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Comparative quantitative proteomics unveils putative mechanisms involved into mercury toxicity and tolerance in *Tigriopus japonicus* under multigenerational exposure scenario☆

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ABSTRACT

In our earlier work, *Tigriopus japonicus* were subjected to different mercuric chloride treatments (0–50 μg/L in the seawater) for five generations (F0–F4), and they were subsequently resumed under clean environments for one generation, i.e., F5. Accumulative effects were hypothesized to participate in mercury (Hg) multigenerational toxicity, however phenotypic plasticity could be responsible for metal resistance in this copepod against the long term exposure. Here, we specifically investigated the proteome profiles in the F0, F2, and F5 copepods of the control and 50 μg/L metal treatment, respectively, so as to elucidate the action mechanisms for Hg toxicity/tolerance in *T. japonicus* under the long term exposure. Functional enrichment analysis showed that a quite different proteomic response was observed in F5 compared with F0 and F2. Namely, the vast majority of enrichments were correlated with the down-regulated proteins in F0 and F2, whereas the enrichments for F5 were mostly attributable to the up-regulated proteins, suggesting that different mechanisms are responsible for Hg toxicity and tolerance (i.e., phenotypic plasticity). Hg toxicity prohibited many proteins in F0 and F2 which are related to several critical processes/pathways, e.g., protein translation, macromolecule metabolic process, DNA replication, cell cycle, cuticle organization, vitellogenesis, etc. In F5, many up-regulated proteins were enriched into compensatory systems, such as carbohydrate metabolism, myosin reorganizations, and stress-related defense pathway. Notably, glycolysis (an oxygen-independent pathway) was enhanced for energy allocation into metal detoxification and tolerance. Taken together, proteomics provides novel mechanistic insights into phenotypic plasticity used by *T. japonicus* when challenged with cumulative effects due to Hg multigenerational toxicity.

1. Introduction

Mercury (Hg) is a widespread and persistent environmental toxicant in aquatic environments (Jiang et al., 2006). Owing to anthropogenic activities, Hg pollution has been a primary environmental problem in China, since it makes an approximately 28% contribution to the atmospheric Hg emitted by the global people. Atmospheric Hg will be deposited and finally aggregate into the marine and coastal environments, thereby causing a heavy Hg contamination for this region in China (Gao et al., 2014; Pan and Wang, 2012; Wang et al., 2009). For instance, the maximum content of total Hg (T-Hg) has been reported as 2.59 μg/L for the seawater in Jinzhou Bay, and it is increased by three orders of magnitude in contrast to the reference level (Wang et al., 2009). In such a seriously polluted environment, the biota may have suffered from the high Hg levels through many generations. Hg toxicity is frequently attributed to their high affinity for the SH groups in endogenous biomolecules (e.g., proteins and enzymes), hence causing their dysfunctions and subsequently producing multiple toxicities in biota including human beings (Castoldi et al., 2001; Wang et al., 2011, 2015). Consequently, an ecotoxicological study, where the important life history traits (e.g., development and

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reproduction) are investigated in organisms under Hg exposure for many generations, is really needed and most valuable, since adaptive or degenerative responses in offspring will show the most crucial effects on marine ecosystems.

Recently, a few studies have been focused on Hg multigenerational effects to the growth and tolerance development in aquatic animals (Li et al., 2015; Tsui and Wang, 2005; Vidal and Horne, 2003), since this long term exposure to environmental toxicants including Hg for many generations may better simulate the environment which the biota confront in situ. To our knowledge, our previous work has firstly examined Hg effects on several important traits in marine copepod *Tigriopus japonicus* subjected to different concentrations of mercuric chloride ($HgCl_2$) in seawater (0, 0.5, 1, 10, and 50 μg/L) for five generations (F0-F4), and afterwards all the treatments are recovered in clean environments for one generation, i.e., F5 (Li et al., 2015). For the six traits, only the number of nauplii/clutch and fecundity are significantly prohibited by Hg exposure, furthermore, the restrained impacts worsening from F0 to F3, and it is probably caused by a tendency that the copepod's metal contents are enhanced with generations. More interesting is that all the six traits insignificantly differ from the control for F5, although the Hg contents in this recovery generation increase with the enhanced metal concentrations used in F0-F4. Thus, we hypothesize that even though accumulative effects might be responsible for Hg multigenerational toxicity to *T. japonicus*, phenotypic plasticity could be involved into Hg tolerance in this copepod against the long term exposure. Nevertheless, little is known about the exact mechanism for phenotypic plasticity acquired by marine copepods to fight against Hg multigenerational effects, despite the fact that it can provide novel mechanistic insights into the underlying process which could render some animals to obtain better fitness under marine environmental stressors including Hg pollution.

Toxicoproteomics is considered as a comparatively new technology that applies proteomics for toxicological researches and consequently targets to elucidate crucial proteins and processes disrupted by environmental toxicants (Alex Merrick, 2006). In recent years, proteomic techniques have well been utilized to provide a mechanistic understanding about Hg toxicity to marine fish in many previous studies (Karlsen et al., 2014; Keyvanshokooh et al., 2009; Nostbakken et al., 2012; Wang et al., 2011, 2013, 2015). It should be emphasized that in those earlier studies two-dimensional gel electrophoresis (2-DE) combined by mass spectrometry (MS) analysis, i.e., gel-based proteomics, is applied for protein separation and identification. Although 2-DE is the most widespread approach employed for protein separation in ecotoxicological studies (Karlsen et al., 2014; Keyvanshokooh et al., 2009; Nostbakken et al., 2012; Wang et al., 2011, 2013, 2015), significant disadvantages (e.g., troublesome, time-consuming and low sensitive) are associated with this approach, therefore resulting in an identification with a limited number of proteins (Roe and Griffin, 2006) and succeedingly impeding to fully understand the biochemical mechanism of toxicants in organisms. As a consequence, gel-free proteomics has increasingly been utilized for ecotoxicological researches in place of 2-DE coupled with MS analysis. Various chemical labeling techniques such as isobaric tandem mass tags (TMT) have been used to examine proteome alterations in the brain and liver of marine fish *Oryzias melastigma* under the exposure against antifouling chemicals (Chen et al., 2014, 2015).

Recalling that, in our previous work, very scare information is revealed about the biochemical mechanism for phenotypic plasticity used by marine copepod *T. japonicus* under multigenerational exposure to Hg contamination (Li et al., 2015); therefore, TMT-based quantitative proteomic analysis was performed in this study. In detail, the protein expression profiles were analyzed in the F0, F2 and F5 of *T. japonicus* which were subjected to the control and 50 μg/L $HgCl_2$ treatments for five generations with F0-F4, and succeedingly resumed under clean conditions for one generation, i.e., F5. The proteomic analysis for F0 and F2 was aimed to explore the action mechanism for Hg multigenerational toxicity, with the analysis in F5 being primarily targeted at providing a mechanistic explanation to metal tolerance (i.e., phenotypic plasticity) acquired by this copepod under the long term exposure. Taken together, our purpose is to provide novel mechanistic insights into phenotypic plasticity used by marine copepod *T. japonicus* to fight against cumulative effects due to Hg multigenerational toxicity.

2. Materials and methods

2.1. Copepod maintenance

Copepods *T. japonicus* were collected from the rocky pools of intertidal zone in Xiamen Bay, People's Republic of China (N 24°25.73'; E 118°6.34′) in 2007, and were remained in the lab until now. All the seawater was collected 20 km offshore in Xiamen Bay, and subsequently filtered through a 0.22 μm polycarbonate membrane for the experiments. The reference content for T-Hg in the seawater was averagely 0.0045 μg/L. The dissolved oxygen levels were 6.2–6.7 mg/L, with salinity for 29–30 PSU and pH for 8.0–8.1. Also, the animals were raised at 22 °C and a 12/12 h light/dark cycle. Three algae, *Isochrysis galbana*, *Platymonas subcordiformis*, and *Thalassiosira pseudonana*, were equally mixed at a density of 8×10^5 cells/L and supplied as prey for *T. japonicus*. All the exposure experiments had the same conditions as depicted for the above copepod culture.

2.2. Multigenerational experiments

To analyze the proteome profiles in the adult animals for the F0, F2 and F5, we carried out a multigenerational life-cycle testing for *T. japonicus* on March 25, 2014. Namely, *T. japonicus* were subjected to the control and 50 μg/L $HgCl_2$ treatment for five generations with F0-F4, and subsequently recovered under “purified” conditions for one generation period, i.e., F5. It should be noted that the relatively high Hg concentration (50 μg/L) was used for proteomic analysis in this study, and the main reason is that, only at this dose, the copepod's offspring production is consistently restrained under the multigenerational exposure but resumes in the recovery generation by contrast with the control according to our earlier work (Li et al., 2015). Meanwhile, the detailed protocol was the same as depicted for the multigenerational exposure experiments in our earlier work (Li et al., 2015). In brief, ten nauplii (<24 h) per concentration treatment were raised in 6-well plates containing 8 mL solution, and it was repeated in 30 replicates. These nauplii were reared under the same conditions as for the above copepod maintenance until they grew up to maturation (i.e., the time for adult females to produce egg sacs). The exposure seawater was renewed for 80% of the solution volume every day, and *P. subcordiformis* was removed from the culture solution by centrifugation and subsequently provided as food at a cell density of approximately 6×10^5 cells/L. As for F1, 10 nauplii yielded from the first or second clutch of each female (F0) were raised in new 6-well plates for each concentration treatment. The detailed procedures were the same as depicted above for the F0 exposure. The following generations were exposed under the same conditions as for F0, and this long term exposure was remained until the F4 nauplii grew up to adults. After the exposure of five generations, the F5 nauplii produced by F4 were recovered under “purified” conditions until they developed to maturation. When the F0, F2 or F5 developed to maturation, roughly 150 copepods from 15 replicates were collected together

and immediately stored at −80 °C for follow-up proteomic analysis. Namely, the proteomic analysis was repeated in duplicate for each treatment.

2.3. Proteomic analysis

2.3.1. Protein preparation

The proteins were obtained from the copepods based on the trichloroacetic acid (TCA)/acetone precipitating method (Görg et al., 1997), which was slightly modified in our earlier study (Wang et al., 2013). In brief, frozen copepods were suspended in 1 mL with 10% ice-cold TCA/acetone (w/v) for 1 h. After centrifuging at 12 000 rpm for 30 min at 4 °C, the deposits were resuspended and subjected for an ultrasonic homogenization in 1.0 mL TCA/acetone (20%, w/v) lysis solution on ice. The supernatants were discarded by centrifuging at 12 000 rpm for 30 min at 4 °C, and the precipitated proteins were rinsed twice in 80% acetone, followed by twice in pre-chilling acetone. The deposited proteins were regained by centrifugation at 12 000 rpm for 30 min at 4 °C each time. Vacuum centrifuging was performed to remove the remnant acetone. The pellets were redissolved in 120 μL buffer (pH 8.0) comprising 8 M urea and 100 mM triethylammonium bicarbonate (TEAB). The supernatants were recovered by centrifugation at 14 000 rpm for 30 min at 10 °C, and subsequently subjected for TMT analysis. The soluble protein contents were calculated by the 2-D Quant commercial kit (GE Healthcare, USA).

2.3.2. TMT labeling and strong cation exchange (SCX) fractionation

For trypsin digestion, each protein solution (100 μg) was reduced by 10 mM dithiothreitol (DTT) at 37 °C for 1 h. The reduced protein was then alkylated by 20 mM iodoacetamide for 45 min in dark room. After diluted by 100 mM TEAB to keep urea concentration less than 2 M, Trypsin Gold (Promega, Madison, WI, USA) was added to digest the proteins at the protein/trypsin ratio (30/1, w/w). The digestion was performed overnight at 37 °C, and terminated by 200 μL of 0.1% trifluoroacetic acid (TFA). The peptide mixture was desalted using Strata X C18 SPE column (Phenomenex) and dried in a SpeedVac.

The peptide mixture was redissolved in 0.5 M TEAB and labeled using the 6-plex TMT kit. Generally speaking, one unit of TMT reagent was redissolved in 24 μL acetonitrile (ACN) to label 100 μg protein for each sample. Samples were labeled with the TMT tags as follows: TMT agent 126 and 127 were for the two biological replicates in the F0 control group, respectively, with 128 and 129 for the two replicates in the F0 treated group; so did the F2 and F5 generations. Overall, the three runs were performed for the F0, F2 and F5 generations, separately. The labeling reaction was conducted for 2 h at room temperature, and quenched by 5% hydroxylamine. Afterwards, all the four samples were pooled together and vacuum-dried in a SpeedVac.

Each sample was then loaded onto the Agilent 300 Extend C_{18} column (5 μm particles, 4.6 mm ID, 250 mm length) for SCX fractionation on high pH reverse-phase HPLC. In brief, the peptides were fractionated into 80 fractions for 80 min under an increased gradient of 2–60% ACN in 10 mM ammonium bicarbonate (pH, 10). After pooling of several fractions, a total of 20 fractions was collected and subsequently vacuum-dried.

2.3.3. LC-MS/MS analysis

The peptide mixtures were redissolved in buffer A (0.1% formic acid in 2% ACN) and immediately separated by an EASY-nLC 1000 UPLC system. Namely, the peptides were fractionated with a 40 min gradient of 6–80% buffer B (0.1% formic acid in 98% ACN) at a fixed flowing rate of 0.28 μL/min on a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific). Afterwards, the peptide eluates were analyzed using a tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo). The MS was processed with a data-dependent mode that automatically switched between MS and MS/MS scans. A survey full scan MS spectra in the Orbitrap (350–1800 *m/z*, 70 000 resolution) was followed by 20 MS/MS scans at NCE 30%. Targeted precursor ions selected for MS/MS were dynamically excluded for 30 s. Fragmented ions were detected in the Orbitrap at a 17 500 resolution. Automatic gain control (AGC) was set as 5×10^4 ions in order to prevent overfilling of the ion trap. Xcalibur software was utilized to acquire all the MS data.

2.3.4. Database searching

Mascot search engine (v.2.3) was processed to search the resulting mass spectra against the NCBI_Tigriopus proteome (1641 sequences) and *T. japonicus* transcriptome (46 369 sequences which are combined from a previous work (Kim et al., 2015) and our unpublished data) database. The cleavage enzyme was defined as trypsin, and two miscleavages were allowable. A mass tolerance of 10 ppm was set for precursor ions, with 0.02 Da for fragmented ions. Carbamidomethylation on cysteine, TMT-6plex (K) and TMT-6plex (*N*-term) were set as fixed modifications, whereas oxidation on methionine was defined as a variable modification. The estimated false discovery rate (FDR) was specified at a maximum of 1% for both peptide and protein identification, and peptide ion score was set > 20.

2.3.5. Bioinformatic analysis

Differentially changed proteins were specified when their normalized fold changes were less than 0.83 (down-regulated) or more than 1.20 (up-regulated), and it was evaluated as a confidence level with 95% according to the pairwise analysis between two experimental replicates (Ren et al., 2013). The proteins were annotated by Gene Ontology (GO) into three groups: cellular component, biological process, and molecular function. Protein domain function was interpreted by InterProScan (http://www.ebi.ac.uk/interpro/). Encyclopedia of Genes and Genomes (KEGG) database was applied for protein pathway annotation. The Database for Annotation, Visualization and Integrated Discovery was utilized to define the enrichments for GO term, protein domain and KEGG pathway. The statistically significant enrichment was determined using Fisher's exact test. Significant enrichments were selected when the Benjamini-Hochberg's p value was adjusted as <0.05. Gene Cluster 3.0 software was operated to construct hierarchical clustering for the differentially changed proteins.

2.4. Biochemical parameter determination

To confirm the proteomic analysis, we specifically detected several biochemical parameters in F0. Namely, the enzyme activities of chymotrypsin-like proteinase and nitric oxide synthase, as well as the contents for reduced glutathione (GSH) and lipid peroxidation (LPO) were examined using the commercial assay kit (Nanjing Jiancheng Bioengineering Institute, China). The detailed procedure was provided in the Supplementary material (Text S1).

3. Results

3.1. Proteome profiles

The analysis for the three runs produced 20 141, 18 920, and 24 958 peptide spectra, and they corresponded to 2827, 2662 and 3373 proteins, respectively (Table S1, Supplementary material). Totally, a merged data from the three runs identified 4581 proteins where 1592 was shared (Fig. 1). Taking the 1.2- and 1.5-fold changes as the criteria, the differentially expressed proteins were quantified

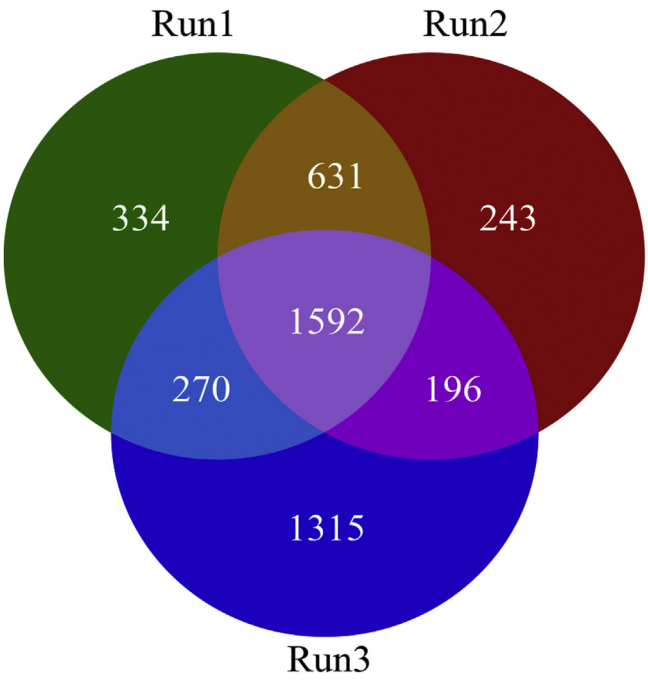

Fig. 1. Venn diagram showing the overlaps of identified proteins of the qualitative comparative proteomics of three *Tigriopus japonicus* proteome sets. One run set represents one generation under two concentration treatments, and two biological replicates were performed for each treatment. Note: run 1 was conducted for F0, run 2 for F2, and run 3 for F5.

for each condition. The numbers of 1.2- and 1.5-fold changed proteins were 195 and 83 for F0, 182 and 57 for F2, 130 and 27 for F5, separately. To maximally explore the potential changes in proteomic profiles between the control and Hg treatment for each generation, we used the 1.2-fold changed proteins in further analysis. For simplicity, throughout the text the terms "induced/up-regulated/overexpressed" and "repressed/down-regulated" are used to describe protein expressions higher or lower than the control, separately. The differentially expressed proteins for F0, F2 and F5 were listed in Tables S2-S7 (Supplementary material). The reproducibility was performed for the two biological replicates per generation using Pearson's correlation analysis (Fig. S1, Supplementary material). Meanwhile, the differentially expressed proteins in F0, F2, and F5 were classified into several groups including metabolic process, single-organism process, cellular process, biological regulation, response to stimulus, and other functions related (Fig. 2).

3.2. Enrichment analysis

To investigate the functional differences of differentially expressed proteins, we performed the enrichment-based clustering analysis for GO, protein domain and KEGG pathway, individually (Figs. 3 and 4, and also the detailed information is provided in Tables S8-S10, Supplementary material).

For F0, only in the cellular component analysis there were some enrichments being attributed to the induced proteins, however all the enrichments were correlated with the repressed proteins in other categories, i.e., molecular function, biological process, protein domain and KEGG pathway. As for cellular component (Fig. 3A), the induced proteins enriched were highly involved into membrane, while the repressed proteins were mainly enriched in ribonucleoprotein complex, ribosome, MCM complex, macromolecular complex, organelle, nucleus, and intracellular part. The molecular function analysis (Fig. 3B) showed that the down-regulated proteins enriched were mostly related to nucleic acid binding, structural constitute of ribosome, DNA binding, heterocyclic compound binding, and lipid transporter activity. In biological process (Fig. 3C), the repressed proteins correlating with cellular biosynthetic process, cellular macromolecule metabolic process, DNA replication, gene expression, translation, lipid transport, and so on were enriched toward Hg toxicity. Also, the protein domain analysis (Fig. 4A) demonstrated that the repressed proteins were primarily enriched into protein domains related to vitellinogen, nucleic acid-binding (OB fold), and mini-chromosome maintenance (DNA-dependent ATPase). In the KEGG pathway category, several pathways exemplified as DNA replication, ribosome, and cell cycle, were enriched in the down-regulated proteins under Hg exposure (Fig. 4B).

In F2, all the enrichments were contributed by the repressed proteins due to Hg toxicity, except that in cellular component nine overexpressed proteins were enriched into intracellular organelle part (Figs. 3 and 4). The cellular component analysis reported that the down-regulated proteins enriched were primarily related to ribosome, and intracellular non-membrane-bounded organelle (Fig. 3A). In molecular function, the proteins involved into structural constitute of cuticle and structural constitute of ribosome were enriched toward Hg treatment (Fig. 3B). In the biological process category, the processes related to macromolecule metabolic process and protein metabolic process were enriched into the repressed proteins due to Hg attack (Fig. 3C). Additionally, the protein domains correlating with peptidase C1A (papain *C*-terminal) and proteinase inhibitor I29 (cathepsin propeptide), as well as the pathway relating to ribosome were enriched in the repressed proteins (Fig. 4).

It should be underlined that a quite different proteomic response was observed in the recovery generation of F5 when compared with F0 and F2 (Figs. 3 and 4). Namely, contrary to F0 and F2, most of the enrichments for F5 were correlated with the overexpressed proteins caused by the pre-exposure to Hg contamination, highlighting that the recovery machinery was effectively initiated in this generation. For cellular component, only two repressed proteins were enriched in cytochrome complex or respiratory chain, while many induced proteins enriched were primarily involved into intracellular, cell part, protein complex, cytoskeleton, intracellular organelle part, myosin complex, and so on (Fig. 3A). In molecular function, the six repressed proteins enriched were involved into serine-type peptidase (or hydrolase) activity, and the other enrichments were attributed to the overexpressed proteins which play roles in intramolecular oxidoreductase activity, hydrolase activity, pyrophosphatase activity, and motor activity (Fig. 3B). As for biological process, all the enrichments were related to the induced proteins, participating in carbohydrate metabolic process and glucose metabolic process (Fig. 3C). The protein domain analysis showed that the overexpressed proteins enriched were involved into myosin and alpha crystallin/Hsp20 domain, while the repressed proteins were principally enriched into trypsin-like cysteine/serine peptidase domain and epidermal growth factor-like domain (Fig. 4A). In KEGG pathway, all the enrichments were ascribed to the up-regulated proteins, which were mainly concerned with glycolysis/gluconeogenesis, microbial metabolism in diverse environments, and biosynthesis of antibiotics (Fig. 4B).

3.3. Biochemical parameters

By contrast to the control, Hg treatment led to a striking increase in the expressions of chymotrypsin-like proteinase (1.87 fold) and nitric oxide synthase (1.50 fold) in F0 (Fig. S2, Supplementary

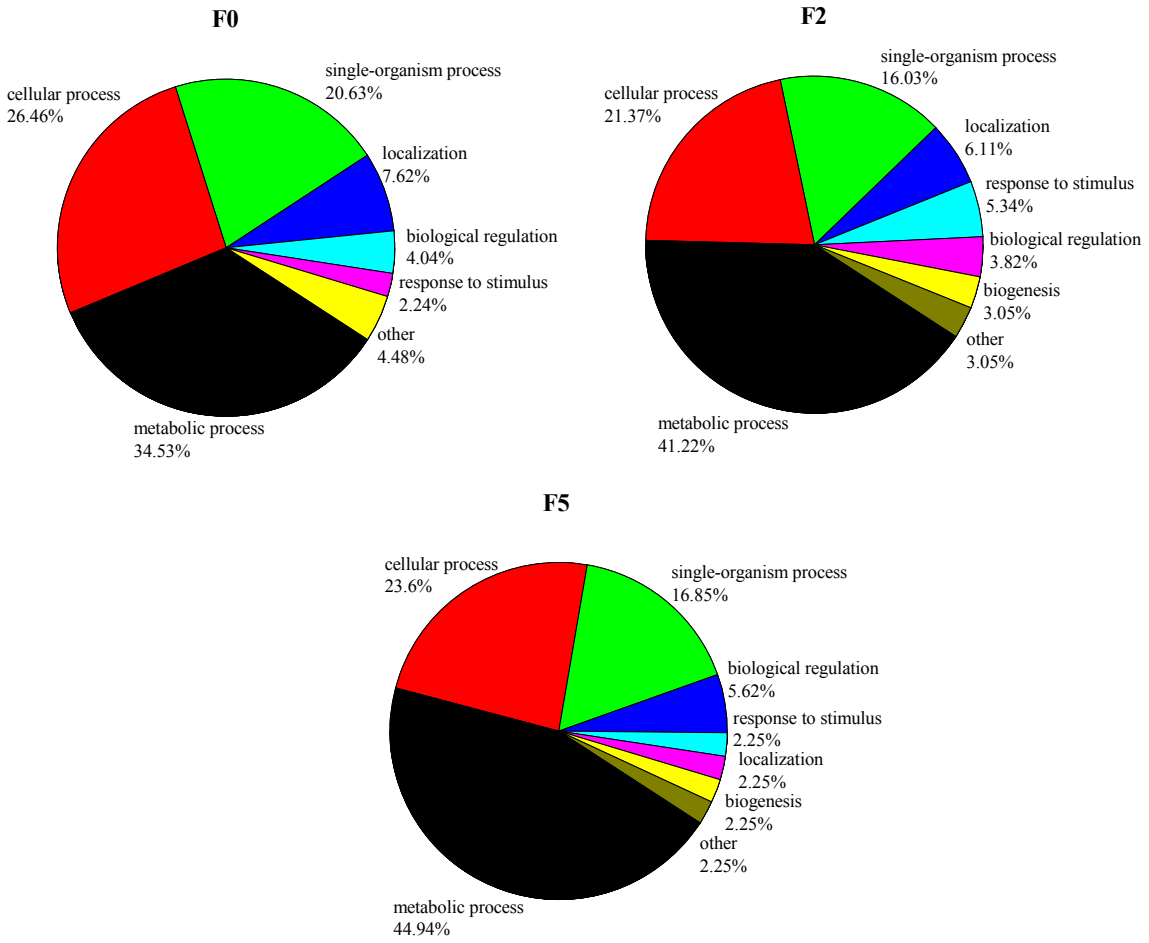

Fig. 2. Functional classifications for differentially expressed proteins in the copepod *Tigriopus japonicus* (i.e., F0, F2, and F5) under five generations of exposure to the control and 50 μg/L Hg treatment, which was followed by one generation recovery in clean seawater.

material). The enzyme activities were induced by 2.04 and 1.32 times, severally, for chymotrypsin-like proteinase and nitric oxide synthase (Fig. S2, Supplementary material), hence confirming the proteomic analysis. In addition, Hg treatment prohibited the GSH content from 55.88 ± 1.00 to 15.70 ± 3.65 μmol/g protein (i.e., decrease to 0.28 fold), and concomitantly enhanced the LPO level by 2.25 times (Fig. S3, Supplementary material). Thus, Hg could cause depletion of endogenous SH groups (e.g., GSH) and subsequently induce several toxic events including oxidative stress, which partially provides a mechanistic explanation to Hg multigenerational toxicity (Fig. 5).

4. Discussion

In recent years, lots of efforts have been poured on examining the effects of heavy metals including Hg on aquatic animals under the multigenerational exposure, nonetheless, very little information is understood about the action mechanism for metal multigenerational toxicity, as well as metal tolerance, in aquatic biota (Contreras et al., 2012; Li et al., 2015; Sun et al., 2014; Tsui and Wang, 2005; Vidal and Horne, 2003). As far as we know, this is the first report using TMT-based proteomic technology to provide a mechanistic explanation into the acclimatory strategy (i.e., phenotypic plasticity) acquired by marine copepod during counteracting Hg multigenerational toxicity in our previous study (Li et al., 2015). Enrichment analysis demonstrated that the proteomic responses for F0 and F2 were clearly different from that for F5. In other words, the vast majority of enrichments were concerned with the down-regulated proteins in F0 and F2, however the enrichments in F5 were mostly attributable to the up-regulated proteins, suggesting that different underlying mechanisms are responsible for Hg toxicity and tolerance (i.e., phenotypic plasticity) in this copepod, separately, during the long term exposure.

4.1. Proteins involved into Hg multigenerational toxicity

Hg displays a highly binding potential for the sulphydryls in cellular biomolecules (e.g., proteins and enzymes), and can subsequently disrupt the cells with thiol-containing proteins/enzymes (Perottoni et al., 2004), consequently disturbing cellular processes/pathways at the global scale, which is exemplified by our proteomic work. Our study clearly indicated that, in F0 and F2, most of the enrichments was related to the repressed proteins caused by Hg treatment, and this is the response of Hg multigenerational toxicity which suppressed many crucial processes/pathways in the copepod, e.g., ribosome, protein translation, macromolecule metabolic process, gene expression, DNA replication, cell cycle, cuticle organization, vitellogenesis, and so on. Meanwhile, we noticed that there was a different response between the F0 and F2 proteomes against Hg exposure. That is to say that more processes/pathways were significantly down-regulated by Hg treatment in F0 when compared with F2, indicating that phenotypic plasticity

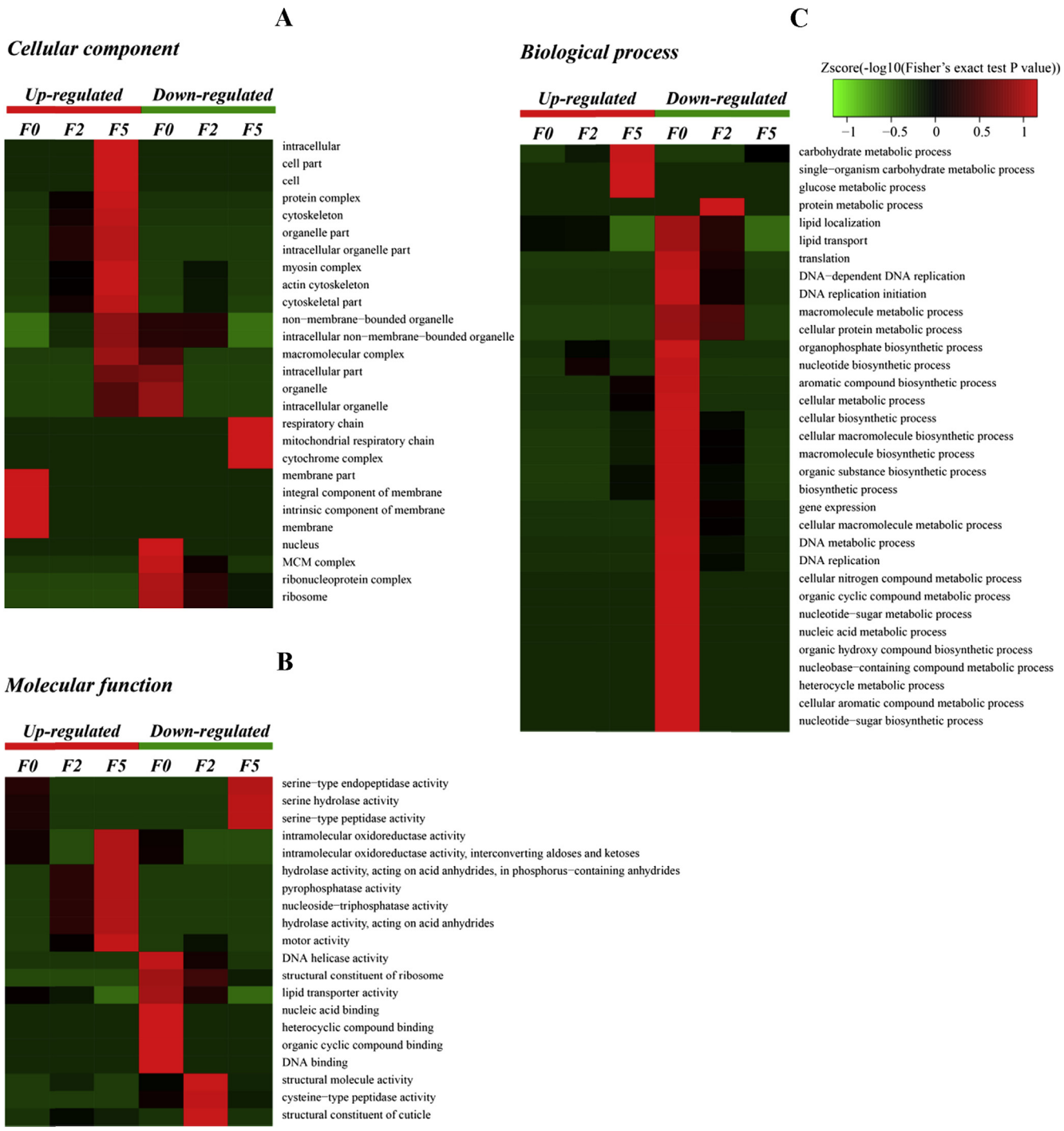

Fig. 3. Hierarchical clustering of the differentially expressed proteins in each generation (F0, F2 and F5) was performed according to Gene Ontology based enrichment: (A) cellular component, (B) molecular function, and (C) biological process. In each GO term, the differentially expressed proteins in response to Hg toxicity were divided into two groups (i.e., up-regulated and down-regulated) for each generation. An enrichment analysis was conducted using the Fisher's exact test with Benjamini-Hochberg adjustment. The *p* values were transformed into Zscores prior to hierarchical clustering analysis. The color index (Zscore) is showed in the legend, and the red color represents the significantly enriched terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

might have been initiated to cope with Hg multigenerational toxicity as early as in the third generation (i.e., F2).

Enrichment analysis via several categories (i.e., cellular component, biological process, molecular function and KEGG pathway) constituently showed that ribosome was enriched by 11 down-regulated proteins in F0 and 8 proteins in F2. Likewise, protein translation (13 proteins) was significantly enriched by the repressed proteins in F0 under Hg treatment. All the down-regulated proteins are the ribosomal subunits (e.g., 40S and 60S subunits) and translated complexes (e.g., threonyl-tRNA synthetase, and eukaryotic translation initiator factor 5A). Therefore, Hg toxicity can target ribosome and concomitantly suppress global translation in the cells, and it is basically in line with other reports (Li et al., 2016; Pytharopoulou et al., 2013). For example, a recent

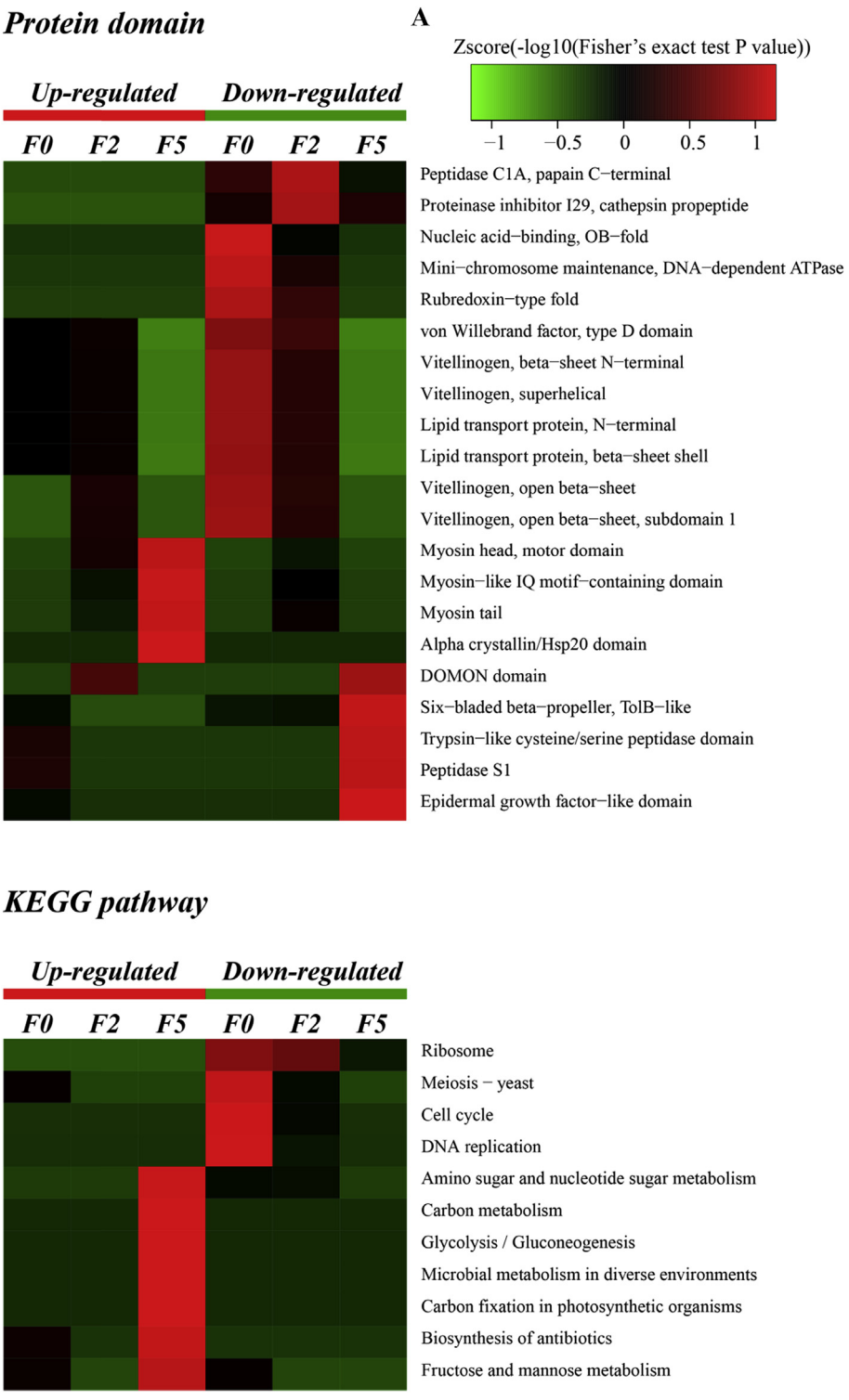

Fig. 4. Hierarchical clustering of the differentially expressed proteins in each generation (F0, F2 and F5) was performed according to KEGG pathway (A) and protein domain (B) based enrichment. In each category, the differentially expressed proteins in response to Hg toxicity were divided into two groups (i.e., up-regulated and down-regulated) for each generation. An enrichment analysis was conducted using the Fisher's exact test with Benjamini-Hochberg adjustment. The *p* values were transformed into Zscores prior to hierarchical clustering analysis. The color index (Zscore) is showed in the legend, and the red color represents the significantly enriched domains/pathways. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metalloproteomic study has been performed to detect Hg-binding proteins, some of which are involved into protein translation, e.g., putative ribosomal protein S15, ribosomal protein S10, and eukaryotic translation initiation factor 5A (Li et al., 2016). Similarly, inorganic Hg can impair each stage for protein synthesis, with particularly exerting severely prohibitive impacts on the formation of the initiation 43S ribosomal complex in the mussel digestive glands (Pytharopoulou et al., 2013). Owing to a global inhibition in

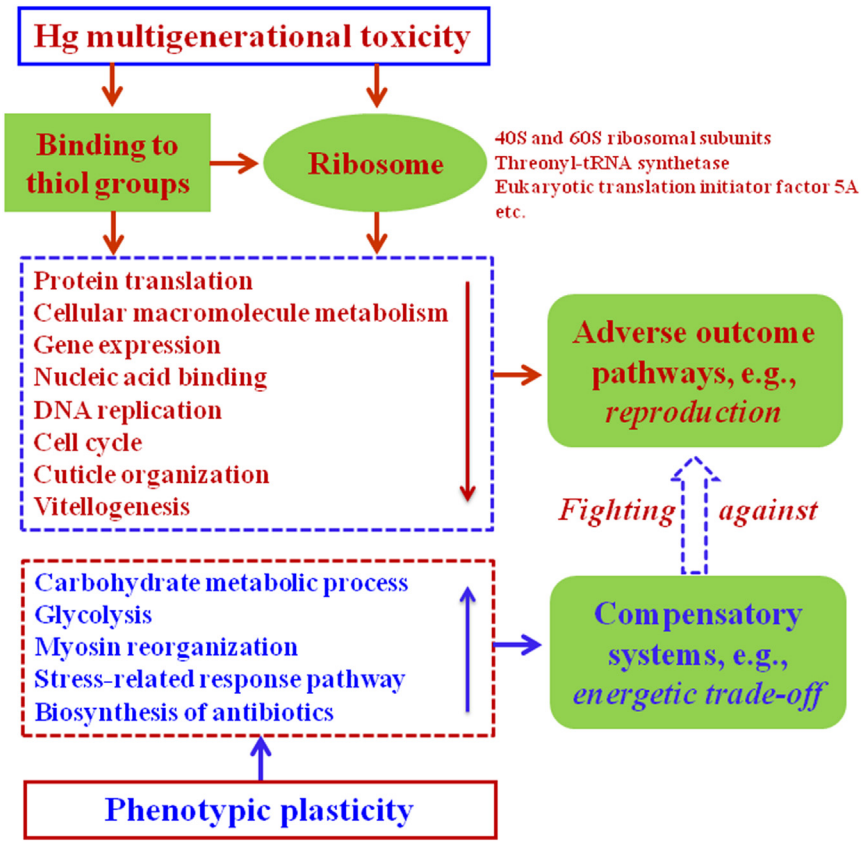

Fig. 5. Proteomics provides a putative mechanism into phenotypic plasticity obtained by *Tigriopus japonicus* against Hg multigenerational toxicity under the long term exposure. Hg toxicity caused depletion of thiol groups and/or targeted ribosome in the cells, hence prohibiting many critical processes including protein translation, macromolecule metabolism, gene expression, DNA replication, cell cycle, cuticle organization, vitellogenesis, and so on. These biochemical events eventually produced adverse outcome pathways, and herein the copepod's reproduction was restrained by Hg multigenerational exposure. Nevertheless, to counter Hg multigenerational toxicity, the copepod might have initiated several effective compensatory systems, e.g., increased carbohydrate metabolism, myosin reorganization, enhanced stress-related defense pathway. Specifically, glycolysis was enhanced to facilitate energetic allocation for metal detoxification and tolerance in the copepod, which is elegantly considered as energetic trade-off. Consequently, Hg accumulation exerted a negligible impact on development and reproduction of the recovery generation (F5). Note: red arrow indicates the repressed processes by Hg toxicity, and blue arrow for the overexpressed processes initiated by phenotypic plasticity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein translation produced by metal attack, it is not surprising that Hg could massively prohibit macromolecule metabolic process (37 and 27 proteins for F0 and F2, respectively), and therein, most of them participated in protein metabolic process (i.e., 26 proteins for F0, and 23 for F2). Meanwhile, the depressed protein metabolism by Hg toxicity mainly includes translation (e.g., ribosomal protein subunits), followed by proteolysis (e.g., cathepsin) and protein folding (e.g., chaperonin containing TCP1). Actually, even in the recovery generation of F5, protein metabolism (e.g., proteolysis) was significantly suppressed by the pre-multigenerational Hg exposure, since six down-regulated proteins were enriched into serine-type endopeptidase activity. Similar effects on protein metabolism have also been shown in a previous proteomic work with somatosensory cortex of rat under methylmercury treatment (Kong et al., 2013), and there might some comparability between inorganic and organic Hg toxicity. To summarize, our proteomic analysis definitely evidences a high sensitivity of cellular metabolism (especially protein metabolic process) to Hg toxicity, and it might partly be attributable to this metal displaying a great affinity to the SH groups (e.g., depletion of GSH caused by Hg attack in this study) and thereby inhibiting the function of various proteins with thiols at a large scale.

In F0, many repressed proteins due to Hg attack were enriched in nucleus (8 proteins), nucleic acid binding (24 proteins), nucleic acid metabolic process (12 proteins), gene expression (18 proteins), DNA replication (6 proteins), and cell cycle (5 proteins). Specifically, six down-regulated proteins where five takes an important role in cell cycle, were concerned with DNA replication, that is, ribonucleoside-diphosphate reductase, proliferating cell nuclear antigen, putative replication factor-a protein 1, minichromosome maintenance 3, mcm4 protein, and putative DNA replication licensing factor mcm4 component (Fragment). Ribonucleoside-diphosphate reductase can catalyze the production of deoxyribonucleotides, and they are prepared for DNA synthesis (Elledge et al., 1992), thereby displaying a crucial part in DNA replication. Proliferating cell nuclear antigen acts as a DNA clamp which facilitates the processivity for DNA polymerase delta in eukaryotic cells and thus takes an essential part in DNA replication and cell cycle regulation (Moldovan et al., 2007). Putative replication factor-a protein 1 can bind to single-stranded DNA in eukaryotic cells, hence participating into DNA replication, repair, and recombination (Wold, 1997). The other three proteins belong to minichromosome maintenance protein complex that is a eukaryotic DNA helicase complex needed for DNA replication. As a result, Hg toxicity induced DNA damage and cell cycle arrest in the copepod partially through inhibiting the activity of DNA synthetic machinery. Several previous reports show that Hg treatment can inhibit cellular DNA replication and cell cycle machinery in *vitro* or

vivo tests (Ferreira et al., 2015; Ponce et al., 1994; Sekowski et al., 1997). In summary, this work showed that Hg toxicity could affect the nucleus and at least prohibit nucleic acid metabolism (e.g., nucleic acid binding, DNA replication, and cell cycle) on a large scale, thus providing a mechanistic insight into Hg genotoxicity to the cells at the proteomic level.

Interestingly, protein domain enrichment analysis reported that three repressed proteins in F0 were related to vitellinogen, superhelical (beta-sheet *N*-terminal). Vitellinogen precursors afford the major egg yolk proteins as nutrient sources for early development in oviparous vertebrates and invertebrates. Vitellogenin synthesis is also inhibited in catfish under the exposure to inorganic and organic Hg (Kirubagaran and Joy, 1995). Moreover, Hook and Fisher (2002) speculate that Hg and other metals could suppress egg reproduction via binding to enzymes involved into vitellogenesis (Hook and Fisher, 2002). Overall, our proteomic work illustrated that Hg multigenerational toxicity might restrain vitellogenesis by repressing vitellogenin expression, and it could result in suppressed reproductive performance, that is, decreased fecundity in the copepod *T. japonicus* under multigenerational exposure to this metal in our earlier study (Li et al., 2015).

For copepods, one remarkable feature is the cuticular covering, i.e., chitinous integument, of the whole body, and it can be divided into the extracellular cuticle and the cellular epidermis. Cuticles are highly organized structure composed of chitin filaments, and they are embedded in a layered, extracellular secretion from the underlying epidermis (Andersen et al., 1995). In our study, many down-regulated proteins in F2 were enriched into structural constitute of cuticle (10 proteins), and all of them are cuticular proteins such as cuticle protein 5a, cuticle protein precursor, BCS-1 protein, pro-resilin and so on. In case of *Tigriopus*, cuticle proteins are likewise repressed by metal exposure (e.g., copper and manganese) in earlier studies (Ki et al., 2009; Kim et al., 2013). In fact, a recent report demonstrates that, besides the digestive epithelium cells, the cuticular covering is regarded as the main sites for metal depositing in the copepod *Tigriopus brevicornis* after the exposure against metals including Hg (Barka, 2007), hence making the copepod cuticle as an important target for Hg attack. In addition, three proteins relating to chitin metabolism were remarkably depressed in F2 under Hg treatment. Therefore, Hg toxicity could strongly down-regulate the expression of cuticular proteins as well as chitin modifying enzymes, with concomitantly damaging cuticle development and formation.

4.2. Proteins involved into phenotypic plasticity

According to our previous study that Hg accumulation for F5 (i.e., the recovery period) increased with the increasing Hg concentrations utilized in F0-F4 (Li et al., 2015), it is conceivable that some cellular processes could be prohibited in this generation by Hg toxicity. For instance, two repressed proteins concerned with respiratory chain are cytochrome *b-c*1 complex subunit 7, and AGAP000851-PA (showing cytochrome-c oxidase activity), indicating that Hg might disrupt respiratory metabolism. Alternatively, considering that electronic transport chains are major sites for reactive oxygen species (ROS) yielding, it is speculated that their inhibition would be the response to depress ROS production and its subsequent oxidative stress, however, with concurrently decreasing energy generation. It is widely reported that one of the mechanisms for metal resistance by aquatic animals is attributed to the increased energetic demand (i.e., energetic trade-offs) in order to facilitate metal detoxification and tolerance, although it has been suggested to be a compensatory response (Uren Webster et al., 2013). Correspondingly, the copepod had probably provided more energetic budget for Hg detoxification and tolerance via enhanced carbohydrate metabolism (the primary source of energy supply), where eight up-regulated proteins were enriched in the recovery generation of F5, and these proteins are described as follows: hexokinase (HK), glucose-6-phosphate isomerase (GPI), fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), D-lactate dehydrogenase (LDH), malate dehydrogenase, glucosamine-6-phosphate isomerase, and poly [ADP-ribose] polymerase 1. Particularly, the first four proteins among them are taking part in glycolysis where HK (the first rate-limiting enzyme) irreversibly catalyzes glucose phosphorylation as the entrance for this pathway. Their overexpression suggests an increased glucose breakdown in the copepod through the glycolytic pathway leading to pyruvate. The pyruvate yielded can subsequently supply energy to cells through the citric acid cycle when oxygen is present, or ferment to produce lactate under anaerobic environments. The lactate pathway is supported by our work that the up-regulated LDH, catalyzing pyruvate into lactate, was probably employed to keep the high rate of glycolysis in *T. japonicus*. In addition, glycolysis is generally regarded as the main pathway to produce energy for crustaceans (Chang and O'Connor, 1983). As a consequence, the copepod might have augmented an oxygen-independent pathway, that is, glycolysis, to facilitate energy allocation for metal detoxification and tolerance, with synchronously decreasing ROS production.

Our proteomic analysis showed that several up-regulated proteins were involved into myosin complex (6 proteins), myosin tail (5 proteins), and myosin head, motor domain (5 proteins), and they are listed as myosin-7, myosin heavy chain isoform 1, myosin heavy chain, myosin heavy chain type b, myosin-9, putative, and myosin heavy chain, nonmuscle or smooth muscle. Myosins constitute a large multigene family that shows actin-based molecular motor activity, and can translocate along microfilaments with an ATP-dependent manner in eukaryotes. Family members are broadly synthesized in the cells and tightly integrated in the biological pathway networks, consequently acting as functional integrators between signaling and the dynamics of cytoskeletal mechanics (Heissler and Sellers, 2016). Several previous studies show that $HgCl_2$ treatment strikingly decreases the myosin ATPase activity in the contractile machinery, however the inhibition can be reversed by cysteine, DTT, or GSH (Moreira et al., 2003; Vassallo et al., 1999). Hypothetically, the thiol groups might be the toxic sites for this metal on myosins, primarily due to several sulphydryls found in their molecules (Flink et al., 1978). Particularly, a recent metallomics study demonstrates that skeletal muscle myosin heavy chain can definitely be detected in the Hg-containing component, thereby rendering this protein to be a potential binding partner of methymercury in fish muscle tissue (Kutscher et al., 2012). Taken together, the overexpression of several myosin proteins in the recovery generation (F5) might be an adaptive response to dysregulation of the actin cytoskeleton in the copepod caused by Hg attack. Alternatively, their up-regulation seems to be a compensatory reaction against Hg toxicity in the copepod's reproduction, since myosins are implicated in spermatogenesis, being essential to sexual development (Li and Yang, 2016). Nonetheless, it would be worthwhile to continuingly examine whether this protein family plays an essential role in Hg tolerance obtained by the copepod against the long term metal exposure.

Special attention was directly concentrated on that two induced proteins, that is, protein lethal(2) essential for life-like and heat shock protein beta-1, and they were enriched into alpha crystallin/hsp20 domain. These two proteins belong to the small heat shock protein (HSP20) family (Kurzik-Dumke and Lohmann, 1995). Heat shock proteins take a critical part in remaining protein homeostasis for the organisms when challenged with sub-lethal stresses (Hofmann et al., 2002). Furthermore, these proteins display

molecular chaperoning especially when protecting other proteins against denaturation and aggregation caused by environmental stressors (Feder and Hofmann, 1999). Several previous studies report that the induction of this protein family is concerned with defense/tolerance response obtained by marine copepods against environmental stressors including heavy metals (Barreto et al., 2015; Ki et al., 2009; Rhee et al., 2009; Won et al., 2015). Particularly, via RNA interference, knock-down of heat shock protein beta-1 expression in *Tigriopus californicus* (a sister species of *T. japonicus* used in this work) leads to clear and dramatic decrease in thermotolerance of this population (Barreto et al., 2015), which partially supports a crucial role of this protein in Hg tolerance in our study. Overall, the overexpression in small heat shock proteins is regarded as an importantly protective strategy for the copepod to counter the long term Hg exposure. Nevertheless, the exact role of small heat shock proteins in Hg tolerance for marine copepods could be validated via RNA interference in our further study. Additionally, we noted that five up-regulated proteins were enriched into biosynthesis of antibiotics, and this increased anti-microbial defense seemed to be correlated with Hg tolerance in the copepod.

5. Conclusions

Our proteomic study clearly demonstrated that Hg multigenerational toxicity could prohibit many critical cellular processes/pathways in *T. japonicus*, e.g., ribosome, protein translation, gene expression, DNA replication, macromolecule metabolic process, cell cycle, cuticle organization, vitellogenesis, and so on, and these biochemical toxic events could successively exert adverse effects at higher levels of organization, that is, the decreased fecundity in this copepod as one of adverse outcome pathways in our earlier study (Li et al., 2015). However, phenotypic plasticity has been used by this copepod to fight against Hg multigenerational toxicity, and it can be explained by our proteomic results. Namely, in the recovery generation (F5), many up-regulated proteins were involved into compensatory mechanisms, such as carbohydrate metabolism, myosin reorganizations, and stress-related defense pathway. Especially, the copepod was hypothesized to enhance an oxygen-independent pathway, i.e., glycolysis, to increase energy budgets for metal detoxification and tolerance, with concurrently reducing ROS production. Meanwhile, it should be underlined that Hg tolerance obtained by *T. japonicus* would rather result from simultaneous cooperation of various processes than from a specific mechanism. In summary, our proteomic study elegantly provides a putative mechanism into phenotypic plasticity used by *T. japonicus* to counteract Hg multigenerational toxicity (i.e., cumulative effects) under the long term exposure (Fig. 5).

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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.envpol.2016.08.087.

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