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Short communication

Proteomic responses reveal the differential effects induced by cadmium in mussels *Mytilus galloprovincialis* at early life stages

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ABSTRACT

Cadmium (Cd) has become an important metal contaminant and posed severe risk on the organisms in the coastal environments of the Bohai Sea. Marine mussel *Mytilus galloprovincialis* is widely distributed along the Bohai coast and consumed as seafood by local residents. Evidences indicate that the early stages of marine organisms are more sensitive to metal contaminants. In this study, we applied two-dimensional electrophoresis-based proteomics to characterize the biological effects of Cd (50 $\mu g\ L^{-1}$) in the early life stages (D-shape larval and juvenile) of mussels. The different proteomic responses demonstrated the differential responsive mechanisms to Cd exposure in these two early life stages of mussels. In details, results indicated that Cd mainly induced immune and oxidative stresses in both D-shape larval and juvenile mussels via different pathways. In addition, the significant up-regulation of triosephosphate isomerase and metallothionein confirmed the enhanced energy demand and mobilized detoxification mechanism in D-shape larval mussels exposed to Cd. In juvenile mussels, Cd exposure also induced clear apoptosis. Overall, this work suggests that Cd is a potential immune toxicant to mussel *M. galloprovincialis* at early life stages.

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1. Introduction

Because of the industrial discharge from numerous metal smelteries, cadmium (Cd) has become an important metal contaminant in the marine and coastal environments along the Bohai Sea. Sun et al. reported that Cd posed prominent risk on the marine organisms and ecosystem due to the high concentrations of Cd in the sediments from the Bohai coast [1]. Our recent research indicated that the dominant species, shrimp *Crangon affinis*, was contaminated by Cd from the Yellow River Estuary along the Bohai Sea [2]. As it is known, Cd can induce multiple adverse effects in organisms. It may induce oxidative stress in organisms by producing excessive reactive oxygen species [3]. Additionally, Cd can disturb the energy metabolism in clam *Ruditapes philippinarum* indicated by the enhanced anaerobic metabolism [4]. Since Cd has become one of the most severe metal contaminants in the Bohai Sea, it is necessary to characterize the biological effects of Cd in marine animals.

The traditional biological approaches basically focus on the test of specific responses, such as the selected gene expression patterns or enzyme activities, to characterize the biological effects of environmental stressors in organisms [5,6]. In recent years, the "–omic" approaches including genomics, transcriptomics, proteomics and metabolomics have been widely used in environmental biology [7–11]. Among these approaches, two-dimensional electrophoresis (2DE)-based proteomics is useful to present complex biologically functional protein networks [12]. Not only is proteomics a powerful tool for describing complete proteomes at organelle, cell, organ or tissue levels, but it can also be used to detect the minor proteomic responses in biological samples under different conditions [13].

Marine mussels are ubiquitous, sedentary filter-feeders that play critical roles in maintaining marine ecosystem health. This species can accumulate high amounts of contaminants from marine

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and coastal environments and therefore is used to monitor marine and coastal environmental contaminants [14]. The mussel *Mytilus galloprovincialis* is widely distributed along the Bohai coast and popularly consumed as seafood by local residents. In this study, mussel *M. galloprovincialis* was used as the experimental animal to investigate the biological effects of Cd. However, evidences indicate that the earlier life stages are more sensitive than the adults to environmental stressors [15]. To compare the differential proteomic responses in the early life stages of mussels *M. galloprovincialis*, the D-shape larval and juvenile mussels were exposed to Cd with an environmentally relevant concentration (50 μg L^{-1}) for 48 h. The 2DE-based proteomics was conducted on the mussel samples to elucidate the differential biological effects of Cd in these two early life stages of mussels *M. galloprovincialis*.

2. Materials and methods

2.1. Larvae breeding and experimental design

Adult mussels *M. galloprovincialis* reached sexual maturity were collected in April 2015 from a pristine site (Yantai, China). All mussels were transported to the laboratory and acclimatized in aerated natural seawater (salinity 31 psu) at 21 °C for 7 days. After acclimatization, these mussels were kept in air in dark place for 2 h. Then each mussel was put into a beaker containing warmer seawater (24 °C). After 2 h, the majority of mussels came to ovulation and spermiation. The 500 mesh sieve screen was used to filter impurities. Then the sperms and eggs were quickly collected, respectively, to fertilize. All the fertilized eggs were then transferred into 50 L of normal filtered seawater (FSW) in a bucket. Continuous aeration was conducted during incubation. After approximately 48 h, the majority of fertilized eggs developed into D-shape larvae (Fig. 1A). Then the D-shape larval mussels were divided into two groups (control and Cd exposure) each containing 6 buckets (30 L) with a density of ~25 D-shape larval mussels per milliliter. The environmentally relevant concentration (50 μg L^{-1}) of Cd was selected for the exposures of D-shape larval mussels *M. galloprovincialis*. During the acclimatization and exposure periods, all the larvae were kept under a photoperiod of 12 h light and 12 h dark, and fed with the *Chlorella vulgaris* daily. After exposure for 48 h, all the larvae (~6.0–7.5 $\times$ 10^5 individuals) from each two bucket were immediately filtered out by 500 mesh sieve screen and collected into one D-shape larval mussel sample. These D-shape larval mussel samples were quickly snap-frozen in liquid nitrogen and stored at −80 °C.

Thirty juvenile mussels (length: ~1.0 cm, Fig. 1B) were purchased from local culturing farm in Yantai, China. After transported to the culture laboratory, the juvenile mussels were allowed to acclimate in aerated seawater (21 °C, 31 psu, collected from pristine environment) in the laboratory for 7 d and fed with the *Chlorella vulgaris Beij* at a ration of 2% tissue dry weight daily. After acclimatization, the mussels were randomly divided into two groups (control and Cd exposure) containing fifteen individuals in 20 L of aerated seawater. The same concentration (50 μg L^{-1}) of Cd was used for the exposure. After exposure for 48 h, the whole soft tissues from juvenile mussels were dissected quickly. The samples of whole soft tissues were flash-frozen in liquid nitrogen and stored at −80 °C before further procedures. During the exposure periods, all the experimental conditions for juvenile mussels were completely identical to those for D-shape larval mussels.

2.2. Protein extraction of mussel samples

Total protein extraction was performed based on previous studies with some modifications [16,17]. Briefly, the mussel

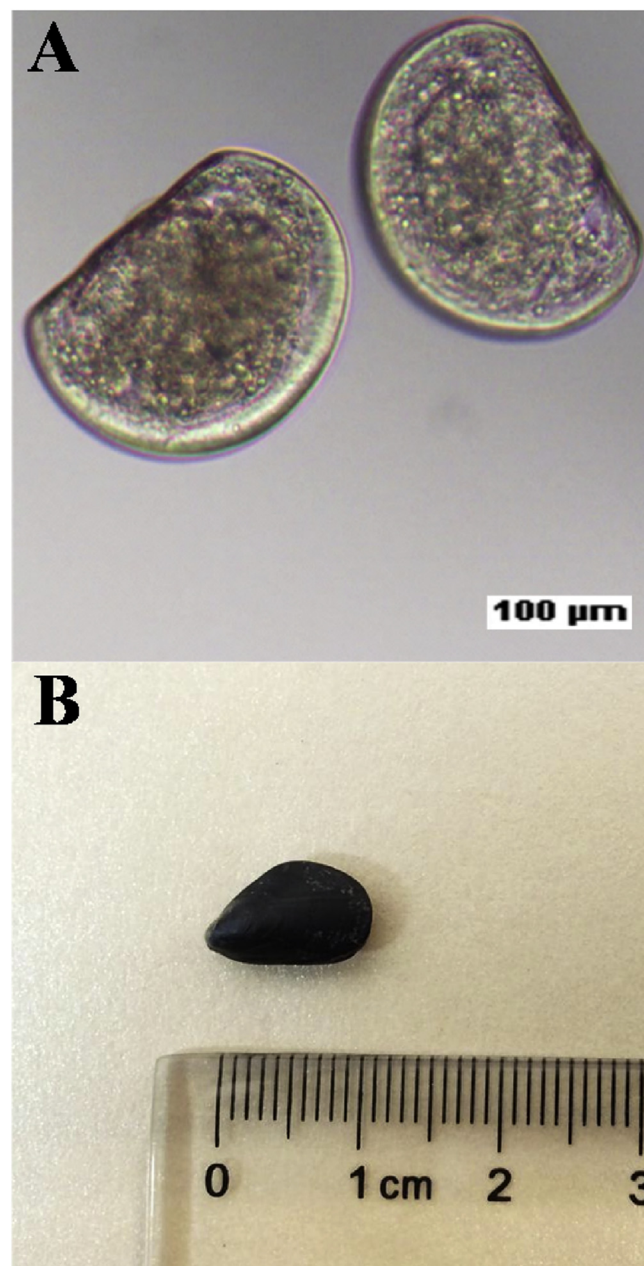

Fig. 1. The mussels *M. galloprovincialis* at early life stages used in this study, (A) D-shape larval and (B) juvenile.

samples were homogenized quickly on ice with 1 mL of TRIzol reagent and centrifuged at 12000 *g* for 5 min at 4 °C. The supernatant was added with 200 μL of chloroform before shaking vigorously for 3 min and precipitating for 3 min. The mixture was centrifuged at 12000 *g* for 15 min at 4 °C, and its upper aqueous layer was discarded. A volume of 300 μL of absolute ethyl alcohol was added and the mixture was allowed to stand for 3 min at room temperature before being centrifuged at 2000 *g* for 5 min at 4 °C. The phenol/ethanol supernatant was precipitated for 30 min at room temperature by the addition of 750 μL of isopropanol prior to centrifugation at 14000*g* for 10 min at 4 °C. The pellets were washed with 1 mL of ethanol (v/v 95%) and centrifuged at 14000 *g* for 10 min at 4 °C. This procedure was repeated twice. The pellets were solubilized in the lysis buffer (7 M urea; 2 M thiourea; 4% m/V CHAPS; 65 mM DTT and 0.2%W/V Bio-lyte buffer) and then incubated for

3 h at room temperature. The homogenate was centrifuged at 15000 *g* for 10 min and the supernatant was applied to electrophoresis. The total concentrations of proteins were determined by Protein Assay Kit of TianGen.

2.3. Two-dimensional gel electrophoresis, image acquisition and data analysis

For the first dimension (IEF), 130 μg of protein was loaded onto IPG strips with a linear pH gradient from 4 to 7 (Immobiline Drystrip TM 24 cm, GE Healthcare, USA) The isoelectric focusing gel solution (7 M urea, 2 M thiourea, 4% m/v CHAPS, 65 mM DTT, 0.001% m/v bromophenol blue and 0.2% W/V Bio-lyte buffer). IEF was conducted at 20 °C with an Ettan IPGphor3 system for a total of 85858 Vh (active rehydration was carried out at 30 V for 12 h, followed by 100 V for 5 h, 500 V for 1 h, 1000 V for 1 h, and a linear increase of voltage to 8000 V for 11 h and stand by 500 V for the second dimension).

After the first dimension, all the strips were placed in equilibration buffer (0.05 M Tris–HCl, pH 8.8; 6 M urea; 30% glycerol; 2% (w/v) SDS; containing 1% (w/v) DTT) and were slowly shaken for 15 min. The strips were then incubated for another 15 min in the equilibration buffer with 2.5% (w/v) iodoacetamide without DTT. The second dimension was conducted on 12.5% SDS-PAGE gels using the Ettan DALTsix system. After electrophoresis, the gels were silver stained by following the method of Mortz and Gharahdaghi [18,19]. Images were captured by ImageScanner III and spots were quantitatively analyzed using ImageMaster 2D Platinum 7.0. For all the matched spots, only protein spots with significant changes of at least 1.2-fold, and deemed significant by Student's *t*-test at a level of 95% were accepted as differentially expressed proteins.

2.4. In gel digestion and MS analysis

In gel digestion was performed according to Katayama et al. [20]. After being completely dried, the samples were re-suspended with 5 μL of 0.1% TFA followed by mixing in 1:1 ratio with a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 50% acetonitrile [21]. One microliter of mixture was analyzed by an ABI 4800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, Foster City, USA), data were acquired in a positive MS reflector using a CalMix5 standard to calibrate the instrument (ABI4800 Calibration Mixture). Both the MS and MS/MS data were integrated and processed using the GPS Explorer V3.6 software (Applied Biosystems, USA) with default parameters. Proteins were successfully identified based on 95% or higher confidence interval of their scores in the MASCOT V2.4 search engine (Matrix Science Ltd., London, U.K.). The following parameters were used in the search: NCBInr Metazoa (Animals) (2861494 sequences) database; trypsin as the digestion enzyme; one missed cleavage site; partial modifications of cysteine carbamidomethylation and methionine oxidization; no fixed modifications; 0.15 Da for precursor ion tolerance and 0.25 Da for fragment ion tolerance. Individual ions scores >40 indicate identity or extensive homology ($p < 0.05$).

3. Results and discussion

Proteomic responses were determined by 2DE-based proteomics in Cd-treated mussel samples at D-shape larval and juvenile stages, respectively (Fig. 2). A total of 16 spots resolved in 2DE gels were differentially expressed (>1.2 folds, $P < 0.05$). Fig. 2 reveals the differential protein spots in Cd-treated mussels *M. galloprovincialis* at D-shape larval and juvenile stages, respectively. For either D-shape larval mussels or juvenile mussels, all the differentially expressed proteins induced by Cd treatment in all three biological replicates of silver-stained gels were analyzed by MALDI-TOF/TOF mass spectrometry. The information of these proteins is summarized in Tables 1 and 2.

In Cd-treated D-shape larval mussel samples, a total of 8 protein spots were significantly differentially expressed, including 5 up-regulated and 3 down-regulated proteins. These proteins were involved in protein homeostasis and immune defensive system (putative ubiquitin thioesterase, ubiquitin, 60S ribosomal protein L13A, ribosomal protein S7, DNA-directed RNA polymerase II and macrophage migration inhibitory factor), energy metabolism (triosephosphate isomerase) and detoxification (metallothionein). Eight differentially expressed proteins (4 up-regulated and 4 down-regulated) were discovered in Cd-treated juvenile mussel samples. These proteins were related to immune and oxidative responses (apextrin-like protein, profilin and NADH dehydrogenase mitochondrial-like), protein translocation (signal sequence receptor beta-like protein) and signal transduction (peptidylprolyl isomerase A, guanine nucleotide-binding protein G(o) subunit alpha, sorting nexin 6 and collagen alpha-6(VI) chain isoform X3).

In D-shape larval mussels, five proteins involved in protein homeostasis were found in response to Cd exposure. Both ubiquitin thioesterase and ubiquitin are regulatory proteins involved in the nonlysosomal degradation of intracellular proteins related to apoptosis induced by stressors [22]. In addition, ubiquitin-related proteins are responsive to immune and oxidative stress, which was observed in bacterial challenged mussel *M. galloprovincialis* and acute free radical-treated mussel *Mytilus edulis* [23,24]. Ribosomal proteins perform the crucial function of protein biosynthesis and have been also recognized as immunogenic proteins [23]. RNA polymerase II is responsible for the transcription of genes coding for protein in the nucleus [25]. Apparently, the alteration of these five proteins indicated that Cd exposure disturbed protein homeostasis and induced immune and oxidative stresses in D-shape larval mussels. Macrophage migration inhibitory factor is an evolutionarily ancient cytokine involved in the host immune response to stress and inflammation, which was confirmed in another marine bivalve, scallop *Chlamys farreri* [26]. This protein was down-regulated in D-shape larval mussels exposed to Cd, which suggested that Cd might inhibit the immune system in D-shape larval mussels. This finding is consistent with above-mentioned immune stress induced by Cd in D-shape larval mussels. Triosephosphate isomerase is an essential enzyme playing an important role in the glycolysis for efficient energy production. The significant up-regulation of triosephosphate isomerase suggested the enhanced energy demand in Cd-treated D-shape larval mussels. Similarly, Cd exposure also significantly enhanced energy demand in adult green mussel *Perna viridis* resulting in reduced level of glucose [10]. Metallothioneins are low molecular proteins rich in SH groups with high affinity for binding heavy metal ions to detoxify the toxicity induced by heavy metals in animals [27]. Therefore, one metallothionein was significantly up-regulated in D-shape larval mussels.

In the Cd-treated juvenile mussels, interestingly, the proteomic responses were completely different compared with those in Cd-treated D-shape larval mussels, which demonstrated the differential responsive mechanisms to Cd exposure in the early life stages of mussels *M. galloprovincialis*. These differential responsive mechanisms may take into consideration behavioral, morphological, physiological and biochemical characteristics which should be different between life stages. Obviously, the D-shape larval and juvenile mussels have different characteristics, such as sizes, organ systems and the time taken for Cd to reach target sites, which might essentially perform differential responsive mechanisms to deal with Cd exposure. Apextrin has an important function in neutralization of pathogens and is immunologically responsive to

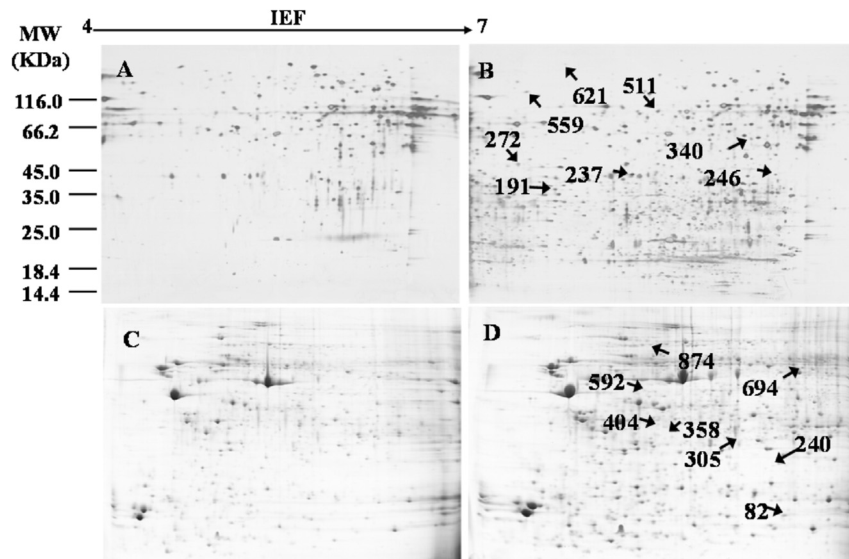

Fig. 2. Representative 2-D gels of proteins extracted from tissues of D-shape larval and juvenile mussels *Mytilus galloprovincialis*. First dimension was performed using 130 μg of total soluble proteins on linear gradient IPG strips with pH 4–7. In the second dimension, 12.5% SDS-PAGE gels were used and proteins were visualized using silver staining. (A) control of D-shape larval mussel group, (B) Cd-exposed D-shape larval mussel group, (C) control of juvenile mussel group and (D) Cd-exposed juvenile mussel group. Arrows show protein spots that changed significantly from Cd-exposed groups compared to the control groups.

Table 1
List of protein spots that differentially expressed in D-shape larval mussels *Mytilus galloprovincialis* exposed to Cd (50 μg L^{-1}).

Match ID[a]	Category and name[b]	Species	gi number[c]	MW/kDa[d]	PI	Protein score[e]	SC[f]	PN[g]	Ratio[h]
191	Triosephosphate isomerase, partial	*Mytilus edulis*	46909461	16.42	4.93	194	27%	1	4.28
237	Putative ubiquitin thioesterase L96	*Crassostrea gigas*	405969289	189.30	5.07	40	10%	1	4.10
246	60S ribosomal protein L13A	*Mytilus edulis*	76573377	23.76	10.61	88	11%	2	2.46
272	Ribosomal protein S7	*Argopecten irradians*	22758886	22.40	9.97	80	10%	2	2.60
340	DNA-directed RNA polymerase II subunit RPB4	*Crassostrea gigas*	405964873	16.74	4.72	63	18%	1	2.20
511	MT-10-IV = 10 kda class I metallothionein	*Mytilus edulis*	455751	8.17	7.62	119	33%	1	−2.55
559	Macrophage migration inhibitory factor	*Mytilus galloprovincialis*	344944171	12.76	6.28	44	8%	1	−2.97
621	Ubiquitin	*Crassostrea hongkongensis*	283459000	15.02	9.94	72	17%	2	−4.86

[a] Assigned spot ID as indicated in Fig. 2.
[b] Identification of differentially expressed proteins in D-shape larval mussels *M. galloprovincialis* between control and Cd-exposed groups.
[c] Database accession numbers after searching against the NCBInr database.
[d] Experimental mass.
[e] Mascot score reported.
[f] Sequence coverage.
[g] Number of peptide sequences.
[h] Fold changes with significances (>1.2 folds and $p < 0.05$) were calculated using ImageMaster 2D Platinum 7.0.

Table 2
List of protein spots that differentially expressed in juvenile mussels *Mytilus galloprovincialis* exposed to Cd (50 μg L^{-1}).

Match ID[a]	Category and name[b]	Species	gi number[c]	MW/kDa[d]	PI	Protein score[e]	SC[f]	PN[g]	Ratio[h]
82	Profilin	*Crassostrea gigas*	405961157	26.96	8.81	336	24%	2	1.22
240	Peptidylprolyl isomerase A, partial	*Mytilus edulis*	672940978	21.74	5.77	562	28%	3	1.55
305	Signal sequence receptor beta-like protein	*Crassostrea gigas*	40643038	24.16	7.89	49	5%	1	−1.78
358	Apextrin-like protein	*Mytilus galloprovincialis*	339785142	30.55	5.16	231	38%	5	−2.20
404	NADH dehydrogenase mitochondrial-like	*Aplysia californica*	524911939	31.22	5.76	273	36%	4	1.30
592	Guanine nucleotide-binding protein G(o) subunit alpha	*Crassostrea gigas*	405970566	40.98	5.22	235	17%	4	−1.30
694	Sorting nexin 6	*Crassostrea gigas*	405952237	31.11	5.16	503	42%	5	1.74
874	Collagen alpha-6(VI) chain isoform X3	*Papio anubis*	685527479	28.05	4.90	475	30%	4	−1.31

[a] Assigned spot ID as indicated in Fig. 2.
[b] Identification of differentially expressed proteins in juvenile mussels *M. galloprovincialis* between control and Cd-exposed groups.
[c] Database accession numbers after searching against the NCBInr database.
[d] Experimental mass.
[e] Mascot score reported.
[f] Sequence coverage.
[g] Number of peptide sequences.
[h] Fold changes with significances (>1.2 folds and $p < 0.05$) were calculated using ImageMaster 2D Platinum 7.0.

pathogens [28]. In Cd-treated juvenile mussels, one down-regulated apextrin-like protein suggested that Cd exposure might inhibit the immune system in juvenile mussels. Profilin is thought to regulate actin polymerization in response to extracellular signals. As a flavoprotein, NADH dehydrogenase contains iron-sulfur centers and is involved in production of reactive oxygen species in mitochondria [29]. These two proteins, profilin and NADH dehydrogenase, are known to be related to oxidative stress [29,30]. Therefore, the alteration of profilin and NADH dehydrogenase confirmed the oxidative stress induced by Cd in juvenile mussels. Previous studies suggested that signal sequence receptor played a role in protein translocation across the endoplasmic reticulum membrane [31]. However, a subsequent study indicated that signal sequence receptor was either not required for protein translocation or was one of a family of functionally redundant components [32]. Therefore, the reason to explain the down-regulation of signal sequence receptor in Cd-treated juvenile mussels remains unclear. Surprisingly, four proteins (peptidylprolyl isomerase A, guanine nucleotide-binding protein G(o) subunit alpha, sorting nexin 6 and collagen alpha-6(VI) chain isoform X3) involved in signal transduction were significantly altered in Cd-treated juvenile mussels. Among these four proteins, guanine nucleotide-binding protein plays an important role in immune functions [33]. The down-regulation of this protein confirmed the immune stress induced by Cd in juvenile mussels, as marked by the down-regulated apextrin-like protein. Peptidylprolyl isomerase A, sorting nexin 6 and collagen alpha-6(VI) chain isoform X3 are known proteins recognized as the markers of apoptosis [30,34,35]. The alterations of these three proteins suggested that Cd exposure caused apoptosis in juvenile mussels.

In summary, the biological effects of an environmentally relevant concentration of Cd (50 $\mu g\ L^{-1}$) were characterized in the early life stages (D-shape larval and juvenile) of marine mussels *M. galloprovincialis* using 2DE-based proteomics. Basically, the different proteomic responses demonstrated the differential responsive mechanisms in the early life stages of mussels treated with Cd. In details, results indicated that Cd mainly induced immune and oxidative stresses in both D-shape larval and juvenile mussels via different pathways. The significant up-regulation of triosephosphate isomerase and metallothionein confirmed the enhanced energy demand and mobilized detoxification mechanism in D-shape larval mussels exposed Cd. In juvenile mussels, Cd exposure also induced clear apoptosis. Overall, this work suggests that Cd is a potential immune toxicant to mussel *M. galloprovincialis* at early life stages because of their immature immune systems.

Acknowledgments

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