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Expression profile analysis of antioxidative stress and developmental pathway genes in the manganese-exposed intertidal copepod *Tigriopus japonicus* with 6K oligochip

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HIGHLIGHTS

- Novel investigation of manganese effects on copepod molecular system.
- Application of extensive copepod genomic information for manganese monitoring.
- Comparative analysis of manganese-induced molecular defense responses in copepod.
- Investigation of detrimental effect of manganese on copepod physiological traits.
- Molecular biomarker development for manganese contamination.

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ABSTRACT

Manganese (Mn) provides one of aquatic pollutants in marine ecosystem. Here we used a 6K oligomicroarray to identify the effect of Mn on transcriptomes in the copepod *Tigriopus japonicus*. A total of 5594 spots were significantly modulated on a 6K oligomicroarray with hierarchical clustering after exposure to Mn over 24 h. Of them, 186 and 489 genes were significantly upregulated and downregulated, respectively. Particularly, several genes involved in stress, detoxification, and developmental functions were significantly modulated in *T. japonicus* exposed for 24 h. In detail, Mn exposure specifically up-regulated genes that were related to intracellular stress, antioxidant, and detoxification pathways such as cytochrome P450s (*CYPs*), glutathione *S*-transferases (*GSTs*), and heat shock proteins (*hsps*), while a majority of downregulated genes was associated with developmental pathways such as cuticle protein, ecdysone receptor, and vitellogenin. These results demonstrated that Mn exposure modulated gene expression in relation to intracellular stress, leading to developmental retardation in the intertidal copepod, *T. japonicus*, and provide a better understanding of mechanistic molecular studies of Mn-induced cellular damage.

1. Introduction

Manganese (Mn) is a trace metal, and is essential for enzymatic and metabolic processes of marine invertebrates. However, elevated levels of Mn would induce detrimental effects by accumulation of Mn on the early development, growth, metabolism, and physiology of animals (Sanchez et al., 1993; Colomina et al., 1996). Free Mn ions (Mn^{2+} and Mn^{4+}) are being released into the aquatic environment by photochemical and chemical reduction of Mn oxides from diverse organic compounds (De Schamphelaire et al., 2007). The transport of Mn ions is modulated by several environmental factors such as temperature, pH, and oxygen concentration. Mn ions induce cytotoxicity and imbalance of homeostasis of organisms (Roth, 2006). However, to date, information on the effects of Mn in aquatic organisms is not well documented, compared to other heavy and trace metals.

Of all marine organisms, crustaceans and molluscs are known as the most manganese-sensitive invertebrates followed by echinoderms (Bryan and Ward, 1965). After Bryan and Ward (1965) firstly reported the absorption and effects of Mn in two

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common lobster species, *Homarus gammarus* and *H. vulgaris*, several researchers reported the effects of manganese. For example, Weinstein et al. (1992) showed a potential correlation between Mn accumulation and shell disease of the blue crab, *Callinectes sapidus*. Holmes et al. (1999) reported that Mn exposure inhibited neuromuscular transmission and muscle excitation as competitive inhibitors of calcium ion channels of the Norway lobster *Nephrops norvegicus*. Different accumulation patterns and the effects of Mn were associated with different seasonal and sex samples of mussels (*Mytilus edulis* and *M. californianus*) and oysters (*Crassostrea gigas* and *C. virginica*) (Nørum et al., 2005). In addition, Mn induced apoptosis of the circulating hemocytes and reduced granular hemocyte maturation in *Nephrops norvegicus* and *M. edulis* (Hernroth et al., 2004; Oweson and Hernroth, 2009). However, to date most studies on Mn have focused on the toxicities and physiological effects of Mn with marine invertebrates. Therefore, the information on gene/protein expressions of Mn-exposed marine invertebrates would be important for a better understanding of the molecular mechanisms of the Mn-induced mode of action.

To date, the potential of the copepod *Tigriopus japonicus* as a model organism has been recognized in environmental monitoring and ecotoxicogenomics (Raisuddin et al., 2007). The general genomic and physiological characters of *T. japonicus* provide several advantages for environmental studies, such as small size (≈1.0 mm in length), distinctive developmental stages, dimorphic sexes, short reproduction period, and extreme tolerance of diverse environmental conditions. Moreover, extensive genomic DNA information (10,894 unigenes) and RNASeq (59,983 assembled ESTs; total length 78.3 Mb; N50 = 2319 as of April 2, 2013) of *T. japonicus* have supported *in vitro* omics experiments (Lee et al., 2010; unpublished data), leading to gene expression-based environmental research with copepods (Kim et al., 2011; Kim et al., 2012). Supportingly, Ki et al. (2009) showed the usage of 6K oligomicroarray in quantifying and qualifying changes of genes modulated by copper (Cu) in *T. japonicus*. Therefore, *in vivo* and *in vitro* advantages with *T. japonicus* would provide many benefits to apply it in omics approaches towards biomonitoring and biomarker development. In this paper, we analyzed mRNA expression profiles of Mn-exposed organisms with a *T. japonicus* 6K oligochip to understand the molecular mechanisms of Mn toxicity.

2. Materials and methods

2.1. Copepods

The intertidal copepod, *T. japonicus* was maintained and reared in 0.2 μm-filtered sea water adjusted to 25 °C temperature, a photoperiod of 12 h:12 h light/dark and a salinity of 30 ppt. Copepods were fed with the algae, *Tetraselmis suecica* (approximately 5×10^4 cells ml^{-1}). Identification of the species was made by morphological characteristics and the sequence identity of the universal barcode marker, mitochondrial DNA COI.

2.2. Mn exposure

A large number (≈2000 adults of both sex) of copepods were exposed to Mn (as 100 μg L $MnSO_4 \cdot H_2O$, Sigma–Aldrich, Inc., St. Louis, MO, USA). To employ the same developmental stage of copepods, all the adult copepods were cultured from the same nauplius stage that was collected with a sieving method. The exposure concentration of Mn was chosen based on acute toxicity values as reported previously (Seo et al., 2006b; Lee et al., 2007). All the experimental conditions including Mn concentration did not induce any acute detrimental effect or mortality in *T. japonicus*. Another group of copepods with a similar number was used as a control. No mortality was observed in both control and Mn-exposed groups for 24 h. At 24 h, copepods were sampled and subjected to total RNA isolation for the microarray experiment. Total RNAs were isolated from *T. japonicus* with conventional TRIzol™ reagent (Invitrogen, USA), and the integrity of isolated total RNA was measured with Agilent's Bioanalyzer 2100 RNA Nano kit (Agilent Technologies). No food was provided and water was not changed during the exposure with the static renewal type.

2.3. Microarray development and analysis

The *T. japonicus* 6K oligomicroarray development was following our previous study (Ki et al., 2009), and data analysis was performed at the E-Biogen Inc. (www.e-biogen.com, Seoul, South Korea). Briefly, hybridization was performed with 5 μg of the labeled target sample per CustomArrayTM 2 × 6K microarray, which were separated from each other for technically duplicated experiments. The scanned images were analyzed by Feature Extraction program (Agilent Technologies). The average fluorescence intensity for each spot was calculated, and then the local background was subtracted. All data manipulation and the selection of fold-changed genes were performed with GeneSpring 7.3.1 (Agilent Technologies). Array points with less than 0.01 signal intensity were transformed to 0.01 (data transformation), and the intensity of the test channel was subtracted by control channel to calculate the normalized ratio values for each spot. More than 2-fold-changed genes were selected and considered as statistically significant. The gene ontology (GO) functional annotations were performed at the NICEM, Seoul National University (Seoul, South Korea).

2.4. Total RNA extraction and single-strand cDNA synthesis for real-time RT-PCR

Entire copepods (≈2000 adults of both sex for microarray experiment; ≈300 adults of both sex for real-time RT-PCR analysis) were homogenized in three volumes of TRIZOL® reagent (Molecular Research Center, Inc.) with a tissue grinder and stored at −80 °C until use. Total RNA was isolated from the tissues according to the manufacturer's instructions, and genomic DNA was removed using DNase I (Sigma, St. Louis, Mo). The quantity of total RNA was measured at 230, 260, and 280 nm with a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience). To check the genomic DNA contamination, we loaded the total RNA in a 1% agarose gel that contained ethidium bromide (EtBr), and was visualized by a UV transilluminator (Wealtec Corp.). Also, to verify the total RNA quality, we loaded the total RNA in a 1% formaldehyde/agarose gel with EtBr staining and checked the 18/28S ribosomal RNAs integrity and band ratio. A single-strand cDNA was synthesized from the total RNA using an oligo$(dT)_{20}$ primer for reverse transcription (SuperScript™ III RT kit, Invitrogen, Carlsbad, CA, USA).

2.5. Real-time RT-PCR

Significant expression changes of interesting genes following microarray analysis were validated using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Copepods (≈300 adults of both sex) were exposed to Mn (as 100 μg L $MnSO_4 \cdot H_2O$, Sigma–Aldrich, Inc., St. Louis, MO, USA) at the same conditions as the microarray experiment. The primers for each gene were designed after the exon/intron boundary was compared to the genomic DNA with GENRUNNER software (Hastings Software, Inc. NY USA), and confirmed by the Primer 3 program (Whitehead Institute/MIT center for Genome Research). The optimized conditions were transferred according to the following CFX96™ real-time PCR protocol (Bio-Rad, Hercules, CA, USA). For RT-PCR amplification, each reaction consisted of 1 μl of cDNA that was reversely

transcribed from 2 μg of total RNA and 0.2 μM primer each of real time RT-F/R and the reference gene primer *18S rRNA* RT-F/R. All the RT-PCRs were carried out in an unskirted low 96-well clear plate (Bio-Rad, Hercules, CA, USA). The reaction conditions were 94 °C/4 min; 35 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/30 s; and 72 °C/10 min. A SYBR® Green (Molecular Probe Inc., Invitrogen) was used to detect the specific PCR products. To confirm the amplification of the specific products, the cycles were continued to check the melting curve under the following conditions: 95 °C/1 min, 55 °C/1 min, and 80 cycles of 55 °C/10 s with a 0.5 °C increase per cycle. Amplification and detection of the SYBR Green-labeled products were performed with the CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). Data from the triplicate experiments were expressed relative to the *18S rRNA* that was used to normalize for any difference in the reverse transcriptase efficiency. Data are means ± S.D. of three replicates of exposed copepods. The fold change for the relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Statistical analysis

The SPSS ver. 17.0 (SPSS Inc., Chicago IL, USA) software package was used for statistical analysis. Data are expressed as means ± S.D. Significant differences between both observations of the control and the exposed groups were analyzed with Student's paired *t*-test and one-way ANOVA followed by Tukey's test. Any difference showing $P < 0.05$ was considered significant.

3. Results

3.1. T. japonicus 6K oligomicroarray

We used *T. japonicus* 6K oligomicroarray as developed previously (Ki et al., 2009). Briefly, 7219 unique expressed sequence tags (ESTs) of *T. japonicus* were identified by pyrosequencing method, and 6006 contigs were used for specific probes in the oligomicroarray. Each probe sequence, annotation information, and gene ontology (GO) functional compositions of 6006 contigs were represented as shown in Ki et al. (2009).

3.2. Analysis of differentially expressed genes in microarray

Exposure to 100 μg L of Mn for 24 h caused significant changes in transcript profiles in *T. japonicus*. Of 5594 spots expressed over control, 3087 spots were upregulated but 2507 spots were downregulated. Gene ontology (GO) analysis for all the modulated genes was represented in Fig. 1. Overall, up- and down-regulated genes showed similar orders in GO composition for each category such as cellular component, molecular function, and biological process category. Particularly, most genes were localized in the cellular function part within the intracellular and extracellular category in both upregulated and downregulated genes (Fig. 1A). As for molecular functions, binding (49.5%) and catalytic activity (25.2%) were predominant in both upregulated and downregulated genes (Fig. 1B). As shown in Fig. 1C, all the modulated genes were almost evenly distributed over diverse biological processes (Fig. 1C). Total 186 genes were upregulated at 24 h of exposure, whereas 489 genes showed downregulation at the 2-fold change ($P < 0.05$).

3.3. Major upregulated genes

Selected 20 upregulated genes (>2.0-fold change, $P < 0.05$) in response to Mn exposure are listed in Table 1. Messenger RNAs of a number of cytochrome P450 (*CYP*) genes showed significant upregulation at 24 h of Mn exposure. As shown in Table 2, *CYP* genes, heat shock proteins (*hsps*), glutathione *S*-transferase (*GST*) genes were upregulated, suggesting that 100 μg L of Mn exposure would indicate a role in the xenobiotic metabolism, detoxification, antioxidant defense or stress response to recover cellular damage at the molecular level.

3.4. Major downregulated genes

After Mn exposure, selected downregulated genes (>2.0-fold change, $P < 0.05$) were listed in Table 3. Interestingly, downregulated genes included cuticle protein, myosin heavy chain, cathepsin z, tubulin alpha, nitric oxide synthase, indicating that Mn exposure would modulate the transcriptional activity of genes that are involved in several physiological metabolisms for trade-off of energy budget with the recovery genes for cellular damage. As shown in Table 4, the downregulated genes revealed that a majority of genes are involved in growth and development, as shown in cuticle protein, ecdysone receptor, and vitellogenin genes.

3.5. Time course real-time RT-PCR

To verify modulation patterns of selected genes over microarray results, we collected several genes and tested their transcript changes over 24 h using real-time RT-PCR. Real-time RT-PCR expression patterns of selected upregulated genes on microarray images are shown in Fig. 2. All the tested genes were upregulated by 100 μg L of Mn exposure at 24 h ($P < 0.05$) in good agreement with those of microarray results. Likewise, we confirmed that real-time RT-PCR of selected downregulated genes was also supported by microarray result (Fig. 3). Thus, most genes showed a similar expression pattern as indicated by the microarray. In case of mRNA expression of *Vtg2* and *EcdR*, they showed a downregulated patterns at 24 h. However, they were not significant in both genes ($P > 0.05$).

4. Discussion

As one of the major environmental pollutants, trace heavy metals are of great concern in freshwater and marine ecosystems (Järup, 2003; Jenssen, 2003), and a lot of efforts are made to provide reliable and ecologically relevant invertebrate toxicity testing models for the monitoring of the aquatic environment. Of several testing species to Mn exposure, copepod species including *Tigriopus* spp. have shown sensitivity to metals (Kusk and Wollenberger, 2007; Raisuddin et al., 2007). Moreover, the Organisation for Economic Co-operation and Development (OECD) has highlighted that *T. japonicus* is useful as a potential model species for toxicity testing and risk assessment of diverse environmental pollutants (OECD, 2006). As yet, several potential biomarkers of *T. japonicus* have tested their expression upon heavy metal exposures as shown previously (Lee et al., 2007; Rhee et al., 2009; Kim et al., 2011, 2012).

Mn is not widely studied in aquatic ecosystems as compared to other metals such as cadmium (Cd), Cu, and zinc (Zn). To date, several studies focused on the accumulation of Mn in the sediment. As one of the essential trace elements, dissolved concentrations of Mn range from 0.01 mg L to 10 mg L in natural waters, while seawater typically contains approximately 2 μg L of Mn (McNeely et al., 1979). However, anthropogenic sources of Mn to aquatic environment, such as domestic wastewater, sewage sludge disposal, and metal refining, induce drastic pollution particularly in freshwater and intertidal regions of the ocean (Nriagu and Pacyna, 1988), leading to an induction of detrimental physiological effects, homeostasis alteration, or molecular responses in aquatic animals. However,

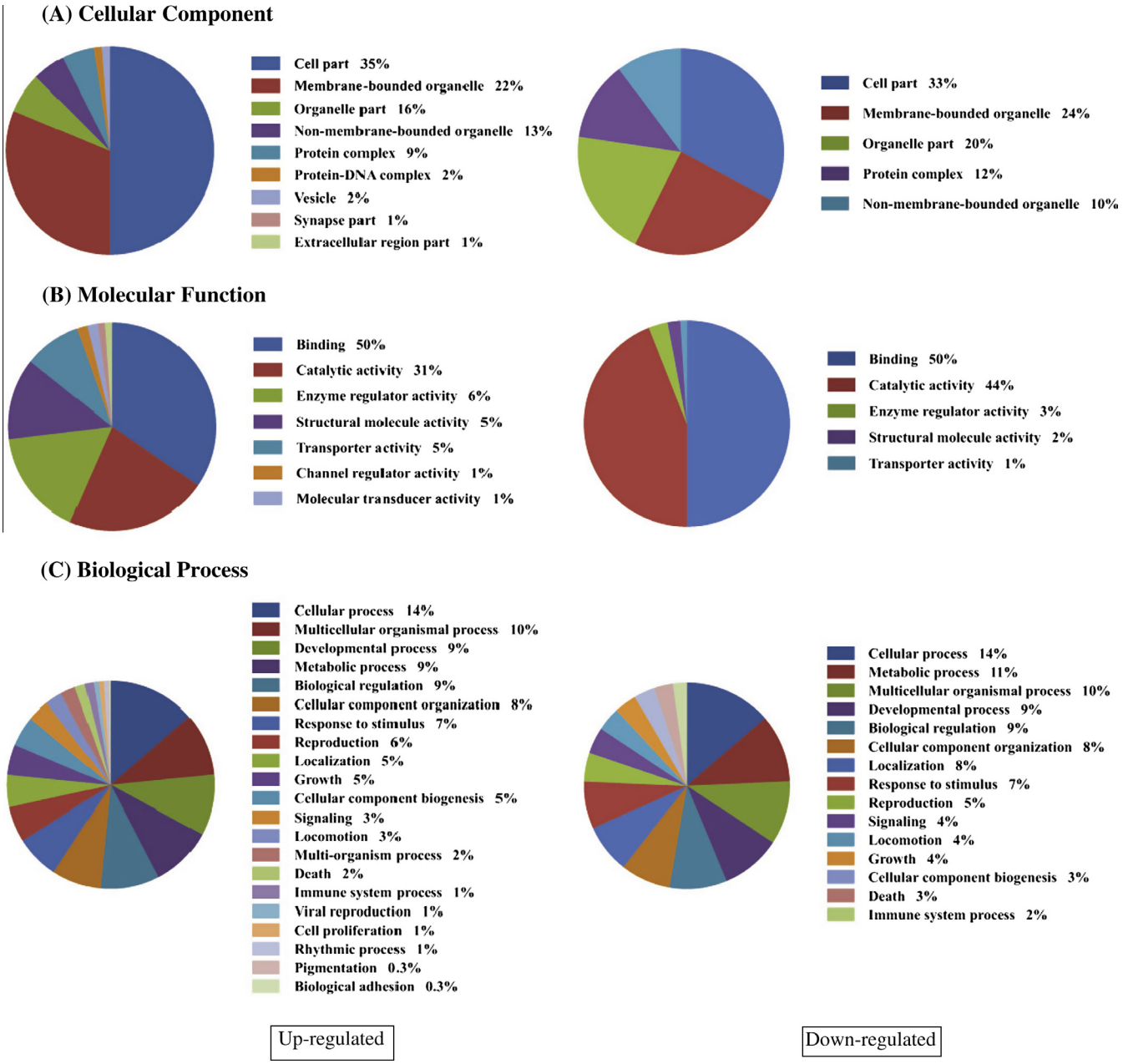

Fig. 1. Categories of gene ontology (GO) for total upregulated and downregulated genes in the *Tigriopus japonicus* 6K microarray. (A) Cellular components, (B) molecular function, (C) biological process.

there is limited information as yet on actual concentrations of Mn in polluted aquatic environments. Only Nriagu and Pacyna (1988) reported that hundreds of Mn pollutants were produced and flowed continuously into freshwater and seawater. Therefore, we hypothesized that acute Mn treatment at relative high concentrations compared to natural levels can induce sublethal toxic effects in *T. japonicus*, although it is an essential trace metal as catalyst to induce or modulate enzymatic activity of living organisms. Subsequently, we selected a high concentration of Mn (100 μg L) as a testing nominal concentration that showed no mortality but would potentially induce transcriptomic modulation in *T. japonicus*.

Among the few experimental aquatic studies on Mn, most studies focused on the acute toxicity of Mn exposure in fish. Among invertebrates, we published effects of Mn from highly contaminated industrial areas using the marine polychaete, *Neanthes succinea*, showing a significant accumulation of Mn in different tissues with various quantities and strong antioxidant responses in *GST* and *SOD* gene inductions (Rhee et al., 2007, 2011). In several aquatic insects, Dittman and Buchwalter (2010) reported that Mn exposure induced transient physiological stress in terms of the molting process. In goldfish *Carassius auratus*, Mn exposure caused an organ-specific antioxidant response via the modulation of the *SOD*, *CAT*, *GPx* activities (Vieira et al., 2012). In fact, 50 and 100 μg L of Mn exposure strongly induced the *GST* transcript for 24 h in *T. japonicus* (Lee et al., 2007), suggesting that Mn has an oxidative stress potential as shown in the present study. Therefore, our finding on effects of Mn is the first step for a better understanding of molecular recovery mechanisms in a copepod after Mn exposure.

The Cytochrome P450 (*CYP*) system is composed of important heme-containing proteins that play critical roles in the metabolism of diverse xenobiotics and endogenous compounds. Certain environmental pollutants including heavy metals interact with

Table 1
List of top 20 up-regulated genes studied by 6K *T. japonicus* microarray in descending order of fold change over controls.

Probe ID	Genbank No.	Gene	Fold change	SD	*P*-value	Closest matched GenBank No. (E-value)
T6K5932	GU213987	Cytochrome P450 9E1	15.83	0.15	0.00	AAR97606 (2.00E−63)
T6K3856	GU213987	Cytochrome P450 9E1	14.98	0.05	0.00	AAR97606 (2.00E−63)
T6K2845	GU213989	Cytochrome P450 9G3	12.04	0.25	0.00	NP_001108456 (5.00E−08)
T6K1978	KC483465	Hypothetical protein CBG05439	9.88	0.42	0.00	CAE61535 (2.00E−15)
T6K5282	GQ144708	Cytochrome P450 13A	9.55	0.15	0.00	ABQ08709 (0.0000)
T6K0943	GU213986	Cytochrome P450 9b1	8.51	0.21	0.00	XP_001846774 (1.00E−39)
T6K1896	KC483466	Hypothetical protein LOC410478	7.41	0.27	0.00	NP_001073028 (2.00E−25)
T6K5210	GQ144716	Heme binding protein 2	6.59	0.30	0.00	AAH45936 (7.00E−09)
T6K3733	GQ144713	Neuronal nicotinic acetylcholine receptor alpha-2	5.82	0.08	0.00	AAD20220 (3.00E−05)
T6K3825	GQ144715	Hsp20/alpha-crystallin	5.17	0.14	0.00	AAS02295 (5.00E−08)
T6K3154	GQ144722	Phenazine biosynthesis protein	4.86	0.33	0.00	YP_001037549 (2.00E−30)
T6K2029	KC483467	Eukaryotic translation initiation factor 3	4.26	0.17	0.00	XP_001663853 (1.00E−18)
T6K3006	KC483468	Y73B6BL.42	4.17	0.26	0.00	NP_001023570 (1.50E−21)
T6K0716	KC483469	ADP-ribosylation factor 4	3.78	0.09	0.00	ACO15315 (4.00E−05)
T6K1997	KC483470	CG6359-PA isoform A isoform 1	3.72	0.20	0.00	XP_392658 (3.00E−25)
T6K3200	AY522572	Heat shock protein 20	3.13	0.24	0.00	BAF03557 (2.00E−17)
T6K0122	GQ144722	Phenazine biosynthesis protein	3.12	0.38	0.00	YP_001037549 (2.00E−30)
T6K3844	KC483471	Werner helicase interacting protein	3.07	0.07	0.00	AAH67729 (5.00E−16)
T6K0219	KC483472	histone h2a	3.07	0.14	0.00	XP_001657152 (1.00E−15)
T6K4046	GQ144723	CG17218-PA	3.04	0.11	0.00	XP 973708 (4.00E−07)

Table 2
List of top 20 down-regulated genes studied by 6K *T. japonicus* microarray is descending order of fold change over controls.

Probe ID	Genbank No.	Gene	Fold change	SD	*P*-value	Closest matched GenBank No. (E-value)
T6K3603	GQ144708	Similar to CG15920-PA isoform A	−10.13	0.08	0.00	XP_001122868 (2.00E−18)
T6K2533	GQ144730	Putative cuticle protein	−9.00	0.23	0.00	ABN13582 (7.00E−03)
T6K3667	GQ144731	Myosin heavy chain CG17927-PF isoform 7	−6.70	0.16	0.00	EAT42858 (8.00E−09)
T6K3693	KC483473	Actin binding protein 280	−5.70	0.27	0.00	CAN88239 (3.00E−15)
T6K4268	GQ144729	Similar to AGAP007114-PA	−5.15	0.34	0.00	EDL08706 (4.00E−06)
T6K1011	GQ144736	Alpha-tubulin	−4.99	0.09	0.00	CAA25855 (3.00E−09)
T6K0839	KC483474	Anion exchange protein	−4.56	0.10	0.00	EEB19670 (4.00E−05)
T6K2332	KC483475	Cathepsin z	−4.55	0.18	0.00	XP_001471259 (2.00E−25)
T6K2871	KC483476	Similar to CG3999 CG3999-PA	−4.52	0.35	0.00	EFA01173 (3.00E−09)
T6K4921	KC483477	Hypothetical protein AaeL	−4.29	0.22	0.00	EAT34115 (2.00E−05)
T6K0508	GQ144742	AGAP011936-PA	−4.26	0.14	0.00	NP_001095946 (1.00E−14)
T6K2291	KC483478	Nitric oxide synthase	−4.18	0.11	0.00	XP_001660328 (3.00E−11)
T6K0544	GQ144735	AGAP011936-PA	−4.16	0.39	0.00	NP_001095946 (6.00E−14)
T6K5682	GQ144734	Hypothetical protein TcasGA2	−4.07	0.52	0.00	TC004645 (3.00E−08)
T6K1159	KC483479	Pyruvate carboxylase	−4.03	0.46	0.00	NP_989677 (2.00E−10)
T6K2279	GQ144741	Flexible cuticle protein 12 precursor	−4.01	0.28	0.00	XP_316784 (6.00E−04)
T6K2089	GQ144743	Mitogen activated protein kinase kinase kinase 1 isoform CRA_b	−4.01	0.16	0.00	EDL18420 (2.00E−04)
T6K2434	T6K5466	Torso-like protein precursor	−4.00	0.24	0.00	NP 001107843 (3.00E−03)
T6K3174	GQ144732	AGAP011936-PA	−4.00	0.30	0.00	NP_001095946 (6.00E−14)
T6K2681	KC483480	ENSANGP00000024129	−3.94	0.17	0.00	XP_319309 (1.00E−09)

xenobiotic metabolizing enzyme (XME) receptors or other reception pathways, leading to the upregulation or downregulation of phases I and II XME gene expression (Nebert and Dalton, 2006). In *T. japonicus*, the regulation of Mn are associated with the alterations of the transcription rate of *CYP* genes, even though there is limited information available on the effects of Mn to *CYP* gene modulation in aquatic invertebrates. After Cu exposure, the *Tigriopus CYP13A* gene was highly induced (Ki et al., 2009), and likely one of potential biomarkers for metal pollution, even though further experiments should be conducted against diverse other heavy and trace metals. Regarding expression of the CYP9 family, induction of *CYP9B1* and *CYP9E1* genes by Mn exposure suggests a potential role of *CYPs* in the metabolism of foreign or excessive innate compounds including metals against diverse environmental xenobiotics in *T. japonicus*, as shown in several invertebrates that had an inducible CYP9 family or CYP6 family (most closely related with CYP9 family in insects) (Stevens et al., 2000; Petersen et al., 2003; Itokawa et al., 2010). In addition, mRNA expressions of both *CYP9* genes within a short time period suggest that they have strong inducibility with a potential involvement in the detoxification or further metabolism in *T. japonicus*. Mn and other heavy metals have been demonstrated to induce or inhibit *CYP* isoforms in several animals. Particularly, Cu and other heavy metals induced aryl hydrocarbon receptor/xenobiotic responsive element (AhR/XRE) binding and *CYP1A* gene expression in cultured hepatoma Hepa1c1c7 cells, implicating that there is an AhR-dependent mechanism in CYP induction by metals (Korashy and El-Kadi, 2004, 2005). Accumulated heavy metals induced CYP1A protein in the marine sea star, *Asterias rubens* (Danis et al., 2006). Cu differently modulated mRNA expression of several *CYP* genes in *T. japonicus* (Ki et al., 2009). Cd (10 and 40 μg L) induced the mRNA expression of *CYP414A1* gene but Cu (10 and 40 μg L) reduced its transcription rate for 96 h in the hepatopancreas of *Venerupis philippinarum* (Zhang et al., 2012).

All known eukaryotes are responding to external stimuli by inducing a group of heat shock proteins (hsps) as a most highly conserved response (Lindquist and Craig, 1988) with persistent *hsp* induction. In the copepod *T. japonicus*, heavy metal-induced intracellular stress would trigger several *hsp* transcripts (Rhee et al., 2009; Ki et al., 2009). Therefore, we can assume that the induction of several *hsps* is a conserved protective response to

Table 3
Detoxification and antioxidant-related genes and development-related genes as potential biomarkers for Mn exposure.

Probe ID	Genbank No.	Gene	Fold change	SD	*P*-value	Closest matched GenBank No. (E-value)
Detoxification and antioxidant-related genes						
T6K5932	GU213987	Cytochrome P450 9E1	15.83	0.15	0.00	AAR97606 (2.00E−63)
T6K3856	GU213987	Cytochrome P450 9E1	14.98	0.05	0.00	AAR97606 2.00E−63
T6K2845	GU213989	Cytochrome P450 9G3	12.04	0.25	0.00	NP_001108456 (5.00E−08)
T6K5282	GQ144708	Cytochrome P450 13A	9.55	0.15	0.00	ABQ08709 (0.0000)
T6K0943	GU213986	Cytochrome P450 9B1	8.51	0.21	0.00	XP_001846774 (1.00E−39)
T6K3825	GQ144715	Hsp20/alpha-crystallin	5.17	0.14	0.00	AAS02295 (5.00E−08)
T6K3200	AY522572	Heat shock protein 20	3.13	0.24	0.00	BAF03557 (2.00E−25)
T6K5958	EU306560	Hsp20.7	2.84	0.19	0.00	ACA03520 (2.00E−19)
T6K5310	EU306560	Hsp20.7	2.63	0.24	0.00	BAF03557 (1.00E−13)
T6K0393	DQ088365	Glutathione *S*-transferase	2.51	0.09	0.00	AAV31410 (7.00E−18)
T6K5328	GU214006	Cytochrome P450 15A1	2.27	0.12	0.00	XP_001945934 (1.00E−19)
Development-related genes						
T6K2533	GQ144727	Insect cuticle protein	−9.00	0.23	0.00	ABN13582 (7.00E−03)
T6K2279	GQ144741	Flexible cuticle protein 12 precursor	−4.01	0.28	0.00	XP_316784 (6.00E−04)
T6K5937	GQ351503	Ecdysone receptor	−3.56	0.15	0.00	BAH56300 (3.00E−15)
T6K4586	EU416312	Vitellogenin 1	−3.27	0.09	0.00	ADD73551 (1.00E−25)
T6K5338	EU416312	Vitellogenin 1	−3.07	0.17	0.00	ADD73551 (1.00E−42)
T6K1365	KC483481	Cuticle protein 6	−2.97	0.21	0.00	ACS36140 (1.00E−36)
T6K5983	ABZ91537	Vitellogenin	−2.89	0.08	0.00	ABZ91537 (0.0000)
T6K5287	GQ351503	Ecdysone receptor	−2.82	0.16	0.00	BAH56300 (3.00E−15)
T6K5288	KC483482	Ecdysone receptor isoform A	−2.63	0.24	0.00	AEH43772 (2.00E−26)
T6K1884	EU831281	Vitellogenin 2	−2.55	0.21	0.00	ACJ12892 (1.00E−22)
T6K1934	KC483483	Vitellogenin-like	−2.26	0.2	0.00	ADD73552 (1.00E−18)
T6K2016	EU831281	Vitellogenin 2	−2.20	0.18	0.00	ACJ12892 (1.00E−33)
T6K2516	KC483484	Vitellogenin-like	−2.00	0.14	0.00	ADD73552 (1.00E−20)

Mn exposure in *T. japonicus*. In fact, in *T. japonicus*, several *hsps* have shown strong biomarker potentials against diverse environmental contaminants including heavy metals (Seo et al., 2006a; Rhee et al., 2009). Also, excessive input of Cu induced apoptotic cell death through reactive oxygen species (ROS)-triggered p38 kinase activation and *hsps* induction in *T. japonicus* (Rhee et al., 2013). Laboratory and field studies alike have shown the potential of *hsp* as an inducible biomarker, and *hsp* inductions upon diverse heavy metal exposures have been reported in several invertebrates (Pyza et al., 1997; Snyder et al., 2001; Piano et al., 2004; Rhee et al., 2009). *T. japonicus* in its intertidal exposed habitat of splash pools with its fluctuating environmental parameters is supposed to be adapted to diverse stressors. It is, therefore, reasonable to assume that *T. japonicus* has been evolving a unique defense system against oxidative stress. This holds primarily for significant variations of osmolarity and/or other factors that would trigger an adaptive on/off switch system of *CYP* and/or *hsps* genes.

To date, *hsp70* has been known to be a generally accepted biomarker for diverse environmental conditions among a wide range of animals. In this study, small *hsps* showed strong sensitivities against Mn exposure, while *hsp70* or relative large molecular weight of *hsps* was not significantly modulated by Mn (data not shown). Regarding small *hsps* expression in response to Mn exposure, only Xiao et al. (2009) reported a similar result that 75 and 200 μmol L of Mn exposure for 48 h induced *hsp16.2* expression in *Caenorhabditis elegans*. Comparative considerations would be necessary on these expression patterns on the potential role of small *hsps*. Firstly, Mn is an essential metal in an organism as we discussed previously, leading to relative lower toxic effects compared to other strong toxic metals such as Cd or Hg. In fact, the concentration of Mn used in this study did not affect any acute hazardous effect or mortality through the above experimental conditions in *T. japonicus*. However, Pinsino et al. (2010) reported that high concentration of Mn (15.4 mg L) induced an upregulation of the *hsc70* and *hsc60* with morphological abnormalities in sea urchin embryos. Therefore, *hsp70* would not respond against relative low concentrations of Mn in this study, while it was strongly induced by other metals such as Cu, Zn, and Ag (Rhee et al., 2009). Secondly, sensitivity of *Tigriopus* hsp20 family and its potential involvement in endocrine disruption would be considered more seriously as homeostatic role of Mn (Roth, 2006). Previously, Seo et al. (2006a) reported strong modulations of *hsp20* gene after exposure to six model endocrine disrupting chemicals (EDCs) in *T. japonicus*. Therefore, other endogenous metabolic aspects of Mn or its excessive metabolites over its internal concentration limit would trigger *hsp20* expression to maintain a physiological balance in *T. japonicus*. Finally, there is a need for more comparable transcript profiling on effects of different concentrations of Mn through time-courses, as transcript expression of certain genes show a sharp bell-type curve or a prolonged expression patterns depending on experimental conditions. These will be tested further to better understand the mode of action of Mn on *Tigriopus hsps* based on integrating the results of multidisciplinary research areas.

With the 6K oligomicroarray, we found that several development-related genes were downregulated by Mn exposure as shown in the expression of vitellogenin, cuticle protein, and ecdysone receptor. In case of *Tigriopus* cuticle protein was previously downregulated by Cu exposure (Ki et al., 2009). Also, there were obvious detrimental effects of Mn on the expression of structural proteins including myosin and tubulin for early development. Like most copepods, *T. japonicus* undergoes 12 distinctive post-embryonic developmental stages including 6 naupliar stages, 5 copepodid stages, and a sexually dimorphic adult stage composed of male and female (Raisuddin et al., 2007). Developmental molting and metamorphosis are regulated by ecdysteroids, and both ecdysteroid and vitellogenin levels are controlled during ovarian maturation which is the period when ecdysteroids stimulate vitellogenesis (Forget-Leray et al., 2005; Dahl et al., 2006). Regarding the effects of heavy and trace metal exposures in copepods, there were consistent findings on the impact of several reproductive parameters of copepods. For example, Sunda et al. (1987) reported that survival and egg laying rates were significantly affected by Zn and Cu exposure in the marine copepod *Acartia tonsa*. Dietary silver (Ag), Cd, mercury (Hg), Mn, and Zn reduced egg production and hatching rate of *Acartia tonsa* and *Acartia hudsonica* (Hook and Fisher, 2002). Actually, Lee et al. (2008) explained that seven physiological traits (nauplius phase, development time, survival, sex ratio, number of clutch, nauplii per clutch and fecundity) would

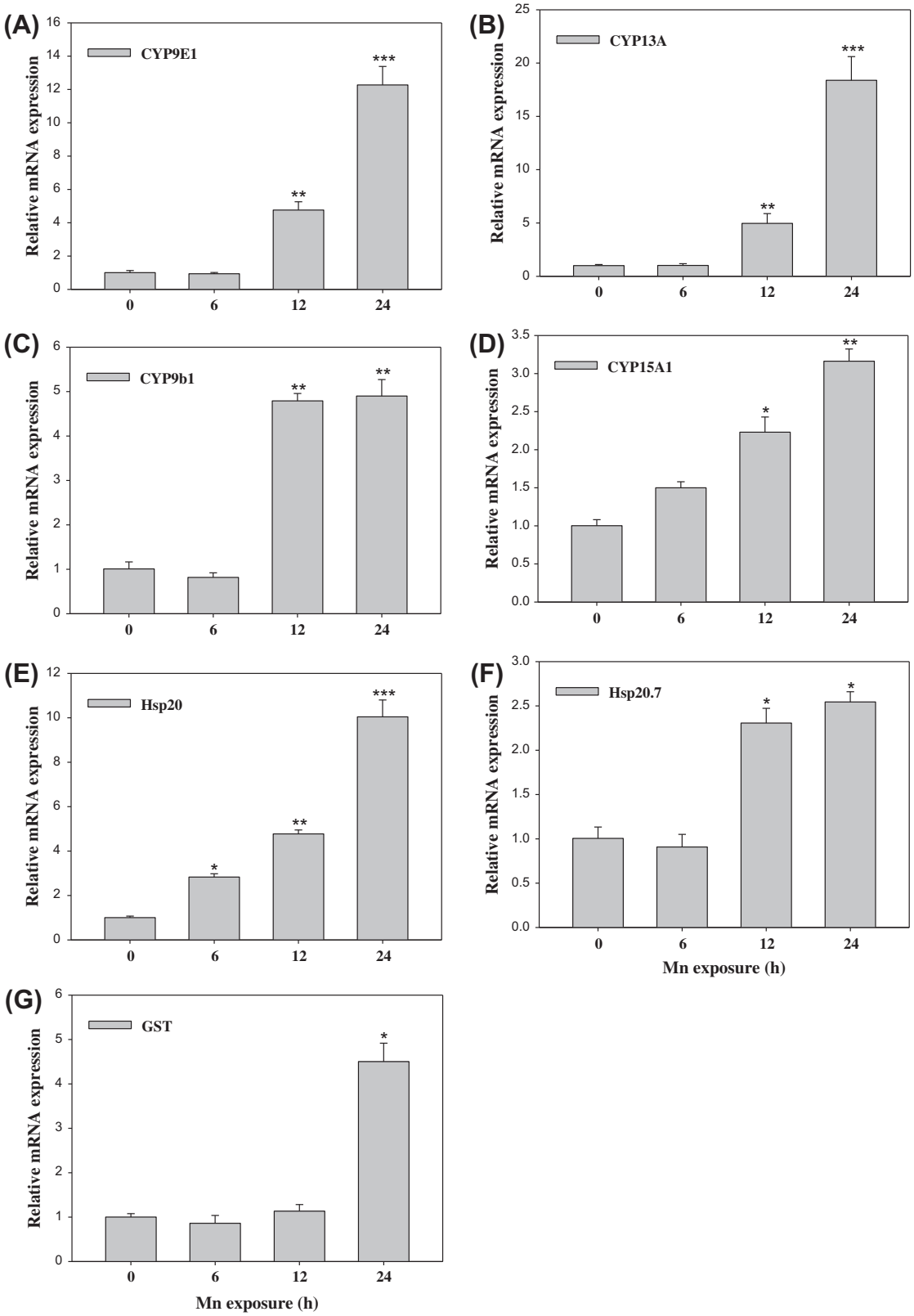

Fig. 2. Time-dependent expression profiles of selected genes as follows: (A) *CYP9E1*, (B) *CYP13A*, (C) *CYP9b1*, (D) *CYP15A1*, (E) *Hsp20*, (F) *Hsp20.7*, and (G) *GST*. The mRNA expressions are shown as relative to *18S rRNA* which was used as a reference housekeeping gene. Data are means ± S.D. of three replicates of exposed copepods. The symbols (*, **, and ***) indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively.

be strongly affected by different metals (Cu, AsIII, AsV) during two generations of *T. japonicus*. Regarding differentially modulated genes by Mn exposure in *T. japonicus*, we suggest that Mn-triggered trade-offs in fitness costs would be strongly associated with the induction of a molecular defense system and the inhibition of several physiological traits in *T. japonicus* to compensate energetic budget for survival. According to Lee et al. (2008), exposure to all metals resulted in an increase in the duration of the nauplius phase

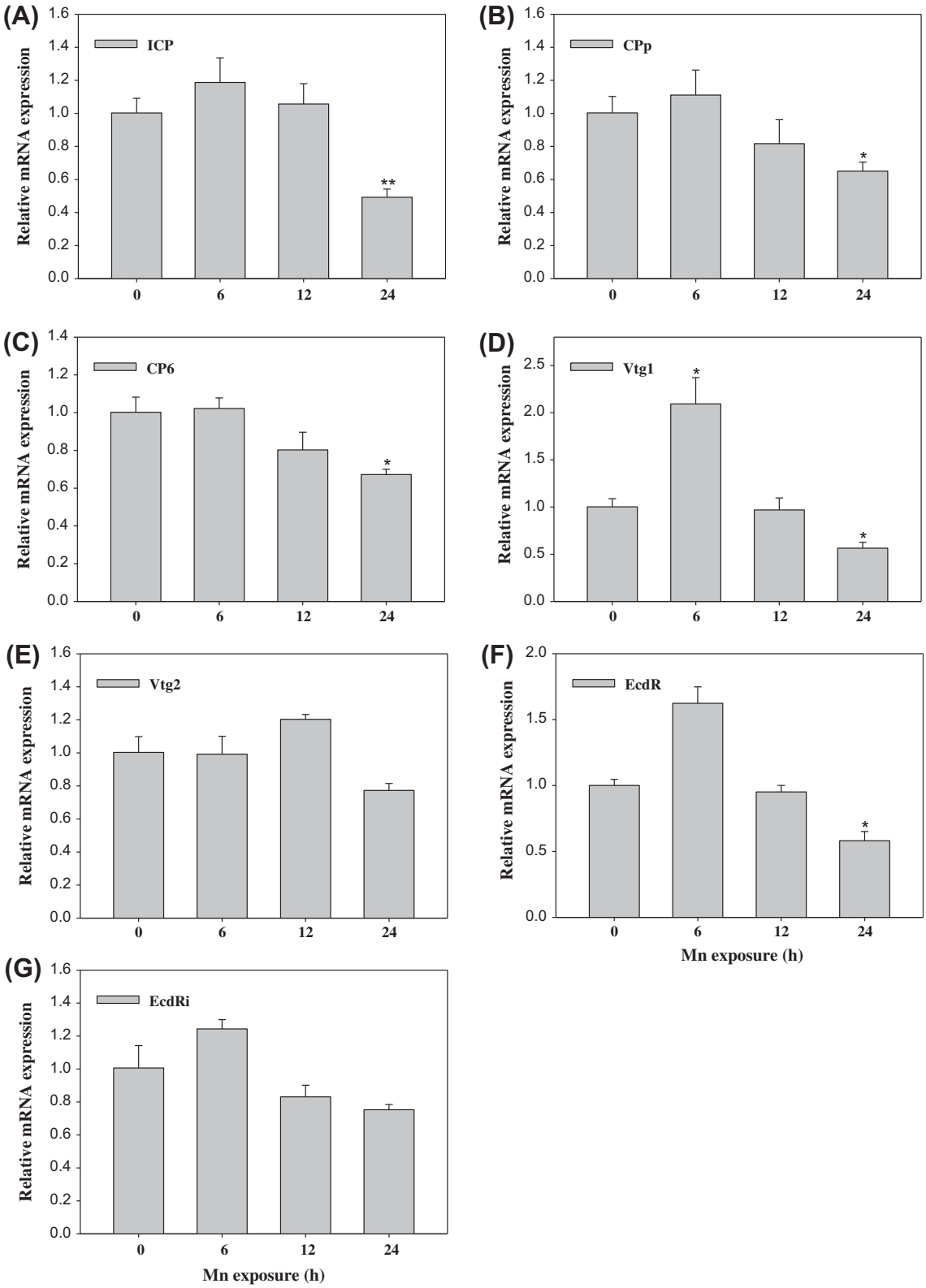

Fig. 3. Time-dependent expression profiles of selected genes are as follows: (A) *ICP*, (B) *CPp*, (C) *CP6*, (D) *Vtg1*, (E) *Vtg2*, (F) *EcdR*, and (G) *EcdRi*. The mRNA expressions are shown as relative to *18S rRNA* which was used as a reference housekeeping gene. Data are means ± S.D. of three replicates of exposed copepods. The symbols (* and **) indicate $P < 0.05$ and $P < 0.01$ respectively.

in the F_0 generation, and significant reductions in nauplius stage and maturation durations were observed in AsIII- and Cu-exposed F_1 generation. Kwok et al. (2009) demonstrated decreased reproductive outputs in Cu-exposed *T. japonicus* in a dose-dependent manner (0–100 μg L). They also suggested that Cu resistance could be developed through multigeneration acclimation against elevated Cu concentrations with a trade-off in fitness cost. In addition, 10 μg L of nickel (Ni) exposure significantly reduced offspring production and egg hatching rate in three marine copepods *T. japonicus*, *Apocyclops borneoensis*, and *Acartia pacifica* (Mohammed et al.,

2010). Regarding the gene expressions of development-related genes, Ki et al. (2009) reported that transcripts of cuticle protein, ecdysone receptor, and vitellogenin were downregulated by Cu exposure in a 6K oligomicroarray of *T. japonicus*. Thus, we can assume that downregulation of the development-related genes by Mn exposure are directly related to abnormal developmental events such as the molting process during embryogenesis, growth, and reproduction of copepods.

In summary, we demonstrate that Mn as an emerging aquatic pollutant can potentially affect the regulation of diverse genes of *T. japonicus* through either inactivation or activation of their transcription rates. To date, the molecular mechanism of toxicity by Mn or endogenous metabolism with Mn or its metabolites are not well understood in invertebrates compared to other trace heavy metals. Therefore, our finding that Mn induces or represses the transcription of certain genes of *T. japonicus* is the first report on the effects of Mn at the level of gene expression in copepods, and indicates that Mn exposure could affect detoxification-, stress-, and development-related pathways in *T. japonicus*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.04.047.

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