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

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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

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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