Aquatic Toxicology 187 (2017) 124–131

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Different transcriptomic responses of two marine copepods, *Tigriopus japonicus* and *Pseudodiaptomus annandalei*, to a low dose of mercury chloride ($HgCl_2$)

Minghua Wang[a], Chang-Bum Jeong[b], Yan Li[a], Jae-Seong Lee[b,*]

[a] Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems/College of the Environment & Ecology, Xiamen University, Xiamen 361102, China
[b] Department of Biological Science, College of Science, Sungkyunkwan University, Suwon 16419, South Korea

ARTICLE INFO

Keywords:
Mercury pollution
Response mechanism
Species-specificity
Transcriptome profiling

ABSTRACT

Mercury (Hg) pollution is a ubiquitous and serious concern in marine environments, but the response mechanisms of marine animals to Hg pollution (i.e., toxicity/tolerance) are poorly understood. To compare the global responses of two marine copepods (*Tigriopus japonicus* and *Pseudodiaptomus annandalei*), we analyzed whole transcriptomes using RNA-seq technology in response to Hg treatment (a nominal 10 μg/L $HgCl_2$ in seawater) for 5 h. Hg was strikingly accumulated in both copepods under treatment. The Hg concentration in *P. annandalei* was higher under metal exposure by approximately 1.4-fold compared with treated *T. japonicus*. Among transcriptomic data, 101 genes in *T. japonicus* and 18 genes in *P. annandalei* were differentially regulated in response to Hg exposure. The up-regulated genes in *T. japonicus* were concerned with stress, growth, and development, while the down-regulated ones were mainly related to immune response. In *P. annandalei*, most of the differentially expressed genes were up-regulated, and all were involved in stress response. Our work indicated that Hg exhibits endocrine-disrupting potential at the transcriptomic level in marine copepods. Overall, our study demonstrates the species-specific molecular responses of these two copepods to Hg pollution.

1. Introduction

Mercury (Hg) is a persistently toxic substance and has been recognized as the most hazardous metal in aquatic organisms (Cargnelutti et al., 2006). China is the greatest source of atmospheric Hg emission in the world (Jiang et al., 2006), and atmospheric Hg has progressively been deposited into coastal and marine environments, resulting in concerning levels of Hg pollution in China and surrounding areas. For example, the maximal concentration of total Hg in seawater reached 2.7 μg/L in the Bohai Sea (Wang et al., 2009).

Hg has a strong affinity for binding thiol groups in enzymes and proteins; this causes toxicities in living creatures including marine organisms (Clarkson and Magos, 2006; Xu et al., 2016). Our understanding of the response mechanisms to Hg contamination of marine organisms (i.e., toxicity/tolerance) are very important, as this can provide novel mechanistic insight into the strategies adopted by biota to cope with the adverse effects of Hg. In this regard, many researchers have sought to investigate the adverse effects of Hg (Mondal et al., 1997; Zhu et al., 2000; Drevnick and Sandheinrich, 2003; Iavicoli et al., 2009). For example, in the fathead minnow *Pimephales promelas*, dietary exposure to methylmercury (MeHg) retarded reproduction and inhibited gonadal development in females, suggesting its role as an endocrine disruptor (Drevnick and Sandheinrich, 2003). Inorganic mercury ($HgCl_2$) exposure induced a high rate of progesterone synthesis with a low rate of conversion to 17β-estradiol in oocytes of the freshwater catfish *Channa punctatus* (Mondal et al., 1997). Recently, in the copepod *T. japonicus*, MeHg exposure induced oxidative stress with developmental and reproductive effects (Lee et al., 2017). Thus, to date, only a few studies have been conducted on Hg toxicity and related defense mechanisms in aquatic invertebrates, despite their importance in the marine ecosystem.

Copepods are the most abundant taxa of marine invertebrates, and they play a critical role in the cycle of substances including toxic pollutants due to its crucial niche in marine ecosystem. In this respect, copepods could be responsible for bioaccumulation and/or biomagnification of environmental pollutants due to their prey-predator relationship in the aquatic food chain. Despite its critical function in the aquatic ecosystem, little is known about the molecular response mechanisms of copepods to Hg pollution. Recently, increasing exploration of genome and transcriptome sequences of copepods has indicated their important role as markers of environmental stressors in marine ecosystems (Hansen et al., 2010; Bron et al., 2011; Jiang et al., 2013; Roncalli

* Corresponding author.
E-mail address: jslee2@skku.edu (J.-S. Lee).

http://dx.doi.org/10.1016/j.aquatox.2017.03.018
Received 22 February 2017; Received in revised form 21 March 2017; Accepted 22 March 2017
Available online 24 March 2017
0166-445X/

et al., 2016). Although sequencing techniques have been applied to profile the transcriptomes of many organisms, relatively few studies have focused on marine copepods (James et al., 1997; Christie et al., 2013; Lenz et al., 2014; Kim et al., 2015). For example, the whole transcriptome of the intertidal benthic copepod *Tigriopus japonicus* (South Korea) was sequenced using next-generation sequencing technology (Kim et al., 2015) with a total of 16,513 unigenes, while no RNA-seq information is available for the marine copepod *Pseudodiaptomus annandalei*.

T. japonicus (Copepoda, Harpacticoid) is an intertidal species with a wide geographic distribution (Raisuddin et al., 2007) and has been regarded as a good model species in marine stress ecology due to the ease of culture, rapid life cycle (~2 weeks), and distinct sex morphology (Raisuddin et al., 2007; Xu et al., 2016). *P. annandalei* (Copepoda, Calanoida) is a widespread and abundant species in coastal, estuarine, and brackish waters in the tropical and subtropical Indo-Pacific (Ramachandran and Swaminathan, 2012; Dhanker et al., 2013). Also, it serves as a common prey item for fish larvae in nature and aquaculture (Chen et al., 2006; Dhanker et al., 2012), and as a good model in marine ecotoxicology (Jiang et al., 2013).

In our study, we exposed copepods (*T. japonicus* and *P. annandalei*) to 10 μg/L $HgCl_2$ for 5 h. To identify differentially expressed genes (DEGs) under Hg exposure, RNA-seq was performed to obtain the whole transcriptomes of these two copepods. In addition, functional enrichment analysis was processed to investigate critical processes/pathways in response to Hg treatment. Overall, this study seeks to unveil and compare the molecular response mechanisms of *T. japonicus* and *P. annandalei* upon exposure to Hg pollution.

2. Materials and methods

2.1. Copepod maintenance

Copepods *T. japonicus* and *P. annandalei* were obtained from rocky intertidal zone pools in Xiamen Bay (24°25′73″N, 118°6′34″E) and coastal waters of Dongshan county (23°35′35″N, 117°19′49″E) (The People's Republic of China), respectively. The copepods were maintained at a temperature of 22 °C under a 12/12-h light/dark cycle, and an equal mixture of three algae (*Isochrysis galbana*, *Platymonas subcordiformis*, and *Thalassiosira pseudonana*) at a density of 8×10^5 cells/L was supplied as their prey. The seawater used in the exposure experiment was collected from 20 km offshore in Xiamen Bay, and was filtered through a 0.22-μm polycarbonate membrane before utilization. The background level for total Hg in the seawater was 3–4 ng/L. Other seawater characteristics were depicted as follows: salinity, 27–28 practical salinity units (PSU); dissolved oxygen, 6.2–6.7 mg/L; and pH, 8.0–8.1.

2.2. Acute exposure

10 μg/L $HgCl_2$ [36.83 nM] was chosen for both copepods in the experiment, since our objective was mainly focused on investigating the molecular mechanism concerning an early response in response to a low dose $HgCl_2$ exposure. A static 48 h acute toxicity testing was performed for both copepods with the survival as the endpoint. In each toxicity testing, there were seven nominal Hg concentrations (i.e., 0.3–0.9 mg/L for *T. japonicus* and 0.10–0.34 mg/L for *P. annandalei*) with a negative control. 20 individuals were used for each replicate of the concentration treatment in triplicates. Both concentrations of no observed effect concentration (NOEC) (300 μg/L $HgCl_2$ for *T. japonicus* and 100 μg/L $HgCl_2$ for *P. annandalei*) and half lethal concentration (LC50) (394 μg/L $HgCl_2$ for *T. japonicus* and 186 μg/L $HgCl_2$ for *P. annandalei*) were considered to choose experimental dose (10 μg/L $HgCl_2$). $HgCl_2$ (Sigma-Aldrich, 99.5%; St. Louis, MO, USA) was added to the seawater to achieve a nominal concentration of 10 μg/L Hg^{2+} for metal treatment. The acute exposure experiment was conducted in an incubator at 22 °C with a 12/12-h light/dark cycle. Prior to exposure, both copepods were acclimated in a glass tank for two days. For the exposure, 150 adult copepods (*T. japonicus* or *P. annandalei*) per treatment were randomly transferred into 500-mL polycarbonate bottles with a 400-mL working solution, and they were exposed to Hg. The exposed groups were maintained for 5 h without feeding, and each treatment was performed with four biological replicates. No mortality was observed in any of the exposed groups.

2.3. Hg accumulation analysis

Approximately 100 adult copepods were used for Hg accumulation analysis and the accumulated Hg concentration was compared between copepod species and with the control group. The experiments were performed with four biological replicates. The copepods were digested in a water bath (95 °C) using concentrated HNO_3 and HCl (1:3, v/v). Hg content in the digested samples was measured using a DMA-80 direct mercury analyzer (Milestone; Sorisole, Italy; referred to EPA Method 7473). The detection limit for Hg analysis was 0.2 ng/g. The Hg standard solutions were measured in each batch of samples with the recovery rates of 85–110% (Zhu et al., 2015). Hg levels in the copepods were described as ng/g dry weight (DW). In addition, to determine the bioaccumulation factors (BCFs) of Hg in both copepods, T-Hg contents in the animals were divided by the nominal metal concentration in the seawater.

2.4. RNA extraction, gene library preparation, and sequencing

Approximately 100 copepods were homogenized in five volumes of TRIZOL® reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) by using a tissue homogenizer and stored at −80 °C until RNA extraction. Total RNA was isolated according to the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels, and its purity was checked using the NanoPhotometer spectrophotometer (IMPLEN; Westlake Village, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies; Carlsbad, CA, USA), and its integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies; Foster City, CA, USA). Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England BioLabs; Ipswich, MA, USA) according to the manufacturer's protocol. In brief, poly-T oligo-attached magnetic beads were applied to purify mRNA from total RNA. Fragmentation was conducted using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer. First-strand cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently carried out using DNA Polymerase I and RNase H. After conversion of the remaining overhangs into blunt ends and ligation of adapters, cDNA fragments preferentially 150–200 bp in length were selected to be enriched in a 10-cycle PCR reaction. PCR products were purified (AMPure XP system, Beckman Coulter; Beverly, MA, USA) and library quality was assessed using the Agilent high-sensitivity DNA assay on the Agilent Bioanalyzer 2100 system (Santa Clara, CA, USA). One individual cDNA library was constructed for each sample, i.e., four libraries per copepod species. Afterwards, the library preparations were sequenced on Illumina HiseqTM 2500 (San Diego, CA, USA), and 100-bp paired-end reads were produced.

2.5. Quality control and de novo assembly

Prior to assembly, raw reads in FASTQ format were processed using in-house Perl scripts. Clean reads were obtained after removing reads with an adapter, reads with poly-N (> 5%) and low-quality raw reads. At the same time, the Q20, GC content and sequence duplication level of the clean data were calculated. Transcriptome assembly was

accomplished based on all of the clean reads using a Trinity *de novo* assembler with min_kmer_cov set to 2 by default and all other parameters were set to the default values (Grabherr et al., 2011).

2.6. Gene function annotation

Gene function was annotated against the following databases (the NCBI non-redundant protein sequences [NR] database, the NCBI non-redundant nucleotide sequences [NT] database, Protein family [Pfam], eukaryotic clusters of Orthologous Groups [KOG], the Swiss-Prot database, Kyoto Encyclopedia of Genes and Genomes [KEGG], and Gene Ontology [GO]) using BLAST (v2.2.26 + x64-linux) with a threshold e-value ≤ $1e^{-5}$. The analysis for DEGs between the control and 10 μg/L Hg exposure was performed using the DEGseq (2010) R package (Wang et al., 2010). The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR) (Benjamini and Hochberg, 1995). A fold-change ≥ 2 and a FDR < 0.05 were set as the threshold for the DEGs caused by Hg treatment. Moreover, corrected *P* value < 0.1 was selected as the threshold for significant enrichment (Nätt et al., 2011).

2.7. Validation of the DEGs by quantitative real-time PCR (qRT-PCR)

To validate the RNA-seq data, qRT-PCR was performed for several DEGs in the copepods. Approximately 100 adult copepods were randomly designated to the control and Hg exposure groups. After the exposure, copepods were collected and total RNA was extracted for each sample as described above, and they were subsequently used for cDNA synthesis. The FastQuant cDNA RT Kit with gDNase (TIANGEN, Beijing, China) was used to synthesize first-strand cDNA. qRT-PCR was performed on an ABI 7500 System (Applied Biosystems; Foster City, CA, USA) using a SuperReal PreMix Plus (SYBR Green) Kit (TIANGEN; Beijing, China). Thermocycling was processed as the followings: 95 °C/min, 55 °C/min, and 40 cycles beginning at 55 °C/10 s with a 0.5 °C increase per cycle. qRT-PCR analysis was performed for cuticle protein 7, heat shock protein 20 (*HSP20*), *HSP70*, *HSP90*, 82-kDa *HSP3*, and vitellogenin genes. The primers designed for qRT-PCR were provided in Table S1. The relative expression of the DEGs was calculated based on the $2^{-\Delta\Delta C}$T relative response method (Livak and Schmittgen, 2001), and β-actin was selected as the internal reference gene.

2.8. Statistical analysis

All of the data were expressed as mean ± standard deviation for both Hg accumulation and qRT-PCR analysis. Data were analyzed via one-way analysis of variance (one-way ANOVA) followed by Student's *t*-test to compare the differences between the control and Hg treatment using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Significant differences were indicated by $P < 0.05$.

3. Results

3.1. Hg accumulation in two copepods

In both copepods, Hg concentrations were significantly increased ($P < 0.05$) over the control after exposure (Fig. 1). In $HgCl_2$-exposed *T. japonicus*, the Hg content was enhanced by roughly 207 times compared to the control (2393.5 ng/g *versus* 11.5 ng/g, respectively). Similarly, in $HgCl_2$-exposed *P. annandalei*, the Hg content was 3340.7 ng/g *versus* 4.0 ng/g for control; thus, Hg accumulation in *P. annandalei* was higher than in *T. japonicus* by approximately 1.4-fold.

3.2. RNA-seq and de novo assembly

Sequencing and assembly results are summarized in Table 1. All raw data were deposited in the SRA database of GenBank under BioProject accession numbers **PRJNA347642** and **PRJNA347881**. A total of 44,298,552–55,891,514 raw reads were generated from four libraries of *T. japonicus*, and 53,827,724–64,281,650 raw reads for *P. annandalei*, respectively. After quality control, 43,596,966–54,937,964 clean reads were produced from four libraries of *T. japonicus* with 52,282,946–63,126,656 clean reads for *P. annandalei*, respectively. The Q20 of the clean reads was calculated to be approximately 96% and the GC content was constant at approximately 46% for all samples. Using Trinity software, a total of 83,191 transcripts were obtained for *T. japonicus*, and all of the transcripts were clustered into 68,473 unigenes. In *P. annandalei*, a total of 165,653 transcripts were obtained, and all transcripts were clustered into 117,021 unigenes.

3.3. Gene functional annotation

In order to obtain comprehensive information regarding gene function, we performed functional annotation of genes in seven databases including the NCBI-NR, NCBI-NT, Pfam, KOG, Swiss-Prot, KEGG, and GO databases. As a consequence, 29,856 unigenes (43.60%) were successfully annotated in at least one database for *T. japonicus*, and 33,659 unigenes (28.76%) were annotated for *P. annandalei* (Table 2).

KEGG annotation revealed that 10,958 (16.00%) and 12,118 (10.36%) unigenes were annotated for *T. japonicus* and *P. annandalei*, respectively, against the KEGG database. Based on the secondary pathway hierarchy, all annotated unigenes were classified into 32 groups. Of these, signal transduction, translation, and the endocrine system were the most abundant functional pathways in both *T. japonicus* and *P. annandalei* (Fig. 2). In addition, the KOG and GO function classifications for two copepods are presented in Figs. S1 and S2.

3.4. Identification of the DEGs of copepods

A total of 101 DEGs (53 up-regulated and 48 down-regulated genes) were obtained from the copepod *T. japonicus* in response to Hg treatment (Fig. 3A), while only 18 DEGs (15 up-regulated and three down-regulated genes) were found in *P. annandalei* (Fig. 3B). All DEGs of both species were shown in Table S2. In *T. japonicus*, the up-regulated genes (e.g., inducible *HSP70*, Hsp20/alpha-crystallin, vitellogenin, vitellogenin 2, α-tubulin 1, β-tubulin, and cuticle protein 7, among others) were involved in stress, growth, and development (Table 3). The down-regulated genes were mainly involved in immune response (e.g., serine protease inhibitor-1S). In *P. annandalei*, most DEGs (e.g., *HSP90*, *HSP70*, and 82-kDa *HSP3*, among others) were up-regulated and all were involved in stress response (Table 3).

Enrichment analysis was conducted for all DEGs in both copepods against the KEGG database (Fig. 4). Eleven KEGG pathways were enriched for *T. japonicus*, while *P. annandalei* had 15 enriched pathways. In *T. japonicus*, the mitogen-activated proteins kinase (*MAPK*) signaling pathway, antigen processing and presentation, and estrogen signaling pathways exhibited significant up-regulation ($P < 0.05$). In *P. annandalei*, aside from the above pathways, protein processing in the endoplasmic reticulum and progesterone-mediated oocyte maturation were significantly increased ($P < 0.05$) in response to Hg exposure. Meanwhile, amino sugar and nucleotide sugar metabolism exhibited significant down-regulation ($P < 0.05$) after Hg treatment.

3.5. QRT-PCR validation

In both copepods, several DEGs obtained via KEGG pathway enrichment analysis were confirmed by qRT-PCR assay and gene transcription levels from qRT-PCR analysis supported the transcriptomic data in terms of both change direction and magnitude (Fig. S3).

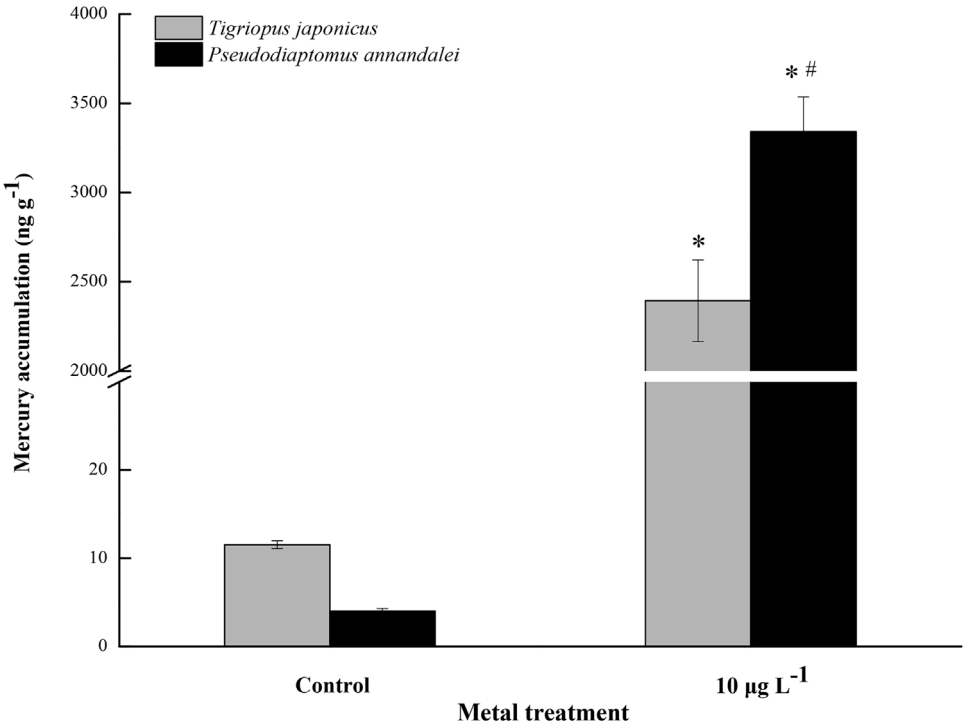

Fig. 1. Mercury content in copepods *Tigriopus japonicus* and *Pseudodiaptomus annandalei* under control conditions and exposed to 10 μg/L mercury. Data are presented as mean ± standard deviation of four replicate samples. Asterisk (*) represents a significant difference in the metal treatment group when compared with the control counterpart in both copepods. # indicates a significant difference between *T. japonicus* and *P. annandalei* under the same metal exposure level.

4. Discussion

Recently, many efforts have focused on investigating the effects of heavy metals on marine copepods, while studies concerning Hg impacts have been very limited in marine copepods. In this study, integrated metal accumulation analysis and transcriptome profiling demonstrated species-specific responses to Hg pollution in two copepods (*T. japonicus* and *P. annandalei*). Our transcriptomic work provided novel mechanistic insights into how these species respond differently to Hg toxicity. Additionally, the transcriptome databases of *T. japonicus* and *P. annandalei* will be helpful in better understanding molecular mechanisms in marine ecotoxicogenomic studies.

4.1. Hg accumulation

Both copepods significantly accumulated Hg in response to $HgCl_2$ exposure. The average BCFs of Hg were calculated as 239.4 and 334.1 L/kg for *T. japonicus* and *P. annandalei*, respectively, under Hg exposure. This difference between the two species is likely related to several factors, including species-specificity. For example, *P. annandalei* is larger than *T. japonicus*; the body length of female *P. annandalei* is approximately 1.28 mm (Dhanker et al., 2012), while that of *T. japonicus*, 0.98 mm (Raisuddin et al., 2007). Therefore *P. annandalei* may be able to retain more Hg in the body than *T. japonicus*. In addition, the calculated BCFs of the two copepod species were lower by 1–3 magnitudes in comparison with those in earlier studies concerning Hg impacts on calanoid copepod (Hook and Fisher, 2001), marine cope-

Table 2
Annotation of unigenes in different databases.

Databases	Number of unigenes	
	Tigriopus japonicus	*Pseudodiaptomus annandalei*
NR	22,016	23,451
NT	7119	7966
Swiss-Prot	18,531	20,506
Pfam	22,410	26,334
GO	22,801	26,701
KOG	14,270	15,680
KEGG	10,958	12,118
In at least one database	29,856	33,659

Note: NR, the NCBI non-redundant protein sequence database; NT, the NCBI non-redundant nucleotide sequences database; Swiss-Prot, A manually annotated and reviewed protein sequence database; Pfam, Protein family; GO: Gene Ontology; KOG, eukaryotic clusters of Orthologous Groups; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table 1
Summary of *Tigriopus japonicus* and *Pseudodiaptomus annandalei* transcriptomes under control and mercury treatment conditions.

Copepod	Samples	Raw reads	Clean reads	Q20 (%)	GC (%)	Total number of unigenes	Mean length of unigenes	N50 length of unigenes
T. japonicus	T_Control-1	44,298,552	43,596,966	96.38	46.23	68,473	820	1820
	T_Control-2	46,780,792	46,112,660	96.1	45.98			
	T_Treatment-1	54,260,034	53,262,214	96.74	46.25			
	T_Treatment-2	55,891,514	54,937,964	96.63	46.53			
P. annandalei	P_Control-1	55,825,612	54,796,480	96.26	48.27	117,021	735	1531
	P_Control-2	54,226,304	53,075,762	96.45	48.58			
	P_Treatment-1	64,281,650	63,126,656	96.47	48.38			
	P_Treatment-2	53,827,724	52,282,946	96.17	41.78			

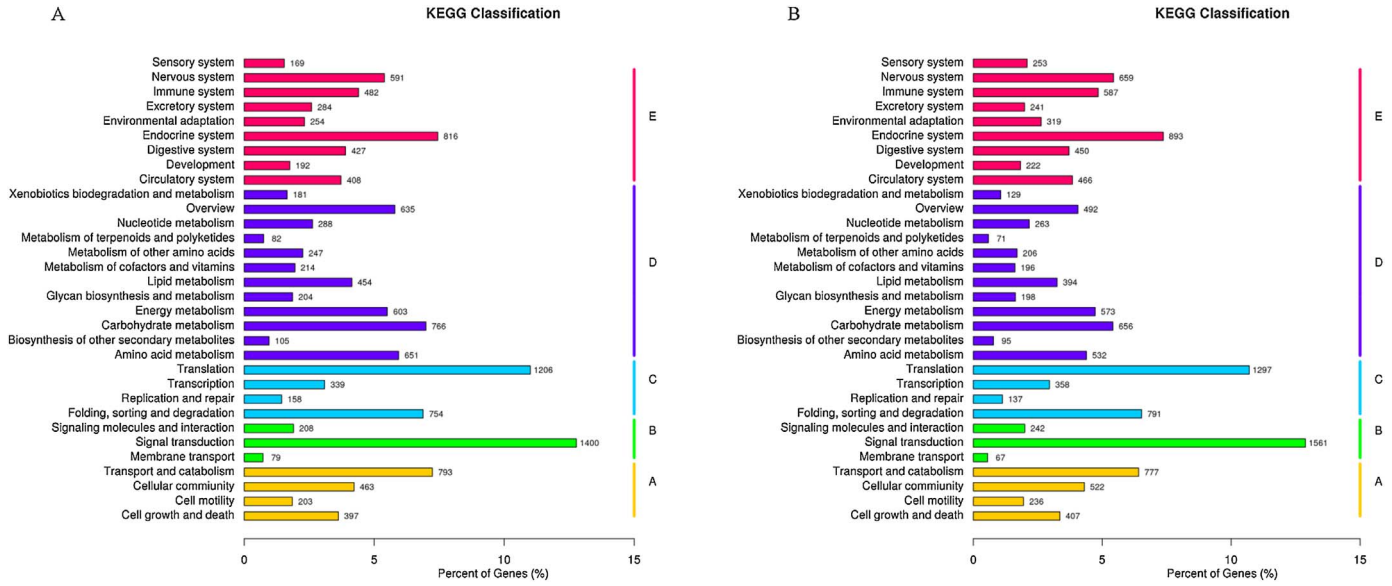

Fig. 2. KEGG classification of *Tigriopus japonicus* (A) and *Pseudodiaptomus annandalei* (B).

pods (Hsiao and Fang, 2013), and Arctic and boreal calanoid copepods (Overjordet et al., 2014). Hg bioaccumulation in copepods around hydrothermal vents and the adjacent marine environment in northeastern Taiwan resulted in 10^3–10^6 L/kg BCFs (Hsiao and Fang, 2013). Thus, Hg accumulation in marine copepods is likely to be dependent on several factors (e.g., species specificity and different exposure conditions). In our study, the metal content in *P. annandalei* was roughly 1.4 times higher than *T. japonicus* at the same Hg exposure level, suggesting that different accumulating mechanism is likely involved in response to Hg pollution. Interestingly, acute toxicity testing showed that the 48-h 50% lethal concentration of $HgCl_2$ was 0.394 mg/L for *T. japonicus* and 0.186 mg/L for *P. annandalei* (unpublished data), indicating that *T. japonicus* is more tolerant to Hg compared to *P. annandalei*. However, it remains unclear whether the lower metal accumulation in *T. japonicus* is likely to confer higher resistance to Hg pollution compared with *P. annandalei*, as well as what variations in transcriptomic response may occur. Overall, Hg accumulation strategies appear to vary depending on species in marine copepods, while the regulatory mechanisms of Hg toxicity require further investigation.

4.2. Stress gene response to $HgCl_2$ in two copepods

Cells have evolved a variety of defense mechanisms in response to environmental stressors (Martindale and Holbrook, 2002). One of the fundamental protection systems is the heat shock response, which is involved in rapid induction of a specific set of genes, including *HSPs*, that encodes cytoprotective proteins (Santoro, 2000). HSPs are highly conserved molecular chaperone proteins produced by cells to defend themselves in response to multiple environmental stressors (e.g., heat, UV light, chemical exposure). The primary toxic effect of Hg is known to be induced by oxidative stresses (Kim et al., 2014; Lushchak, 2011). The oxidative environment in the cell may lead to misfolding of proteins from their native folded conformation due to changes in the intracellular redox state (Fehrenbach and Northo, 2001). In fact, HSP70

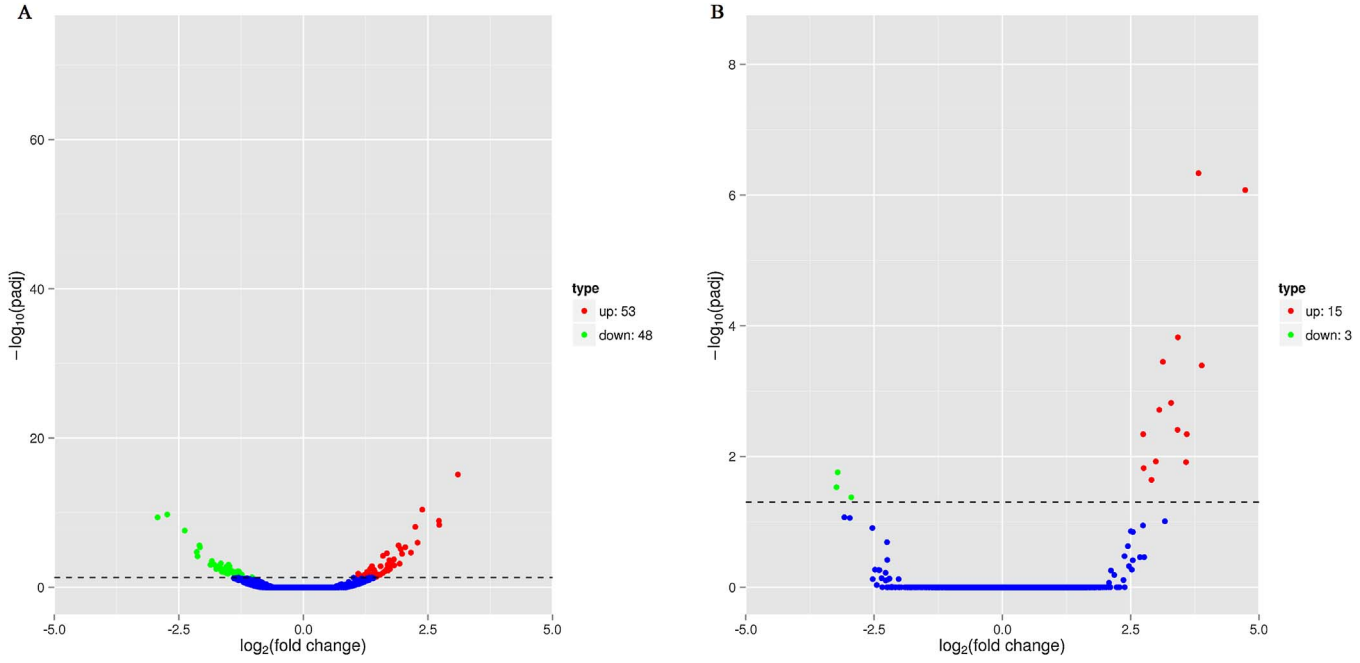

Fig. 3. Volcano plots of gene transcription under mercury treatment with comparison to the control (*Tigriopus japonicus* [A] and *Pseudodiaptomus annandalei* [B]).

Table 3
Differential expression of genes in *Tigriopus japonicus* and *Pseudodiaptomus annandalei* under mercury treatment.

Gene_id	Treatment_ read count	Control _ read count	log2 fold change	FDR	Gene description
Up-regulated in *T. japonicus*					
c12770_g1	557.7798592	106.5889232	1.9092	2.51E-06	inducible HSP 70 [*Tigriopus japonicus*]
c10265_g1	1399.106203	370.2216103	1.597	6.17E-05	tyrosine decarboxylase [*Nicrophorus vespilloides*]
c20036_g1	491.77693	101.8707585	1.7264	0.0002381	Hsp20/alpha-crystallin [*Tigriopus japonicus*]
c23782_g1	651.9967895	207.4980023	1.3757	0.001508	vitellogenin [*Tigriopus japonicus*]
c39810_g1	931.878982	230.3226216	1.5491	0.0015233	Hsp20/alpha-crystallin [*Tigriopus japonicus*]
c19262_g1	398.4648267	114.2473497	1.4164	0.0044387	alpha tubulin 1 [*Bigelowiella natans*]
c23471_g1	191.4774604	52.03176269	1.4236	0.007796	vitellogenin 2 [*Pseudodiaptomus annandalei*]
c20260_g1	178.1428592	47.56889898	1.4304	0.0081858	vitellogenin 2 [*Pseudodiaptomus annandalei*]
c19920_g1	2500.438333	1031.782254	1.0991	0.015039	inducible HSP 70 [*Tigriopus japonicus*]
c21839_g2	256.02624	80.95599329	1.2923	0.019719	beta-tubulin [*Oxytricha granulifera*]
c23367_g1	969.3306714	332.1979821	1.2365	0.020867	dynein heavy chain 3, axonemal [*Crassostrea gigas*]
c23563_g2	855.4697101	343.0703794	1.1061	0.028841	putative cuticle protein [*Lepeophtheirus salmonis*]
c23563_g5	444.1374082	163.0835496	1.173	0.029189	cuticle protein 7 [*Lepeophtheirus salmonis*]
c12796_g1	508.0563622	191.7708871	1.1407	0.041235	putative cuticle protein [*Lepeophtheirus salmonis*]
Down-regulated in *T. japonicus*					
c8810_g1	52.60169068	198.3353818	−1.3327	0.042603	serine protease inhibitor-1S [*Azumapecten farreri*]
Up-regulated in *P. annandalei*					
c40314_g1	381.594799	21.44351368	3.4197	0.0001504	HSP 90 [*Pseudodiaptomus annandalei*]
c53771_g1	1222.02614	94.87895788	3.1271	0.0003563	HSP 70 [Oreochromis mossambicus]
c60218_g1	947.0568759	53.98386475	3.2869	0.0015136	Hsp70 [*Eunicella cavolinii*]
c60218_g2	2525.691936	193.9883578	3.0575	0.0019361	Hsp70 [*Eunicella cavolinii*]
c38761_g1	166.7429938	11.9097423	2.9902	0.011915	HSP 90 [*Paracyclopina nana*]
c38761_g2	153.6216323	11.44351443	2.9054	0.022812	82-kDa HSP 3 [*Philodina roseola*]
Down-regulated in *P. annandalei*					
c62019_g1	2.044746	69.10193	−3.2316	0.029493	chitinase [*Monochamus alternatus*]

and HSP90 are involved in restoring misfolded or damaged proteins by chaperoning and/or transporting proteins to the proteasomes (Schlesinger, 1990), therefore protecting cells from oxidative stress (Lindquist and Craig, 1988; Nathan and Lindquist, 1995). For example, heavy metals (arsenic, cadmium, copper, silver, and zinc) induce up-regulation of *HSPs* including *HSP70* in *T. japonicus* (Rhee et al., 2009; Kim et al., 2014). In the present study, the expression of *HSP70* was significantly increased ($P < 0.05$) in both *T. japonicus* and *P. annandalei*, while *HSP90* was induced *P. annandalei* in response to Hg exposure, suggesting that HSP70 and HSP90 can be associated with detoxification processes. Moreover, previous reports have demonstrated the up-regulation of *HSP70* in response to metal exposure (Rhee et al., 2009; Kim et al., 2014). Thus, it can be considered as a useful biomarker for monitoring metal pollutions in the aquatic environment. In the case of *Hsp20/α-crystallin* gene, it was significantly up-regulated ($P < 0.05$) by Hg exposure in *T. japonicus*. Small HSPs play as an antioxidant agent by holding glutathione in reduced form, maintaining the redox homeostasis under oxidative condition and thereby protecting cells from oxidative stress (Preville et al., 1999; Arrigo et al., 2005; Arrigo, 2007). This suggests that the induction of *Hsp20/α-crystallin* in *T. japonicus* in

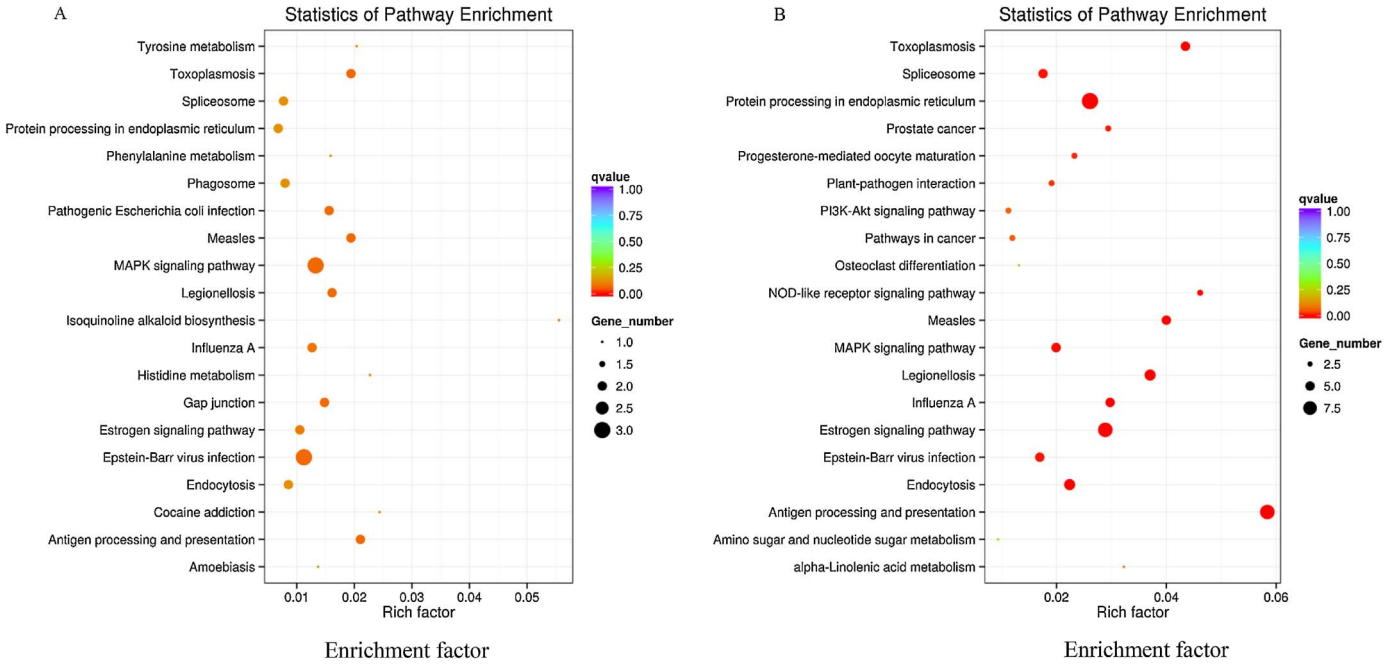

Fig. 4. Scatter plot of differentially expressed genes (DEGs) enriched in the KEGG pathways of *Tigriopus japonicus* (A) and *Pseudodiaptomus annandalei* (B). The enrichment factor indicates the ratio of DEGs to the number of unigenes in the pathway, and the q value represents the corrected *P* value.

response to Hg exposure is possibly associated with the prevention of protein denaturation/damage by Hg toxicity. The up-regulation of *Hsp20/α-crystallin* gene in *T. japonicus* is likely conferring relatively higher Hg tolerance to *T. japonicus* than *P. annandalei*, as *T. japonicus* showed comparatively lower LC50 value than *P. annandalei*. Taken together, the increased expression of several *HSPs* observed in both copepod species in response to Hg, likely helps to protect cells from oxidative stress-induced damages (e.g., misfolded or denatured proteins) caused by Hg toxicity. Also, the copepods could achieve tolerance to Hg pollution via the induction of *HSPs* through universal adaptability in response to environmental stressors (Morimoto et al., 1994). Thus, heat shock response in two marine copepods is an important mechanism of coping with Hg pollution and an adaptive strategy at the cellular level.

In *T. japonicus* and *P. annandalei*, KEGG pathway enrichment analysis indicated that HSPs were also involved in several significantly up-regulated enrichment pathways including the MAPK signaling pathway, antigen processing and presentation, and the estrogen signaling pathway. These pathways are primarily related to the immune system, endocrine-disrupting effects, and signal transduction, suggesting that Hg exposure induced toxic effects in these copepods at a global transcriptomic level. In a previous study, the phosphorylation status of the MAPK signaling pathway was examined in response to MeHg, and the phosphorylation of p38 and JNK varied. Thus, p-JNK was activated at a relatively low MeHg concentration (0–100 ng/L), while p-p38 was activated at high concentrations (500 and 1000 ng/L), suggesting that MAPK pathways are closely linked to the regulation of MeHg-responsive pathways (Lee et al., 2017). The expression of the estrogen signaling pathway in these two copepods is supporting that Hg is one of EDCs as shown in previous studies of the fathead minnow *P. promelas* (Drevnick and Sandheinrich, 2003) and the freshwater catfish *C. punctatus* (Mondal et al., 1997). For example, both *in vitro* and *in vivo* $HgCl_2$ treatment significantly increased progesterone synthesis in *C. punctatus* oocytes (Mondal et al., 1997), indicating the endocrine disrupting potential of Hg via interference with estrogen activity.

Taken together, our transcriptomic data of these two copepod species demonstrates the signaling pathways potentially activated in response to Hg exposure, providing some insights into Hg toxicity and its molecular mechanism in copepods.

4.3. Growth and development proteins involved in specific responses in *T. japonicus*

In *T. japonicus*, growth and development-associated genes (e.g., vitellogenin, vitellogenin 2, cuticle protein 7, α- tubulin 1, and β-tubulin) were upregulated in response to $HgCl_2$. Vitellogenin is the primary precursor of egg-yolk proteins, which provide an energy storage for embryonic development in oviparous organisms (Matozzo et al., 2008), and acts as a sensitive biomarker for heavy metal exposure in marine copepods (Lee et al., 2008; Lauritano et al., 2012). Vitellogenin mRNA expression was significantly enhanced 96 h after cadmium exposure in *T. japonicus* (Lee et al., 2008), implying that there is an interaction between metal and vitellogenin. In catfish, vitellogenin synthesis was inhibited in response to inorganic and organic Hg (Kirubagaran and Joy, 1995). Moreover, Hg and other metals suppressed egg production by binding to enzymes involved in vitellogenesis (Hook and Fisher, 2002). Thus, the increased expression of the *T. japonicus* vitellogenin gene in response to Hg exposure is likely a mechanism to facilitate vitellogenesis.

Cuticle mainly functions as an external skeleton that maintains body structure in order to resist environmental stressors. Aquatic organisms are in constant contact with the ambient water column, which contains several environmental pollutants. Therefore, cuticles in copepods are an important defensive barrier to environmental pollutants. Previously, the cuticle was revealed to be a major site of heavy metal accumulation including Hg, as shown in copepods *T. brevicornis* (Barka, 2007) and *T. japonicus* (Xu et al., 2016). In many invertebrates, the cuticle protein is up-regulated during diapause (Macrae, 2010), presumably reducing cuticle permeability (Schoville et al., 2012). Thus, the increased expression of several cuticle proteins is beneficial to cuticle formation and growth. α-tubulin proteins are the building blocks of microtubules, and act as an active component of the cytoskeleton. Tubulins work together with microtubule binding proteins and associated proteins in many cellular functions including maintenance of microtubule polymerization and depolymerization, cell shaping, growth, signaling, and protein movement (Calligaris et al., 2010; Lauritano et al., 2011). α-tubulin proteins are involved in tolerance to heavy metals (Mattingly et al., 2001; Mireji et al., 2010; Wang et al., 2011), and also exhibit up-regulation in response to heavy metal (e.g., cadmium, copper, and nickel) exposure (Stillitano et al., 2007; Zapata et al., 2009; Jiang et al., 2013). For example, cadmium is toxic via oxidative stress induction and binds to the α-tubulin sulfhydryl-group, resulting in cellular disruption (Silvestre et al., 2006; Stillitano et al., 2007). Thus, in *T. japonicus* and *P. annandalei*, the significant up-regulation of the α-tubulin 1 gene is likely to be linked to protection against and/or repairing damage to the microtubule network from oxidative disruption. Overall, our trancriptomic response studies and accumulation analysis of Hg indicate that two copepod species, *T. japonicus* and *P. annandalei*, exhibit species-specific molecular response mechanisms to Hg exposure.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (No.41476094).

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.2017.03.018.

References

Arrigo, A.P., Virot, S., Chaufour, S., Firdaus, W., Kretz-Remy, C., Diaz-Latoud, C., 2005. Hsp27 consolidates intracellular redox homeostasis by upholding glutathione in its reduced form and by decreasing iron intracellular levels. Antioxid. Redox Signal. 7, 414–422.

Arrigo, A.P., 2007. The cellular networking of mammalian Hsp27 and its functions in the control of protein folding: redox state and apoptosis. Adv. Exp. Med. Biol. 594, 14–26.

Barka, S., 2007. Insoluble detoxification of trace metals in a marine copepod *Tigriopus brevicornis* (Müller) exposed to copper, zinc, nickel, cadmium, silver and mercury. Ecotoxicology 16, 491–502.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate – a practical and powerful approach to multiple testing. J. R. Stat. Soc. 57, 289–300.

Bron, J.E., Frisch, D., Goetze, E., Johnson, S.C., Lee, C.E., Wyngaard, G.A., 2011. Observing copepods through a genomic lens. Front. Zool. 8, 1–15.

Calligaris, D., Verdier-Pinard, P., Devred, F., Villard, C., Braguer, D., Lafitte, D., 2010. Microtubule targeting agents: from biophysics to proteomics. Cell. Mol. Life Sci. 67, 1089–1104.

Cargnelutti, D., Tabaldi, L.A., Spanevello, R.M., de Oliveira Jucoski, G., Battisti, V., Redin, M., Linares, C.E.B., Dressler, V.L., de Moraes Flores, E.M., Nicoloso, F.T., 2006. Mercury toxicity induces oxidative stress in growing cucumber seedlings. Chemosphere 65, 999–1006.

Chen, Q., Sheng, J., Lin, Q., Gao, Y., Lv, J., 2006. Effect of salinity on reproduction and survival of the copepod *Pseudodiaptomus annandalei* Sewell, 1919. Aquaculture 258, 575–582.

Christie, A.E., Fontanilla, T.M., Nesbit, K.T., Lenz, P.H., 2013. Prediction of the protein components of a putative *Calanus finmarchicus* (Crustacea, Copepoda) circadian signaling system using a *de novo* assembled transcriptome. Comp. Biochem. Physiol. D 8, 165–193.

Clarkson, T.W., Magos, L., 2006. The toxicology of mercury and its chemical compounds. Crit. Rev. Toxicol. 36, 609–662.

Dhanker, R., Kumar, R., Hwang, J.S., 2012. Predation by *Pseudodiaptomus annandalei* (Copepoda: Calanoida) on rotifer prey: size selection, egg predation and effect of algal diet. J. Exp. Mar. Biol. Ecol. 414/415, 44–53.

Dhanker, R., Kumar, R., Tseng, L.C., Hwang, J.-S., 2013. Ciliate (*Euplotes* sp.) predation by *Pseudodiaptomus annandalei* (Copepoda: Calanoida) and the effects of mono-algal and pluri-algal diets. Zool. Stud. 52, 1293–1295.

Drevnick, P.E., Sandheinrich, M.B., 2003. Effects of dietary methylmercury on reproductive endocrinology of fathead minnows. Environ. Sci. Technol. 37, 4390–4396.

Fehrenbach, E., Northo, H., 2001. Free radicals exercise apoptosis and heat shock proteins. Immunol. Rev. 7, 66–89.
Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644–652.
Hansen, B.H., Altin, D., Booth, A., Vang, S.-H., Frenzel, M., Sørheim, K.R., Brakstad, O.G., Størseth, T.R., 2010. Molecular effects of diethanolamine exposure on *Calanus finmarchicus* (Crustacea: Copepoda). Aquat. Toxicol. 99, 212–222.
Hook, S.E., Fisher, N.S., 2001. Reproductive toxicity of metals in calanoid copepods. Mar. Biol. 138, 1131–1140.
Hook, S.E., Fisher, N.S., 2002. Relating the reproductive toxicity of five ingested metals in calanoid copepods with sulfur affinity. Mar. Environ. Res. 53, 161–174.
Hsiao, S.H., Fang, T.H., 2013. Hg bioaccumulation in marine copepods around hydrothermal vents and the adjacent marine environment in northeastern Taiwan. Mar. Pollut. Bull. 74, 175–182.
Iavicoli, I., Fontana, L., Bergamaschi, A., 2009. The effects of metals as endocrine disruptors. J. Toxicol. Environ. Health B 12, 206–223.
James, A.C., Azevedo, R.B.R., Partridge, L., 1997. Genetic and environmental responses to temperature of *Drosophila melanogaster* from a latitudinal cline. Genetics 146, 881–890.
Jiang, G.B., Shi, J.B., Feng, X.B., 2006. Mercury pollution in China. Environ. Sci. Technol. 40, 3672–3678.
Jiang, J.L., Wang, G.Z., Mao, M.G., Wang, K.J., Li, S.J., Zeng, C.S., 2013. Differential gene expression profile of the calanoid copepod, *Pseudodiaptomus annandalei*, in response to nickel exposure. Comp. Biochem. Physiol. C 157, 203–211.
Kim, B.-M., Rhee, J.-S., Jeong, C.-B., Seo, J.S., Park, G.S., Lee, Y.-M., Lee, J.-S., 2014. Heavy metals induce oxidative stress and trigger oxidative stress-mediated heat shock protein (*hsp*) modulation in the intertidal copepod *Tigriopus japonicus*. Comp. Biochem. Physiol. C 166, 65–74.
Kim, H.-S., Lee, B.-Y., Won, E.-J., Han, J., Hwang, D.-S., Park, H.G., Lee, J.-S., 2015. Identification of xenobiotic biodegradation and metabolism-related genes in the copepod *Tigriopus japonicus* whole transcriptome analysis. Mar. Genomics 24, 207–208.
Kirubagaran, R., Joy, K.P., 1995. Changes in lipid profiles and ^{32}P-uptake into phosphoprotein (vitellogenin) content of the ovary and liver in the female catfish, *Clarias batrachus*, exposed to mercury. Biomed. Environ. Sci. 8, 35–44.
Lauritano, C., Borra, M., Carotenuto, Y., Biffali, E., Miralto, A., Procaccini, G., Ianora, A., 2011. First molecular evidence of diatom effects in the copepod *Calanus helgolandicus*. J. Exp. Mar. Biol. Ecol. 404, 79–86.
Lauritano, C., Procaccini, G., Ianora, A., 2012. Gene expression patterns and stress response in marine copepods. Mar. Environ. Res. 76, 22–31.
Lee, K.-W., Hwang, D.-S., Rhee, J.-S., Ki, J.-S., Park, H.G., Ryu, J.C., Raisuddin, S., Lee, J.-S., 2008. Molecular cloning, phylogenetic analysis and developmental expression of a vitellogenin (*Vg*) gene from the intertidal copepod *Tigriopus japonicus*. Comp. Biochem. Physiol. B 150, 395–402.
Lee, Y.H., Kang, H.-M., Kim, D.-H., Wang, M., Jeong, C.-B., Lee, J.-S., 2017. Adverse effects of methylmercury (MeHg) on life parameters, antioxidant systems, and MAPK signaling pathways in the copepod *Tigriopus japonicus*. Aquat. Toxicol. 184, 133–141.
Lenz, P.H., Roncalli, V., Hassett, R.P., Wu, L.S., Cieslak, M.C., Hartline, D.K., Christie, A.E., 2014. *De novo* assembly of a transcriptome for *Calanus finmarchicus* (Crustacea, Copepoda) – the dominant zooplankter of the North Atlantic Ocean. PLoS One 9, e88589.
Lindquist, S., Craig, E.A., 1988. The heat-shock proteins. Ann. Rev. Genet. 22, 133–138.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta C}$t method. Methods 25, 402–408.
Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.
Macrae, T.H., 2010. Gene expression, metabolic regulation and stress tolerance during diapause. Cell. Mol. Life Sci. 67, 2405–2424.
Martindale, J.L., Holbrook, N.J., 2002. Cellular response to oxidative stress: signaling for suicide and survival. J. Cell Physiol. 192, 1–15.
Matozzo, V., Gagné, F., Marin, M.G., Ricciardi, F., Blaise, C., 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. Environ. Int. 34, 531–545.
Mattingly, K.S., Beaty, B.J., Mackie, R.S., McGaw, M., Carlson, J.O., Rayms-Keller, A., 2001. Molecular cloning and characterization of a metal responsive *Chironomus tentans* α-tubulin cDNA. Aquat. Toxicol. 54, 249–260.
Mireji, P.O., Keating, J., Hassanali, A., Impoinvil, D.E., Mbogo, C.M., Muturi, M.N., Nyambaka, H., Kenya, E.U., Githure, J.I., Beier, J.C., 2010. Expression of metallothionein and α-tubulin in heavy metal-tolerant *Anopheles gambiae* sensu stricto (Diptera: Culicidae). Ecotoxicol. Environ. Saf. 73, 46–50.
Mondal, S., Mukhopadhyay, B., Bhattacharya, S., 1997. Inorganic mercury binding to fish oocyte plasma membrane induces steroidogenesis and translatable messenger RNA synthesis. Biometals 10, 285–290.
Morimoto, R.I., Tissières, A., Georgopoulos, C., 1994. The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Monogr. 26, 457–494.
Nätt, D., Rubin, C.J., Wright, D., Johnsson, M., Beltéky, J., Andersson, L., Jensen, P., 2011. Heritable genome-wide variation of gene expression and promoter methylation between wild and domesticated chickens. BMC Genomics 13, 59.
Nathan, D.F., Lindquist, S., 1995. Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. Mol. Cell. Biol. 15, 3917–3925.
Overjordet, I.B., Altin, D., Berg, T., Jenssen, B.M., Gabrielsen, G.W., Hansen, B.H., 2014. Acute and sub-lethal response to mercury in Arctic and boreal calanoid copepods. Aquat. Toxicol. 155, 160–165.
Preville, X., Salvemini, F., Giraud, S., Chaufour, S., Paul, C., Stepien, G., Ursini, M.V., Arrigo, A.P., 1999. Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. Exp. Cell Res. 247, 61–78.
Raisuddin, S., Kwok, K.W.H., Leung, K.M.Y., Schlenk, D., Lee, J.-S., 2007. The copepod *Tigriopus*: a promising marine model organism for ecotoxicology and environmental genomics. Aquat. Toxicol. 83, 161–173.
Ramachandran, G., Swaminathan, S., 2012. Sex ratio and mating behaviour in the calanoid copepod *Pseudodiaptomus annandalei*. Zool. Stud. 51, 89–98.
Rhee, J.-S., Raisuddin, S., Lee, K.-W., Seo, J.S., Ki, J.-S., Kim, I.-C., Park, H.G., Lee, J.-S., 2009. Heat shock protein (*Hsp*) gene responses of the intertidal copepod *Tigriopus japonicus* to environmental toxicants. Comp. Biochem. Physiol. C 149, 104–112.
Roncalli, V., Cieslak, M.C., Lenz, P.H., 2016. Transcriptomic responses of the calanoid copepod *Calanus finmarchicus* to the saxitoxin producing dinoflagellate *Alexandrium fundyense*. Sci. Rep. 6, 25708.
Santoro, M.G., 2000. Heat shock factors and the control of the stress response. Biochem. Pharmacol. 59, 55–63.
Schlesinger, M.J., 1990. Heat shock proteins. J. Biol. Chem. 265, 12111–12114.
Schoville, S.D., Barreto, F.S., Moy, G.W., Wolff, A., Burton, R.S., 2012. Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod *Tigriopus californicus*. BMC Evol. Biol. 12, 170.
Silvestre, F., Dierick, J.F., Dumont, V., Dieu, M., Raes, M., Devos, P., 2006. Differential protein expression profiles in anterior gills of *Eriocheir sinensis* during acclimation to cadmium. Aquat. Toxicol. 76, 46–58.
Stillitano, F., Mugelli, A., Cerbai, E., Vanucci, S., 2007. Quantification of midkine gene expression in *Patella caerulea* (Mollusca, Gastropoda) exposed to cadmium. Estuar. Coast. Shelf Sci. 75, 120–124.
Wang, S., Jia, Y., Wang, S., Wang, X., Wang, H., Zhao, Z., Liu, B., 2009. Total mercury and monomethylmercury in water sediments, and hydrophytes from the rivers, estuary, and bay along the Bohai Sea coast, northeastern China. Appl. Geochem. 24, 1702–1711.
Wang, L., Feng, Z., Wang, X., Wang, X., Zhang, X., 2010. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics 26, 136–138.
Wang, M., Wang, Y., Wang, J., Lin, L., Hong, H., Wang, D., 2011. Proteome profiles in medaka (*Oryzias melastigma*) liver and brain experimentally exposed to acute inorganic mercury. Aquat. Toxicol. 103, 129–139.
Xu, X., Lin, S., Wang, M., 2016. Comparative quantitative proteomics unveils putative mechanisms involved into mercury toxicity and tolerance in *Tigriopus japonicus* under multigenerational exposure scenario. Environ. Pollut. 218, 1287–1297.
Zapata, M., Tanguy, A., David, E., Moraga, D., Riquelme, C., 2009. Transcriptomic response of *Argopecten purpuratus* post-larvae to copper exposure under experimental conditions. Gene 442, 37–46.
Zhu, X., Kusaka, Y., Sato, K., Zhang, Q., 2000. The endocrine disruptive effects of mercury. Environ. Health Prev. Med. 4, 174–183.
Zhu, H., Zhong, H., Evans, D., Hintelmann, H., 2015. Effects of rice residue incorporation on the speciation, potential bioavailability and risk of mercury in a contaminated paddy soil. J. Hazard. Mater. 293, 64–71.