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RESEARCH REPORT

Di-(2-ethylhexyl) phthalate mediates glycolysis and the TCA cycle in clam *Venerupis philippinarum*

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Abstract

Di-(2-ethylhexyl) phthalate (DEHP) has many adverse effects on immunity and metabolic states. However, scarce information is available on its connection with toxicologically relevant proteomics response in marine invertebrates. In this study, GS-MS was employed to determine the bio-accumulated levels of DEHP in clam *Venerupis philippinarum*. After exposure to 0.4 mg L^{-1} and 4mg L^{-1} DEHP, the bio-accumulated DEHP in the clam foot was significantly increased in the first 24 h, and then sharply decreased from 0.203 ± 0.022 μg g^{-1} to 0.104 ± 0.011 μg g^{-1} , and from 1.689 ± 0.018 μg g^{-1} to 1.172 ± 0.012 μg g^{-1}, respectively. Comparative proteomic was conducted to investigate the global protein expression changes towards this contaminant exposure. Twenty-eight proteins with significant differences in abundance were identified and characterized, among them six enzymes related to the glycolysis pathway were suppressed, and two members of TCA cycle were induced. The activity and mRNA expression level of malate dehydrogenase (MDH) were further assessed using qPCR and an enzymatic assay. The MDH activity and mRNA transcript levels were both elevated compared to those in the ethanol control group. Our findings indicated that a DEHP-treated clam modulated host toxicological effect through depressing glycogen synthesis and activating TCA cycle.

Key Words: *Venerupis philippinarum*; Di-(2-ethylhexyl)phthalate; 2-dimensional electrophoresis ; glycolysis; TCA cycle

Introduction

The industrial revolution unleashes a vast variety of new chemicals into the environment, and it has been roughly estimated that approximately 1,000 - 2,000 new chemicals are produced each year (Wei *et al*., 2010). Phthalates are among these industrial chemicals and have been used for a variety of purposes, such as plasticizers that impart flexibility and durability to polyvinyl chloride products. Among them, high molecular weight di-(2-ethylhexyl) phthalate (DEHP) is gaining focus in the marine environment, as it is increasingly being used in construction materials and in numerous PVC products (Lu *et al*., 2013). It was estimated that the concentrations of DEHP in river water and sediments typically range from 8 to 25 mg L^{-1} and 1000 to 2000 mg kg^{-1}, respectively (Park and Kwak, 2008). Zhuang *et al* (2011) reported that the content of phthalate esters (PAEs) ranged from 18.77 to 191.51 ng L^{-1} and from 171.50 to 1435.61 μg kg^{-1} in seawater and sediments, respectively, in the Quanzhou Bay of China, where DEHP is a major component.

Cumulative evidence has revealed that DEHP, as an estrogenic chemical, poses multiple health risks due to its ability to bind and activate estrogen receptors (ER) *in vitro* (Oh and Lim, 2009; Uren-Webster *et al*., 2010). In rodents, DEHP has been shown to decrease fetal testis testosterone production and reduce the expression of steroidogenic genes (Borch *et al*., 2006). DEHP has also been widely reported to produce reproductive and developmental toxicities, in mammals and fish, depending on the dose and exposure time (Park and Choi, 2007; Svechnikova *et al*., 2007; Paola *et al*., 2012).

In aquatic animals, DEHP can be absorbed, metabolized and largely accumulated in the tissue of a penaied shrimp (Hobson *et al*., 1984). It can also affect or disrupt endocrine hormones (Oh and Lim, 2009) and interfere with the delicate balance of the immune system (Chalubinski and Kowalski, 2006). In zebrafish, DEHP reduces fertilization success in oocytes spawned by increasing the levels of two

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Table 1 The concentration of clam foot DEHP determined by GC-MS

Exposure doses	Exposure time	Foot DEHP concentration
0.4 mg L^{-1}	24 h	0.203±0.022 μg g^{-1}
	96 h	0.104±0.011 μg g^{-1}
4 mg L^{-1}	24 h	1.689±0.018 μg g^{-1}
	96 h	1.172±0.012 μg g^{-1}

peroxisome proliferator activated receptor (PPAR) responsive genes (Uren-Webster *et al*., 2010). In our previous study, DEHP is found to mediate immune responses in clams by triggering the production of reactive oxygen species and nitric oxide (Lu *et al*., 2013). During the experiment, a strong decrease in movement is observed in DEHP-treated clam compared to that of the control group. However its toxicological effects are still largely unknown in clams, although DEHP has been shown to influence aquatic animals' energy metabolism.

Recently, proteomic analysis has emerged as an approach for the analysis of differential gene expression at the protein level and seems to be an effective way for analyzing the delicate effects of environmental toxicants based on comparisons of the patterns of proteomes after exposure to compounds of toxicological relevance (Bandara and Kennedy, 2002; Kennedy, 2002; Wetmore and Merrick, 2004; Dowling *et al*., 2006; Benninghoff, 2007). The purposes of the present study were: (1) to determine the accumulated levels of DEHP in clam foot; (2) to identify differentially expressed proteins in a DEHP-treated clam foot; and (3) to clarify the expression and activity of malate dehydrogenase at mRNA and protein levels.

Materials and Methods

Sample preparation

Clams (*Venerupis philippinarum*; 8.65 ± 1.24 g in weight) were purchased from Ningbo, Zhejiang Province, China. The clams were acclimated in glass tank for a week before commencing the experiment. The temperature was maintained at 20 - 22 °C throughout the experiment, and the salinity of the supplied seawater was maintained at 30 ppt. The clams were randomly divided into four groups (50 individuals for each group). Firstly, 200 mg DEHP (Sigma, Shanghai, China, analytical purity >99.0 %) was dissolved in 25 mL absolute ethanol, then subsequently diluted with 25 mL seawater to obtain a stock solution of 4 g L^{-1}. The DEHP stress experiment was performed by exposing the clams to DEHP with two final concentrations of 0.4 mg L^{-1} (4 mL stock solution + 18 mL absolute ethanol) and 4.0 mg L^{-1} (40 mL stock solution) in 40 L seawater. The control group was exposed to the same volume of absolute ethanol (20 mL). After 96 h of exposure, the clam foot from 20 randomly selected individuals were ground in liquid nitrogen for DEHP concentration determination and protein extraction.

Assay of DEHP richness in foot by GS-MS

Ten grams of foot samples from treated and control groups were collected, and then homogenized, respectively. The pellets were obtained by centrifuging at 5,000 rpm for 10 min. Subsequently, DEHP was extracted with 20 mL hexane for three times, then mixed together and dried in nitrogen conditions. The extracted DEHP was dissolved into 1 mL hexane for GS-MS analysis. There were three biological replications for each sample. A working standard solution was prepared by ten times dilution of the stock solution (212 mg L^{-1}) with hexane. We performed GC-MS analysis in Trace DSQ α MS (Thermo) with TR-1 column (30.0 mm×0.25 mm×0.25 μm). The inlet was set at 250 °C and automatic injections of 1μl of extracts were performed in a splitless mode. The helium carrier gas flow was set at 1 mL min^{-1}. The oven temperature began at 80 °C, then increased to 280 °C at 10 °C min^{-1}, and kept finally at this temperature for 5 min. The MS detection was in selective ion monitoring operating mode (SIM) at an electron impact energy of 70 eV. Two or three mass fragments were selected for each compound. The most intense ion was used for quantification and the other ions were used for confirmation the presence of the compounds. The concentrations were expressed as (means±SD) μg DEHP per gram of wet weight of clam foot.

Sample preparation for electrophoresis analysis

The foot tissue (approximately 600 mg) was first dissolved in 1 mL lysis buffer (7 M urea, 2 M thiourea, 65 mM DTT, 4 % w/v CHAPS, 20 mM Tris, pH 8.5, and 0.2 % v/v Bio-lyte) and then homogenized at 20 kw for 2 min with a electric homogenizer (Xinzhi, Ningbo). The resulting mix was incubated on ice for 2 h, with vortex for 20 - 30 s every 15 min, and it was sonicated for 3 min with 5 s of ultrasound and 5 s resting intervals. After centrifugation at 12,000 g for 60 min at 4 °C, the supernatant was collected for desalt treatment. The protein samples were purified using a 2-D Clean-up kit (GE Healthcare, USA) according to the manufacturer's instructions. Protein was re-suspended in rehydration solution containing 7 M urea, 2 M thiourea, 4 % w/v CHAPS, 65 mM DTT, and 0.2 % v/v Bio-lyte. The concentration of protein was determined using a protein quantification kit with BSA as a standard (Sangon) and was further confirmed by 12 % SDS-PAGE.

Two-dimensional gel electrophoresis (2-DE)

The first-dimension of isoelectric focusing (IEF) was performed with a PowerPac Basic/Mini-Protean® Tetra Cell System (Bio-Rad, USA) according to the ReadyStrip™ IPG Strip instruction. Briefly, proteins (300 μg) were diluted in 150 μL of rehydration buffer and loaded onto a pH 3 - 10 immobilize Dry Strip (IPG strip) (7 cm, linear,

Bio-Rad). Next, the strips were covered with mineral oil and rehydrated for 13 h at 20 °C. The voltage settings for IEF were 250 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, 4,000 V for 3 h, 4,000 V for 5 h and 6,500 V, to a total of 80 kVh. Following the IEF, strips were equilibrated for 15 min at room temperature in 2.5 mL of SDS equilibration buffer (375 mM Tris-HCl, pH 8.8, 6 M urea, 20 % glycerol, and 2 % SDS) containing 20 mg/ml dithiothreitol for the first equilibration and 25 mg/mL iodoacetamide for the second equilibration. The gel strips were then washed with electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS) and placed on a 12 % polyacrylamide gel containing 0.1 % SDS. The gels were sealed with low melting point agarose, and the second dimension of gel electrophoresis was conducted at 70 v for 30 min and then 130 v for approximately 90 min until the dye front reached the end of the gel. The protein spots on 2-DE gels were visualized by Coomassie Brilliant Blue staining. The images of the gels were scanned using an Image Scanner GS-800 (Bio-Rad, USA). Gel evaluation and data analysis were performed using PDQuest 8.0 (Bio-Rad, USA). Three replicates were conducted per sample to ensure gel reproducibility.

Mass spectrometry and protein identification

Spots with significant and reproducible changes (average protein spot intensities that changed 1.5-fold between control and treated gel sets) were manually excised from stained gels and subjected to in-gel trypsin digestion. After digestion, the supernatants were transferred into another tube, and the gels were extracted once with 50 μL extraction buffer (67 % ACN and 5 % TFA). The peptide extracts and the supernatant of the gel spot were combined and then completely dried. Samples were re-suspended with 5 μL 0.1 % TFA followed by mixing in 1:1 ratio with a matrix consisting of a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 50 % ACN, 0.1 % TFA. 1 ul mixture was spotted on a stainless steel sample target plate. Peptides were analyzed by matrix-assisted laser desorption/ionization tandem time-of flight (MALDI-TOF/TOF) mass spectrometry using the ABI5800 Proteomics Analyzer. Proteins were unambiguously identified by searching against the non-redundant sequence database (NCBInr) and EST database of clams and molluscs via the MASCOT program (http://www.matrixscience.com).

Enzymatic assays of malate dehydrogenase

Malate dehydrogenase (MDH) activity was determined by oxaloacetate reduction via the decrease in absorbance for NADH at 340 nm according to the MDH assay kit (Jian cheng, Nanjing). The assays were performed in 50 mM potassium phosphate buffer, pH 7.5, 1 mM oxaloacetate, 0.15 mM NADH, and the appropriate volume of the fraction (Šukalović *et al*., 2011). Activity was expressed as the mean values ± S.D U/mg (n = 5). The data were subjected to a *t*-test analysis , and $p < 0.05$ was considered to be a significant difference.

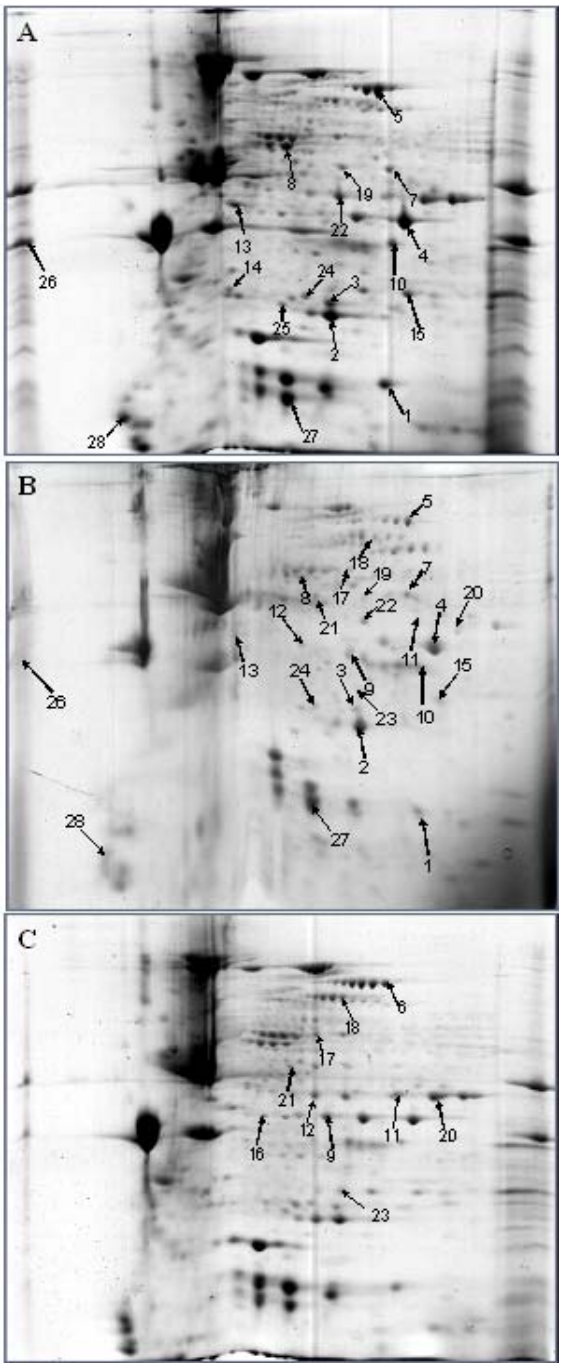

Fig. 1 Protein expression patterns of *Venerupis philippinarum* foot under different concentrations of DEHP exposure: ethanol group (A); 0.4 mg L^{-1} DEHP (B); 4 mg L^{-1} DEHP (C). Proteins were visualized by CBB R250 staining. Arrows and spot numbers refer to differentially expressed proteins.

Confirmation of differential expressed protein by qPCR

The publically registered clam MDH was selected for expression analysis. RNA extraction and complementary DNA (cDNA) synthesis were described previously (Lu *et al*., 2013). Two clam 18S

Table 2 List of spots/proteins identified by MS analysis from *Venerupis philippinarum* foot by 2-DE following DEHP treatment.

Spot no.	Protein name	Accession no.[a]	Matching species	Threshold (P<0.05)[b]	Score	Mr	pI	Regulation[c]
1	Transgelin	AM872804	*Crassostrea gigas*	62	120	24944	8.32	↓
2	Ltap	XP_002121953	*Ciona intestinalis*	49	50	63623	8.45	↓
3	Triosephosphate isomerase	ADO27908	*Ictalurus furcatus*	49	203	26824	6.90	↓
4	Glyceraldehyde-3 phosphate dehydrogenase	NP_001080567	*Xenopus laevis*	46	185	36017	7.60	↓
5	Arginine kinase	ACP43447	*Pholas orientalis*	47	735	81948	6.66	↓
6	Arginine kinase	ACP43447	*Pholas orientalis*	47	417	81948	6.66	↑
7	Phosphoglycerate kinase	BAD17914	*Amia calva*	48	151	42091	5.76	↓
8	Enolase	O02654	*Doryteuthis pealeii*	49	138	47738	5.78	↓
9	Glyceraldehyde-3 phosphate dehydrogenase	NP_001080567	*Xenopus laevis*	47	190	36017	7.60	↑
10	Voltage-dependent anion channel protein	ADI56517	*Haliotis diversicolor*	48	207	30670	6.84	↓
11	Fructose bisphosphate aldolase	P53448	*Carassius auratus*	49	117	39849	6.41	↑
12	Malate dehydrogenase	AM874213	*Ruditapes philippinarum*	59	97	24356	7.69	↑
13	Coiled-coil domain protein	XP_002154202	*Hydra magnipapillata*	48	59	61209	8.95	↓
14	Actin	1101351B	*Pecten sp*	49	340	41827	5.30	↓
15	Phosphoglycerate mutase	AAX29976	*Clonorchis sinensis*	49	99	29250	7.66	↑
16	Malate dehydrogenase	EJW88983	*Wuchereria bancrofti*	49	258	36191	8.92	↑
17	H^+-transporting ATP synthase alpha subunit isoform 1	AAM93478	*Petromyzon marinus*	49	242	39603	9.53	↑
18	Unknown protein							↑
19	Phosphoglycerate kinase	BAD17914	*Amia calva*	48	113	42091	5.76	↓

20	Fructose bisphosphate aldolase C	P53448	*Carassius auratus*	47	113	39849	6.41	↓
21	Phosphoglycerate kinase	CAA45574	*Macropus eugenii*	49	132	45274	7.98	↓
22	Unknown protein							↓
23	Phosphoglycerate mutase	EKC26210	*Crassostrea gigas*	49	79	28792	7.66	↓
24	ATP-dependent RNA helicase	XP_792082	*Strongylocentrotus purpuratus*	49	51	90593	8.91	↓
25	Unnamed protein product	CBY21625	*Oikopleura dioica*	49	53	51639	5.80	↓
26	Tropomyosin	BAF46896	*Balanus rostratus*	49	453	32706	4.61	↓
27	Unknown protein							↓
28	Myosin	1803425C	*Mercenaria mercenariav*	47	210	18105	4.47	↓

a. Database accession numbers according to NCBInr
b. Results of Mascot searching with scores higher than the thresholds indicated P<0.05
c. "↑" denotes that up-regulated spots, "↓" denotes that down-regulated spots

rDNA primers, CGTCTTTCAAATGTCTGCCCTATC and GCCGTATCTCATGCTCCCTCTCC, were used to amplify a 115-bp fragment as an internal control, which verified successful reverse transcription and calibrated the cDNA template. Two gene specific primers for MDH (ATCTATGGCTGTTATTTCCGAC and TCTCTCTCTTCTTCAAGCTCCT) were designed to amplify an 196 bp product. Real-time PCR amplification was performed using a Rotor-Gene 6000 real-time PCR detection system. We utilized the reaction components and thermal profiles suggested by the manufacturer. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of the candidate genes, and the value obtained denoted the n-fold difference relative to the calibrator (ethanol group). The data were presented as relative mRNA expression levels (means ± SD, n = 5), and subjected to a *t*-test analysis.

Results and Discussion

DEHP accumulation in clam foot

Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer and a ubiquitous environmental contaminant that may have adverse effects on organisms. The accumulated DEHP concentrations in clam's foot were presented in Table 1. The higher DEHP concentration was found in the first 24 h with 0.203 ± 0.022 μg g^{-1} and 1.689 ± 0.018μg g^{-1} at 0.4 mg L^{-1} and 4 mg L^{-1} exposure doses, respectively. No DEHP was detected in the control group, indicating that clam foot had the powerful capacity for DEHP enrichment at the first stage. As time progressed, the concentrations of DEHP in the two exposure doses were sharply decreased, and arrived to 0.104 ± 0.011 μg g^{-1} and 1.172 ± 0.012 μg g^{-1} at the 96 h, respectively. This decrease of DEHP informed that clam might have the special abilities to degrade or squeeze DEHP from host by certain molecular mechanisms.

2-DE image patterns

In order to identify the proteins involved into eliminating DEHP in foot tissue, the proteomic expression map under different concentrations of DEHP exposure was investigated (Fig. 1). Within the 9 analytical gels, approximately 600 spots in each group were detected by PDQuest 8.0. The analysis of protein data sets enabled the selection of 28 protein spots that are commonly altered in their expression between the experimental and ethanol control groups (Fig. 1, arrow indicated), among which 20 protein spots displayed increased abundance and 8 showed decreased expression profiles (Table 2).

Characterization of differentially expressed proteins

A series of processes for protein spot picking, in-gel digestion and application to the target plate for ABI5800 were automatically performed to characterize these differentially expressed proteins. As a result, 28 proteins with average protein spot

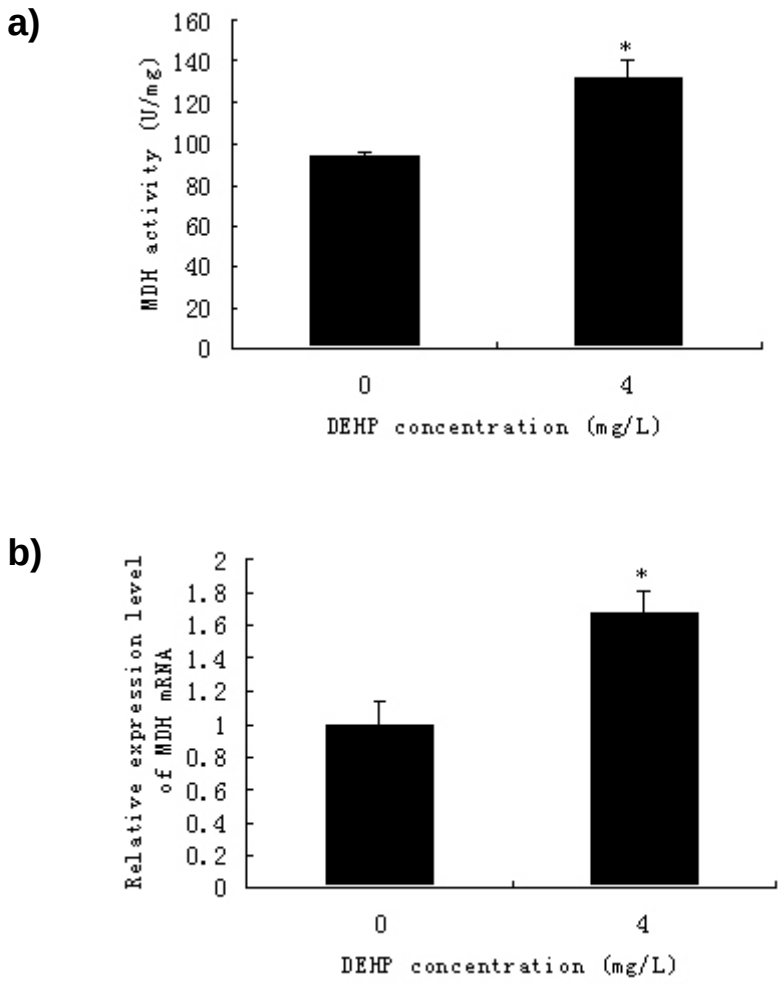

Fig. 2 Enzymatic activity (A) and mRNA expression level (B) of MDH before and after exposure to 0.4 and 4 mg L^{-1} DEHP.

intensities were altered in the DEHP-treated group and were successfully identified . Among these proteins, 25 were successfully annotated and 3 did not yield unambiguous protein identification. Among the identified proteins, 11 were involved in glycolysis (spot 3, 4, 7, 8, 9, 11, 15, 19, 20, 21, 23), and 10 members had at least a 1.5-fold decrease in intensity. An exception was observed for spot 9, glyceraldehyde-3-phosphate dehydrogenase (GAPD), which had a 3.8-fold increase compared to its expression in ethanol control group (Table 2). However, another GAPD (spot 4) showed opposite expression patterns with 6.4-fold decrease towards DEHP challenge. The distinct expression profiles of the two GAPDs indicated that they might be participated in the different metabolic pathways for DEHP detoxication. Mammals were known to possess two tissue-specific GAPD isoenzymes of GAPD-1 and GAPD-2, which served as classical metabolic proteins involved in glycolytic energy production and maintenance of sperm motility (Kuravsky *et al*., 2011). The decrease expression patterns of another GAPD (spot 4) indicated a possible redirection of carbon flux from glycolysis to the pentose phosphate pathway for NADPH regeneration. This mechanism had been proposed to be beneficial during oxidative stress due to the use of NADPH as reducing equivalents by the thioredoxin and glutaredoxin antioxidant systems (Costa *et al.,* 2002; Shenton and Grant, 2003; Shanmuganathan *et al*., 2004). Meantime, an increasing number of diverse non-glycolytic activities of GAPDH have been reported, including macromolecular transport, microtubule bundling, nuclear tRNA transport and apoptosis (Ishitani and Chuang, 1996; Nahlik *et al*., 2003). Ellington also reported that GAPD could be up-regulated in apoptotic cells to 3-fold higher than that in non-apoptotic cells (Ellington, 2001). The up-regulation of spot 9 indicated that cell apoptosis were induced on DEHP exposure through unknown GAPD-based regulatory network.

It is known that the ATP generated in the glycolytic pathway is the major energy source for several metabolic reactions. In DEHP-fed rats, there is a significant suppression of glucose metabolism

observed in liver and muscle tissues (Martinelli *et al*., 2006). In our study, 6 of the 11 glycolysis enzymes were detected with significantly down-regulated expression in clams under DEHP stress, in which phosphoglycerate kinases and phospheglycerate mutases were identified two or three times (Table 2). It was speculated that impaired glycolysis in the clam was linked to an abnormal glucose intermediate flux in the foot (Mushtaq *et al*., 1980). The content of glucose-6-phosphate (G-6-P), fructose-6-phosphate, pyruvate, lactate, glucose-1-phosphate and glycogen in the foot will be addressed in our future work.

DEHP exposure also impacted expression levels of other energy metabolism-related proteins in our study, such as arginine kinase and ATP synthase. Arginine kinase, the equivalent of mammalian creatine kinase, can phosphorylate or dephosphorylate a phosphagen molecule, thus allowing the formation or consumption of ATP. Hence, arginine kinase regulates ATP and proton buffering and, consequently, metabolism and energetic transport (Ellington, 2011). The distinct expression profiles of two arginine kinases perhaps indicated that there was functional diversity among these enzymes (Uda *et al*., 2012). The H^+-transporting ATP synthase alpha subunit isoform (spot 17) had significantly increased levels, which would directly increase the ATP level. These up-regulations could compensate the significant down-regulation of the ATP synthesis in glycolysis in response to the DEHP exposure.

MDH expression at protein and mRNA levels towards DEHP exposure

The TCA cycle, a sub-pathway of glycolysis, is a series of chemical reactions used by all aerobic living organisms to generate energy, and it provides precursors for the biosynthesis of compounds (Chen and Russo, 2012). Two isoforms of MDHs, reversibly catalyzing the oxidation of malate to oxaloacetate in the TCA cycle (spots 12, 16), were also identified with 1.97- or 1.54-fold increases in expression compared to those of the ethanol group, respectively (Table 1). To further elucidate its expression change in response to DEHP exposure, MDH activity and mRNA expression level were characterized and the results were shown in Figure 2. DEHP treatment increased malate dehydrogenase activities from 96.05 to 125.62 U/mg at 96 h (Fig. 2A). The qPCR results revealed that the mRNA expression level of MDH was elevated, with a 1.67-fold increase compared to the ethanol control group (Fig. 2B). These results were consistent with the protein expression patterns revealed by 2-DE analysis (Fig. 1). A significant difference was observed not only in the enzymatic assay but also in mRNA expression, according to the *t*-test analysis of the control and challenged groups.

Overall, the reference map reported here provided a useful tool for identifying protein pattern changes in the clam and clearly demonstrated the cellular response to DEHP exposure. DEHP triggered adjustments of the glycolysis pathway and the TCA cycle, which are crucial for energy production and the maintenance of sufficient ATP levels.

Acknowledgments

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References

Bandara LR, Kennedy S. Toxicoproteomics - a new preclinical tool. Drug Discov. Today 7: 411-418, 2002.

Benninghoff AD. Toxicoproteomics-the next step in the evolution of environmental biomarkers. Toxicol. Sci. 95: 1-4, 2007.

Borch J, Metzdorff SB, Vinggaard AM, Brokken L, Dalgaard M. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology 223: 144-155, 2006.

Chalubinski M, Kowalski ML. Endocrine disrupters potential modulators of the immune system and allergic response. Allergy 61: 1326-1335, 2006.

Chen J, Russo J. Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells, Biochim. Biophys. Acta 1826: 370-384, 2012.

Costa V, Amorim MA, Quintanilha A, Moradas-Ferreira P. Hydrogen peroxide-induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae* : the involvement of the oxidative stress response regulators Yap1 and Skn7. Free Radical Bio. Med. 33: 1507-1515, 2002.

Dowling VA, Sheehan D. Proteomics as a route to identification of toxicity targets in environmental toxicology. Proteomics 6: 5597-5604, 2006.

Ellington WR. Evolution and physiological roles of phosphagen systems. Annu. Rev. Physiol. 63: 289-325, 2001.

Hobson JF, Carter DE, Lightner DV. Toxicity of a phthalate ester in the diet of a penaied shrimp. J. Toxicol. Environ. Health 13: 959-968, 1984.

Ishitani R, Chuang DM. Glyceraldehyde-3-phosphate dehydrogenase antisense oligodeoxynucleotides protect against cytosine arabinonucleoside-induced apoptosis in cultured cerebellar neurons. Proc. Natl. Acad. Sci. 93: 9937-9941, 1996.

Kennedy A. The role of proteomics in toxicology: identification of biomarkers of toxicity by protein expression analysis. Biomarkers 7: 269-290, 2002.

Kuravsky ML, Aleshin VV, Frishman D, Muronetz VI. Testis-specific glyceraldehyde-3-phosphate dehydrogenase: origin and evolution. BMC Evol.. Biol. 11: 160, 2011.

Kuravsky ML, Muronetz VI. Somatic and sperm-specific isoenzymes of glyceraldehyde-3-phosphate dehydrogenase: comparative analysis of primary structures and

functional features. Biochemistry 72: 744-749, 2007.

Lu Y, Zhang P, Li C, Su X, Jin C, Li Y, *et al.* Characterisation of immune-related gene expression in clam (*Venerupis philippinarum*) under exposure to di(2-ethylhexyl) phthalate. Fish Shellfish Immunol. 34: 142-146, 2013.

Martinelli M, Mocchiutti NO, Bernal CA. Dietary di(2-ethylhexyl) phthalate-impaired glucose metabolism in experimental animals. Hum. Exp. Toxicol. 25: 531-538, 2006.

Mushtaq M, Srivastava SP, Seth P. Effect of di-2-ethyl hexyl phthalate (DEHP) on glycogen metabolism in rat liver. Toxicology 16: 153-161, 1980.

Nahlik KW, Mleczko AK, Gawlik MK, Rokita HB. Modulation of GAPDH expression and cellular localization after vaccinia virus infection of human adherent monocytes. Acta. Biochim.. Pol. 50: 667-676, 2003.

Oh PS, Lim KT. Phytoglycoprotein (75 kDa) isolated from *Cudrania tricuspidata* Bureau inhibits expression of interleukin-4 in the presence of di (2-ethylhexyl) phthalate via modulation of p38 mitogen-activated protein kinase in primary-cultured mouse thymocytes. J. Appl. Toxicol. 29: 496-504, 2009.

Paola P, Fiandanese N, Secchi C, Berrini A, Fischer B, Schmidt JS, *et al.* Exposure to Di(2-ethyl-hexyl) phthalate (DEHP) in Utero and during lactation causes long-term pituitary-gonadal axis disruption in male and female mouse offspring. Endocrinology 153: 937-948, 2012.

Park SY, Choi J. Cytotoxicity, genotoxicity and ecotoxicity assay using human cell and environmental species for the screening of the risk from pollutant exposure. Environ. Int. 33: 817-822, 2007.

Park K, Kwak IS. Characterization of heat shock protein 40 and 90 in *Chironomus riparius* larvae: effects of di(2-ethylhexyl) phthalate exposure on gene expressions and mouthpart deformities. Chemosphere 74: 89-95, 2008.

Shanmuganathan A, Avery SV, Willetts A, Houghton JE. Copper-induced oxidative stress in *Saccharomyces cerevisiae* targets enzymes of the glycolytic pathway. FEBS Lett. 556: 253-259, 2004.

Shenton D, Grant C. Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*. Biochem. J. 374: 513-519, 2003.

Šukalović VH, Vuletić M, Marković K, Vučinić Z. Cell wall-associated malate dehydrogenase activity from maize roots. Plant Sci. 181: 465-470, 2011.

Svechnikova I, Svechnikov K, Söder O. The influence of di-(2-ethylhexyl) phthalate on steroidogenesis by the ovarian granulosa cells of immature female rats. J. Endocrinol. 194: 603-609, 2007.

Uda K, Ellington WR, Suzuki T. A diverse array of creatine kinase and arginine kinase isoform genes is present in the starlet sea anemone *Nematostella vectensis*, a cnidarian model system for studying developmental evolution. Gene 497: 214-227, 2012.

Uren-Webster TM, Lewis C, Filby AL, Paull GC, Santos EM. Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. Aquat. Toxicol. 99: 360-369, 2010.

Wei X, Ching L, Cheng S, Wong M, Wong CK. The detection of dioxin- and estrogenic-like pollutants in marine and freshwater fishes cultivated in Pearl River Delta, China. Environ. Pollution 158: 2302-2309, 2010.

Wetmore BA, Merrick BA. Toxicoproteomics: proteomics applied to toxicology and pathology. Toxicol. Pathol. 32: 619-642, 2001.

Zhuang W, Yao W, Wang X, Huang D, Gong Z. Distribution and chemical composition of phthalate esters in seawater and sediments in Quanzhou bay, China. J. Environ. Health 28: 898-902, 2011.