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Differential Expression of Physiological Genes of *Neocaridina Denticulate* at the Transcriptional Level Following Short-Term Exposure to Non-Lethal Concentrations of Phthalate Esters

Su CH, Tsai YC and Sung HH*

Department of Microbiology, Soochow University, Taipei 111, Taiwan

Abstract

Xenobiotic phthalate esters (PAEs) are considered endocrine disrupting chemicals and are known to cause immunotoxicity, having been linked with the susceptibility of shrimp to pathogens; however, the effect on other physiological functions has not been explored. This study sought to understand the genetic responses of *Neocaridina denticulate*, a common freshwater shrimp, to three different PAEs, i.e., diethyl phthalate (DEP), dipropyl phthalate (DPrP), and diphenyl phthalate (DPP). The differential expression of 10 functional known expressed sequence tags derived from *N. denticulate* were analyzed by semi-quantitative RT-PCR after 24 h of exposure to non-lethal concentrations (0, 0.1, 1.0, and 10 mg/L) of PAEs in pond water. Compared with the control group, nine genes were differentially expressed in the DEP-treated groups, seven genes were differentially expressed in the DPrP-treated groups, and five genes were differentially expressed in the DPP-treated groups. These PAE-affected genes primarily belonged to three functional classes: defense-related genes (*qm*: *QM protein hc: hemocyanin*), metabolism-related genes (*cathepsin-L* like *cysteine protease*, *catl; glutamate dehydrogenase, gdh*; and *inosine monophosphate dehydrogenase 1, impdh-1*) and stress-related genes (*hsp70*: *70 kDa-heat shock protein*; *gst*: *glutathione S-transferase*, and *tps*: *trehalose-6-phosphate synthase*). Among these, the stress-related genes were significantly up-regulated by DEP, DPrP, and DPP. These effects of PAEs on the expression of genes required for multiple physiological functions suggest that even with non-lethal concentrations of PAEs, a polluted aquatic environment may still present a potential risk to *N. denticulate.*

Keywords: Phthalate esters; *Neocaridina denticulate*; Aquatic crustacean; Expressed Sequence Tag (EST); Differential gene expression

Introduction

Increasing evidence indicates that many xenobiotics, i.e., products of chemical pollutants that are degraded but not biologically decomposed in sewage treatment systems, are often not acutely toxic for exposed aquatic animals but instead lead to chronic intoxication, resulting in tissue alterations. There is a developing awareness that diseases in both fish and mollusk populations are linked to environmental changes or coastal marine pollution. A considerable amount of evidence supports the links among environmental changes (including contaminants), non-infectious diseases, and the deterioration of the immune system [1,2]. Phthalate esters (PAEs) are widely used industrial chemicals that serve as important additives to impart flexibility to polyvinyl chloride (PVC) resins and have become widely diffused in the environment [3] via the manufacturing process. In Taiwan, PAEs have been found to be widely distributed in river water, sediment, and soil [4] and have been found to accumulate in fish [5].

Numerous experiments have shown that the bioaccumulation of PAEs occurs in aquatic and terrestrial food chains due to their low solubility and degradation and to their high hydrophobicity and adhesion [3]. The accumulation of PAEs interferes considerably with the propagation and size of populations as a result of changes to sexual development and alterations in reproductive function, including changes in embryo development, sperm maturation, reproductive organs and hormones, and arachidonate metabolism [6-9]. Autian indicated that the human male fetus can become feminized in the course of development when PAEs accumulate during pregnancy [10]. Singh reported that some PAEs can induce teratogenic effects (such as anophthalmia, twisted hind legs, and hemangiomas), fetal death, and fetal resorption in pregnant rats [11]. Additionally, PAEs can be hydrolyzed to phthalic acids; phthalic acids have been found to act as germ-cell mutagens in rats [9] and have inhibitory effects on arachidonate metabolism in rat peritoneal leucocytes and human peritoneal T-lymphocytes [7,8]. Phthalic acids have also been found to influence prostanoid output [12,13]. The United States Environmental Protection Agency (EPA) and its counterparts in several other countries have classified the most commonly occurring PAEs as priority pollutants and endocrine-disrupting compounds. Therefore, evaluating PAE pollution in the environment and its associated toxicity is particularly important.

Many invertebrate toxicity test protocols are routinely used in regulatory toxicity testing [14], and freshwater crustaceans are currently employed to monitor environmental pollution because they possess a number of advantageous features: they are the major invertebrate component in most aquatic ecosystems, their populations are often numerous, and they are easily cultured in the laboratory [15-17]. *Neocaridina denticulate* (De Haan, 1844, Crustacea, Decapoda) is distributed in rivers throughout eastern Asia and the Hawaiian islands and is a common shrimp in the fresh water ecosystems of Taiwan. Several characteristics of *N. denticulate* are beneficial as aquatic indicators for assessing environmental pollution: they have a small size (2-3 cm), undergo spontaneous interbreeding, and lack a metamorphosis

***Corresponding author:** Hung-Hung Sung, Department of Microbiology, Soochow University, Taipei 111, Taiwan, Tel: 886228819471; E-mail: hhsung@scu.edu.tw

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stage during development. In 2005, the Environmental Protection Administration of Taiwan announced this organism as an indicator for use in acute toxicity assays to assess whether various bodies of water or effluents from industrial districts are qualified for some degree of environmental protection. However, *N. denticulate* has been used in only a few studies to date to determine the toxicity of pesticides, including ammonium chloride, copper sulfate, cadmium chloride, mercuric chloride, and zinc sulfate [18], and the effects of pollutants, such as chlordane and lindane, on growth and reproductive hormones [19].

Previous *in vitro* exposure experiments conducted by our group demonstrated that xenobiotic PAEs, including dipropyl phthalate (DPrP), can damage hemocytes and impact the cellular immunity of the giant freshwater prawn (*Macrobranchium rosenbergii*) [20]. We also found that the increased susceptibility of prawns to pathogens by *in vivo* exposure to PAEs may be caused not only by changes in immunity but also by changes to other physiological functions [21]. Furthermore, we found that the increased susceptibility of *N. denticulate* exposed to a non-lethal concentration of DPrP was short-term and may be related to the increased expression of DPrP-induced immune mediators, including acid phosphatase, β-glucuronidase, phenoloxidase, superoxide dismutase, and hemocyanin [22]. Therefore, one must determine what effect PAEs at a non-lethal dosage have on the global physiology of *N. denticulate*.

This study aimed to determine the expression of a set of multi-functional genes of *N. denticulate* after exposure to three different PAEs, i.e., diethyl phthalate (DEP), dipropyl phthalate (DPrP), and diphenyl phthalate (DPP). In this study, the expressed sequence tags (ESTs) derived from either DPrP- or nonylphenol-treated shrimps [23,24] were used to determine the messenger RNA expression of selected genes associated with various physiological functions and to assess the differences in gene expression caused by PAEs. Our results are useful for assessing the feasibility of employing *N. denticulate* as an aquatic indicator, and the ESTs found to be differentially expressed may serve as potential genomic biomarkers for the biomonitoring of hidden risks to aquatic organisms caused by pollutants in the environment.

Materials and Methods

Acclimation of animal

Green-neon shrimp (*Neocaridina denticulate*) with body lengths of 2-3 cm were purchased on separate days from a private aquarium in Taipei, Taiwan, and were acclimated in 20 l glass aquaria containing fresh pond water and water plants (*Egeria densa* Planch.) at 28°C, pH 6.9~7.5, an oxygen concentration >4 ppm, and a 12 h light-dark photoperiod. The shrimp were acclimated for at least one week prior to the experiments, and the expression of the prophenoloxidase gene (*propo*) was used as a parameter to determine whether the batch of shrimp was usable. The animals were considered usable only when the ratio of the mRNA levels of *propo* to that of *actin* (as an internal control) was 0.1-0.3 [23]. The shrimp were fed with mosquito larvae twice per day. The stocking densities were maintained at 100 individuals per aquarium.

Immersion treatment

The three PAEs, diethyl phthalate (DEP, Chem Service O-525), dipropyl phthalate (DPrP, Chem Service F-2158), and diphenyl phthalate (DPP, Chem Service 10588-0), were dissolved in acetone to a concentration of 10,000 mg/L as a stock solution and stored at room temperature. Prior to immersion treatment, the stock solution was serially diluted into different concentrations with fresh pond water. Preliminary tests showed that the mortality of DPrP-treated shrimp subjected to concentrations of 10 mg/L continuously for 10 days was less than 5%, and the mortality of the control (acetone-treated) group was less than 2% [22]. Therefore, in these experiments, shrimp were treated with the three PAEs at three concentrations (0.1, 1.0, and 1.0 mg/L), with a density of 60 individuals in 20-L glass aquaria containing 18 L of fresh pond water for 24 h. The control shrimp were treated with pond water containing a 1,000-fold dilution of acetone.

Preparation of tissue homogenates

Because these shrimp are too small to collect sufficient amounts of different tissues for different experiments, tissue homogenates were prepared from at least five whole individuals. Briefly, after the shrimp were immersed in ice-cold PBS for 1-2 min, they were ground into powder using a liquid nitrogen-chilled mortar and Teflon pestle (Kontes, Vineland, NJ, USA). The resulting tissue homogenate could be directly used to isolate RNA.

RNA isolation

One tissue homogenate sample was prepared from five individuals. For RNA isolation, 50-100 mg of the tissue homogenate was resuspended in 1 mL of Trizol reagent (Invitrogen, USA) and incubated at room temperature for 5 min. The solution was mixed vigorously for 15 s with 0.2 mL of chloroform and incubated at room temperature for 10 min. After the mixture was centrifuged at 12,000 xg for 15 min at 4°C, the uppermost layer was collected and mixed with 0.5 mL of isopropanol at room temperature for 10 min. Following a second centrifugation, the RNA pellet was desalted using 75% (v/v) ethanol prepared with diethylpyrocarbonate (DEPC; Sigma)-treated sterile water and dissolved in 10 μL of DEPC-treated water. PolyA$^+$ RNA was purified from this preparation with a Dynabeads mRNA purification kit (Invitrogen). Prior to the following experiments, the concentration of RNA was quantified by measuring the absorbance of the RNA solution at a wavelength of 260:280 nm.

Semi-quantitative RT-PCR

After treatment with different concentrations of DEP, DPrP, or DPP, the differential gene expression pattern was verified by semi-quantitative RT-PCR using total RNA (0.25 μg cDNA). In this study, 10 ESTs of known function (Table 1) were isolated from either DPrP-treated [24] or nonylphenol-treated shrimp [23] and used as the target genes. Primers for different genes were designed and used in PCR (Table 2). Beta-actin was used as an internal control for this experiment, and its expression was determined by PCR amplification with a pair of specific primers, actin-F (5'-CCCAGAGCAAGAGAGGTA-3') and actin-R (5'-GCGTATCCTTCGTAGATGGG-3'), which yielded a 309 bp fragment (GenBank accession number: HO762521). The PCR conditions were as follows: a denaturing step at 94°C for 1 min, an annealing step at 51°C or 55°C for 1 min, and a final extension at 72°C for 1 min. All PCR products were analyzed on a 1% agarose gel and quantified using the ImageMaster™ software (TotalLab Software v. 2.00, Amersham). The intensity of bands was expressed in relative absorbance units. Semi-quantitative determinations were expressed as a ratio of the absorbance units of the target gene band to the absorbance units of the actin band in the same sample. The data shown in figures from this study are expressed as a "ratio of control", which was calculated as the ratio of the level of one target gene from the PAE-treated group to the level of the same target gene from the control group.

EST ID	Accession number	Gene identified	Accession number	Species	EST size (bp)
Immune-related genes					
DPrP9	FL592852	QM protein	EU004069.1	*Marsupenaeus japonicus*	398
NP2	FL640908	Hemocyanin mRNA	AF431737.1	*Penaeus monodon*	361
Respiration-related genes					
NP7	FL640914	Mitochondrial COI gene for cytochrome oxidase subunit I	AB300187.1	*Neocaridina denticulata sinensis*	693
NP11	FL640918	Mitochondrion	DQ656600.1	*Fenneropenaeus chinensis*	612
Metabolism-related genes					
NP8	FL640915	Cathepsin-L like cysteine protease mRNA	X85127.1	*Penaeus vannamei*	438
NP10	FL640917	glutamate dehydrogenase	AM076955.1	*Litopenaeus vannamei*	607
NP16	FL640932	Inosine monophosphate dehydrogenase 1	NM 001017283.2	*Xenopus tropicalis*	552
Stress-related genes					
NP14	FL640930	70 kD heat shock protein cognate	NM 001043372.1	*Bombyx mori*	307
NP15	FL640931	Glutathione S-transferase class mu	EU008563.1	*Cyphoma gibbosum*	285
NP18	FL640934	Trehalose-6-phosphate synthase 1	XM 392397.3	*Apis mellifera*	576
When the E-value is less than 10^{-25}, the EST is considered a known functional EST.					

Table 1: Characteristics of the functional ESTs used in this study.

EST ID	Accession no.	Primer (5'→3')	Size (bp)
DPrP9	FL592852	F: GCCTTGTTACGGTCGTGAGT	330
		R:GGGAAGAAAGAAGGCTGACG	
NP2	FL640908	F: CAGCCCAAATGTCCGTTACT	600
		R: CCTTGAAGCTTGGCACATC	
NP7	FL640914	F: GGCTCGTGTGTCAACATCC	522
		R: CCCCCATTAGCAAGAGGAA	
NP8	FL640915	F: CTTCAACCCAGCCAATGTG	337
		R: GGTAGCAATGCCGCAGTTA	
NP10	FL640917	F: GTCGTGGATTGCTGACACC	531
		R: GTTGGGCCATTAGCAGCTT	
NP11	FL640918	F: CACACCTCGCGGGTAGTAGT	510
		R: ACCAAGCTGCTGCTTCAAA	
NP14	FL640930	F: TGTGGGTGGAGTGATGACA	209
		R: CAATCTGGGGCACACCTC	
NP15	FL640931	F: TCTCAGTGGTGCCACACAA	201
		R: GCGATGAGTTCGAGGACAA	
NP16	FL640932	F: CGTGACCCAGTTCGTTTGA	460
		R: CATCAGCACCTGCATTTACC	
NP18	FL640934	F: AGTGCAAGGCGAGAGGATT	482
		R: ACGCCAAATTGGTCTCCA	

Table 2: Specific primers for ESTs used in this study.

Statistical analysis

Because outbred animals were used as samples in this study, the phenotypic background most likely varied significantly between individual animals. The means of the average relative value of at least five replicates from 25 individuals were statistically analyzed to examine the effect of PAE on mRNA expression using an ANOVA and Duncan's multiple range tests, with a specified significance level of $P<0.05$.

Results and Discussion

PAEs, which are considered endocrine disrupting chemicals (EDCs), have been widely used in aquatic ecosystems to study toxicity in algae, invertebrates, and fish. Because the regulatory mechanisms of an organism are very complex, vary throughout the lifecycle of the organism, and increase in complexity with evolution, the toxicity of PAEs is a multifaceted issue that depends on many factors. Gene expression analysis offers mechanistic value and provides more comprehensive insight into toxicity [25]; in addition, gene expression analysis is being increasingly used in the diagnosis of environmental contamination because it may be more sensitive and less time-consuming than conventional toxicology endpoints. Due to the lack of information concerning the biological effects of PAEs and the advent of new technologies in functional genomic analysis, monitoring the expression of multiple genes involved in a wide spectrum of cellular processes and signaling pathways offers a potentially powerful method to study the mechanistic basis of toxic action and unravel the global effect underlying the observed adverse physiological effects of PAEs. A handful of studies have used gene expression analysis to assess the ecotoxicity of PAE exposure in aquatic wildlife invertebrates, such as *Tigriopus japonicus* and *Chironomus riparius* [26,27]. The high sensitivity of the *N. denticulate hc* gene to DPrP reported in a previous study has suggested that a gene expression profile may be potentially used as a biomarker tool for evaluating aquatic contamination by DPrP or other phthalate esters [22].

In this study, to understand the global effect of PAEs on *N. denticulate*, an EST analysis developed from the subtractive library [23,24] was used to determine differentially expressed genes after short-term exposure to DEP, DPrP, or DPP exposure for 24 h. The 10 functional target genes were classified into four different groups according to their physiological functions (Table 1): immune-related genes (QM protein and hemocyanin), respiration-related genes (cytochrome oxidase subunit I and mitochondrion), metabolism-related genes (cathepsin-L-like cysteine protease, and glutamate dehydrogenase) and stress genes (70-kDa heat shock protein, glutathione S-transferase, and trehalose-6-phosphate synthase).

Effects of three PAEs on the expression of immune genes

Previous studies have indicated that four PAEs can reduce cellular immunity and increase the susceptibility of an economically important freshwater prawn *Macrobrachium rosenbergii* to infection [20,21]. In arthropods, except hemocyanin protein (Hc) is produced in the hepatopancreas and serves as an oxygen carrier, several studies have demonstrated that the physicochemical properties of Hc are very similar to those of phenoloxidase after proteolytic cleavage at the amino-terminal of Hc, such as the spider *Eurypelma californicum* [28,29] and horseshoe crab *Tachypleus tridentatus* [30]. Under normal conditions, Hc functions as an oxygen carrier, but it may be converted to phenoloxidase after infection to prevent microbial invasion. In addition, crustacean Hc can be processed by a cysteine-like proteinase to generate an antimicrobial peptide [31,32]. In crustaceans, Xu et al.

[33] found that the shrimp QM gene (*qm*) was up-regulated in virus-resistant shrimp and that the QM protein could participate in the prophenoloxidase activation system by interacting with Hc and myosin and regulating the phenoloxidase activity of Hc [33].

In this study, the transcription of the two immune-related genes *qm* (DPrP9) and *hemocyainin* (NP2) was selected as a measure of immunity provoked by exposure to the three xenobiotic chemicals. The mRNA expression of the gene *qm* in the PAE-exposed shrimps was significantly inhibited at a high concentration (10 mg/L) of both DEP and DPP (Figure 1) but increased at a low concentration (0.1 mg/L) of DEP (Figure 1). Compared with the controls, the different concentrations (0.1, 1, and 10 mg/L) of DPrP treatments did not alter the mRNA levels of the *qm* gene in any of the concentrations assayed

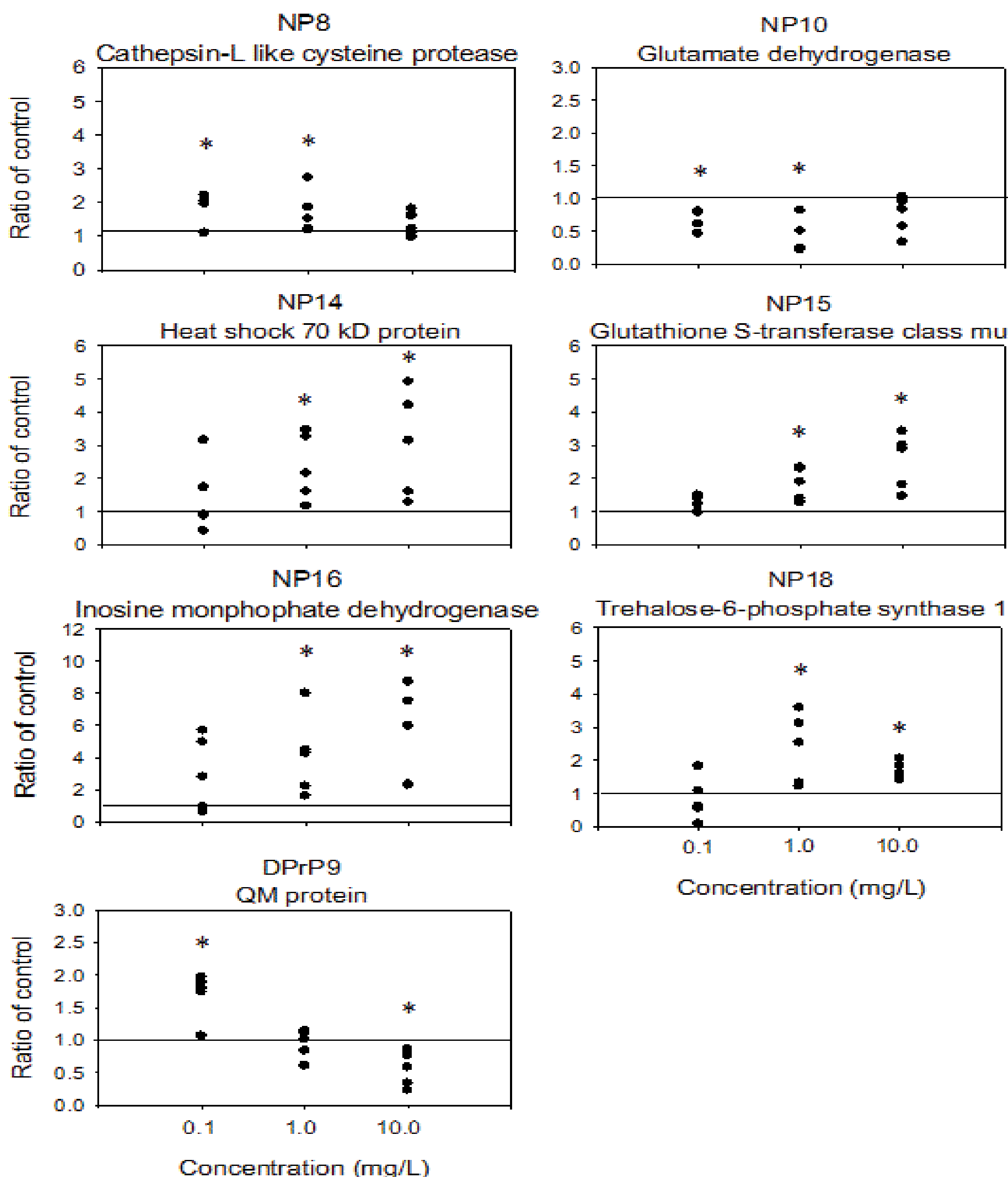

Figure 1: Messenger RNA expression of genes of *Neocaridina denticulate* treated with various concentrations of diethyl phthalate (DEP) for one day. A semi-quantitative determination of mRNA levels of target genes and an internal control (actin) was conducted. The data are expressed as the "ratio of control," which was calculated as the ratio of the level of the target gene (X) from the DEP-treated group to the level of that target gene (X) from an acetone-treated control group. Significant differences in mRNA expression between the DEP-treated group and control group are indicated with a single asterisk ($P<0.05$).

(Figure 2). An increase in *hc* mRNA levels was detected following DPrP treatment with all three concentrations (Figure 2), but the mRNA levels of *hc* in DPP-treated groups were significantly reduced (Figure 3). The results indicated that the effect of the three PAEs on *hc* expression was completely different, and that gene *qm* can be modulated by either DEP or DPP (Figures 1 and 3). Previous studies have demonstrated that the *hc* gene was up-regulated by DPrP [22] but down-regulated by nonylphenol [23]. The results of this study from the *hc* and *qm* expression analyses suggest that PAEs may interfere with the immunity of *N. denticulate* to increase the risk of infection.

Effects of three PAEs on the expression of metabolism- and respiration-related genes

Metabolism is the set of life-sustaining chemical transformations that manage the material and energy resources of the cells of living organisms; respiration is one of the key methods a cell gains useful energy to fuel cellular activity. One of these genes, *gdh*: *glutamate dehydrogenase*; NP10, was affected by all three PAEs (Figures 1-3); *gdh* was up-regulated by DEP (0.1 mg/L and 1 mg/L) and DPrP (10 mg/L) but down-regulated by DPP (10 mg/L). The mRNA levels of the other two genes, *cathepsin-L* like *cysteine protease* (*catl;* NP8) and *inosine monophosphate dehydrogenase 1 (impdh-1;* NP16), were significantly elevated by DEP (Figure 1); DPrP increased the expression of *catl* but did not affect the expression of *impdh-1* (Figure 2). As shown in Figures 1 and 2, *catl* was up-regulated by both DEP and DPrP at 0.1 and 1 mg/L, and *impdh-1* was affected by DEP (1 and 10 mg/L). Furthermore, two respiration-related genes, *cytochrome oxidase subunit I* (*cos*; NP7) and *mitochondrion* (*mit*; NP11), were selected as measures of the

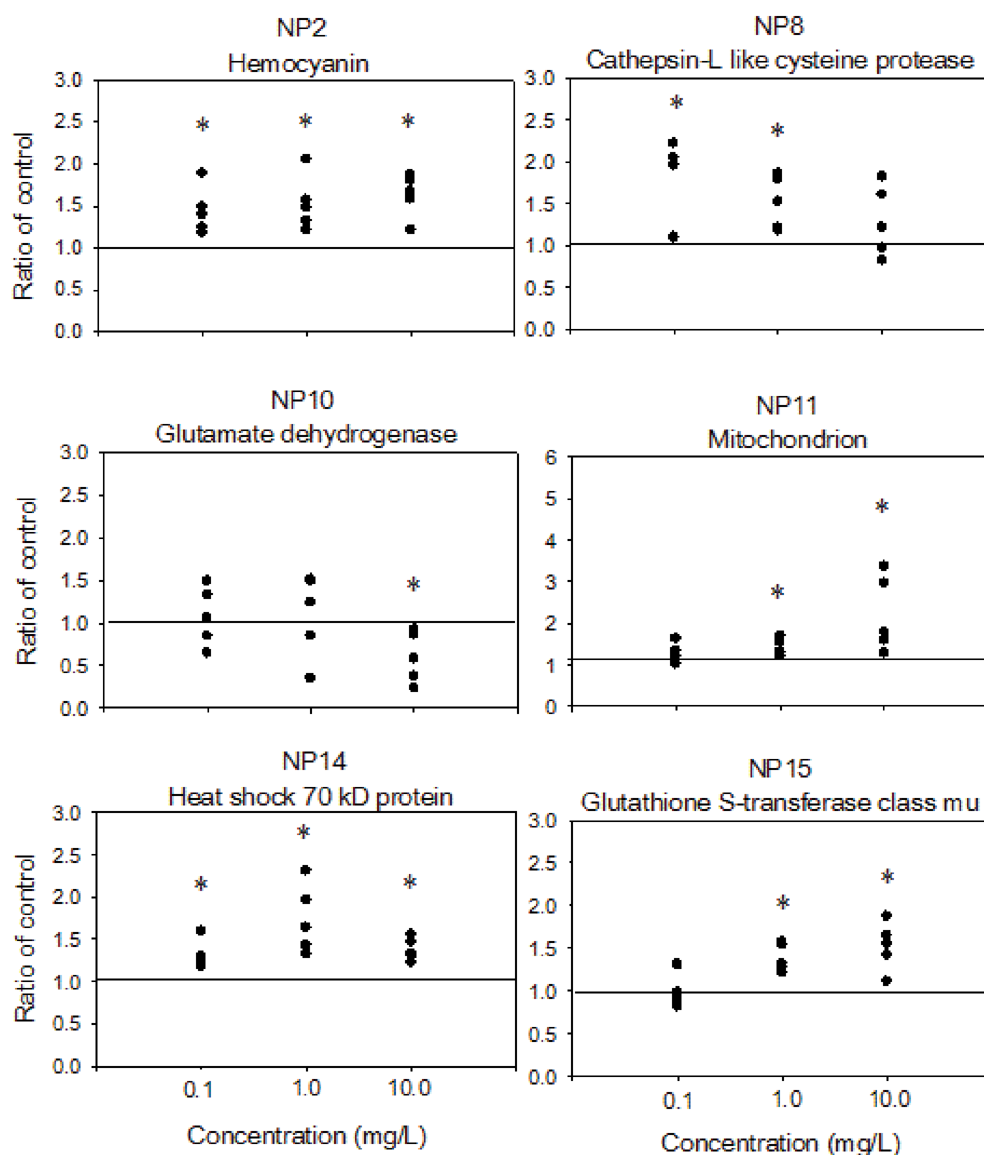

Figure 2: Messenger RNA expression of genes of *Neocaridina denticulate* treated with various concentrations of dipropyl phthalate (DPrP) for one day. A semi-quantitative determination of the mRNA levels of target genes and an internal control (actin) was conducted. The data are expressed as "ratio of control," which was calculated as the ratio of the level of target gene (X) from the DEP-treated group to the level of that target gene (X) from an acetone-treated control group. Significant differences in mRNA expression between the DEP-treated group and control group are indicated with a single asterisk (P<0.05).

effects on the energy supply caused by the exposure to PAEs. Only the DPrP significantly increased the mRNA levels of *mit* at the different concentrations (Figure 2), and the three PAEs did not affect the mRNA levels of the *cos* gene in shrimp following PAE exposure (Figures 1-3).

In marine invertebrates, GDH plays a role in the hyperosmotic stress; GDH activity decreases when NaCl concentration is increased in *Tigriopus californicus* [34]. In addition, GDH is capable of regulating the hemolymph ammonia concentration and hemolymph protein level in *Litopenaeus vannamei* [35]. These results suggest that organisms with high GDH activity levels reduce their energy for growth by wasting more energy in oxidation of the excess proteins. However, this mechanism is unlikely to be involved in freshwater amphipod *Gammarus pulex* exposed to polychlorinated biphenyls (PCBs) because a previous study observed a decrease in GDH activity after PCB exposure [36]. In this study, the *gdh* gene was down-regulated in *N. denticulate* exposed to DEP and DPrP (Table 3). These results suggest that a decrease of GDH may reduce energy consumption to facilitate the detoxification and survival of *N. denticulate* in a polluted environment.

In crustaceans, cathepsin-L cysteine proteases (CatLs) were found to play important roles during development in *Artemia franciscana* [37] and in the hepatopancreas of the shrimp, where they are involved in food digestion [38]. Furthermore, several studies have indicated that CatL affects the innate immune system of crustaceans, including shrimp [39]. In the study, we found that the *catl* gene was up-regulated by DEP and DPrP, but another study found that the *catl* mRNA level was decreased in shrimp exposed to nonylphenol [23]. The product of *impdh-1,* the other metabolic gene that was up-regulated by DEP in this study, is an essential, rate-limiting enzyme in the *de novo* guanine nucleotide synthetic pathway. IMPD has been studied in humans but is poorly understood in other organisms. Furthermore, the important role of IMPDH activity in the immune response has been demonstrated; IMPDH types I and II are expressed during T-lymphocyte activation and are associated with lymphocyte proliferation, and IMPDH type I mRNA levels increase in response to a variety of stimuli [40]. Based on the functions of CatL and IMPDH that have been previously demonstrated, we hypothesize that the functions of CatL and IMPDH in crustaceans (including *N. denticulate*) are related to either metabolism or immunity. Based on the results of the expression of *catl*, *gdh*, and *impdh-1*, we propose that PAEs have multi-modulating effects on either the metabolic activities or immune response of *N. denticulate.*

Effects of three PAEs on the expression of stress-related genes

The cellular response to stress is characterized by the activation of a set of genes to counteract the physiological disturbance induced by physical or xenobiotic agents; a variety of enzymes and other proteins

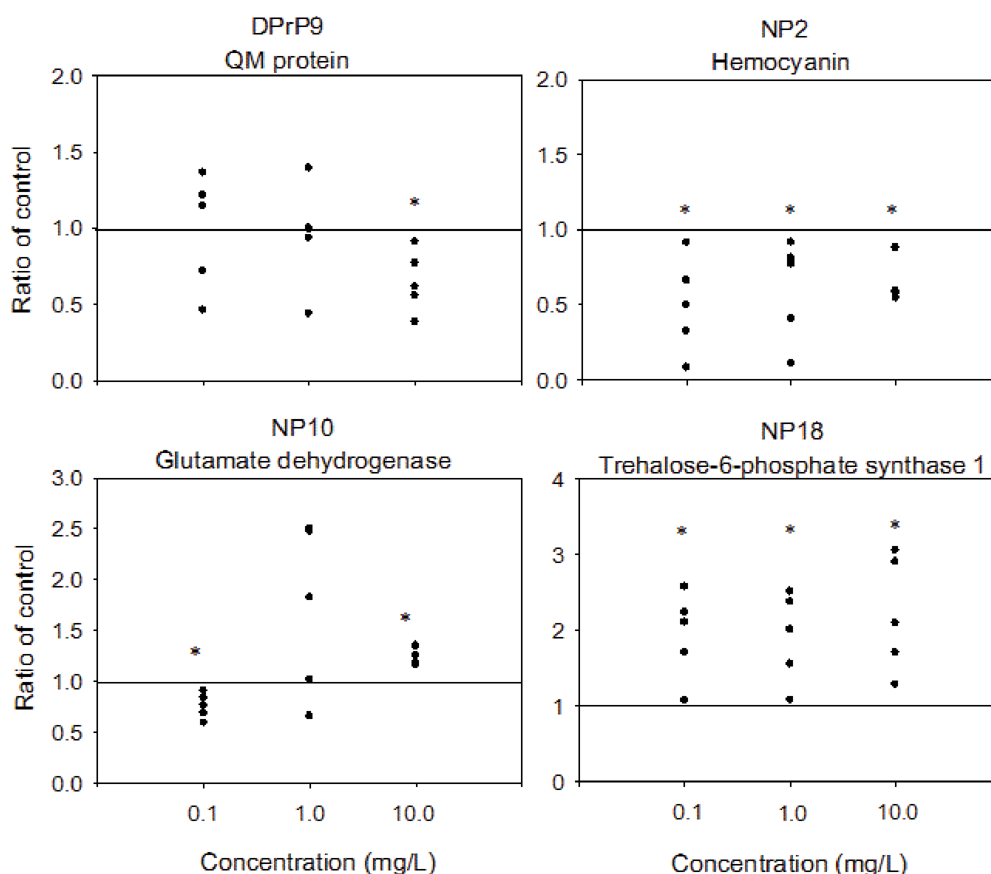

Figure 3: Messenger RNA expression of genes of *Neocaridina denticulate* treated with various concentrations of diphenyl phthalate (DPP) for one day. A semi-quantitative determination of mRNA levels of target genes and an internal control (actin) was conducted. The data are expressed as "ratio of control," which was calculated as the ratio of the level of a target gene (X) from the DEP-treated group to the level of that target gene (X) from the acetone-treated control group. Significant differences in mRNA expression between the DEP-treated group and control group are indicated with a single asterisk (P<0.05).

EST ID	Known function	Phthalate Concentration (mg/L)								
		DEP			DPrP			DPP		
		0.1	1	10	0.1	1	10	0.1	1	10
Immune-related genes										
DPrP9	QM protein	↑		↓						↓
NP2	Hemocyanin mRNA				↑	↑	↑	↓	↓	↓
Respiration-related genes										
NP7	Cytochrome oxidase subunit I									
NP11	Mitochondrion				↑	↑	↑			
Metabolism-related genes										
NP8	Cathepsin-L like cysteine protease	↑	↑		↑	↑				
NP10	Glutamate dehydrogenase	↓	↓				↓	↓		↑
NP16	Inosine monophosphate dehydrogenase 1		↑	↑						
Stress-related genes										
NP14	70 kD heat shock protein		↑	↑	↑	↑	↑			
NP15	Glutathione S-transferase		↑	↑		↑	↑			
NP18	Trehalose-6-phosphate synthase 1		↑	↑				↑	↑	↑

↑ and ↓, Significant positive and negative effects on the gene expression of the experimental group (P<0.05).

Table 3: Summary of the effects of three phthalate esters on the differential expression of functional genes of *Neocaridina denticulate* via immersion treatment.

are produced by organisms in response to xenobiotic exposure. This study selected three stress-related ESTs, NP14, NP15, and NP18, hereafter referred to as *70-kDa heat shock protein* (*hsp70*), *glutathione S-transferase* (*gst*), and *trehalose-6-phosphate synthase* (*tps*), respectively, to determine the effect of PAEs on the stress response of shrimp. The mRNA levels of these three genes were significantly elevated after exposure to DEP (1 and 10 mg/L) (Figure 1). The *hsp70* gene was up-regulated after exposure at the three concentrations (0.1, 1, and 10 mg/L) of DPrP, and the mRNA levels of *gst* were significantly elevated after treatment with 1 and 10 mg/L DPrP (Figure 2). The mRNA levels of the *tps* gene were increased by DEP treatment (1 and 10 mg/L) (Figure 1) and up-regulated by DPP (0.1, 1, and 10 mg/L) (Figure 3).

The HSP70 family of proteins differs from other classes of HSPs because it is one of the most highly conserved families and the first to be induced under stress conditions [41]. The induction of *hsp70* gene expression was detected in the harlequin fly (*Chironomus riparius*) after exposure to bisphenol A [42], butyl benzyl phthalate (BBP), and di(2-ethylhexyl) phthalate (DEHP) [27]. We observed an elevation of the *hsp* gene in both DEP- and DPrP-treated groups, suggesting that HSP70 protein may increase to aid in refolding damaged proteins and be responsible for survival and adaption of organisms during xenobiotic exposure. Similar to the *hsp* gene, the *gst* gene was also up-regulated after exposure to DEP and DPrP but was not affected by DPP (Figures 1-3).

In invertebrates, most studies have demonstrated that glutathione S-transferases (GSTs) function in antioxidant defense systems and the detoxification of insecticides and xenobiotics are often induced, e.g., after exposure to toxins [43-47]. The third stress-related gene examined in this study, *tsp* was up-regulated by DEP and DPP and highly sensitive to DPP (Figures 1 and 2). The accumulation of trehalose in chironomidae implies a role in physiological and biochemical adaptations in extreme environmental conditions [48]. Chung provided evidence of presence of trehalose in hemocytes and TPS in the tissues of the blue crab (*Callinectes sapidus*) and suggested its functional role in energy metabolism and physiological adaptation [49]. The up-regulation of the *tps* gene following exposure to PAEs may help shrimp adapt to and survive in a polluted environment.

In the study, as a further examination of the responses of these target genes to the three PAEs, the mRNA levels of most genes were differentially expressed after the shrimp were exposed to DEP and DPrP, but only three genes were affected by DPP, including two down-regulated immune-related genes (*qm* and *hc*), and one up-regulated stress-related gene (*tps)*. These results suggest that the higher water solubility of DEP and DPrP may help both PAEs enter the organisms and modulate gene expression. Our previous study demonstrated that nonylphenol, an EDC, reduced *hc*, eg-1α, and *catl* expressions and elevated the expression of *tps* [23]. The different effects of xenobiotic chemicals on the same target genes may suggest that various genes in *N. denticulate* can be affected by a variety of chemicals in the aquatic environment, but the impact on the gene level is associated with the structure of the chemical. In addition, together with the results of previous studies [23,27,50], our study suggests that although non-lethal concentrations of EDCs (for example, phthalate esters and nonylphenol) may allow aquatic organisms to survive, the polluted aquatic environment may still present a potential risk to these organisms by either interfering with their immunity and metabolism or increasing the stress response.

Conclusion

The ecotoxigenicity of PAEs to *N. denticulate* is caused by broad changes in multi-functional genes at the transcriptional level (Table 3). Aside from the *hsp70*, *gst*, and *tsp* responses of *N. denticulate* related to stress due to the presence of three PAEs, the expression of several genes concerning metabolism- and immunity-related functions varied at the RNA level caused by the different chemicals. Because *N. denticulate* has been previously used in Taiwan to monitor bodies of water and due to the results observed here, we suggest that an aquatic indicator system may be established using *N. denticulate* to assess the risks of aquatic pollution to aquatic environments or organisms and that ESTs with differential expression derived from *N. denticulate* may be employed as potential genomic biomarkers. There is little DNA sequence information available for *N. denticulate*, and this work reinforces the need to identify more specific and sensitive genes in the species.

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