β-Naphthoflavone Induces Oxidative Stress in the Intertidal Copepod, *Tigriopus japonicus*

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ABSTRACT: β-Naphtoflavone (β-NF) is a flavonoid and enhances oxidative stress in vertebrates with little information from aquatic invertebrates as yet. In this study, we investigated the effects of β-NF on the antioxidant defense systems of the intertidal copepod *Tigriopus japonicus*. To measure the β-NF-triggered changes in oxidative stress markers, such as intracellular reactive oxygen species (ROS), glutathione (GSH) concentration, residual glutathione *S*-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD) activity, *T. japonicus* were exposed to β-NF (0.5 and 1 mg/L) for 72 h. Significant ($P < 0.05$) induction of the intracellular ROS content (%) was observed in 1 mg/L of β-NF exposed *T. japonicus*, compared to the negative control and H_2O_2-exposed group. The GSH levels were significantly increased in the 0.5 mg/L of β-NF-exposed group for 12 h and 1 mg/L of β-NF-exposed groups for 12–24 h. GPx, GST, and GR activities showed a significant increase in the 1 mg/L β-NF-exposed group, indicating that β-NF induces oxidative stress in *T. japonicus*. To understand the effects of β-NF at the level of transcript expression, a 6K microarray analysis was employed. Transcript profiles of selected antioxidant-related genes were modulated after 72 h exposure to 1 mg/L of β-NF. From microarray data, 10 *GST* isoforms, *GR, GPx, PH-GPx*, and *Se-GPx* were chosen for a time-course test by real-time RT-PCR. *T. japonicus GST-S*, *GST-O, GST-M*, and *GST-D1* were significantly increased in a 1 mg/L β-NF-exposed group. *T. japonicus GPx*, *GR*, and *Se-GPx* mRNA levels were also significantly increased at both concentrations. Our results revealed that oxidative stress was induced by β-NF exposure in *T. japonicus*. Environ Toxicol 30: 332–342, 2015.

Keywords: *Tigriopus japonicus*; oxidative stress; biomarkers; β-naphthoflavone; β-NF

INTRODUCTION

β-Naphthoflavone (β-NF) is a synthetic derivative of a naturally occurring flavonoid and is known as an aryl hydrocarbon receptor (AhR) agonist which can strongly induce the cytochrome P4501A enzyme system (Ahmad et al., 2006;

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Aboutabl and El-Kadi, 2007; Zordoky and El-Kadi, 2007). β-NF has also the potential for a promutagen that can cause damage to DNA in fish (Johnson, 1992; Maria et al., 2002). Previously, Rushmore et al. (1991) suggested the bifunctional induction of intracellular oxidative stress by β-NF treatment in human hepatoma cells, HepG2. To date, many researchers demonstrated that PAHs including β-NF enhance the oxidative stress response via the generation of ROS in mammals (Elbekai et al., 2004; Dewa et al., 2009), fishes (Bacanskas et al., 2004; Bauder et al., 2005; Oliveira et al., 2008; Timme-Laragy et al., 2009), and a water flea *Daphnia magna* (Feldmannova et al., 2006). However, there is little information regarding the oxidative stress potential elicited by β-NF in marine invertebrates.

Oxidative stress causes a variety of diseases such as cancer, inflammatory and neurodegenerative disease as well as aging (Finkel and Holbrook, 2000). ROS provide reactive molecules and a source of oxidative stress, and has important roles in the redox cycling mediated by metabolites induced by xenobiotics. Overproduction of ROS can damage cellular macromolecules such as proteins, DNA, and lipids (Marnett, 2000). PAHs activate the expression of cytochrome P450 which is involved in the detoxification of xenobiotic compounds by transferring an oxygen radical to a substrate. One of the toxic mechanisms of PAHs is supposed to be the result of ROS generated by CYP1A-produced PAH metabolites, although PAHs have been known to elicit much of their toxicities through AhR pathways (Livingstone, 2001; Schlezinger and Stegeman, 2001; Meyer et al., 2002). The equilibrium between the amounts of free radicals and the antioxidant scavenging capacity could be disturbed by ROS-triggered oxidative stress (Lesser, 2006). Thus, involvement of several antioxidant enzymes and/or antioxidant molecules (e.g., GSH) against ROS and oxidative stress are the key protection mechanisms of aquatic organisms that are exposed to a wide range of environmental chemicals.

Tigriopus japonicus (Copepoda, Harpactidae) is an intertidal benthic copepod and has been used as a laboratory model animal because it is small in size, sexually dimorphic, showing high fecundity, short reproduction time (~14 days), being quite hardy (can survive broad ranges of temperature, salinity, pH), and easy culture under laboratory condition (Raisuddin et al., 2007). In addition, *Tigriopus* is easy to be collected from splash pools, and no specialized sampling strategy or device is needed. These characteristics make it a potential sentinel species for aquatic ecotoxicological studies (Raisuddin et al., 2007). The use of *T. japonicus* in acute ecotoxicity studies, transgenerational toxicity, and gene expression studies have demonstrated its potentials as a model organism (Lee et al., 2008; Ki et al., 2009; Rhee et al., 2009; Guo et al., 2012; Kim et al., 2013). However, there is a paucity of information regarding the toxic effect of β-NF in *T. japonicus* and other copepods with reference to oxidative stress, although extensive ecotoxicological studies using *T. japonicus* have been performed with several environmental pollutants (Marcial et al., 2003; Kwok and Leung, 2005; Lee et al., 2007; Raisuddin et al., 2007). In this paper, we analyzed antioxidant defense systems with transcript profiling to better understand the oxidative stress response patterns in *T. japonicus* upon β-NF exposure.

MATERIALS AND METHODS

Copepods

The intertidal copepod, *T. japonicus* was maintained and reared in 0.2-μm filtered artificial sea water (TetraMarine Salt Pro, Tetra™, OH) adjusted to 25°C, a photoperiod of 12 h:12 h light/dark, and a salinity of 30 practical salinity unit (psu). The copepods were fed with the algae *Tetraselmis suecica* ($\sim 0.4 \times 10^9$/L/day). Identification of the species was made by morphological characteristics and the sequence identity of the universal barcode marker, mitochondrial DNA COI.

β-NF Exposure

A large number (≈300 in each group) of adult *T. japonicus* (mixed gender) were exposed to β-NF (0.5 and 1 mg/L; Sigma-Aldrich, St. Louis, MO). Two control groups of *T. japonicus* were prepared with control and solvent (DMSO, >0.001%) control. The exposure concentration of β-NF was selected based on acute toxicity values reported earlier in several aquatic invertebrates (Snyder, 1998; Reichert and Menzel, 2005; Watanabe et al., 2008). Copepods were exposed for up to 72 h. At the interval of 0, 6, 12, and 24 h, *T. japonicus* was sampled from each group and used for total RNA isolation and for further biomarker analysis. During exposure, the copepods were cultured in natural seawater at a density of four copepods per milliliter in a glass vessel (32 practical salinity unit (psu), dissolved oxygen (DO) 3.9 ± 0.15 mg/L) at 25 ± 1°C under 12 h light: 12 h dark cycle. The copepods were not fed during the exposure period.

Total RNA Isolation

Copepods (≈300 in each group) were gently sampled from the experimental chambers and immediately grounded in liquid nitrogen using glass mortar and pestle in three volumes of TRIZOL® (MRC, USA) reagent with a tissue grinder. Total RNA was extracted according to the manufacturers' protocol and stored at −80°C until use. DNA digestion was performed using DNase I (Sigma, St. Louis, Mo). Total RNA was quantified by absorption of light at 230, 260, and 280 nm (A230/260, A260/280) using 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 on 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. After determining the RNA quality, a single-strand cDNA was synthesized from 2 μg of total RNA from each sample using an oligo$(dT)_{20}$ primer for reverse transcription in 20 μl reactions (SuperScript™ III RT kit, Invitrogen, Carlsbad, CA).

Gene Expression Analysis Using a *T. japonicus* 6K Oligomicroarray

T. japonicus 6K oligomicroarray was developed according to the procedures described by Ki et al. (2009). A total of 6K oligonucleotide probes (each of 35 nucleotides in length) were designed by comparison of all available EST sequences in a web-based tool (CombiMatrix Corp., Mukilteo, WA). Subsequently, the 6K oligonucleotide probes were synthesized onto a plate provided by CombiMatrix following the manufacturers' protocol. We used MessageAmpTM II-Biotin Enhanced Single Round aRNA Amplification Kit (Ambion, Austin, TX) which is based on the RNA amplification protocol. Ambion's MEGAscript® IVT technology was used to generate hundreds to thousands of antisense RNA copies of each mRNA in the sample. RNA labeling and hybridization were carried out following a protocol for hybridization and imaging of CustomArray™ 12K Microarrays provided by CombiMatrix. Hybridized microarrays were scanned at PMT 500–700 (pixel size 5, focus position 130) using a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA). After scanning was completed the resulting images were visualized and processed for data extraction as per the standard procedure (https://webapps.combimatrix.com). Microarray data for individual samples were normalized by a global normalization using the probes with a signal value greater than zero, lesser than 60,000 (saturation value) and cumulatively greater than the lowest 5% of each sample's signal value. Finally, a total of 5463 spots remained for the subsequent analysis. Fold-change was applied to determine differentially expressed sets of genes across three experimental samples. The significance level of fold-change was tested by a local pooled-error (LPE) test of significance analysis in R package (version 2.6.1). In addition, the complete linkage hierarchical clustering was performed based on Euclidean distance measure of samples using the normalized significant genes. Avadis Prophetic version 3.3 (Strand Genomics, Bangalore, India) and Arrayassit 5.5.1 (Agilent Technologies) were used for data analysis and statistics.

Real-Time RT-PCR Analysis After β-NF Exposure

The mRNA expression of antioxidant genes was measured using real-time RT-PCR. The primer sequences were the same as being used in our previous study (Lee et al., 2008). Each reaction included 1 μl of cDNA which was reversely transcribed from 2 μg of total RNA and a 0.2 μM primer (real-time RT-F/R for each antioxidant gene and *18S rRNA* RT-F/R). The optimized conditions were transferred according to the following CFX96™ real-time PCR system protocol (Bio-Rad). The reaction conditions were as follows: 95°C/3 min; 40 cycles of 95°C/30 s, 55°C/30 s, and 72°C/30 s. To confirm the amplification of 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. SYBR® Green (Molecular Probes, Invitrogen) was used to detect the specific amplified products. Amplification and detection of the SYBR® Green-labeled products were performed using a CFX96™ real-time PCR system (Bio-Rad, Hercules, CA). The data from each experiment were expressed relative to expression levels of the *18S rRNA* gene to normalize the expression levels between samples. All experiments were done in triplicate. Data were collected as threshold cycle (C_T) values (PCR cycle number where fluorescence was detected above a threshold and decreased linearly with increasing input target quantity), and used to calculate the ΔC_T values of each sample. The fold change in the relative gene expression was calculated by the $2^{-\Delta\Delta C_T}$ method.

ROS Measurement

After β-NF exposure (0.5 and 1 mg/L) for 72 h, intracellular reactive oxygen species (ROS) was measured using 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probe, Invitrogen™, Eugene, Oregon, USA). The overall experiment was performed with minor modifications by the procedures of LeBel et al. (1992). After exposed to β-NF, ~500 individuals were washed with PBS (pH 7.4) twice, and then homogenized in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of PMSF, pH 7.4) using a Teflon homogenizer. The homogenate was centrifuged at 15,000*g* for 15 min at 4°C. Upper aqueous layer was collected for ROS measurement and incubated at 28°C for 30 min in PBS containing the H_2DCF-DA at a final concentration of 40 μM. We checked the increased fluorescence intensity of DCF by Varioskan® FLASH (Thermo Electron Corporation) with appropriate wavelengths (excitation at 485 nm, emission at 520 nm). Hydrogen peroxide (1 mM) exposed group was used as a positive control for the induction of ROS. The ROS measurements were normalized by total protein and represented as a percentage of fluorescence of DCF.

Measurement of Glutathione Content and Glutathione-Related Enzymes' Activities

To check the induction of oxidative stress by exposure to β-NF, the change of oxidized glutathione (GSH) to reduced GSH was determined by an enzymatic method using

TABLE I. List of antioxidant related genes, which were upregulated in β-NF-exposed *T. japonicus* studied by 6K microarray

Probe No.	Probe Name	Gene Name	Fold Change (> ±1.5)
5975	T6K5282	Cytochrome P450 13A	2.34
5325	T6K5283	Cytochrome P450 25A	2.02
5398	T6K5356	GST-sigma	73.52
6040	T6K5998	GST-sigma	50.04
5344	T6K5302	GST-mu4	3.92
3522	T6K3480	UDP-glucuronosyl transferase	3.61
5993	T6K5951	GST-mu5	3.03
5345	T6K5303	GST-mu5	2.75
5986	T6K5944	GST-delta-epsilon 1	2.50
5991	T6K5949	GST-mu2	2.45
5336	T6K5294	GST-delta-epsilon 1	2.31
2340	T6K2298	Brain type mu-glutathione S-transferase	2.31
5987	T6K5945	GST-delta-epsilon 2	2.17
5992	T6K5950	GST-mu4	2.17
1028	T6K0986	Glutathione peroxidase 1	2.15
288	T6K0288	Glutathione peroxidase	2.03
2757	T6K2715	Manganese superoxide dismutase	1.99
5333	T6K5291	Glutathione peroxidase 1	1.98
5983	T6K5941	Glutathione peroxidase 1	1.94
2272	T6K2230	Glutathione peroxidase	1.93
5988	T6K5946	GST-theta	1.89
3855	T6K3813	GST-1-1 (GST class-theta)	1.85
5338	T6K5296	GST-theta	1.85
3695	T6K3653	Superoxide dismutase [Cu-Zn]	1.81
5375	T6K5333	Se-dependent glutathione peroxidase	1.78
3595	T6K3553	Selenium dependent salivary glutathione peroxidase	1.74
2017	T6K1975	GST	1.74
5985	T6K5943	Glutathione peroxidase 7	1.72
4579	T6K4537	Glutathione peroxidase	1.72
5335	T6K5293	Glutathione peroxidase 7	1.69
2250	T6K2208	Glutathione peroxidase	1.64
393	T6K0393	GST-like protein	1.64
66	T6K0066	Glutathione peroxidase	1.61
5337	T6K5295	GST-delta-epsilon 2	1.61
5984	T6K5942	Glutathione peroxidase 6	1.59
5272	T6K5230	GST-kappa 1	1.58
5066	T6K5024	Phospholipid hydroperoxide glutathione peroxidase	1.55

BIOXYTECH® GSH-420™ kit (*Oxis*Research®, Portland, USA). After β-NF (0.5 and 1 mg/L) treatment for 36 and 72 h, *T. japonicus* was washed in 0.9% NaCl. Rinsed sample was homogenized in trichloroacetic acid at a ratio of 1–20 (w/v) using a Teflon homogenizer. The homogenate was centrifuged at 3000*g* for 10 min at 4°C. The upper aqueous layer was collected for a GSH content assay according to the manufacturer's protocol. GSH content was measured at an absorbance of 420 nm using spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience), and standard curves were generated using GSH equivalents (0, 150, and 350 μM).

Glutathione peroxidase (GPx) and glutathione reductase (GR) activities were measured by an enzymatic method using BIOXYTECH® GPx-340™ and GR-340™ kits, respectively (*Oxis*Research®, Portland, USA). After β-NF (0.5 and 1 mg/L) treatment for 36 and 72 h, *T. japonicus* were homogenized in cold buffer (50 mM Tris-Cl, 5 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.5) at a ratio of 1–4 (w/v) using a Teflon homogenizer. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C. The upper aqueous layer containing the enzyme was collected for an enzymatic assay. GPx and GR activities were measured at an absorbance of 340 nm using a spectrophotometer at 25°C.

Glutathione *S*-transferase (GST) activity was measured following our previous report (Rhee et al., 2007). After β-NF (0.5 and 1 mg/L) treatment for 36 and 72 h, *T. japonicus* was homogenized in cold buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, 0.2 mM DTT, and 0.1 mM PMSF, pH 7.4) at a ratio of 1–4 (w/v) using a Teflon homogenizer. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C.

The cytosolic fraction containing the enzyme was collected for an enzymatic assay using 1-chloro-2, 4-dintitrobenzene (CDNB) as a substrate. An enzymatic assay monitored the conjugation of CDNB and GSH at 340 nm using a spectrophotometer at 25°C.

Overall SOD enzyme activities were prepared according to our previous study (Kim et al., 2011). Total SOD activities were measured with an enzymatic method using SOD assay kit (Sigma-Aldrich Chemie, Switzerland). After β-NF (0.5 and 1 mg/L) treatment for 36 and 72 h, the copepods were homogenized in ice-cold buffer (0.25 M sucrose, 0.5% triton X-100, pH 7.5) at a ratio of 1–4 (w/v) using a Teflon homogenizer. The homogenate was centrifuged at 30,000*g* for 30 min at 4°C. The upper aqueous layer containing the enzyme was collected for the enzymatic assay according to the manufacturer's protocol. Total SOD activities were then measured at an absorbance of 440 nm using a spectrophotometer at 25°C. Enzyme activities were normalized by total protein and represented as % of control.

Statistical Analysis

SPSS version 17.0 (SPSS, Chicago, IL) software package was used for statistical analysis. All data were expressed as mean ± SD. Significant differences between the observations of control and exposed groups in β-NF exposure study were analyzed using one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered as significant.

RESULTS

Analysis of Antioxidant-Related Genes in Microarray

From whole microarray results, oxidative stress-related genes were selected and analyzed in this study as a Minimum Information About a Microarray Experiment (MIAME). *T. japonicus* exposed to β-NF (1 mg/L) showed a significant increase of several antioxidant-related genes in mRNA expression profiles using 6K cDNA oligochip (-Table I). Two cytochrome P450 (*CYP*) genes, *CYP13A* and *CYP25A*, were upregulated 2.34 and 2.02-fold. Real-time RT-PCR analysis confirmed the transcriptional expression of *CYP13A* (4.56-fold) and *CYP25A* (3.89-fold). Among *GSTs* increased, the transcript of *GST-S* showed the highest expression (50.04- and 73.52-fold).

β-NF-Induced Modulation of GSTs in *T. japonicus*

The expression level of 10 *GST* genes (*mGST2, mGST1, GST-Z1, GST-T3, GST-S, GST-O, GST-M5, GST-K, GST-D2*, and *GST-D1*) was measured by real-time RT-PCR in β-NF (0.5 and 1 mg/L)-exposed *T. japonicus* [Fig. 1(A,B)] for 72 h. Each value and their statistical analysis were represented in Table II. The expression pattern of *GST*s mRNA depended on the type of isoforms. GST sigma (*GST-S*) and omega (*GST-O*) increased 12 h after exposure of 0.5 mg/L of β-NF in a time-dependent manner, and showed the highest expression level after 72 h exposure at both concentrations of β-NF. GST-delta-epsilon 1 (*GST-D1*), GST-mu 5 (*GST-M5*), and GST-theta 3 (*GST-T3*) slightly increased from 24 h after β-NF exposure. Other *GST* isoforms did not change significantly.

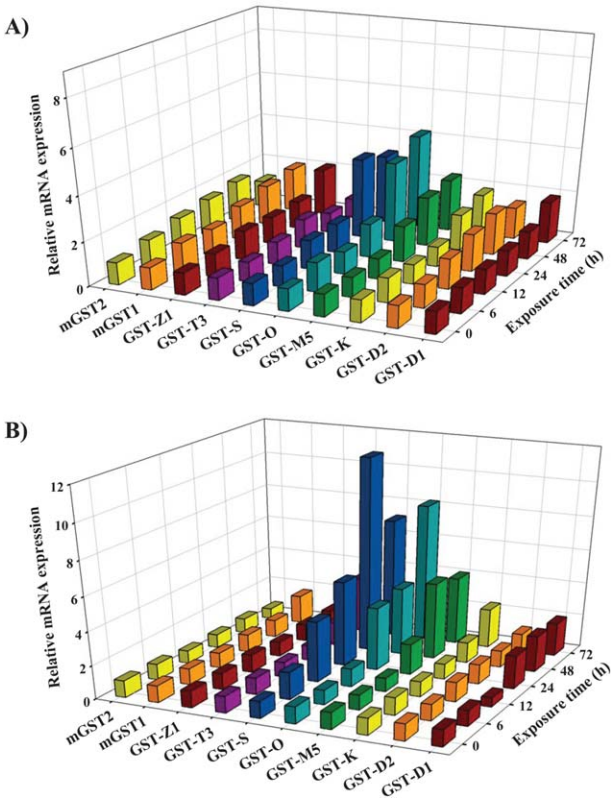

Fig. 1. Time-course mRNA expression of *GST* gene family after exposure to (A) 0.5 and (B) 1mg/L β-NF for 72 h. Each value and their statistical analysis were represented in Table II. *T. japonicus 18S rRNA* gene was used as a reference gene to normalize the expression. Each value represents the mean of three replicate samples compared to the control group (0 h). Abbreviation of *GST* isoforms: *mGST*, microsomal; *GST-Z*, zeta; *GST-T*, theta; *GST-S*, sigma; *GST-O*, omega; *GST-M*, mu; *GST-K*, kappa; *GST-D*, delta–epsilon. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Time Course Expression of Oxidative Stress Biomarkers

As a biomarker of oxidative stress, the transcript expression levels of *GR* and three types of *GPx* were measured after exposure to 0.5 [Fig. 2(A)] and 1 [Fig. 2(B)] mg/L of β-NF for 72 h by real-time RT-PCR. In 0.5 mg/L of the β-NF-exposed group, transcripts of *GR*, *GPx*, and *Se-GPx* were

TABLE II. Expression patterns of *GST* genes in *T. japonicus* exposed to different concentrations of β-NF[a]

β-NF Concentration / Genes	Exposure Time (h)					
	Normalized Transcript Value					
β-NF 0.5 mg/L	0	6	12	24	48	72
GST-D1	1.00	1.15	1.13	1.15	1.26	1.90
GST-D2	1.00	1.05	1.37	1.68	1.90	1.44
GST-K	1.00	1.11	0.88	0.89	1.63	1.85
GST-M5	1.00	0.90	0.93	1.63	2.23	2.36[b]
GST-O	1.00	1.33	1.01	1.52	3.63[c]	4.24[d]
GST-S	1.00	0.96	1.33	1.33	3.83[c]	3.12[b]
GST-T3	1.01	0.90	1.03	1.33	0.91	0.89
GST-Z1	1.00	1.06	1.33	1.23	1.25	2.11
mGST1	1.00	1.33	1.20	1.56	1.82	1.98
mGST2	1.00	1.25	1.52	1.69	1.85	1.20
β-NF 1 mg/L	0	6	12	24	48	72
GST-D1	1.00	0.95	0.55[b]	1.99	2.16	1.99
GST-D2	1.00	0.97	1.16	1.17	0.99	1.06
GST-K	1.01	1.06	0.91	0.92	1.23	2.35[b]
GST-M5	1.01	0.89	0.81	1.83	4.59[d]	4.05[d]
GST-O	1.01	0.87	1.08	3.77[b]	4.04[b]	8.24[d]
GST-S	1.01	1.63	3.63[b]	5.06[b]	11.52[d]	7.14[d]
GST-T3	1.00	0.98	0.90	0.92	1.40	3.33[b]
GST-Z1	1.00	0.99	1.07	0.87	1.00	0.97
mGST1	1.00	0.96	0.90	1.06	1.03	1.70
mGST2	1.00	0.95	0.76[b]	0.81[b]	0.88	0.63

[a]All the mRNA expression values were expressed as the fold change.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

induced after 48 h till 72 h. However, after exposure to 1 mg/L of β-NF, transcripts of *GR*, *GPx*, and *PH-GPx* significantly increased relatively earlier, compared to 0.5 mg/L of β-NF exposure.

Effect of *β*-NF on the Generation of ROS of *T. japonicus*

ROS level was measured in β-NF (0.5 and 1 mg/L)-exposed *T. japonicus* for 72 h. As a result, intracellular ROS levels were elevated in 1 mg/L of β-NF-exposed *T. japonicus* for 72 h. Particularly, an induction (173%) of ROS was observed at high dose (1 mg/L) when compared to control group (100%) with a significance ($P < 0.05$) (Fig. 3). As a positive control, 1 mM of H_2O_2-exposed *T. japonicus* showed a highly significant induction of ROS (354%) compared to the control ($P < 0.001$).

GSH Content, GST, GPx, GR, and SOD Activity After Exposure to *β*-NF

Antioxidant enzyme activities were measured at 0.5 and 1 mg/L of β-NF-exposed groups for 36 and 72 h, respectively, and compared to negative control and solvent (DMSO) control. As a result, significant changes in GSH level were observed for 12–24 h (Fig. 4). All the residual GST, GPx, GR, and SOD activities were significantly induced at 1 mg/L of β-NF-exposed group for 72 h (Fig. 5). GST and GPx activity were significantly induced at 1 mg/L of β-NF-exposed group at 36 h. Group exposed to 0.5 mg/L of β-NF had no effect to antioxidant enzyme activities except for GPx activity at 72 h.

DISCUSSION

β-NF is known as a strong inducer of cytochrome P450 (*CYP*) gene in vertebrates. Particularly, β-NF promoted cytochrome P4501A-dependent ethoxyresorufin *O*-deethylase (EROD) activities in aquatic animals (Pacheco and Santos, 1998). In *T. japonicus*, two CYP genes (*CYP13A* and *CYP25A*) were upregulated as shown in Table I, although invertebrates have no CYP1A as vertebrates have in their genome. Using *T. japonicus* 6K gene chip, we successfully discovered several upregulated antioxidant-related genes as shown in Table I, supporting our previous findings (Figs. 1 and 2). Thus, a microarray approach provides a sensitive, fast, and useful methodology to detect molecular biomarkers and to better understand the molecular mode of action of defense mechanisms upon environmental pollution. In general, an organism's genome responds to toxicity induced by environmental contaminants by the expression of responsive

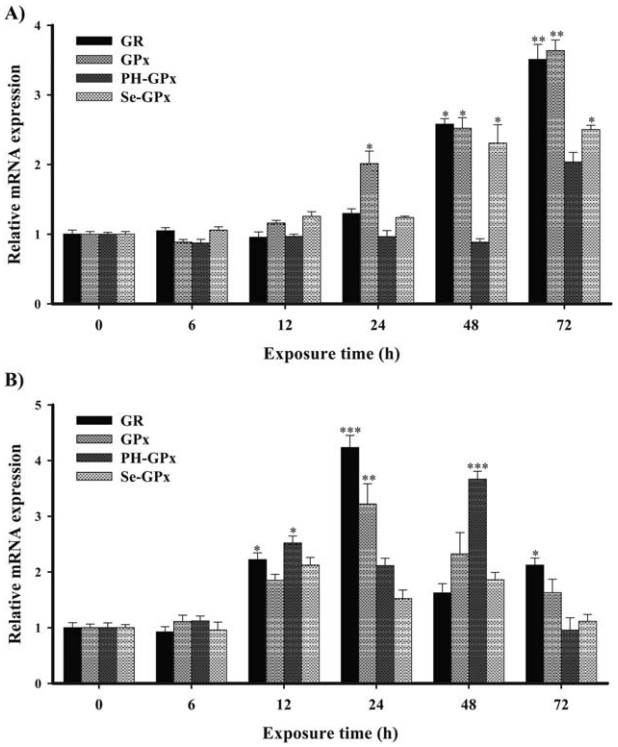

Fig. 2. Time-course mRNA expression of antioxidant enzymes (*GR*, GPx, *PH-GPx*, and *Se-GPx*) after exposure to (A) 0.5 and (B) 1mg/L β-NF for 72 h. *T. japonicus 18S rRNA* gene was used as a reference gene to normalize the expression. Each value is the mean of three replicate samples compared to the control (0 h). Data are shown as means ± SD. Significant differences between the means of mRNA level were analyzed by one-way ANOVA followed by Tukey's test. Asterisks (*, **, and ***) indicate significant change with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

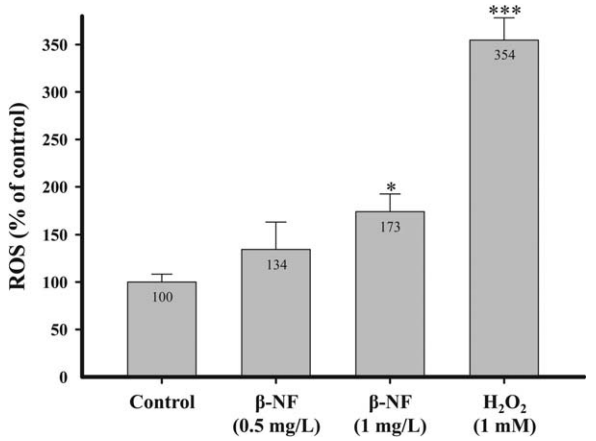

Fig. 3. Change of reactive oxygen species (ROS) level after exposure to β-NF (0.5 or 1 mg/L) and H_2O_2 (1 mM) for 72 h relative to control (100%). Each value is the mean of three replicate samples and data are shown as means ± SD. Significant differences between the means of ROS levels were analyzed by one-way ANOVA followed by Tukey's test. Asterisks (* and ***) indicate a significant change with $P < 0.05$ and $P < 0.001$, respectively.

genes that can be used as molecular biomarkers (Snape et al., 2004). Therefore, ecotoxicogenomic tools provide a mechanistic understanding of the mode of action in aquatic ecotoxicology. In fact, the application of microarray techniques to the copepod *T. japonicus* after exposure to Cu, Mn, and UV-B represented that microarrays may also be helpful in delineating the mechanism of adaptation and resistance in copepods exposed to recurrent pollutants or environmental changes in the marine environment (Ki et al., 2009; Rhee et al., 2012; Kim et al., 2013). Thus, application of ecotoxicogenomic tools will provide useful insights for a better understanding on the fate and mechanism of the mode of action of defense induced by environmental pollutants.

The mRNA expression of *GST*s in *T. japonicus* showed different expression patterns during a time-course upon β-NF exposure. Among 10 *GST* genes, five *GSTs* (*GST-S, GST-O, GST-M5*, *GST-T3*, and *GST-D1*) showed a significant correlation with β-NF exposure, whereas expression of other *GSTs* did not change significantly over an exposure period of 72 h. We can assume that several reasons can be proposed to these kinds of up- or downregulation of different *GST* genes in *T. japonicus*. Firstly, in case of upregulation, β-NF exposure influenced the transcription-regulating factors of some *GST* promoter regions, mediating the transcription of specific *GST* isoenzymes. Secondly, in case of downregulation, *GST* expression was selectively associated with the accumulation of GSH conjugates under β-NF exposure. Several reports from different aquatic animals exposed to PAHs supported these controversial findings (Henson et al., 2001, Teles et al., 2003; Lu et al., 2009). Thus, since GST catalyzes the conjugation of certain xenobiotics to reduced GSH, its expression is likely induced as a result of

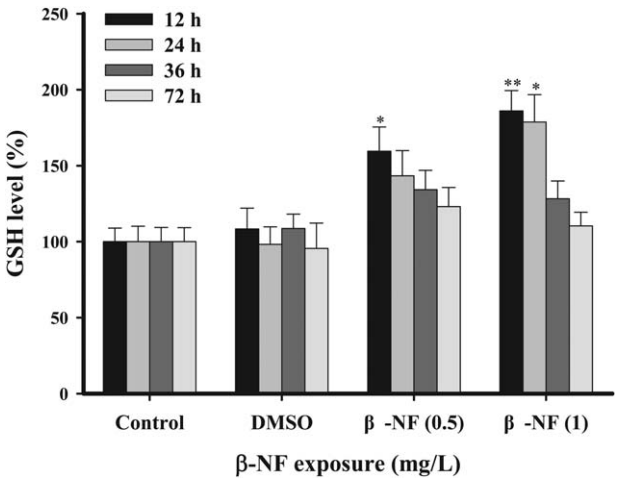

Fig. 4. GSH content measured after exposure to β-NF (0.5 or 1 mg/L) for 36 and 72 h. Each value is the mean of three replicate samples and data are shown as means ± SD.

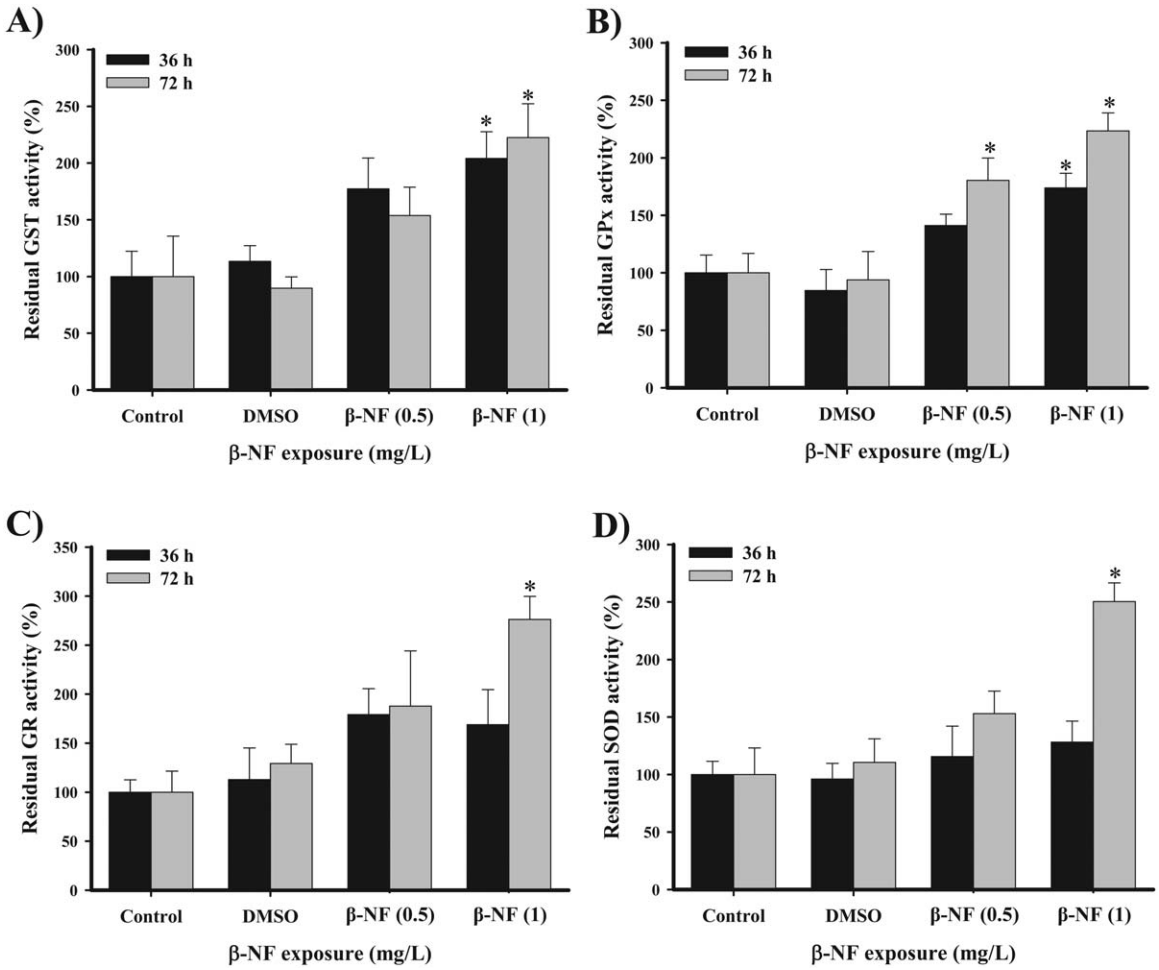

Fig. 5. GSH-related enzyme activities measured after exposure to β-NF (0.5 or 1 mg/L) for 36 and 72 h. GST (A), GPx (B), GR (C), and SOD (D). Each value is the mean of three replicate samples and data are shown as means ± SD. Significant differences between the means of activities were analyzed by one-way ANOVA followed by Tukey's test. Asterisks (*) indicate a significant change with $P < 0.05$.

cytosolic GSH depletion following PAH-induced GST-catalyzed conjugation. In *T. japonicus*, five specific *GST* genes showed significant sensitivity and upregulation after 24 h β-NF exposure. Particularly, *T. japonicus GST-S* and *GST-O* were expressed highest among the upregulated *GSTs*. Lee et al. (2007) reported that the sigma-class of GST, non-mammalian GST, had a possible antioxidant function in *in vitro* recombinant proteins in *T. japonicus*. Also, Lee et al. (2008) demonstrated that the expression of *GST-S* mRNA was highly upregulated in a dose- and/or time-dependent manner in *T. japonicus* exposed to H_2O_2 and heavy metals such as Cd, As, and Cu which are known as oxidative stress inducers, and also showed that the expression level of *T. japonicus GST-D1* and *GST-O* mRNA was also significantly increased in H_2O_2, suggesting that these GST isoforms played a main role in the antioxidant defense in *T. japonicus*. Taken together, these results indicate that *GST* genes, particularly *GST-S* and *GST-O* isotype, would provide suitable biomarkers for the monitoring of environmental oxidative stressors in *T. japonicus*.

The relative mRNA expression of antioxidative genes was shown as a time-dependent induction. We assumed that the *GR* induction over time was potentially influenced by excessive generation of ROS during the β-NF metabolism. The relative expression of *GPx* in the 1 mg/L of β-NF-exposed *T. japonicus* was upregulated over the exposure time. Nahrgang et al. (2009) demonstrated before that the *GPx* mRNA level was upregulated in polar cod exposed to benzo[*a*]pyrene (BaP). In the Pacific oyster, *GPx* mRNA expression was significantly increased with exposure to cadmium (Jo et al., 2008). Thus, induction of *GPx* against oxidative stress provides its strong involvement in the antioxidant defense mechanism of aquatic organisms.

Phospholipid hydroperoxide glutathione peroxidase (PH-GPx) is an important ubiquitous antioxidant enzyme involved in the removal of lipid hydroperoxides (LOOHs)

from cell membranes (Pradhan et al., 1990). The formation of LOOHs within the cellular membrane bilayer disrupts structure and function of the membrane leading to subsequent cellular injury and death (Pradhan et al., 1990). The repair of intracellular LOOHs is generally mediated by cytosolic GPx and PH-GPx. However, PH-GPx directly reduces both phospholipid- and cholesterol-hydroperoxides in cell membranes (Thomas et al., 1990; Hu et al., 2010). In β-NF-exposed *T. japonicus*, the expression of *PH-GPx* mRNA was upregulated for 72 h (significant at $P < 0.01$), compared to 0 h. This result supported the findings of other researchers, even though we could not measure the LOOHs in *T. japonicus*. For example, the increase of *PH-GPx* mRNA was shown in H_2O_2-exposed plants (Li et al., 2000) and insects (Hu et al., 2010), and γ-irradiation- and metal-exposed fish (Olsvik et al., 2010).

Se-GPx utilizes the reduced glutathione for catalyzing the reduction of hydrogen peroxide and organic hydroperoxides to alcohol and water (Birringer et al., 2002). Therefore, Se-GPx protects cellular organelles from oxidative damage by scavenging the potentially harmful peroxides generated by normal cellular metabolism. In β-NF-exposed *T. japonicus*, significant induction of *Se-GPx* gene expression was found over OR after/at 72 h. Our finding is consistent with previous studies. For example, an increase of Se-GPx activity by oxidative damage was reported in mammals (El-Bayoumy, 2001; Liu et al., 2004), some fish species (Watanabe et al., 1997), and mussel (Chatziargyriou and Dailianis, 2010). de Zoysa et al. (2009) demonstrated that the disk abalone (*Haliotis discus discus*) *Se-GPx* mRNA level was induced significantly under oxidative stress such as different salinity and hypoxia, suggesting that *Se-GPx* may be a potential indicator gene for oxidative stress.

Under normal physiological conditions, there is a cellular balance between the amount of ROS produced and the cellular antioxidant systems. ROS plays a crucial role in initiating and catalyzing a variety of radical reactions. When ROS production becomes excessive or if the cellular antioxidant defense is impaired, then cellular oxidative stress is generated. In this study, *T. japonicus* exposed to 1 mg/L of β-NF led to a significant induction of ROS, compared to the control. ROS are harmful byproducts of oxidative metabolism that cause molecular damage in living systems. Dewa et al. (2008) suggested that the administration of β-NF enhances oxidative stress responses which may be involved in the induction of hepatocellular carcinoma in rats. β-NF-exposed rats showed a dose-dependent increase of ROS production. Oxidative stress was elevated by high levels of intracellular ROS after exposure of a kind of PAH, BaP in human skin (Costa et al., 2010). This is in accordance with our finding that this flavonoid β-NF can also induce oxidative stress by generating ROS in *T. japonicus*.

In this study, significant changes in the GSH level were observed in early time-courses in both concentrations. Oliveira et al. (2008) reported that the GSH content was significantly elevated in the golden grey mullet exposed to the lowest dose of phenanthrene. Feldmannova et al. (2006) demonstrated a significant decline of GSH content in *Daphnia magna* exposed to N-PAHs. Although our results showed no statistical significance, the increase of the level of GSH in β-NF-exposed *T. japonicus* may likely be an adaptive response to oxidative stress, whereas the reduction of the GSH level seems to be related to its consumption in GPx and GST activities with its insufficient regeneration (Ahmad et al., 2006). Exposure of β-NF (5 mg/kg) to rainbow trout or striped bass was not affected by GST activity (Lemaire et al., 1996), while longer exposure to β-NF (50 mg/kg) induced GST activity in rainbow trout after 7 days (Fenet et al., 1998) and 14 days (Zhang et al., 1990). In *D. magna*, GST activity was different depending on the kinds of N-PAHs (Feldmannova et al., 2006). Although information between GST activity and β-NF in marine invertebrates is still lacking, GST activity may be closely related to β-NF exposure over time and concentration of exposed xenobiotics. In *T. japonicus*, induction of GPx and GR activity in the β-NF-exposed group goes along with a reduction of GSSG to GSH. In β-NF-exposed rainbow trout, GR induction was also detected after 5 days, suggesting that GR may be an indicator for oxidative stress (Stephensen et al., 2002).

Responsive genes of a cell or organism exposed to toxic chemicals can be considered as toxicological endpoints. The results of overall gene expression studies with ROS, GSH, and enzyme activity measurements revealed that oxidative stress can be induced by β-NF exposure in *T. japonicus*. From the foregoing account, it can be clearly concluded that the above measured oxidative stress parameters are very well used as a signature tool for assessing early stress in the ecophysiology of *T. japonicus* with reference to β-NF toxicity.

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