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Short communication

The impact of ocean acidification and cadmium on the immune responses of Pacific oyster, *Crassostrea gigas*

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ABSTRACT

Seawater acidification (OA) and cadmium (Cd) has the potential to lead to immunosuppression effect on marine bivalves. However, the interaction between these two environmental stressors on immune system of marine bivalves has received limited attention. In order to evaluate the defense responses of oysters under the combined exposure to OA and cadmium, the oysters *Crassostrea gigas* were exposed to 10 μg/L Cd at three pH levels (8.1, 7.8 and 7.6) for 31 days. Results showed that OA exposure alone led to increased DNA damage, apoptosis rate and ROS production of hemocytes. However, inhibited phagocytosis rate, combined with increased DNA damage, apoptosis rate and ROS production of hemocytes were observed in oysters under exposure to Cd exposure alone or combined with OA. Significant interactive effects between OA and Cd were observed on ROS production and DNA damage of hemocytes. In addition, there is generally significant increase in the mRNA expression of genes related to immune-related TLR pathway and two immune factors (TNF and integrin beta-1B) in Cd-exposed oysters at pH 7.6. The results revealed that even though the mRNA expression of genes related to immune responses (TLR pathway and immune factors) was stimulated to counteract the immunosuppression caused by acidified seawater and Cd, depressed hemocyte function perhaps sensitized oysters to potential pathogen infection.

1. Introduction

Since industrial revolution, sustained absorption of anthropogenic derived CO_2 leads to ocean acidification (OA), which has already led to a reduction of 0.1 units in global surface seawater pH [1]. With continuous and increasing release of anthropogenic CO_2, ocean pH is projected to reduce by a further 0.3–0.5 units by 2100 [1,2]. Meanwhile, seasonal upwelling of hypercapnic seawater and eutrophication could further reduce seawater pH in coastal and estuaries regions [3,4]. In the meantime, large amounts of cadmium (Cd) enter the marine environment mainly from anthropogenic activities such as refining, copper and nickel smelting, fossil fuel combustion and municipal wastes, leading to Cd pollution in coastal and estuaries regions [5]. It has been reported that Cd is one of the dominant metal pollutants in the Bohai Sea, and the concentration of Cd could reach as high as 5 μg/L in the coastal waters of Bohai sea [6].

CO_2-driven ocean acidification has demonstrated adverse effects on immune responses of marine invertebrates [7–13]. Moreover, numerous studies have shown that Cd could lead to immunotoxicity in marine invertebrates [14–17]. In a heterogeneous and naturally variable marine environment, anthropogenic stressors seldom occur in isolation. Thus, investigation of the impacts of multiple environmental stressors on the immune responses of marine invertebrates is crucial to better understand the environmental risk posed by these stressors [18]. To date, few studies have examined the combined effect of elevated pCO_2 combined with Cd exposure on bivalve immune responses. For example [19], has investigated the phagocytotic activity, adhesion capability and lysozyme activity of clam and oyster hemocytes, and the results show that elevated pCO_2 (~800–2000 μatm pCO_2) potentiates the negative effects of Cd (50 μg/L) on immunity of clam *Mercenaria*

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Table 1
Measured and calculated carbonate chemistry parameters of seawater for the exposure experiment. The pH is monitored daily with a pH electrode calibrated with NBS standard pH solutions, and total alkalinity (TA) is determined weekly. pCO_2 and total dissolved inorganic carbon (DIC) are calculated using CO_2SYS software.

Parameters	pH 8.1	pH 7.8	pH 7.6	pH 8.1 + Cd	pH 7.8 + Cd	pH 7.6 + Cd
Temperature (°C)	17.6 ± 0.4	17.3 ± 0.6	17.3 ± 0.6	17.9 ± 0.5	17.8 ± 0.4	17.3 ± 0.4
Salinity (‰)	31.2 ± 0.5	31.2 ± 0.5	31.3 ± 0.5	31.2 ± 0.5	31.0 ± 0.5	31.3 ± 0.5
pH (NBS scale)	8.15 ± 0.02	7.81 ± 0.04	7.54 ± 0.04	8.17 ± 0.02	7.78 ± 0.03	7.57 ± 0.02
TA (μmol/kg)	2257.13 ± 41.14	2300.17 ± 45.33	2279.39 ± 25.16	2275.07 ± 55.08	2258.65 ± 33.26	2297.44 ± 28.95
DIC (μmol/kg)	2056.59 ± 9.15	2229.05 ± 12.97	2291.28 ± 12.29	2067.06 ± 4.68	2198.27 ± 7.83	2300.28 ± 6.12
pCO_2 (ppm)	435.19 ± 23.26	1064.74 ± 105.58	2038.22 ± 195.56	427.460 ± 11.47	1143.67 ± 70.70	2136.70 ± 139.95

Table 2
Primers used in this study.

	Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Genbank accession number
Reference gene	Elongation factor 1 alpha (EF1α)	AGTCACCAAGGCTGCACAGAAAG	TCCGACGTATTTCTTTGCGATGT	AB122066.1
TLR-pathway related proteins	Inhibitor of NF-κB (IκB)	GAAAAAGTGGCAAGAGTGTC	GAAGAGTCATCGAAAGCAAC	DQ250326
	Transcription factor Rel1	GCTGAACCAGAACCTCATGA	CGAAGGACATGTTCTGATCC	AAK72690
	Toll-like receptor (Toll-1)	AAGTTCTCGTGAAGTCCCATCC	ATACAGGCGTCTCGTAACCATC	XM_020066660.1
	Myeloid differentiation factor88 (MyD88)	AGGTACCGGCTGTGATACGA	TTCAAACGCCACCAAGACTG	DQ530619
	TNF receptor associated factor 3 (TRAF3)	CAAGCAACGAAAACAAAGCATTA	CTTAGGCTGGTGTTCAACCATTC	BQ426746
Antioxidant enzyme	Extracellular superoxide dismutase (EcSOD)	ACGGACTTCAGATCCACGAG	GGCCAAGAATTCCGTCTGTA	DQ010420.1
Stress protein	Heat Shock Protein 90 (HSP90)	AGCAGGGAAGTGGTTCAGTCG	TGACTTTGCACAATCCCTCGTAC	EF687776.1
Immune factors	Integrin beta-1B	CCTCGTAAAGAGCAGGGATG	CCATTGAGTTTGAGAGGTCCAT	AB066348.1
	Tumor necrosis factor (TNF)	AGACTGGGAGGATGTCCTGGAG	GTCAAAAACTTCTGGCTGTCAC	JH816585.1

mercenaria and oyster *Crassostrea virginica* [19]. Also, in mussel *Mytilus edulis* L, phagocytosis levels were found declined with decreasing pH (from 8.2 to 6.2) with Cd exposure on 35 days [20].

As sessile marine animals living in estuarine and intertidal regions, the Pacific oyster *Crassostrea gigas* has great economic and ecological value. Like other invertebrate, oysters rely solely on their innate immune system mediated by both cellular and humoral components to defend themselves against pathogens [21,22]. In this study, the phagocytosis rate, apoptosis rate, ROS production and DNA damage of oyster hemocytes were investigated to evaluate the impact of reduced seawater pH and environmental realistic concentration of Cd pollution (10 μg/L) posed on hemocyte function of marine invertebrates. Furthermore, Toll-like receptor (TLR) signaling pathway was an important and evolutionarily conserved immune pathway [23,24]. Once activated, the TLR signaling pathway could subsequently induce a core set of immune responses [25,26]. In addition, as immune factors, the tumor necrosis factor (TNF) plays important roles in phagocytosis, apoptosis, and anti-bacterial activity of hemocytes in *C. gigas* [27], and the integrin provides a significant contribution to immune responses such as attachment, spreading, and phagocytosis in invertebrate hemocytes [28]. Thus, the mRNA expression level of genes related to TLR signaling pathway and immune factors were also investigated in this study to dig out how immune system of *C. gigas* respond to OA and Cd exposure from molecular level.

2. Materials and methods

2.1. Experimental design

The Pacific oysters *Crassostrea gigas* were purchased from a local oyster farm. Before commencement of the experiment, animals were acclimated in aerated seawater for 2 weeks. After acclimation, oysters were exposed to six different treatments for 31 days using three levels of pCO_2 (pH 8.10, pH 7.80 and pH 7.60) and either no metal addition or 10 μg/L Cd. The selected pH levels were representative of the present-day condition (pH 8.10) and the predicted condition by the moderate scenarios of IPCC 2007 [45] for the year 2100 (pH 7.80) and the year 2250 (pH 7.60). For each treatment, three replicate aquaria were used with 30 organisms per aquarium (90 individuals per treatment). The concentration of Cd (10 μg/L) was set according to the environmental concentrations of the coastal areas of Bohai Sea [6]. Seawater bubbled with atmospheric air was used as the control throughout the exposure experiment, while seawater acidification treatments were bubbled with air-CO_2 mixtures, which were adjusted through an air and CO_2 gas flow adjustment system. Animals were feed daily with commercial algal blend containing *Chlorella vulgaris* Beij and *Phaeodactylum tricornutum* at a concentration of 1×10^4 cells mL^{-1}. Seawater was renewed every other day using pre-equilibrated seawater during exposure. No oyster mortality was recorded in any of the treatments during the exposure period.

The pH was monitored daily with a pH electrode (pH meter PB-10, Sartorius Instruments, Germany) calibrated with NBS standard pH solution. Salinity, temperature and dissolved oxygen (DO) was measured daily using an YSI meter (YSI® model 85, Yellow Springs, OH, USA). Total alkalinity (TA) were determined per week by potentiometric titration [29]. For the calculation of other related parameters of the carbonate chemistry, TA values and measured parameters (temperature, salinity and pH values) were plotted in CO_2SYS software with the constants for seawater pH from Ref. [30] and for KSO_4^-, from Ref. [31]. The environmental parameters of all the treatments are provided in Table 1.

2.2. Assays of hemocyte-mediated parameters

After experimental exposure, 1 mL of hemolymph was extracted from the pericardial cavity of each oyster using a sterile 2-mL syringe equipped with a 22 G needle. The syringe was filled with 1 mL of ice-cold Modified Alsever's Solution (27 mM of sodium citrate, 336 mM of sodium chloride, 115 mM of glucose and 9 mM of EDTA, pH 7.5) to prevent aggregation of the hemocytes. At the end of the experiment, a total of 30 oysters were sampled after 31 days' exposure to OA and/or Cd. Hemolymph extracted from five individuals were pooled together, and six randomly constituted hemolymph pools were prepared for the determination of immune-related parameters. The hemolymph sample was filtered through an 80-μm mesh sieve, centrifuged at 4 °C, and then washed twice with phosphate-buffered saline (PBS) buffer (137 mM

Table 3
Two-way ANOVA: Effects of elevated pCO_2 and Cd exposure on the immune-related parameters and gene expression alterations in *C. gigas*. Significant effects are highlighted in bold.

		Factors/interaction		
		CO_2	Cd	$CO_2 \times$ Cd
Immune-related parameters	Phagocytosis	**F (2, 12) = 7.77 P = 0.007**	**F (1, 12) = 8.72 P = 0.012**	F (2, 12) = 2.98 P = 0.089
	ROS	**F (2, 24) = 14.97 P < 0.0001**	**F (1, 24) = 31.68 P < 0.0001**	**F (2, 24) = 7.57 P = 0.003**
	Apoptosis	**F (2, 30) = 7.87 P = 0.002**	**F (1, 30) = 13.88 P = 0.0008**	F (2, 30) = 0.54 P = 0.589
	Comet assay	**F (2, 595) = 108.70 P < 0.0001**	**F (1, 595) = 263.00 P < 0.0001**	**F (2, 595) = 20.29 P < 0.0001**
Gene expression in hemocytes	IkB	**F (2, 30) = 4.80 P = 0.015**	**F (1, 30) = 8.95 P = 0.005**	F (2, 30) = 1.73 P = 0.194
	Rel	**F (2, 30) = 3.15 P = 0.054**	F (1, 30) = 2.96 P = 0.093	F (2, 30) = 1.46 P = 0.245
	Toll-1	F (2, 30) = 1.12 P = 0.336	**F (1, 30) = 16.47 P = 0.0002**	**F (2, 30) = 5.31 P = 0.009**
	MyD88	**F (2, 30) = 23.16 P < 0.0001**	F (1, 30) = 0.09 P = 0.761	**F (2, 30) = 5.47 P = 0.008**
	TRAF3	**F (2, 30) = 20.61 P < 0.0001**	**F (1, 30) = 21.51 P < 0.0001**	**F (2, 30) = 20.32 P < 0.0001**
	EcSOD	**F (2, 30) = 23.16 P < 0.0001**	F (1, 30) = 0.09 P = 0.761	**F (2, 30) = 5.47 P = 0.008**
	HSP90	**F (2, 30) = 6.24 P = 0.005**	F (1, 30) = 3.81 P = 0.059	**F (2, 30) = 24.41 P < 0.0001**
	Integrin	**F (2, 30) = 7.71 P = 0.002**	F (1, 30) = 1.89 P = 0.179	F (2, 30) = 0.07 P = 0.93
	TNF	**F (2, 30) = 3.59 P = 0.04**	**F (1, 30) = 6.32 P = 0.018**	**F (2, 30) = 7.49 P = 0.002**

NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). The cells were re-suspended in 500 μL of PBS as working solution.

2.2.1. Apoptosis

Apoptotic cells were identified and quantified using the Annexin V-FITC/PI detection kit (Invitrogen, Gaithersburg, MD, USA). Briefly, 100 μL of hemocyte sample was re-suspended with Annexin V binding buffer, and incubated with 5 μL of Annexin V-FITC and 10 μL of PI working solution for 15 min in the dark. After that, 400 μL of 1 × Annexin V binding buffer was added into each tube, and the cells were immediately analyzed by flow cytometry (FACSAria, Becton Dickinson, Franklin Lakes, NJ, USA) for fluorescence in the FL-1 (Annexin V) and FL-2 (PI) channels.

2.2.2. ROS production

The intracellular ROS content was determined by incubating hemocytes with 5 μL of fluorescent probe DCFH-DA (0.01 mM) at 18 °C for 1 h in the dark. Three replicate hemocyte samples with no DCFH-DA addition were set as negative control. At the end of the incubation, the hemocytes were analyzed by flow cytometry with the excitation and emission wavelength at 488 and 530 nm, respectively. The results were analyzed with Cell Quest software and expressed as the geometric mean of the fluorescence (in arbitrary units, AU) detected in each hemolymph sample.

2.2.3. Phagocytosis

The phagocytosis assay was adapted from the method of [32] with minor modification. The hemolymph was mixed with 2.3% yellow-green FluoSpheres (YG 2.0 microns, Polyscience, Eppelheilm, Germany), and then incubated at 18 °C for 1 h in the dark, followed by addition of 6% formalin solution to terminate the reaction. The redundant beads were removed by centrifugation at 4 °C, 800 g for 10 min, and the hemocytes were resuspended with PBS buffer for flow cytometry (FCM) analysis. Hemocytes were analyzed by flow cytometry using the FL-1 tunnel, and the phagocytic rate was expressed as the percentage of hemocytes that had engulfed three or more beads.

2.2.4. Comet assay

The comet assay was performed as described by Ref. [33] with slight modification. 40 μL of hemocyte suspension and 75 μL of 1.0% low melting point agar were mixed, spread onto glass slides pre-coated with 2.0% normal melting point agarose, and then covered with coverslips. After solidification, the slides were immersed into freshly prepared lysis buffer (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris, 1% Triton X-100, 10% DMSO, pH 10.0) for 1 h at 4 °C. Following lysis, the slides were aligned in a horizontal electrophoresis tray and immersed in an alkaline electrophoresis buffer (300 mM of NaOH and 1 mM of EDTA, pH = 13.0) for 20 min at 4 °C. After electrophoresis (25 V, 300 mA for 10 min), slides were neutralized in Tris buffer (0.4 M of Tris-HCl, pH 7.5). DNA was stained with SYBR Green I (Molecular Probe, Oregon, USA) and examined with a fluorescence microscope (Olympus FV 1000, Tokyo, Japan). DNA damage was expressed as the percentage of DNA in the comet tail (% DNA in tail).

2.3. RNA extraction and qRT-PCR

At the end of the exposure experiment, six replicates of hemolymph were sampled for each treatment. Hemolymph samples were centrifuged at 1000 g for 10 min, and the resultant hemocyte pellet was subjected to total RNA extraction and cDNA synthesis as described previously [13]. The mRNA expression of TLR pathway-related genes (Toll-1, IκB, Rel, MyD88 and TRAF3), stress protein HSP90, oxidative stress-related protein EcSOD and two immune factors (TNF and integrin beta-1B) was determined using the ABI 7500 Real-Time Detection System (Applied Biosystems, Foster City, CA, USA). The comparative C_T method ($2^{-\Delta\Delta CT}$ method) was used to analyze the relative expression level of the candidate genes [34]. The detailed information on primer sequences is provided in Table 2.

2.4. Statistical analysis

All statistical analyses were performed using SPSS 23.0. Data were represented as the means ± deviation (SD). Raw data were assessed for normality and homogeneity of variances using the Shapiro-Wilk test and Bartlett's test, respectively. Data were analyzed using two-way analysis of variance (ANOVA) followed by post hoc tests (Fisher's least significant difference). ANOVA results for all studied traits are given in Table 3. Differences were considered significant if $p < 0.05$. Principal component analysis (PCA) was performed with CANOCO 5.0 software (Microcomputer Power Inc., NY, USA).

3. Results

3.1. Hemocyte-mediated parameters

Generally, ROS production, apoptotic rate and DNA damage of hemocytes were stimulated after 31 days of OA and/or Cd exposure (Fig. 1a, Fig. 1b and c). Significant increase was observed in ROS production at pH 7.80 in non-Cd exposed oysters (Fig. 1a). Moreover, at pH 7.60, significant elevation of hemocyte ROS production were observed in oysters in response to Cd exposure. ANOVA results suggested there

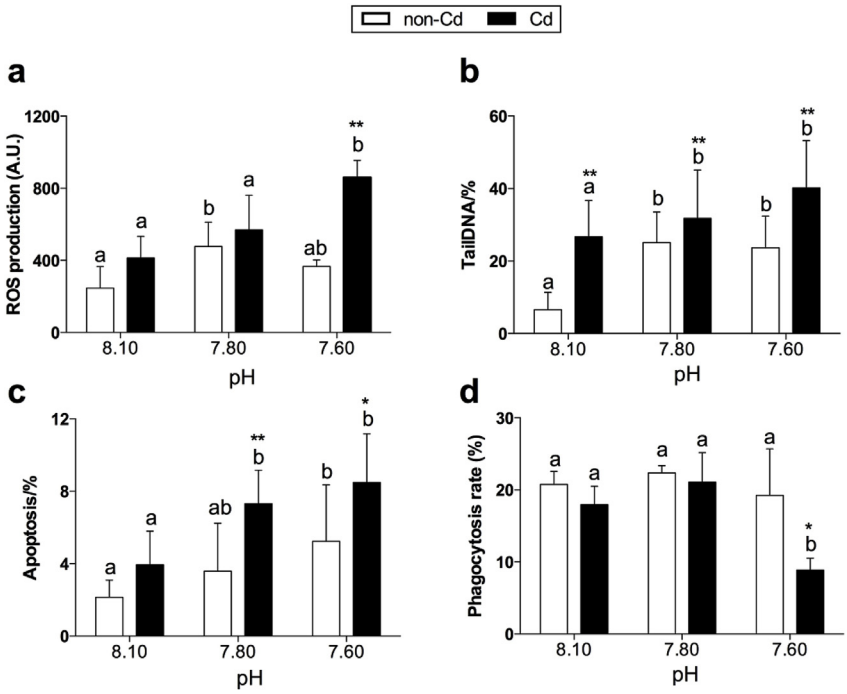

Fig. 1. The immune-related parameters and genetic damage evaluated in hemocytes of *C. gigas* after elevated pCO_2 and/or Cd exposure. Effect of OA and Cd exposure on the ROS production (a), DNA damage (b), apoptosis rate (c), and phagocytic rate (d) of oyster hemocytes. Different letters indicate significant differences among treatments at the same concentration of Cd exposure ($p < 0.05$). * indicates significant difference between non-Cd exposure and Cd exposure at the same pH level. ** indicates extremely significant difference between non-Cd exposure and Cd exposure at the same pH level.

was significant interaction between OA and Cd on hemocyte ROS production of *C. gigas* (Table 3).

There is significant increase in DNA damage values of the oyster hemocytes after exposure to OA for 31 days irrespective of Cd exposure (Fig. 1b). Meanwhile, significant increase in DNA damage values were found in Cd-exposed oysters compared with non-Cd exposed oysters under each level of pCO_2 exposure. Significant interaction between OA and Cd on hemocyte DNA damage was observed in two-way ANOVA results (Table 3).

Compared with control treatment, significantly enhanced apoptosis rate of hemocytes was observed at pH 7.60 in non-Cd exposed oysters (Fig. 1c). In addition, significant increase in hemocyte apoptosis rate was found in Cd-exposed oysters at both pH 7.80 and pH 7.60 compared with individuals at pH 8.10.

There was no alteration in the phagocytosis rate of non-Cd exposed oysters after 31 days' exposure to decreased pH. However, the phagocytosis rate of hemocytes was significantly decreased in Cd-exposed oysters at pH 7.60 compared to individuals from other treatments (Fig. 1d).

3.2. mRNA expression of immune-related genes

Despite the suppression of hemocyte function, the expression of TLR signaling pathway-related genes was generally stimulated in Cd-exposed oysters at pH 7.60 (Fig. 2a–e). Significant stimulation in the mRNA expression of IκB, Rel, Myd88 and TRAF3 were observed in Cd-exposed oysters at pH 7.60 compared with other treatments (Fig. 2a, b, e, f). In addition, there was significant increase in the expression of Toll-1 transcripts in Cd-exposed oysters at pH 7.60 compared to non Cd-exposed individuals and Cd-exposed individuals at pH 8.10 (Fig. 2a). However, significant inhibition in the mRNA expression of Toll-1 was observed in oysters at pH 7.60 without Cd exposure compared to individuals at pH 7.80 (Fig. 2d).

In addition, the expressions of two immune factors (TNF and integrin beta-1B) were stimulated under both OA and Cd exposure (Fig. 2h and i). However, the expression of antioxidant enzyme EcSOD transcripts decreased in response to elevated pCO_2 and Cd exposure (Fig. 2g). The expression of HSP90 was increased in non-Cd-exposed oysters at pH 7.80 compared to other OA groups yet was decreased following exposure to the combination of increased pCO_2 and Cd (Fig. 2j). Significant interaction between OA and Cd on most of the investigated genes (Toll-1, MyD88, TRAF3, SOD, HSP90 and TNF) was observed in two-way ANOVA results (Table 3).

3.3. Principal component analysis (PCA)

PCA showed that 81.47% of total variance was explained by the two principal components (Fig. 3). PC1 accounted for 66.35% of the total variance, and the most distinct response referred to the separation between the Cd-OA and other treatments. PC2 explained 15.12% of the total variance, and separated decrease pH (pH 7.80 and pH 7.60) from pH 8.10 (Fig. 3). The PCA analysis results strongly indicate the positive correlation between OA-Cd exposure and stimulated hemocyte DNA damage, apoptosis rate and ROS production, as well as stimulated immune-related gene expression (IκB, Rel, Myd88 and TRAF3, TNF and integrin beta-1B).

4. Discussion

In the present study, without Cd exposure, elevated pCO_2 alone did not have a significant impact on the phagocytic activity of hemocytes but induced the increase in the apoptosis rate and ROS production after exposure for 31 days. Meanwhile, Cd leads to decreased phagocytosis rate and increased ROS production, DNA damage and apoptosis of oyster hemocytes. The results of the hemocyte function in present study suggested that there was a mild negative effect of OA on the immune function compared to the Cd exposure. Furthermore, significant interaction of OA and Cd exposure was observed on the immune parameters (ROS production and DNA damage), indicating that elevated pCO_2 enhanced the immunosuppressive effects of Cd in *C. gigas*. We suppose the synergetic inhibition effect of OA and Cd on the oyster immune system could be attributed to the increased oxidative stress caused by those two stressors. An earlier proteomics study on the adult oyster *Crassostrea virginica* has suggested that the increased oxidative stress by OA may possibly be due to CO_2 reacting with ROS to form other additional radicals, or due to induced Fenton reaction by lower pH values through affecting the activity of the electron transport chain [35]. Similarly, oxidative stress are often implicated in Cd toxicity [36–38]. In

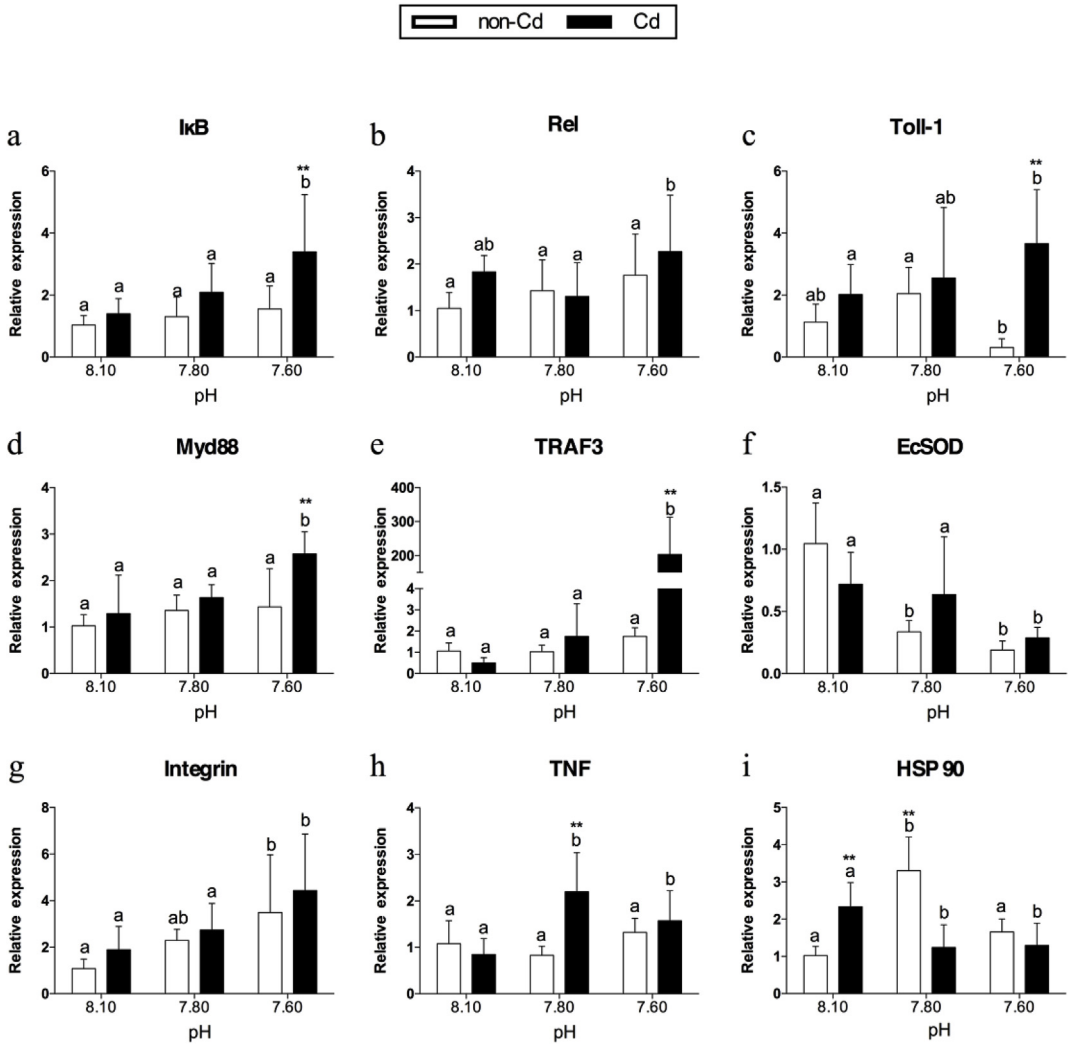

Fig. 2. The mRNA expression profiles of immune-related genes in the hemocytes of oysters post elevated pCO_2 and Cd exposure. a—IκB, b–Rel, c–Toll-1, d–Myd88, e–TRAF3, f–EcSOD, g–Integrin, h–TNF, i–HSP 90. Each bar represented mean ± SD (n = 6). Different letters indicated significant differences among treatments at the same concentration of Cd exposure ($p < 0.05$). * indicated significant difference between non-Cd exposure and Cd exposure at the same pH level. ** indicated extremely significant difference between non-Cd exposure and Cd exposure at the same pH level.

the present study, the depressed expression of EcSOD transcripts also indicated the failure of the antioxidant defense system in oysters. Previous studies have also suggested the correlation between DNA damage and oxidative stress in marine invertebrates under exposure to seawater acidification or Cd [39,40]. Moreover, the increased oxidative stress in Cd- and/or OA-exposed oysters might associate with the increased DNA damage in this study.

[41] has found that the elevated CO_2 could activate the mitochondria-CgsAC pathway of apoptosis in oyster *C. gigas*. Also [17], found that disturbed cellular energy balance could lead to cadmium-induced apoptosis in oyster hemocytes. Accordingly, stimulated hemocyte apoptosis rate was found in OA or Cd exposed oysters in this study. Furthermore, the increased apoptosis rate perhaps associated with increased ROS production, as ROS production could act as a signaling molecules in the induction of apoptosis [38,42]. Moreover, previous study has found decreased hemocyte viability in organisms under Cd or OA exposure [14,43], which perhaps linked with the observed phagocytosis rate reduction in oysters after exposure to Cd alone or combined with OA in this study. In general, our results suggested that the increased oxidative stress caused by OA and/or Cd exposure might lead to the malfunction of the oyster immune system.

In this study, an up-regulation of the TLR signaling pathway was observed in oysters exposed to Cd at pH 7.6. However, our data showed an increase in the expression of two immune factors (TNF and integrin beta-1B). Generally, there is significant interaction between OA and Cd on the mRNA expression of most of the immune-related genes investigated in this study, which suggested much higher stress level for oysters under the combined exposure to OA and Cd compared with each single stressor.

To summarize, our results strongly demonstrate that ocean acidification aggravated the toxicity of environmental relevant concentrations of Cd on hemocytes of *C. gigas*, as revealed by the synergistic effect of these two factors on ROS production and DNA damage of oyster hemocytes. We suppose that the increased Cd accumulation in oysters exposed to OA discovered by our previous research might contribute to the increased immune toxicity of Cd under acidified seawater [44]. However, from the molecular level, the expression of TLR pathway-related genes were generally stimulated in Cd exposed oysters at pH 7.60, and the expression of two immune effectors (TNF and integrin beta-1B) were stimulated at both OA and Cd exposure. Even though the immune TLR pathway was stimulated in oysters to counteract the immunosuppression caused by acidified seawater and Cd, depressed hemocyte function perhaps sensitized oysters to upcoming pathogen infection. This study further confirms the hypothesis that organisms may be more vulnerable to multiple environmental stressors than single stressor alone.

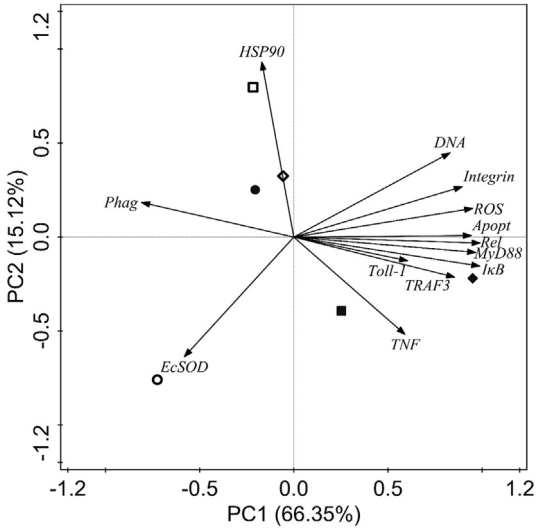

Fig. 3. Principle component analysis (PCA) ordination biplot of ocean acidification and/or Cd treatments for *C. gigas,* using data of cellular and molecular hemocyte responses at day 31 (○: pH 8.10 ●: Cd × pH 8.10 □: pH 7.80 ■: Cd × pH 7.80 ◊: pH 7.60 ◆: Cd × pH 7.60). Abbreviation: Phag, phagocytosis; ROS, ROS production; DNA, DNA damage; Apopt, apoptosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2018.07.055.

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