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Research Article

Assessment of Clam *Ruditapes philippinarum* as Heavy Metal Bioindicators Using NMR-Based Metabolomics

There are mainly distributed three pedigrees (White, Liangdao Red, and Zebra) of Manila clam *Ruditapes philippinarum* in Yantai population along the Bohai marine and coast. However, the biological differences to environmental stressors have been ignored in toxicology studies, which could lead to the distortion of biological interpretations of toxicological effects induced by environmental contaminants. In this study, we applied a system biology approach, metabolomics to compare the metabolic profiles in digestive gland from three pedigrees of clam and characterize and compare the metabolic responses induced by mercury in clam digestive gland tissues to determine a sensitive pedigree of clam as a preferable bioindicator for metal pollution monitoring and toxicology research. The most abundant metabolites, respectively, included branched-chain amino acids, alanine, and arginine in White samples, glutamate, dimethylglycine, and glycine in Zebra clams and acetylcholine, betaine, glucose, and glycogen in Liangdao Red clams. After 48 h exposure of 20 $\mu g\,L^{-1}$ Hg^{2+}, the metabolic profiles from the three pedigrees of clams showed differentially significant changes in alanine, glutamate, succinate, taurine, hypotaurine, glycine, arginine, glucose, etc. Our findings indicate the toxicological effects of mercury exposure in Manila clams including the neurotoxicity, disturbances in energetic metabolisms and osmoregulation in the digestive glands and suggest that Liangdao Red pedigree of clam could be a preferable bioindicator for the metal pollution monitoring based on the more sensitive classes of metabolic changes from digestive glands compared with other two (White and Zebra) pedigrees of clams.

Keywords: Bioindicator; Manila clam; Mercury; Metabolomics; NMR

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1 Introduction

Manila clam *Ruditapes philippinarum* is widely distributed and can been found at high densities in intertidal areas along the Chinese Bohai coast. This species is relatively tolerant to a wide range of temperature and salinity [1–3], and is readily sampled throughout the whole year and maintained in the laboratory. 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 therefore meets most of the criteria that define a bioindicator for metal pollution monitoring and hence has been considered a good bioindicator in marine and coast ecotoxicology [4, 5]. Recent toxicological studies have elucidated distinct biochemical and genetic responses of *R. philippinarum* to heavy metal exposures and other toxic contaminants, which supports the proposition that *R. philippinarum* could be a useful biomonitor for marine and coastal pollutions [6–9]. However, there are dominantly distributed three pedigrees (White, Liangdao Red, and White) of Manila clams in Yantai population along the Bohai coast. To our knowledge, there is a paucity of studies on the biological and biochemical differences between various pedigrees of Manila clams, which could induce distortion to the biological interpretations of toxicological effects. Thus it is extremely necessary to characterize the biological and/or biochemical differences and the sensitivity to the marine and coastal environmental contaminants to determine a preferable pedigree of clam as bioindicator for metal pollution monitoring.

Traditionally, toxicological approaches focus on the measure of specific responses, such as the lysosomal membrane stability to test for activation of catabolic processes or the glutathione peroxidase levels to test for oxidative stress [10]. Recent developments in genomics have greatly expanded the single biomarker approaches [11–13]. Proton NMR (^{1}H-NMR) spectroscopy-based metabolomics is a post-genomic approach that combines the high throughput meta-

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Abbreviations: AChE, acetylcholinesterase; **ADP**, adenosine diphosphate; **ATP**, adenosine triphosphate; **BCAA**, branched chain amino acids; **LV**, latent variable; **PC**, principal component; **PCA**, principal components analysis; **PLS-DA**, partial least squares-discriminant analysis; **PR**, pattern recognition

bolic profiling capabilities of ^{1}H-NMR with pattern recognition (PR) techniques to identify the metabolic differences (metabolic biomarkers) between samples [14]. Such study of the low molecular weight (<1000 Da) metabolite profiles has been widely used in drug toxicity, disease diagnosis, functional genomics, and environmental toxicology [18, 19]. It is well established and developed that NMR-based metabolomics can provide valuable biochemical information on the perturbations that are induced by both endogenous (e.g., inborn diseases) and exogenous factors (e.g., environmental contaminants), through the analysis of biofluids and tissues [20, 21].

Mercury is a hazardous heavy metal pollutant in the soil, air, and water due to its high toxicity to the organisms and subsequent ecological risk [22–26]. Along the Bohai marine and coastal environments, mercury pollution has induced extremely high risk to the ecosystems and human health because of the high mercury concentration up to 100 $\mu g\,L^{-1}$ sea water in certain polluted coastal environment [22]. The predominant form of mercury contamination is Hg^{2+} that is highly water soluble and readily accumulates in marine invertebrates. In this limited study, the digestive gland tissue of clam was chosen for analysis since it is the main organ utilized for the long-term storage of persistent toxicants (such as heavy metals) and is also involved in detoxification of pollutants [27]. One of the approaches of systems biology, ^{1}H-NMR-based metabolomics was applied to Manila clams *R. philippinarum* to 1) compare the differences between the metabolic profiles of digestive gland tissue extracts from three pedigrees (White, Liangdao Red, and Zebra) of clams and 2) characterize and compare the metabolic responses (molecular biomarkers) to the acute waterborne Hg^{2+} exposure to determine a sensitive pedigree of clam as bioindicator for the metal pollution monitoring of the Bohai marine and coastal environments.

2 Materials and methods

2.1 Experimental design

All the adult Manila clams *R. 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 and fed with *Chlorella vulgaris* Beij at a ratio of 2% tissue dry weight per day. After acclimatization for 1 wk, 15 clams ($n = 5$ from White, Liangdao Red, and Zebra pedigrees, respectively) were sacrificed and the digestive gland 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 $\mu g\,L^{-1}$ 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 the Bohai Sea in which the mercury concentrations are ranged from 0.2 to 166 $\mu g\,L^{-1}$ sea water [22]. After 48 h of exposure, all the individual clams from both control and exposed groups were immediately dissected for the digestive gland tissues. All the digestive gland tissues were first frozen in liquid nitrogen, and then stored at −80 °C prior to the NMR analysis.

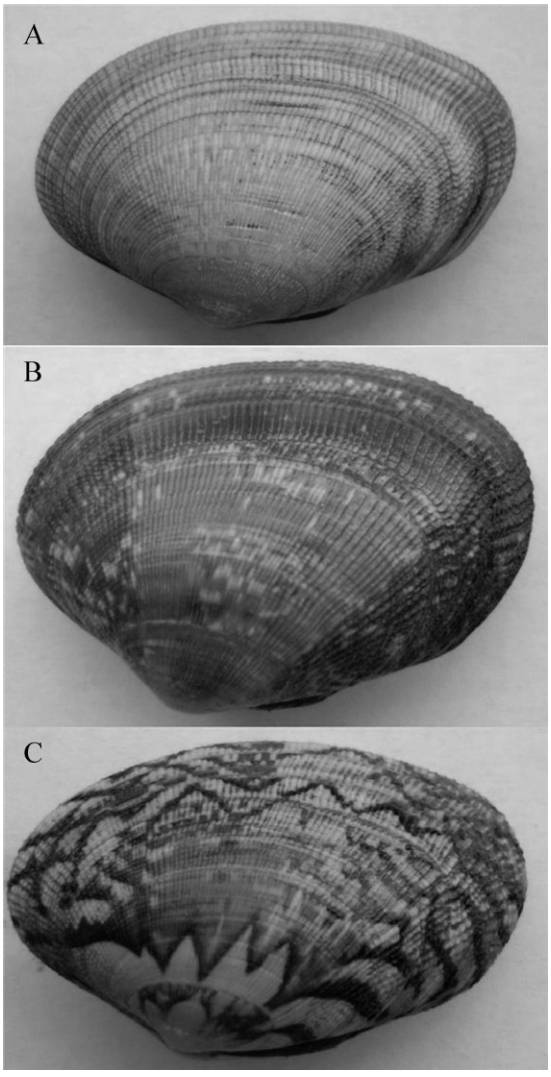

Figure 1. Representative Manila clams *R. philippinarum* of White (A), Liangdao Red (B), and Zebra (C) pedigrees with the shell length ranging from 3.4 to 3.8 cm.

2.2 Metabolite extraction

Polar metabolites were extracted from digestive gland tissues of clams by a modified extraction protocol using methanol/chloroform [28–30]. Briefly, the digestive gland tissue (approx. 100 mg) was homogenized 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 × *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 × *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, including 0.5 mM TSP, pH 7.0) in D_2O. 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 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). One-

dimensional (1D) ^{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 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 [31, 32] and some of them confirmed by the 2D-NMR method, ^{1}H-^{1}H homonuclear correlation spectroscopy (COSY).

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). 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 8.57 and 8.60 ppm, between 7.13 and 7.20 ppm, and between 7.67 and 7.69 ppm containing pH-sensitive NMR peaks were compressed into single bins. 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 with transformation parameter $\lambda = 1.4 \times 10^{-9}$ [33, 34] to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks.

The two well-developed PR techniques, principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), were used in this work for the separation of sample groups. PCA is an exploratory unsupervised PR method since it detects inherent variation within the dataset and takes no account of class membership. The algorithm of this PR 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 [35]. PLS-DA is a supervised PR method to maximize the separation between the biological samples [35, 36]. In PLS-DA, the *X* matrix is the measured matrix, i.e., the NMR data, and the *Y* matrix is made of dummy variables consisting of ones and zeros that indicate the class for each treatment [37]. The quality of the PLS-DA model was assessed using cross-validation with five-way split Venetian blinds [38]. A Q2 score of >0.08 indicates that the model is significantly better than chance, while a score between 0.7 and 1.0 indicates that the model is highly robust [39]. Data were mean-centered before PCA and PLS-DA using PLS Toolbox (version 4.0, Eigenvector Research, Manson, WA).

3 Results

3.1 ^{1}H-NMR spectroscopy of digestive gland tissue extracts

A representative ^{1}H-NMR spectrum and corresponding 2D COSY spectrum of digestive gland tissue extracts from a White clam are shown in Figs. 2 and 3. 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 and citrate). However, all spectra were found to be dominated mainly by the organic osmo-

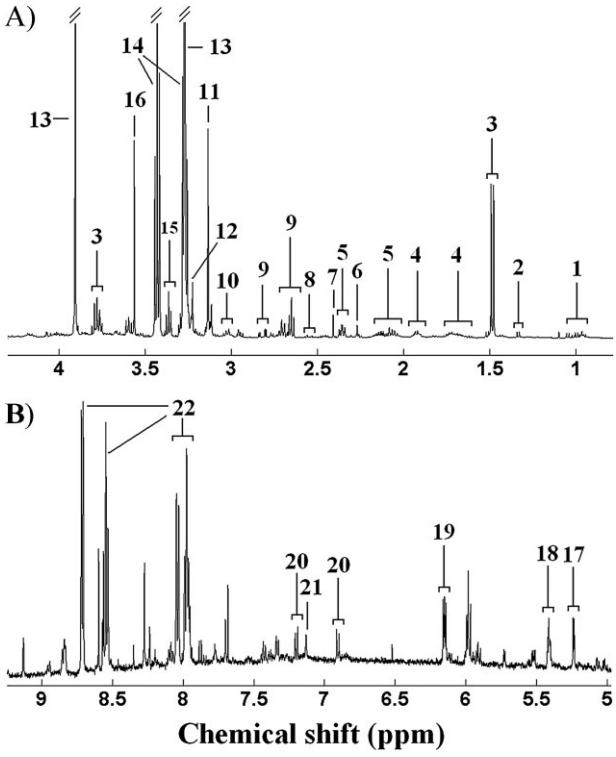

Figure 2. A representative one-dimensional 600 MHz ^{1}H-NMR spectrum of digestive gland tissue extracts from a White clam (A) vertical expansion of the aromatic region (B). Keys: (1) Branched chain amino acids: isoleucine, leucine, and valine, (2) lactate, (3) alanine, (4) arginine, (5) glutamate, (6) acetoacetate, (7) succinate, (8) citrate, (9) aspartate, (10) α-ketoglutarate, (11) malonate, (12) acetylcholine, (13) betaine, (14) taurine, (15) hypotaurine (16) glycine, (17) glucose, (18) glycogen, (19) ATP/ADP, (20) tyrosine, (21) histidine, and (22) homarine.

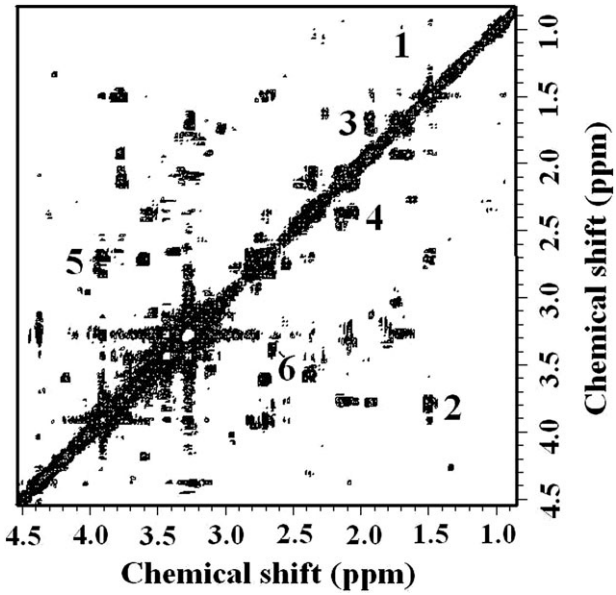

Figure 3. A two-dimensional ^{1}H-^{1}H homonuclear COSY NMR spectrum of digestive gland extracts from a White clam used to confirm metabolite identification. Keys: (1) Leucine, (2) alanine, (3) arginine, (4) glutamate, (5) aspartate, and (6) hypotaurine.

lytes, alanine, betaine, taurine, and glycine, in which alanine is also involved in anaerobic metabolism [40] (Fig. 2A).

3.2 Pattern recognition analysis on the ^{1}H NMR spectra of digestive gland tissue extracts from untreated clams

Principal components analysis was initially applied to the NMR spectral datasets of digestive gland tissues from three untreated pedigrees (White, Liangdao Red, and Zebra) of clams to compare the metabolic profiles between pedigrees. However, no significant difference ($p > 0.05$) was found between the groups using PCA (data not shown), hence the supervised technique PLS-DA was used to classify the three groups of clam samples (Fig. 4). PLS-DA resulted in greater separation between the groups (Fig. 4A) than PCA and presented the differences between the metabolic profiles based on the weights plots which conveniently facilitated the identification of metabolic changes between the three pedigrees of clam groups (Fig. 4B and C). The latent variable (LV) score plots showed that NMR spectra from clams of Liangdao Red (green cycles) along negative LV 1, while both White (red triangles) pedigrees, and Zebra pedigree (blue squares) clustered mainly along positive LV 1, and Zebra and White pedigrees were located at positive and negative LV2, respectively (Fig. 4A). These results indicated that the metabolic profiles of three pedigrees were inherently different to each other, and the detailed differences between the metabolic phenotypes were discovered using PLS-DA.

From the LV weights plots (Fig. 4B and C), the significant differences (the ratio of representative peak area of significant metabolites to total spectral area, $p < 0.05$) between the metabolic profiles of three various pedigrees of clams were observed and listed in Tab. 1. The most abundant metabolites in White clam samples were branched-chain amino acids (leucine, isoleucine, and valine), alanine, and arginine while the metabolite profile of Liangdao Red clam samples comprises highest level of acetylcholine, betaine, glucose, and glycogen. For Zebra clam samples, the metabolite profile exhibited highest amounts of glutamate, dimethylglycine, and glycine.

3.3 Pattern recognition analysis on the ^{1}H-NMR spectra of digestive gland tissue extracts from control and mercury-exposed clams

Principal components analysis 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 (inverted red triangles) and exposed (green cycles) groups were obviously observed from the White and Zebra samples based on the PC scores plots (Fig. 5A and B), ($P < 0.05$). For the Liangdao Red samples, no separation was found between control and mercury-treated groups by PCA (Fig. 6A), while the LV scores plot revealed clear classification after PLS-DA (Fig. 6B).

From either corresponding PC loading plots (Fig. 5C and D) or LV weights plot (Fig. 6C), the metabolic profiles of digestive gland extracts from Hg^{2+}-exposed White pedigree of clams showed significantly increase in 3-hydroxybutyrate, alanine, glutamate, succinate, and taurine and decrease in acetoacetate, hypotaurine, and glycine (Tab. 2, Fig. 5C). The distinguishable metabolic changes caused by Hg^{2+} exposure in Liangdao red clam digestive gland tissues included the increased branched-chain amino acids (valine, leucine, and isoleucine), alanine, glutamate, succinate, aspartate, betaine, and homarine, together with the decreased arginine, glucose, and ATP/ADP

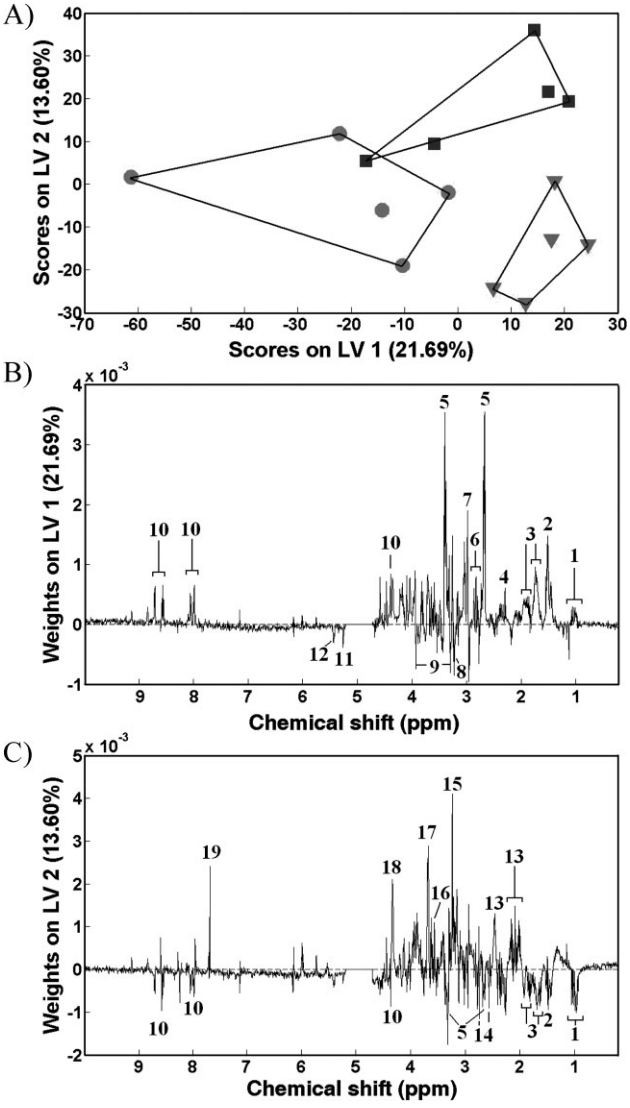

Figure 4. Partial least-squares discriminant analysis (PLS-DA) model showing (A) separations between untreated White (▼), Liangdao Red (●), and Zebra (■) clam samples ($Q^2 = 0.456$); and corresponding LV1 (B) and LV2 (C) weights plots showing the metabolic differences between the different pedigrees of clam. Keys: (1) Branched chain amino acids: Isoleucine, leucine, and valine, (2) alanine, (3) arginine, (4) acetoacetate, (5) hypotaurine, (6) aspartate, (7) unknown 1 (2.96 ppm), (8) acetylcholine, (9) betaine, (10) homarine (11) glucose, (12) glycogen, (13) glutamate, (14) citrate, (15) unknown 2 (3.24 ppm), (16) glycine, (17) unknown 3 (3.68 ppm), (18) unknown 4 (4.33 ppm), and (19) unknown 5 (7.68 ppm).

(Tab. 2, Fig. 6C). For the Zebra pedigree, the elevated citrate, taurine, and homarine were found as well as the reduced alanine, arginine, glutamate, acetylcholine, ATP/ADP, and glycine (Tab. 2, Fig. 5D).

4 Discussion

4.1 Comparison between the metabolic profiles of digestive gland extracts from untreated White, Liangdao Red, and Zebra clams

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

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)
	Alanine (1.48, 3.78)
	Arginine (1.73, 1.91)
Liangdao Red	Acetylcholine (3.20)
	Betaine (3.27, 3.91)
	Glucose (5.24)
	Glycogen (5.41)
Zebra	Glutamate (2.10, 2.36)
	Dimethylglycine (2.74, 2.94)
	Glycine (3.57)

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

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 invertebrates. Those osmolytes can be actively accumulated in high salinity environments and released when the salinity decreases. Therefore, organic osmolytes play key physiological roles in osmotic regulation of invertebrates and hence were detected at higher levels than other metaboites in the digestive glands of clams [41].

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

4.2 Toxicological effects induced by mercury in digestive glands from White, Liangdao Red, and Zebra clams

Alanine is a known important organic osmolyte like betaine, taurine, homarine, and glycine in many invertebrates [42], and has been found in high concentrations in the clams poisoned with mercury in this work, as well as being produced in anaerobic metabolism in which alanine and succinate constitute the major portion of end-products of glucose breakdown anaerobically [40, 43]. In some of these studies, the increase in alanine caused by anoxia was correlated with an increase in succinate that is a clear biomarker of anaerobiosis in molluscs [44], the consistent elevation of succinate was observed in the clam digestive glands from mercury-exposed White and Liangdao Red clams in the current study, which suggested that the elevation of alanine should be related to the disturbances in the anaerobic metabolism together with the increase of succinate. However, these metabolic changes were not detected in the Zebra samples. Contrarily, the alanine was declined and succinate was at

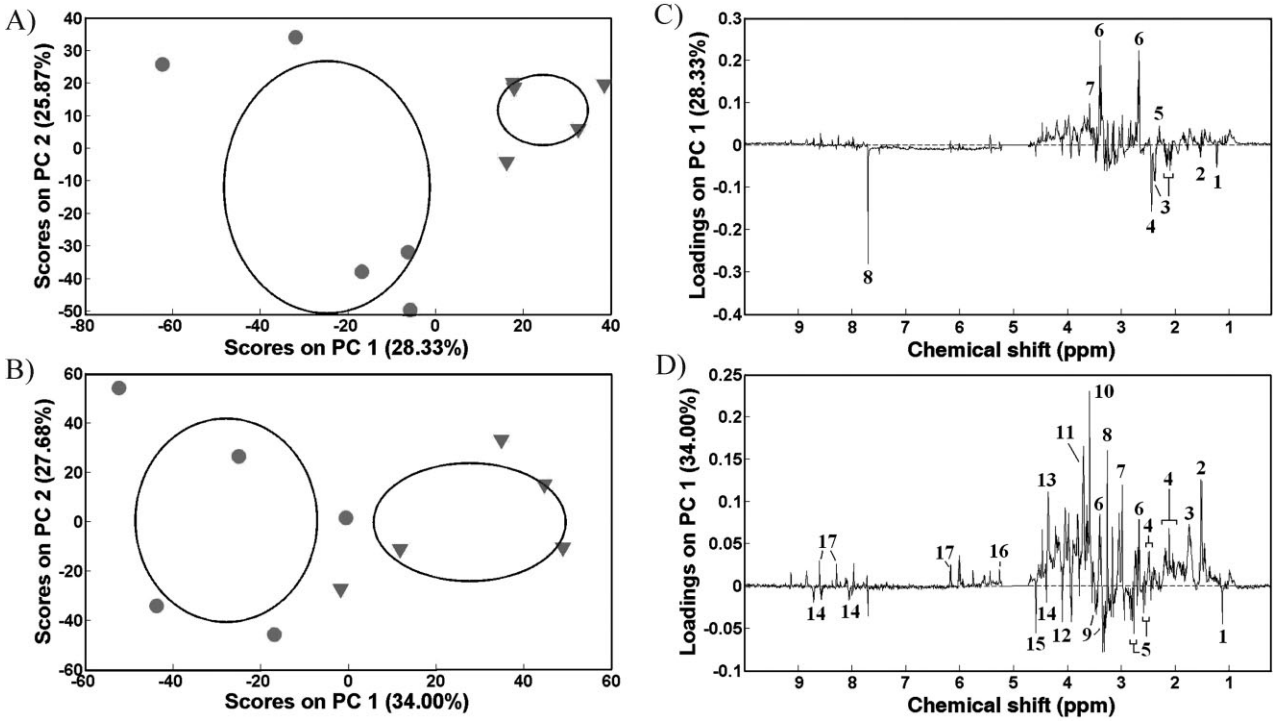

Figure 5. Principal components analysis (PCA) on the ^{1}H-NMR spectra of both control (▼) and Hg^{2+}-exposed (●) clams from White (A) and Zebra (B) pedigrees, and corresponding PC loadings plots, (C) and (D) showing the metabolic differences between the control and Hg^{2+}-exposed clams after 48 h of exposure. Keys in (C): (1) 3-Hydroxybutyrate, (2) alanine, (3) glutamate, (4) succinate, (5) acetoacetate, (6) hypotaurine, (7) glycine, and (8) unknown (7.68 ppm); and keys in (D): (1) Unknown 1 (1.10 ppm), (2) alanine, (3) arginine, (4) glutamate, (5) citrate, (6) hypotaurine, (7) unknown 2 (2.96 ppm), (8) acetylcholine, (9) taurine, (10) glycine, (11) unknown 3 (3.67 ppm), (12) unknown 4 (4.07 ppm), (13) unknown 5 (4.33 ppm), (14) homarine, (15) unknown 5 (4.56 ppm), (16) glucose, and (17) ATP/ADP.

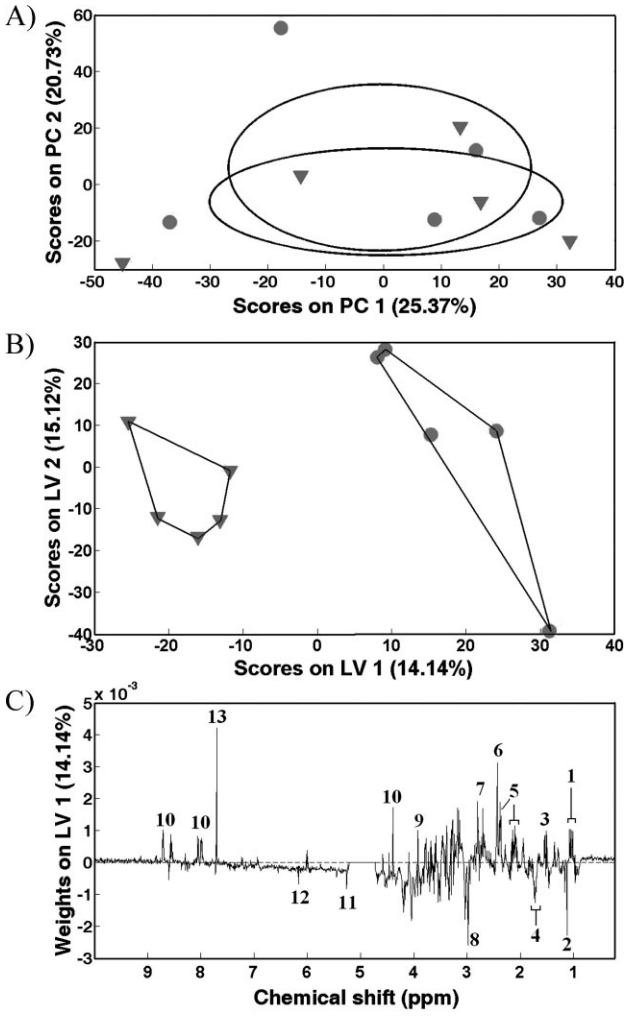

Figure 6. Principal components analysis (A) and PLS-DA model showing (B) separations between control (▼) and mercury-exposed Liangdao Red (●) ($Q^2 = 0.576$); and corresponding LV1 (C) weights plots showing the metabolic differences between control and mercury exposed clams. Keys in (C): (1) Branched chain amino acids: Isoleucine, leucine, and valine, (2) unknown 1 (1.10 ppm), (3) alanine, (4) arginine, (5) glutamate, (6) succinate, (7) aspartate, (8) unknown 2 (2.96 ppm), (9) betaine, (10) homarine, (11) glucose, (12) ATP/ADP, and (13) unknown 3.

the control level in the Zebra clams that could imply no disturbance in the energetic metabolism induced by mercury in Zebra pedigree. Since aniline is also an osmolyte in invertebrates, the decrease of alanine in Zebra clams should be related to the disturbance in osmotic regulation in this pedigree of clams.

From both Liangdao Red and Zebra samples, the depletions of ATP/ADP and arginine were the key metabolic changes induced by mercury exposure. Arginine and ATP are the end products of:

$$\text{Phosphoarginine} + \text{ADP} \rightarrow \text{Arginine} + \text{ATP}$$

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 depletion in the intensities of overlapped NMR peaks should be generated from the decrease of ATP. Therefore, both arginine and ATP decreased in the digestive glands from mercury exposed Liangdao Red and Zebra clams in our study. The process of:

$$\text{Phosphoarginine} + \text{ADP} \rightarrow \text{Arginine} + \text{ATP}$$

is catalyzed by arginine kinase, which exchanges a phosphate from phosphoarginine to ADP, thus maintaining a stable ATP concentration [45]. In this study, the depletion of arginine and ATP/ADP in the White clams might be the biomarker of disorder of energy metabolism. However, Viant et al. [45] reported contrarily 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 in their study, this could be accounted for the various toxicological mechanisms between copper and mercury.

The metabolic changes in organic osmolytes including betaine, hypotaurine, homarine, taurine, and glycine were differentially adjusted in White, Liangdao Red, and Zebra clams after mercury exposure, which included the increase in taurine and decreased hypotaurine and glycine in White clams, increased betaine and homarine in Liangdao Red clams, and elevated taurine and homarine and declined glycine in Zebra samples. These detectable metabolic changes in osmolytes implied the disturbances in osmotic balances, however, the metabolic differences in osmotic metabolites showed different toxicological mechanisms induced by mercury exposure in various pedigrees of clams.

Citrate is an important intermediate of Krebs cycle that includes the key metabolic pathways of energy metabolisms. In this study, the elevated citrate in mercury exposed Zebra clams should be the key biomarker of disturbances in Krebs cycle in energy metabolisms caused by mercury in Zebra clam digestive glands.

In Liangdao Red clam samples, the elevation of branched-chain amino acids was obviously observed. Recent studies have reported that some marine mollusks used high intracellular concentrations of free amino acids to balance their intracellular osmolarity with the environment [32], 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 betaine and homarine showed that the more severe disturbance in osmoregulation in Liangdao red clams.

Glutamate is a transmitter of central nervous system, involved in a series of pathways, in which glutamate can be converted to glutamine by glutamate synthetase. Thus, the increase of glutamate could be the biomarker of neurotoxic effects induced by mercury in White and Liangdao Red clams. However, the level of glutamate was declined in the Zebra digestive glands and another neural transmitter was declined as well, which was different compared with that of White and Liangdao red samples. Acetylcholine can be degraded to choline in cholinergic synapses and neuromuscular junctions by acetylcholinesterase (AChE) [46]. In this study, the decreased acetylcholine could be related to the neurotoxicity caused by mercury in Zebra clams with different mechanism. A further study is necessary to elucidate the toxicological mechanisms of neurotoxic effects in various pedigrees of Manila clams.

On the basis of metabolic changes in the digestive glands, Liangdao red clam exhibited more sensitive disturbances in the osmoregulation (BCAAs, betaine, and homarine), energetic metabolisms (alanine, succinate, ATP, and arginine), and neurotoxicities (acetylcholine and glutamate) to the mercury exposure, and there-

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	3-Hydroxybutyrate (1.20) Alanine (1.48) Glutamate (2.10, 2.36) Succinate (2.41) Taurine (3.27, 3.43)	Acetoacetate (2.26) Hypotaurine (2.66, 3.38) Glycine (3.57)
Liangdao Red	Branched-chain amino acids (0.94-1.04) Alanine (1.48) Glutamate (2.10, 2.36) Succinate (2.41) Aspartate (2.68, 2.81, 3.89) Betaine (3.27, 3.91) Homarine (4.37, 7.97, 8.04, 8.55, 8.72)	Arginine (1.73, 1.91) Glucose (5.24) ATP/ADP (6.15, 8.27, 8.54)
Zebra	Citrate (2.55, 2.74) Taurine (3.27, 3.43) Homarine (4.37, 7.97, 8.04, 8.55, 8.72)	Alanine (1.48) Arginine (1.73, 1.91) Glutamate (2.10, 2.36) Acetylcholine (3.20) ATP/ADP (6.15, 8.27, 8.54) Glycine (3.57)

a) *p*-values determined using one-way ANOVA on the bin areas from the representative peak of corresponding metabolite.

fore the Liangdao red pedigree of Manila clam could be the preferable bioindicator in the marine and coastal ecotoxicology.

5 Concluding remarks

In this limited study, we focused on the differences of metabolic profiles from White, Liangdao Red, and Zebra clams and the toxicological effects in various pedigrees of clam digestive glands to the acute mercury exposure to determine a suitable pedigree of clam as bioindicator for the heavy metal monitoring and marine and coastal ecotoxicology. 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), alanine, and arginine in White clam samples, highest levels of acetylcholine, betaine, glucose, and glycogen in Liangdao Red samples, and highest amounts of glutamate, dimethylglycine, and glycine in Zebra samples. On the basis of metabolic changes in the digestive glands induced by mercury, Liangdao Red clam exhibited more sensitive disturbances in the osmoregulation (BCAAs and betaine), energetic metabolisms (succinate) and neurotoxicities (acetylcholine) to the mercury exposure, and therefore the Liangdao red pedigree of Manila clam could be the preferable bioindicator in the marine and coastal ecotoxicology when digestive gland tissue is used as target organ.

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