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Role of crustacean hyperglycemic hormone (*CHH*) in the environmental stressor-exposed intertidal copepod *Tigriopus japonicus*

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ABSTRACT

To identify and characterize *CHH* (*TJ-CHH*) gene in the copepod *Tigriopus japonicus*, we analyzed the full-length cDNA sequence, genomic structure, and promoter region. The full-length *TJ-CHH* cDNA was 716 bp in length, encoding 136 amino acid residues. The deduced amino acid sequences of TJ-CHH showed a high similarity of the CHH mature domain to other crustaceans. Six conserved cysteine residues and five conserved structural motifs in the CHH mature peptide domain were also observed. The genomic structure of the *TJ-CHH* gene contained three exons and two introns in its open reading frame (ORF), and several transcriptional elements were detected in the promoter region of the *TJ-CHH* gene. To investigate transcriptional change of *TJ-CHH* under environmental stress, *T. japonicus* were exposed to heat treatment, UV-B radiation, heavy metals, and water-accommodated fractions (WAFs) of Iranian crude oil. Upon heat stress, *TJ-CHH* transcripts were elevated at 30 °C and 35 °C for 96 h in a time-course experiment. UV-B radiation led to a decreased pattern of the *TJ-CHH* transcript 48 h and more after radiation (12 kJ/m^2). After exposure of a fixed dose (12 kJ/m^2) in a time-course experiment, *TJ-CHH* transcript was down-regulated in time-dependent manner with a lowest value at 12 h. However, the *TJ-CHH* transcript level was increased in response to five heavy metal exposures for 96 h. Also, the level of the *TJ-CHH* transcript was significantly up-regulated at 20% of WAFs after exposure to WAFs for 48 h and then remarkably reduced in a dose-dependent manner. These findings suggest that the enhanced *TJ-CHH* transcript level is associated with a cellular stress response of the *TJ-CHH* gene as shown in decapod crustaceans. This study is also helpful for a better understanding of the detrimental effects of environmental changes on the CHH-triggered copepod metabolism.

1. Introduction

Crustacean hyperglycemic hormone (CHH) plays key roles in a wide range of biological processes including stress response in crustaceans and is responsible for the regulation of hemolymph glucose in crustaceans (Böcking et al., 2002). To date, several reports suggested that the CHH has different roles with diverse physiological functions such as lipid metabolism (Santos et al., 1997), molting (Chung et al., 1999), osmoregulation (Spanings-Pierrot et al., 2000; Serrano et al., 2003; Chung and Webster, 2006), and reproduction (De Kleijn and Van Herp, 1998), although the physiological function of CHH is known to be regulated by the hemolymphatic glucose level. Moreover, in decapod crustaceans, hyperglycemia is a response to various kinds of environmental stressors including hypoxia (Webster, 1996; Chang et al., 1998; Chung and Zmora, 2008), light/dark cycle (Kallen et al., 1990), parasite infection (Stentiford et al., 2001), salinity (Chang et al., 1998), thermal shock (Chang et al., 1998; Kuo and Yang, 1999; Chung and Zmora, 2008), and environmental pollutants (Reddy et al., 1996; Lorenzon et al., 2000, 2004).

To date, the gene information of *CHH* has been widely documented in several decapods. In decapods, hemolymph glucose level is known to be controlled by CHH concentration that is synthesized in the X-organ-sinus-gland (XO-SG) complex in the medulla terminalis of the eyestalk (Fingerman, 1987). CHH family is composed by 72 to 78 amino acids and is divided into two subfamilies based on their peptide structures (Lacombe et al., 1999). For example, Type I peptides are pleiotropic hormones, while Type II peptides (molt-inhibiting hormone, MIH; vitellogenesis-inhibiting hormone, VIH; mandibular organ-inhibiting hormone; MOIH) are functionally more specialized than CHH (Fanjul-Moles, 2006; Montagné et al., 2010). As mentioned above, the CHH family is an important hormone in development, reproduction, and innate metabolism. The importance of CHH as an effector of a homeostatic control mediator in the stress response in decapod has been discussed (Fanjul-Moles, 2006). Failure of the

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integrated homeostatic response may lead to physiological disturbance in an organism. Thus, CHH is useful in predicting the health status of crustaceans as an indicator of environmental stress response. However, there is no information available on *CHH* gene and its function in copepods as yet. We tried to obtain gene information and gene response to stressors for a better understanding of homeostatic metabolism in copepods.

The intertidal copepod, *Tigriopus japonicus* (Copepoda, Harpactidae), has a worldwide distribution with its congeneric species and shows peculiar adaptations to its upper intertidal splashpool habitat. *Tigriopus* has been used as a potential model animal in aquatic ecotoxicological studies due to several advantages such as easy maintenance under controlled laboratory conditions, small body size (adults are about 1.0 mm in length), sexual dimorphism, high fecundity, and a short life cycle (Raisuddin et al., 2007). More recently, extensive genomic DNA information (10,894 unigenes) and RNA-seq (59,983 assembled ESTs; total length 78.3 Mb; N50 = 2,319) were obtained by using GS-FLX titanium and Illumina in this species (Lee et al., 2010; unpublished data).

In the present study, we firstly cloned and sequenced full-length *CHH* cDNA and its corresponding genomic DNA and analyzed the promoter region from the intertidal copepod *T. japonicus*. We also investigated transcriptional changes of *TJ-CHH* gene after exposure to several environmental stressors such as heat shock, UV-B radiation, heavy metals, and WAFs. This study is useful to better understand the molecular response mechanisms against various environmental stressors in aquatic invertebrates.

2. Materials and methods

2.1. Culture and maintenance

The copepod, *T. japonicus* was originally collected from a single rockpool of Haeundae beach (35°9′29.57″N, 129°9′36.60″E) in Busan (South Korea) in 2003, and since then, we cultured them in the laboratory (Hanyang University, Seoul, South Korea) continuously with 0.2-μm-filtered artificial sea water (TetraMarine Salt Pro, Tetra™, OH, USA) adjusted to 25 °C temperature, a photoperiod of 12 h:12 h light/dark with a salinity of 30 practical salinity units (psu). The copepods were fed with the green algae, *Chlorella* sp. (approximately 6×10^4 cells/mL). Identification of the species was made using morphological characteristics and sequence identity of the universal barcode marker, mitochondrial DNA COI. To analyze the *TJ-CHH* transcript level of different stages, we prepared distinctive post-embryonic developmental stages for naupliar, copepodid, and adults using sieving method and stereomicroscopy observation based on our previous studies (Seo et al., 2006; Raisuddin et al., 2007).

2.2. Total RNA extraction and single-strand cDNA synthesis

Entire copepods (≈300 adult copepods) were homogenized in 3 volumes of TRIZOL® reagent (Molecular Research Center, Inc., OH, USA) with a tissue grinder and stored at −80 °C until use. Total RNA was isolated from the tissues according to the manufacturer's instructions. Genomic DNA was removed using DNase I (Sigma-Aldrich, St. Louis, Mo, USA). The quantity of total RNA was measured at 230, 260, and 280 nm with a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience, Freiburg, Germany). 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., Sparks, NV, USA). 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).

2.3. Cloning and characterization of T. japonicus CHH gene

Partial sequences of the *TJ-CHH* cDNA and genomic DNA were obtained from a database of genomic DNA information from *T. japonicus* (Lee et al., 2010). To identify the exon/intron boundary and transcript sequences, we designed 5′- and 3′-RACE primers (Table 1) and obtained RACE products according to the manufacturer's instructions. To obtain the full-length *TJ-CHH* cDNA, the GeneRacer kit (Invitrogen) was used according to the manufacturer's instructions. Serial steps of RACE were performed under the following conditions: 94 °C/4 min; 40 cycles of 98 °C/25 s, 55 °C/30 s, 72 °C/60 s; and 72 °C/10 min. The final PCR products were isolated from 1% agarose/TBE gel, cloned into pCR2.1 TA vectors (Invitrogen), and sequenced with an ABI PRISM 3700 DNA analyzer (Bionics Co., Seoul, South Korea). The promoter region of *TJ-CHH* was screened using Genetyx version 7.0 software.

2.4. Phylogenetic analysis

To place the identified TJ-CHH protein into its phylogenetic position, we aligned it with other CHHs, ion transport peptides (ITPs) and molt-inhibiting hormone (MIHs) proteins by Clustal X 1.83. The pairwise alignment parameter were set as 10 of gap opening and 0.1 of gap extension, and multiple alignment parameters were set as 10 of gap opening and 0.2 of gap extension. In total, 73 sequences were retrieved from Montagné et al. (2010) and GenBank/DDBJ/EMBL databases. Gaps and missing data were excluded from the analysis. The generated data matrix was converted to the nexus format, and the data matrix was analyzed with Mr. Bayes v3.1.2 program using a general time-reversible model. Four parallel Monte Carlo Markov chains of differentially heated chains were run for 1,000,000 generations with the posterior probabilities, and the sampling frequency assigned every 100 generations using the Jones, Taylor and Thornton amino acid substitution matrix. After analysis, the first 1,000 generations were deleted as a burn-in process. A consensus tree was constructed, and then visualized with Tree View of PHYLIP. Bayesian posterior probabilities (0.50) were indicated at each branch node.

2.5. Heat shock, UV-B irradiation, heavy metal treatment, and water-accommodated fraction (WAFs) exposure

The heat exposure procedure followed generally our previous studies on *T. japonicus* (Rhee et al., 2009). The habitats of *T. japonicus* were distributed from South Korea to Japan and Hong Kong with a temperature range from 13.2 °C to 30.4 °C in a year (Ki et al., 2009). Therefore, we set the experimental temperature range from 10 °C to 35 °C, as temperature in rockpools drastically changes daily and annually. Adult copepods ($n \approx 300$ for both sexes) were heat stressed at 10 °C, 30 °C, and 35 °C and were collected at 0, 3, 6, 12, 24, 48, 72, and 96 h. During the experiment, we supplied an algal diet of *Chlorella* every 24 h. Any significant mortality in experimental temperatures was not observed for 96 h except for naturally dying copepods as revealed by comparing with the control, and all dead copepods were excluded from subsequent experiments.

Table 1
Primer list used in this study.

Gene	Oligo name	Sequence (5′– > 3′)	Remarks	Efficiency (%)
TJ-CHH	5GSP1	CAATACCATCGCATTTACTGGGACTG	5′-RACE	
	5GSP2	GCAATGCGACAACACGCAGCAATG		
	3GSP1	GCGATGGTATTGGAATAGGGTTGTTC	3′-RACE	
	3GSP2	CCTCTATCTTTCCGACGAGGTCACCG		
	RT-F	GTCACCGGGATTTGCAGATTTAACTG	Real-time PCR	98.7
	RT-R	CCAAGGACACCAGTTGCTCAGAGTC	amplification	
18S rRNA	RT-F	GACTCAACACGGGAAATCTCACC	Real-time PCR	101.6
	RT-R	ACCAACTAAGAACGGCCATGCAC	amplification	

The overall exposure method of UV-B was based on our previous studies on *T. japonicus* (Kim et al., 2012; Rhee et al., 2012). We placed 300 adult copepods (both sexes) into a Petridish (150 mm; working volume, 20 mL) and exposed them to UV-B radiation under dark conditions at 3, 6, 9, 12, 15, 18, 21, and 24 kJ/m^2 using a UV-B lamp (wavelength range of 280–360 nm; G15T8E, Sankyo Denki, Japan). The copepods were harvested at 6 h after UV-B irradiation, and total RNAs were extracted for further analysis. Significant mortalities for each dose of UV-B radiation were not observed during 6 h. UV-B was irradiated in Petridish with a quartz cover (90T1, Taemin Science, Korea) to allow UV-B transparency and to prevent evaporation during UV-B exposure. The intensity of UV-B was measured by a UVX radiometer (Model M007-043; loaded Mid Range UVX 300-nm Probe, Model M007-045, CON-TROL-CURE®, USA). For a time-course experiment, copepods were exposed to UV-B (12 kJ/m^2) and sampled for 48 h. During the experiment, we supplied an algal diet of *Chlorella* every 24 h.

To study effects of environmental toxicant exposure on *TJ-CHH* transcript change, we exposed five metals (viz., Ag, silver; As, arsenic; Cd, cadmium; Cu, copper; Zn, zinc) to *T. japonicus*. All the chemicals were purchased from Sigma-Aldrich (purity > 99%). Stock solutions of metals were prepared in ultrapure water. The exposed concentrations of environmental toxicants were based on our previous studies on NOEC, LC_{10}, and LC_{50} of *T. japonicus* (Lee et al., 2007; Rhee et al., 2009). The following concentrations were used in all heavy metal exposures: 5, 10, 50, and 100 μg/L. The exposures were given for 96 h at a static renewal culture. Three replicates were used for each concentration containing approximately 300 adult copepods (both sexes) in each container. Fifty percent of culture water was renewed after 24 h and the desired concentrations of toxicants were maintained accordingly.

WAFs were employed from our previous study (Kim et al., 2013). Briefly, overall WAFs preparation and exposure method was followed by the CROSERF (The Chemical Response to Oil Spills: Ecological Research Forum) protocol that was established as a standardized method for preparing WAF of physically and chemically dispersed oil products, laboratory exposures to aquatic organism, and analytical chemistry measurement. To make WAFs standard, we used Iranian heavy crude (IHC) oil supplied from the Korea Institute of Ocean Science and Technology (KIOST), Geoje, South Korea. The WAF was made by mixing stabilized crude oil and seawater (25 g crude oil/L of 30 psu salinity) in a vessel over Teflon-coated cap with 20 ± 25% depth for 18 h at 25 °C. To obtain the water fraction, a screw bottle was allowed to settle for 6 h prior to the isolation of the WAF followed by the CROSERF guideline. The polyaromatic hydorcarbons (PAHs) of WAF were analyzed by gas chromatography mass spectrometry (GC-MS) and inductively coupled plasma mass spectrometry (ICP-MS) at KIOST and their composition are listed in supplementary table (Supplementary Table 1). Adult copepods ($n = 300$ of both sexes for each group) were exposed to three different diluted WAFs (40%, 60%, and 80%) for 48 h by static renewal maintenance. Significant mortalities for each WAF concentration were not observed during 48 h. In each group, copepods were quickly removed, and total RNAs were extracted as described above. Three replicates were used for each time-course experiment. Fifty percent of culture water was renewed after 24 h and the desired concentrations of WAFs were maintained accordingly.

2.6. Real-time RT-PCR

To investigate specific transcript patterns of *TJ-CHH* gene, real-time RT-PCR was performed with a comparative C_T method. Primer efficiency was calculated with the slope of the standard curve plot for each gene in a 10-fold dilution series. The slope values were −3.284 for *18S rRNA* gene and −3.3533 for the *TJ-CHH* gene, respectively. Each reaction included 1 μl of cDNA and 0.2 μM primer (real-time RT-F/R and *18S rRNA* RT-F/R) as shown in Table 1. Primers were designed after comparing exon/intron boundary to genomic DNA using GENRUNNER software (Hastings Software, Inc., NY, USA) and confirmed by Primo 3 program (Whitehead Institute/MIT center for Genome Research). Optimized conditions were transferred according to the following CFX96™ real-time PCR system protocol (Bio-Rad). Reaction conditions were as follows: 95 °C/3 min; 40 cycles of 95 °C/30 s, 55 °C/30 s, 72 °C/30 s. To confirm the amplification of specific products, cycles were continued to check the melting curve under the following conditions: 95 °C/1 min, 55 °C/1 min, and 80 cycles at 55 °C/10 s with 0.5 °C increase per cycle. SYBR® Green (Molecular Probes Inc., Invitrogen) was used to detect specific amplified products. Amplification and detection of SYBR® Green-labeled products were performed using CFX96™ real-time PCR system (Bio-Rad, Hercules, CA, USA). Data from each experiment were expressed relative to transcript levels of the *18S rRNA* gene to normalize the transcript levels of *TJ-CHH* gene between samples. All experiments were done in triplicate. Data were collected as threshold cycle (C_T) values (i.e., PCR cycle numbers where fluorescence was detected above a threshold and decreased linearly with increasing input target quantity) and used to calculate ΔC_T values of each sample. The fold change in the relative transcript level was calculated by the $2^{-\Delta\Delta_{CT}}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The SPSS ver. 17.0 (SPSS Inc., IL, USA) software package was used for statistical analysis. Data are expressed as means ± SD. Significant differences between the observations of control and the exposed groups were analyzed with one-way ANOVA followed by Tukey's test. Any difference showing $P < 0.05$ was considered significant.

3. Results

3.1. Sequence analysis of TJ-CHH cDNA, gDNA, and amino acid

This is the first report on the cloning and characterization of *CHH* gene in copepods. The full-length cDNA of *TJ-CHH* gene was completely sequenced (Supplementary Fig. 1) and deposited to GenBank (accession no. KC694109). The complete cDNA sequence of *TJ-CHH* was 716 bp in length, including 126 bp of 5′-untranslated region (UTR), 408 bp of the open reading frame (ORF), and 182 bp of 3′-UTR with a poly (A) tail.

TJ-CHH genomic DNA was 4,593 bp in length containing three exons and two introns (Fig. 1A). The splicing donor and acceptor sequence of the two introns was 5′-GT/AG-3′. The promoter region contained one estrogen response (ER) element, five heat shock response elements (HREs), two metal response elements (MREs), two steroidogenic factor 1 (SF-1) elements, one xenobiotic response element (XRE), one p53 binding site, and many putative transcription factor binding sites (data not shown).

The ORF encodes a polypeptide of 136 amino acids (Fig. 1B). Non-redundant (NR) BLASTX comparison showed the highest amino acid similarity (39% in identities and 45% in positives; E-value 2e-08) to CHH protein of the freshwater crab *Ptychognathus pusillus* (AER27833). Comparison of the deduced amino acid to those of other aquatic crustaceans revealed that TJ-CHH contains an identical CHH mature peptide region but showed a low similarity in the 5′-flanking polypeptide. The predicted molecular weight (MW; kilo Dalton; kDa) and theoretical p*I* of CHH protein were calculated in 14.9 kDa and 8.11, respectively. A putative signal peptide and CHH precursor-related peptide (CPRP) were observed. A dibasic processing site (Lys-Arg) was identified in K^{56} and R^{57} residues. The mature CHH amino acid contained six cysteine residues (C^{64}, C^{79}, C^{82}, C^{95}, C^{99}, and C^{108}) and five structural motifs (A1–A5) that are important characteristics of the CHH protein family.

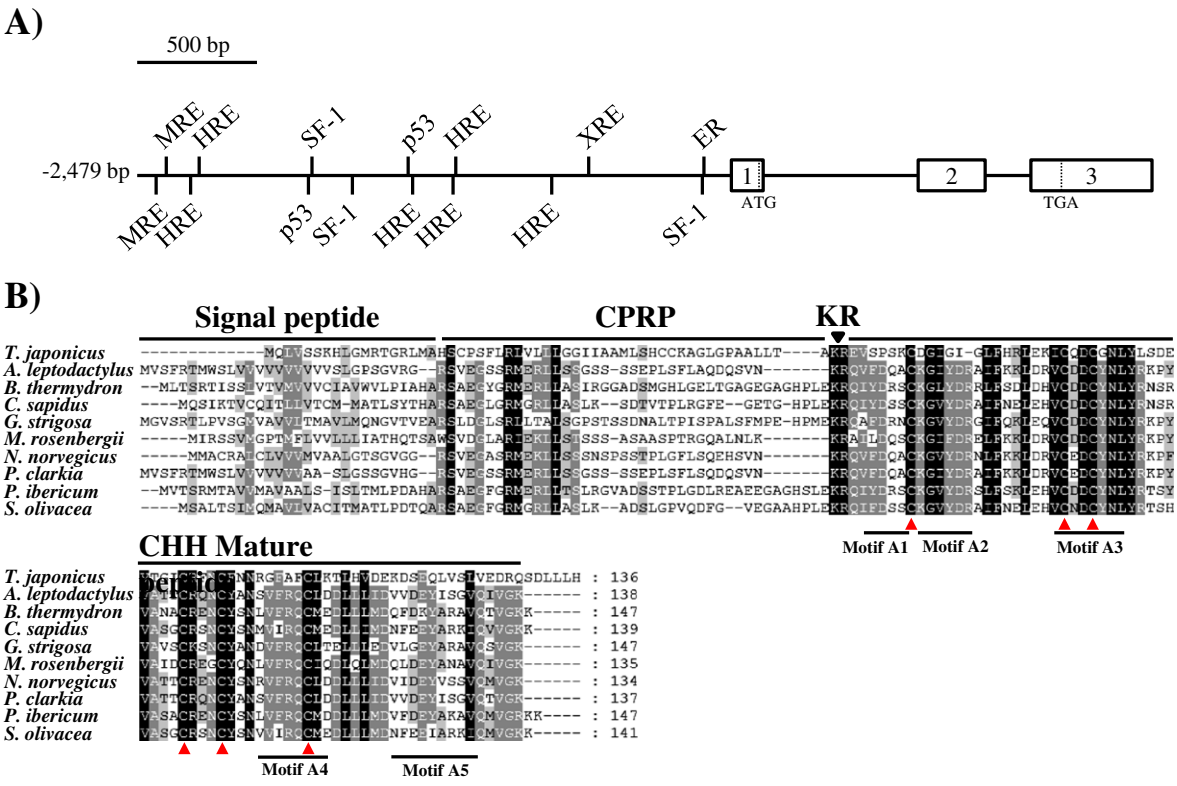

Fig. 1. (A) Genomic structure of *TJ-CHH* gene. Box represents exon and both exon and intron drawn to scale. Each length is indicated in nucleotides. Translation start codon (ATG) and termination codon are marked with a vertical dotted line. (B) Multiple alignments for amino acid sequence of *TJ-CHH* gene with decapod CHHs. Identical residues are boxed in black. The signal peptide and CHH precursor-related peptide (CPRP) are marked. The dibasic processing site is labeled by inverted triangles. The six cysteine residues are labeled with inverted red triangle. The conserved CHH mature peptide and five structural motifs (A1–A5) are marked. The sequences used were belonging to the following crustaceans: *Astacus leptodactylus* (AAX09331), *Bythograea thermydron* (AAK28329), *Callinectes sapidus* (AAS45136), *Galathea strigosa* (ABS01332), *Macrobrachium rosenbergii* (AAL40915), *Nephrops norvegicus* (AAQ22391), *Procambarus clarkia* (BAA89003), *Potamon ibericum* (ABA70560), and *Scylla olivacea* (AAQ75760).

3.2. Phylogenetic analysis

In our preliminary study, four CHH mature peptide-containing genes were cloned and tested to analyze whether these genes could be allocated either to the CHH family or to ion transport peptides (ITPs). A phylogenetic analysis revealed that three CHH mature peptide-containing genes (accession nos.: ITP1, KC694108; ITP2, KC694110; ITP3, KC694111) were closely related to insect ITPs, while one gene (*TJ-CHH* gene used in this study) formed a clade with decapod CHHs (data not shown). In this study, based on the formed clade, the phylogenetic topology of TJ-CHH proteins was more closely related to CHHs from other crustaceans such as astacurans (lobsters and crayfish), brachyurans (crabs), and palaemonids (shrimp). Our phylogenetic analysis showed that TJ-CHH protein was separated from insect ITPs and MIHs that are functionally more specialized than CHHs are (Fig. 2).

3.3. Analysis of the TJ-CHH transcript in different developmental stages

TJ-CHH transcripts were detected throughout various stages of development (Fig. 3). Of whole stages, early copepodids (C1-2) showed the highest level and adult males and females without eggs showed the lowest transcript levels. Although the transcript level of the *TJ-CHH* gene was slightly higher in males than in females, this difference was not significant. There was a significant difference ($P < 0.05$) between the levels of adult males and females with eggs.

3.4. Analysis of TJ-CHH transcript level with different heat treatments

The transcript level of *TJ-CHH* gene in a time-course experiment revealed that its highest abundance at 35 °C for 24 h (Fig. 4). The levels at 6 h and 48 h were still significantly higher than the control, although elevated levels were back to control levels 72 h after exposure. When *T. japonicus* were exposed to different temperatures such as below and above ambient culture temperature (25 °C), the *TJ-CHH* transcript increased only at 30 °C after 72 h.

3.5. Analysis of TJ-CHH transcript level after exposure to UV-B radiation

Specific LD_{10}, LD_{50}, and LD_{95} were calculated in our preliminary study as 5.62, 23.12, and 113.55 kJ/m^2 for males copepods and 5.89, 26.42, and 142.95 kJ/m^2 for female copepods, respectively (unpublished data). The transcript level of *TJ-CHH* gene was dose-dependently down-regulated after exposure to UV-B radiation, and then remarkably reduced at 24 kJ/m^2 (Fig. 5A). After checking the transcript levels of *TJ-CHH* at different doses of UV-B radiation, we analyzed *TJ-CHH* level at NOEC level (12 kJ/m^2) as a time-course experiment for 48 h. UV-B radiation significantly decreased *TJ-CHH* transcript for 12 h ($P < 0.05$) and slightly down-regulated it for 48 h (Fig. 5B).

3.6. Analysis of TJ-CHH transcript level after exposure to heavy metals

The transcript level of *TJ-CHH* gene was investigated in five heavy metal-exposed *T. japonicus* (Fig. 6). All the five tested metals caused an up-regulation of *TJ-CHH* transcript at 96 h. Of them, As and Zn significantly induced *TJ-CHH* transcription at 100 μg/L exposure at 96 h (Fig. 6B, E). The transcript level of *TJ-CHH* gene was changed approximately 2.2-fold ($P < 0.05$) at 10 μg/L of Cd exposure (Fig. 6C).

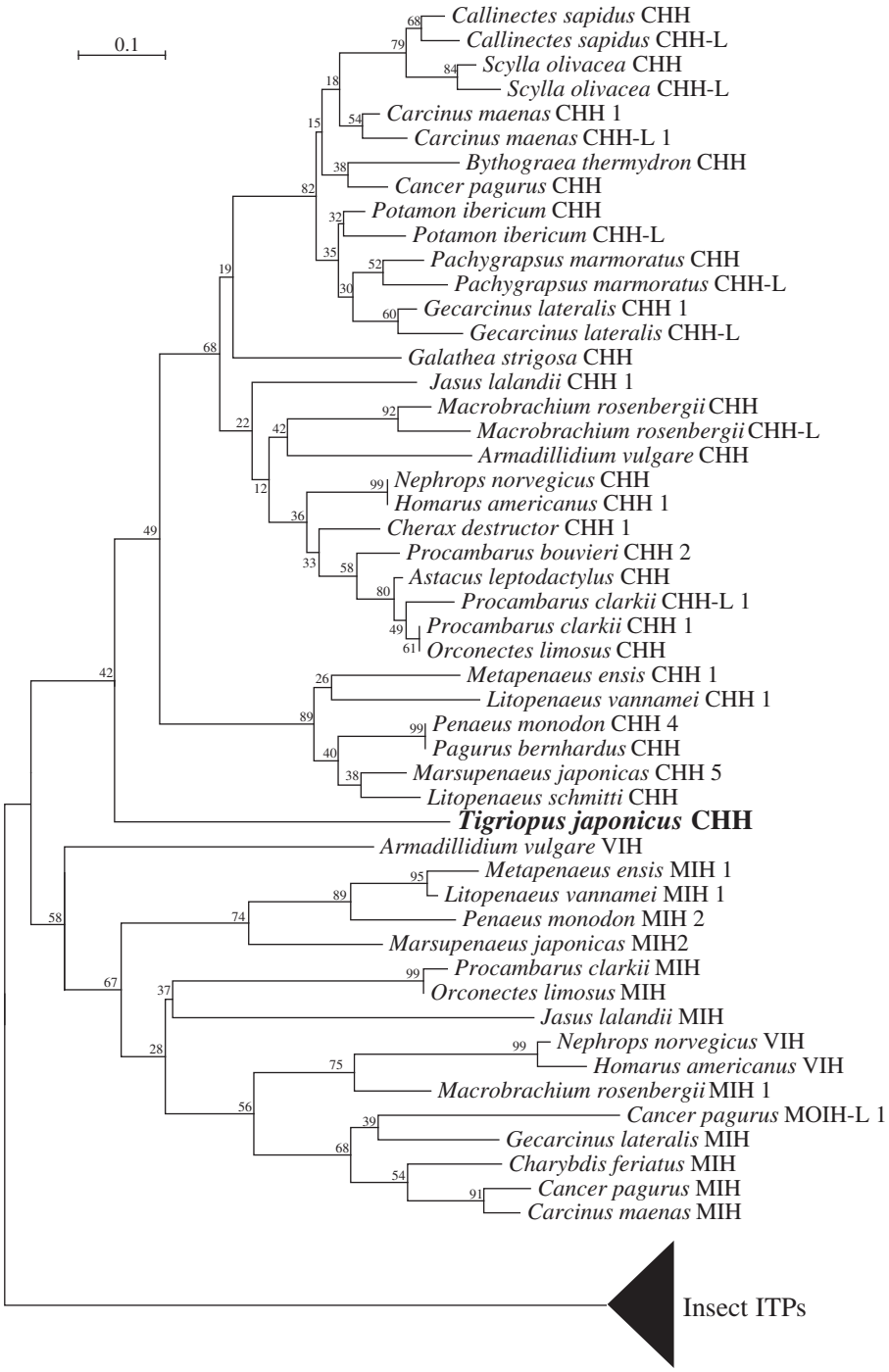

Fig. 2. Phylogenetic analysis of TJ-CHH compared to other 73 sequences that were retrieved from Montagné et al. (2010). Numbers at branch nodes represent the confidence levels of posterior probabilities. The scale bar represents genetic distance.

3.7. Analysis of TJ-CHH transcript level after exposure to WAFs

Exposure to different concentrations of WAFs (40%, 60%, and 80%) induced a dose-specific modulation of the transcript level of *TJ-CHH* gene for 48 h (Fig. 7). In case of 40% WAFs exposure, its transcript level was significantly increased at 6 h but down-regulated to a control level afterwards (Fig. 7A). After exposure to 80% WAFs, *TJ-CHH* mRNA time-dependently decreased for 48 h (Fig. 7C), while its transcript was down-regulated only at 24 h in 60% WAFs-exposed copepods (Fig. 7B).

4. Discussion

CHH, belonging to an important metabolic homeostasis hormone family, has a crucial role as an adaptive hormone involved in several physiologically relevant metabolisms. Despite its importance in numerous mechanisms, gene information and expression pattern of *CHH* gene were documented as yet only in decapods among the crustaceans. As a first report for a copepod crustacean, we identified and characterized the *CHH* gene here from the intertidal copepod, *T. japonicus*. In the promoter region of the *TJ-CHH* gene, several important transcription factors

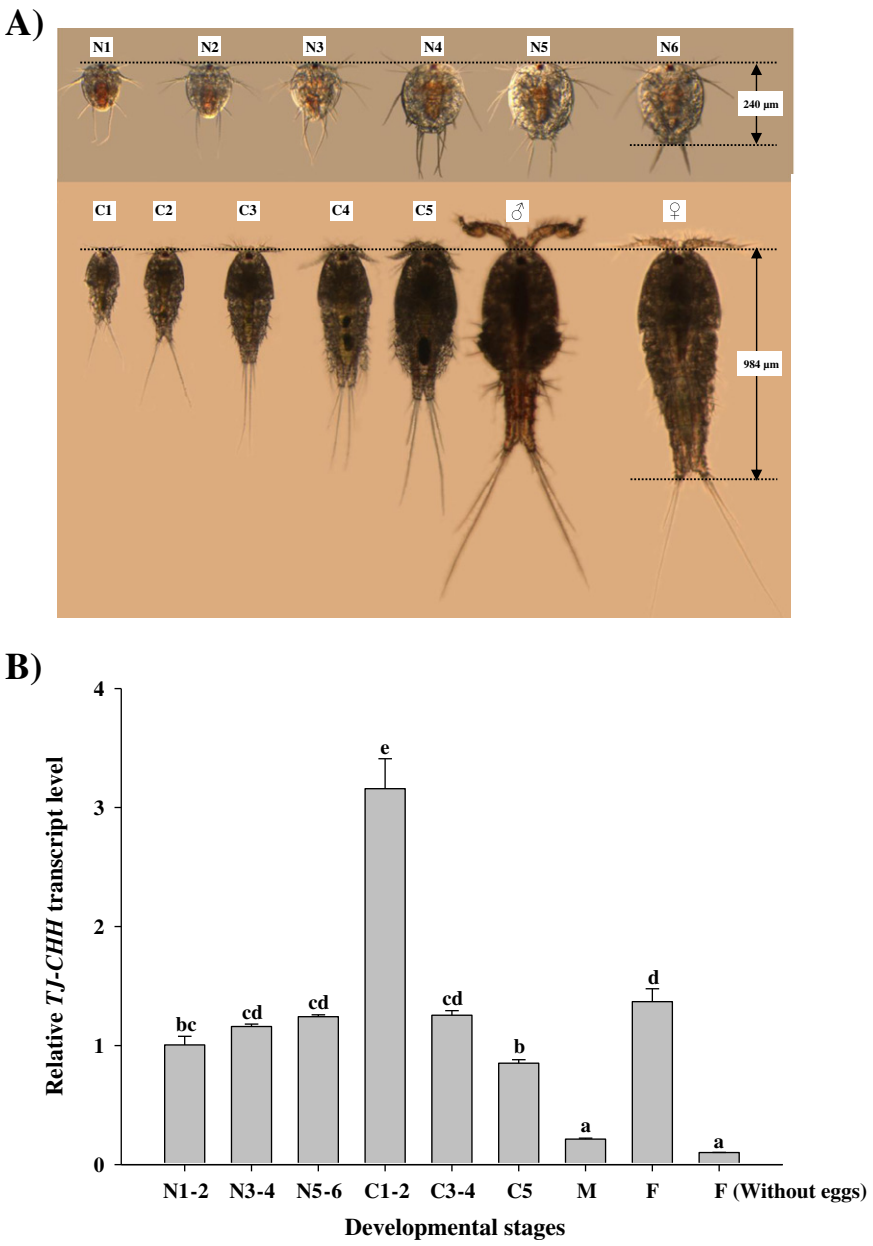

Fig. 3. (A) Different stages of development of the intertidal harpacticoid copepod, *Tigriopus japonicus*. Stages 1–6 are nauplius stages, and 5 stage in the second row (from 7 to 11) represent copepodite stages (figure reproduced with permission from Raisuddin et al., 2007). (B) Levels of *CHH* transcripts at various stages of development of *T. japonicus*. The values are means of three replicates. The *T. japonicus 18S rRNA* gene was used as a reference gene to normalize the transcript level. Significant differences were analyzed using Tukey's multiple-comparison test. Different letters above columns indicate significant differences. Significant difference was ascertained at $P < 0.05$.

such as ER, HREs, MREs, SF-1, XRE, and p53 binding site were detected, suggesting that this gene can be modulated by diverse environmental stressors. Structural characteristics also support the gene ontology of CHH as previously reported for crustacean CHH by Lacombe et al. (1999) using the nucleotide and amino acid sequence of *TJ-CHH*: a signal peptide, CPRP domain, a dibasic processing site, and a highly conserved CHH mature peptide domain that contains six conserved cysteine residues and five structural motifs. The phylogenetic analysis supported that TJ-CHH clustered with other decapod CHHs. However, we could not find CHH orthologs in other experimental copepod species due to a lack of gene information.

The life cycle of *T. japonicus* consists of 6 naupliar stages, 5 copepodid stages, and sexually dimorphic adults (Fig. 3A). Among the developmental stages of *T. japonicus*, the *TJ-CHH* transcript showed a consistent level. Even though there was a highest level detectable at C1-2 stage, a broad spectrum of the *TJ-CHH* gene over developmental stages indicated that this gene probably plays an important functional role in each stage. Among the developmental stages, the C1-2 stages are important for metamorphosing copepodid larvae, and therefore we assumed that *TJ-CHH* gene would be strongly involved in successful onset of copepodid stages to reach adult copepods. Regarding the role of CHH in crustacean, previously, in different decapods, Gorgels-Kallen and Meij (1985) identified CHH-producing cells in the eyestalk from crayfish post-embryonic stages with *in situ* hybridization and immunohistological assays, suggesting that CHH could be involved in naupliar and copepodid developmental stages of copepods as well. Fanjul-Moles (2006) reported a review on CHH that juvenile decapods show higher levels of CHH compared to adults, supporting the notion that *CHH* transcription could be higher in early developmental stages rather than adult stages in copepod as well. Zheng et al. (2010) also showed that two *CHH* transcripts in the eyestalk ganglia of the blue crab *Callinectes sapidus* dramatically increased in premolt stages

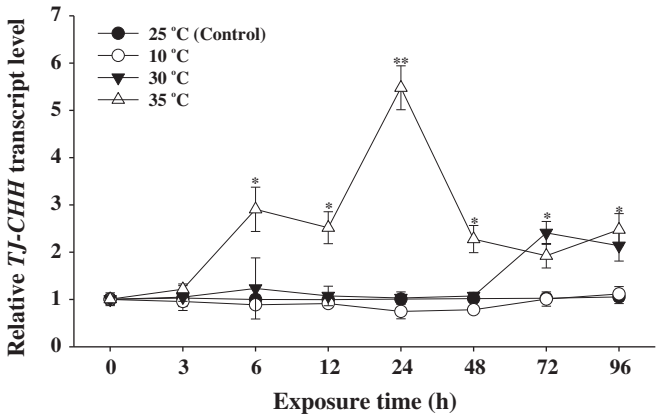

Fig. 4. Effects of different heat treatments for 96 h on *TJ-CHH* transcript level in *T. japonicus*. The *T. japonicus 18S rRNA* gene was used as a reference gene to normalize the transcript level. Data are means ± SD of three replicates of treated copepods. Significant differences between the means of transcript levels were analyzed by one-way ANOVA followed by Tukey's test. The symbols (* and **) indicate $P < 0.05$ and $P < 0.01$, respectively.

compared to intermolt and postmolt stages. The molting or ecdysis demands among others an enhanced metabolism due to massive ion and water uptake for body expansion (Chung et al., 1999). Therefore, another obvious role of CHH could be the regulation of stressful ecdysis-related hyperglycemia in the C1-2 stages of *T. japonicus*. In addition, express adult males and females without eggs lowest levels of *CHH* gene transcripts, suggesting that *CHH* is maintained at low level and functions differently as a hormonal metabolic regulator in the adult stage of *T. japonicus* than in development or sexual maturation. Regarding the relatively high level of *CHH* transcripts in ovigerous females of *T. japonicus* (Fig. 3B), we assume that one of the pleiotropic properties of CHH is associated with copepod ovarian physiology as observed in decapods (Webster, 1993; Fanjul-Moles, 2006). However, there is no report among crustaceans on differential *CHH* expression in males and females. Chung and Webster (2004) investigated that CHH was released in the embryogenesis of the neurosecretory complex in the green shore crab, *Carcinus maenas*, suggesting that CHH might have a function in the embryogenesis of copepods as well.

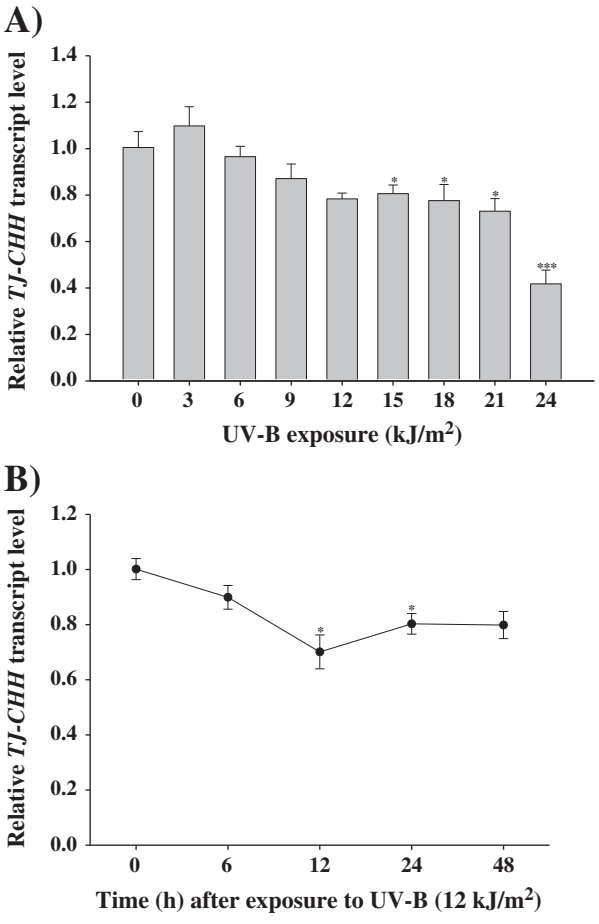

Fig. 5. (A) Dose–response change of *CHH* transcript at 48 h after exposure to UV-B radiation in *T. japonicus*. Transcript levels were analyzed at 0, 3, 6, 9, 12, 15, 18, 21, and 24 kJ/m^2 by real-time RT-PCR. (B) Effect of UV-B (12 kJ/m^2) exposure on the time-course of *CHH* transcript level in *T. japonicus*. Transcript levels were analyzed at 0, 6, 12, 24, and 48 h by real-time RT-PCR. The *T. japonicus 18S rRNA* gene was used as a reference gene to normalize the transcript level. Data are means ± SD of three replicates of treated copepods. Significant differences between the means of transcript level were analyzed by one-way ANOVA followed by Tukey's test. The symbols (* and ***) indicate $P < 0.05$ and $P < 0.001$, respectively.

Hyperglycemia is a common stress response of many aquatic animals that is regulated by CHH. To investigate the transcriptional response of *TJ-CHH* gene under natural environmental stressors, we used heat and UV-B radiation on *T. japonicus*. Relative high temperature (30 °C and 35 °C) enhanced levels of *TJ-CHH* transcript for 96 h in *T. japonicus*, indicating that the increase of *CHH* transcripts is associated with the general and physiological metabolism including crustacean hyperglycemia that is reported to be modulated by temperature change (Dean and Vernberg, 1965; Salminen and Lindquist, 1975; Keller and Orth, 1990). Previously, Chang et al. (1998) reported that 10 °C elevated temperature to 23 °C caused a significant increase of the hemolymph CHH level as a thermal stress response in the crayfish, *Orconectes limosus*. In the blue crab, *C. sapidus*, Chung and Zmora (2008) also showed a slight increase of *CHH* mRNA by temperature elevation from 22 °C to 29 °C in eyestalk and the pericardial organ. In fact, the intertidal copepods are continuously exposed to numerous hydrographic conditions including daily temperature changes. Based on our previous study (Rhee et al., 2009) on the strong induction of *T. japonicus Hsp70* (*TJ-Hsp70*) at the same temperature conditions, temperature change represents an obvious stressful factor. Previously, *T. japonicus* corticotropin-releasing hormone binding protein (*TJ-CRH-BP*) and ecdysone receptor (*TJ-EcR*) gene were significantly induced by elevated temperature to 35 °C within a short time period (90 min) (Lee et al., 2008a,b; Hwang et al., 2010). Taken together, we concluded that *T. japonicus* has sensitive endocrine receptors that induce a complex hormonal metabolism including CHH modulation to adapt to thermal stress. In addition, the presence of heat shock response elements (HREs) in the promoter region of *TJ-CHH* gene indicates that the transactivation of *TJ-CHH* gene would be regulated by a heat shock transcription factor (HSF), which is involved in the stress response by diverse environmental factors, suggesting that the *TJ-CHH* gene is responsive to thermal stress. We conclude, therefore, that changes of the *TJ-CHH* transcript are strongly related to an adaptive role of the CHH hormone in *T. japonicus*.

UV-B radiation represents one important environmental stressor in aquatic ecosystems. Exposure to high UV-B doses induces adverse effects on survival, enzyme activity, and movements (Dahms and Lee, 2010). Among crustaceans, the intertidal copepods are highly exposed to daytime UV-B radiation due to their habitat in the intertidal zone. Regarding the adaptive role of CHH, we suggest that the *TJ-CHH* transcript shows a stress response to UV-B radiation as an environmental stressor. In *T. japonicus*, the *CHH* transcript was down-regulated over LD_{10} value of UV-B radiation in a dose-dependent manner, indicating that elevated UV-B radiation induces detrimental effects on CHH-triggered physiological metabolism. Previous studies showed that UV-B causes direct damage to macromolecules such as DNA, protein, and lipid, and consequently leads to metabolic imbalance, mutation, and aging in aquatic animals (Häder et al., 2007; Dahms and Lee, 2010). In the crab *C. maenas*, Chung et al. (1999) reported that precisely regulated release of CHH is strongly associated with successful ecdysis. In gravid females and eggs of the grass shrimp *Palaemonetes pugio*, UV radiation

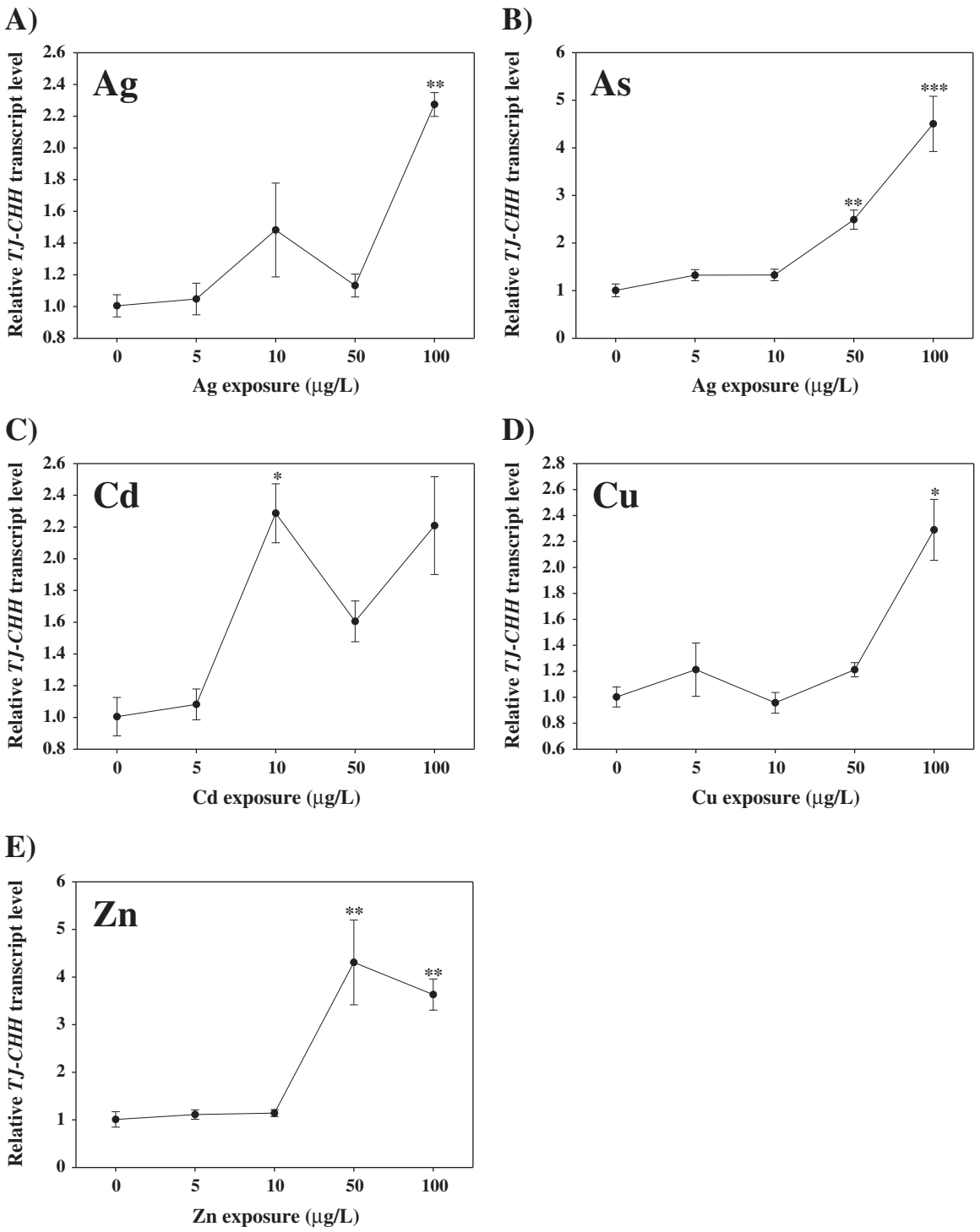

Fig. 6. Effects of (A) Ag, (B) As, (C) Cd, (D) Cu, and (E) Zn for 96 h on *TJ-CHH* transcript level in the copepod, *T. japonicus*. The *T. japonicus 18S rRNA* gene was used as reference gene to normalize the transcript level. Data are means ± SD of three replicates of treated copepods. Significant differences between the means of transcript levels were analyzed by one-way ANOVA followed by Tukey's test. The symbols (*, **, and **) indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

decreased the ecdysteroid level (Volz et al., 2002), suggesting that UV radiation induces detrimental effects on ecdysis and CHH-involved physiological development. Based on *T. japonicus*, mortalities against UV-B radiation, we demonstrated that decreased *TJ-CHH* levels induce molting inhibition or developmental retardation and finally decreases survival rate, even though the exact role of CHH in the ecdysis of *T. japonicus* is yet to be defined. In the monogonont rotifer, R.-O. Kim et al. (2011) reported that UV-B radiation induces growth retardation and decreases fecundity. Indeed, a higher dose over 12 kJ/m^2 of UV-B-induced oxidative stress, leading to the active involvement of antioxidant enzymes, DNA repair-related genes, and cell stress proteins upon UV-B damage in *T. japonicus* (Kim et al., 2012; Rhee et al., 2012; unpublished data). Therefore, the dose-dependent decrease of *TJ-CHH* transcript is correlated with the balanced repair capacity of cellular metabolism. Also, this clearly showed a correlation between high dose of UV-B-induced hazardous damage such as

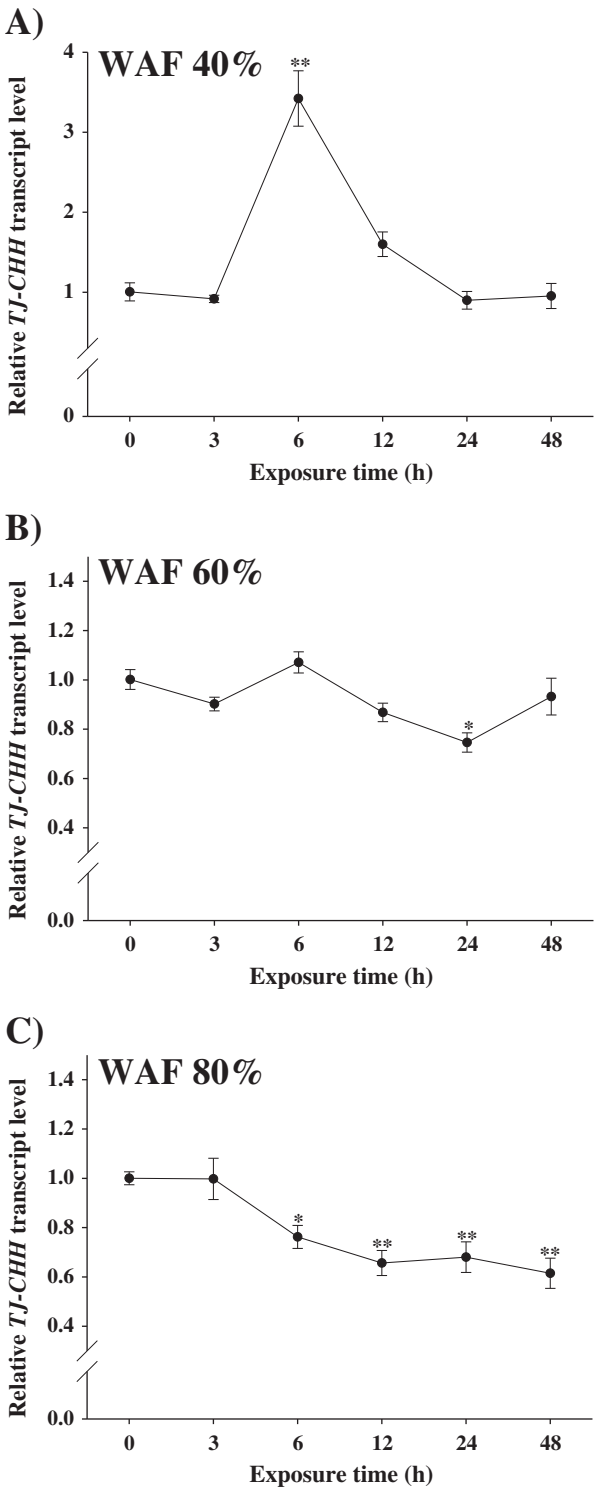

Fig. 7. Time-course effects of different concentrations of WAF, (A) 40%, (B) 60%, and (C) 80%, on *CHH* transcript level over time course (0, 3, 6, 12, 24, and 48 h) in the copepod, *T. japonicus*. The *T. japonicus 18S rRNA* gene was used as a reference gene to normalize the transcript level. Data are means ± SD of three replicates of treated copepods. Significant differences between the means of transcript level were analyzed by one-way ANOVA followed by Tukey's test. The symbols (* and **) indicate $P < 0.05$ and $P < 0.01$, respectively.

mortality increase and the presumable inhibited function via *CHH* transcript down-regulation.

Upon five heavy metal exposures, the *TJ-CHH* transcript level was significantly increased at high dose (50 and 100 μg/L) of As and Zn, while it was slightly increased in the Ag-, Cd-, and Cu-treated groups. In several decapods, Cd-, Cu-, and Hg-induced hyperglycemia (reviewed in Reddy et al., 1996; Lorenzon, 2005). In the shrimp *Palaemon elegans*, exposure to 0.1–5 mg/L of Cu and Hg induced a dose-related rapid release of CHH from the eyestalk into the hemolymph within 3 h (Lorenzon et al., 2004). Previously, *T. japonicus* has shown a strong tolerance against several heavy metals (Lee et al., 2007; Rhee et al., 2013). In a two-generation study of *T. japonicus* upon metal exposure significant modulations of seven life history traits (nauplius phase, development time, survival, sex ratio, number of clutch, nauplii per clutch, and fecundity) were revealed (Lee et al., 2008a,b). These results demonstrated that CHH-involved metal tolerance and CHH-mediated physiological development is modulated by chronic exposure of metals in *T. japonicus*. Regarding CHH-mediated mitigation of metal-stress, Kwok et al. (2009) suggested that Cu tolerance of *T. japonicus* would provide a multigeneration response through metabolic trade-offs in fitness costs as an adaptive physiological response. Taken together, we suggest that *TJ-CHH* is also involved as a trade-off mechanism for metal-triggered energetic budget balancing. Moreover, the presence of the metal response element (MRE) sites on the promoter region of the *TJ-CHH* gene is closely related to the transcript change of *TJ-CHH* gene upon heavy metal exposure. It became obvious that *T. japonicus*, which can remarkably tolerate metal-stressed conditions, adopt several strategies to maintain tolerance by CHH increase as well as by the development of cell chaperones and an antioxidant defense system (Seo et al., 2006; Rhee et al., 2009; Wang and Wang, 2009). We assume, therefore, that heavy metals cause an increase of *TJ-CHH* transcripts. This mechanism plays an adaptive role in heavy metal-induced cellular damage.

Our time-course experiment after WAF exposure demonstrated that the transcript level of *TJ-CHH* gene was modulated by the exposure to different WAFs concentrations. However, high concentrations of WAF exposure led to the inactivation of the *TJ-CHH* gene by toxic effect of WAFs, disrupting the general physiological metabolism or inducing physiological alterations. Among the several components of WAFs, naphthalene and its alkylated forms seem to be the main agents for toxicity. Reddy et al. (1996) reported naphthalene-induced hyperglycemia in the fiddler crab, *Uca pugilator*. However, limited information is available from WAFs or its components as anthropogenic chemicals as yet. In addition, several transcription factor binding sites such as xenobiotic response element (XRE) and estrogen response element (ERE) were also detected in the promoter region of *TJ-CHH* gene. XRE is a binding site of the aryl hydrocarbon receptor (AhR) and is involved in the regulation of gene expression in response to diverse xenobiotic compounds exposure. The presence of ERE is regulated by ER which is involved in the estrogen action by endocrine disrupting chemicals or xenobiotics. Therefore, our results suggested that *CHH* transcription is also regulated by estrogenic compounds or xenobiotics in *T. japonicus*. In fact, environmentally relevant concentrations of several estrogenic compounds (17β-estradiol, bisphenol A, 4-nonylphenol, *p-t*-octylphenol, and 20-hydroxyecdysone) induced endocrine disruption and delayed significantly the completion of the naupliar stages in the parental generation of *T. japonicus* (Marcial et al., 2003). Previously, EcR, a molecular target of the molting hormone ecdysteroid, was significantly down-regulated by the NOEC concentration (100 μg/L) of bisphenol A (BPA) exposure for 24 h (Hwang et al., 2010), as the *TJ-EcR* gene contained an XRE element in its promoter region, suggesting that xenobiotics affect ecdysteroid-regulated and CHH-involved mechanisms via transcriptional regulation. Also, our finding suggests that the *TJ-CHH* gene is modulated by WAFs and its components that may be regulated by the interaction of these chemicals with XRE and ERE.

Like most crustaceans, copepods may modulate energy metabolism through CHH-mediated glycolysis to overcome stressful environmental and physiological conditions. Therefore, we assume that changes of environmental conditions may be direct or indirect involved in the modulation of *CHH* transcription or hormone release as shown in previous studies (Webster, 1996; Chang et al., 1998; Chung and

Zmora, 2008). In fact, in *T. japonicus*, *TJ-CRH-BP*, and *TJ-EcR* were significantly modulated by several environmental stressors including pollutants (Lee et al., 2008a,b; Hwang et al., 2010), suggesting that this species has a conserved and adaptive endocrine system that reacts to environmental changes as shown by the CHH response. Regarding the endocrine metabolic energy regulation, CHH plays a critical role to meet energy requirements by regulating glucose and lipid levels (Webster, 1996; Fanjul-Moles, 2006). However, further study will be necessary to reveal the molecular mechanism of *TJ-CHH* gene regulation and on circulating metabolites of CHH by diverse environmental stressors in copepods.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2013.06.001.

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