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Effect of cadmium on the defense response of Pacific oyster *Crassostrea gigas* to *Listonella anguillarum* challenge*

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Abstract Heavy metal pollution can affect the immune capability of organisms. We evaluated the effect of cadmium (Cd) on the defense responses of the Pacific oyster *Crassostrea gigas* to *Listonella anguillarum* challenge. The activities of several important defensive enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), acid phosphatase (ACP), Na^+, K^+-ATPase in gills and hepatopancreas, and phenoloxidase-like (POL) enzyme in hemolymph were assayed. In addition, the expression levels of several genes, including heat shock protein 90 (HSP90), metallothionein (MT), and bactericidal/permeability increasing (BPI) protein were quantified by fluorescent quantitative PCR. The enzyme activities of SOD, ACP, POL, and GPx in hepatopancreas, and the expression of HSP90 were down-regulated, whereas GPx activity in the gill, Na^+, K^+-ATPase activities in both tissues, and MT expression was increased in Cd-exposed oysters post *L. anguillarum* challenge. However, BPI expression was not significantly altered by co-stress of *L. anguillarum* infection and cadmium exposure. Our results suggest that cadmium exposure alters the oysters' immune responses and energy metabolism following vibrio infection.

Keyword: cadmium; *Listonella anguillarum*; *Crassostrea gigas*; defensive enzyme; gene expression

1 INTRODUCTION

In recent years, intensive anthropogenic activity around the Bohai Sea has resulted in severe pollution from heavy metals, including cadmium, lead, mercury, and arsenic (Zhang, 2001). Cadmium, a non-essential metal element for organisms, has become increasingly prevalent because of unregulated discharges from zinc mining operations along the Bohai coast (Zhang, 2001). Cadmium concentrations in the tidal zone of the Bohai Sea are ~20 mg/kg in the sediment, which is 100× higher than background levels (Qin et al., 2006). Furthermore, Cd concentrations of up to 50 μg/L have been recorded in heavily polluted estuaries or harbors (Chester, 1990; Zhang, 2001). Cadmium is toxic to organisms and interferes with a number of physiological processes by causing oxidative stress, metabolic disturbance, and hemocyte apoptosis (Sokolova et al., 2004; Li et al., 2011; Zhang et al., 2011; Sokolova et al., 2012). Given these effects and the high concentrations in Bohai Bay, there are likely significant, but currently undocumented, environmental effects.

In addition to being stressed by heavy metal contaminants, marine organisms in the Bohai sea are also exposed to a variety of pathogens, including trematodes and *vibrios*. *Vibrios* are relatively common in estuarine environments and are one of the primary pathogens infecting aquatic organisms (Deng et al., 1992; Flick, 2007). *Listonella* (=*Vibrio*) *anguillarum* (Thompson et al., 2011) was identified as the causative agent of mass mortality in oysters. The complex interaction between vibrio infections and exposure to

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environmental pollutants results in constant outbreaks of oyster vibriosis and has a significant impact on oyster production throughout the world (Deng et al., 1992; Hauton et al., 2000; Flick, 2007).

The Pacific oyster *Crassostrea gigas*, is an economically important shellfish in the coastal provinces of North China and is widely cultured along the Bohai coast (Pauley et al., 1988). Because of its high tolerance to contaminants, *C. gigas* is thought to be an ideal sentinel for pollution monitoring programs (Bouilly et al., 2006; Bado-Nilles et al., 2010). However, few studies have evaluated the influence of cadmium exposure on the immune responses of the Pacific oyster to *L. anguillarum* challenge. Because of the presence of both cadmium pollution and *vibrio* pathogens in the marine environment, there is a need to determine the sublethal effects of cadmium exposure on the immune response of *C. gigas* to *L. anguillarum* challenge.

The immune response of mollusks involves a series of cytoprotective measures to minimize the adverse effects of external stressors. Excessive ROS induced by heavy metal contaminants and pathogen infection causes oxidative damage to cell components such as lipids, proteins, and nucleic acids (Kannan and Jain, 2000; Pena-Llopis et al., 2003). Antioxidant enzymes, including SOD and GPx, play an important role in removing ROS (Jing et al., 2007; Jo et al., 2008). In addition, heat shock proteins (HSPs) and metallothioneins (MTs) represent two forms of stress-defense proteins that maintain cellular homeostasis (Wang et al., 2009; Zhang and Zhang, 2012). The activity of acid phosphatase (ACP) and phenoloxidase-like (POL) enzymes is often used to evaluate the immune state of mollusks (Verma et al., 1980; Cong et al., 2008). The bactericidal/permeability increasing protein (BPI) plays a critical role in the defense against Gram-negative bacteria (Elsbach and Weiss, 1993). In addition, Na^+, K^+-ATPase is an important membrane enzyme responsible for energy expenditure during the immune response and detoxification (Jorgensen and Pedersen, 2001). Our objective was to determine the effect of cadmium exposure on the defense responses of the Pacific oyster to *L. anguillarum* invasion. The Pacific oysters were pre-exposed to cadmium and then co-challenged with *L. anguillarum*. The activities of antioxidant enzymes (SOD, GPx), energy metabolism-related enzyme (Na^+, K^+-ATPase), immune-related enzymes (ACP, POL), and the expression profiles of defense-related genes (HSP 90, MT and BPI) were subsequently measured.

2 MATERIAL AND METHOD

2.1 Animals and treatments

L. anguillarum M3 was cultured and harvested as described previously (Cong et al., 2008). The concentration in the final exposure experiment was 5×10^6 CFU/mL (Brown, 1981). To minimize trauma to the oysters, *L. anguillarum* was added to the seawater following pre-exposure to cadmium instead of being injected into the muscle.

One hundred and eighty *C. gigas* (mean weight=54.1 g) were purchased from an unpolluted culture facility (0.1–0.2 μg cadmium/L seawater) on Yangma Island near Yantai City. The animals were acclimated in aerated seawater (25±2°C, salinity of 32; collected from an unpolluted area) in plastic tanks under laboratory conditions for 14 d and fed with the *Isochrysis galbana*. Seawater was changed entirely daily. After acclimation, the oysters were divided into three groups, each consisting of three tanks. The first group were held in normal seawater throughout the experiment (control). The second group (*La*-exposed group) were held in normal seawater for the first 48 h, then challenged with *L. anguillarum* (5×10^6 CFU/mL). The third group (Cd+*La*-exposed group) were exposed to Cd^{2+} (20 μg/L) throughout the experiment and challenged with *L. anguillarum* (5×10^6 CFU/mL) after 48 h. The concentration of Cd used in the experiment (20 μg/L) is similar to that at several points along the Bohai coast and has been used by many researchers to study the adverse effects of cadmium on the mollusks (Zhang, 2001; Wu and Wang, 2011; Liu et al., 2012). When appropriate, Cd^{2+} and *L. anguillarum* were added to the tanks daily following seawater exchange. There was no mortality during the experiment. Five oysters were removed randomly from each group and dissected immediately at time 0 (time of first exposure to Cd in group 3), 48 (time of initial exposure to *L. anguillarum* in groups 2 and 3), 52, 56, 60, 72, and 96 h. The gill and hepatopancreas tissues were flash-frozen in liquid nitrogen after dissection. A slice of hepatopancreas was removed and immediately immersed into Trizol reagent (Invitrogen) for RNA extraction. The hemolymph was sampled from the pericardium of oysters using a syringe. All the samples were stored at -80°C before further processing.

2.2 Enzyme assays

The gill and hepatopancreas tissues were ground in

Table 1 The primers* and amplicons of cDNA sequences from *C. gigas*

Gene	GenBank accession #	Forward primer (5′–3′)	Reverse primer (5′–3′)	Length of the amplicon (bp)
HSP90	EF687776	TACTCCGCCTACTTGGTTGC	GGGATAGCCGATGAACTGAC	237
MT	AJ243263, AJ242657, AJ297818	ATGTGTCTGCTCTGATTCGTGTC	ACTTGACTTTGCAGCCTGAAC	98
BPI	AY165040, FJ669296–FJ669322	GTCGAACCAAAACTTAATGAACTC	TGCAATCAAAAGTGTGTCCTTG	114
β-actin	AF026063	GCCCTGGACTTCGAACAA	CGTTGCCAATGGTGATGA	100

* Primers specific to the selected genes were used to amplify products (90 to 300 bp in length) to check mRNA expression of the target genes.

liquid nitrogen, and the enzymatic activities of SOD, GPx, Na^+, K^+-ATPase, and ACP were assayed using a multiskan spectrum microplate spectrophotometer (Infinite M200, TECAN) according to the manufacturer's protocols (Jiancheng, Nanjing, China). The hemolymph was centrifuged at 3 000 r/min at 4°C for 5 min, after which the supernatant was collected. L-DOPA (Sigma) was used as the substrate to assay POL activity, as described by Cong et al. (2008). Total protein concentration was measured using a BCA assay kit (Pierce). All enzyme activities were measured in triplicate and expressed as unit activity per mg of protein, where one unit represents the change in absorbance mg/protein.

2.3 RNA extraction and gene quantification

Total RNA from the hepatopancreas was isolated following the manufacturer's directions (Invitrogen), and first-strand cDNA synthesis was carried out according to M-MLV RT Usage information (Promega) using oligo (dT)-adaptor (5′-CTCGAGATCGATGCGGCCGCT$_{17}$-3′) as the primer and the DNase I-treated (Promega) total RNA as template. Gene-specific primers for HSP90, MT, BPI, and the internal control β-actin (Elsbach and Weiss, 1993; Ivanina et al., 2010) were used to amplify amplicons specific for *C. gigas*. The sequences of primers and the length of amplicons are given in Table 1. The fluorescent real-time quantitative PCR amplifications were carried out in triplicate, and the program and computational method were the same as described previously (Livak and Schmittgen, 2001; Cong et al., 2008).

2.4 Statistical analysis

Differences between the control, *La*-, and Cd+*La*-exposed groups were assessed using a one-way ANOVA followed by Tukey's test. All tests were conducted using Minitab 15.0 (TechMax. Information Technology Co. Ltd.). All data are presented as the mean±standard deviation (n=5 individuals).

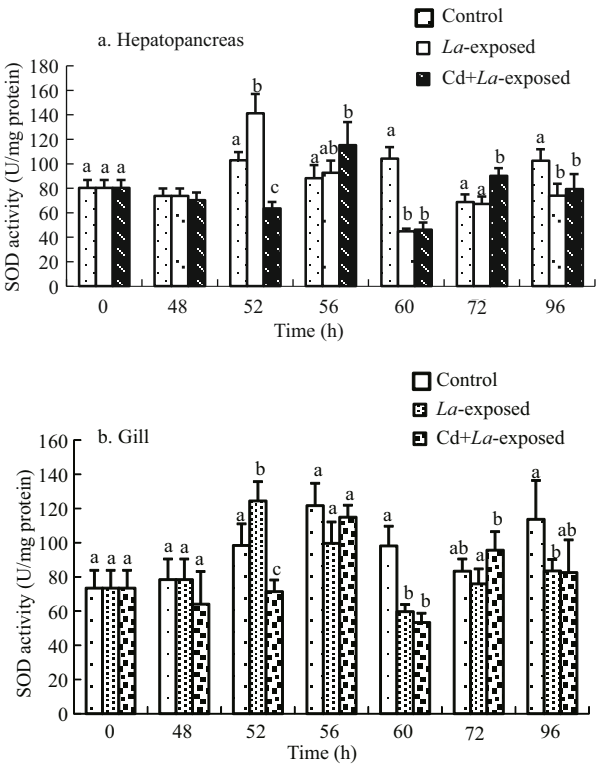

Fig.1 SOD enzyme activity in the hepatopancreas (a) and gills (b)

Data are expressed as mean±SD (n=5 individuals) and were analyzed by one-way ANOVA. Columns with different letters are significantly different (P<0.05).

3 RESULT AND DISCUSSION

3.1 Enzyme activities

Both cadmium exposure and *L. anguillarum* challenge induce an imbalance of oxygen metabolism in living organisms by formation of reactive oxygen species (ROS) (Fridovich, 1975; Sokolova et al., 2004; Labreuche et al., 2006; Zhang et al., 2011). Among the antioxidant enzymes, SOD is the first defensive enzyme to neutralize superoxide and helps to protect against cell destruction (Fridovich, 1975). In our study, pre-exposure to cadmium did not affect SOD activity in either the hepatopancreas or gill tissues (Fig.1). SOD activity was significantly

elevated in the hepatopancreas of the *La*-exposed group at 52, 72, and 96 h but significantly lower than the control at 56 and 60 h (P<0.01). Conversely, SOD activity was lower than the control group at 52, 60, and 96 h (P<0.01) in the Cd+*La*-exposed group but higher at 56 h (P<0.05) and 72 h (P<0.01). In gill tissue, SOD activity was significantly higher in the *La*-exposed group at 52 h (P<0.01), but significantly lower at 60 and 96 h (P<0.01) in comparison with the control. The changes in gill SOD activity in the Cd+*La*-exposed group were similar to those in the hepatopancreas. SOD activity was elevated at 52 h, post *L. anguillarum* exposure, and peaked at 56 h. Our results suggest that exposure of cadmium inhibits the SOD response against vibrio challenge.

The pattern of change in GPx differed between the hepatopancreas and gill tissues (Fig.2). In the hepatopancreas, GPx activity was significantly higher in the *La*-exposed oysters than in the Cd+*La*-exposed oysters (P<0.01) at 52 h, and both were significantly higher than in the control group (P<0.01). At 60 h, GPx activity was significantly lower in the two challenged groups relative to the control group (P<0.01). Pre-exposure to cadmium induced a significant decrease in gill GPx activity at 48 h (P<0.01) compared with the other groups, suggesting that gill GPx activity was highly susceptible to cadmium exposure. Our observations are consistent with a previous study which concluded that inhibition of GPx was more pronounced in the gill than in the hepatopancreas (Cossu et al., 1997). We hypothesize that this difference reflects the gills' role at the interface between the internal and external environments. Thus, adverse changes in the ambient environment likely elicit a more rapid response in the gills. The addition of *L. anguillarum* at 60 h, resulted in a significant increase in GPx activity in the Cd+*La*-group relative to the other groups (P<0.01), and a decrease in *La*-exposed oysters. Thus, pre-exposure to cadmium appears to induce an increase in GPx activity to combat subsequent exposures to *L. anguillarum*. This may be explained by the hormesis phenomenon, in which pre-exposure to a low dose of toxicant enhances the production of defense substances to additional stimuli (Calabrese, 2008; Mattson, 2008). Alternatively, the response may represent a compensatory mechanism for the Cd+*La*-exposed oysters to neutralize excess superoxide given the inhibition of SOD activity, particularly as GPx operates downstream of SOD during the detoxification of reactive oxygen species (McCord, 1993). At 96 h,

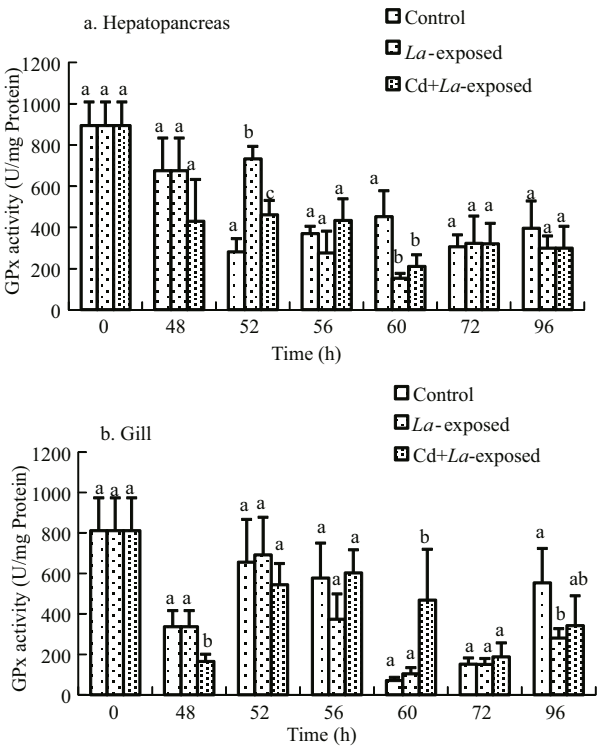

Fig.2 GPx enzyme activity in the hepatopancreas (a) and gills (b)

Data are expressed as mean±SD (n=5 individuals) and were analyzed by one-way ANOVA. Columns with different letters are significantly different (P<0.05).

GPx activity was significantly lower in the gill of the *La*- and Cd+*La*-exposed oysters than in the control group, but were not different from each other.

ACP and POL are enzymes that play a role in the cellular immunity of invertebrates. ACP activity was significantly higher in the hepatopancreas of the *La*- and Cd+*La*-exposed oysters than in the control group at 60 h (P<0.01, Fig.3). Conversely, *L. anguillarum* challenge resulted in a decrease in gill ACP activity in the Cd+*La*-exposed oysters at 52 h (P<0.05), and in the *La*-exposed oysters later at 56 h (P<0.05). This suggests that pre-exposure to cadmium attenuates the ability of ACP to defend against pathogen invasions. Phenoloxidase (or phenoloxidase-like) is responsible for the synthesis of melanin and plays an important role in encapsulation of foreign materials such as pathogens (Aladaileh et al., 2007; Luna-Acosta et al., 2010). POL activity was significantly higher in the Cd+*La*-exposed group than in the control after 56 h (P<0.05) whereas there was no difference between the *La*-exposed and control groups. Thus, POL activity wasn't affected by vibrio challenge alone, though pre-exposure to Cd resulted in down-regulation of POL after the addition of vibrio. The

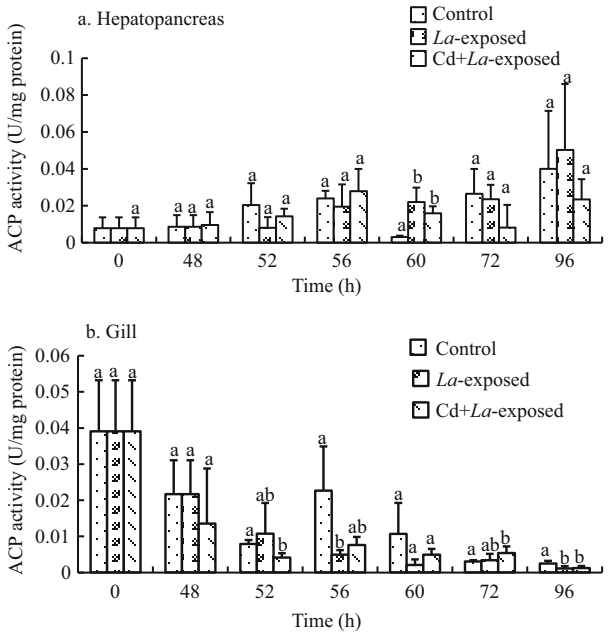

Fig.3 ACP enzyme activity in the hepatopancreas (a) and gills (b)

Data are expressed as mean±SD (n=5 individuals) and were analyzed by one-way ANOVA. Columns with different letters are significantly different (P<0.05).

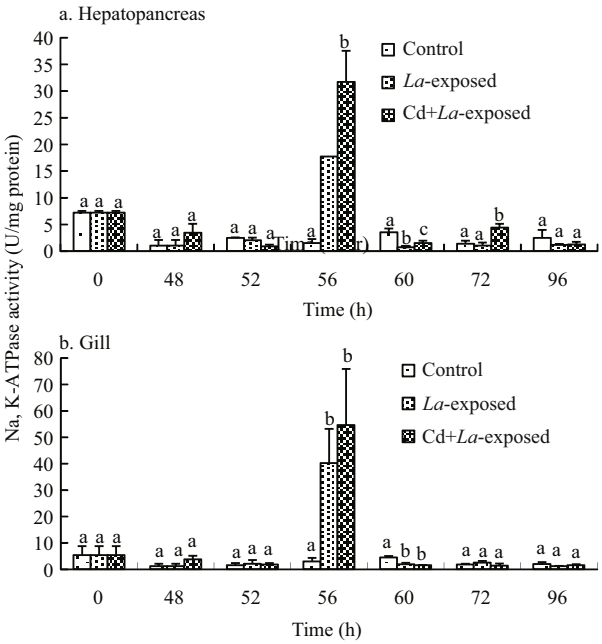

Fig.4 Na^+, K^+-ATPase activity in the hepatopancreas (a) and gills (b)

Data are expressed as mean±SD (n=5 individuals) and were analyzed by one-way ANOVA. Columns with different letters are significantly different (P<0.05).

rapid decrease in ACP activity and reduction in POL activity suggests that Cd exposure reduces cellular immunity in oysters.

The Na^+, K^+-ATPase plays a role in a number of processes, including energy expenditure and ion regulation (Jorgensen and Pedersen, 2003). We observed a significant reduction in Na^+, K^+-ATPase in the hepatopancreas of both of *La*- and Cd+*La*-exposed groups relative to the control (P<0.01, Fig.5). Furthermore, Na^+, K^+-ATPase activity was lower in the Cd+*La*-exposed group than in the *La*-exposed group (P<0.01) at 52 h. Interestingly though, the levels increased by 18 times and 31 times in the *La*- and Cd+*La*-exposed groups at 56 h and were both higher than the control (P<0.01). Furthermore, the levels were higher in the Cd+*La*-exposed oysters than in the *La*-exposed group (P<0.05, Fig.5). After 60 h, the Na^+, K^+-ATPase activity had decreased and was significantly lower in both the *La*- and Cd+*La*-exposed groups compared with the control (P<0.01), with levels in the *La*-exposed oysters being lower than those in the Cd+*La*-exposed group (P<0.05). Taken together, these observations suggested that exposure to cadmium altered the Na^+, K^+-ATPase reponse to *L. anguillarum* challenge. At 72 h, Na^+, K^+-ATPase activity was significantly higher in the Cd+*La*-stressed oysters than in the remaining two groups (P<0.01).

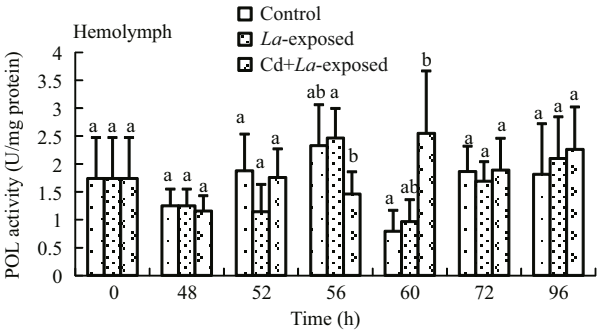

Fig.5 POL enzyme activity in hemolymph

Data are expressed as mean±SD (n=5 individuals) and were analyzed by one-way ANOVA. Columns with different letters are significantly different (P<0.05).

The pattern of change in Na^+, K^+-ATPase activity was similar between the gill and hepatopancreas. The up-regulation of Na^+, K^+-ATPase activity in the gill and hepatopancreas of Cd+*La*-exposed oysters suggests that more energy is expended resisting the co-stress of *L. anguillarum* infection and Cd exposure. This reflects a disturbance of energy metabolism caused by Cd-exposure. Our observations are consistent with previous studies which showed that cadmium had a singificant effect on energy demand and supply, and resulted in increased energy expenditure (Cherkasov et al., 2006; Ivanina et al., 2010).

Table 2 Expression levels of several genes at different time-points

Genes	Exposures	0 h	48 h	52 h	56 h	60 h	72 h	96 h
HSP90	Control	1.23±0.69	1.56±1.34	15.08±11.15	6.14±2.31	16.51±4.22	11.96±4.65	20.56±7.98
	La-exposed	1.23±0.69	1.45±1.26	9.95±7.09	14.19±11.86	394.89±57.20*	49.52±5.45*	30.60±19.30
	Cd+*La*-exposed	1.23±0.69	23.37±9.01**	4.16±1.65	13.65±9.04	121.44±33.11**	51.65±16.72*	24.42±13.40
MT	Control	1.02±0.18	0.44±0.09	4.75±2.35	0.62±0.31	0.92±0.02	0.60±0.13	0.35±0.17
	La-exposed	1.02±0.18	1.07±0.21	2.59±1.59	0.57±0.52	28.04±7.59*	6.27±1.16*	0.43±0.08
	Cd+*La*-exposed	1.02±0.18	1.15±0.20	1.16±0.14	2.79±2.04	37.63±13.04*	27.24±9.35**	1.00±0.47
BPI	Control	1.35±1.09	0.82±0.19	0.72±0.18	0.36±0.18	0.57±0.26	0.43±0.28	0.63±0.06
	La-exposed	1.35±1.09	0.79±0.21	10.03±6.20	0.29±0.04	62.78±21.09*	8.00±0.52*	0.72±0.25
	Cd+*La*-exposed	1.35±1.09	5.64±4.29	0.20±0.06	1.15±1.03	31.43±13.41*	6.23±0.93*	0.52±0.05

Asterisks represent a significant difference compared with the control. *: $P<0.05$; **: $P<0.01$.

3.2 Gene quantification

The variation in gene expression levels is illustrated in Table 2. We observed a significant increase in HSP90 expression in the Cd+*La*-exposed group at 48 h ($P<0.01$) following a single exposure to cadmium. The addition of vibrio resulted in significant increases in HSP90 expression in the *La*- and Cd+*La*-exposed groups ($P<0.01$) at 60 h. The relative expression of HSP90 in the *La*-exposed group was almost 3-times higher than in the Cd+*La*-exposed group ($P<0.01$). At 72 h, there was a slight increase in HSP90 expression in both the *La*- and Cd+*La*-exposed groups ($P<0.01$). HSPs are a class of molecular chaperones and are responsible for protein folding, assembly, and translocation in prokaryotic and eukaryotic organisms (Zhang and Zhang, 2012). *L. anguillarum* challenge was assoicated with a significantly lower HSP90 response in Cd+*La*-exposed group than in *La*-exposed group. This suggests that Cd-exposure inhibits the response of HSP90 following *L. anguillarum* invasion. The partial depression of HSP90 up-regulation suggests limitation of cytoprotection by molecular chaperones, which is likely to cause an increase in the number of misfolded proteins and subsequently result in disease or death (Barral et al., 2004; Masters and O'Neill, 2011).

MTs are a cysteine-rich protein that can help to protect against metal toxicity and oxidative stress (Fang et al., 2009; Wang et al., 2009). The expression of MT was significantly higher in both the *La*- and Cd+*La*-exposed groups than in the control group at 60 and 72 h ($P<0.01$). In addition, MT was higher in the Cd+*La*-exposed group than in the *La*-exposed group at 72 h ($P<0.01$). The higher number of MT mRNAs expressed in the Cd+*La*-exposed group after the addition of *L. anguillrum* suggests that Cd-exposure and vibrio challenge had an interactive effect on MT induction. Interestingly, the induction of MT after heavy metal exposure appears to decrease expression of most immune effectors in invertebrates. This phenomena is thought to represent a trade-off (Brulle et al., 2007) and may also explain why enhanced MT expression was accompanied with detrimental immune effects in the Cd-exposed oysters following exposure to *L. anguillarum*.

BPI expression was significantly higher in the *La*- and Cd+*La*-exposed groups compared with the control group at 60 and 72 h ($P<0.01$). However, there was no difference between the former two groups suggesting that Cd-exposure has little effect on BPI mRNA expression.

Based on our results, we hypothesize that exposure to cadmium modulates the oysters' antioxidant system (Fridovich, 1975; Sokolova et al., 2004; Zhang et al., 2011) which in turn slows the clearance rate of ROS produced by other external stimuli (Labreuche et al., 2006) as well as *L. anguillarum* infection. Excessive ROSs are harmful to immune cells, and cause disturbances in immune response and energy metabolism (Kannan and Jain, 2000; Pena-Llopis et al., 2003; Giannapas et al., 2012). The inhibition of POL and ACP activity likely reflects a reduced capability for pathogen encapsulation and destruction. Furthermore, the partial depression of the molecular chaperon (HSP90) likely results in an increase in misfolded proteins and leads to abnormalities in immune cells (Zhang and Zhang, 2012). Prior research suggests that cadmium challenge disturbs energy balance in oysters (Ivanina et al., 2010). In our study, pre-exposure to cadmium study increased energy consumption during the defense response against vibrio.

4 CONCLUSION

We evaluated the effect of cadmium exposure on a range of defense parameters in the Pacific oyster to *Listonella anguillarum* challenge. The gill SOD, POL, and ACP activity, hepatopancreas GPx activity, and HSP90 expression were down-regulated, in Cd-exposed oysters compared with *La*-exposed oysters when both were challenged by *L. anguillarum*. Conversely, gill GPx activity, Na^+, K^+-ATPase, and MT expression were up-regulated. Thus, cadmium exposure modulates the defense response of oysters by depressing the immune response and increasing energy expenditure. Our results provide insight into the joint effects of heavy metal contaminants and pathogens on sublethal responses in shellfish.

References

Aladaileh S, Nair S V, Birch D, Raftos D A. 2007. Sydney rock oyster (*Saccostrea glomerata*) hemocytes: morphology and function. *J. Invertebr. Pathol*., **96**: 48-63.

Bado-Nilles A, Renault T, Faury N, Le Floch S, Quentel C, Auffret M, Thomas-Guyon H. 2010. *In vivo* effects of LCO soluble fraction on immune-related functions and gene transcription in the Pacific oyster, *Crassostrea gigas* (Thunberg). *Aquat. Toxicol*., **97**: 196-203.

Barral J M, Broadley S A, Schaffar G, Hartl F U. 2004. Roles of molecular chaperones in protein misfolding diseases, *Semin. Cell Dev. Biol*., **15**: 17-29.

Bouilly K, Gagnaire B, Bonnard M, Thomas-Guyon H, Renault T, Miramand P, Lapègue S. 2006. Effects of cadmium on aneuploidy and hemocyte parameters in the Pacific oyster, *Crassostrea gigas*. *Aquat. Toxicol*., **78**: 149-156.

Brown C. 1981. A prodiginine pigment toxic to embryos and larvae of *Crassostrea virginica*. *J. Invertebr. Pathol*., **38**: 281-293.

Brulle F, Mitta G, Leroux R, Lemière S, Leprêtre A, Vandenbulcke F. 2007. The strong induction of metallothionein gene following cadmium exposure transiently affects the expression of many genes in *Eisenia Fetida*: a trade-off mechanism? *Comp. Biochem. Physiol. C*, **144**: 334-341.

Calabrese E J. 2008. Hormesis: why it is important to toxicology and toxicologists. *Environ. Toxicol. Chem*., **27**: 1 451-1 474.

Cherkasov A S, Biswas P K, Ridings D M, Ringwood A H, Sokolova I M. 2006. Effects of acclimation temperature and cadmium exposure on cellular energy budgets in a marine mollusk *Crassostrea virginica*: linking cellular and mitochondrial responses. *J. Exp. Biol*., **209**: 1 274-1 284.

Chester R. 1990. Marine Geochemistry, 1st edn. Allen & Unwin, London.

Cong M, Song L, Wang L, Zhao J, Qiu L, Li L, Zhang H. 2008. The enhanced immune protection of zhikong scallop *Chlamys farreri* on the secondary encounter with *Listonella anguillarum*. *Comp. Biochem. Physiol. B*, **151**: 191-196.

Cossu C, Doyotte A, Jacquin M C, Babut M, Exinger A, Vasseur P. 1997. Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels and lipid peroxidation in freshwater bivalves, *Unio turmidus* as biomarkers of aquatic contamination in field studies. *Ecotox. Environ. Saf.*, **38**: 122-131.

Deng H, Sui X L, Wang Z S. 1992. Primary study on vibrios of the Pacific oyster, *Crassostrea gigas*. *Fish. Sci*., **11**: 1-4. (in Chinese with English abstract)

Elsbach P, Weiss J. 1993. Bactericida/permeablity increasing protein and host defense against gram-negative bacteria and endotoxin. *Curr. Opin. Immunol*., **5**: 103-107.

Fang Y, Yang H, Wang T, Liu B, Zhao H, Chen M. 2009. Metallothionein and superoxide dismutase responses to sublethal cadmium exposure in the clam *Mactra veneriformis*. *Comp. Biochem. Physiol. C*, **151**: 325-333.

Flick J G J. 2007. Pathogenic vibrios in shellfish. *Global Aquaculture Advocate*, **10**: 46-48.

Fridovich I. 1975. Superoxide dismutases. *Annu. Rev. Biochem*., **44**: 147-159.

Giannapas M, Karnis L, Dailianis S. 2012. Generation of free radicals in haemocytes of mussels after exposure to low molecular weight PAH components: immune activation, oxidative and genotoxic effects. *Comp. Biochem. Physiol. C*, **155**: 182-189.

Hauton C, Hawkins L E, Hutchinson S. 2000. The effects of salinity on the interaction between a pathogen (*Listonella anguillarum*) and components of a host (*Ostrea edulis*) immune system. *Comp. Biochem. Physiol. B*, **127**: 203-212.

Ivanina A V, Eilers S, Kurochkin I O, Chung J S, Techa S, Piontkivska H, Sokolov E P, Sokolova I M. 2010. Effects of cadmium exposure and intermittent anoxia on nitric oxide metabolism in eastern oysters, *Crassostrea virginica*. *J. Exp. Biol*., **213**: 433-444.

Jing G, Li Y, Xie L, Zhang R. 2007. Different effects of Pb^{2+} and Cu^{2+} on immune and antioxidant enzyme activities in the mantle of *Pinctada fucata*. *Environ. Toxicol. Phar*., **24**: 122-128.

Jo P G, Choi Y K, Choi C Y. 2008. Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, *Crassostrea gigas* in response to cadmium exposure. *Comp. Biochem. Physiol. C*, **147**: 460-469.

Jorgensen P L, Pedersen P A. 2001. Structure-function relationships of Na^+, K^+, ATP, or Mg^{2+} binding and energy transduction in Na, K-ATPase. *Biochim. Biophys. Acta*, **1505**: 57-74.

Kannan K, Jain S K. 2000. Oxidative stress and apoptosis. *Pathophysiology*, **7**: 153-163.

Labreuche Y, Lambert C, Soudant P, Boulo V, Huvet A, Nicolas J. 2006. Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. *Dev.*

Comp. *Immunol*., **8**: 2 715-2 724.

Li R, Zhou Y, Ji J, Wang L. 2011. Oxidative damages by cadmium and the protective effects of low-molecular-weight chitosan in the freshwater crab (*Sinopotamon yangtsekiense* Bott 1967). *Aquac*. *Res*., **42**: 506-515.

Liu F, Wang D Z, Wang W X. 2012. Cadmium-induced changes in trace element bioaccumulation and proteomics perspective in four marine bivalves. *Environ*. *Toxicol*. *Chem*., **31**: 1 292-1 300.

Livak K J, Schmittgen T D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, **25**: 402-408.

Luna-Acosta A, Bustamante P, Godefroy J, Fruitier-Arnaudin I, Thomas-Guyon H. 2010. Seasonal variation of pollution biomarkers to assess the impact on the health status of juvenile Pacific oysters *Crassostrea gigas* exposed in situ. *Environ*. *Sci*. *Pollut*. *Res*. *Int*., **17**: 999-1 008.

Masters S L, O'Neill L A J. 2011. Disease-associated amyloid and misfolded protein aggregates activate the inflammasome. *Trends Mol*. *Med*., **17**: 276-282.

Mattson M P. 2008. Hormesis defined. *Ageing Res*. *Rev*., **7**: 1-7.

McCord J M. 1993. Human disease, free radicals, and the oxidant/antioxidant balance. *Clin*. *Biochem*., **26**: 351-357.

Sokolova I M, Evans S, Hughes F M. 2004. Cadmium-induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition. *J*. *Exp*. *Biol*., **207**: 3 369-3 380.

Sokolova I M, Frederich M, Bagwe R, Lannig G, Sukhotin A A. 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Mar*. *Environ*. *Res*., **79**: 1-15.

Pena-Llopis S, Ferrando M D. 2003. Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by NH_2-acetylcysteine. *Aquat*. *Toxicol*., **65**: 337-360.

Pauley G B, Van Der Raay B M, Troutt D. 1988. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Pacific Northwest): Pacific oyster. *Fish and Wildlife Service Biological Report*, **82**(11.85): 28.

Qin Y, Meng W, Zheng B, Su Y. 2006. Heavy metal pollution in tidal zones of Bohai Bay using the dated sediment cores. *J*. *Environ*. *Sci*., **18**: 610-615. (in Chinese with English abstract)

Sies H. 1993. Strategies of antioxidant defense. *Eur*. *J*. *Biochem*., **215**: 213-219.

Thompson F L, Thompson C C, Dias G M, Naka H, Dubay C, Crosa J H. 2011. The genus *Listonella* Macdonell and Colwell 1986 is a later heterotypic synonym of the genus *Vibrio* Pacini 1854 (approved lists 1980)—a taxonomic opinion. *Int*. *J*. *Syst*. *Evol*. *Micr*., **61**: 3 023-3 027.

Verma S R, Rani S, Dalela R C. 1980. Effects of phenol and dinitrophenol on acid and alkaline phosphatases in tissues of a fish, *Notopterus notopterus*. *Arch*. *Environ*. *Contam*. *Toxicol*., **9**: 451-459.

Wang L, Song L, Ni D, Zhang H, Liu W. 2009. Alteration of metallothionein mrna in bay scallop *Argopecten irradians* under cadmium exposure and bacteria challenge. *Comp*. *Biochem*. *Physiol*. *C*, **149**: 50-57.

Wu H, Wang W X. 2011. Tissue-specific toxicological effects of cadmium in green mussels (*Perna viridis*): nuclear magnetic resonance-based metabolomics study. *Environ*. *Toxicol*. *Chem*., **30**: 806-812.

Zhang L, Liu X, You L, Zhou D, Yu J, Zhao J, Feng J, Wu H. 2011. Toxicological effects induced by cadmium in gills of manila clam *Ruditapes philippinarum* using NMR-based metabolomics. *Clean Soil Air Water*, **39**: 989-995.

Zhang X. 2001. Investigation of pollution of Pb, Cd, Hg, as in sea water and deposit of Bohai Sea area. *Heilongjiang Environ*. *J*., **25**: 87-90. (in Chinese with an English abstract)

Zhang Z, Zhang Q. 2012. Molecular cloning, characterization and expression of heat shock protein 70 gene from the oyster *Crassostrea hongkongensis* responding to thermal stress and exposure of Cu^{2+} and malachite green. *Gene*, **497**: 172-180.