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The influence of salinity on toxicological effects of arsenic in digestive gland of clam *Ruditapes philippinarum* using metabolomics*

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Abstract *Ruditapes philippinarum*, a clam that thrives in intertidal zones of various salinities, is a useful biomonitor to marine contaminants. We investigated the influence of dilution to 75% and 50% of normal seawater salinity (31.1) on the responses of the digestive gland of *R. philippinarum* to arsenic exposure (20 μg/L), using nuclear magnetic resonance (NMR)-based metabolomics. After acute arsenic exposure for 48 h, salinity-dependent differential metabolic responses were detected. In normal seawater, arsenic exposure increased the concentrations of branched-chain amino acids, and of threonine, proline, phosphocholine and adenosine, and it decreased the levels of alanine, hypotaurine, glucose, glycogen and ATP in the digestive glands. Differential changes in metabolic biomarkers observed at lower salinity (~23.3) included elevation of succinate, taurine and ATP, and depletion of branched-chain amino acids, threonine and glutamine. Unique effects of arsenic at the lowest salinity (~15.6) included down-regulation of glutamate, succinate and ADP, and up-regulation of phosphocholine. We conclude that salinity influences the metabolic responses of this clam to arsenic.

Keyword: *Ruditapes philippinarum*; salinity; arsenic; toxicological effects; nuclear magnetic resonance (NMR); metabolomics

1 INTRODUCTION

Arsenic (As) is a toxic element that is widespread in estuarine and coastal environments. Although some arsenic is released into the environment from natural geogenic sources, e.g., volcanic activity (World Health Organization, 1981; Sanders et al., 1994), the major contributors to arsenic contamination of environment are anthropogenic activities, such as mining, smelting, and the production of fertilizers and pesticides (Agency for Toxic Substances and Disease Registry, 2007; Irving et al., 2008; Chen et al., 2009). Arsenic is toxic to most organisms and in mammals it causes diverse effects, including cancer and cardiovascular disease (Hughs, 2002). In an aquatic environment, arsenic may occur in the inorganic forms of arsenate As(V) and arsenite As(III), and in organic forms, including arsenoribosides, arsenobetaine, monomethylarsonic acid, and dimethylarsinic acid (Harrington et al., 1997; Sohrin et al., 1997). In heavily-polluted estuaries along the Bohai Bay, North China, concentrations of total arsenic in water as high as 400 μg/L have been recorded (Meng et al., 2004). The main form of arsenic in aquatic environments is arsenate As(V) (Maeda, 1994; Suhendrayatna et al., 2002). Arsenic is readily accumulated by marine organisms, where it may induce a range of toxicological effects. Along the

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Bohai Sea coast, North China, salinity changes from 0 to 33 between estuaries and marine environments Therefore, to better understand the toxicological mechanisms of environmental contaminants, it is important to investigate the influence of salinity on these processes.

Previous research has focused on the toxic effects of arsenic in fish (Gilderhus, 1996; Larsen and Francesconi, 2003; Liao et al., 2003) but the mechanisms of arsenic toxicity in marine mollusks have not been well elucidated. Along the Bohai Sea coast, the clam *Ruditapes philippinarum* inhabits both low intertidal and subtidal zones, where salinities change rapidly during the entry and exit of fresh water. Because of its high tolerance of salinity changes and contaminants, *R. philippinarum* has frequently been used as a bioindicator in the "Mussel Watch Program" and in marine environmental toxicology (Laing and Child, 1996; Moraga et al., 2002; Matozzo et al., 2004; Ji et al., 2006; Hegaret et al., 2007; Liu et al., 2011a; Zhang et al., 2011a). Consequently, *R. philippinarum* was selected as the experimental animal for this study.

In the post-genomic era, metabolomics represents a systems biology approach that has been successfully applied in multiple studies, including drug toxicity, and disease diagnosis toxicology (Brindle et al., 2002; Bundy et al., 2004; Viant et al., 2006a, 2006b; Wu et al., 2005a, 2005b). Metabolomics focuses on all of the low molecular weight (<1 000 Da) metabolites, which are the end-products of metabolism, and which represent the functional responses of biological systems (e.g., in cells, tissues, urine, or plasma) (Lindon et al., 2000; Viant et al., 2001). Several modern analytical techniques, particularly nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), have been successfully used in metabolomics (Nicholson et al., 1985; Plumb et al., 2003; Wang et al., 2003; Wu and Wang, 2010). High resolution proton nuclear magnetic resonance (HR-^{1}H NMR) spectroscopy is particularly suitable for the measurement of a large number of metabolites in biological samples because it is rapid, non-invasive and is rich in structural and quantitative information (Brindle et al., 2002; Lindon et al., 2000). NMR-based metabolomics has been efficiently used in several recent studies in marine environmental toxicology (Viant et al., 2001; Viant et al., 2006a, 2006b; Jones et al., 2008; Williams et al., 2009; Gordon and Leggat, 2010; Lannig et al., 2010; Santos et al., 2010; Tikunov et al., 2010).

The primary aim of this work was to illustrate the influences of reduced seawater salinities on arsenic-induced toxicological effects in *R. philippinarum*, using NMR-based metabolomics. Three experimental salinities lying within the normal range experienced by *R. philippinarum* (14.0–33.5) were used in this study: normal seawater, 75% seawater, and 50% seawater.

2 MATERIAL AND METHOD

2.1 Chemicals

Sodium dihydrogen phosphate (Na_2HPO_4), disodium hydrogen phosphate (NaH_2PO_4) and sodium arsenate (Na_2HAsO_4) (all analytical grade) were purchased from Guoyao Chemical Co. Ltd. (Shanghai, China). Extraction solvents, methanol and chloroform (HPLC grade) were purchased from Guoyao Chemical Co. Ltd. (Shanghai, China). Deuterium oxide (D_2O, 99.9% in D) and sodium 3-trimethlysilyl [2,2,3,3-D4] propionate (TSP) were purchased from Cambridge Isotope Laboratories (Miami, FL, USA).

2.2 Clam exposure

Adult clams *R. philippinarum* (shell length: 3.4–3.8 cm, white pedigree, n=72) were purchased from a local culturing farm. Animals were transported to the laboratory and kept for 1 d in natural seawater (salinity 31.1) collected from a pristine environment. The clams were then acclimated to either normal seawater (salinity 31.1), or 75% seawater (salinity 23.3) or 50% seawater (salinity 15.6). Gradual dilution to the experimental salinities was achieved by adding de-ionized water over a period of 3 d and measured daily (Fig.1). Clams were further acclimated at each salinity for another 3 d, refreshing the water daily. Six treatments were designed consisting of three unexposed control groups (normal, 75% and 50% seawater) and three corresponding arsenic-exposed groups. The nominal concentration of arsenic was 20 μg/L (measured concentrations, 21.1–22.0 μg/L) and was prepared from a stock solution of Na_2HAsO_4 (200 mg/L As). This represents an environmentally realistic concentration in polluted sites along the Bohai coast (Meng et al., 2004). In each treatment, 12 individuals were maintained in a tank containing 10 L of the experimental seawater. During the acclimation and exposure periods, clams were kept at 22°C under a photoperiod of 12 h light and 12 h dark, and fed with the *Chlorella vulgaris* Beij at a ration of 2% tissue dry weight daily. After 48 h of exposure, all clams were

immediately dissected for digestive gland tissues, which were flash frozen in liquid nitrogen and then stored at -80°C before further processing (n=12).

2.3 Metabolite extraction, NMR spectroscopy and data analysis

Polar metabolites in digestive glands of clams were extracted using a modified extraction protocol as described previously (Liu et al., 2011b, 2011c; Zhang et al., 2011b).

Extracts of digestive gland from clams were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K), as described previously (Liu et al., 2011a, 2011b, 2011c; Zhang et al., 2011b). NMR spectral peaks were identified from tabulated chemical shifts (Fan, 1996; Viant et al., 2003), using the software, Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada).

As described previously, all one-dimensional proton NMR spectra were converted to a format using custom-written ProMetab software in Matlab (version 7.0; The MathsWorks, Natick, MA, USA) to generate NMR spectral data (Purohit et al., 2004; Parsons et al., 2007; Katsiadaki et al., 2009). NMR spectral data were mean-centered before principal components analysis (PCA) using PLS Toolbox (version 4.0, Eigenvector Research, Manson, WA, USA).

3 RESULT AND DISCUSSION

3.1 Metabolic differences in the digestive glands of clams exposed to reduced salinity

As reported previously, the white pedigree of the clam *R. philippinarum* is relatively sensitive to heavy metal contaminants (Liu et al., 2011b, 2011c), which was the reason for its use as the experimental animal in this work. A representative raw ^{1}H NMR spectrum of a digestive gland tissue extract from a control clam is shown in Fig.2a, and as the generalized log (glog)-transformed form in Fig.2b, with identified metabolites labeled in Arabic numerals.

The unsupervised pattern recognition method, principal components analysis (PCA), was conducted on the NMR spectral datasets of digestive gland extracts from the three control groups to compare their metabolic profiles (Fig.3a; normal seawater salinity 31.1, inverted red triangles; reduced salinity groups 23.3, green circles; and 15.6, blue squares). Significant differences (P<0.001) were found between the groups, which are also shown in a PC1 loadings plot (Fig.3b). It is apparent that the reduced salinities up-regulated branched-chain amino acids (valine, leucine and isoleucine), arginine, succinate,

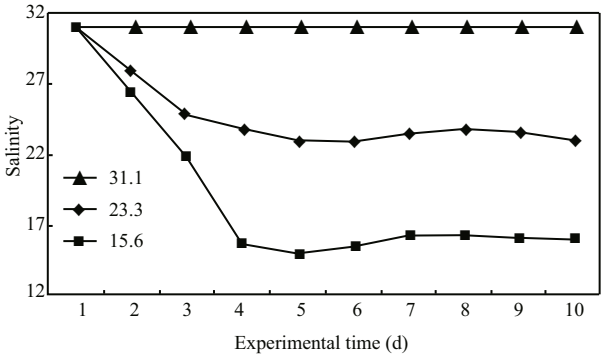

Fig.1 Time course of the salinity change for both the unexposed clams and the arsenic-exposed clams during the acclimation (7 d) and exposure (2 d) periods

Labeled values indicate the nominal final salinities of the three dilutions of seawater.

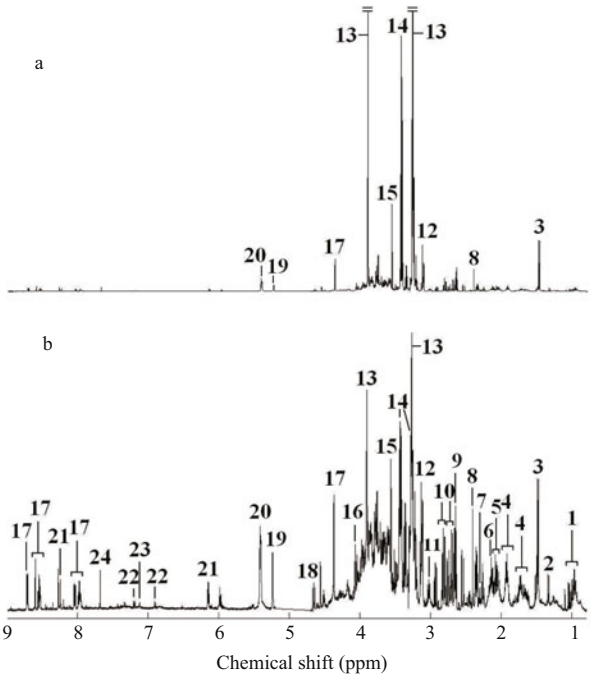

Fig.2 A representative 1-dimensional 500 MHz ^{1}H NMR spectrum of digestive gland tissue extracts from a white clam of control group in raw (a) and generalized log transformed (λ=1.0×10^{-9}) (b) forms

Keys: (1) branched-chain amino acids: isoleucine, leucine and valine; (2) threonine; (3) alanine; (4) arginine; (5) glutamate; (6) glutamine; (7) acetoacetate; (8) succinate; (9) hypotaurine; (10) aspartate; (11) 4-aminobutyrate; (12) malonate; (13) betaine; (14) taurine; (15) glycine; (16) unknown 1 (4.07 ppm); (17) homarine; (18) β-glucose; (19) α-glucose; (20) glycogen; (21) ATP; (22) tyrosine; (23) histidine; (24) unknown (7.68 ppm).

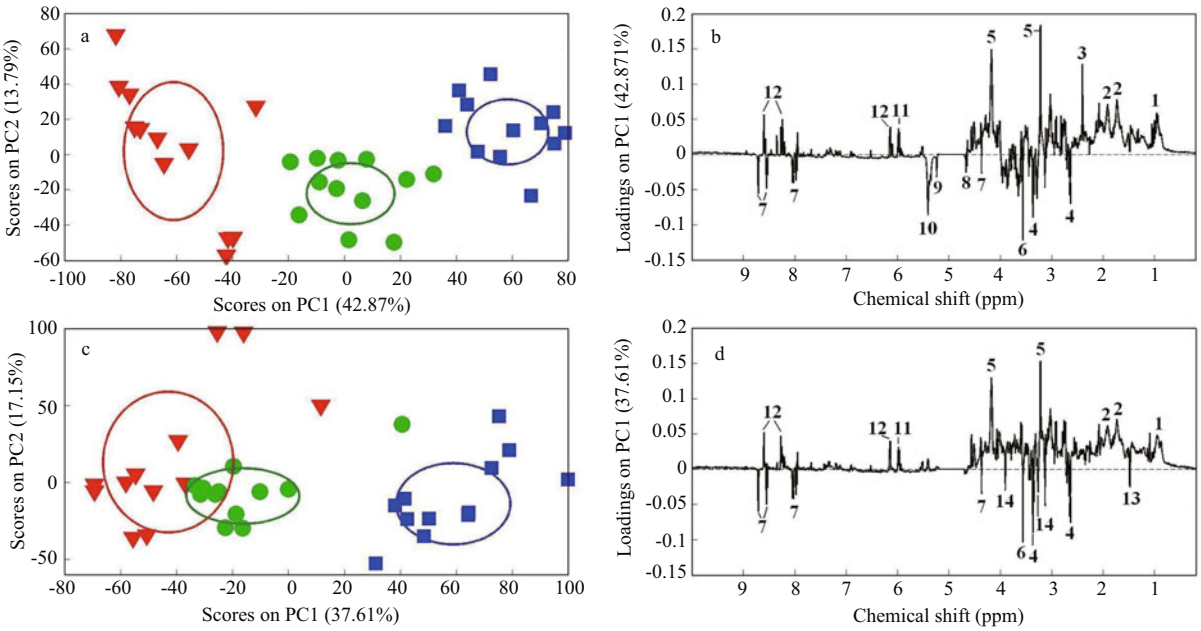

Fig.3 Principal components analysis (PCA) on the ^{1}H NMR spectra showing significant separations among (a) the normal seawater salinity (▼) and reduced salinity-treated (75% salinity, ●; 50% salinity, ■) control groups; and (c) significant separations between the normal salinity (▼) and reduced salinity-treated (75% salinity, ●; 50% salinity, ■) groups exposed to 20 μg/L arsenic for 48 h

Ellipses represented mean±standard deviation of PC scores for each group. (b) and (d) are the corresponding PC1 loadings plots. Key: (1) branched-chain amino acids: isoleucine, leucine and valine, (2) arginine, (3) succinate, (4) hypotaurine, (5) phosphocholine, (6) glycine, (7) homarine, (8) β-glucose, (9) α-glucose, (10) glycogen, (11) unknown 1 (5.99 ppm), (12) ATP, (13) alanine and (14) betaine.

phosphocholine and ATP, and down-regulated hypotaurine, glycine, homarine, glucose and glycogen. Hypotaurine and homarine are organic osmolytes that help to balance the intracellular osmolarity (Viant et al., 2003). They may be accumulated or released, respectively, in response to increased or decreased seawater salinity. Accordingly, we observed that these two osmolytes were decreased in the digestive glands of the clams under reduced seawater salinities. The levels of glycogen and glucose also fell, which is a common observation in mollusks experiencing food restriction (Viant et al., 2003; Jones et al., 2008). Since reduced salinities can significantly decrease the food intake of clams, the depletion of glycogen and glucose may have been metabolic responses to starvation induced by reduced salinity. As discussed by Jones et al. (2008) and Viant et al. (2003), some marine mollusks maintain high intracellular concentrations of amino acids to match their intracellular osmolarity to the high osmolarity of the environment. These oxidizable amino acids are also used extensively in cellular energy metabolism (Moyes et al., 1990). Therefore, the decreased glycine concentration in the digestive glands of clams under reduced seawater salinities was probably concerned with osmotic regulation, and is consistent with the decreased levels of homarine and hypotaurine. Conversely, the increased concentrations of branched-chain amino acids (especially valine and leucine) and arginine could be related to energy metabolism. As reported by De Zwaan et al. (1976), mussels are able to use an unusual carbohydrate fermentation process, with a non-oxidative tricarboxylic acid (TCA) cycle that works in reverse, from pyruvate up to succinate. Therefore, we infer that the increase in succinate observed here under reduced salinities was a biomarker for anaerobic processes in the clam. Phosphocholine is catalyzed by choline kinase that converts ATP and choline into phosphocholine and ADP. The accumulation of phosphocholine and ATP in clam digestive glands at reduced salinities implies that stimulation of energy metabolism was associated with the hypoosmotic stress.

3.2 Differential metabolic responses in digestive gland from clams exposed to arsenic under reduced seawater salinities

Differences were observed in the metabolic profiles of the digestive glands of clams exposed to both arsenic and reduced seawater salinities relative to the

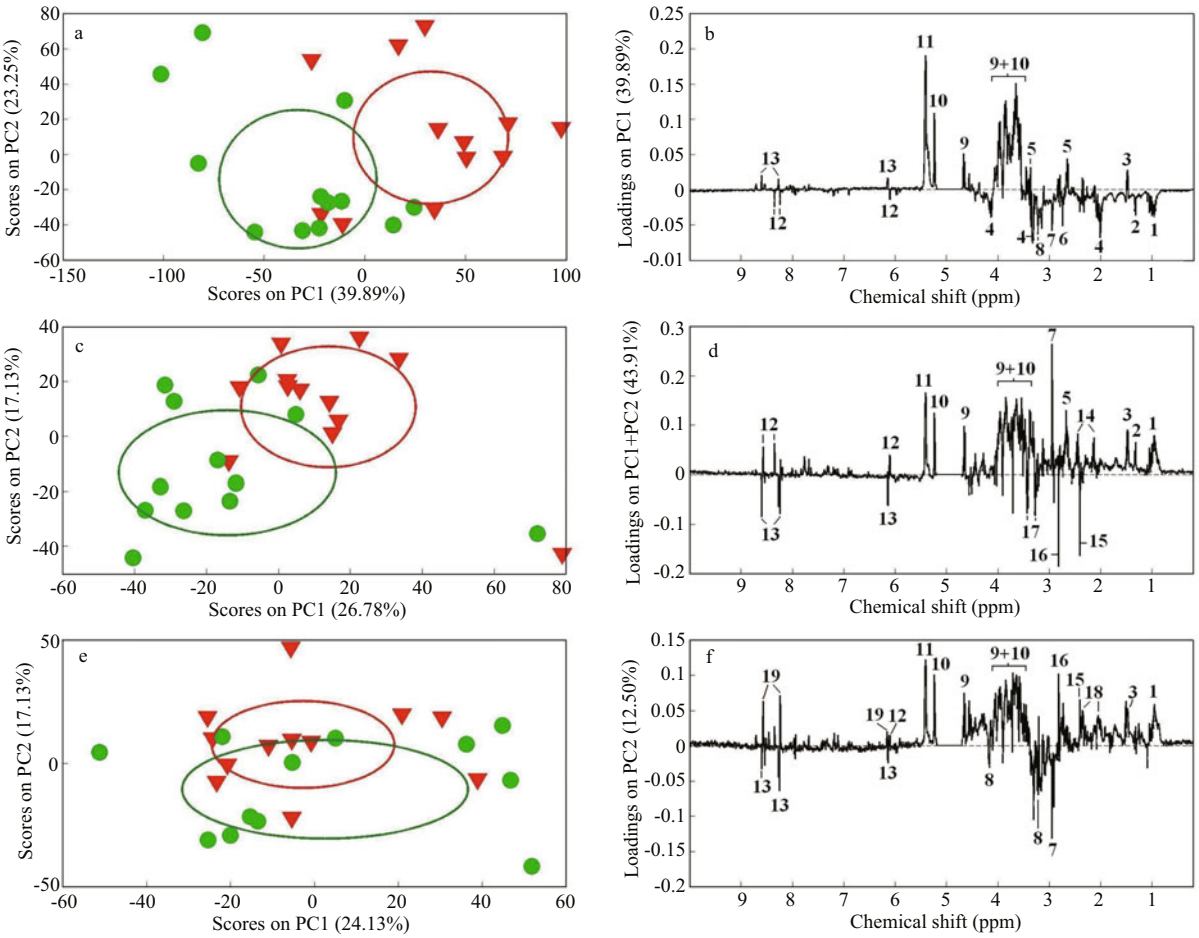

Fig.4 Principal components analysis (PCA) of the ^{1}H NMR spectra of the arsenic-free (▼) and arsenic-exposed (●) clam samples under various salinities (a. normal seawater; c. 75% seawater; e. 50% seawater), and their corresponding PC loadings plots (b, d, and f) showing the metabolic differences between the arsenic-free and arsenic-exposed clams after 48 h of exposure

Ellipses represented mean±SD of PC scores for each group. Keys: (1) branched-chain amino acids: isoleucine, leucine and valine, (2) threonine, (3) alanine, (4) proline, (5) hypotaurine, (6) unknown 1 (2.74 ppm), (7) unknown 2 (2.95 ppm), (8) phosphocholine, (9) β-glucose, (10) α-glucose, (11) glycogen, (12) adenosine, (13) ATP, (14) glutamine, (15) succinate, (16) unknown 3 (2.82 ppm), (17) taurine, (18) glutamate and (19) ADP.

profiles of clams exposed only to reduced salinity (Fig.3). Decreased alanine and betaine levels were observed (Fig.3d) although the level of succinate was not altered. These differences suggest that arsenic exposure modifies the metabolic responses induced by reduced seawater salinity, i.e., reduced salinity and arsenic toxicities must interact.

Therefore, PCA was performed on the ^{1}H NMR spectral datasets generated from the control (untreated with arsenic) and arsenic-treated groups of clams under reduced seawater salinities (Fig.4). For the groups under normal (31.1) and reduced seawater salinities (23.3 and 15.6), the control (inverted red triangles) and arsenic-exposed groups (green circles) were all significantly (P<0.05) separated along various PC axes (Fig.4a, 4c and 4e). Significant metabolic responses induced by arsenic at normal seawater salinity included the elevation of branched-chain amino acids (valine, leucine and isoleucine), threonine, proline, phosphocholine and adenosine, and the depletion of alanine, hypotaurine, glucose, glycogen and ATP. At the reduced seawater salinity of 23.3 (75% seawater), arsenic exposure caused significant increases of succinate, taurine and ATP, and decreases in branched-chain amino acids, threonine, alanine, glutamine, hypotaurine, glucose, glycogen and adenosine. Essentially similar metabolic profiles were observed in clam samples from 15.6 salinity (50% seawater) although some specific differences were detected, including elevated phosphocholine, and depletion of glutamate and succinate. Similar changes in taurine and homarine

levels were observed in clam samples from salinities 23.3 and 15.6.

The differential metabolic responses to arsenic exposure show that reduced seawater salinity affects the metabolic responses to arsenic exposure in the digestive glands of clams. In normal seawater, the metabolic biomarkers including threonine, glucose, glycogen, phosphocholine, branched-chain amino acids and ATP indicated disturbances to energy metabolism, as mentioned above. Changes in other metabolites, such as hypotaurine and proline, were linked to the disturbances in osmotic regulation. The change of adenosine, a nucleotide, may be related to peroxisome proliferation, as reported by Ringeissen et al. (2003). At the reduced salinity of 23.3, differential metabolic responses relative to the metabolic profiles of samples from normal seawater were detected after arsenic exposure, including decreased branched-chain amino acids, threonine, ATP and adenosine. In addition, the level of succinate, a biomarker of anaerobic processes, was elevated in the clam samples from salinity 23.3. The decreased levels of branched-chain amino acids and increased taurine levels at this salinity presumably reflected disturbance of osmotic regulation. The metabolic profiles of clam samples from the lowest salinity (15.6) were similar to those from the 23.3 salinity group, except for the changes in succinate, phosphocholine, glutamate, ADP, homarine and taurine. Succinate, phosphocholine and ADP were clearly biomarkers of disturbed energy metabolism, while the change in the amino acid glutamate may have been concerned with osmotic regulation. These findings imply that the two reduced seawater salinities (75% and 50% seawater) had similar influences on the toxicological effects induced by arsenic exposure in clam digestive glands. However, at the lowest salinity, arsenic induced more severe disturbances in energy metabolism. Possibly, the weaker physiological status of clams at the lowest salinity reduced their tolerance to arsenic.

4 CONCLUSION

There is a lack of information on the influences of environmental factors (e.g., temperature, salinity) on the metabolic responses of marine organisms used as environmental bioindicators and biomonitors for contaminants. Here, we investigated the influences of reduced seawater salinities (75% and 50% seawater) on the metabolic responses within the digestive gland of *R. philippinarum* exposed to an environmentally relevant level of arsenic (20 μg/L), using NMR-based metabolomics. Acute exposure for to arsenic for 48 h caused disturbances in energy metabolism and osmotic regulation in the digestive glands of clams at normal seawater salinity (31.1) and at reduced salinities (23.3 and 15.6). However, a number of differential metabolic biomarkers were detected in the digestive glands of clams at a salinity of 23.3, including elevated levels of succinate, taurine and ATP, and depletion of branched-chain amino acids, threonine and glutamine. At a salinity of 15.6, further distinct metabolic biomarkers induced by arsenic included the down-regulation of glutamate, succinate, phosphocholine and ADP. These observations demonstrate that metabolomics is able to provide important insights into the mechanisms of response to arsenic under changing salinities.

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Certain units appeared in this paper, such as ‘ppm’, ‘cm^{-1}’, ‘‰’, are used upon authors’ request, though not recommended by CJOL editorial office.