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# Differential toxicological effects induced by mercury in gills from three pedigrees of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics

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**Abstract** Mercury is a hazardous pollutant in the Bohai marine environments due to its high toxicity to the marine organisms and subsequent ecological risk. Manila clam *Ruditapes philippinarum* is one of important sentinel organisms in 'Mussel Watch Program' launched in China and therefore used as a bioindicator in marine and coastal ecotoxicology. There are dominantly distributed three pedigrees of clam (White, Liangdao Red and Zebra) in Yantai population endowed with different tolerances to environmental stressors. In this study, gill tissues were collected from both untreated and mercury exposed White, Liangdao Red and Zebra clams, and the extracts were analyzed by NMR-based metabolomics to compare the original metabolomes and the toxicological effects induced by mercury exposure in three pedigrees. The major abundant metabolites in White clam sample were branched-chain amino acids, lactate, alanine, arginine, acetoacetate, glutamate, succinate, citrate, malonate and taurine, while the metabolite profile of Liangdao Red clam sample comprises relative high levels of alanine, arginine, glutamate, succinate and glycogen. For Zebra clam sample, the metabolite profile exhibited relatively high amount of aspartate, acetylcholine and homarine. After 48 h exposure of 20 μg $l^{-1}$ $Hg^{2+}$, the metabolic profiles from all the three pedigrees of clams commonly showed significant increases in alanine, arginine, glutamate, aspartate, α-ketoglutarate, glycine and ATP/ADP, and decreases in citrate, taurine and homarine. The unique metabolic differences between the metabolomes of gill tissues from $Hg^{2+}$-exposed White, Liangdao Red and Zebra clams were found, including elevated acetylcholine and branched-chain amino acids in White clams, and the declined succinate in both White and Liangdao Red samples as well as the declined betaine in Zebra and White clams. Overall, our findings showed the differential toxicological responses to mercury exposure and that White clams could be a preferable bioindicator for the metal pollution monitoring based on the metabolic changes from gill compared with other two (Liangdao Red and Zebra) pedigrees of clams.

**Keywords** Manila clam · Bioindicator · Mercury · NMR · Metabolomics

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## Introduction

Heavy metal contamination has been of great concern in marine and coastal ecotoxicology. Among the heavy metals, mercury is a hazardous contaminant in the marine and coastal environments due to its high toxicity to the organisms and subsequent ecological risk (Zhang 2001; Beiras et al. 2002). It has been reported that the mercury concentration has been up to 100 μg $l^{-1}$ sea water in some extremely heavily polluted sites along Bohai marine and coastal environments (Zhang 2001). Inorganic mercury of $Hg^{2+}$ is the predominant form of mercury contamination that is highly water soluble and readily accumulates in marine invertebrates by associating with colloid and other ultrafine

materials in water and therefore presents a risk to organisms such as filter-feeders, e.g. Manila clam, by binding to sulfhydryl that are abundant in proteins and polypeptides of organism (Clarkson 1997). Thus, it is often found in cells and tissues bound with thiol-containing proteins and small-molecular weight thiols such as cysteine and glutathione (GSH). In addition, mercury can induce oxidative stresses and hence lipid, protein, and DNA damages by generating production of reactive oxygen species (ROS) and lipid peroxides in organisms (Lund et al. 1993; Clarkson 1997).

Manila clam *Ruditapes philippinarum* is one of important sentinel organisms in 'Mussel Watch Program' launched in China in 2004. Along Bohai marine and coastal ecosystems, there are mainly distributed three pedigrees of clam (White, Liangdao Red and Zebra) in Yantai population endowed with different tolerances to environmental stressors. Due to the wide distribution, long life cycle, high tolerance to salinity and temperature, ease of collection and high bioaccumulation of heavy metals, Manila clam has been considered a good bioindicator in marine and coast ecotoxicology (Park et al. 2008; Park et al. 2006; Ji et al. 2006; Xu et al. 2010; Liu et al. 2010; Wang et al. 2009). To our knowledge, however, few studies have focused on the differences of toxicological effects induced by heavy metal contaminants in clams from various pedigrees, which makes it extremely necessary to define one sensitive pedigree of clam as sentinel for the heavy metal monitoring of Bohai marine and coastal ecosystems.

Traditional toxicological approaches attempt to measure the specific responses based on the selected biomarkers to characterize the biological stresses induced by heavy metals in marine organisms, such as the activity of acetylcholinesterase (AChE) to test for the neurotoxicity or the superoxide dismutase levels to test for oxidative stress (Matozzo et al. 2005; Elbaz et al. 2010). Metabolomics is a recent developed a system biology tool that has greatly expanded the single biomarker approaches, focusing on the systematic study of global metabolic profile, especially the low molecular-weight metabolites (<1000 Da), left behind by the specific cellular processes (Davis 2005; Saavedra and Bachere 2006). Such study of metabolic profiling has been widely used in drug toxicity, inborn disease diagnosis, functional genomics, and ecotoxicology (Brindle et al. 2002; Bundy et al. 2004; Wu et al. 2005a; Viant et al. 2006a, b). $^{1}$H NMR spectroscopy is applicable to analyze a wide range of endogenous metabolites from biological samples including intact tissues or tissue extracts to provide valuable biochemical information on the physiological perturbations induced by both endogenous and exogenous factors since this technique is rapid and rich in structural and quantitative information and allows the metabolites to be analyzed simultaneously (Lindon et al. 2000; Wu et al. 2005b).

Gill tissue is a main target tissue for heavy metal accumulation in marine bivalve invertebrates (Panfoli et al. 2000; Viarengo et al. 1994). In this study, we apply $^{1}$H NMR-based metabolomics to Manila clam (*Ruditapes philippinarum*) gill tissue to 1) compare the differences between the metabolic profiles in gill tissues from three pedigrees (White, Liangdao Red and Zebra) of clams distributed along the marine and coastal environments in the Bohai Sea, and 2) characterize and distinguish the toxicological effects induced by acute waterborne $Hg^{2+}$ exposure in various pedigrees of clam based on the metabolic fingerprinting (molecular biomarkers) to determine a sensitive pedigree of clam as sentinel organism for the metal pollution monitoring of Bohai marine and coastal environments.

## Materials and methods

### Experimental design

All the adult Manila clams *Ruditapes philippinarum* (shell length: 3.4–3.8 cm, from White, Liangdao Red and Zebra pedigrees, Fig. 1) were purchased from local culturing farm. They were allowed to acclimate in aerated seawater (25°C, 33 psu, collected from pristine environment) in the laboratory for 1 week and fed with the *Chlorella vulgaris Beij* at a ration of 2% tissue dry weight per day. After acclimatization, 15 clams ($n = 5$ from White, Liangdao Red and Zebra pedigrees, respectively) were sacrificed and the gill tissues dissected from each individual. The remaining 30 clams ($n = 10$ from White, Liangdao Red and Zebra pedigrees) were divided into two tanks (one control and one mercury exposed) containing five White, Liangdao Red and Zebra clams, respectively, and exposed to dissolved 20 μg $l^{-1}$ $Hg^{2+}$ for 48 h. Mercury was prepared from $HgCl_2$ (analytical grades). The experimental concentration of $Hg^{2+}$ can be found in heavily polluted sites of Bohai Sea in which the mercury concentrations are ranged from 0.2 to 133 μg $l^{-1}$ sea water and the average concentration of mercury was ca. 27 μg $l^{-1}$ sea water (Zhang 2001). After 48 h of exposure, all the clams from both control and mercury-exposed groups were immediately dissected for the gill tissues. All the gill tissues were flash frozen in liquid nitrogen, and then stored at −80°C prior to the metabolite extraction.

### Metabolite extraction

Polar metabolites were extracted from gill tissues of clams by a modified extraction protocol using methanol/chloroform (Bligh and Dyer 1959; Lin et al. 2007; Wu et al. 2008). Briefly, the gill tissue (approx. 100 mg) was

**Fig. 1** Representative Manila clams *Ruditapes philippinarum* of White (**a**), Liangdao Red (**b**) and Zebra (**c**) pedigrees with the shell length ranging from 3.4 to 3.8 cm

homogenized using a high throughput homogenizer (Precellys 24, Bertin Technologies, France) and extracted in 4 ml $g^{-1}$ of methanol, 0.85 ml $g^{-1}$ of water, and 2 ml $g^{-1}$ of chloroform. The mixture was shaken and centrifuged (5 min, $3000\times g$, at 4°C), and the supernatant substance was removed. A total of 2 ml $g^{-1}$ of chloroform and 2 ml $g^{-1}$ of water was added to the supernatant, and the mixture was vortexed and then centrifuged again (10 min, $3000\times g$, 4°C). The methanol/water layer with polar metabolites was transferred to a glass vial. The sample was dried in a centrifugal concentrator and stored at −80°C. It was subsequently resuspended in 600 µl of 100 mM of phosphate buffer ($Na_2HPO_4$ and $NaH_2PO_4$ with 0.5 mM TSP, pH 7.0) in $D_2O$. The mixture was vortexed and then centrifuged at $3000\times g$ for 5 min at 4°C. The supernatant substance (550 µl) was then pipetted into a 5 mm NMR tube for NMR analysis.

## NMR spectroscopy

Extracts of gill tissue from clams were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K). Basic one-dimensional (1-D) $^1$H 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 then 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 (Fan 1996; Viant et al. 2003) and some of them confirmed by the 2D NMR method, $^1$H–$^1$H homonuclear correlation spectroscopy (COSY).

## 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). Each spectrum was segmented into 0.005 ppm bins between 0.2 and 10.0 ppm with bins from 4.72 to 4.96 ppm (water) excluded from all the NMR spectra. Bins between 8.59 and 8.61 ppm, between 8.56 and 8.58 ppm, between 7.67 and 7.69 ppm, and between 3.56 and 3.57 ppm containing pH-sensitive NMR peaks were compressed into single bins. The total spectral area of both compressed and uncompressed bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed (glog) with transformation parameter $\lambda = 1.4 \times 10^{-9}$ (Purohit et al. 2004; Parsons et al. 2007) to stabilize the variance across the spectral bins and to increase the weighings of the less intense peaks. For multivariate analysis, principal components analysis (PCA) was used in this work for the separation of sample groups and detection of differences between various groups of samples. PCA is an exploratory unsupervised pattern recognition (PR) method since it detects inherent variation within the dataset and takes no account of class membership. 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. For each model built, the loading vector for the PC was examined to identify the metabolites which contributed to the clusters (Xu 2004). One way ANOVA (analysis of variance) was performed on the ratio of representative bin area (peak intensity) to the total spectral area which was contributive for the separation between control and heavy metal treated groups and was used as the "concentration" of corresponding metabolite. A $P$ value of 0.05 was considered significant for the multiple comparisons on the metabolites between control and exposed samples.

## Results

### $^{1}H$ NMR spectroscopy of gill tissue extracts

A representative $^{1}H$ NMR spectrum of gill tissue extracts from a White clam is shown in Fig. 2. Several metabolite classes were observed, including amino acids (branched-chain amino acids, arginine, and glutamate, etc.), energy storage compounds (ATP/ADP and glycogen), and Krebs cycle intermediates (succinate, citrate and α-ketoglutarate).

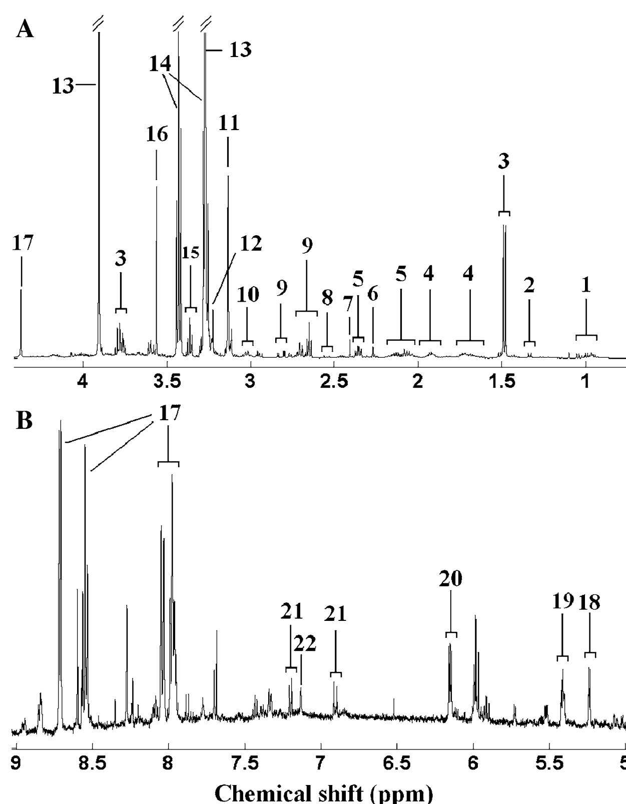


**Fig. 2** A representative one dimensional 500 MHz $^{1}H$ NMR spectrum of gill tissue extracts from a White clam (**a**) vertical expansion of the aromatic region (**b**). Keys: *1* Branched chain amino acids: isoleucine, leucine and valine, *2* lactate, *3* alanine, *4* arginine, *5* glutamate, *6* acetoacetate, *7* succinate, *8* citrate, *9* aspartate, *10* α-ketoglutarate, *11* malonate, *12* acetylcholine, *13* betaine, *14* taurine, *15* hypotaurine *16* glycine, *17* homarine, *18* glucose, *19* glycogen, *20* ATP/ADP, *21* tyrosine, and *22* histidine

### Principal components analysis of $^{1}H$ NMR spectra of gill tissue extracts from untreated clams

PCA was applied for the separation between untreated White, Liangdao Red and Zebra clam samples, and significant separations ($P < 0.05$, based on the PC1 and PC2 scores of each sample) were found along both PC1 and PC2 axes (Fig. 3a). The PC1 versus PC2 scores plot showed that NMR spectra from clams of Zebra pedigree (blue squares) along negative PC1, while both Liangdao Red (green cycles) and White (red inverted triangles) pedigrees clustered mainly along positive PC1, and White and Liangdao Red pedigrees were located at positive and negative PC2, respectively (Fig. 3a).

Basically, the significantly (the ratio of representative peak area of significant metabolites to total spectral area, $P < 0.05$) abundant metabolites in White clam sample were branched-chain amino acids (leucine, isoleucine and valine), lactate, alanine, arginine, acetoacetate, glutamate, succinate, citrate, malonate and taurine, while the metabolite profile of Liangdao Red clam sample comprises relative high levels of alanine, arginine, glutamate, succinate, glycogen and 2 unknown metabolites (1.51 and 7.68 ppm) Table 1. For Zebra clam sample, the metabolite profile exhibited relatively high amount of aspartate, acetylcholine and homarine (Fig. 3b,c). Overall, there exhibited the highest levels of branched-chain amino acids (leucine, isoleucine and valine) and malonate in White clam samples, highest levels of alanine and succinate in Liangdao Red samples, and highest amounts of aspartate, acetylcholine and homarine in Zebra samples.

### PCA of $^{1}H$ NMR spectra of gill tissue extracts from control and mercury-exposed clams

PCA was conducted on the $^{1}H$ NMR spectral data sets generated from the control and $Hg^{2+}$-exposed groups of clams from White, Liangdao Red and Zebra pedigrees, respectively, and the separations between the control (green cycles) and exposed (red inverted triangles) were obviously observed from the PC scores plots (Fig. 4a, b, c) ($P < 0.05$). From the corresponding PC1 loading plots, the metabolic profiles of gill extracts from White pedigree of clams showed significantly increase in branched-chain amino acids (valine, leucine and isoleucine), alanine, arginine, glutamate, α-ketoglutarate, acetylcholine, aspartate, glycine, ATP/ADP and an unknown metabolite at 2.96 ppm and decrease in

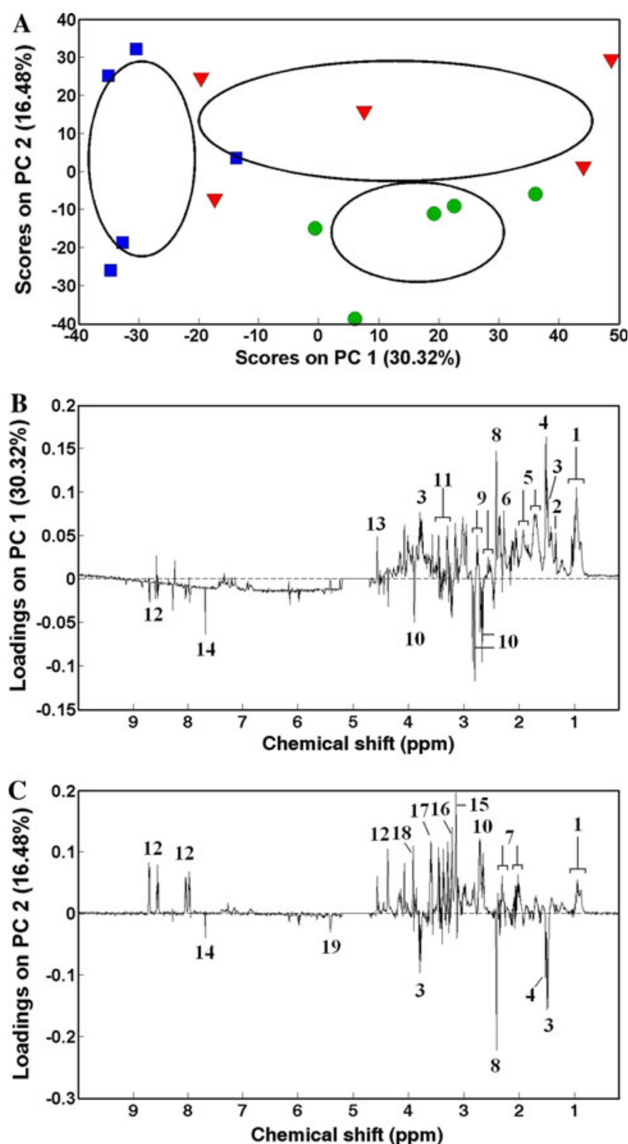


**Fig. 3** Principal components analysis (PCA) showing (**a**) separations between untreated White (*red inverted triangle*), Liangdao Red (*green circle*) and Zebra (*blue square*) clam samples**,** and corresponding PC1 (**b**) and PC2 (**c**) loadings plots showing the metabolic differences between the different pedigrees of clam. *Ellipses* represented mean ± SD of PC scores along both PC1 and PC2 axes for each group. Keys in (**b, c**): *1* branched chain amino acids: isoleucine, leucine and valine, *2* lactate, *3* alanine, *4* unknown 1 (1.51 ppm), *5* arginine, *6* acetoacetate, *7* glutamate, *8* succinate, *9* citrate, *10* aspartate, *11* taurine, *12* homarine, *13* unknown 2 (4.57 ppm), *14* unknown 3 (7.68 ppm), *15* malonate, *16* acetylcholine, *17* hypotaurine, *18* betaine and *19* glycogen. (Color figure online)

succinate, citrate, taurine, betaine, homarine and an unassigned metabolite at 1.51 ppm (Table 2, Fig. 4d). The distinguishable metabolic changes caused by $Hg^{2+}$ exposure in Liangdao Red clam gill tissues included increased alanine, arginine, glutamate, aspartate, α-ketoglutarate, glycine and ATP/ADP, together with the decreased succinate, citrate, taurine and homarine (Table 2, Fig. 4e). For the Zebra pedigree, the elevated alanine, arginine, glutamate, α-ketoglutarate, aspartate, glycine, ATP/ADP and the unknown metabolite (2.96 ppm) were found as well as the reduced citrate, betaine, taurine, homarine and 2 unidentified metabolites at 1.51 and 4.57 ppm (Table 2, Fig. 4f). Although the metabolic profiles from all the three pedigrees of clams commonly showed significant increases in alanine, arginine, glutamate, aspartate, α-ketoglutarate, glycine and ATP/ADP, as well as decreases in citrate, taurine and homarine, there were unique detailed metabolic differences between the metabolomes of gill tissues from $Hg^{2+}$-exposed White, Liangdao Red and Zebra clams, which included elevated acetylcholine and branched-chain amino acids in White clams. Also, declined succinate was found in both White and Liangdao Red samples as well as the declined betaine in Zebra and White clams.

**Table 1** List of the significantly abundant metabolites in three pedigrees of clam ($P < 0.05$[a])

| Clam pedigree | Abundant metabolites (ppm) |
|---|---|
| White | Branched-chain amino acid (0.94–1.04) |
| | Lactate (1.33) |
| | Alanine (1.48, 3.78) |
| | Arginine (1.73, 1.91) |
| | Acetoacetate (2.27) |
| | Glutamate (2.10, 2.36) |
| | Succinate (2.41) |
| | Citrate (2.55, 2.74) |
| | Malonate (3.13) |
| | Taurine (3.27, 3.43) |
| Liangdao Red | Alanine (1.48, 3.78) |
| | Arginine (1.73, 1.91) |
| | Glutamate (2.10, 2.36) |
| | Succinate (2.41) |
| | Glycogen (5.41) |
| | Unknown 1 (1.51) |
| | Unknown 2 (7.68) |
| Zebra | Aspartate (2.68, 2.81, 3.89) |
| | Acetylcholine (3.20) |
| | Homarine (4.37, 7.97, 8.04, 8.55, 8.72) |

[a] *P* values determined using one-way ANOVA on the bin areas from the representative peak of corresponding metabolite

## Discussion

### Comparison between the metabolic profiles of gill extracts from untreated White, Liangdao Red and Zebra clams

The original NMR spectrum (Fig. 2a) is dominated by several organic osmolytes, betaine (3.27 and 3.91 ppm),

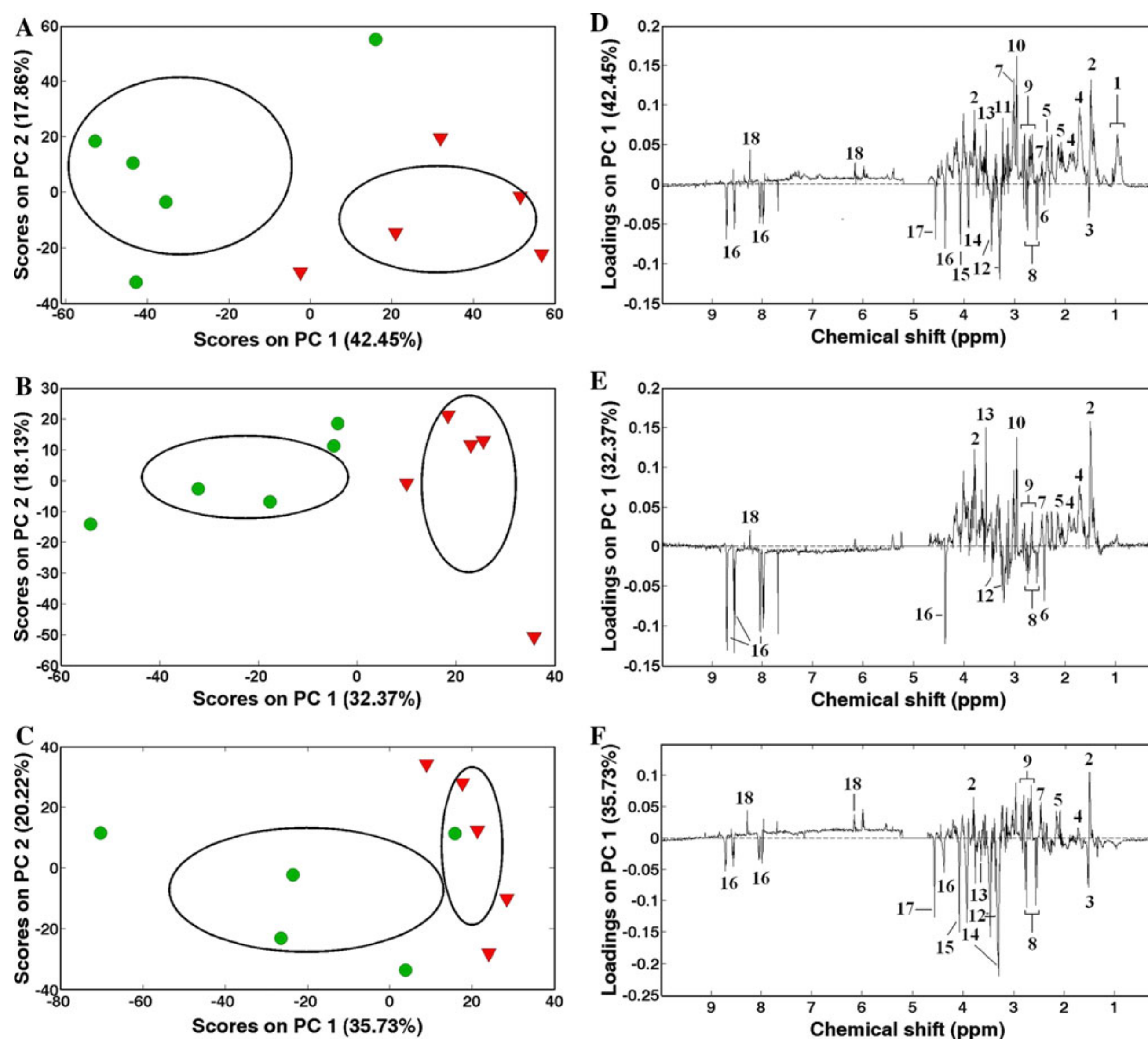


**Fig. 4** Principal components analysis (PCA) on the $^{1}H$ NMR spectra of both control (*green circle*) and $Hg^{2+}$-exposed (*red inverted triangle*) clams from White (**a**), Liangdao Red (**b**) and Zebra (**c**) pedigrees, and corresponding PC loadings plots, (**d**, **e** and **f**) showing the metabolic differences between the control and $Hg^{2+}$-exposed clams after 48 h of exposure. *Ellipses* represented mean ± SD of PC scores along both PC1 and PC2 axes for each group. Keys: *1* branched chain amino acids: isoleucine, leucine and valine, *2* alanine, *3* unknown 1 (1.51 ppm), *4* arginine, *5* glutamate, *6* succinate, acetoacetate, *7* α-ketoglutarate, *8* citrate, *9* aspartate, *10* unknown 2 (2.96 ppm), *11* acetylcholine, *12* taurine, *13* glycine, *14* betaine, *15* unknown 3 (4.07 ppm), *16* homarine, *17* unknown 4 (4.57 ppm) and *18* ATP/ADP. (Color figure online)

taurine (3.27 and 3.45 ppm), glycine (3.57 ppm) and alanine (1.48 ppm) (Fig. 2a), which is approx.10 times intense than other metabolites. Organic osmolytes such as betaine, homarine, and taurine are small organic molecules to regulate the osmotic balance in marine organisms. Those osmolytes can be actively accumulated in high salinity environments and released when the salinity decreases. Therefore, organic osmolytes play important physiological roles in osmotic regulation of invertebrates and hence were detected at higher levels than other metabolites in clams (Preston 2005).

Although these metabolic differences between the pedigrees are related to complex metabolic pathways and physiological regulations, all the three pedigrees of clam are from the same species Manila clam *R. philippinarum* sharing the similar genotypic milieu. Therefore the differences of phenotypic fingerprinting (e.g., metabolic differences) between various pedigrees of clam might be

**Table 2** Significantly up- or down-regulated metabolites ($P < 0.05$[a]) from three pedigrees of clams with acute (48 h) mercury exposure

| Clam pedigree | Metabolites increased in exposed samples (ppm) | Metabolites decreased in exposed samples (ppm) |
|---|---|---|
| White | Branched-chain amino acids (0.94–1.04) | Succinate (2.41) |
| | Alanine (1.48) | Citrate (2.55, 2.74) |
| | Arginine (1.73, 1.91) | Unknown 1 (1.51, 3.63) |
| | Glutamate (2.10, 2.36) | Taurine (3.27, 3.43) |
| | Aspartate (2.68, 2.81, 3.89) | Betaine (3.27, 3.91) |
| | α-ketoglutarate (2.43, 3.01) | Homarine (4.37, 7.97, 8.04, 8.55, 8.72) |
| | Acetylcholine (3.20) | Unknown 3 (4.07) |
| | Glycine (3.57) | Unknown 4 (4.57) |
| | ATP/ADP (6.15, 8.27, 8.54) | |
| | Unknown 2 (2.96) | |
| Liangdao Red | Alanine (1.48) | Succinate (2.41) |
| | Arginine (1.73, 1.91) | Citrate (2.55, 2.74) |
| | Glutamate (2.10, 2.36) | Taurine (3.27, 3.43) |
| | Aspartate (2.68, 2.81, 3.89) | Homarine (4.37, 7.97, 8.04, 8.55, 8.72) |
| | α-ketoglutarate (2.43, 3.01) | |
| | Unknown 2 (2.96) | |
| | Glycine (3.57) | |
| | ATP/ADP (6.15, 8.27, 8.54) | |
| Zebra | Alanine (1.48) | Citrate (2.55, 2.74) |
| | Arginine (1.73, 1.91) | Betaine (3.27, 3.91) |
| | Glutamate (2.10, 2.36) | Taurine (3.27, 3.43) |
| | Aspartate (2.68, 2.81, 3.89) | Homarine (4.37, 7.97, 8.04, 8.55, 8.72) |
| | α-ketoglutarate (2.43, 3.01) | Unknown 1 (1.51) |
| | Glycine (3.57) | Unknown 4 (4.57) |
| | ATP/ADP (6.15, 8.27, 8.54) | |

[a] $P$ values determined using one-way ANOVA on the bin areas from the representative peak of corresponding metabolite

generated from the differential gene expressions and consequent amounts of enzymes related to the corresponding metabolisms such as osmotic regulations. However, further studies on the mechanisms of metabolic differences between the pedigrees are necessary.

## Toxicological effects induced by mercury in White, Liangdao Red and Zebra clam gills

Although the metabolic profiles from all the three pedigrees of clams commonly showed significant increases in alanine, arginine, glutamate, aspartate, α-ketoglutarate, glycine and ATP/ADP, and decreases in citrate, taurine and homarine, the unique detailed metabolic differences (e.g., elevated acetylcholine and branched-chain amino acids in White clams, declined succinate in both White and Liangdao Red samples and decreased betaine in Zebra and White clams) between the metabolomes of gill tissues from $Hg^{2+}$-exposed White, Liangdao Red and Zebra clams indicated the differential toxicological effects induced by mercury in various pedigrees of clams.

In all the three mercury-exposed (White, Liangdao Red and Zebra) pedigrees of clams, the increased glycine and decreased taurine and homarine were commonly found, which indicated disturbances in the osmotic regulation induced by mercury. Citrate and α-ketoglutarate are two important intermediates of Krebs cycle that includes the key metabolic pathways of energy metabolisms. In this study, the elevated α-ketoglutarate and declined citrate should be the key biomarkers of disturbances in Krebs cycle in energy metabolisms caused by mercury in clam gills. Arginine and ATP are the end products of phosphoarginine + ADP → arginine + ATP, in which phosphoarginine is the primary phosphagen in invertebrates, serving as both a spatial and temporal energy buffer in tissues with high-energy demands. Since the concentration of ATP is usually approx. 1000 times higher than that of ADP in the living organisms, the elevation in the intensities of overlapped NMR peaks should be generated from the increase of ATP. Therefore, both arginine and ATP increased in the gills of clams in our study. The process of 'phosphoarginine + ADP → arginine + ATP' is catalyzed by arginine kinase, which exchanges a phosphate from phosphoarginine to ADP, thus maintaining a stable ATP concentration (Viant et al. 2001). Viant et al. (2001) reported similar findings that the arginine and ATP would

be elevated with a depletion of phosphoarginine in the gill tissue of abalone exposed to 66 μg/l copper after 8 h of waterborne exposure (Viant et al. 2001). Hereby, the increase in arginine and ATP was the metabolic biomarkers of energetic disturbance induced by the metals as well.

Alanine is another important organic osmolyte like betaine, taurine, homarine and glycine in many invertebrates (Abe et al. 2005). It has also been reported that alanine constitutes the major portion of end-product of glucose breakdown anaerobically, together with the metabolite of succinate in invertebrate (Carlsson and Gade 1986; Stokes and Awapara 1968). In this work, the high level of alanine was found in the clams poisoned with mercury. In some studies the increase in alanine caused by anoxia was correlated with an increase in succinate that is a clear biomarker of anaerobiosis in mollusks (De Zwaan et al. 1976), while the elevation of succinate was not observed in the clam gills in the current study. Contrarily, the increase in alanine was accompanied by a decrease in succinate in White and Liangdao Red samples, which suggested that the elevation of alanine should be related to the disturbances in osmotic balance but in the anaerobic metabolism. In another metabolic pathway, there was substantial conversion of aspartate to succinate with no detectable enrichment of other compounds under anoxic conditions in mollusk hence with decreased aspartate and increased succinate (Graham and Ellington 1985). Interestingly, the contrary result of increased aspartate as well as decreased succinate was found in the mercury exposed clams in our work, which denied the occurrence of anaerobiosis metabolism induced by mercury. Since the levels of two intermediates (α-ketoglutarate and citrate) in Krebs cycle were altered in the mercury poisoned clams, the decreased succinate (another intermediate in Krebs cycle) could also be reasonably associated with the disturbance in the Krebs cycle in White and Liangdao Red clams.

The metabolism of glutamate that is a transmitter of central nervous system involves a series of pathways, in which glutamate is taken up by astrocytes and converted to glutamine by glutamate synthetase. Glutamine is then released from astrocytes, and transported into neurons, where it is converted back to glutamate by glutaminase. Excessive synaptic accumulation of glutamate can cause neuronal over activation, stimulating a cascade of cellular events that lead ultimately to cell death, a phenomenon termed glutamate neurotoxicity (Choi 1988). In the present study, the altered levels of glutamate implies that when the clams were exposed to $Hg^{2+}$, the Hg ions could enter the central nervous system, causing disturbed glutamate-glutamine metabolism, hence leading to neurotoxicity, which was also observed in the freshwater oligochaete (*Lumbrculus variegates*) after exposure to copper (O'Gara et al. 2004).

Specially, the elevated branched-chain amino acids and acetylcholine were detected in White clams. Recent studies have reported that some marine mollusks used high intracellular concentrations of free amino acids to balance their intracellular osmolarity with the environment (Viant et al. 2003), and these pools of oxidizable amino acids were also used extensively in cellular energy metabolism. Therefore the elevated levels of amino acids, together with the altered glycine, betaine, taurine and homarine showed that the more severe disturbance in osmoregulation in White clams. Acetylcholine is a neural transmitter that can be degraded to choline in cholinergic synapses and neuromuscular junctions by acetylcholinesterase (AChE) (Matozzo et al. 2005). Indeed, $Hg^{2+}$ is a known neurotoxic substance to the animals by interrupting the nervous transmission. In some studies, the measurement of AChE activity was demonstrated useful as a biomarker of neurotoxic compounds in aquatic organisms and has been successfully applied to the monitoring of neurotoxic contaminations (Cajaraville et al. 2000; Matozzo et al. 2005). For example, Matozzo et al. (2005) reported that clams collected at Marghera, a highly polluted area, showed lower AChE activity than that of animals from both Campalto and Poveglia (prinstine sites) indicating that enzyme inhibition was due to exposure to neurotoxic substances. As a matter of fact, the inhibition of AChE is followed by accumulation of acetylcholine, therefore, the elevation of acetylcholine could be a metabolic biomarker of neurotoxicity of $Hg^{2+}$ induced by in White clams.

## Conclusions

In this limited study, the significant differences between the original metabolic profiles of White, Liangdao Red and Zebra clams were found including highest levels of branched-chain amino acids (leucine, isoleucine and valine) and malonate in White clam samples, highest levels of alanine and succinate in Liangdao Red samples, and highest amounts of aspartate, acetylcholine and homarine in Zebra samples using NMR-based metabolomics. Since the White clam exhibited some unique metabolic changes including branched chain amino acids, acetylcholine and succinate, which implied more sensitive disturbances in the osmoregulation (BCAAs), energetic metabolisms (succinate) and neurotoxicities (acetylcholine) to mercury exposure, we concluded that White pedigree of Manila clam could be the preferable bioindicator in the marine and coastal ecotoxicology.

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