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Toxicological evaluation of two pedigrees of clam *Ruditapes philippinarum* as bioindicators of heavy metal contaminants using metabolomics

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ABSTRACT

Heavy metal pollution has been of great concern in the Bohai marine environment. Manila clam *Ruditapes philippinarum* has been used as a bioindicator in marine toxicology. In this study, NMR-based metabolomics was used to ascertain whether there were significant biological differences between two dominant pedigrees (White and Zebra) of clam and evaluate the suitability of two pedigrees for marine environmental toxicology, together with antioxidant enzymatic analysis. Our results indicated that there were significant biological differences between White and Zebra clams based on the metabolic profiles and antioxidant enzyme activities. In details, the metabolic profiles showed higher levels of amino acids and succinate in Zebra clam digestive glands and higher levels of ATP in White clam digestive glands, respectively. The superoxide dismutase activities in control White and Zebra clam samples were significantly different. Additionally, White clam was more sensitive to Cd based on the significant accumulation of Cd, antioxidant enzymatic alterations and sensitive metabolic changes. Overall, we concluded that White clam could be a preferable bioindicator for marine environmental toxicology.

1. Introduction

Due to the anthropogenic activities, the Bohai Sea has been seriously polluted by diverse heavy metals such as mercury (Hg), lead (Pb), arsenic (As), cadmium (Cd) and zinc (Zn) (Mao et al., 2009). These excessive heavy metals can induce adverse effects to organisms and pose risks on marine environments (Lavery et al., 2009; Romeo et al., 2000). Therefore, it is necessary to carry out biomonitoring for heavy metal pollution in the Bohai Sea. Although mussels and oysters have been frequently used as bioindicators in many countries for marine pollution monitoring in 'Mussel Watch Programs' (Cong et al., 2012; Goldberg, 1975; Regoli and Orlando, 1993; You et al., 2013; Wang et al., 2012; Wang et al., 2013a,b,c; Wei et al., 2014; Wu and Wang, 2010; Zhang et al., 2014), Manila clam *Ruditapes philippinarum* is the preferred sentinel species in 'Mussel Watch Programs' in China due to its wide geographic distribution. As a matter of fact, *R. philippinarum* has been suggested a good bioindicator for the marine heavy metal

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pollution on the basis of high accumulation of various heavy metals including Cd, Zn, Cu, Pb, Fe, Cr, Co and Ni (Ji et al., 2006). Thus *R. philippinarum* has been not only used for heavy metal pollution monitoring, but also widely employed as a suitable bioindicator in marine toxicology (Li et al., 2010; Liu et al., 2013; Matozzo et al., 2004; Wang et al., 2011; Wu and Wang, 2011a; Wu et al., 2011b; Zhao et al., 2010; Zhang et al., 2012). For example, Matozzo et al. (2004) reported the oxidative stresses of 4-nonylphenol in both gills and digestive gland of *R. philippinarum* and suggested that toxicology studies should be carried out at environmentally realistic concentrations of contaminants.

There are two main pedigrees (White and Zebra) of *R. philippinarum* distributed along the Bohai Sea. In the biomonitoring programs and previous aquatic toxicology studies, little attention has been paid on the potential biological differences between various pedigrees of clams. However, some evidences indicated that different pedigrees of clams had differential tolerances to environmental stressors (Yan et al., 2005). Yan et al. (2005) reported that Zebra pedigree of clam had the highest survival rate and tolerance to environmental stressors (e.g., high temperature) than other pedigrees. Hereby, a question has arisen: can we use mixed pedigrees of Manila clams as biomonitor or bioindicator for the heavy metal pollution biomonitoring or toxicology studies? In other words, we should characterize the toxicological indices such as uptake rates, bioaccumulation and efflux rates, biochemical indices (e.g., antioxidant enzyme activity), or molecular biomarkers (e.g., metabolites) of different pedigrees of clams to heavy metal stresses to define a sensitive pedigree of clam as bioindicator for heavy metal monitoring of the Bohai marine environment and toxicology.

Previously, the differential metabolic responses were investigated in gill tissues from various pedigrees of clams exposed to mercury and White clam was recommended to be the preferable bioindicator for marine mercury monitoring (Liu et al., 2011a). However, it is necessary to further study the differential effects of various pedigrees of clams to either non-essential or essential heavy metals. On the basis of metal bioaccumulations, antioxidant enzyme activities and metabolic responses, the further studies can provide more evidences for the selection of one suitable pedigree of clam for heavy metal pollution monitoring and toxicology study. In this work, Cd and Zn were used as non-essential and essential metals for clam exposures, respectively. Cd and Zn are commonly found as contaminants in the Bohai marine and coastal environments (Mao et al., 2009). Here we need to answer three questions. Firstly, are there significant biological differences between two dominant pedigrees (White and Zebra) of clams along the Bohai marine and coastal environments based on the metabolic profiles and antioxidant enzyme activities? Secondly, are the toxicological responses (e.g., metabolic changes, antioxidant enzyme activities and bioaccumulation of heavy metals) different between White and Zebra clams? Thirdly, which pedigree is more sensitive to heavy metal contaminants, Cd and Zn? In order to answer these three questions, the metabolic differences in digestive glands of clams between the control and heavy metal-exposed groups were determined by NMR-based metabolomics that has been widely used in multiple areas, such as drug toxicity, plant sciences and environmental sciences (Feng et al., 2013; Katsiadaki et al., 2010; Santos et al., 2010; Viant et al., 2009; Williams et al., 2011; Wu et al., 2012a,b,c,d; Wu et al., 2013). In addition, the antioxidant enzyme activities (superoxide dismutase, glutathione peroxidase and glutathione S-transferases) and bioaccumulation of Cd and Zn were measured as well.

2. Materials and methods

2.1. Clam exposure

Two hundred adult clams *R. philippinarum* (shell length: 3.4–3.8 cm, $n = 100$ from White and Zebra pedigrees, respectively) were purchased from local culturing farm. The clams were allowed to acclimate in aerated seawater (25 °C, 33 psu, collected from pristine environment in Yangma Island, Yantai) in the laboratory for 1 week and fed with the *Chlorella vulgaris Beij* at a ration of 2% tissue dry weight daily. After acclimatization, the clams were randomly divided into four tanks (one control and three heavy metal exposures). Each tank contained 25 White and 25 Zebra clams which were exposed to dissolved Cd^{2+} (20 μg L^{-1}), Zn^{2+} (50 μg L^{-1}), or a mixture of Cd^{2+} and Zn^{2+} for 48 h. Cadmium and zinc were prepared from $CdCl_2$ and $ZnCl_2$ (analytical grades). The experimental concentrations of Cd^{2+} and Zn^{2+} can be found in heavily polluted sites along the Bohai Sea (Mao et al., 2009). After 48 h of exposure, all the clams were immediately dissected for the digestive gland tissues which were flash frozen in liquid nitrogen, and then stored at −80 °C before metabolite extraction ($n = 10$), antioxidant enzyme activity measurement ($n = 8$) and metal determination ($n = 5$).

2.2. Metabolite extraction

Polar metabolites in digestive gland tissues of clams were extracted by the modified extraction protocol as described previously (Lin et al., 2007; Liu et al., 2011b; Zhang et al., 2011a,b). Briefly, the digestive gland tissue (ca. 100 mg) was homogenized and extracted in 4 mL g^{-1} of methanol, 5.25 mL g^{-1} of water and 2 mL g^{-1} of chloroform. The methanol/water layer with polar metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The extracts of digestive gland tissue were subsequently re-suspended in 600 μL of 100 mM of phosphate buffer (Na_2HPO_4 and NaH_2PO_4, pH 7.0) in D_2O containing 0.5 mM Sodium 3-trimethylsilyl-2,2,3,3-d4-propionate (TSP) as internal reference. The mixture was vortexed and then centrifuged at 3000 × g for 5 min at 4 °C. The supernatant substance (550 μL) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

2.3. High resolution one dimensional ^1H NMR spectroscopy

Extracts of digestive gland tissue from clams were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K) as described previously (Zhang et al., 2011c). Briefly, one-dimensional ^1H NMR spectra were obtained using a 11.9 μs pulse, 6009.6 Hz spectral width, mixing time 0.1 s, and 3.0 s relaxation delay with standard 1D NOESY pulse

sequence, with 128 transients collected into 16, 384 data points. Datasets were zero-filled to 32, 768 points, and exponential line-broadenings of 0.3 Hz were applied before Fourier transformation. All ^{1}H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker). NMR spectral peaks were assigned following tabulated chemical shifts (Zhang et al., 2011d) and using the software, Chenomx (Evaluation Version, Chenomx Inc., Canada).

2.4. *Spectral pre-processing and multivariate data analysis*

One dimensional proton NMR spectra were converted to a format for multivariate analysis using custom-written ProMetab software in Matlab (version 7.0; The MathsWorks, Natick, MA) (Zhang et al., 2011c). Each spectrum was segmented into 0.005 ppm bins between 0.2 and 10.0 ppm with bins from 4.70 to 5.20 ppm (water) excluded from all the NMR spectra. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed (glog) with transformation parameter $\lambda = 1.0 \times 10^{-8}$ (Zhang et al., 2011d) to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks.

The unsupervised pattern recognition method, PCA was used to reduce the dimensionality of the data and summarize the similarities and differences between multiple NMR spectra (Zhang et al., 2011c). The algorithm of this pattern recognition method calculates the highest amount of correlated variation along PC1, with subsequent PCs containing correspondingly smaller amounts of variance. One-way analysis of variance (ANOVA) was conducted on the PC scores from each group to test the statistical significance ($p < 0.05$) of separations. Chenomx software (Evaluation version, Chenomx Inc., Canada) was then used to identify and quantify the potentially significant metabolites between various groups. The metabolite concentrations were normalized to the mass of digestive gland tissue by calculating the concentration of metabolites in each NMR tube.

2.5. *Measurement of antioxidant enzyme activities*

The antioxidant enzyme activities in the digestive gland tissues ($n = 8$) of *R. philippinarum* were assayed using a multiskan spectrum microplate spectrophotometer (Infinite M200, TECAN) according to the manufacturer's protocols of enzyme kits (Jiancheng, Nanjing, China). In this work, the antioxidant enzymes for the activity measurement included superoxide dismutase (SOD, EC 1.15.1.1), glutathione peroxidase (GPx, EC 1.11.1.9) and glutathione S-transferases (GST, EC 2.5.1.18), and all of them were performed in microplates. In details, SOD activity was assayed spectrophotometrically at 550 nm by use of a xanthine and xanthine oxide system. One unit of SOD activity was defined as the amount of SOD required for 50% inhibition of the xanthine and xanthine oxidase system reaction in 1 mL enzyme extract per milligram of protein. GPx was assayed spectrophotometrically by measuring the decrease of enzymatic reaction of GSH at 412 nm. One unit of GPx activity was defined as the decrease of GSH content in system of enzymatic reaction of per milligram of protein per minute, by excluding non-enzymatic reaction. GST activity was measured by evaluating the conjugation of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB). One unit of GST activity was defined as the amount of enzyme depleting 1 μmol GSH per milligram of protein per minute. Protein concentration was determined by the Coomassic brilliant blue G-250 dye-binding method with bovine serum albumin as standard (Bradford, 1976; Liu et al., 2011b). All the enzyme activities were expressed as U mg^{-1} protein.

2.6. *Cadmium and zinc concentrations in digestive gland tissues*

The digestive gland tissue samples ($n = 5$) of *R. philippinarum* were dried at 80 °C to the constant weights. The dried tissues were digested in concentrated nitric acid (70%, Fisher Scientific) using a microwave digestion system (CEM, MAR5). The samples were heated in the microwave oven (program: heating to 200 °C and holding at 200 °C for 15 min). All completely digested samples were diluted appropriately with ultra pure water for the quantification of Cd and Zn using ICP-MS technique (Agilent 7500i, Agilent Technologies Co. Ltd, USA).

2.7. *Statistical analysis*

Data of metabolite concentrations, antioxidant enzyme activities and metal concentrations in the digestive gland tissues were expressed as the mean ± standard deviation. The data were statistically analyzed using the statistical software, Statistics toolbox in Matlab (version 7.0; The MathsWorks, Natick, MA). One way ANOVA with Tukey's test was conducted on the data, and significant difference was defined at $p < 0.05$.

3. Results

3.1. *Metabolic differences in digestive glands between White and Zebra clams*

A representative ^{1}H NMR spectrum of digestive gland tissue extracts from a White clam is shown in Fig. 1. Several metabolite classes were identified, including amino acids (branched chain amino acids: valine, leucine and isoleucine, alanine, aspartate, glutamate, glycine, etc.), energy storage compounds (ATP, glucose and glycogen), Krebs cycle intermediates (succinate and fumurate), phosphagen (phosphocholine) and osmolytes (betaine, taurine, hypotaurine and homarine).

PCA was initially applied to the ^{1}H NMR spectral data from both White control and Zebra control clam samples to examine the significance of metabolic profiles in the digestive glands (Fig. 2). From the PC scores plot (PC1 vs. PC2), the White control and Zebra control samples were significantly ($p < 0.05$) separated along PC2 axis (Fig. 2). The significant metabolic differences were detected after statistical analysis. Basically, there were several significantly ($p < 0.05$) abundant metabolites including valine, isoleucine, leucine and histidine in digestive gland from Zebra clams (Table 1). The metabolic profile of White clam samples showed high level of ATP compared with that of Zebra clam samples.

Table 1 – Metabolite concentrations (μmol g^{-1} wet tissue) in digestive gland tissues from *R. philippinarum* exposed to heavy metals. Values are presented as mean ± standard deviation. Statistical significances ($p < 0.05$, *, $p < 0.01$, **) between control and heavy metal-exposed *R. philippinarum* samples were determined by one–way ANOVA. $^{\#}$ means that the statistical significance approached 0.05 ($p < 0.1$). s = singlet, d = doublet, t = triplet, m = multiplet, ABX = complex multiplet involving 2 protons (A and B) and a heavy atom (X).

Metabolites	Chemical shift	White clam				Zebra clam			
	(ppm, multiplicity)	Control	Cd	Zn	Cd + Zn	Control	Cd	Zn	Cd + Zn
Valine	1.05 (d)	0.11 ± 0.02	0.18 ± 0.03**	0.16 ± 0.03*	0.16 ± 0.04*	0.14 ± 0.02	0.15 ± 0.04	0.13 ± 0.02	0.14 ± 0.02
Isoleucine	1.00 (d)	0.07 ± 0.02	0.11 ± 0.02**	0.10 ± 0.02$^{\#}$	0.11 ± 0.02$^{\#}$	0.09 ± 0.01	0.09 ± 0.03	0.09 ± 0.01	0.08 ± 0.01
Leucine	0.94 (t)	0.11 ± 0.02	0.15 ± 0.03*	0.16 ± 0.03*	0.16 ± 0.03*	0.14 ± 0.02	0.12 ± 0.05	0.12 ± 0.03	0.14 ± 0.02
Threonine	1.34 (d)	0.20 ± 0.06	0.24 ± 0.05	0.24 ± 0.04	0.27 ± 0.06	0.22 ± 0.04	0.20 ± 0.05	0.21 ± 0.05	0.19 ± 0.04
Alanine	1.48 (d)	2.67 ± 0.62	3.51 ± 1.24	3.85 ± 1.19	2.57 ± 0.62	3.27 ± 0.58	3.45 ± 0.69	3.16 ± 0.53	4.79 ± 1.34*
Arginine	1.70 (m)	1.14 ± 0.16	1.48 ± 0.35$^{\#}$	0.96 ± 0.44	1.20 ± 0.27	1.24 ± 0.45	1.22 ± 0.28	1.19 ± 0.33	1.77 ± 0.31$^{\#}$
Glutamate	2.05 (m)	1.67 ± 0.24	2.13 ± 0.50$^{\#}$	1.97 ± 0.29	1.63 ± 0.32	1.90 ± 0.15	1.91 ± 0.18	1.96 ± 0.32	2.18 ± 0.23
Glutamine	2.14 (m)	0.57 ± 0.14	0.54 ± 0.13	0.60 ± 0.10	0.53 ± 0.12	0.51 ± 0.16	0.66 ± 0.12	0.61 ± 0.12	0.68 ± 0.21
Acetoacetate	2.26 (s)	0.23 ± 0.08	0.35 ± 0.10$^{\#}$	0.31 ± 0.09	0.36 ± 0.11$^{\#}$	0.27 ± 0.07	0.24 ± 0.13	0.38 ± 0.22	0.40 ± 0.11
Succinate	2.41 (s)	0.27 ± 0.34	1.04 ± 0.86$^{\#}$	0.50 ± 0.28	0.53 ± 0.44	0.82 ± 0.36	0.68 ± 0.69	0.61 ± 0.27	0.41 ± 0.22
Hypotaurine	2.66 (t)	3.12 ± 0.11	2.32 ± 0.67*	2.92 ± 0.84	2.67 ± 0.23	3.17 ± 0.97	3.05 ± 0.76	3.71 ± 0.74	3.11 ± 0.68
Aspartate	2.68 (ABX)	1.71 ± 0.38	1.65 ± 0.38	1.32 ± 0.36	1.12 ± 0.16*	1.36 ± 0.27	1.29 ± 0.36	1.11 ± 0.11	1.75 ± 0.41
Phosphocholine	3.21 (s)	0.66 ± 0.16	0.57 ± 0.23	0.63 ± 0.28	0.85 ± 0.17	0.61 ± 0.21	0.64 ± 0.17	0.78 ± 0.34	0.55 ± 0.20
Taurine	3.45 (t)	38.56 ± 1.60	38.95 ± 1.85	37.48 ± 2.17	35.77 ± 1.74*	37.45 ± 2.11	36.78 ± 3.16	39.14 ± 2.32	36.71 ± 2.64
Betaine	3.27 (s)	31.29 ± 1.59	30.99 ± 1.94	30.85 ± 1.94	27.72 ± 2.46*	31.00 ± 1.57	30.26 ± 2.81	28.69 ± 1.41	29.44 ± 0.58
Glycine	3.57 (s)	4.76 ± 1.37	4.65 ± 1.38	5.57 ± 1.09	4.07 ± 1.41	4.13 ± 0.71	3.96 ± 1.02	4.62 ± 1.44	6.89 ± 1.91*
Glucose	4.64 (d), 5.23 (d)	2.60 ± 0.88	4.33 ± 2.18	3.25 ± 0.17	5.73 ± 1.04**	2.46 ± 0.38	3.93 ± 0.89	4.58 ± 1.61*	5.22 ± 2.06*
Homarine	4.37 (s)	9.10 ± 2.32	8.28 ± 0.99	8.07 ± 2.01	7.07 ± 1.37	7.74 ± 1.78	5.96 ± 1.07$^{\#}$	6.88 ± 1.30	8.56 ± 0.33
ATP	6.14 (d)	0.43 ± 0.03	0.37 ± 0.04*	0.48 ± 0.06	0.34 ± 0.10$^{\#}$	0.30 ± 0.07	0.44 ± 0.11*	0.41 ± 0.20	0.48 ± 0.10**
Fumarate	6.52 (s)	0.012 ± 0.003	0.012 ± 0.005	0.011 ± 0.004	0.008 ± 0.003	0.009 ± 0.003	0.010 ± 0.002	0.009 ± 0.004	0.010 ± 0.004
Tyrosine	6.91 (d)	0.08 ± 0.01	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.03	0.08 ± 0.02	0.09 ± 0.01
Histidine	7.10 (d)	0.06 ± 0.01	0.08 ± 0.03	0.07 ± 0.01*	0.07 ± 0.01	0.09 ± 0.01	0.06 ± 0.02$^{\#}$	0.07 ± 0.03	0.08 ± 0.01

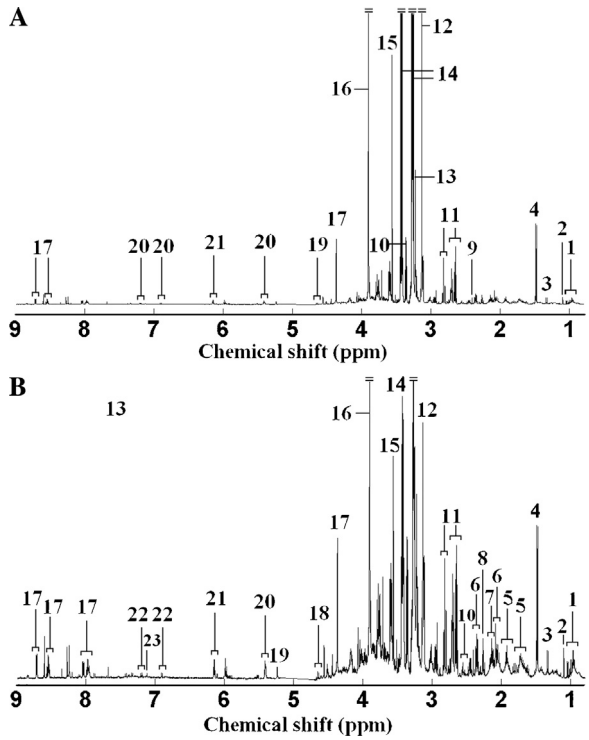

Fig. 1 – A representative 1-dimensional 500 MHz ^{1}H NMR spectrum of digestive gland tissue extracts from a White clam of control group in original (A) and generalized log transformed ($\lambda = 1.0 \times 10^{-8}$) (B) forms. Keys: (1) branched chain amino acids: isoleucine, leucine and valine, (2) unknown, (3) threonine, (4) alanine, (5) arginine, (6) glutamate, (7) glutamine, (8) acetoacetate, (9) succinate, (10) hypotaurine, (11) aspartate, (12) malonate, (13) phosphocholine, (14) taurine, (15) glycine, (16) betaine, (17) homarine, (18) β–glucose, (19) α–glucose, (20) glycogen, (21) ATP, (22) tyrosine and (23) histidine.

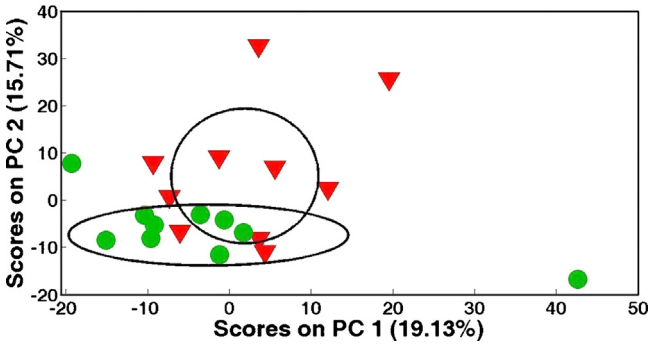

Fig. 2 – Principal components analysis (PCA) showing significant separation ($p < 0.05$) between control White (●) and Zebra (▼) clam samples. Ellipses represented mean ± standard deviation of PC scores along both PC1 and PC2 axes for each group.

3.2. *Metabolic changes induced by heavy metals in digestive glands from White and Zebra clams*

PCA was performed on the ^{1}H NMR spectral data sets generated from control and heavy metal-exposed groups of clams from White and Zebra pedigrees, respectively (Figs. 3 and 4). For the White clam samples, the control and heavy metal exposed groups were significantly ($p < 0.05$) separated along various PC axes (Fig. 3A–C). The significant metabolic biomarkers induced by Cd in White clam digestive glands included the elevated valine, isoleucine, leucine, arginine, glutamate, acetoacetate and succinate, and depleted hypotaurine and ATP (Table 1). In Zn-treated White clams, significantly increased valine, isoleucine, leucine and histidine in the digestive gland were found. For the exposure of mixed Cd and Zn, the inducible metabolic biomarkers comprised the increased valine, isoleucine, leucine, acetoacetate and glucose and decreased aspartate, taurine, betaine and ATP in White clam digestive glands.

Based on the PC scores plots (Fig. 4A and B), separations between control and Cd or Zn treated Zebra clam samples were not significant. However, metabolic effects induced by

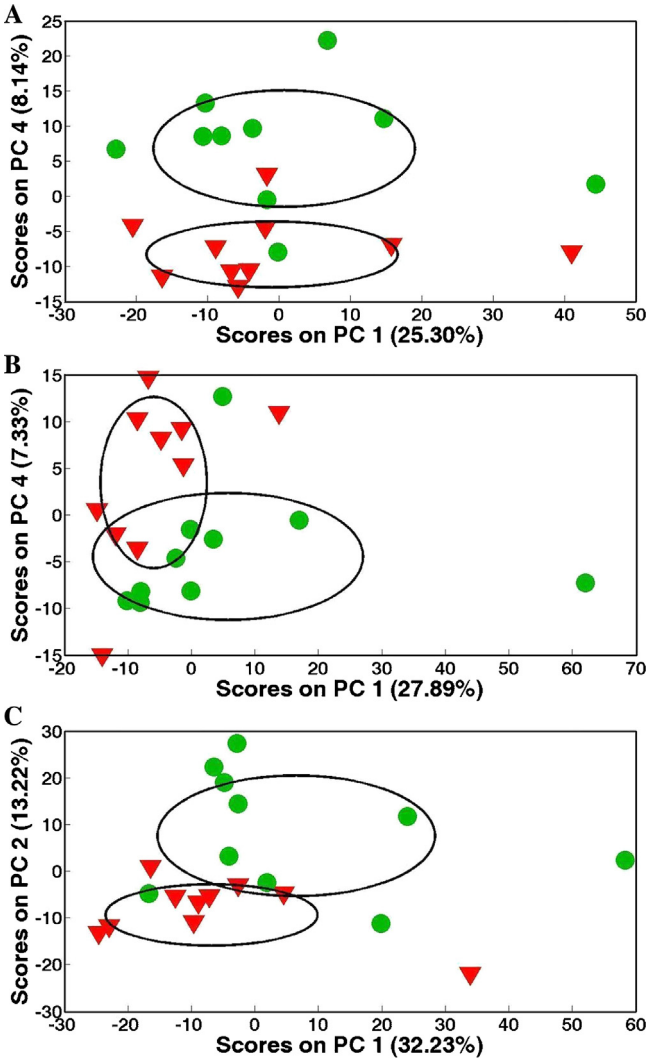

Fig. 3 – Principal components analysis (PCA) on the ^{1}H NMR spectra showing significant separations between control (▼) and Cd (A), Zn (B) and mixed Cd and Zn (C) exposed (●) White clams. Ellipses represented mean ± standard deviation of PC scores for each group.

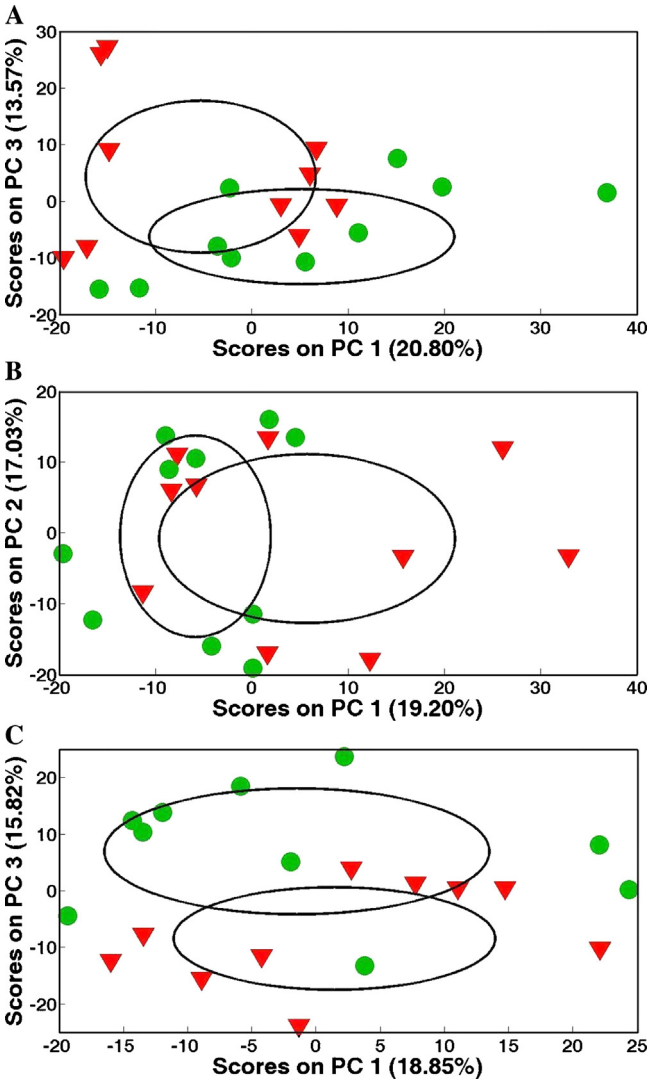

Fig. 4 – Principal components analysis (PCA) on the ^{1}H NMR spectra showing separations between control (▼) and Cd (A), Zn (B) and mixed Cd and Zn (C) exposed (●) Zebra clams. Ellipses represent mean ± standard deviation of PC scores for each group.

Cd exposure along PC3 (ANOVA, $p = 0.051$) and by Zn exposure along PC1 (ANOVA, $p = 0.051$) approached statistical significance (<0.05). To identify the significant metabolites, one way ANOVA was conducted on the metabolite concentrations in digestive glands. The significant metabolic responses to Cd exposure were the increased ATP, and decreased homarine and histidine in Zebra clam digestive glands. Zinc exposure induced clear increase in glucose. For the mixed heavy metal exposure, PCA resulted in highly significant ($p < 0.01$) separation along PC3 (Fig. 4C), which indicated obvious metabolic differences between control and mixed heavy metal exposed Zebra clam samples. As shown in Table 1, elevated alanine, arginine, glycine, glucose and ATP were apparently observed in mixed heavy metal exposed-Zebra clam samples.

3.3. *Antioxidant enzyme activities in digestive glands from White and Zebra clams exposed to heavy metals*

After exposure to heavy metals for 48 h, SOD activities in Cd-treated White clam samples were inhibited with a statistical significance approaching 0.05 (ANOVA, $p = 0.093$) (Table 2). However, SOD activities in mixed heavy metal exposed White clams were increased with a statistical significance approaching 0.05 (ANOVA, $p = 0.083$). For zinc exposed White clam samples, no obvious alterations in SOD activities were found. Interestingly, the activities of SOD between control White and control Zebra clam samples were significantly different ($p < 0.05$). Both Cd and Zn exposures caused obvious decreases in GPx activities in White clam digestive glands. GST activities in mixed heavy metal treated White clam samples were increased with a statistical significance approaching 0.05 (ANOVA, $p = 0.081$). For the Zebra clam samples, no significant changes of antioxidant enzyme activities were observed in all heavy metal treated groups.

3.4. *Cadmium and zinc concentrations in digestive glands from White and Zebra clams exposed to heavy metals*

Table 3 illustrates the metal accumulations in two pedigrees of clams. After exposure with heavy metals for 48 h, the concentrations of Cd in White clam digestive gland varied significantly (Table 3). Apparently, the average Cd concentrations in both Cd- and mixed heavy metal-treated White clam samples were significantly higher ($p < 0.05$) than that of control or Zn-treated samples. However, Zebra clams showed no significant accumulation of Cd in digestive gland tissues from all heavy metal-exposed groups. There were no significant elevations of Zn content in both White and Zebra clam samples exposed to heavy metals.

4. Discussion

4.1. *Biological differences between White and Zebra clams*

In current marine biomonitoring programs in China, Manila clam *R. philippinarum* is a common biomonitor species. However, the potential biological differences between different pedigrees of clams are ignored in routine sampling and analysis, which could introduce biologically statistical variations for pollution biomonitoring. In addition, the biological differences can also distort biological interpretation due to the different tolerances and sensitivities of clams to environmental contaminants in marine toxicology studies. As a system biology approach, metabolomics has been widely used in toxicology (Liu et al., 2011c; Wei et al., 2008; Wei et al., 2009; Williams et al., 2009, 2014; Zhang et al., 2006;).

Based on the PCA results, metabolic differences between White and Zebra clam samples were clearly found (Fig. 2). Since both White and Zebra clams are from the same species *R. philippinarum* sharing the similar *genotypic milieu*, the differential phenotypic fingerprints (e.g., metabolic differences) might be generated from the differential gene expressions and

Table 2 – The activities (U mg^{-1} protein) of SOD, GPx and GST in digestive glands from White and Zebra pedigrees of Manila clams *Ruditapes philippinarum* after exposure to Cd, Zn and the mixture of Cd and Zn for 48 h. Values are presented as the mean ± standard deviation. Significant difference (* $p < 0.05$) between control and heavy metal treated–groups and significant difference ($^{\#}$ $p < 0.05$) between control groups of White and Zebra pedigrees were tested by one–way analysis of variance. ^{a} meant the statistical differences between control and heavy metal treated–groups approached 0.05 (< 0.1).

Treatment	White pedigree			Zebra pedigree		
	SOD	GPx	GST	SOD	GPx	GST
Control	155.48 ± 23.83$^{\#}$	478.60 ± 134.52	433.57 ± 91.15	108.57 ± 40.60$^{\#}$	577.47 ± 40.16	462.62 ± 158.32
Cd	133.77 ± 28.53^{a}	345.71 ± 135.81^{a}	361.06 ± 135.31	92.19 ± 24.16	511.38 ± 166.89	344.89 ± 111.31
Zn	151.78 ± 33.46	306.08 ± 170.44*	498.40 ± 270.43	100.59 ± 9.77	533.76 ± 133.81	424.30 ± 29.18
Cd + Zn	232.99 ± 115.07^{a}	563.81 ± 407.98	584.08 ± 207.54^{a}	104.36 ± 10.39	621.02 ±122.53	363.92 ± 59.03

consequent amounts of enzymes related to various metabolisms. The major metabolic differences between White and Zebra clam samples lied in amino acids (valine, isoleucine, leucine and histidine) and energy metabolism-related metabolites (succinate and ATP). As discussed by Viant et al. (2009), some marine mollusks could use high intracellular concentrations of free amino acids to balance their intracellular osmolarity, the differential amino acids profiles could also be related to the different strategies to regulate osmotic balance in these two pedigrees of clams (Zhang et al., 2011c). The relatively high level of succinate meant an activated anaerobic process in Zebra clam. Interestingly, the activities of SOD between control White and control Zebra clam samples were significantly different ($p < 0.05$). Therefore, it would undoubtedly distort the toxicological interpretation when testing oxidative stresses induced by contaminants (e.g., heavy metals) in mixed pedigrees of clams. It is necessary to select a sensitive pedigree of clam for marine heavy metal pollution biomonitoring and marine toxicology study.

4.2. *Differential responses to heavy metal exposures*

The bioaccumulation of heavy metals in soft tissues of bivalves is the key index in heavy metal pollution biomonitoring. Since digestive glands of bivalves are main target organ for heavy metal accumulation (Panfoli et al., 2000), it was used for determination of heavy metals accumulated by clams. Although both White and Zebra clams revealed a similar baseline level of Cd in control groups (Table 3), White clams accumulated significant ($p < 0.05$) amounts of Cd in digestive gland tissues after Cd and mixed Cd and Zn exposures. For Cd and mixed heavy metal exposed-Zebra clam samples, the average amounts of Cd were higher than that of control samples. However, the statistical significances were larger than 0.25. In both White and Zebra clam samples, no significant accumulations of Zn were detected in Zn and Cd + Zn-exposed groups. Since Zn is an essential element for organisms, the baseline levels of Zn were *approx.* 200 times higher than Cd in clam digestive gland tissues. Therefore, the accumulation of environmentally relevant Zn (50 μg L^{-1}) was unapparent in clams with a short-term exposure of Zn. These findings indicated that White clam was a relatively preferable marine pollution biomonitor, at least for Cd pollution.

Antioxidant enzyme activities are routinely used for testing oxidative stresses induced by toxicants (Regoli and Principato, 1995). In White clam samples, SOD activities in Cd and Cd + Zn-exposed groups differed from that of control group with statistical significances approaching 0.05. GPx activities in Cd and Zn-exposed groups and GST activities in Cd + Zn-exposed group were obviously altered. However, no obvious alterations in these antioxidant enzyme activities were found in heavy metal exposed-Zebra clam samples. These findings suggested that White pedigree of clam was more sensitive to heavy metal exposures than Zebra pedigree clam.

Metabolomics has been successfully employed in environmental toxicology based on the metabolite fingerprinting to environmental pollutants (Bundy et al., 2004; Viant et al., 2006a, b). In this work, heavy metal exposures (Cd, Zn and Cd + Zn) induced significant metabolic changes in both White and Zebra clam digestive glands. However, the altered metabolic profiles differed between White and Zebra clam samples exposed to single heavy metal or a mixture of heavy metals. The significant metabolic biomarkers induced by Cd in White clam digestive glands included the elevated valine, isoleucine, leucine, arginine, glutamate, acetoacetate and succinate, and depleted hypotaurine and ATP (Table 1). However, Cd exposure induced high level of ATP and decreased homarine and histidine in Zebra clam samples,

Table 3 – The accumulated concentrations (μg g^{-1}) of cadmium and zinc in digestive glands from White and Zebra pedigrees of Manila clams *Ruditapes philippinarum* after exposure to Cd, Zn and the mixture of Cd and Zn for 48 h. Values are presented as the mean ± standard deviation. Significant differences (* $p < 0.05$ and ** $p < 0.01$) between control and heavy metal treated-groups were tested by one-way analysis of variance.

Treatment	Cd concentration		Zn concentration	
	White pedigree	Zebra pedigree	White pedigree	Zebra pedigree
Control	0.60 ± 0.16	0.79 ± 0.22	137.48 ± 20.28	162.09 ± 50.02
Cd	0.95 ± 0.28*	1.41 ± 1.10	162.19 ± 33.56	141.36 ± 9.11
Zn	0.74 ± 0.23	0.63 ± 0.40	141.36 ± 18.93	118.58 ± 15.65
Cd + Zn	1.41 ± 0.51**	1.01 ± 0.38	152.59 ± 18.34	129.25 ± 27.08

which was completely different to the metabolic biomarkers in White clam samples. In Zn-treated White clams, significantly increased valine, isoleucine, leucine and histidine in the digestive gland were found. Comparatively, only glucose was elevated in Zn-treated Zebra clam digestive glands. For the exposure of mixed Cd and Zn, the inducible metabolic biomarkers between White and Zebra clams were also different except increased glucose. These distinctive metabolic biomarkers between White and Zebra clam samples meant differential toxicological mechanisms in White and Zebra clam samples. For example, elevated valine, isoleucine, leucine, arginine and glutamate indicated osmotic stress by Cd in White clam samples, which was not observed in Zebra clam samples. For Zn exposure, the metabolic profiles in White clam samples clearly showed the osmotic stress due to the elevation of amino acids (valine, isoleucine, leucine and histidine). The elevation of glucose implied the up-regulated gluconeogenesis by Zn in Zebra clam digestive gland. The mechanisms were unclear, however, the differential responsive mechanisms suggested that one pure pedigree of clams should be used in toxicology study. After exposure with mixed Cd and Zn, the metabolic responses including valine, isoleucine, leucine, acetoacetate and ATP in White clam samples were similar to those of Cd-treated White clam samples, which indicated that Cd induced the dominant toxicological effects in White clams exposed to mixed heavy metals (Cd and Zn). However, in Zebra clam digestive, mixed heavy metals induced dissimilar metabolic profiles to either Cd- or Zn-treated samples, except elevated ATP and glucose, which illustrated the synergistic effects of Cd and Zn in Zebra clams.

In conclusion, this study focused on the selection of one suitable pedigree of clam *Ruditapes philippinarum* for marine heavy metal pollution biomonitoring and marine environmental toxicology study. In this work, we set out to answer three questions. Hereby, the answer to the first question: there were significant biological differences between White and Zebra pedigrees of clams based on the metabolic profiles and antioxidant enzyme activities. The metabolic profiles showed higher levels of valine, isoleucine, leucine, histidine and succinate in Zebra clam digestive glands and higher level ATP in White clam digestive glands, respectively. Additionally, the activities of SOD in control White and Zebra clam digestive gland tissues were significantly ($p < 0.05$) different. The answer to the second question: the toxicological responses (e.g., metabolic changes, antioxidant enzyme activities and bioaccumulation of heavy metals) were different between White and Zebra clams. The answer to the third question: White clam was more sensitive to all heavy metal exposures based on the significant ($p < 0.05$) accumulation of Cd, obvious antioxidant enzymatic regulations and sensitive metabolic changes in digestive glands. Overall, we concluded that White pedigree of clam could be a preferable bioindicator used for marine heavy metal pollution biomonitoring and marine environmental toxicology.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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