Proteomic analysis revealed gender-specific responses of mussels (*Mytilus galloprovincialis*) to trichloropropyl phosphate (TCPP) exposure

Mingyu Zhong, Huifeng Wu, Fei Li, Xiujuan Shan, Chenglong Ji

CAS Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research (YIC), Chinese Academy of Sciences (CAS); Shandong Key Laboratory of Coastal Environmental Processes, YICCAS, Yantai, 264003, PR China

Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266237, PR China

Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, 266071, PR China

**Abstract**

Trichloropropyl phosphate (TCPP) is a halogenated organophosphate ester that is widely used as flame retardants and plasticizers. In this study, gender-specific accumulation and responses in mussel *Mytilus galloprovincialis* to TCPP exposure were focused and highlighted. After TCPP (100 nmol L\(^{-1}\)) exposure for 42 days, male mussels showed similar average bioaccumulation (37.14 ± 6.09 nmol g\(^{-1}\) fat weight (fw)) of TCPP with that in female mussels (32.28 ± 4.49 nmol g\(^{-1}\) fw). Proteomic analysis identified 219 differentially expressed proteins (DEPs) between male and female mussels in control group. There were 52 and 54 DEPs induced by TCPP in male and female mussels, respectively. Interestingly, gender-specific DEPs included 37 and 41 DEPs induced by TCPP in male and female mussels, respectively. The proteomic differences between male and female mussels were related to protein synthesis and degradation, energy metabolism, and functions of cytoskeleton and motor proteins. TCPP influenced protein synthesis, energy metabolism, cytoskeleton functions, immunity, and reproduction in both male and female mussels, respectively. The proteomic differences between male and female mussels were related to protein synthesis and degradation, energy metabolism, and functions of cytoskeleton and motor proteins. TCPP influenced protein synthesis, energy metabolism, cytoskeleton functions, immunity, and reproduction in both male and female mussels. Protein-protein interaction (PPI) networks indicated that protein synthesis and energy metabolism were the main biological processes influenced by TCPP. However, DEPs involved in these processes and their interaction patterns were quite different between male and female mussels. Basically, twelve ribosome DEPs which directly or indirectly interacted were found in protein synthesis in TCPP-exposed male mussels, while only 3 ribosome DEPs (not interacted) in TCPP-exposed female mussels. In energy metabolism, only 4 DEPs (with the relatively simple interaction pattern) mainly resided in fatty acid metabolism, butanoate/propanoate metabolism and glucose metabolism were discovered in TCPP-exposed male mussels, and more DEPs (with multiple interactions) functioned in TCA cycle and pyruvate/glyoxylate/dicarboxylate metabolism were found in TCPP-exposed female mussels. Taken together, TCPP induced gender-specific toxicological effects in mussels, which may shed new lights on further understanding the toxicological mechanisms of TCPP in aquatic organisms.

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This study elucidated the gender-specific toxicological effects of trichloropropyl phosphate (TCPP) in mussel *Mytilus galloprovincialis*. 

1. **Introduction**

Organophosphate esters (OPEs) are organic compounds with a phosphorus atom center and a variety of substituent groups (Zhong et al., 2018). Since usage of some brominated flame retardants (BFRs) was banned, OPEs, as alternatives, took the position of BFRs to meet flammability standards and the production and usage of OPEs increased significantly (Shoeib et al., 2019; Wang et al., 2015). It was reported that the usage of OPEs increased to 680,000 t in 2015 which accounted for 30% of flame retardants (FRs) worldwide.
As FRs and plasticizers, OPEs bond to the added materials non-covalently, thus they are liable to be released to the environment (Wei et al., 2015). Furthermore, OPEs, especially halogenated ones, are resistant to degradation, and hence can exist persistently in the environment (Liagkouridis et al., 2015; Wei et al., 2015; Zhang et al., 2016). Actually, OPEs are detected in a variety of environmental matrices (Ali et al., 2012; Fromme et al., 2014; Gao et al., 2012; Mihajlovic et al., 2011) and in biota (Chen et al., 2000; Kim et al., 2011; Ma et al., 2013), with trichloropropyl phosphate (TCP) being the main compound. For instance, the concentration of TCP is up to 31.4 ng L\(^{-1}\) in the Bohai Sea (BS) which is the semi-closed inner sea of China (Shen et al., 2013; Zhong et al., 2017). The concentration of TCP in the fishes of the Pearl River reaches 4690 ng g\(^{-1}\), indicating high accumulation of TCP in biota (Liu et al., 2019).

Due to the frequent detection in the environment and biota, more and more attentions have been paid to the toxicities of TCP. The neurotoxicity to zebra fish and Cuernohabitis elegans (Disshaw et al., 2014; Noyes et al., 2015; Xu et al., 2017), cytotoxicity to avian hepatocytes (Crump et al., 2012), developmental toxicity to chicken embryos (Farhat et al., 2013), and endocrine disruption to H295R and CHO–K1 cells (Kojima et al., 2013; Liu et al., 2012) of TCP have been reported extensively in recent years, suggesting that TCP may influence multiple biological processes in different species.

However, most of these studies applied bottom-up strategies to detect the toxicities of TCP in which only limited genes, proteins, and biological pathways could be monitored and analyzed at one time (Garcia-Reyero and Perkins, 2011). Furthermore, it was also difficult to discover new biological molecules related to toxicological effects as these studies mainly focused on known sorts of toxicity-responsive molecules (Ji et al., 2016). To solve these problems, high-throughput omics techniques which can present global profiles of organisms’ responses to certain pollutants have been widely employed in modern toxicological researches (Weckwerth, 2011). Gender should be taken into consideration since gender-specific effects were identified in mussels exposed to certain pollutants (Anantharaman and Craft, 20102). Ji et al. (2013a, 2016) discussed the gender-specific responses in the gills of \(M. \) galloprovincialis to tetrabromobisphenol A (TBBPA) and 2,2',4,4'-tetrabromodiphenylether (BDE 47) using proteomics, which revealed differential responses in energy metabolism, osmotic regulation, protein homeostasis and cell apoptosis between male and female mussels. These studies indicated the importance of gender to accurately elucidate the toxicity of organic pollutants.

\(M. \) galloprovincialis belongs to a group of closely related species (family Mytilidae) found in marine habitats all over the world and is an important species in marine aquaculture due to its edible and nutritional values (Ji et al., 2013b). As a filter-feeder, \(M. \) galloprovincialis has the ability to accumulate and tolerate huge amounts of pollutants. Thus, this bivalve is often used as a preferable bioindicator for marine environmental pollutants and frequently investigated as an experimental species in ecotoxicology (Ciacci et al., 2012; Li et al., 2012). In China, \(M. \) galloprovincialis is ubiquitous along the coast of the BS and may be influenced by high concentration of TCP due to dumping to the BS (Wu et al., 2013). Thus, aiming to elucidate the gender-specific responses of \(M. \) galloprovincialis towards a chronic TCP exposure (42 days), we applied iTRAQ (isobaric tags for relative and absolute quantification)-based proteomics to characterize the differentially expressed proteins (DEPs) between control and TCP-exposed groups for male and female mussels, respectively.

\section{2. Materials and methods}

\subsection{2.1. Animal culture, exposure and sample collection}

Sexually matured adult mussels \(M. \) galloprovincialis (shell length: 4–6 cm) were collected from a local farm (Yantai, China). After cleaned with fresh seawater, the mussels were acclimatized in aerated fresh seawater for 7 days and then were divided into two groups (solvent control and TCP exposure), with each group containing two replicate tanks (length: 60 cm; width: 32 cm; height: 38 cm). Approximately 50 mussels were placed in each tank containing 40 L of filtered seawater. The mussels cultured in the filtered seawater containing 0.00025% dimethylsulfoxide (DMSO, v/v) were used as solvent control group, with another group being exposed to 100 nmol L\(^{-1}\) TCP. The concentration of TCP stock solution was 10 mg mL\(^{-1}\) in DMSO to ensure the same DMSO concentration in TCP-exposed group and solvent control group. During the periods of acclimation and exposure, all the animals were kept at 18–20 °C under a photoperiod of 12 h light and 12 h dark, and fed with 1.5 g (dry weight) Chlorella vulgaris for the mussels in each tank per day. After exposure for 42 days, whole tissues (except byssus and shell) of 24 mussels (12 males (six from each tank) and 12 females (six from each tank)) in each group were collected randomly. Then, these 12 mussels from each group were randomly divided into three biological replicates (each containing 4 individual mussels) and ground into powder in liquid nitrogen. The ground samples were stored at −80 °C for further iTRAQ-based proteomic analysis. Meanwhile, whole tissues (except byssus and shell) of another 24 mussels (12 males (six from each tank) and 12 females (six from each tank)) in each group were also randomly collected, cleaned, dissected and frozen-dried for 72 h. Then, the frozen-dried tissues were stored at −20 °C for determination of TCP bioaccumulation. Genders of mussels were identified by observing their gonads under a microscope.

\subsection{2.2. Measurement of TCP concentrations in seawater and in mussels}

For measurement of TCP concentrations in seawater, method proposed by Zhong et al. (2017) was referred and employed in the present study. Detailed information was described in the Supporting Information.

For measurement of TCP concentrations in mussels, the frozen-dried tissues were ground into powder in clean porcelain mortars. Each ground tissue sample (approximately, 0.3 g) was collected and spiked with 40 \(\mu\)L of \(d_{12}\)-TCP at a concentration of 1000 pg \(\mu\)L\(^{-1}\). The spiked tissue sample was subjected to soxhlet extraction for 24 h in 200 mL of dichloromethane at 52 °C. The 200 mL of extracts were evaporated and determined to 10 mL using hexane as the keeper. A volume of 1 mL of the concentrated extracts were taken out for fat weight determination and the remaining 9 mL of the extracts were further concentrated to about 2 mL in methanol under gentle nitrogen flows in Teflon tubes. The extracts (~2 mL) were frozen at −20 °C overnight and centrifuged at 5000 g for 5 min in a precooling centrifuge (Eppendorf, Germany). The supernatants were transferred into separating funnels, diluted by 500 mL of ultra pure water and subjected to solid phase extraction by gravity with HLB (hydrophilic–lipophilic balance) columns (6 cm\(^3\), 200 mg). Then, the columns were drained and eluted with 10 mL of ethyl acetate and the elutes were frozen at −20 °C for 24 h.
to remove water and the residual water was removed again by adding 3 g of baked Na2SO4. The dewatered eluates were evaporated to around 200 μL under gentle nitrogen flows and transferred to new vials. Finally, 20 ng of polychlorinated biphenyl 208 (PCB 208) was added as an injection standard before instrumental analysis.

The analytical method for the determination of OPEs was described previously (Zhong et al., 2017). In the current study, an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer (GC–MS) was used to analyze the samples and detailed information were described in Supporting Information. The method blank and recovery for TCPP was 0.0025 ± 0.00014 nmol L\(^{-1}\) and 88.99 ± 15.22% \((n = 3)\). All the data related to TCPP concentrations in the mussel tissues were processed and analyzed by IBM SPSS statistics 22 while the TCPP concentrations in the mussel tissues were not corrected by the method recovery.

### 2.3. Quantitative proteomic analysis

Quantitative proteomic analysis was conducted by using iTRAQ strategy on the whole tissue (except byssus and shell) of mussels from each group. Main procedures of quantitative proteomic analysis included protein extraction, protein digestion, iTRAQ labeling, and instrumental analysis. The protein extraction was conducted by using sodium dodecyl sulfate (SDS) extraction. The main procedures of protein digestion were referred to Wisniewski et al. (2009) and the processing of iTRAQ labeling was conducted according to the manufacturer’s instructions (Applied Biosystems Incorporation, USA). An Agilent 1200 HPLC System (Agilent, USA) with an Eksigent nano LC-1D plus system (SCIEX, USA) coupled with a Triple TOF 5600 mass spectrometer (SCIEX, USA) equipped with a Nanospray III source (SCIEX, USA) was used in the instrumental analysis of all proteomes. Quantitative analysis of protein expression was based on the labeling of peptides and conducted as follows: In mass spectrometry analysis, different samples with the same peptide which had the same mass to charge ratio were selected for secondary fragmentation at the same time to produce different quality of report ions and realize the quantification of proteins among different products by the abundance of the report ions. Protein Pilot 5.0 (SCIEX, USA) was used to align proteomic data with the Mollusk Protein Database (mollusca.fasta) from the Uniprot (https://www.uniprot.org/) using the Paragon algorithm to conduct protein identification. The Screening criteria of credible proteins were as follows: Unused >1.3 and unique peptide >1. remove invalid values and the anti-library data. The screening of DEPs was based on the results of three replicates of the identified credible proteins. Detailed information was described in the Supporting Information.

### 2.4. Protein-protein interaction analysis

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is a widely used biological database and web resource of known and predicted protein-protein interactions. DEPs were imported to the string website (http://string-db.org/cgi/input.pl), then the network file generated from the imported DEPs (string_ interactions.tsv) was downloaded. Subsequently, the file was imported into the cytoscape 3.5 to make a network diagram (Figs. 6 and 7). The network diagram was based on information gained up to 3 level of functional analysis: fold change of protein, protein-protein interaction, and KEGG pathway enrichment.

### 3. Results and discussion

#### 3.1. TCPP accumulation in mussels

The waterborne concentrations of TCPP were 0.03 ± 0.0024 and 96.40 ± 2.36 nmol L\(^{-1}\) in control and TCPP-exposed group, respectively. The actual concentration of TCPP for exposure was close to the nominal concentration (100 nmol L\(^{-1}\)) of TCPP. TCPP concentrations in tissues were 0.10 ± 0.02 and 0.07 ± 0.02 nmol g\(^{-1}\) fat weight (fw) in male and female mussels from the control group \((n = 12)\), respectively (Table 1). For the TCPP-exposed group, the accumulated TCPP were 37.14 ± 6.09 and 32.28 ± 4.49 nmol g\(^{-1}\) fw in tissues from male and female mussels \((n = 12)\), respectively. Although the average TCPP concentration in male mussels was slightly higher than that in female mussels with TCPP exposure, there was no statistical significance between them. The significant \((P < 0.05)\) accumulations of TCPP in TCPP-exposed group could induce toxicological effects in both male and female mussels.

#### 3.2. Proteomic characterization

Overall, 55206, 52104 and 53217 peptide spectra were identified corresponding to 3156, 2991 and 3113 proteins in the three biological replicates for the four subgroups (male control, male treatment, male female control treatment). Finally, a total of 4976 proteins were identified from 160527 peptides among which 22005 were unique, and the average coverage of the peptides matched was 16.53%. Only those proteins (754) that presented in all the three biological replicates were used for quantification.

Significantly differentially expressed proteins \((P < 0.05)\) with fold change (FC) lower than 0.77 or higher than 1.30 were designated as DEPs. On the whole, a total of 243 DEPs were detected among which 219 DEPs were found between male control and female control mussels. And 52 and 54 DEPs were respectively induced by TCPP in male and female mussels (Fig. 1, Table S1, S2 and S3). As shown in the Venn diagram (Fig. 1B and Table S1), 37 and 41 DEPs among gender-specific proteomic differences between male control and female control mussels were found to be differentially expressed in TCPP-exposed male and female mussels, respectively. It implied that the gender-specific proteomic differences might be the biological foundation for varied responses to TCPP exposure. However, only 13 DEPs were commonly found in both TCPP-exposed male and female mussels (Fig. 1C and D), indicating that proteomic responses to TCPP were mostly gender-specific.

#### 3.3. Proteomic differences between male and female mussels

A total of 219 DEPs were found between male and female mussels from the control group. These gender-specific DEPs were mainly involved in protein synthesis and degradation, energy metabolism, and functions of cytoskeleton and motor proteins (Fig. 2 and Table S1).

#### 3.3.1. Protein synthesis and degradation

Generally, 40 ribosome proteins, 3 aminoacyl-tRNA synthetases,

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<td>TCPP concentrations (nmol g(^{-1}) fat weight (fw)) detected in mussel tissues from control and TCPP-exposed groups ((n = 12)).</td>
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<tr>
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Values labeled with black asterisks mean statistical significance \((P < 0.01)\) of TCPP accumulation between control and TCPP-exposed mussels.
4 spliceosome proteins, 7 protein synthesis factors, 11 molecular chaperones, and 4 proteasome proteins were DEPs functioning in protein synthesis and degradation (Fig. 3). Most of the ribosome proteins, aminoacyl-tRNA synthetase, spliceosome proteins and protein synthesis factors playing important roles in protein synthesis showed lower expression in male mussels. On the other hand, male mussels also presented lower expression of molecular chaperones and proteasome proteins which functioned in degradation of misfolded proteins. In other words, male and female mussels might use different strategies to maintain protein homeostasis and male mussels showed less activated protein metabolisms than female mussels. Taken together, all the DEPs mentioned above might imply different strategies in male and female mussels to maintain protein homeostasis and more activated protein metabolisms of female mussels (Fig. 3).

3.3.2. Energy metabolism

The DEPs mainly involved in energy generation (glycolysis/
gluconeogenesis, tricarboxylic acid (TCA) cycle and lipid metabolism, together with DEPs of electron transfer chain showed a generally higher expression tendency in male mussels (Fig. 4). This pattern indicated more energy demands in male mussels during individual maintenance and growth. Interestingly, adenylate kinase 8 (K1PRS8) mainly functioning in metabolic and energy monitoring by sensing the levels of AMP in cells had higher expression levels in male mussels as well (Dzeya and Terzic, 2009). According to Panayiotos et al. (2011), adenylate kinase 8 can phosphorylate AMP, CMP, dAMP and dCMP with ATP and GTP as the phosphate donors hence regulating the energy balance among different cell compartment and/or even in different tissues. Thus, the higher abundance of adenylate kinase 8 suggested the enhanced metabolic regulation corresponding to enhanced energy demands in male mussels in comparison with female mussels.

3.3.3. Functions of cytoskeleton and motor proteins

Cytoskeleton and motor proteins not only provide mechanical supports and conduct substance transportation but also play essential roles in the functions of sperm flagella and muscle contraction (Wickstead and Gull, 2011). The DEPs of cytoskeleton and motor proteins primarily consisted of 11 dyneins, 2 tubulins, 1 myosin and their related proteins. These DEPs might influence flagella motility of sperms and contraction of anterior byssus retractor muscle (ABRM) which was important in reproduction and adaption of intertidal environment. Detailed functions of these DEPs were discussed in Supporting Information and DEPs associated with flagella motility of sperms were also summarized in Fig. 5. In addition, the dyneins and tubulins mentioned above might also function in cilia functions of gill cells, since mussels were typical filter feeding marine animals and the cilia movement of gill cells played essential roles in their food taking (Jorgensen et al., 1984). Thus, these up-regulated cytoskeleton and motor proteins also suggested the stronger feeding ability of male mussels.

3.4. Proteomic responses of male mussels to TCPP exposure

The TCPP treatment to male mussels elicited 30 up-regulated and 12 down-regulated proteins (Fig. 1A and C) mainly involved in protein synthesis, energy metabolism, cytoskeleton, immunity, and reproduction (Fig. 2B and Table S2). DEPs involved in protein synthesis (nine ribosome proteins and a eukaryotic translation elongation factor 2, Table S2) showed an incongruous alteration pattern, in which 6 proteins (60S ribosomal protein L27, A0A077GVV6; 60S ribosomal protein L10, A0A077H3K9; 60S ribosomal protein L5, A0A194ANG4; 40S ribosomal protein S26, A0A0L8HXQ8; 40S ribosomal protein S23, K1RK12 and eukaryotic translation elongation factor 2, K1QFW9) were up-regulated and 4 proteins (60S ribosomal protein L13, A0A077GZL7; 60S ribosomal protein L35, A0A077H0Q5; 60S ribosomal protein L38, A0A077B5S4 and 40S ribosomal protein S2, A0A077GZK0) were down-regulated. These DEPs implied that TCPP exposure disturbed protein synthesis in male mussels.

For DEPs involved in energy metabolism, the up-regulated glyceraldehyde-3-phosphate dehydrogenase (A0A076V8B6) resides on the central position of glycolysis producing ATP, reductive force and pyruvate. Additionally, a tri-functional enzyme (A0A0B7B943) and an acetyl-CoA acetyl transferase (V4AZG4) mainly functioning in fatty acid degradation were up-regulated as well. Over expression of these three enzymes and the two components of electron transfer chain (cytochrome c, B6CMY0 and ATP synthase subunit beta, Q6PHT6) meant the enhanced energy metabolism in TCPP-exposed male mussels. However, other two enzymes (glycogen phosphorylase, V4ALJ5 and glycerol-3-phosphate dehydrogenase 1b, V4AL14) related to glycogen and fat degradation were down-regulated, respectively. The differential expressions of these enzymes involved in glucose and lipid metabolisms suggested that TCPP disturbed energy metabolism in male mussels.

Six cytoskeleton related DEPs were differentially expressed in TCPP-treated male mussels. The up-regulation of the dynein light chain (A0A0B7BT39) meant that TCPP could activate microtubule based transportation and flagella movement as mentioned above. One actin (O76784) was up-regulated as well, suggesting that TCPP influenced the microfilament (MF) related functions. According to Michelot and Drubin (2011), MF provides force-generating system in many different processes, such as cell motility, cell adhesion, endocytosis, cytokinesis, or more generally, for the control of cell morphology. However, two cytoskeleton associated DEPs (transgelin-like protein-4, A0A0KOYB26 and shell myostracum Ser-rich protein 1, A0A0K0PUQ1) primarily involved in adductor muscle–shell attachment which allowed the mussels to open and close their shells were down-regulated (Gao et al., 2015; Liao et al., 2015). Furthermore, two proteins from the category of extracellular matrix (ECM) (collagen-like protein 1 and 7, A0A0K0PU6 and A0A0K0YB43) also functioning in adductor muscle–shell

Fig. 2. Categories of DEPs between male control and female control mussels (A), in TCPP-exposed male mussels (B), and in TCPP-exposed female mussels (C).
attachment were similarly altered (Gao et al., 2015). Additionally, mitochondrial phosphate carrier protein (A0A077GYV9) in the category of transportation proteins was up-regulated. This protein had a high similarity with human solute carrier family 25A3 (SLC25A3) expressed exclusively in muscle lacking energy (Fiermonte et al., 1998). Therefore, the up-regulation of mitochondrial phosphate carrier protein suggested that TCPP could impair energy supporting in muscle cells of male mussels and their contraction. These findings demonstrated TCPP-induced impairment in the formation of adductor muscle–shell attachment and muscle contraction and therefore weaken the control of shell opening and closing which were important for food taking and survival of sessile mussels.

Two proteins (MgC1q, E1UJ66 and apextrin-like protein 1, A0A0K0PV08) belonging to the category of ECM presented a down-regulated pattern. According to Kishore and Reid (2000), C1q is considered to be a major link between classical pathway-driven innate immunity and acquired immunity in vertebrates. In mussels, MgC1q was reported to be a pattern recognition molecule being able to recognize pathogens during innate immune responses. Furthermore, it highly expressed in hemocytes and could be responsive to bacteria exposure sensitively (Gestal et al., 2010). The apextrin-like protein 1 was reported to be up-regulated in bacteria-challenged mussels, indicating its immunity related functions (Estévez-Calvar et al., 2011). The down-regulation of these two proteins suggested the immunotoxicity induced by TCPP in male mussels.

The over expression of gamete related proteins (tektin 3, V4BBB3; vitelline coat lysine (VEL)M6, Q86LZ9; vitelline envelope zona pellucida domain protein (VEZPD), G0YPE8 and putative vitelline envelop receptor for lysine (VERL), B9WP6) suggested the reproductive toxicity induced by TCPP in male mussels. Tektin 3 was proposed to influence reproduction through its interaction with axonemal dyneins and tubulins in flagella. VELM6, showing strong egg vitelline coat lysin and first polar body releasing activities, was firstly identified in Mediterranean mussels (Takagi et al., 1994). VERL and VEZPD were supposed to mediate fertilization by forming a cognate pair with lysin and provided mechanical protection for the eggshell (Arukwe et al., 1997; Galindo et al., 2002). These two proteins should be exclusively expressed in female mussels for its location on eggs (Farcy et al., 2013). However, VERL and VEZPD in M. edulis testis were also up-regulated under the exposure of exogenous estrogen which was a well-known hormone in reproductive endocrinology (Ciocan et al., 2011). Therefore, these DEPs suggested the potential reproductive endocrine disruption effects induced by TCPP in male mussels.

3.5. Proteomic responses of female mussels to TCPP exposure

There were 54 DEPs (38 up-regulated and 16 down-regulated) induced by TCPP in female mussels (Fig. 1A and D). The proteomic responses in TCPP-treated female mussels were also basically related to protein synthesis, energy metabolism, cytoskeleton, immunity and reproduction (Fig. 2C and Table S3).

Three altered ribosome proteins (A0A077GY50, down-regulated; Q3HM58, down-regulated; and K1P8W6, up-regulated) and two aminoacyl-tRNA synthetases (A0A194AMH4, up-regulated and V4BBR9, down-regulated) indicated that TCPP...
Fig. 4. DEPs involved in energy metabolism between male control and female control mussels.

Fig. 5. DEPs involved in sperm flagella between male control and female control mussels.
influenced the process of protein synthesis in female mussels as well. However, two proteasome proteins (20S proteasome subunit alpha 4, A0A182Z8R9 and proteasome 26S subunit, ATPase 1a, K1QLT5) were down-regulated, which suggested the lowered ability of degrading misfolded proteins in TCPP-exposed female mussels.

For energy metabolism, six metabolic enzymes clustering in glycolysis and citrate circle presented an overall up-regulated pattern (A0A076V861, A0A0K0YB40, K1R2Q9, V4A6K4 and Q9NHX3) with the mitochondrial isocitrate dehydrogenase (IDH) subunit alpha (K1Q9G3) being an exception. In addition, the glycerol-3-phosphate dehydrogenase 1b (V4A114) bringing glycerol (metabolic products of fat degradation) into the glycol metabolism was significantly up-regulated. These DEPs indicated an enhanced degradation of sugar and fat with robust energy demands which might be utilized to cope with TCPP stress in female mussels (Ji et al., 2016). However, in the category of electron transfer chain components, electron transfer flavoprotein beta polypeptide (A0A0B6YS36, up-regulated) and NADH-ubiquinone oxidoreductase 75 kDa subunit (V3ZZE4, down-regulated) showed an opposite
regulation. It is well-known that citrate cycle coupled oxidative phosphorylation is efficient in energy generation. Interestingly, though the degradation of sugar and fat might be enhanced, the down-regulated IDH and NADH-ubiquinone oxidoreductase 75 kDa subunit implied blocked citrate cycle and oxidative phosphorylation. Therefore, TCPP exposure might cause disorders in energy metabolism mainly through suppressing IDH expression and resulting in excessive glycolysis and fat degradation.

For cytoskeleton and motor proteins, DEPs like tubulin beta (G3ETB4), axonemal dynein heavy chain 7 (K1QK11), axonemal dynein heavy chain 5-like (V4AFP1) and tetratricopeptide repeat protein 25 (K1PYM8) were involved in cilium functions. The up-regulations of these proteins suggested an enhanced cilium functions in food taking and environment sensing to cope with TCPP stress in female mussels (Wickstead and Gull et al., 2011). However, proteins involved in byssus retractor and adductor muscle contraction (pedal retractor muscle myosin heavy chain (9QU056), paramyosin (O960G4), and shell myostracum Ser-rich protein 1 (A0A0K0PUQ1)) were down-regulated, which indicated the impaired controlling of shell opening and closing induced by TCPP in female mussels (Funabara et al., 2001, 2003; Yamada et al., 2000). In addition, the Rab14 (K1Q7C8) in the category of signaling proteins showed lowered expression as well. Functions of Rab 14 were not (Anantharaman and Craft, 2010) characterized in (Cao et al., 2000) details in mollusks. However, this (Chen et al., 2012) protein (Dishaw et al.) showed high similarity with its homolog in human. According to Guo et al. (2017) human Rab 14 mediates (Wisniewski et al., 2009) glucose uptake by increasing the number of GLUT4/SLC2A4 glucose transporters at the muscle cell surface. Down-regulated expression of Rab 14 meant the insufficient glucose intake and then impaired energy generation in adductor muscles of TCPP-exposed female mussels. Interestingly, transgelin-like protein-4 (A0A0K0K26) and two proteins of extracellular matrix (collagen-like protein 1 and 7 (A0A0K0PUP6 and A0A0K0YB43)) mainly functioning in adductor muscle—shell attachment were over expressed (Gao et al., 2015), which might be considered as a compensation of impaired adductor muscle function.

Unlike immune inhibition effects of TCPP in male mussels, three C1q domain containing proteins (E1UJ66, abbreviated as MgC1q; F0V443, named C1q containing protein; and F0V452, named cerebellin7) and one galectin 2 (A0A0C5Q4G0) showed general over expressions. As discussed above, C1q domain containing proteins can be responsive to bacterial infection and play important roles in immune system (Gestal et al., 2010), while galectin 2 functions as an immune molecule being able to signal the pathogen-associated tissue damage in bivalves (Gerdol and Venier, 2015). Therefore, up-regulations of these proteins suggested that TCPP could activate immune response in female mussels.

Two gamete related proteins (B9WP6, abbreviated as VERL and U3Q6X6, abbreviated as VEL M3) were up-regulated, indicating that TCPP influenced the reproduction of female mussels as well. Another protein (ADP, ATP carrier protein, K1R0Y9) showing sequence similarity with human solute carrier family 25A6 (SLC25A6) also over expressed. According to Palmieri (2004), SLC25A6 abundantly expresses in highly proliferative cells with quite low expression in the main tissues. It is well known that ADP, ATP carrier protein (or proteins in SLC25 family) is located on the mitochondrial inner membrane and mainly involves in ATP generation (Palmieri, 2004). That was to say, the up-regulated ADP, ATP carrier protein also affected gamete proliferation to a certain extent through energy supply. These DEPs meant that TCPP exposure could influence reproduction of female mussels through different ways compared with those in male mussels.

3.6. TCPP influenced protein synthesis and energy metabolism in both male and female mussels

In order to elucidate the main influence of TCPP exposure and relationships among DEPs, all the DEPs induced by TCPP in male and female mussels were mapped to Danio rerio protein database for Danio rerio, the most studied model spices in marine animals so far. KEGG analysis and String database based protein-protein interaction (PPI) networks were constructed and visualized by Cytoscape 3.5 as mentioned in section 2.4. As shown in Figs. 6 and 7, a total of 29 DEPs in male mussels and 35 DEPs in female mussels were presented in the PPI networks with protein synthesis and energy metabolism being the main biological processes influenced by TCPP for both male and female mussels.

In TCPP-exposed male mussels, ee212, rps26, rpl6, rpl5a, cct6a, rpl38, rpl13, rps23, ddx6, rps2, rpl10, and rpl35 were closely clustered and associated with the KEGG term of ribosome. However, in TCPP-exposed female mussels, only rpl4, rps4x and rpl5a directly linked to ribosome while rpl4 indirectly associated with faua which interacted with psma1b and psma8, two components of the proteasome. There was no common DEPs in protein synthesis between TCPP-exposed male and female mussels.

For DEPs involved in energy metabolism in the male mussels, hadhaa and acat1 seemed to reside in the central position of fatty acid, butanoate, propanoate and amino acid (lysine, tryptophan, valine, leucine, and isoleucine) metabolism. These two enzymes were also associated with gapdh and idh3a, two key enzymes in glucose metabolism, through the KEGG term of carbon metabolism. Meanwhile, hadhaa was associated with atp6v1a1, a component in the electron transfer chain. Compared with male mussels, more DEPs functioning in energy metabolism such as etfb, acat1, mdh2, gpd1a, aldob, gapdh, idh3a, got2a and txnr1 were associated directly or indirectly with each other and involved in the KEGG term of pyruvate metabolism, glyoxylate and dicarboxylate metabolism, TCA cycle, carbon metabolism, and amino acid (phenylalanine, tyrosine, tryptophan, cysteine, and methionine) metabolism. Taken together, although TCPP mainly influenced protein synthesis and energy metabolism in male and female mussels, the DEPs involved in these processes and their interaction relationships were quite different, which indicated gender-specific responses of mussels to TCPP exposure.

4. Conclusion

This study focused on the responses of mussels to TCPP exposure by taking gender into consideration. Male and female mussels in the control group showed the most varied proteomic differences, which could be viewed as the biological foundation for the gender-specific response of mussels to TCPP exposure. With the similar accumulating capacity, TCPP altered biological processes like protein synthesis, energy metabolism, cytoskeleton functions, immunity and reproduction in both male and female mussels. Meanwhile, PPI analysis implied that protein synthesis and energy metabolism were likely to be the main processes influenced by TCPP for both male and female mussels. Nevertheless, DEPs involved in the mentioned processes and their interaction relationships were quite different. These results suggested dissimilar toxic mechanisms of TCPP to male and female mussels and that gender-specific responses should be viewed as an important factor in toxicology.

CRediT authorship contribution statement

Mingyu Zhong: Formal analysis, Writing – original draft. Hui-feng Wu: Funding acquisition, Writing - review & editing, Project
administration. **Fei Li**: Funding acquisition, Writing - review & editing. **Xiujuan Shan**: Funding acquisition, Writing - review & editing. **Chenglong Ji**: Writing - review & editing.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (21677173, 41530642, 41976152), the Marine S&T Fund of Shandong Province for Pilot National Laboratory for Marine Science and Technology (Qingdao, 2018SDKJ0501-1) and the Youth Innovation Promotion Association CAS (2017255).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115537.

Proteins labeled with red triangles meant that they were up-regulated. G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; F-1,6-2P: fructose-1,6-diphosphate; DHAP: dihydroxyacetone phosphate; PGAL: 3-glyceraldehyde phosphate; 1,3-BPG: 1,3-diphosphoglycerate; 3-PCA: 3-phosphoglycerate; 2-PCA: 2-phosphoglycerate; PEP: phosphoenolpyruvate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; CS: citrate synthetase; IDH: isocitrate dehydrogenase; KDH: 2-ketoglutarate dehydrogenase; SDH: succinic dehydrogenase; MDH: malate dehydrogenase.

IDH3A: inner dynein arms; ODA: outer dynein arms; RS: radical spoke; CP: central pair; PKA: protein kinase A; PKC: protein kinase C; AKAP: A kinase anchor protein; cAMP: cyclic adenosine monophosphate; ATP: adenosine triphosphate.

The circle nodes represented DEPs and the rectangle represented KEGG pathways. Pathways were colored with gradient color from yellow to blue, yellow for smaller P-value, and blue for bigger P-value. In case of fold change analysis, proteins were colored in red (up-regulation) and green (down-regulation). Interactions with bigger confident score were shown as solid lines between proteins, otherwise in dashed lines.

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