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An integrated proteomic and metabolomic study on the gender-specific responses of mussels *Mytilus galloprovincialis* to tetrabromobisphenol A (TBBPA)

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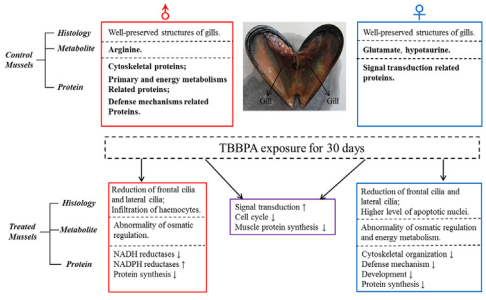
HIGHLIGHTS

- An integrated -omics approach was used to explore the toxicological effects of TBBPA.
- Biological differences were found between non-exposed male and female mussels.
- TBBPA induced gender-specific responses in mussels.
- TBBPA was found to be an endocrine disruptor in mussel.

GRAPHICAL ABSTRACT



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ABSTRACT

Tetrabromobisphenol A (TBBPA), accounting for the largest production of brominated flame-retardants (BFRs) along the Laizhou Bay in China, is of great concern due to its diverse toxicities. In this study, we focused on the gender-specific responses of TBBPA in mussel *Mytilus galloprovincialis* using an integrated proteomic and metabolomic approach. After exposure of TBBPA (10 μg L^{-1}) for one month, a total of 9 metabolites and 67 proteins were altered in mussel gills from exposed group. The significant changes of metabolites in female mussel gills from exposed group exhibited the disturbances in energy metabolism and osmotic regulation, while in male samples only be found the variation of metabolites related to osmotic regulation. iTRAQ-based proteomic analysis showed biological differences between male and female mussel gills from solvent control group. The higher levels of proteins related to primary and energy metabolism and defense mechanisms in male mussel gills meant a greater anti-stress capability of male mussels. Further analysis revealed that TBBPA exposure affected multiple biological processes consisting of production and development, material and energy metabolism, signal transduction, gene expression, defense mechanisms and apoptosis in both male and female mussels with different mechanisms. Specially, the responsive proteins of TBBPA in male mussels signified higher tolerance limits than those in female individuals, which was consistent with the biological differences between male and female mussel gills from solvent control group. This work suggested that the gender differences should be considered in ecotoxicology.

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Abbreviation: A2I, apoptosis 2 inhibitor; ALP, apextrin-like protein; BFRs, brominated flame-retardants; CMD, cytosolic malate dehydrogenase; GSTA, glutathione S-transferase A; O-PLS-DA, orthogonal partial least squares discriminant analysis; PAIHFST, putative alpha-isopropylmalate/homocitrate synthase family transferase; PECSR, putative epidermal cell surface receptor; PLS-DA, partial least squares discriminant analysis; QLP, qm-like protein; RAKC, receptor of activated kinase C; TBBPA, tetrabromobisphenol A; TUNEL, terminal dUTP nick-end labeling.

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1. Introduction

Brominated flame-retardants (BFRs) have been widely used in a diverse array of industrial products, such as plastics, textiles, upholstery foam and electronic equipment, to reduce the risk of fire (Pan et al., 2010). As a reactive flame retardant, TBBPA has been the most frequently used BFR, with a global market of 170,000 tons in 2004 (Makinen et al., 2009), and TBBPA consumption nowadays shows an increasing tendency. Due to the abundant resources of seawater and underground brine, there are many industrial manufacturing plants of brominated flame-retardants (BFRs) along the Laizhou Bay of the Bohai Sea in China. As it is known that TBBPA could be released into environments during its production and disposal processes, which leads to manufacturing plants of TBBPA being a main source of TBBPA pollution. In addition, like other BFRs, TBBPA is a lipophilic substance with a high persistency in the environment and therefore is traceable in air conditioning filter dust ranging from 30 to 140 ng g^{-1} (Ni and Zeng, 2013), waters up to 4.87 μg L^{-1} (Morris et al., 2004; Yang et al., 2012), sediments up to 1420 mg kg^{-1} dry weight (Morf et al., 2005) and organisms ranging from 28.5 to 39.4 ng g^{-1} (Yang et al., 2012). In the water of Mihe estuaries near the Laizhou Bay, the concentrations of TBBPA has been up to 0.67 μg L^{-1}, which poses a potential risk on the marine animals (Zhang et al., 2011b). In addition, derivatives of TBBPA, such as TBBPA bis (allyl) ether (TBBPA BAE) and TBBPA bis (2, 3-dibromopropyl) ether (TBBPA BDBPE) have also been detected in various environmental compartments near the Laizhou Bay (Qu et al., 2013). In this case, more attention should be paid to the environmental risk of the increasing release of TBBPA.

It is reported that TBBPA has diverse toxicities including cytotoxicity (Strack et al., 2007), hepatotoxicity (Szymanska et al., 2000), nephrotoxicity and neurotoxicity (Birnbaum and Staskal, 2004). Additionally, due to the molecular similarity to the natural thyroid hormones (THs), TBBPA is suggested to have the potential to impact several components of the thyroid system (Jagnytsch et al., 2006). Previous studies have revealed the TBBPA toxicity in aquatic organisms such as zebrafish (Kling and Forlin, 2009), sea urchin (Anselmo et al., 2011), freshwater fish (Feng et al., 2013) and rainbow trout (Ronisz et al., 2004). Traditional ecotoxicology has focused on a bottom-up approach to understand stressor effects, in which a few molecules including genes and proteins, biochemical reactions and histological studies are studied at a time (Garcia-Reyero and Perkins, 2011). Since researchers often target a known class of toxicity-responsive molecules, it is difficult to discover new molecules related to toxicological effects. Therefore, there are few opportunities to present a comprehensive understanding of toxicological responses of organisms to contaminants if relying only on these bottom-up approaches in ecotoxicology researches.

With the rapid development of -omic techniques including genomics, transcriptomics, proteomics and metabolomics, a top-down approach has quickly evolved in the biomedical arena (Garcia-Reyero and Perkins, 2011), by which researchers can focus on a global profile of one type of molecules such as genes, proteins and metabolites and their alterations with high-throughput analyses. The -omic technologies allow researchers to more deeply investigate and unravel toxicological effects and mechanisms of environmental contaminants (Weckwerth, 2011). Among these technologies, proteomics is a large-scale study of proteins encoded by the given genome in an organism (Anderson and Anderson, 1988), it can describe complete proteomes at tissue, cell, or organelle levels as well as be used to analyze proteomes under environmental contaminant stress (Ahsan et al., 2009). Metabolomics usually focuses on the whole set of low molecular weight (<1000 Da) metabolites that are the end products in multiple biological systems including organs, tissues, biofluids, or even whole organisms. Since metabolomics and proteomics can directly characterize the perturbations of metabolic pathways and linked enzymes and stress-responsive proteins, an integration of these two -omic techniques may present an insightful view into the stressor-induced responses (Zhang et al., 2011a).

Mussel *Mytilus galloprovincialis* is a frequently used bioindicator in marine ecotoxicology because of its high tolerance and accumulation of contaminants (Ciacci et al., 2012). Additionally, *M. galloprovincialis* can be easily sampled along the Bohai coast where is of the biggest manufacturing base of TBBPA in China. Since TBBPA is a known endocrine disrupting chemical to animals, the gender-specific effects of TBBPA should be considered in ecotoxicology, which may better understand the responsive mechanisms in toxicology bioindicators towards TBBPA exposure. To our knowledge, no studies attempted to compare the gender-specific responses induced by TBBPA in marine mussel at protein and metabolite levels. In this study, we combined proteomics and metabolomics to characterize the differential responses and elucidate the differential toxicological effects of TBBPA in male and female mussels.

2. Materials and methods

2.1. Animals

Adult mussels *M. galloprovincialis* (shell length: 5.5–6.0 cm, $n = 30$) were collected in July 2012 in an aquaculture farm (37°36′21″ N, 121°90′12″ E) where the marine water met the first category quality standard according to "Marine water quality standard of the People's Republic of China" (Yantai, China). After transported to the laboratory, the animals were acclimatized in aerated natural seawater (salinity 31 psu) for 7 d and were then divided into two groups (solvent control and treatment) each containing 15 mussels to ensure at least six female and six male individuals in each group. Dimethyl sulfoxide (DMSO) was used as the solvent for the dissolution of TBBPA (Guoyao, Shanghai, China). Since our previous study confirmed that there was no significant proteomic and metabolomic difference in mussel *M. galloprovincialis* samples between seawater control (mussels cultured in the filtered seawater) and solvent (DMSO) control groups, we did not set the seawater control group in this study (Ji et al., 2013a). The mussels in the treatment group were exposed to one sublethal concentration (10 μg L^{-1}) of TBBPA. The concentration of TBBPA stock solution was 5 mg L^{-1} in DMSO, ensuring the same DMSO concentration (0.002%, v/v) in the TBBPA-exposed group to that of solvent control group. During the acclimation and exposure periods, mussels were kept at 25 °C under a photoperiod of 12 h light and 12 h dark, and fed with the *Chlorella vulgaris* at a ration of 2% of tissue per dry weight daily. After exposure for 30 days, all the mussels were immediately dissected for gill and gonad tissues. The gonad tissues were used for sex determination. Each gill tissue sample was divided into three parts that were used for histological observation, metabolite extraction and protein and RNA extraction. Those gill samples for metabolite, protein and RNA extraction were snap-frozen in liquid nitrogen, and then stored at −80 °C. For iTRAQ-based proteomics, either solvent control group or TBBPA-exposed group consisted of two (three pooled into one) biological replicates for both male and female mussels, respectively.

2.2. Histology

Both gonad and gill tissues were carefully fixed in the Bouin's fixative solution after dissection from the mussels. The histology of gonad tissues was simply used for sex determination (Fig. S1). Tissues from control and TBBPA treatment (10 μg L^{-1}) were pro-

cessed together in batches to remove artefacts between control and TBBPA treatment. Histological sections (6–8 μm thickness) were stained with hematoxylin-eosine (HE) and observed under a light microscope (Olympus BX61, Tokyo, Japan) at × 200 magnification (zoom on the camera was × 2.5). The injury of mussel gills was measured according to previous study with some modifications (Sheir and Handy, 2010). The detailed information on making sections and histological observations were described in the Supporting Information.

Terminal dUTP nick-end labeling technique (TUNEL-technique) was applied to detect the apoptosis in gills and performed with one step TUNEL kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) according to the manufacturer's instructions. For each slide, five fields on each section were randomly chosen under the microscope. Assays were conducted in a blinded manner. More details about TUNEL technique were described in the Supporting Information.

2.3. Metabolomic analysis

Polar metabolites in mussel gill tissues ($n = 6$ for each treatment) were extracted in 4 mL g^{-1} of methanol, 5.25 mL g^{-1} of water and 2 mL g^{-1} of chloroform as described previously and analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 25 °C) as described previously (Liu et al., 2011).

All one dimensional ^{1}H NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab version 7.0 (The MathsWorks, Natick, MA, USA) and then segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. All the NMR spectra were generalized log transformed with a transformation parameter $\lambda = 1.0 \times 10^{-8}$ and subsequently data were mean-centered before data analysis. More details about metabolomic analysis were described in the Supporting Information.

2.4. Quantitative proteomic analysis

Each pooled gill sample ($n = 2$) was ground into powder in liquid nitrogen and then dissolved in lysis buffer (9 M urea, 4% CHAPS, 1% w/v DTT and 1% IPG buffer) with protease inhibitor (Roche Applied Science, Mannheim, Germany) to extract proteins. The concentrations of the protein extracts were determined using the Bradford method (Bradford, 1976).

iTRAQ technique was applied to quantitative proteomic analysis. The iTRAQ labeling of peptides from mussel samples of solvent control and TBBPA exposure were performed using iTRAQ 8-plex reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Eight samples (two biological replicates per group) were labeled with the iTRAQ tags as female control, female TBBPA treatment, male control and male TBBPA treatment. Protein Pilot Software 4.0 (AB SCIEX, Framingham, MA, USA) was used to process proteomic data against a marine bivalve-EST-Translated protein database (*M. galloprovincialis*, *Mytilus edulis*, *Ruditapes philippinarum* and *Crassostrea gigas*, 238,224 sequences) using the Paragon algorithm (Shilov et al., 2007). The protein ratios in each replicate were then quantified based on the summed intensities of the matched spectrum. These ratios from the biological replicates were evaluated by using a Student's t-test combined with the Benjamini–Hochberg correction (Han et al., 2013). Proteins with corrected p values less than 0.05 and fold changes larger than 1.20 or smaller than 0.83 were considered to be significantly differential. More details about quantitative proteomic analysis were described in the Supporting Information.

2.5. RNA extraction and qRT-PCR

In order to evaluate the correlation between mRNA expression and protein abundances, qRT-PCR was used to determine the expression levels of mRNA. More details of RNA extraction and qRT-PCR were described in the Supporting Information.

3. Results

3.1. Histological observation and apoptosis of gills from mussels exposed to TBBPA

HE staining and TUNEL technique were performed on gill tissue sections to assess the pathological changes and occurrences of apoptotic nuclei in individual cells. Gill sections from control and TBBPA-exposed mussels were exhibited in Fig. 1. In solvent control group, gill sections from both female and male mussels presented well-preserved structures, with no evidence of necrosis or lesions of the gill filaments. Meanwhile, the TUNEL test showed no obvious apoptotic nuclei in the solvent control mussels either. In the TBBPA treatment group, by contrast, a rather abnormal morphology and apparent apoptotic nuclei of the gill filaments were noted in both male and female individuals. Through the HE staining gill sections, both genders showed a reduction of frontal cilia and lateral cilia. Moreover, gill filaments of male individuals presented infiltration of haemocytes in the abfrontal. The TUNEL test showed that apoptosis occurred in the abfrontal zone of gill filaments of both male and female mussels from TBBPA treatment group.

3.2. Effects of TBBPA on the metabolome of mussel gills

O-PLS-DA (orthogonal partial least squares discriminant analysis) demonstrated the significant ($p < 0.05$) metabolic differences between male and female mussel samples from solvent control group with a reliable Q^2 value of 0.485 (Fig. 2). As shown in Fig. 2, female mussel samples had significantly ($p < 0.05$) higher levels of glutamate and hypotaurine, and lower level of arginine than those in male mussel samples from solvent control group.

Further O-PLS-DA analysis indicated that TBBPA induced differential metabolic responses between male and female mussels (Fig. 3). In male mussel samples, five metabolites were significantly altered including decreased phosphocholine and homarine and increased dimethylglycine (DMG), choline and betaine in TBBPA-exposed group. Except the elevated choline and depleted homarine, the metabolic responses induced by TBBPA in female mussel samples were different with those in male mussel samples. As shown in Fig. 3, TBBPA induced increased aspartate and decreased betaine, taurine, ATP, homarine and glycine in female mussel gills after TBBPA exposure for 30 days.

3.3. Effects of TBBPA on the proteome of mussel gills

The protein expression profiles from male and female solvent control groups were compared. Each group comprised two biological replicates that were from three pooled individual mussel samples with an equal mass (50 mg wet weight) from each sample. The biological replicates were used to determine the significance of the protein abundances between male and female solvent control groups with a 95% confidences level (Benjamini and Hochberg corrected T-test at $p < 0.05$). In total, 1017 proteins were identified on the basis of 24536 highly confident spectra, of which peptides were unique. On average, each protein was quantified using 25.0

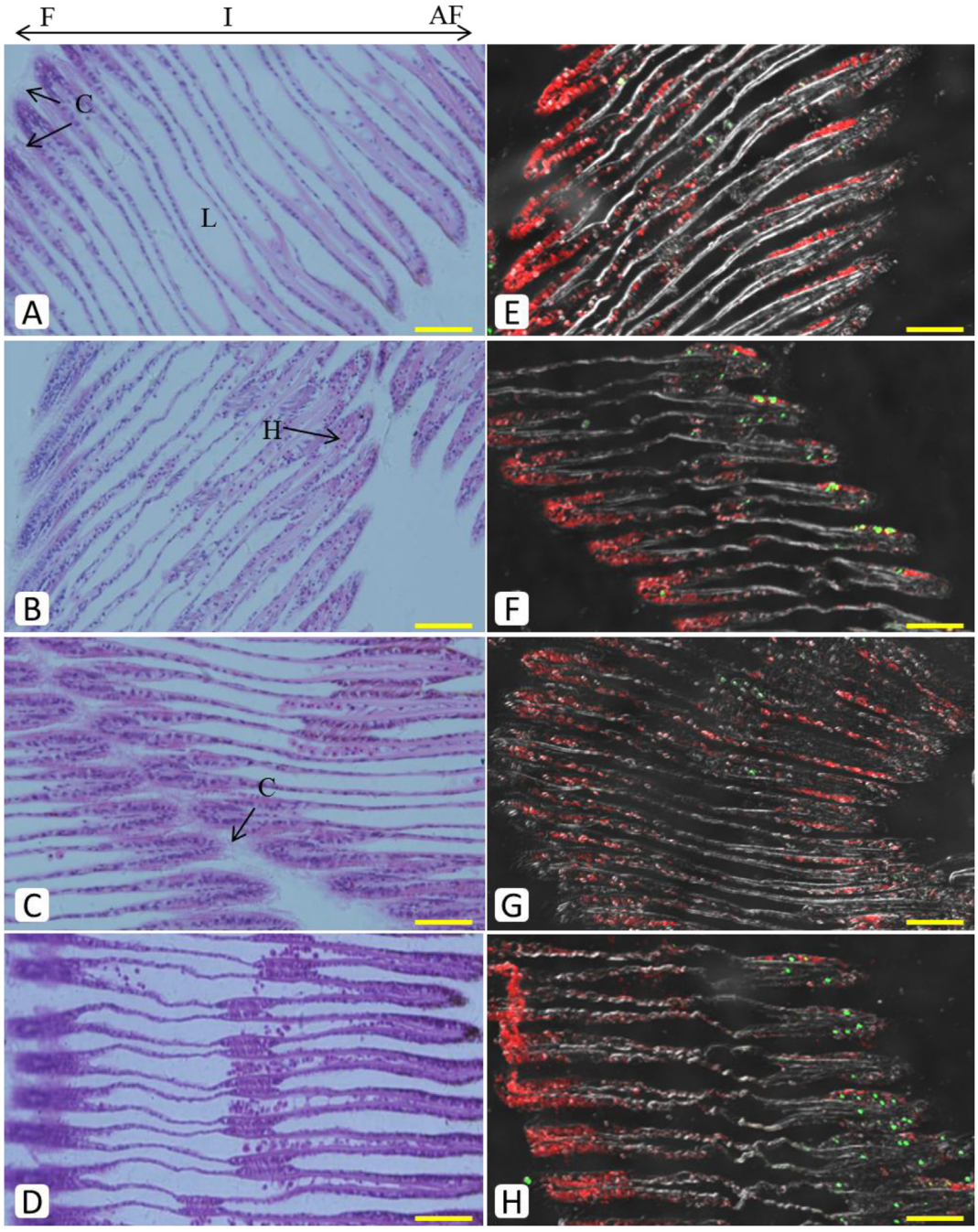

Fig. 1. H/E stained (A, B, C, D) and TUNEL (green)/PI (red) (E, F, G, H) sections through gill tissues of mussels. (A, E) female individual from solvent control group; (B, F) female individual from 10 μg L^{-1} TBBPA treatment group; (C, G) male individual from solvent control group; (D, H) male individual from 10 μg L^{-1} TBBPA treatment group. **F**, frontal cilia; **I**, intermediate filament; **AF**, abfrontal cilia; **L**, lumen; **C**, cilia; **H**, haemocytes. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spectra. Among the 1017 proteins, a total of 55 proteins showed significantly ($p < 0.05$) different abundances in gills between male and female mussels from solvent control group (Table 1), including 34 and 21 abundant proteins in male and female mussel samples, respectively. According to the KEGG pathway analysis, these proteins from solvent control group were basically involved in cytoskeleton, reproduction and development, material and energy metabolism, signal transduction, protein synthesis, chaperones, defense mechanisms and apoptosis.

Further studies on comparison between solvent control and TBBPA-exposed mussel groups indicated that 67 proteins were significantly ($p < 0.05$) changed in expression from both male and female mussel samples. These differentially expressed proteins were mainly involved in multiple biological processes and cellular components including material and energy metabolism (17%), ribosomal proteins (14%), signal transduction and transport (13) and cytoskeleton (13%). As shown in Table 2, in female mussel samples from TBBPA treatment group, a total of 36 proteins displayed sig-

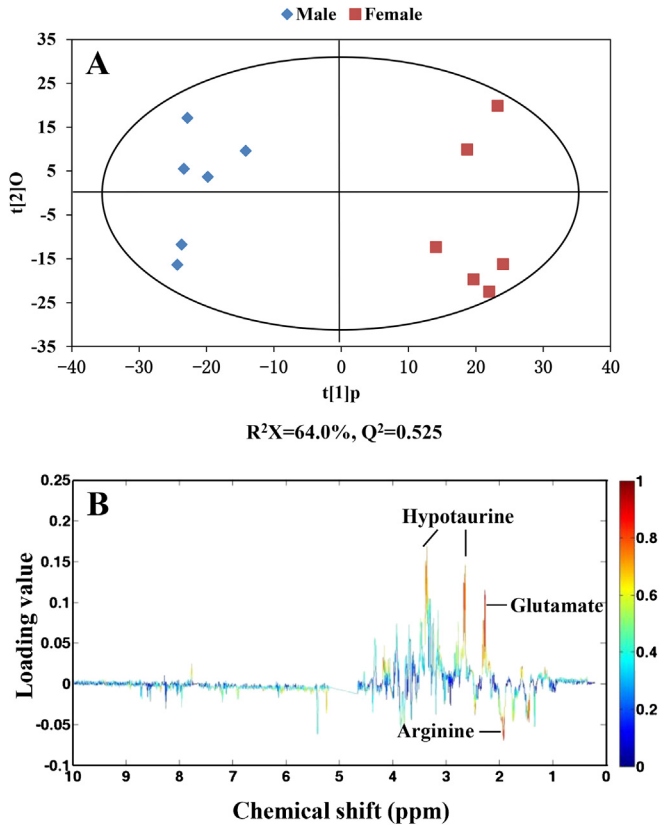

Fig. 2. O-PLS-DA scores derived from ^{1}H NMR spectra of gill extracts of *M. galloprovincialis* from male control (♦) and female control (■) groups (A) and corresponding coefficient plot (B). The color map shows the significance of metabolite variations between the two classes (male control and female control). Peaks in the positive direction indicate metabolites that are more abundant in the female solvent control group. Consequently, metabolites that are more abundant in the male solvent control group are presented as peaks in the negative direction.

nificant changes in expression, among which 11 proteins showed increased expressions and 25 presented decreased expressions. For the male mussel samples, there were 42 proteins altered in TBBPA-treated group, including 18 up-regulated and 24 down-regulated proteins. Interestingly, only eleven proteins (16.4%) were commonly changed in expression from both male and female mussel samples exposed to TBBPA.

3.4. Correlation between gene expressions and protein abundances

To further verify the results of protein expressions and compare the correlation between protein and gene expressions, seven representative genes related to altered proteins, including apoptosis 2 inhibitor (A2I), putative alpha-isopropylmalate/homocitrate synthase family transferase (PAIHFST) and receptor of activated kinase C (RAKC) in both male and female group, putative epidermal cell surface receptor (PECSR) and apextrin-like protein (ALP) in female group, cytosolic malate dehydrogenase (CMD) and glutathione S-transferase A (GSTA) in male group, were quantified using qRT-PCR. The results indicated that the mRNA expression levels of A2I, PAIHFST and RAKC had consistent alteration tendencies with corresponding proteins in both male and female TBBPA-treatment group. Additionally, PECSR and GSTA demonstrated positive correlation between mRNA and protein expressions in male and female TBBPA treatment group, respectively, while ALP and CMD presented disparities between mRNA expressions and corresponding protein abundances, respectively (Fig. 4).

4. Discussion

4.1. TBBPA induced damages and apoptosis in mussel gills

Mussel gill is a suitable organ for ecotoxicological studies because it is the first uptake site for many toxicants in the aquatic environment (Gomez-Mendikute et al., 2005). In this study, we have distinguished three different zones in mussel gill filaments: frontal, intermediate and abfrontal, as described in a previous study (Gomez-Mendikute et al., 2005). The frontal zone, with ciliated and non-ciliated cells, is involved in transporting and uptaking of nutrients and contaminants (Owen, 1974). The so-called endothelial cells are flattened non-ciliated cells in the intermediate zone of gill filament in which gas exchange and interactions between the external medium and haemolymph occur (Gomez-Mendikute et al., 2005). Both ciliated and non-ciliated cells are also found in the abfrontal zone, whose functions are not completely clear. In this study, gills presented loss of frontal cilia and lateral cilia after TBBPA treatment for 30 days, which suggested that TBBPA might affect the normal respiration and nutrient uptake. Similar phenomena have been reported in mussels exposed to tri-n-butyltin, Cd and mussels from natural polluted environments polluted by various heavy metals (Micic et al., 2001; Sheir and Handy, 2010; Fasulo et al., 2008). Due to a lack of adaptive immunity in mussels, the hemocytes are very important for immune defense, and the circulating hemocytes are able to penetrate tissue in response to antigens. In TBBPA treatment group, male mussels presented infiltration of haemocytes in the abfrontal zone of gill filaments, which was also found in *M. edulis* exposed to a mixture of diesel oil and copper and in *Perna viridis* gills from polluted sites (Auffret, 1988; Arockia et al., 2012).

TUNEL test was used to assess apoptosis of gills from TBBPA-exposed mussels. Interestingly, the distinct apoptotic nuclei were only found in the abfrontal zone of gill filaments, whose functions are not completely clear (Gomez-Mendikute et al., 2005). Lipofuscin granules that are symptoms of damage to membrane, mitochondria and lysosomes and contribute to apoptosis were found to be of greater abundance in the abfrontal zone of gill filaments (Powell et al., 2005). That is to say, abfrontal cells undergo apoptosis more easily than other cells in the gill filaments, which are consistent with the observations of apoptosis in gills with TBBPA treatment in this study. Histological observations and TUNEL assay indicated that TBBPA treatment could result in losses of frontal and lateral cilia and apoptosis in abfrontal cells, respectively, in both female and male mussels. To further elucidate the responsive mechanisms, an integration of metabolomics and proteomics was used to quantitatively profile *M. galloprovincialis* metabolites and proteins in response to TBBPA treatment.

4.2. Differential metabolites detected in female and male mussel gills

Three metabolites consisting of glutamate, hypotaurine and arginine exhibited significantly different concentrations between male and female mussel samples from solvent control group. Arginine is the main constituent of sperm proteins and is therefore observed at a higher (~1.5 times) level in male mussels than that in female mussel samples. Free amino acids can be involved in both osmotic regulation and energy metabolism (Viant et al., 2003). The higher level of glutamate probably meant that female mussels could use more glutamate to regulate osmolarity, together with the

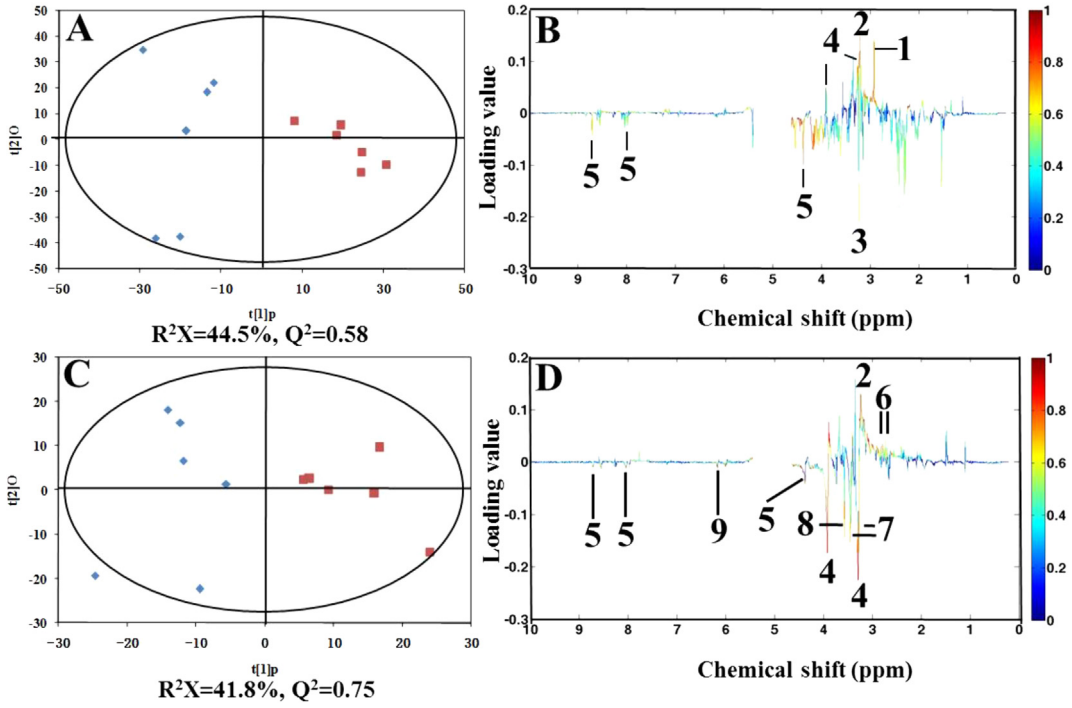

Fig. 3. O-PLS-DA scores derived from ^{1}H NMR spectra of tissue extracts from control (♦) and 10 μg L^{-1} TBBPA-exposed groups (■), (A) male and (C) female, and corresponding coefficient plots (B) and (D). The color map shows the significance of metabolite variations between the two classes (solvent control and TBBPA treatment). Peaks in the positive direction indicate metabolites that are more abundant in the TBBPA-exposed group. Consequently, metabolites that are more abundant in the solvent control group are presented as peaks in the negative direction. 1: dimethylglycine, 2: choline, 3: phosphorylcholine, 4: betaine, 5: homarine, 6: aspartate, 7: taurine, 8: glycine, 9: ATP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

organic osmolyte, hypotaurine. These metabolic differences indicated that there were intrinsic (gender-specific) biological differences between male and female mussel gills from solvent control group. Therefore there could be gender-specific responses in male and female mussels to toxicants due to the intrinsic biological differences between male and female mussels.

4.3. Effects of TBBPA treatment on the metabolome of gills in male and female mussels

As shown in Fig. 3, five metabolites consisting of dimethylglycine, choline, betaine, homarine and phosphocholine were significantly altered in gills from male mussels after TBBPA exposure for 30 days. Choline is the key component of phosphatidylcholine and sphingomyelin, two classes of phospholipid that are abundant in cell membrane, and betaine and dimethylglycine are important intermediates in choline-glycine pathway (Friesen et al., 2007). The significant up-regulation of choline, betaine and dimethylglycine suggested that TBBPA might promote the choline-glycine pathway aiming to produce more betaine, dimethylglycine and glycine, which was also attributed to the down-regulation of phosphocholine that is the intermediate in the synthesis of phosphatidylcholine. Marine bivalves accumulate osmoprotectants or osmolytes such as betaine, homarine and dimethylglycine to maintain osmotic balances and stabilities of cytomembrane under environmental stress (Shen et al., 2002). Zhang et al. (2011c) and Liu et al. (2010) found the similar phenomenon in bivalve *Ruditapes philippinarum* exposed to heavy metals.

Differing from male mussels, female individuals presented increases in choline and aspartate and decreases in betaine, homarine, taurine, glycine and ATP. The alterations of osmolytes including choline, homarine, taurine and glycine suggested the disturbance in osmotic regulation. Aspartate, an important intermediate in amino acids metabolism, involved in multiple biological processes including biosynthesis of amino acids, urea cycle and gluconeogenesis. The elevated aspartate and depleted ATP revealed the abnormality of energy metabolism. It is hypothesized that a new trade-off between energy production and consumption was established (Tomanek, 2011). Our previous study showed that 2,2′,4,4′-Tetrabromodiphenyl ether (BDE 47) induced the similar alterations such as disturbance of energy metabolism and osmotic regulation in mussel *M. galloprovincialis* (Ji et al., 2013a).

4.4. Differential proteins expressed in non-exposed male and female mussel gills

The iTRAQ combined with 2D-LC-MS/MS analysis identified 1017 proteins, of which 55 were of significantly ($p < 0.05$) different abundances in gills between male and female mussels from solvent control group (Table 1). According to the KEGG pathways analysis, these 55 significantly expressed proteins were divided into several pathways including cytoskeleton, reproduction and development, material and energy metabolism, signal transduction, gene expression, stress response and apoptosis.

Gill of mussel *M. galloprovincialis* plays an important role not only in feeding and breathing, but also in sensing the external

Table 1
Differentially expressed proteins between male and female groups.

Accession	Description	No. of unique peptides	*p* value[a]	Ratio (male/female)
Cytoskeleton				
FL497795	Paramyosin	5	0.026	4.41
FL494855	Myosin heavy chain, non-muscle, partial	15	0.0001	0.31
AJ624701	Transgelin-2	14	0.0007	7.59
AJ624239	Tropomyosin	40	0.0323	1.69
FL491653	Troponin T	10	0.0467	2.88
FL497768	Sarcoplasmic calcium-binding protein	15	0	9.29
FL493105	Calponin-like protein	14	0.0009	4.41
Production and development				
FL633554	vdg3	5	0.0037	1.85
FL491843	Nacre protein	5	0.028	5.45
AM879763	Tumor protein D54	9	0.0315	0.58
FL501181	Prohibitin2	5	0.007	0.43
Material and energy metabolism				
FL593791	Cytosolic malate dehydrogenase	25	0.0032	2.09
FL501267	Fructose-bisphosphate aldolase	30	0.0168	2.33
FL498012	Cathepsin L	9	0.0357	1.21
FL493697	Carboxypeptidase B	8	0.0035	4.7
FL491755	isocitrate dehydrogenase	9	0.0213	1.39
FL489629	Arginine kinase	33	0.0237	11.38
EH663547	V-type proton ATPase subunit E	5	0.0336	0.37
AM879488	Peroxisomal trans-2-enoyl-CoA reductase	1	0.0027	10.28
FL593628	CoA-disulfide reductase	2	0.0364	1.61
FL498079	putative alpha-isopropylmalate/homocitrate synthase family transferase	21	0	3.08
FL491608	PREDICTED: phytanoyl-CoA dioxygenase, peroxisomal-like	1	0.0031	6.19
Signal transduction and transport				
FL498212	GTPase	2	0.0394	0.02
FL496303	Neuronal acetylcholine receptor subunit alpha-10	13	0.0249	0.06
AJ624341	Putative epidermal cell surface receptor	2	0.0168	0.64
FL493905	EF-hand domain-containing protein 1	6	0.0094	1.92
Ribosomal proteins				
FL495623	60S ribosomal protein L7a	3	0.0465	0.67
FL495604	Ribosomal protein S5	9	0.033	4.41
FL492455	40S ribosomal protein SA	12	0.0227	0.19
FL492189	Ribosomal protein L9	9	0.0007	0.3
AJ626134	Ribosomal protein S10	4	0.0141	0.13
AJ516626	Ribosome-binding protein 1	7	0.0499	4.74
Transcription and translation				
FL494458	Glucose-repressible alcohol Dehydrogenase transcriptional effector	3	0.0159	0.25
AM878774	Elongation factor 2	8	0.0108	2.21
AJ624855	PREDICTED: eukaryotic translation initiation factor 3 subunit H-like	4	0.0439	1.84
Chaperone				
FL501012	Protein SET	4	0.003	0.17
FL499562	Hsc70-interacting protein	8	0.0434	0.38
Defense mechanisms				
FL495425	Astacin	22	0.0033	1.75
FL500303	Fibrinogen-related protein 5	7	0.0215	2.09
FL496423	Apextrin-like protein	5	0.0284	20.32
FL494057	Glutathione S-transferase A	9	0.0062	4.25
FL492661	Lectin	20	0.0018	0.37
AJ624097	Chitotriosidase	133	0.049	0.14
FL496472	Putative C1q domain containing protein MgC1q5	5	0.0309	2.36
FL491045	Coagulation factor V, partial	6	0.0146	0.5
Apoptosis				
FL500771	Apoptosis 2 inhibitor	7	0.0245	1.72
FL491544	PREDICTED: cell death protein 3-like	4	0.0455	0.42
Others				
FL594915	Hypothetical protein CGI_10019787	3	0.0206	0.21
FL497934	Kyphoscoliosis peptidase	7	0.0145	4.92
FL496999	Hypothetical protein CGI_10027602	6	0.0119	6.61
FL492936	none	33	0.0479	5.6
AJ625534	PREDICTED: uncharacterized protein LOC100179434	19	0.011	28.58
AJ625057	Hypothetical protein EAG_11213	11	0.0427	7.73
FL499014	Endothelin-converting enzyme 1	10	0.033	0.41
FL491471	Canopy-like protein 3	7	0.035	0.31

[a] Means *p* values obtained by using a Student's t-test have been corrected using the Benjamini–Hochberg method.

Table 2
Differentially expressed proteins in response to TBBPA treatment in male and female mussel gills.

Accession	Description	No. of unique peptides	*p* value[a]	Fold change[b]
Cytoskeleton				
FL497199	Non-neuronal cytoplasmic intermediate filament protein	43	0.0002	3.31[c]
				2.13[d]
FL492506	Fascin	19	0.0031	−2.11[c]
AJ624239	Tropomyosin	40	0.0272	1.36[c]
				−2.07[d]
FL494855	Myosin heavy chain, non-muscle, partial	15	0.0029	2.21[d]
AJ624420	Actin	112	0.0025	3.08[d]
AJ624701	Transgelin-2	14	0.0262	−1.45[d]
FL489346	Paramyosin	5	0.0161	1.84[d]
FL497768	Sarcoplasmic calcium-binding protein	15	0	−10.67[d]
FL493105	Calponin-like protein	14	0.0051	−4.06[d]
Reproduction and development				
FL633554	vdg3	5	0.0166	−4.49[c]
FL500578	Bindin	6	0.0028	1.89[c]
				−12.02[d]
FL491843	Nacre protein	5	0.0268	5.25[c]
FL489696	Leukocyte cell derived chemotaxin 1-like protein	6	0.015	−3.66[c]
				14.06[d]
Material and energy metabolism				
FL593791	Cytosolic malate dehydrogenase	25	0.0244	−2.36[c]
FL498079	putative alpha-isopropylmalate/homocitrate synthase family transferase	21	0.0498	−6.19[c]
				−2.56[d]
FL493697	Carboxypeptidase B	8	0.0052	1.45[c]
FL493457	Alcohol dehydrogenase class-3	9	0.0051	−6.31[c]
FL489629	Arginine kinase	33	0.0355	−4.79[c]
AM879488	peroxisomal trans-2-enoyl-CoA reductase	1	0.0132	1.36[c]
AJ625708	Putative mitochondrial ATP synthase F chain	4	0.0397	−2.07[c]
FL491911	Perlucin	3	0.0058	−1.25[d]
FL501230	Procollagen-proline dioxygenase beta subunit	18	0.0068	−2.05[d]
FL493384	PREDICTED: Protein ADP-ribosylarginine hydrolase-like isoform X2	2	0.0323	18.18[d]
Signal transduction and transport				
FL490621	Kielin/chordin-like protein	2	0.0125	−1.66[c]
				1.84[d]
FL497578	Receptor of activated kinase C	15	0.0126	−1.84[c]
				−1.24[d]
FL496506	Calcyphosin-like protein	12	0.0461	1.43[c]
FL496303	Neuronal acetylcholine receptor subunit alpha-10	13	0.0472	2.51[c]
AJ624341	Putative epidermal cell surface receptor	2	0.021	−3.98[c]
AJ624260	Rab GDP dissociation inhibitor beta	4	0.0279	−3.19[c]
FL493905	EF-hand domain-containing protein 1	6	0.0067	2.21[c]
FL498212	GTPase	2	0.0255	37.04[d]
FL498029	Axonemal dynein light chain p33	9	0.0466	1.94[d]
Ribosomal proteins				
AJ626278	PREDICTED: 60S ribosomal protein L5-like	6	0.0487	−2.21[c]
FL593537	Ribosomal protein L28	5	0.0389	−1.41[c]
				−1.32[d]
FL497628	60S ribosomal protein L6	11	0.0222	−1.57[c]
FL495584	Ribosomal protein L	5	0.0017	−4.83[c]
AJ625324	Ribosomal protein S27	12	0.0198	−2.56[c]
FL492837	40S ribosomal protein S4, X isoform	8	0.0423	−1.98[d]
FL492224	60S ribosomal protein L14	9	0.0198	−2.94[d]
FL491967	Ribosomal protein S3	11	0.0409	2.00[d]
AM879598	Ribosomal protein L19	6	0.006	−1.71[d]
AM881638	40S ribosomal protein S17	11	0.0215	−1.66[d]
Transcription and translation				
FL492906	Putative period clock protein	2	0.0385	3.84[d]
AM880346	qm-like protein	9	0.0237	−3.47[c]
				−2.17[d]
AM878774	Elongation factor 2	8	0.0076	−2.75[c]
FL497275	PREDICTED: elongation factor-1, delta, a isoform X3	11	0.049	−1.66[d]
AJ624855	PREDICTED: eukaryotic Translation initiation factor 3 subunit H-like	4	0.0478	−1.85[d]
Chaperones				
FL501012	Protein SET	4	0.0445	−1.56[c]
EH663477	Coactosin-like protein	19	0.0175	2.40[c]
EH662751	Calreticulin	33	0.0192	1.58[d]
AJ624092	Excinuclease ABC subunit B	138	0.0092	−4.97[d]
FL496057	Small heat shock protein 24.1	51	0.0333	2.15[d]
Defense mechanisms				
FL496423	Apextrin-like protein	5	0.0363	2.56[c]
FL495433	Cathepsin L2 cysteine protease	16	0.0383	6.25[d]
FL496702	Lysozyme	7	0.0419	−1.87[d]
FL495401	Chitotriosidase-1	104	0.0113	−9.04[d]
FL494057	Glutathione S-transferase A	9	0.0073	−3.22[d]

(*continued on next page*)

Table 2 (*continued*)

Accession	Description	No. of unique peptides	*p* value[a]	Fold change[b]
FL491817	Mitochondrial manganese superoxide dismutase	8	0.0143	−3.98[d]
AJ625233	PREDICTED: acidic mammalian chitinase isoform X2	43	0.0176	−2.01[d]
Apoptosis				
FL497051	Apoptosis 2 inhibitor	13	0.0017	2.23[c] −2.68[d]
FL494240	Endophilin-B1	11	0.021	−2.38[c]
Others				
FL594915	Hypothetical protein CGI_10019787	3	0.0275	−3.34[c] 2.09[d]
FL594693	Hypothetical protein CGI_10010255	5	0.0199	3.94[c]
FL594962	none	4	0.007	−3.80[c]
FL497934	Kyphoscoliosis peptidase	7	0.0297	−2.83[d]
FL500225	Hypothetical protein CGI_10021532	8	0.0086	3.22[d]
FL496051	Hypothetical protein, partial	3	0.0169	4.92[d]

[a] Means *p* values obtained by using a Student's t-test have been corrected using the Benjamini–Hochberg method.
[b] Plus (+) which is omitted represents up-regulated expression, and minus (−) represents down-regulated expression.
[c] Means that altered proteins in female mussels.
[d] Means that altered proteins in male mussels.

environment. Cytoskeletal components such as actin and microtubulin are compactly distributed on the surface of the epithelial cells of gill filament, which contribute to the sensitivity of gill to environmental stress (Reed et al., 1984). The higher levels of cytoskeletal proteins including tropomyosin, troponin T, paramyosin, sarcoplasmic calcium-binding protein and calponin-like proteins in gills of male mussels presumably implied the greater abilities of sensing and adapting environmental stress. Thirteen proteins involved in material and energy metabolism presented different expression levels, of which 12 proteins were of higher expression levels in male mussel samples. These proteins function as key enzymes in multiple metabolic pathways including glycolysis, proteolysis, Krebs cycle, lipid metabolism and energy metabolism. It is worth noting that reductases such as CoA-disulfide reductase and peroxisomal trans-2-enoyl-CoA reductase were also expressed higher in male mussel gills. These reductases defend body against cell damage by producing NADPH which can eliminate the extra ROS induced by environmental stress (Kültz, 2005; Go and Jones, 2008). The higher levels of proteins related to material and energy metabolism in male mussel gills meant greater anti-stress capability of male individuals. Additionally, stimuli from external environment can induce defense responses in antioxidant enzyme system and immune system and apoptosis in organisms. Nine differentially expressed proteins are related to defense mechanisms, of which 6 were of higher expression levels in male mussel samples. Proteins related to defense mechanisms help cells to monitor damage and act directly on xenobiotics aiming at temporarily increasing tolerance limits (Kültz, 2003). Specially, as a member of anti-apoptotic family of proteins, apoptosis 2 inhibitor can inhibit the downstream components of the caspase activation pathways in the regulation of apoptosis and plays important roles in regulating the progress of apoptosis in many species (Wei et al., 2008). The higher abundance of apoptosis 2 inhibitor in male mussel gills may explain the observation in TUNEL assay that male samples showed lower levels of apoptotic nuclei slightly than those of female samples.

Overall, these proteins from solvent control group with different abundances clearly indicated the significant biological differences at protein level between male and female mussel samples. Therefore, there might be differential responses to TBBPA exposure in mussels due to the biological differences.

4.5. Effects of TBBPA treatment on the proteome of gills in male and female mussels

A total of 67 proteins in TBBPA treatment group were significantly altered in abundances. These proteins were involved in multiple biological processes including cytoskeleton, reproduction and development, material and energy metabolism, signal transduction, gene expression, defense mechanisms and apoptosis. Among the altered proteins, 11 proteins were commonly altered in both male and female TBBPA-exposed samples with similar or contrary alteration tendencies, of which non-neuronal cytoplasmic intermediate filament protein, receptor of activated kinase C (RAKC), qm-like protein (QLP), putative alpha-isopropylmalate/homocitrate synthase family transferase (PAIHSFT) showed consistent expression tendencies in both male and female mussel gills. Theoretically, these 4 altered proteins can serve as non-gender-specific proteomic biomarkers of TBBPA exposure. Intermediate filament proteins (IFP) are important components of cytoskeleton, functioning as providing structural support for cells (Snider and Omary, 2014). The increasing IFP may be used to maintain the stability of cells. The signal pathways were influenced by TBBPA treatment, evidenced by the reduction of RAKC, which is an important anchored protein in protein kinase C signal pathway and plays key roles in signal transduction (Wei et al., 2008). Wu et al. found the same phenomenon in the *M. galloprovincialis* treated with injection of *Vibrio anguillarum* (Wu et al., 2013). QM, a gene that was originally identified as a tumor suppressor gene, has been reported to be involved in processes such as regulating cell cycle and differentiation of specific tissues during embryogenesis (Mills et al., 1999). The QLP was down-regulated which indicated the impact of TBBPA on cell cycle in *M. galloprovincialis* gills. Previous studies both *in vivo* and *in vitro* have shown that leucine at a very high dose can stimulate muscle protein synthesis and trigger muscle growth (Garlick, 2005). As a key enzyme in leucine synthesis, the PAIHSFT was down-regulated to reduce the level of leucine and consequently suppress muscle protein synthesis. As we known, the muscles of mussel gill play key role in regulating water flow, which is a process of ATP consumption (Medler and Silverman, 1997). The suspension of muscle growth presumably aimed to save more energy in response to TBBPA stress. Additionally, though there were several other proteins altered in both male and female mussel samples, the expression tendencies were inconsistent.

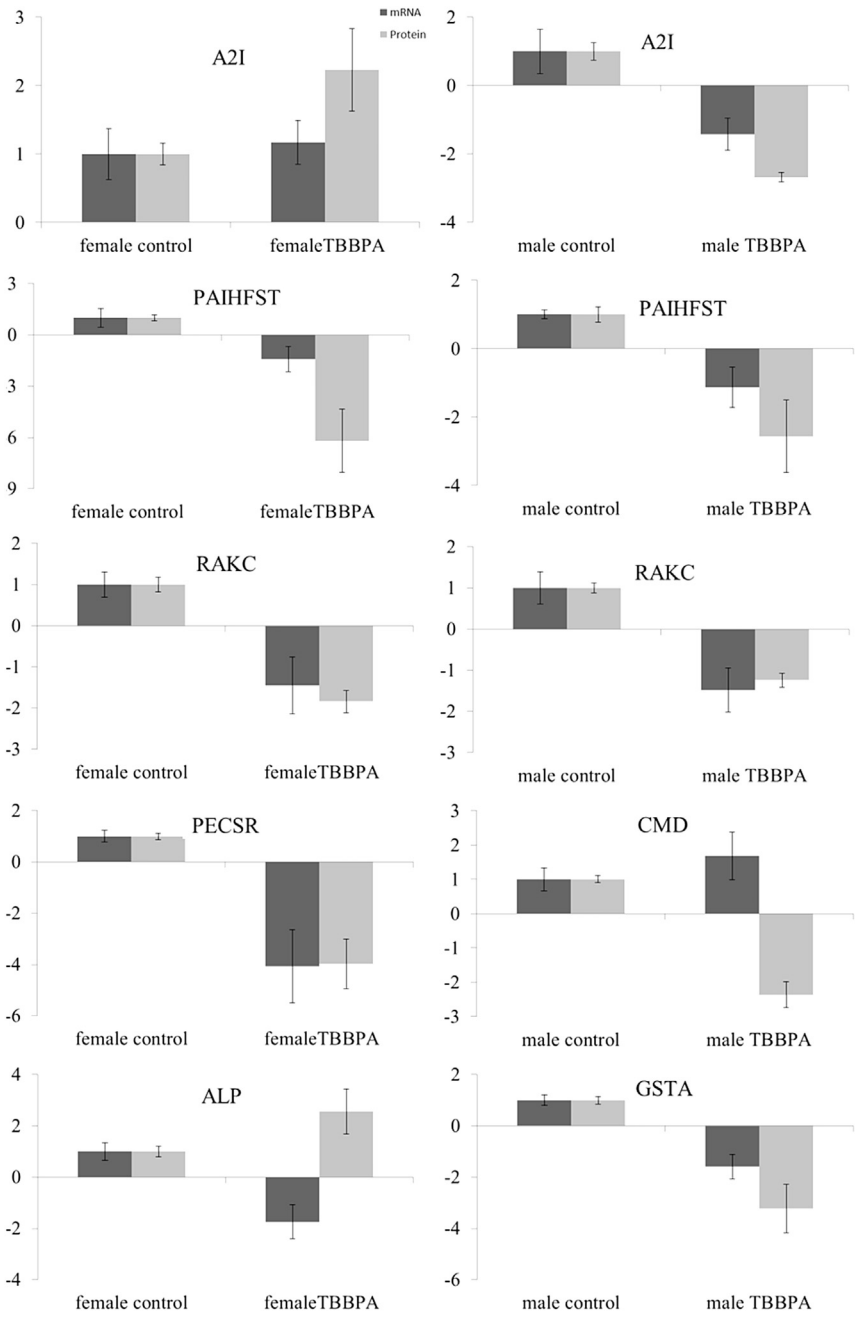

Fig. 4. Comparison of seven genes at the mRNA and protein levels in male and female mussels exposed to TBBPA. **A2I**: apoptosis 2 inhibitor; **ALP**: apextrin-like protein; **CMD**: cytosolic malate dehydrogenase; **GSTA**: Glutathione S transferase A; **PAIHFST**: putative alpha-isopropylmalate/homocitrate synthase family transferase; **PECSR**: epidermal cell surface receptor; **RAKC**: receptor of activated kinase C.

Except for the 11 proteins, other differentially expressed proteins were only observed in either male mussels or female mussels. In TBBPA-treated male group, several proteins involved in material and energy metabolism were significantly altered in mussel gills. Malate dehydrogenase and ethanol dehydrogenase, two key metabolic enzymes in Krebs cycle and glycolysis respectively, can provide NADH for redox reaction. The down-regulation of the two enzymes suggested the reduction of redox reaction, which may aim to lower the generation of ROS and ultimately re-establish cellular homeostasis in response to TBBPA treatment. Moreover, production of reducing equivalents such as NADPH can also reduce the excess ROS (Tomanek, 2011). Peroxisomal trans-2-enoyl-CoA reductase plays an important role in the process of fatty acids synthesis, during which numerous NADPH is produced. Increasing level of peroxisomal trans-2-enoyl-CoA reductase could generate more reducing equivalents to respond to TBBPA exposure. Other material and energy metabolism such as protein synthesis were also influenced by TBBPA treatment, as evidenced by the down-regulation of multiple ribosomal proteins. Previous study has indicated that environmental stress could contribute to reduction of ribosomal proteins in *Arabidopsis* roots, which may be explained as decreasing protein synthesis in order to save energy (Wang et al., 2013). Additionally, the alteration of apextrin-like protein and apoptosis 2 inhibitor revealed the effect of TBBPA on immunization and apoptosis in male mussel gills. Overall, we speculated that TBBPA exposure induced increases of tolerance limits in male mussel samples by lowering the generation of ROS and protein synthesis and increasing the production of NADPH.

Due to the biological differences, the proteomic responses in female TBBPA-exposed mussel samples were different with those in male TBBPA-exposed mussel samples. Sarcoplasmic calcium-binding protein and calponin-like proteins has been implicated in the regulation of smooth muscle contractions and cytoskeletal organization (Yang et al., 1999; Gao et al., 2006). The significant down-regulation of these two proteins could impact the normal motor ability of female mussel gills. A significant decrease of perculin and procollagen-proline dioxygenase beta subunit suggested that TBBPA affected the normal development of female mussels. Due to a lack of adaptive immunity in mussels, innate immunity based on innate defenses and on other humoral immune system plays key roles in response to environmental stresses. Lysozyme is an important defense molecule of the innate immune system (Gao et al., 2006), the decrease of which might be resulted from the impairment of the normal stress system caused by the long-time exposure to TBBPA. Another result of long-time exposure to TBBPA was the reduction of GST and SOD, which was also found in mussels exposed to BDE 47 (Ji et al., 2013a). Moreover, similar to male mussels with TBBPA exposure, the down-regulation of several ribosomal proteins, together with elongation factor and translation initiation factor, indicated that TBBPA treatment remarkably influenced the protein synthesis. One more interesting thing was that histological observations showed that apoptosis happened in both female and male mussel gills, while the proteomic analysis revealed apoptosis occurred in female and male mussels with differential responsive mechanisms, which implied the necessity of integrity of omic analyses and histological observations. In summary, female mussels re-established energy homeostasis in response to TBBPA exposure, which was also verified by the altered metabolites in gills. In addition, the down-regulated expressions of cytoskeletal proteins and defense mechanisms related proteins may reveal that TBBPA exposure has caused cellular damage beyond the tolerance limits.

4.6. Correlation between gene expressions and protein abundances

The expression of seven representative genes corresponding (A2I, PAIHFST, RAKC, PECSR, CMD, ALP and GSTA) were quantified using qRT-PCR to explore the correlation between protein abundances and mRNA expression levels. The results indicated that not all mRNA expressions correlate well with the protein abundances. The disparity between mRNA expression and corresponding proteins was not surprising, since mRNA expression means the tendency of the corresponding encode protein which, however, does not always happen due to the posttranscriptional and posttranslational modifications and differential degradation rates between mRNA and protein (Ji et al., 2013a, 2013b).

5. Conclusions

In this study, a combined metabolomic and proteomic approach was used to investigate the gender-specific responses in mussel *M. galloprovincialis* exposed to tetrabromobisphenol A (TBBPA). We confirmed the biological differences in gills between male and female mussels evidenced by differentially expressed proteins and metabolites with different levels. Further analyses revealed the gender-specific responses, at protein and metabolite levels, between male and female mussel gills exposed to TBBPA. In details, the significant changes of metabolites including betaine, homarine, taurine, glycine and ATP suggested the disturbance in energy metabolism and osmotic regulation in female mussel gills, while in male mussel samples only be found the variation of osmolytes such as choline and betaine, which implied the influence of TBBPA on osmotic regulation in male mussels. The proteomic analyses revealed that TBBPA exposure may affect multiple biological processes consisting of reproduction and development, material and energy metabolism, signal transduction, gene expression, defense mechanisms and apoptosis in both male and female mussels with different mechanisms. In special, the responsive proteins of TBBPA in male mussels signified higher tolerance limits than those in female individuals. As shown in Fig. 5, a combined metabolomic and proteomic approach could provide an important insight into the toxicological effects of environmental pollutant in organisms, and also suggested that the gender differences should be considered in ecotoxicology.

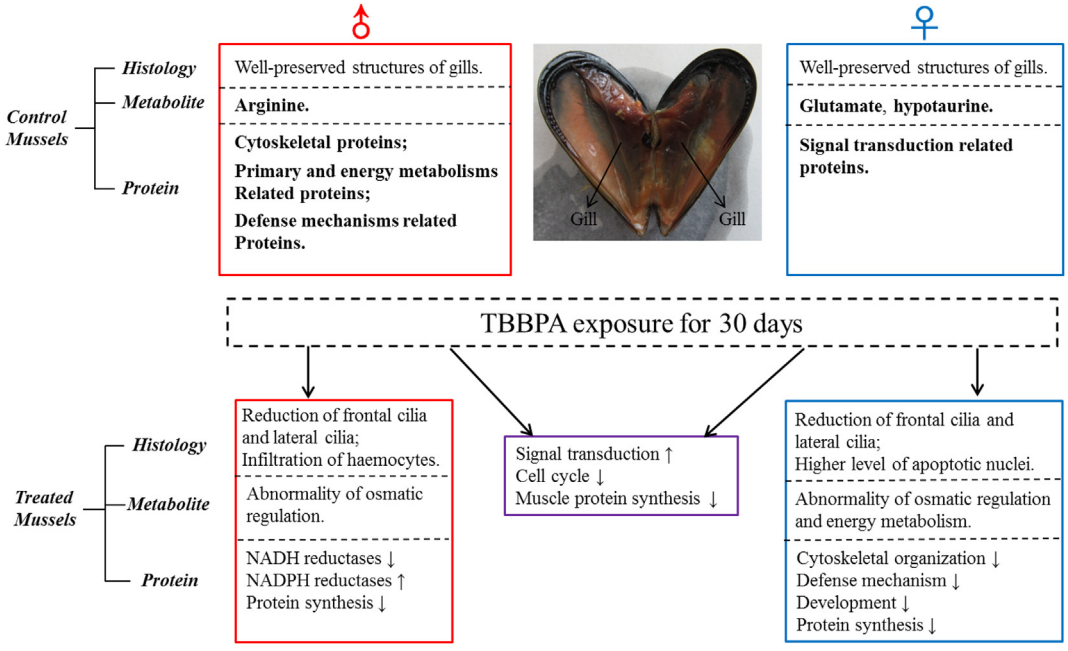

Fig. 5. The profile of alterations at histological, metabolomic and proteomic levels in gills from male and female mussel exposed to TBBPA. The bolds mean the proteins or metabolites are abundant. The up arrow and down arrow represent promotion and suppression of the biological processes, respectively.

Conflicts of interest

The authors have declared on conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.08.052.

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