; Xu et al., 2014; Duan et al., 2018; Jiang et al., 2019; Hu et al., 2021). Similar effects were also detected in the shrimp *Litopenaeus vannamei* and crab *Charybdis japonica* (Cheng et al., 2007; Xu et al., 2014; Duan et al., 2018; Jiang et al., 2019; Hu et al., 2021). Although many researchers have treated sulfide as an adverse factor for aquatic animals, others found some macrobenthos make use of sulfide. Recently, increasing evidences confirm the essential roles of mitochondrial alternative respiration and sulfide oxidation chains in adaption to sulfide exposure in benthic invertebrates, including polychaetes, crustaceans and bivalves (Grieshaber and Völkel, 1998; Hildebrandt and Grieshaber, 2008a; Hildebrandt and Grieshaber, 2008b; Huang et al., 2013; Ren et al., 2015). Studies in the clam *Anadara broughtonii* revealed that the toxicity of sulfide could be mitigated by its own physiologic functions including energy-balancing in the mitochondrial alternative chains, ROS cleansing, and apoptosis regulating (Wang and Zhang, 2021). Even though it is still not certain how sulfide mediate the oxidative or immune stress in many of these sulfide-tolerant invertebrates, sulfide is widely accepted to be a signaling molecule that exhibits some potentially beneficial therapeutic effects at physiological concentrations, including attenuating oxidative

stresses (Kimura and Kimura, 2004; Kimura et al., 2010; Lan et al., 2011; Yang et al., 2011; Sun et al., 2012; Hu et al., 2018; Kimura et al., 2019). Most recently, the important roles of sulfide in inflammation and immunity-related processes have also been noticed. Researchers put forward new therapeutic approaches using H₂S donors in inflammation and immune response, regarding H₂S as not only a gasotransmitter, but also a key immunomodulatory factor (Yuan et al., 2017; Li et al., 2021). The effects of sulfide on marine invertebrate species have not been adequately investigated, and its impacts on the immunotoxicity of invertebrates are still unknown and need further investigation.

The blood clam *Tegillarca granosa*, a typical marine bivalve that is of commercial importance and widely distributed in the West Pacific coasts, inhabits flow-retarding mudflat or enclosing mud ponds (Shi et al., 2017; Zha et al., 2019; Zhai et al., 2019; Bao et al., 2021). The muddy feature of the habitat makes this species easy to suffer from hypoxia and sulfide exposure. According to the latest study, the sulfide concentration of the sediment porewater was normally 100–200 µmol/L in Chinese typical habitat of blood clams (Liu et al., 2022). While the concentration could be higher in hot weather. In other words, this species must find ways to live with hypoxia and sulfide. In bivalves, the non-specific immune system plays vital roles in resisting disease and keeping healthy. However, the immune responses can be greatly affected by the environmental factors through either ameliorating or exacerbating the disease (Mishra et al., 2015; Sui et al., 2016; Zanuzzo et al., 2020; Pozzi et al., 2022). Hence, it is meaningful to uncover how the immune responses to hypoxia and sulfide happen in this species. Like the other invertebrates that are lack of specific immune system, *T. granosa* depends on the haemocyte phagocytosis and various active factors released from haemolymph to mediate non-specific immune responses to virus, bacteria, and pollutants (Su et al., 2018). The latest study on the clams showed that high sulfide concentration (1 mM, 7 days) induced hemocyte toxicity and microbiota dysbiosis, as indicated by THC, cell viability, ROS levels, phagocytic activities, and microbial community structure (Liu et al., 2022). In the studies on other aquatic invertebrates, the humoral factors including lysozyme (LZM), peroxidase (POD), acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD), catalase (CAT) are commonly used indicators of the immunocompetence (Li et al., 2009; Xia and Wu, 2018; Bao et al., 2020). These antioxidant, phagocytosis and antibacterial defense systems coordinate the immune responses to biotic and abiotic factors (Jiang et al., 2017; Han et al., 2021b; Zhang et al., 2021). Evidences in some aquatic invertebrates also indicated that immune responses including phagocytosis of haemocytes could be regulated by several crosslinked molecular pathways (Huang et al., 2019; Nie et al., 2020). For instances, activation of mammalian NFκB signaling pathway not only enhance immune responses, but also counteract with haemocytes apoptosis signaling in haemocytes by activating B-cell lymphoma-2 (Bcl-2), which inhibits the activation of apoptosis executor, the caspases (Vallabhapurapu and Karin, 2009). The study on the clam *A. broughtonii* found that exposure to sulfide significantly enriched genes related to apoptosis, TNF and NFκB signaling pathways which could mediate the inflammation and immune defense, and two-sided effects of sulfide on

mitochondrial apoptotic cascade were also detected (Wang et al., 2019; Wang et al., 2021). These cases provide references for the studies of immune responses to hypoxia and sulfide in the clam *T. granosa*.

Therefore, to better understand the immunotoxic effects of sulfide and hypoxia on *T. granosa*, we evaluated the haemocyte immune function using the cellular and humoral indicators. We also investigated the underlying molecular mechanisms using transcriptomic analysis.

2 Materials and methods

2.1 Animal collection and acclimation conditions

Adult 2-year old clams (shell length of 31.13 ± 0.37 mm, mean \pm SE) were obtained from Xiangshan, China ($29^{\circ}24'$ N and $121^{\circ}25'$ E) in April 2021. The individuals were acclimated in circulating seawater systems, which were filled with filtered natural seawater (temperature 19.93 ± 0.22 °C, salinity 25 ± 0.1 ‰, dissolved oxygen 9.05 ± 0.07 mg/L, PH = 8.1). During the acclimation period, the clams were fed twice a day with diatom *Cylindrotheca fusiformis* at a rate of 5% of the tissue dry weight until one day before the experiment. The clams were not fed during the experiment, and three hundred individuals were randomly and evenly assigned to twelve experimental buckets (25 individuals in each experimental bucket). Each bucket was sealable and filled with five liters seawater that has been disinfected and filtered through 0.45 μ m filter membrane.

2.2 Experimental design

The clams were exposed to hypoxia alone or sulfide in combination for 96 h. According to the results of pre-experiment and studies on the clams, 0.1mM and 0.5mM was defined as the mild and moderate level of sulfide, respectively (Liu et al., 2022). Four experimental groups were designed in triplicate, including (1) control group (DO = 9 mg/L, C); (2) hypoxia group (DO \leq 0.5 mg/L, Q); (3) hypoxia and mild-sulfide group (DO \leq 0.5 mg/L, 0.1 mM sulfide, QD); (4) hypoxia and moderate-sulfide group (DO \leq 0.5 mg/L, 0.5 mM sulfide, QG). The hypoxic seawater was prepared by bubbling with pure nitrogen until DO \leq 0.5 mg/L in a storage tank which was connected to the experimental buckets. The concentration of sulfide was acquired by adding the sulfide stock solution (0.1 M, pH = 8.0 ± 0.1), which was prepared with Na₂S·9H₂O in oxygen-free water and were determined by the methylene blue method (Reese et al., 2011). Based on our preliminary experiment on the clam, to maintain target DO and sulfide levels (100% \pm 10% of target concentration), and water quality (nitrite < 0.01 mg/L, ammonia nitrogen < 0.2 mg/L) at the given loading density with clams, the experimental water was completely renewed every 4–6 h.

2.3 Analyses of THC and phagocytic activity

After 96 h of exposure, three individual clams from each replicate bucket were randomly selected to collect the haemolymph. The samples were immediately used for measurements of THC and phagocytic activity by using the method described by Tang et al. (Tang et al., 2020). Briefly, a volume of 100 μ L haemolymph extracted individually from the cavity using a 1 milliliter syringe. The extract that was used for the THC was diluted with 100 μ L 2.5% glutaraldehyde and 800 μ L of phosphate buffered saline (PBS, pH at 7.4) in a 1.5 mL centrifuge tube. The THC was estimated with a hemocytometer under an microscope at magnification of 400 \times . The extract that was used for phagocytic analysis was mixed with 100 μ L Alsever's solution. The mixtures was added yeast suspensions ($5.80 \pm 0.03 \times 10^7$ /mL) at a haemocyte: yeast ratio of 10: 1 and followed by an incubation at 25° C for 30 min. After fixation with 100 μ L 2.5% glutaraldehyde, blood smears were made and stained with Wright's Gimesa stain, and the phagocytic rate and phagocytic index of haemocytes were determined at magnification of 400 \times under a microscope. To ensure data accuracy of experiments, more than 200 haemocytes were calculated for each sample. The phagocytic rate refers to the number of 200 haemocytes participating in phagocytosis divided by the total number of haemocytes in the visual field. The phagocytic index refers to the total number of phagocytosed yeast divided by the total number of haemocytes participating in phagocytosis in the visual field.

2.4 Measurement of immune-related enzyme activities

The haemolymph samples that were extracted from nine individuals in each group were centrifuged at 800 g for 10 min. The supernatant was collected to determine the activities of immune-related enzymes including SOD, CAT, ACP, AKP, POD, LZM. The supernatant was either immediately used for measurements or snap-frozen in liquid nitrogen and stored at -80 °C for later usage. According to the methods described and tested by Bao et al. (Bao et al., 2020), Nan Xiang et al. (Xiang et al., 2017) and Cui et al. (Cui et al., 2020), we use spectrophotometric assays (Jiancheng Bioengineering Institute, Nanjing, China) with a microplate reader (SPARK, Groding, Austria) to determine the activities or contents. SOD, CAT and POD were expressed as U/mL, ACP and AKP activities were expressed as U/L, LZM contents were expressed as μ g/mL, respectively.

2.5 Integrated biological response index analysis on the biomarkers

The integrated biomarker response (IBR) index analysis was performed for all immune-related biomarkers measured, and the IBR was calculated according to the method described by Beliaeff and

Burgeot (Beliaeff and Burgeot, 2002). The *IBR* index was computed as follows: (1) Calculation of the mean value (X) for per biomarker at each treatment and sampling time, as well as the total mean value (m) and standard deviation (s) for each biomarker. (2) Standardization of the response data for per biomarker: $Y = (X - m)/s$, where Y is the standardized value of the biomarker. (3) Use standardized value, the Z_i was defined as $+Y_i$ or $-Y_i$ if a biomarker in the case of activation or inhibition, and the absolute minimum value ($|Z_{min}|$) of per biomarker at all sampling times was obtained. (4) The score S_i for per biomarker was calculated as $S_i = Y + |Z_{min}|$, where S_i can be shown by the length of the radiation line in the star plots. (5) The corresponding *IBR* value was calculated according to the following formula:

$$A_i = \frac{S_i}{2} \sin \beta (S_i \cos \beta + S_i + 1 \sin \beta), \quad \beta = \arctan \left(\frac{S_i + 1 \sin \alpha}{S_i - S_i + 1 \cos \alpha} \right) \quad \text{and} \quad \alpha = \frac{2\pi}{n}, \quad IBR = \sum A_i,$$

where n represented the number of biomarkers.

2.6 RNA extraction and samples preparation

The haemolymph from three individuals of *C* (here in after referred to as N), Q, QG groups were sampled after 24 h challenge, respectively. The total RNA was extracted following the manufacturer's instructions (Invitrogen) and treated with DNase (Promega RQ1 DNase I, Madison, WI, USA) to remove genomic DNA contamination. RNA degradation and contamination were monitored on 1% agarose gels. RNA concentration and quality were determined using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA).

2.7 Library construction and Illumina sequencing

Nine cDNA libraries were constructed from *T. granosa* in the sulfide challenged group QG (QG1, QG2 and QG3), hypoxia challenged group Q (Q1, Q2 and Q3), and normoxia control group N (N1, N2 and N3) for RNA-seq analysis. The RNA-seq libraries were performed at Biomarker Technologies Co., Ltd. (Beijing, China) following the manufacturer's recommendations. The mRNA was isolated by Oligo (dT) and fragmented by NEBNext First Strand Synthesis Reaction Buffer. The fragments were amplified for the synthesis of first- and second-strand cDNA using random hexamers. The cDNA libraries were built after the PCR products being purified using the AMPure XP system and were then assessed on the Agilent Bioanalyzer 2100 system. At last, the libraries were sequenced on an Illumina HiSeq 2500 platform.

2.8 Assembling and functional annotation

Clean reads were obtained by removing reads containing adapters, ploy-N and low-quality reads from raw data. The remaining high quality clean reads were assembled using Trinity for transcriptome assembly without reference genome (Grabherr et al., 2011). The longest transcript of each single gene was selected as a unigene. For annotation analysis, unigenes were BLASTX-searched against eight databases, including the NCBI non-redundant protein sequence (Nr), Protein family (Pfam), Clusters of Orthologous Groups (KOG/COG), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO), Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggNOG), TrEMBL, and the Swiss-prot database, using a cut-off E-value of 10^{-5} .

2.9 Differential expression and enrichment analyses

Differentially expressed genes (DEGs) were measured by counting tags from hypoxia/sulfide treated samples against the control, which were normalized using the RNA Sequence Expected Maximization (RSEM) method (Li and Dewey, 2011). Unigenes with adjusted P -value (P_{adj}) < 0.05 and $|\log_2$ (fold change)| > 0 were set as the DEGs. The annotated DEGs were further assigned to GO, KEGG enrichment analysis, and the annotated unigenes were performed gene set enrichment analysis (GSEA) based on the GO and KEGG database. GSEA ranks genes based on the degree of differential expression between two groups, followed by testing whether the gene set is significantly enriched at the top or bottom of the ranking list. Rather than focusing on individual genes, this analysis evaluates the expression of the whole transcriptome, which enables the inclusion and analysis of genes with more subtle changes (Aravind Subramanian et al., 2005).

2.10 Validation of DEGs by quantitative real-time PCR

Six DEGs were randomly selected for the validation of DGE data by quantitative real-time PCR (qRT-PCR) in the QG vs. C groups. The 18s rRNA of *T. granosa* (GenBank accession no. JN974506.1) was used as the internal control (Han et al., 2021a). Each sample with a total mixture of 20 μ l was reacted by the following program: denaturation at 95°C for 5 min, followed by 40 cycles of amplification (95°C for 10 s, 60°C for 20 s for annealing, and 72°C for 30 s for extension). The primer sequences of the tested genes used in the qRT-PCR study were designed by Primer Premier 5 software (Table 1). The expression level of different genes was analyzed using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

TABLE 1 Primer sequences and internal reference used for qRT-PCR validation.

Genes	Forward/Reverse Sequences (5' to 3')	Log2(fold change) of qRT-PCR	Log2(fold change) of RNA-seq
18S	CTTTCAAATGTCTGCCCTATCAACT	None	None
	TCCCGTATTGTTATTTTCGTCCT		
Sequestosome-1-like	CAGTGGATGTAGGTGGGG	1.48	1.59
	GCTGTGTGCCCTGGGTCT		
homocysteine S-methyltransferase	CACCAAGACATCCTACAGGC	2.25	2.05
	AAAGAAACGATTCATCAACA		
Protein toll	GGAGAGAAAACGCCGAAC	3.61	3.42
	GCAGAGAAACACGAAACC		
Thioredoxin reductase (NADPH)	ATCATACCAACTTTCAACC	1.63	1.49
	CTATCACTTTTTCCTTTTC		
Baculoviral IAP repeat-containing protein 2/3	GGGACGAGGTTGTCTGTT	2.53	1.73
	ACTTCTGCTTCGGTTAG		
Apoptosis-inducing factor 3	TGCTGCTGGTGATGTTGT	2.18	1.29
	TGCTGTAGGCTGCTGTTC		

2.11 Statistical analysis

Indicators of the humoral factors were analysed using one-way ANOVA followed by Tukey's post-hoc tests. For all analyses, Levene's tests and Shapiro-Wilk's tests were used to verify the homogeneity of variances and normality, respectively. The statistical analyses were carried out using Origin-Pro 8.0 software package, and P value < 0.05 was described as significant difference.

3 Results

3.1 Effects of hypoxia and sulfide on the THC and phagocytic activity of haemocytes

THC levels in all treatment groups showed similar temporal trends. In Q, QD, and QG group, THC reached to the maximum level during 12 h - 24 h and was significantly higher than initial level ($P < 0.05$). Since then, THC gradually dropped to the minimum level at 96 h and was significantly lower than the control level ($P < 0.05$) (Figure 1A).

In the C and Q groups, the phagocytic rate and phagocytic index showed no significant difference. However, the phagocytic rate and phagocytic index in the QD group significantly increased during 12 h - 24 h. The phagocytic rate and phagocytic index in the QG group significantly increased during 24 h - 48 h ($P < 0.05$) (Figures 1B, C).

3.2 Effects of hypoxia and sulfide on the activities of immune-related enzymes

SOD activities in the C and Q groups remained stable in 96 h (28.66 ± 0.31). SOD in the QD group gradually increased since 24 h and was significantly higher than the Q and QG levels at 96 h ($P < 0.05$). SOD in the QG group only increased at 24 h and was significantly higher than that in Q group ($P < 0.05$) (Figure 2A).

CAT activities in all treatment groups reached to their maximum levels at 24 h and have significant differences with the initial levels ($P < 0.05$). Besides, CAT in the QD group also significantly increased at 96 h ($P < 0.05$). At 48 h, CAT in the QG group was significantly lower than that in the other groups (Figure 2B).

POD activities were significantly inhibited in all treatment groups at 12 h ($P < 0.05$). Since then, POD in the Q group was stable at 7.19 ± 0.11 , and that in the QD and QG groups gradually increased to initial levels. At 96 h, POD in the QD group was significantly higher than that in the Q group ($P < 0.05$) (Figure 2C).

ACP activity in the Q group was significantly inhibited at 12 h, 24 h, 48 h ($P < 0.05$), while was recovered at 96 h. ACP in the QD group gradually decreased to the minimum level (0.45 folds, $P < 0.05$) at 24 h and was recovered to initial level since then. ACP in the QG group was significantly inhibited since 12 h ($P < 0.05$) and remained at relatively low level (0.37 folds - 0.48 folds) (Figure 2D).

AKP activity in the Q group was relatively stable in 48 h (5.42 - 7.66 U/L), but significantly dropped to 0.55 folds lower than the initial level at 96 h ($P < 0.05$). AKP in the QD group was relatively stable (5.74 - 8.02 U/L) while that in the QG group significantly dropped at 12 h (0.42 folds) and 48 h (0.45 folds) ($P < 0.05$) (Figure 2E).

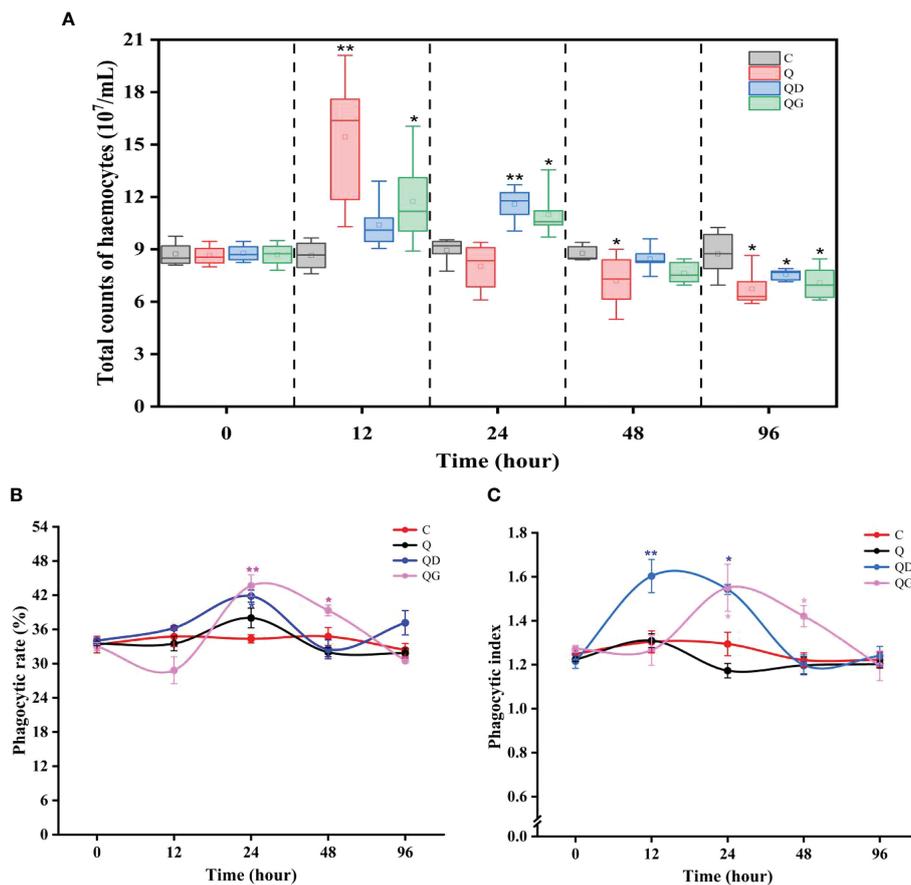


FIGURE 1 Total count of haemocytes (A), phagocytic rate of haemocytes (B), and phagocytic index of haemocytes (C). C, Q, QD, and QG stand for the control group, hypoxia group, hypoxia and mild-sulfide group, hypoxia and high-sulfide group, respectively. The asterisk * and ** indicate significant difference ($P < 0.05$) and highly significant difference ($P < 0.01$) compare to the control.

LZM activity in the Q group had a significant decrease at 12 h (0.67 folds, $P < 0.05$). In the QD group, LZM significantly increased since 48 h (1.25 - 1.76 folds, $P < 0.05$). In the QG group, LZM increased significantly at 12 h (1.86 folds) and 48 h - 96 h (1.42 - 1.56 folds) ($P < 0.05$) (Figure 2F). At 24 h, LZM in the QD and QG groups were significantly higher than that in the Q group ($P < 0.05$).

3.3 Integration of biomarker responses in immune-related biomarkers

By comparing the coverage areas of the star plots, the *IBR* indexes of Q and QG groups (the control value was standardized to 0) fluctuated with increasing time (Figure 3). To further investigate the integrated responses, the biomarkers were assembled into 3 categories, including cellular immune indexes (THC, PR, PI), antioxidant indexes (SOD, CAT, POD), and antibacterial indexes (ACP, AKP, LZM). The *IBR* values for cellular immune, antioxidant, and antibacterial responses in the Q group stay low for 96 h. While in the QD and QG groups, the *IBR* values for the cellular immune indexes were remarkably high at 24 h (QD=2.13, QG=2.45). In the QG group, antibacterial response was inhibited at 12 h and the

antioxidant response was also decreased at 48 - 96 h. At 96 h, the cellular immune responses, antibacterial responses, and antioxidant responses in the QD group were much higher than those in other groups.

3.4 Sequencing assessment, gene identification, and annotation of DEGs

To better understand the underlying molecular mechanism of the immunostimulation that was induced by moderate sulfide (0.5 mM, 24 h), a transcriptomic analysis was carried out. The Illumina sequencing yielded more than 60 million reads from the control, hypoxia and moderate-sulfide groups (Table S1). A total of 57,583 unigenes were obtained with an average length of 1417 bp (N50 length: 2351 bp). The statistics of the unigenes annotated in public databases were shown in Table 2. In the distribution of annotated unigenes in Nr, 22.60% unigenes have similarities with *Pecten maximus*, followed by *Mizuhopecten yessoensis* (21.04%), *Mytilus coruscus* (14.92%), *Crassostrea gigas* (14.66%) (Figure 4A). A total of 536 DEGs were detected from all the stressed groups. Among them, 102 DEGs were detected between N vs. Q groups, 423 DEGs

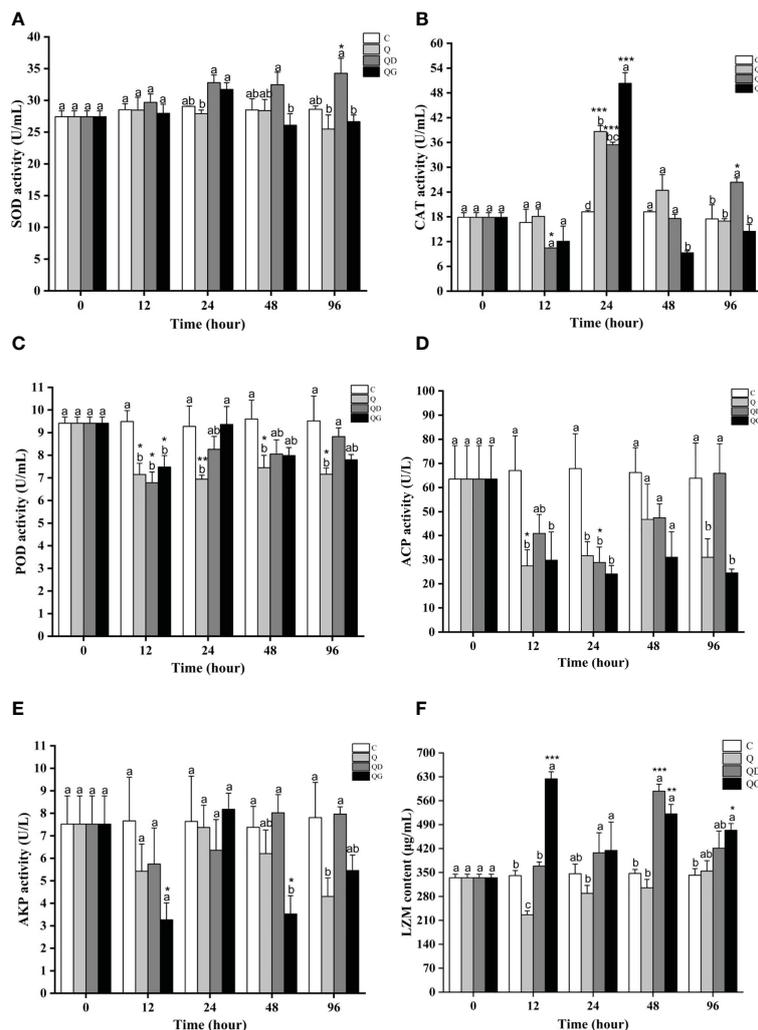


FIGURE 2 The activities of immune-related enzymes after 96 h of exposure to hypoxia alone or sulfide in combination (C, Q, QD, and QG stand for the control group, hypoxia group, hypoxia and mild-sulfide group, hypoxia and high-sulfide group, respectively). Different letters indicate values that are significantly different among the treatments within each time point, and the asterisk *, ** and *** indicate significant difference ($P < 0.05$) and highly significant difference ($P < 0.01$, $P < 0.001$) under the same treatment conditions. (A): SOD activity; (B): CAT activity; (C): POD activity; (D): ACP activity; (E): AKP activity; (F): LZN content.

were detected between N vs. QG groups, 123 DEGs were detected between Q vs. QG groups, and only 7 DEGs were commonly annotated in the stressed groups (Figure 4B). The heatmap and hierarchical clustering of the DEGs revealed differences between the control, hypoxia, and sulfide groups (Figure 4C).

3.5 Enrichment analysis of DEGs

Many genes related to immuno-regulation and apoptotic process were detected in the DEGs (Additional file 1). In N vs. Q groups, we found immuno-related gene *tollo*, which plays key role in Toll and Imd signaling pathway, was significantly up-regulated. While the immuno-related genes *nlp3*, *hmcn1* were significantly down-regulated. We also noticed that calcium-binding related gene *eip63F-1* was significantly inhibited. In N vs. QG groups, many immuno-related genes, including *cd22*, *tollo*, *foxo*, *gimap4*, *sting*,

ileu, *birc2/3*, *gadd45a*, *src*, *sqstm1*, *trim56*, *dhx15*, *lbp*, *irf2*, and *klhl20*, were significantly up-regulated. Besides, genes involved in MAPK signaling pathway (*dusp1/10*, *gadd45a*, *jun*, *aifm3*) and calcium signaling pathway (*cckar*, *octb2*) were significantly up-regulated. In Q vs. QG groups, genes involved in immunity (*cd22*, *tlr13*, *irf1/2*, *birc2/3*, *sqstm1*), MAPK signaling pathway (*dusp1*, *gadd45a*, *aifm3*) and calcium signaling pathway (*cckar*) were significantly up-regulated.

GO classification was performed to identify the cellular, biological and molecular processes associated with hypoxia and sulfide exposure in *T. granosa*. DEGs of three comparisons (N vs. Q; N vs. QG; and Q vs. QG) were assigned to three GO categories (biological process, BP; cellular components, CC; molecular functions, MF) and 60 GO terms (Figure S1). In N vs. QG and Q vs. QG groups, cellular process, integral component of membrane and protein binding significantly enriched in the biological process, cellular components, molecular functions, respectively.

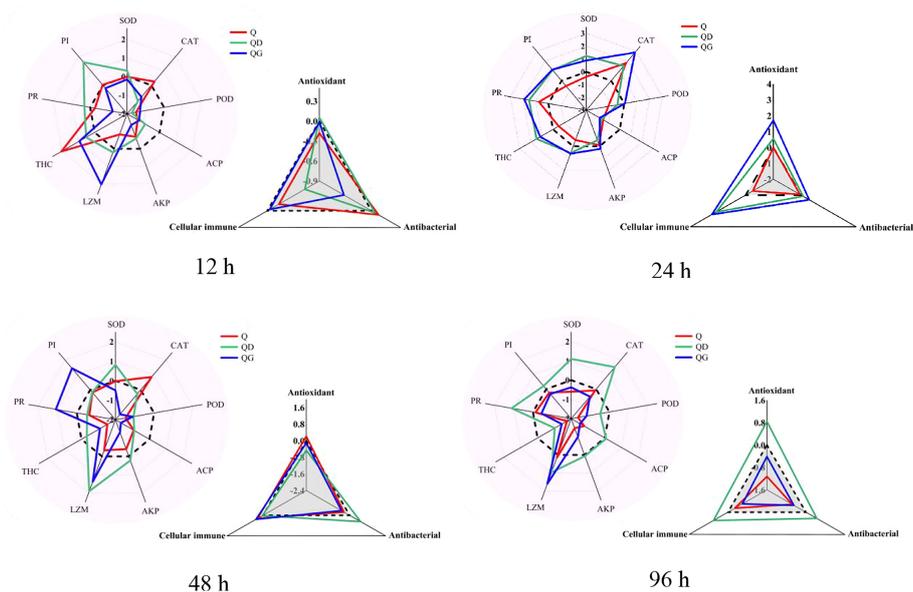


FIGURE 3 Star plots and the integrated biomarker response (IBR) of immune-related biomarkers in the adult *Tegillarca granosa* exposed to hypoxia and sulfide. Zero, Q, QD, QG, stand for the control level, hypoxia group, hypoxia and mild-sulfide group, hypoxia and high-sulfide group; THC, Total count of haemocytes; PR, phagocytic rate; PI, phagocytic index; SOD, superoxide dismutases; CAT, catalase; POD, peroxidase; ACP, acid phosphatase; AKP, alkaline phosphatase; LZM, lysozyme.

KEGG pathway analysis was performed to identify the broad functional categories of DEGs. Several signaling pathways were enriched in glutathione metabolism, arachidonic acid metabolism, FoxO, MAPK, NOD-like receptor (NLR), and Toll and Imd (Figure S2). These pathways were related to processes of metabolism, apoptosis, and immunity.

We used GSEA analysis to dig the responsive immuno-related pathways based on the KEGG and GO annotation on the gene set of unigenes. In the GO database, the gene set was significantly

enriched ($|NES| > 1$, $Pvalue < 0.05$, $qvalue < 0.25$) in many immuno-related “biological process” (Additional file 2). In N vs. Q group, a total of 10 immuno-related terms, involving in autophagy, macrophage activation, and toll-like receptor signaling pathway (Toll), were identified in 78 positively and significantly enriched terms ($NES > 1$, $Pvalue < 0.05$, $qvalue < 0.25$). Moreover, the cellular response to virus (GO:0098586) was identified as negatively and significantly enriched terms ($NES < -1$, $Pvalue < 0.05$, $qvalue < 0.25$). In N vs. QG group, over a third (125/353) of positively and significantly enriched terms had clear connection with immune responses, involving the responses of antimicrobial/antibacterial peptide, interferon, interleukin, leukocyte, lymphocyte, and Toll. Moreover, only two immuno-related terms were identified in the negatively and significantly enriched terms. In Q vs. QG group, over a fourth (119/440) of positively and significantly enriched terms involved in the similar immune responses to those in N vs. QG group, and only six immuno-related terms were identified in the negatively and significantly enriched terms. In the N vs. QG and Q vs. QG groups, many terms related to MAPK, TNF, and Apoptotic signaling pathways were also positively and significantly enriched. The top 10 most enriched immuno-related “biological process” were shown in the Figure 5.

In the KEGG database, the gene set was significantly enriched in FoxO signaling pathway (FoxO, ko04068) in all compared groups (Figure 6). In Q vs. QG groups, the gene set was also enriched in NOD-like receptor signaling pathway (NOD, ko04621) and Apoptosis (ko04210). The FoxO signaling pathway was predicted to be the main immuno-related pathway in the clam in response to hypoxia and sulfide.

TABLE 2 Summary statistics of functional annotation of *Tegillarca granosa* unigenes in public databases.

Public database	Annotated Number	300≤length	Length≥1000
COG	5,204	876	4,328
GO	17,937	3,746	14,180
KEGG	15,443	2,854	12,589
KOG	11,951	2,004	9,947
Pfam	17,155	3,118	14,037
Swissprot	8,999	1,474	7,525
TrEMBL	21,034	4,651	16,383
eggNOG	16,301	2,978	13,323
Nr	21,650	4,920	16,730
All Annotated	22,515	5,463	17,041

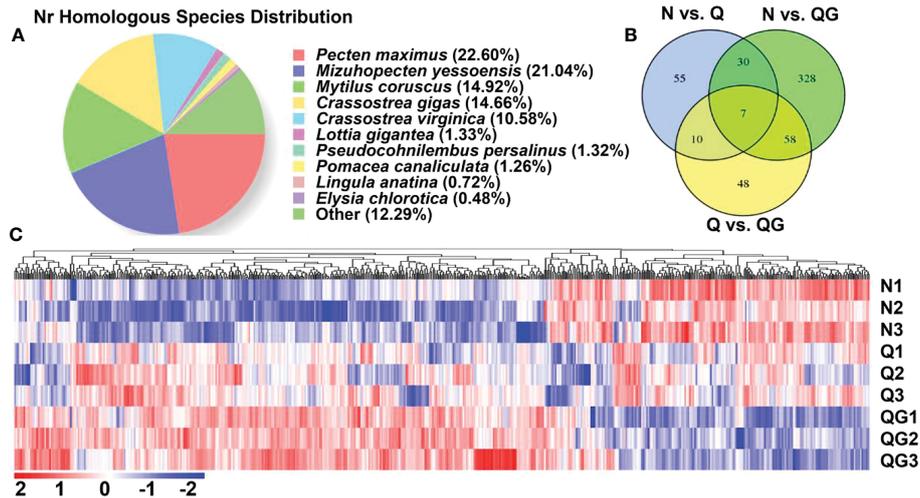


FIGURE 4 Nr homologous species distribution of the annotated unigenes (A); Venn diagram of the differentially expressed unigenes (DEGs) (B); Hierarchical cluster of the DEGs (C).

4 Discussion

4.1 Cellular and humoral immune responses to hypoxia and sulfide

In marine invertebrates, cellular and humoral immune systems play crucial roles in antimicrobial defense and immuno-regulation. The THC and phagocytosis of haemocytes are the commonly used indicators of cellular immune responses in invertebrates (Loker et al., 2004; Allam and Raftos, 2015). Previous studies on many marine invertebrates, including the bivalves *Perna perna*, *M. coruscus*, *Perna viridis* (Wang et al., 2014; Sui et al., 2016;

Nogueira et al., 2017; Shen et al., 2019) and the crustaceans *Eriocheir sinensis*, *Carcinus aestuarii*, *Nephrops norvegicus*, *Macrobrachium rosenbergii* (Cheng et al., 2002; Hernroth et al., 2015; Qin et al., 2016; Qyli et al., 2020), showed that exposure to prolonged hypoxia could decrease THC and phagocytic activities. The results indicated that the cellular immune responses were intervened by hypoxia exposure. In the present study on *T. granosa*, THC might be rapidly stimulated by hypoxia but the stimulation did not last more than 24 h. Interestingly, hypoxia with additional sulfide significantly induced both the THC and phagocytic levels around 24 h, which indicated that the cellular immune response of *T. granosa* was stimulated by certain

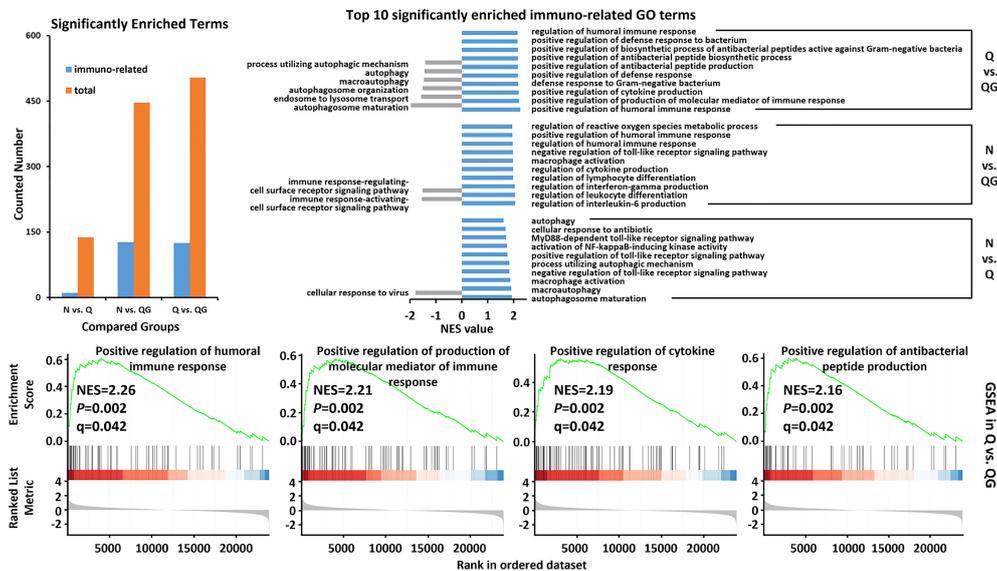


FIGURE 5 The critical GO pathways associated with immune response to hypoxia and sulfide in haemolymph of *Tegillarca granosa* were explored by gene set enrichment analysis.

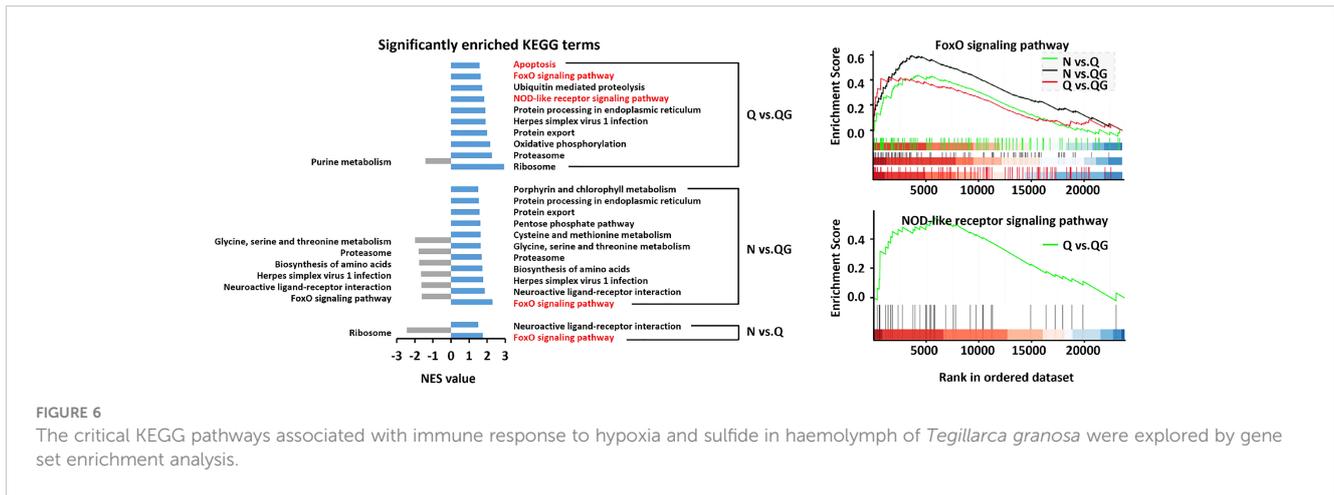


FIGURE 6 The critical KEGG pathways associated with immune response to hypoxia and sulfide in haemolymph of *Tegillarca granosa* were explored by gene set enrichment analysis.

concentration of sulfide. This finding was different from previous reports in various marine species (Cheng et al., 2007; Sun et al., 2014; Xu et al., 2014).

Humoral factors such as agglutinins (e.g., lectins), lysosomal enzymes (e.g., acid phosphatase, lysozyme), and various antimicrobial peptides (Canesi et al., 2002) are considered as important indicators to investigate the humoral immunity of invertebrates. Prolonged hypoxia causes significant decrease of antioxidant and other humoral immune responses has been demonstrated in many invertebrates including the bivalves *Arctica islandica*, *P. perna*, *M. coruscus* and *Hyriopsis cumingii* (Philipp et al., 2012; Hu et al., 2015; Sui et al., 2016; Nogueira et al., 2017; Li et al., 2022b), as well as the crabs *M. japonicus*, *Callinectes sapidus*, and *Scylla paramamosain* (Tanner et al., 2006; Cheng et al., 2020; Wang et al., 2022). In this study, hypoxia significantly increased CAT at 24 h. However, POD and ACP in hypoxia group significantly decreased in 12-96 h. Besides, AKP and LZM in hypoxia group also decreased to a certain extent. The results indicated that hypoxia significantly enhanced the CAT activity at 24 h, but the overall humoral immunity might be suppressed within 96 h. After adding sulfide, SOD (24-96 h), CAT (24 h, 96 h), POD (48 h, 96 h), ACP (96 h), AKP (96 h), and LZM (12-96 h) significantly increased compared to the hypoxia group. The results indicated that sulfide could stimulate the humoral immunity to some extent under hypoxic conditions. However, after adding sulfide, only SOD (24-96 h), CAT (24 h, 96 h), and LZM (12 h, 48 h, 96 h) significantly increased compared to the normoxia control group, CAT (48 h), POD (12 h), ACP (12-96 h), and AKP (48 h) were still significantly lower than the normoxia control group. This indicated that sulfide have certain limitations and specificity in stimulating the humoral immunity under hypoxic conditions.

We inferred that moderate exogenous sulfide attenuated the immunosuppression caused by hypoxia through both cellular and humoral immune responses. The attenuation through cellular way happened around 24 h while the humoral way took effects mainly at 48 - 96 h.

4.2 Integration of biomarkers using IBR

To support our inferences, we induce IBR method. The IBR index proposed by Beliaeff and Burgeot (2002) (Benoit Beliaeff and Burgeot, 2002) novelly integrated biomarkers of CAT, GST and AchE in *Mytilus edulis* and showed diverse stress levels along the coasts of Baltic Sea. Recently, the IBR has been addressed as an integrative method for the assessment of environmental stress, as well as for the measurement of the sensitivity of organisms after exposed to toxicants (Broeg and Lehtonen, 2006; Pytharopoulou et al., 2008; Raftopoulou and Dimitriadis, 2010; Ji et al., 2018; Chang et al., 2020). By using IBR method, we found that CAT and LZM were the most sensitive antioxidant and antibacterial indexes in the haemolymph of *T. granosa* in response to hypoxia and sulfide. The IBR analysis also demonstrated that moderate sulfide had a remarkable effect in attenuating the immunosuppression caused by hypoxia exposure through multiple ways, including stimulating the cellular immune response at around 24 h, and stimulating the antibacterial and antioxidant responses by low level sulfide at around 96 h. Although no similar studies of sulfide have been reported in invertebrates, H₂S has been regarded as not only a gas transmitter, but also a key factor in immunity-related processes in clinical medicine (Yuan et al., 2017; Li et al., 2021).

Even though IBR discriminates the stress types or levels, it provides a simple method for the quantitative evaluation of the combination effects of hypoxia and sulfide in this study, and it can be recommended for future quantified risk assessment of hypoxia and sulfide on aquatic organisms.

4.3 Transcriptomic analysis revealed immunomodulatory effects of sulfide

Studies had confirmed that the clams had extremely tolerance to hypoxia and sulfide with different adaptive mechanisms (Joyner-Matos et al., 2006; Kodama et al., 2018; Wang et al., 2019; Wang and Zhang, 2021; Wang et al., 2021). In the present study, our findings

supplemented the immune response mechanisms to hypoxia and sulfide in the clam *T. granosa*. During the hypoxia, the clam downregulated DEGs involved in NLR signaling pathway (*nlrp3*) and activated autophagy (*mTOR*), which might alleviate cell damages and inflammation (Cosin-Roger et al., 2017). Hypoxic challenge also inhibited DEGs involved in calcium-binding signaling pathways (*eip63F-1*), while hypoxic challenge with additional sulfide significantly induced both NLR and calcium-binding signaling pathways. The result suggested the potential immunostimulation effects of sulfide, as it has been reported that NLR and calcium-binding signaling pathway play important roles in recognizing pathogen-associated molecular patterns (PAMPs) and regulation immune responses (Wen et al., 2013; Horng, 2014; Zhao and Zhao, 2020).

After treated with exogenous sulfide, the clam positively enriched many biological processes that were involved in antimicrobial/antibacterial peptide, interferon, interleukin, leukocyte and lymphocyte, according to the GSEA analysis. Those factors play significant roles in the defense against pathogens infection and elimination of bacterial action. For instance, antimicrobial peptides (AMPs) are important component of the innate immune system and executive antibacterial activity in mollusks. AMP that was isolated from *M. coruscus* displayed strong antimicrobial activity against gram-positive bacteria (Liao et al., 2013; Oh et al., 2020). Interferon is a cytokine mainly produced by lymphocytes, involving the activation of macrophages and control of cell proliferation and apoptosis (Schoenbom and Wilson, 2007; Pereiro et al., 2019). The activation of those biological processes explained the stimulation of THC, phagocytic activity, and LZM after the 24 h exposure to sulfide.

We also identified many other stimulated immuno-related signaling pathways and genes such as Toll and Imd (*tollo*), FoxO (*foxo*, *gadd45a*, *klhl20*, *egfr*), TLR (*tlr13*), NF-κB (*vps9d1*), TNF (*c1ql3*), MAPK (*dusp1/10*, *jun*), apoptosis (*aifm3*, *birc2/3*). Toll and Imd signaling pathway have been paid great attention in immunomodulatory (Tanji and Ip, 2005; Rances et al., 2013). These pathways are critical to the expression of antimicrobial peptides, which fight against fungi, Gram-negative bacterial and Gram-positive bacterial (Tanji and Ip, 2005; Tanji et al., 2007). In the mud crab *S. paramamosain* and the Pacific white shrimp *L.*

vannamei, Toll and Imd signaling pathways are activated in innate immune responses to white spot syndrome virus (WSSV) and sulfide exposure (Li and Xiang, 2013; Duan et al., 2017; Chen and Wang, 2019; Li et al., 2022a). FoxO signaling pathway is involved in mitochondria-dependent processes of apoptosis by triggering the expression of TNF apoptosis ligand and Bcl-X_L from Bcl-2 family members (Farhan et al., 2017). In aquatic animals, such as grass carp (*Ctenopharyngodon idellus*), Asian clam (*Corbicula fluminea*), and sea cucumber (*Apostichopus japonicus*), genetic and transcriptomic analysis revealed that activation of FoxO could regulate antioxidant and apoptosis pathways (Wang et al., 2015; Jin et al., 2017; Zhang et al., 2019). Toll-like receptors (TLRs) signaling pathway also plays an important role in innate immunity in invertebrates, and the activation of TLR can activate multiple inflammatory pathways (Brown et al., 2011). In the present study, a variety of genes related to TLRs and NF-κB, TNF and MAPK signaling pathway was significantly upregulated by exposing to sulfide, which indicated that TLRs signaling pathway activated the downstream MAPK, NF-κB, TNF signaling pathways (Figure 7). The present results were consistent with the transcriptomic profile of the clam *A. broughtonii* that was exposed to hypoxia and sulfide for 24 h (Wang et al., 2019), in which MAPK and TNF signaling pathways were significantly enriched, and some genes related to TLRs and NF-κB signaling pathways were also upregulated. In the clam, the active apoptosis signaling pathway indicated that sulfide might regulate cell apoptosis through TLR, MAPK, NF-κB, and TNF, as the connection of apoptosis and TLR, MAPK, NF-κB, TNF has been described in many invertebrates (Cao et al., 2018; Wang et al., 2020). Wang et al. (2021) also suggested the possible ways of MAPK that regulated sulfide-induced apoptosis in the clam *A. broughtonii*.

5 Conclusion

In conclusion, our study found that hypoxia alone suppressed the overall immune response, while additional sulfide attenuated the immunosuppression through multiple cellular and humoral ways. Besides, Toll, NOD, FoxO, MAPK, TNF, and Apoptosis signaling pathways might play critical roles in the sulfide-mediated immune responses. Our study would provide useful

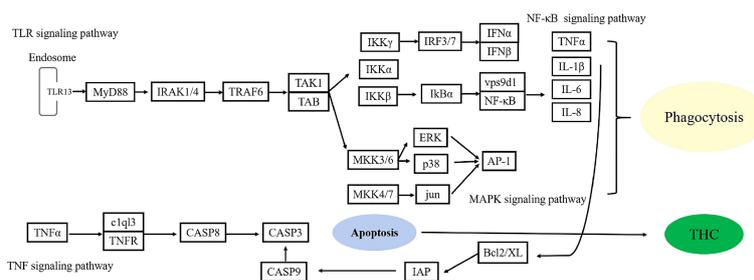


FIGURE 7 The possible signal transduction of immunostimulation mediated by sulfide.

tools for assessing the integrated biological and transcriptomic impacts of hypoxia and sulfide on *T. granosa* and offer deep insight into the sulfide-tolerant mechanisms in shellfish.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA931778. <https://www.ncbi.nlm.nih.gov/sra/PRJNA931778>.

Author contributions

ZY: conceptualization, experimental design, carry out experiments, writing-original draft. YW: Verification of the results and revision of the manuscript. PJ: visualization, data curation. FX: investigation, methodology, software. YX: software, data curation. XT: data curation, validation. XZ: funding acquisition, writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1139973/full#supplementary-material>

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