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iTRAQ-based quantitative proteomic analyses on the gender-specific responses in mussel *Mytilus galloprovincialis* to tetrabromobisphenol A

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ABSTRACT

Tetrabromobisphenol A (TBBPA) accounts for the largest production of brominated flame-retardants (BFRs) along the Laizhou Bay in China and is the most widely used BFR in industrial products. It can induce diverse toxicities including hepatotoxicity, nephrotoxicity, neurotoxicity and endocrine disrupting effects in mammalian and fish models. In this work, we applied iTRAQ-based proteomics to investigate the gender-specific responses in mussel *Mytilus galloprovincialis* to TBBPA. Thirty-one proteins were differentially expressed in hepatopancreas between male and female mussels, which clearly indicated the biological differences between male and female mussels at the protein level. After exposure of TBBPA (18.4 nmol/L) for one month, a total of 60 proteins were differentially expressed in response to the TBBPA treatment in mussel hepatopancreas, among which 33 and 29 proteins were significantly altered in TBBPA-treated male and female mussel samples, respectively. Only two of the 60 proteins were commonly altered in both male and female mussel samples exposed to TBBPA. Based on KEGG analysis, these differentially expressed proteins of TBBPA-induced effects were assigned to several groups, including cytoskeleton, reproduction and development, metabolism, signal transduction, gene expression, stress response and apoptosis. Overall, results indicated that TBBPA exposure could induce apoptosis, oxidative and immune stresses and disruption in energy, protein and lipid metabolisms in both male and female mussels with different mechanisms. This work suggested that the gender differences should be considered in ecotoxicoproteomics.

1. Introduction

Brominated flame retardants (BFRs) have been extensively used in a variety of industrial products, such as plastics, electronic equipment, textiles and upholstery foam, to reduce the risk of fire (de Wit, 2002). Among the BFRs, tetrabromobisphenol A (TBBPA) is nowadays the most frequently used BFR and can be released into marine environment (de Wit, 2002). Like other BFRs, TBBPA is a lipophilic substance and has a high persistency in the environment and therefore is globally traceable in multiple environmental samples including sediments and soils, marine and fresh water animals (Pan et al., 2010). Due to the abundant resources of seawater and underground brine, there are many industrial manufacturing plants of BFRs along the Laizhou Bay (Fig. S1) (Pan et al., 2010). The biggest manufacturing base for BFRs in China has been constructed and located in Weifang Binhai Economic Development Zone along the south coast of the Laizhou Bay (Pan et al., 2010). Zhang et al. (2011) reported that the TBBPA concentration in Mihe estuary was up to 1.24 nmol/L, which contributed directly to the TBBPA pollution in the Laizhou Bay. Additionally, the released TBBPA can be potentially bioaccumulated by animals via the food chain in the aquatic environment (Morris et al., 2004). Therefore, the increasing release of TBBPA has attracted great attention worldwide because of its environmental persistence and potential toxicity to organisms (Johnson-Restrepoa et al., 2008).

TBBPA has diverse toxicities including hepatotoxicity, nephrotoxicity, neurotoxicity and cytotoxicity, which have been well documented (Szymanska et al., 1999; Birnbaum and Staskal, 2004; Strack et al., 2007). Szymanska et al. (1999) reported the hepatotoxicity of TBBPA in rat liver, indicated by the significant changes of biochemical indicators including glutathione (GSH), malondialdehyde (MDA) and 5-aminolevulinate (ALA-D). In addition, the

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endocrine disrupting effects induced by TBBPA were also found in different models including mammalian and fish (Meerts et al., 2000; Huang et al., 2013). The tissue of hepatopancreas is an important digestive and immune organ in bivalve functioning as innate immunity and detoxification (Du et al., 2013). Traditional toxicological approaches usually consist of histological studies on the lesions and measurement of specific responses such as the antioxidant activities for the test of oxidative stress or specifically expressed genes and proteins to test for certain toxicities without high-throughput analyses (Ji et al., 2013). Since researchers often target a known class of toxicity-responsive molecules, there are few opportunities to discover new molecules related to toxicological effects. These researches obviously presented a primary and less comprehensive understanding of toxicological responses of organisms to contaminants.

Mussel *Mytilus galloprovincialis* is frequently used as a good bioindicator in marine ecotoxicology because of its high tolerance and accumulation of contaminants (Ciacci et al., 2012). In addition, *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 level. In this study, we used iTRAQ coupled with two-dimensional liquid chromatography–tandem mass spectrometry to characterize the differential proteomic changes 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 a pristine site (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 male and six female individuals in each group. Dimethyl sulfoxide (DMSO) was used as the solvent for the dissolution of TBBPA (Guoyao, Shanghai, China). The mussel group cultured in the normal filtered seawater (FSW) containing 0.002% DMSO (v/v) were used as solvent control group. Since our previous experiment confirmed that there was no significant proteomic difference in mussel *M. galloprovincialis* samples between seawater control (mussels cultured in the FSW) and solvent (DMSO) control groups (Ji et al., 2013), we did not set the seawater control group in this study. The mussels in the treatment group were exposed to one sublethal concentration (18.4 nmol/L) of TBBPA. The concentration of TBBPA stock solution was 9.20 mmol/L 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 1 month, all the mussels were immediately dissected for hepatopancreas and gonad tissues. The gonad tissues were used for sex determination. Each hepatopancreas tissue sample was divided into three parts that were used for histological observation, TUNEL assay and protein and RNA extraction. Those hepatopancreas samples for 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 hepatopancreas 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. S2). After fixation for 24 h, the tissues were then dehydrated in a progressive series of ethanol and embedded in paraffin. Tissues from control and TBBPA treatment (18.4 nmol/L) were processed together in batches to remove artefacts between control and TBBPA treatment. Histological sections (6–8 μm thickness) were cut from the paraffin embedded tissues and mounted on slides which 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). For each individual animal, there were three sections examined. The histological observations were randomly made on five fields of one section per sample. A mean for each section was derived by randomly counting at least 10 tubules.

2.3. TUNEL assay

Terminal dUTP nick-end labeling technique (TUNEL-technique) was applied to detect the apoptosis in hepatopancreas and performed with one step TUNEL kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) according to the manufacturer's instructions. More details about TUNEL technique were described in the Supporting Information.

2.4. Quantitative proteomic analysis

Each pooled hepatopancreas sample 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 tags). Protein Pilot Software 4.0 (AB SCIEX, Framingham, MA, USA) was used to process proteomic data against a marine bivalve-EST-Translated protein database (*Mytilus galloprovincialis*, *Mytilus edulis*, *Ruditapes philippinarum* and *Crassostrea gigas*, 238224 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.

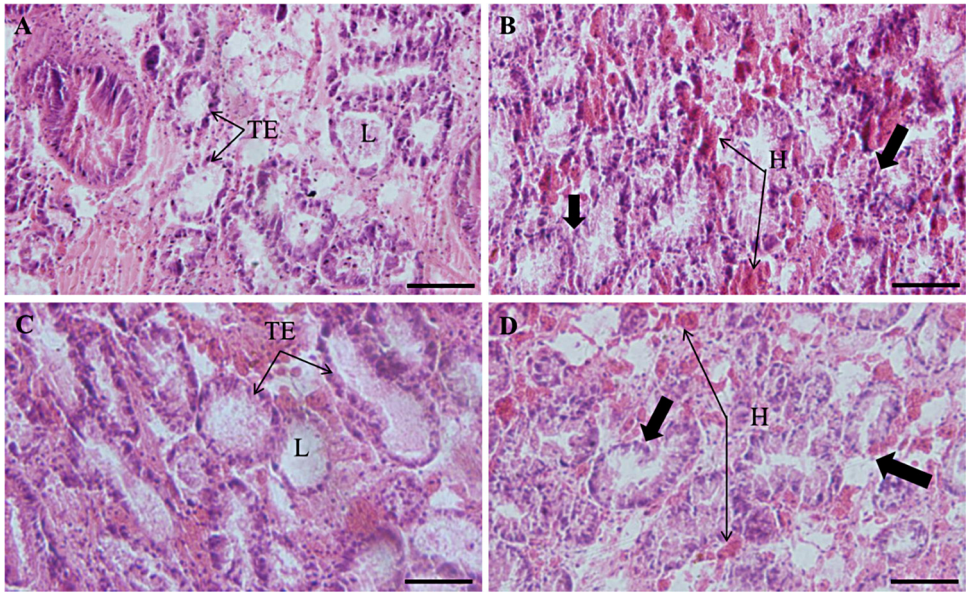

Fig. 1. H/E stained sections through hepatopancreas tissue of mussels. (A) female individual from control group; (B) female individual from 10 μg/L TBBPA treatment group; (C) male individual from control group; (D) male individual from 10 μg/L TBBPA treatment group. TE, tubular epithelium; L, lumen; H, infiltration of haemocytes; deformed tubules (bold arrow). Scale bar: 40 μm.

3. Results

3.1. Histological observation of hepatopancreas from mussels exposed to TBBPA

Sections of hepatopancreas from control and TBBPA-exposed mussels are exhibited in Fig. 1. No significant histological differences were observed between female control (Fig. 1A) and male control (Fig. 1C) hepatopancreas samples presenting normal architecture of hepatopancreatic tubule and lumen. In contrast, the hepatopancreas tissues showed infiltration of haemocytes as well as deformation of tubules in TBBPA-exposed female mussels (Fig. 1B). The similar appearances were also observed in hepatopancreas from male mussel samples with TBBPA exposure (Fig. 1D).

3.2. Apoptosis in the hepatopancreas from mussels exposed to TBBPA

TUNEL technique was performed on hepatopancreas tissue sections to assess the occurrences of apoptotic nuclei in individual cells. In the control groups, as shown in Fig. 2A andC, no obvious apoptotic nuclei were found in either epithelial cells or interstitial connective tissue (ICT) in both male and female mussel hepatopancreas tissues from control groups. In TBBPA treatment group, epithelial cells apoptosis was not found in either female or male mussel samples. However, the ICT of hepatopancreas from both male and female TBBPA treatment groups showed significant higher levels of apoptotic nuclei than those from control groups (Fig. 2B and D), which meant the occurrences of apoptosis induced by TBBPA exposure in ICT of hepatopancreas from both male and female TBBPA treatment groups. The apoptosis appearances did not demonstrate gender-specific responses to TBBPA treatment in mussel hepatopancreas tissues.

3.3. Proteomic differences in hepatopancreas between male and female mussels

In total, 1043 proteins were quantified on the basis of 23376 highly confident spectra, of which 10231 peptides were unique. On average, each protein was quantified using 22.4 spectra. Among the 1043 proteins, 31 proteins showed significantly ($p < 0.05$) different abundances in hepatopancreas between male and female mussels from control group (Table 1), including 15 and 16 abundant proteins in male and female mussel samples, respectively. Pathways of these differential proteins were analyzed using the KEGG database. According to the KEGG pathway analysis, these proteins were basically involved in cytoskeleton, reproduction and development, energy and primary metabolism, translation, stress response and signal transduction.

3.4. Differential proteomic responses in hepatopancreas between male and female mussels exposed to TBBPA

Comparison between control and TBBPA-exposed mussel groups indicated that 60 proteins were significantly ($p < 0.05$) changed in expression from both male and female mussel samples. A total of 33 proteins displayed significant changes in expression, in which 8 proteins showed increased expression, while 25 showed decreased expression in male mussel samples exposed to TBBPA for one month (Table 2 and Fig. 3). Among these 33 significantly altered proteins in male TBBPA-treated mussel group, only three (9.1%) of them were also found to be of significant differences between male control and female control groups. For the female mussel samples, there were 29 proteins altered in TBBPA-treated group, including 19 up-regulated and 10 down-regulated proteins. Interestingly, twelve proteins (41.4%) among these 29 proteins in response to TBBPA exposure were gender-specific in female mussel samples with significantly different abundances compared to those in male mussel samples (Fig. 3). However, only two proteins (3.3%) were commonly changed in expression from both male and female mussel samples exposed to TBBPA. Fig. 4 summarized the pathways related to the proteomic response of *M. galloprovincialis* to the TBBPA treatment.

3.5. 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

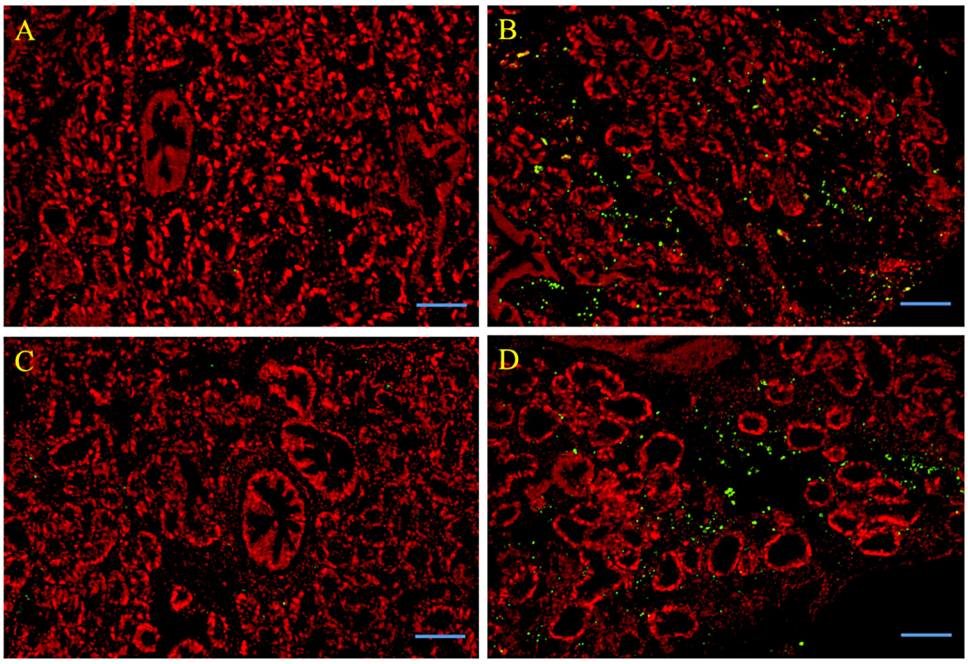

Fig. 2. TUNEL (green)/PI (red) stained sections through hepatopancreas tissue of mussels. (A) female individual from control group; (B) female individual from 10 μg/L TBBPA treatment group; (C) male individual from control group; (D) male individual from 10 μg/L TBBPA treatment group. Scale bar: 50 μm.

Table 1
Differentially expressed proteins between male and female mussel groups.

Accession	Description	No. of unique peptides	*p* value[a]	Ratio (male/female)
Cytoskeleton				
FL499829	β-actin	134	0.0136	5.10
FL494680	Suprabasin	2	0.0232	0.82
FL489343	RecName: Tropomyosin >gb|AAA82259.1|	40	0.0444	0.59
FL489336	Cytoplasmic dynein 1 light intermediate chain 1	1	0.0389	1.39
AJ625699	Tropomyosin >dbj|BAA19209.1|	31	0.0238	0.48
Reproduction and development				
FL594305	Protamine-like PL-II/PL-IV precursor	2	0.0359	2.13
FL493691	Meiosis-specific nuclear structural protein 1	5	0.0030	8.24
FL493456	Yolk ferritin	4	0.0470	0.44
FL491285	Zonadhesin	3	0.0034	4.09
AJ625943	Putative vitelline envelop receptor for lysin	1	0.0310	0.29
Metabolism				
FL497290	PREDICTED: L-xylulose reductase-like isoform X1	10	0.0228	0.47
FL496349	Glyceraldehyde-3-phosphate dehydrogenase	56	0.0291	13.68
FL494162	Glycosyl hydrolase	2	0.0347	13.30
FL492895	Low-density lipoprotein receptor-related protein 6	5	0.0338	0.23
FL489629	Arginine kinase	33	0.0015	6.61
AJ625434	PREDICTED: glucose dehydrogenase [acceptor]-like	14	0.0440	0.47
AJ624848	Pyruvate carboxylase, mitochondrial	1	0.0031	1.68
Signal transduction				
FL493105	Calponin-like protein	14	0.0078	2.01
AJ624341	Putative epidermal cell surface receptor	2	0.0194	3.60
AJ623579	Sarcoplasmic calcium-binding protein	14	0.0171	0.58
Translation				
FL495584	Ribosomal protein L	5	0.0406	0.72
AM879598	Ribosomal protein L19	6	0.0344	0.73
FL492189	Ribosomal protein L9	9	0.0221	0.72
Stress response				
FL499875	78 kDa glucose-regulated protein, partial	63	0.0367	0.44
FL496423	Apextrin-like protein	5	0.0308	10.96
FL495401	Chitotriosidase-1	104	0.0260	0.74
FL494057	Glutathione S-transferase A	9	0.0409	1.58
AJ626434	Chitotriosidase	133	0.0354	0.55
Others				
FL497051	Hypothetical protein CGI_10021496	13	0.0062	0.42
FL489129	Hypothetical protein	1	0.0036	1.25
AJ625534	PREDICTED: uncharacterized protein LOC100179434	19	0.0027	5.60

[a] *p* values have been corrected using the Benjamini–Hochberg method.

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

Accession	Description	No. of unique peptides	*p* value[a]	Fold change[b]
Cytoskeleton				
FL594178	Profilin	34	0.0065	−3.13[c]
FL499932	Kinesin-related protein 1	1	0.0343	−7.45[c]
FL497207	Tubulin A	51	0.0029	−2.94[c]
FL494680	Suprabasin	2	0.0122	1.64[c]
AJ624420	Actin	112	0.0372	3.10[c]
FL500031	Septin-2	2	0.0449	3.10[d]
FL497199	Non-neuronal cytoplasmic intermediate filament protein	43	0.0322	2.09[d]
FL492621	Catchin protein	9	0.0452	−2.07[d]
AJ625699	Tropomyosin >dbj|BAA19209.1|	31	0.0495	−1.99[d]
Reproduction and development				
FL633554	Vdg3	5	0.0498	−4.61[c]
FL491285	Zonadhesin	3	0.0047	1.49[d]
AJ625943	Putative vitelline envelop receptor for lysin	1	0.0297	−3.80[d]
FL493691	Meiosis-specific nuclear structural protein 1	5	0.0047	1.66[d]
Metabolism				
FL501230	Procollagen-proline dioxygenase beta subunit	18	0.0060	−4.29[c]
FL495257	Ganglioside GM2 activator	2	0.0493	−2.61[c]
FL492537	Proteasome subunit beta type-1	3	0.0348	1.94[c]
FL490476	Isoamyl acetate-hydrolyzing esterase 1-like protein	1	0.0370	−5.35[c]
FL490188	Cob(I)yrinic acid a,c-diamide adenosyltransferase, mitochondrial-like	1	0.0357	−1.20[c]
AM879460	Fatty acid-binding protein	10	0.0295	−1.77[c]
AJ625020	Electron transfer flavoprotein subunit alpha, mitochondrial-like isoform X1	7	0.0282	−1.61[c]
FL594008	ATP synthase beta subunit, partial	46	0.0336	−3.50[c]
FL496349	Glyceraldehyde-3-phosphate dehydrogenase	56	0.0344	2.75[d]
J625434	Glucose dehydrogenase [acceptor]-like	14	0.0265	2.07[d]
FL489629	Arginine kinase	33	0.0017	4.88[d]
Signal transduction				
FL497430	Collagen alpha-5(VI) chain	3	0.0196	7.73[c]
AJ626069	Collagen alpha-1(XII) chain	87	0.0113	2.78[c]
			0.0001	1.80[d]
FL494852	Tyrosine-protein phosphatase non-receptor type 6	1	0.0275	−4.66[c]
AJ516340	Calcium binding protein 1	18	0.0347	−2.09[c]
AJ624341	Putative epidermal cell surface receptor	2	0.0234	3.08[d]
Gene expression				
FL496796	Elongation factor 1-gamma	7	0.0288	−2.25[c]
EH663238	Ribosomal protein	16	0.0083	−6.49[c]
AM878418	Nucleoprotein TPR	1[e]	0.0034	−1.26[c]
			0.0089	−2.54[d]
AJ624092	Excinuclease ABC subunit B	138	0.0074	2.27[c]
FL496868	Transcription activator	3	0.0285	−5.20[d]
FL495374	Elongation factor 2	22	0.0216	3.08[d]
FL492837	40S ribosomal protein S4, X isoform	8	0.0135	−2.28[d]
FL489105	PREDICTED: Serine/arginine-rich splicing factor 4-like isoform X1	3	0.0193	2.91[d]
AM880346	QM-like protein	9	0.0326	2.01[d]
AJ625362	60S ribosomal protein L24-like	5	0.0197	2.29[d]
Stress response				
FL595030	Selenium-binding protein 1, partial	7	0.0128	−2.65[c]
FL499875	78 kDa glucose-regulated protein, partial	63	0.0007	−4.41[c]
FL499581	Peroxiredoxin 6	17	0.0296	−10.19[c]
FL499562	Hsc70-interacting protein	8	0.0022	−5.75[c]
FL491817	Manganese superoxide dismutase	8	0.0020	−5.06[c]
FL492107	TCTP	12	0.0343	−3.50[c]
FL593839	Calreticulin	35	0.0234	−3.28[c]
AJ625233	Acidic mammalian chitinase isoform X2	43	0.0349	3.87[c]
AJ624097	Chitotriosidase	133	0.0293	1.36[c]
FL492661	Lectin	20	0.0459	−2.94[c]
FL496702	Lysozyme	7	0.0357	8.39[d]
FL496423	Apextrin-like protein	5	0.0336	8.47[d]
FL495401	Chitotriosidase-1	104	0.0002	2.18[d]
Apoptosis				
FL497051	Apoptosis 2 inhibitor	13	0.0036	−12.24[d]
FL491544	Cell death protein 3-like	4	0.0247	−8.71[d]
Others				
FL489129	Hypothetical protein	1	0.0036	−1.25[c]
FL497768	Hypothetical protein CGI_10012958	15	0.0122	−1.44[d]
FL494257	Hypothetical protein CGI_10009690	23	0.0271	−4.09[d]
FL494162	Hypothetical protein	2	0.0357	11.37[d]
AJ625057	Hypothetical protein EAG_11213	11	0.0221	2.85[d]
AJ625534	PREDICTED: uncharacterized protein LOC100179434	19	0.0023	3.41[d]

[a] *p* values have been corrected using the Benjamini–Hochberg method.
[b] Plus (+) which is omitted represents up-regulated expression, and minus (−) represents down-regulated expression.
[c] Altered proteins in male mussels.
[d] Altered proteins female mussels.

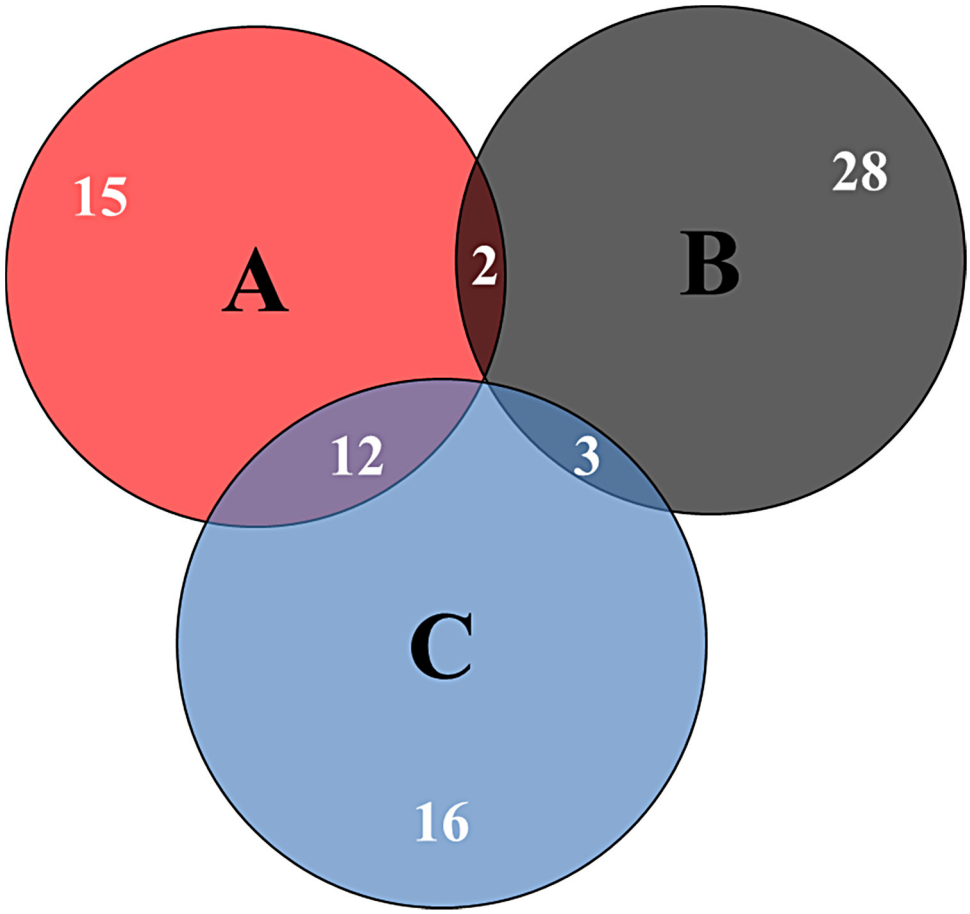

Fig. 3. A Venn diagram showing the overlaps between TBBPA-related proteins from females (A) and males (B), and gender-specific proteins (C).

nucleoprotein translocated promoter region protein (TPR) and 78 kDa glucose-regulated protein (GRP78) in male group and TPR, glucose dehydrogenase [acceptor]-like (GDA), epidermal cell surface receptor (ECSR), arginine kinase (AK), apextrin-like protein (ALP) and apoptosis 2 inhibitor (A2I) in female group, were quantified using qRT-PCR. The results indicated that the mRNA expression levels of both TPR and GRP78 had consistent alteration tendency with corresponding proteins in male TBBPA-treatment group. In female TBBPA-treatment group, TPR, GDA, ECSR and A2I demonstrated positive correlation between mRNA and protein expressions, while AK and ALP presented disparity between mRNA expression and corresponding protein abundance (Fig. 5).

4. Discussion

With the rapid development of -omic techniques including genomics, transcriptomics, proteomics and metabolomics, 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, which offer great potential in unraveling toxicological effects and mechanisms of environmental contaminants (Weckwerth, 2011; Fasulo et al., 2012; Cappello et al., 2013a,b; Ji et al., 2013). Among these approaches, proteomics is a large-scale study of proteins encoded by the given genome in an organism (Anderson and Anderson, 1998). Not only can proteomics describe complete proteomes at tissue, cell, or organelle levels, but it can also be used to compare proteomes under environmental contaminant stresses (Ahsan et al., 2009). iTRAQ is a gel-free technique used to quantify proteins from different samples in a single experiment by using isotope-coded covalent tags (Pütz et al., 2012; Han et al., 2013) and has been popularly employed in quantitative proteomic studies due to its relatively high sensitivity and reproducibility (Martyniuk et al., 2012; Han et al., 2013). In this study, we explored the gender-specific response in hepatopancreas of mussel *M. galloprovincialis* to TBBPA by using the iTRAQ-based proteomics.

4.1. TBBPA induced damages and apoptosis in mussel hepatopancreas

Histological observations may provide straightforward evidences on the tissue disorders related to the status of organisms. In both male and female samples with the TBBPA treatment, the hepatopancreas displayed conspicuous haemocytic infiltration and severe lesions of tubules. Infiltration of haemocytes in response to exposure to organic pollutants, such as PAHs and a mixture of diesel oil, has been reported in marine mussels (Auffert, 1988; Cappello et al., 2013a,b) and was interpreted as a repair process following tissue damage (Garmendia et al., 2011). Moore (1985) assumed that autolytic process of the epithelia of hepatopancreas tubule was a consequence of full lysosomal destabilization, which was confirmed by Auffert (1988) who found the incidence of lysosomes was inversely correlated to the extent of tubule lesions.

TUNEL technique was used to assess apoptosis of hepatopancreas from TBBPA-exposed mussels. Results indicated that ICT represented the distinct apoptotic nuclei. As a part of hepatopancreas, ICT surrounding the digestive tubules plays an important role in fixing and protecting tubules. Minguez et al. (2013) found that zebra mussels infected by bacterium showed apoptotic nuclei

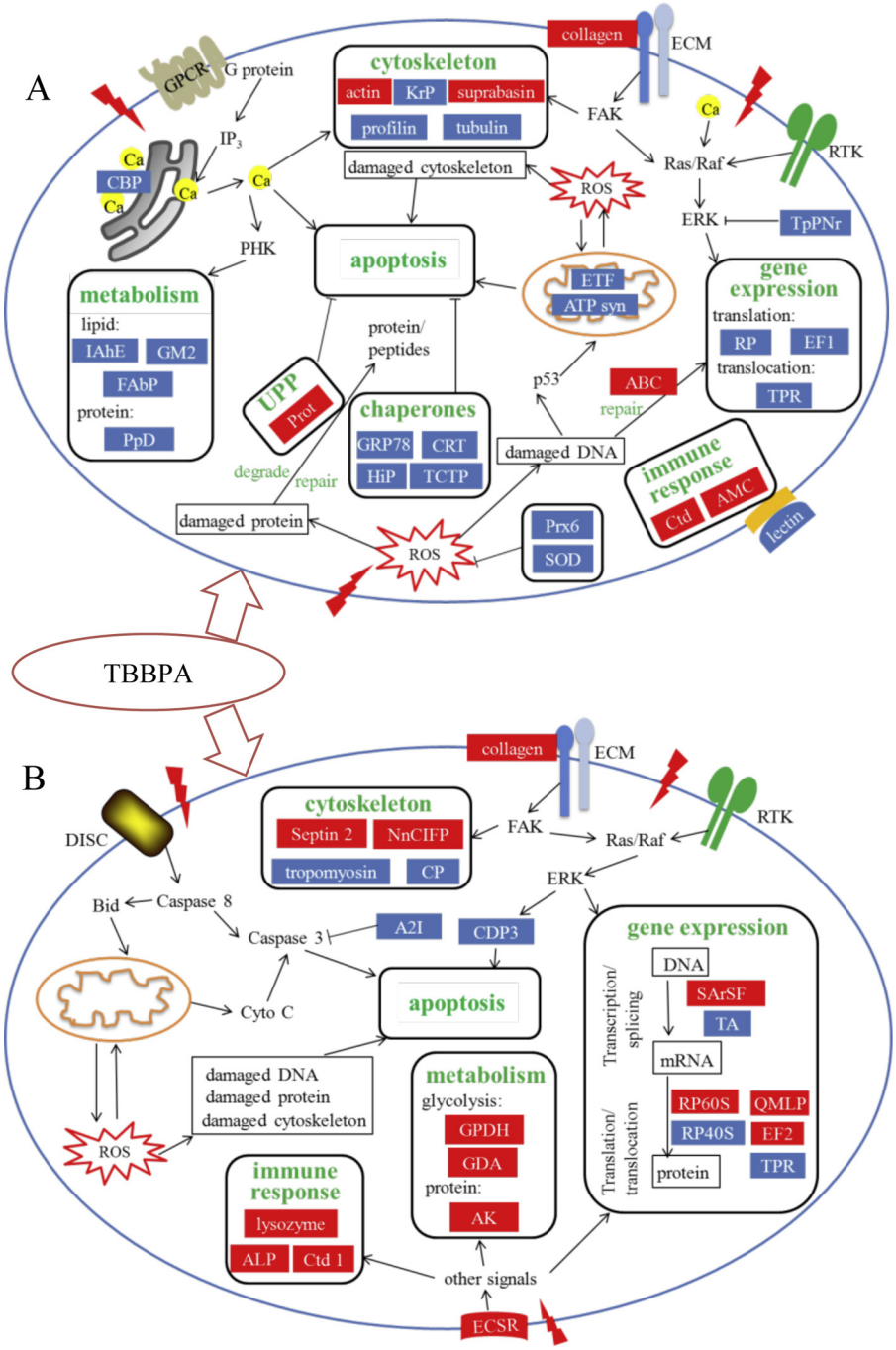

Fig. 4. A schematic presentation of pathways indicated by altered proteins in male (A) and female (B) mussels exposed to TBBPA. The identified proteins involved in different pathways were marked in red (increased) and blue (decreased). Abbreviation: A2I, Apoptosis 2 inhibitor; ABC, excinuclease ABC; AK, arginine kinase; ALP, apextrin-like protein; AMC, acidic mammalian chitinase; ATP syn, ATP synthase; CBP, calcium binding protein; CDP3, cell death protein 3-like; CRT, calreticulin; Ctd, chitotriosidase; DISC, death-inducing signaling complex; ECM, Extracellular matrix; ECSR, epidermal cell surface receptor; EF1, Elongation factor 1-gamma; EF2, elongation factor 2; ERK, Extracellular signal-regulated kinases; ETF, electron transfer flavoprotein; FAbP, Fatty acid-binding protein; FAK, Focal Adhesion Kinase; GDA, glucose dehydrogenase [acceptor]-like; GM2, Ganglioside GM2 activator; GPCR, G protein-coupled receptor; GPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP78, 78 kDa glucose-regulated protein; HiP, Hsc70-interacting protein; IAhE, Isoamyl acetate-hydrolyzing esterase IP3: Inositol trisphosphate; KrP, Kinesin-related protein 1; NnCIFP, Non-neuronal cytoplasmic intermediate filament protein; PHK, Phosphorylase kinase; PpD, procollagen-proline dioxygenase; Prot, Proteasome; Prx6, peroxiredoxin 6; QMLP, qm-like protein; RP, ribosomal protein; RP40S, 40S ribosomal protein S4; RP60S, 60S ribosomal protein L24; RTK, Receptor tyrosine kinase; SArSF, serine/arginine-rich splicing factor 4-like; SOD, manganese superoxide dismutase; TA, transcription activator; TCTP, translationally controlled tumour protein; TpPNr, Tyrosine-protein phosphatase non-receptor type 6; TPR, Nucleoprotein translocated promoter region protein; UPP, ubiquitin-proteasome pathway.

near the digestive gland, whereas the digestive tubules showed no stained cells, which was similar to TBBPA-induced apoptosis in hepatopancreas in marine mussels in this study.

Both histological observations and TUNEL assay indicated that TBBPA treatment could induce clear haemocytic infiltration, lesions of tubules and apoptosis in mussel hepatopancreas. However, these results did not present potentially differential responses between male and female mussel samples towards TBBPA exposure. To elucidate the gender-specific responses and molecular mechanisms, an iTRAQ-based proteomic approach was used to quantitatively profile *M. galloprovincialis* proteins in response to TBBPA treatment.

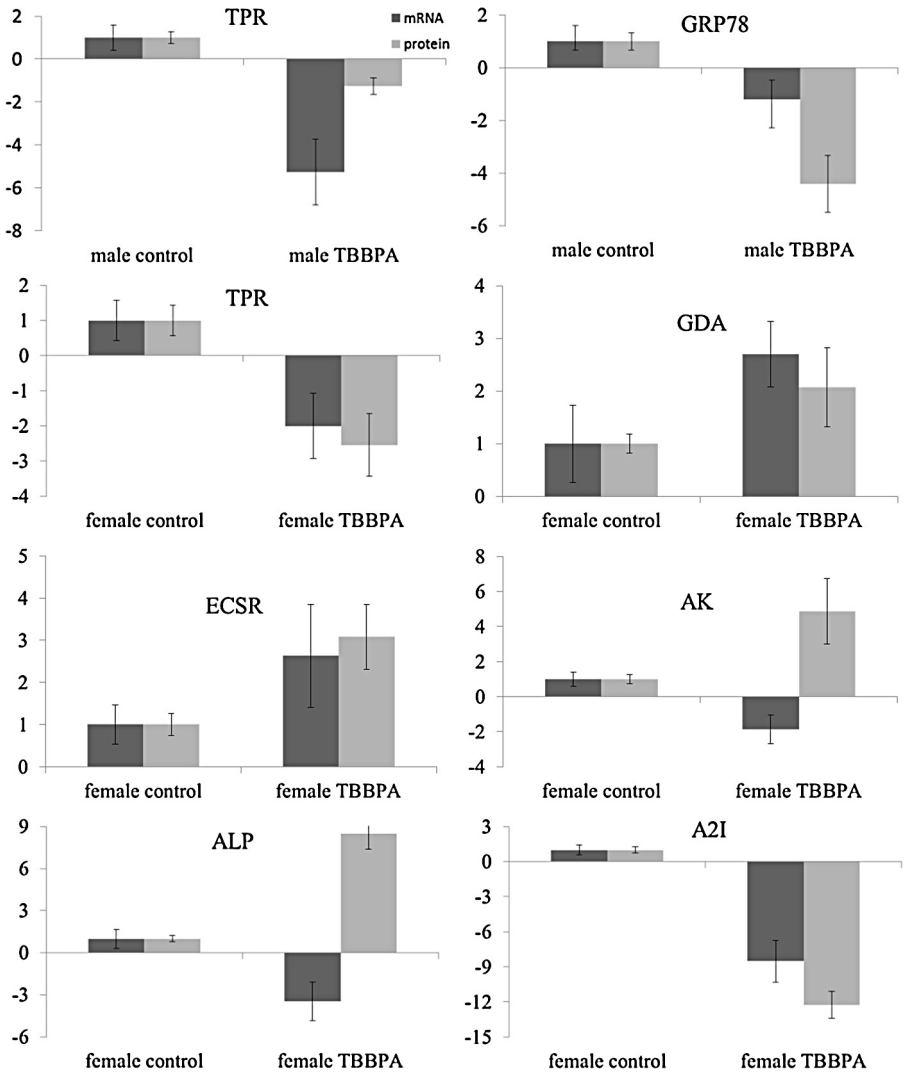

Fig. 5. Comparison of seven genes at the mRNA and protein levels in male and female mussels exposed to TBBPA. The mRNA and protein values of the ratio of treatment group to the control are plotted. Abbreviations: TPR, nucleoprotein translocated promoter region protein; GRP78, 78 kDa glucose-regulated protein; GDA, glucose dehydrogenase [acceptor]-like; ECSR, epidermal cell surface receptor; AK, arginine kinase; ALP, apextrin-like protein; A2I, apoptosis 2 inhibitor.

4.2. Differential proteins expressed in male and female mussel hepatopancreas

The iTRAQ coupled to 2D-LC-MS/MS analysis identified 1043 proteins, of which 31 were of significantly ($p < 0.05$) different abundances in hepatopancreas between male and female mussels. According to the KEGG pathway analysis, these 31 significantly expressed proteins were divided into several pathways including cytoskeleton, reproduction and development, energy and primary metabolism, gene expression, stress response and signal transduction.

Cytoskeleton consisting mostly of microtubules, microfilaments and intermediate filaments plays multiple functions, including cell shape, cell movement, cytokinesis, cell signaling and muscle contraction (Wickstead and Gull, 2011). The higher levels of actin and cytoplasmic dynein combined the lower levels of tropomyosins could imply the differential activity of muscle contraction between male and female mussel samples, while the different levels of suprabasin meant the different processes of epidermal differentiation.

The significant differences of proteins involved directly or indirectly in glucose metabolism implied the metabolic differences between male and female mussels in hepatopancreas. Especially, glyceraldehyde-3-phosphate dehydrogenase and glycosyl hydrolase in male mussel hepatopancreas were more than 13 times higher than those in female mussel hepatopancreas. In addition, arginine kinase and low-density lipoprotein receptor-related protein, related to energy metabolism and chylomicron catabolism respectively (Alonso et al., 2011; Beisiegel et al., 1991), also exhibited differential expression levels between female and male mussel hepatopancreas.

Three differentially expressed proteins including calponin-like protein, epidermal cell surface receptor (ESCR) and sarcoplasmic calcium-binding protein combined with three ribosomal proteins implied the differences in the cellular processes of transduction and translation between male and female mussel samples. Moreover, differential stress-responsive proteins such as apextrin, chitotriosidases glutathione S-transferases (GSTs) and glucose regulated protein (GRP78) suggested that male and female mussel might have different responsive mechanisms to stressors.

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

4.3. Gender-specific proteins responded to TBBPA in male and female mussel hepatopancreas

Among the 60 proteins responded to TBBPA exposure, only two of them (3.3%) were commonly changed in expression from both male and female mussel samples exposed to TBBPA, which clearly indicated the differential proteomic responses towards TBBPA treatment between male and female mussel samples. These proteins were classified into several groups according to the KEGG database.

4.3.1. Cytoskeleton

In TBBPA-treated male mussel hepatopancreas, both actin and tubulin were significantly down-regulated. The similar alteration of actin and tubulin has been also observed in response to cellular stress and apoptosis in *M. galloprovincialis* exposed to *Cylindrospermopsis raciborskii* cells (Puerto et al., 2011). Therefore, the alterations of actin and tubulin suggested the cellular stress and apoptosis in TBBPA-treated male mussel samples, which was also evidenced by TUNEL assay. Moreover, suprabasin involved in epidermal differentiation was present in higher concentration in TBBPA-exposed male individuals, suggesting the inhibition of epidermal differentiation by TBBPA exposure. In contrast, the proteomic responses related to cytoskeleton in female mussel samples with TBBPA treatment were completely different with those in male mussel samples. The alterations of septin, catchin and tropomyosin meant the disruption in muscle contraction and subsequent apoptosis induced by TBBPA in female mussel samples, together with the up-regulated cytoplasmic intermediate filament protein.

4.3.2. Reproduction and development

Vdg3 and meiosis-specific nuclear structural protein, being of significantly higher expressions in male mussel hepatopancreas as mentioned above, were both significantly up-regulated in female mussel samples, which probably meant that TBBPA induced reproductive toxicities in female mussels, together with the down-regulated vitelline envelop receptor for lysin.

4.3.3. Metabolism

Ganglioside GM2 activator is a glycolipid transport protein having multiple functions, including innate immunity and lipid metabolism (Klima et al., 1993). Both ganglioside GM2 activator and fatty acid binding protein were down-regulated in male mussel samples after TBBPA exposure, which suggested the disturbance in lipid metabolism induced by TBBPA. The beta subunit of procollagen-proline dioxygenase belongs to the family of protein disulfide isomerases (PDI) catalyzing the formation and breakage of disulfide bonds between sulfides (S-) in cysteine residues of folding proteins. The alterations of procollagen-proline dioxygenase and proteasomes indicated the disturbance in protein metabolism induced by TBBPA. Additionally, the altered cob(I)yrinic acid a,c-diamide adenosyltransferase, electron transfer flavoprotein (ETF) and ATP synthase demonstrated the disturbance in cobalamin metabolism and energy metabolism in male mussel group with TBBPA exposure (Sheppard et al., 2004; Watmough and Frerman, 2010).

In the female mussel samples treated with TBBPA, glyceraldehyde-3-phosphate dehydrogenase, glucose dehydrogenase (acceptor) and arginine kinase were significantly up-regulated, which suggested that TBBPA mainly disturbed energy metabolisms via metabolic pathways of glucose and arginine in female mussel hepatopancreas. In addition, the up-regulation of glyceraldehyde-3-phosphate dehydrogenase probably implied that female mussels used extra glyceraldehyde-3-phosphate dehydrogenase to prevent them from apoptosis and oxidative stress induced by TBBPA exposure (Chandramouli et al., 2013).

4.3.4. Signal transduction

In both male and female mussel samples treated with TBBPA, two collagens and one of them were up-regulated with statistical significances, respectively. Collagens are not only essential for the mechanical resistance and resilience of multicellular organisms, but are also signaling molecules defining cellular shape and behavior (Daley et al., 2008). Especially in the female mussel samples exposed to TBBPA, one epidermal cell surface receptor was significantly up-regulated together with collagen alpha-1(XII) chain, which clearly confirmed the disruption in signaling pathways via the extracellular matrix induced by TBBPA. In addition to the alterations of collagens, tyrosine-protein phosphatase non-receptor was down-regulated in male mussel samples treated with TBBPA. Tyrosine-protein phosphatase non-receptor is known to act as a negative regulator of the JAK/STAT signaling pathways downstream of cytokines (Zikherman and Weiss, 2011). Calcium binding proteins are proteins involved in calcium cell signaling pathways by binding to Ca^{2+} and play important roles in numerous biological processes including muscle contraction, cellular metabolism, cell proliferation, differentiation and apoptosis (Yui et al., 1995). The down-regulation of calcium binding protein probably meant the apoptosis induced by TBBPA in male mussel samples, which was consistent to the alteration of suprabasin.

4.3.5. Gene expression

Nucleoprotein TPR, the component of the cytoplasmic fibrils of the nuclear pore complex in nuclear protein import, was reported to be related to apoptosis and oxidative stress (Ferrando-May et al., 2001). In this work, nucleoprotein TPR was significantly down-regulated in both male and female mussel hepatopancreas, indicating the apoptosis induced by TBBPA in mussel samples, as mentioned above. The elongation factors and ribosomal proteins are universal proteins involved in protein biosynthesis occurring in the ribosome, whose proteomic differences suggested the disturbance in protein biosynthesis with differential mechanisms between male and female mussel samples. The up-regulated ABC excinuclease likely was the indicator of oxidative stress induced by TBBPA in female mussel hepatopancreas, since ABC excinuclease is an ATP-dependent DNA repair enzyme that removes the damaged nucleotides that are often generated by excessive ROS-induced oxidative stress (Orren and Sancar, 1989). In addition, the significant alterations of transcription activator and serine/arginine-rich protein probably meant the disruption in the process of gene expression in female mussels.

4.3.6. Stress response

TCTP has multiple functions in diverse organisms and was down-regulated in TBBPA-treated male mussel samples. Recently, evidences have indicated the responses of TCTP to oxidative stresses, such as heavy metals and pathogens, to prevent cell from apoptosis (Li et al., 2010). Heat shock cognate 70 (HSC70) usually functions as a molecular chaperon and exerts important roles in folding of newly synthesized polypeptides, membrane translocation and degradation of misfolded proteins (Gachet et al., 1999). The Hsc70-interacting protein was found to be down-regulated in TBBPA-treated male mussel samples, which probably meant the disruption in protein stability. Similar to Hsc 70, glucose regulated protein (GRP78) is a stress protein belonging to the 70 kDa heat shock protein family and was also down-regulated in TBBPA-treated male mussel samples. Peroxiredoxin, an antioxidant enzyme reducing hydrogen peroxide (H_2O_2) and alkyl hydroperoxides treatments, regulates peroxide-mediated signaling cascades, while superoxide dismutase catalyzes the

dismutation of superoxide into oxygen and hydrogen peroxide. Therefore, these altered proteins could be reflected in stronger oxidative stress conditions in the hepatopancreas of male mussels treated with TBBPA than in female mussel samples. In addition, the alterations of selenium binding protein, calreticulin and other stress-response proteins including acidic mammalian chitinase, chitotriosidase and lectin suggested that TBBPA also induced immune responses in male mussel hepatopancreas.

In female mussel hepatopancreas samples, the expressions of lysozyme, apextrin-like protein and chitotriosidase were increased in response to TBBPA exposure. Lysozymes are antibacterial enzymes in the immune system by cleaving the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan layer of bacterial cell walls. Our results demonstrated that the goose-type lysozyme played an important role in digestion indicated by the significantly increased expression level after bacterial challenges (Wang et al., 2012). Apextrin has an important function in neutralization of pathogens and is immunologically responsive to pathogens (Dheilly et al., 2011). Therefore, the significant up-regulation of lysozyme, apextrin-like protein and chitotriosidase clearly indicated the immune responses induced by TBBPA in female mussels.

Clearly, these differential proteins suggested that TBBPA induced oxidative and immune stresses in both male and female mussel hepatopancreas via different metabolic pathways, together with other altered proteins such as ABC excinuclease and glyceraldehyde-3-phosphate dehydrogenase.

4.3.7. Apoptosis

In the TBBPA-treated female mussel samples, however, both apoptosis 2 inhibitor (A2I) and cell death protein (CDP) were remarkably down-regulated (>8.0 folds). As members of the anti-apoptotic family of proteins, apoptosis inhibitors can inhibit the downstream components of the caspase activation pathways in the regulation of apoptosis in many species (Wei et al., 2008). Cell death proteins play important roles in apoptosis and can be up-regulated during cell death responses in various tissues and after various toxic stress signals (Lankat-Buttgereit and Göke, 2003). Some confirmatory data suggest that cell death proteins act as molecular chaperones to scavenge denatured proteins outside cells following specific stress-induced cellular injuries. The down-regulation of apoptosis inhibitor and cell death protein likely implied that TBBPA could inhibit the biosynthesis of these two proteins and therefore apoptosis occurred in female mussel hepatopancreas after TBBPA exposure for one month.

4.4. Correlation between gene and protein expressions

To further evaluate the correlation between gene expression and protein abundances, the expressions of seven genes corresponding to TPR, GRP78, GDA, ECSR, AK, ALP and A2I in *M. galloprovincialis* were quantified to explore the correlation between protein and mRNA expression levels. Results indicated that the mRNA expressions of both AK and ALP did not correlate with protein expressions. The disparity between mRNA and corresponding protein expressions was not surprising (Wang et al., 2010), since mRNA expression means the tendency of the corresponding encoded protein which does not always happen due to the posttranscriptional and post-translational modifications (Wang et al., 2010).

5. Conclusion

In this study, we used iTRAQ coupled with 2D LC-MS/MS proteomic analysis to investigate the gender-specific responses in mussel *Mytilus galloprovincialis* exposed to tetrabromobisphenol A (TBBPA). We confirmed the proteomic differences in hepatopancreas between male and female mussels indicated by 31 differentially expressed proteins. The further proteomic analysis revealed the gender-specific responses between male and female mussel samples to TBBPA exposure, as shown by a total of 60 altered proteins. In details, results indicated that TBBPA exposure could induce apoptosis, immune and oxidative stress and disruption in energy metabolisms in both male and female mussels via different metabolic pathways. In addition, TBBPA exposure could induce disturbances in cell developmental process, lipid and protein metabolisms in male mussel hepatopancreas. In female mussel hepatopancreas, differentially, TBBPA induced reproductive toxicities and disruption in muscle contraction. This work suggested that the gender differences should be considered in ecotoxicoproteomics.

Supporting Information

The location of the Laizhou Bay in China (Fig. S1), the histological results of gonad for sex determination (Fig. S2), the primer sequences for the determination of housekeeping genes (Table S1), and primer sequences of selected genes for qRT-PCR (Table S2).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2014.09.008.

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