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Long-term effects of di-octyl phthalate on the expression of immune-related genes in *Tegillarca granosa**

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Abstract Di-octyl phthalate (DOP) is widely used as a plasticizer in the plastics industry. As a result, DOP is often found in marine water ecosystems where many species are exposed to it. Our objective was to evaluate the effect of long-term (14 d) DOP exposure (2.6, 7.8, or 31.2 mg/L) on the expression of immune-related genes in *Tegillarca granosa*. The expression of small heat shock protein (sHSPs) and tissue inhibitor of metalloproteinase (TIMP) were highest in clams exposed to 31.2 mg/L DOP on days 7 and 14. The relative expression of Tg-ferritin, superoxide dismutase (SOD), and metallothionein (MT) increased initially then decreased as the concentration of DOP increased. The hemoglobin of *T. granosa* (Tg-HbI) exhibited two distinct expression patterns at two time points. Our results suggest that the immune response of *T. granosa* against DOP pollution varies depending on the dose. Additionally, we identified some immune-related genes that are promising candidates for biomarkers of DOP.

Keyword: *Tegillarca granosa*; di-octyl phthalate; immune response

1 INTRODUCTION

Phthalate esters (PAEs) are an important additive that increase flexibility in polyvinyl chloride (PVC) resins. PAEs are increasingly used as plasticizers, wire insulators, and pesticide carriers. As a result of their widespread use, PAEs are commonly found in the environment (Jobling et al., 1995). For example, the concentration of PAEs ranges from 0.1–300 μg/L at the seawater surface (Mayer et al., 1972; Giam et al., 1978; Fatoki and Vernon, 1990; Gledhill et al., 1980) and 0.1 ng/g–100 μg/g in river sediments (Thurén, 1986; Tan, 1995). Di-octyl phthalate (DOP) is a major component of PAEs and is widely used in several industries (e.g., food packaging materials, medical devices, etc.) because of its utility and cost effectiveness. Because DOP is not chemically bound to PVC resin, it can leak from disposable plastic products into the environment. Thus, DOP is frequently adsorbed into soil, sediment, and suspended solids because of its low water solubility (approximately 0.003 mg/L) and high octanol/water partition coefficient (log*K*ow=7.94) (Staples et al., 1997). The estimated concentration of DOP in Laizhou Bay seawater and sediments typically ranged from 2.002 to 43.891×10^{-9} g/L and 4.454 to $4\,389.243\times10^{-9}$ g/kg, respectively. In Quanzhou Bay, the concentration of DOP in seawater and sediments ranged from 18.77 to 191.51 ng/L and 171.50 to 1 435.61 μg/kg, respectively (Sung et al., 2003; Zhuang et al., 2011).

In recent years, a number of researchers have evaluated the effects of DOP pollution on marine animals. Hobson et al. (1984) reported that DOP could be absorbed, metabolized, and accumulated at high levels in the tissue of penaeid shrimp. Sung et al. (2003) demonstrated that DOP damages the hemocytes of prawns and impairs their immune defense system. Additionally, Park and Kwak (2009, 2012) and Caldwell (2012) found that DOP can alter the activity of some proteins involved in apoptosis, signaling, and metabolism. Furthermore, DOP

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induces the expression of many genes, including cytoskeletal proteins, alcohol dehydrogenases, and heat shock proteins in lower aquatic animals such as *Chironomus riparius* and *Tigriopus japonicas* (de Lafontaine et al., 2000; Oh and Lim, 2009; Li et al., 2010).

T. granosa is an economically important bivalve shellfish that is found on beaches along the Chinese coast. Outbreaks of pathogens and environmental degradation have resulted in dramatically decreased production of *T. granosa*. Immune-related genes are thought to be promising biomarkers for early monitoring of environmental conditions because of their rapid response to environmental perturbations and contamination (Brown et al., 1995; de Lafontaine et al., 2000; Lyons et al., 2003).

Although the acute toxicity of PAHs has been evaluated in marine animals (Sung et al., 2003; Chen and Sung, 2005; Lu et al., 2013), there is little information about the long-term immune responses of organisms to DOP exposure. To address this, we evaluated the in vivo effects of long-term DOP exposure on the expression of immune-related genes in *T. granosa*. We identified several genes that were promising candidates for biomarkers of DOP.

2 MATERIAL AND METHOD

2.1 Clams

Clams (*T. granosa*, average weight being 8.12±0.36 g) were purchased from a clam farm in Ningbo (Zhejiang Province, China). The clams were acclimated for a week before beginning the experiment. The temperature was maintained at 20–22°C throughout the experiment, and the salinity of the seawater was maintained at 30.

2.2 Treatment protocols

DOP (analytical purity >99.0%) was first dissolved in absolute ethanol and subsequently diluted with an equal volume of seawater to obtain a stock solution of 4 g/L. The clams were assigned randomly into four groups (30 individuals/group). The DOP stress experiments were performed by continuously feeding the clams one of three doses of DOP (final concentrations of 2.6, 7.8, or 31.2 mg/L). The control group was exposed to the same volume of ethanol and seawater. Water was charged twice within 1 week and the corresponding volume of DOP was supplied. We collected individuals from the experimental and control groups on days 7 and 14 and removed the visceral mass. We performed five replicates of each experimental and control group.

Table 1 Sequences of oligonucleotide primers used for qRT-PCR

Gene	Accession number	Forward and reverse primers
sHSP	HM125895	F: 5'-CCCAAGTAATGCCCTTTTCG-3' R: 5'-CGTTCAGTTCCTCGGGTTCA-3'
TIMP	JN663889	F: 5'-TGGAAATGGATGTAACAACCCCTG-3' R: 5'-AGCGTAACAGTCGCCAGGTAATC-3'
Tg-ferritin	GU078657	F: 5'-TGTGGCTCTGCCTAGTTTTGC-3' R: 5'-TGCCTGGTTGACACTTTTCTC-3'
SOD	GU078656	F: 5'-TCCAAATAGCTTCCGTTTACGTC-3' R: 5'-CACTGTTTAGCAGCAGCAAGTC-3'
MT	AAS753181	F: 5'-TGCCGGCGATAACTGCCGAT-3' R: 5'-TGTCACTGGTTACACGGACACGA-3'
Tg-HbI	HQ149305	F: 5'-TAAACTCGCAACAATGCCTTCC-3' R: 5'-TTGCCTTGTTTGATGTCTCCC-3'

2.3 Analysis of expression profiles of candidate genes using quantitative real-time PCR

Total RNA was isolated from the visceral mass of *T. granosa* using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using an MMLV First-Strand cDNA Synthesis Kit according to the manufacturer's instructions (Bio Basic Inc.). The expression levels of candidate genes of clams exposed to DOP were measured by quantitative real-time PCR (qRT PCR). We used β-actin as an internal control to verify successful reverse transcription and to calibrate the cDNA template. Two specific primers for each gene were designed to amplify the desired product (Table 1). Real-time PCR amplification was performed using a Rotor-Gene 6000 real-time PCR detection system. We used the reaction components and thermal profiles suggested by the manufacturer. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. Finally, the C_t values for the target amplified genes and for the internal control (β-actin) were determined for each sample, then converted into relative expression levels using the $2^{-\Delta\Delta Ct}$ method. All data are expressed as mean±SD. Differences between groups were analyzed by one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P<0.05$.

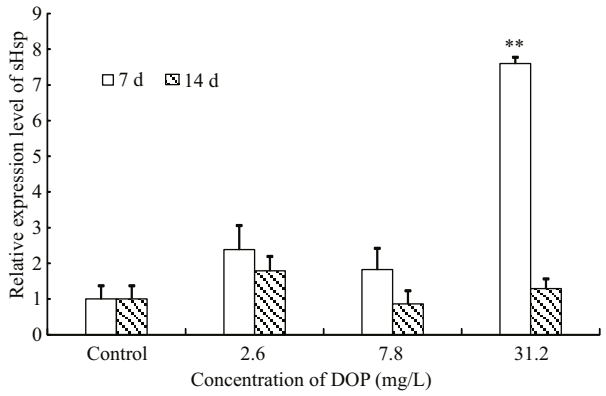

Fig.1 Mean±SD (*n*=5) expression of sHsp in the viscera of *T. granosa* after exposure to DOP for 7 and 14 days

Significant differences between the means of expression level were analyzed by one-way ANOVA followed by Tukey's test. The symbols * and ** indicate $P<0.05$ and $P<0.01$, respectively.

3 RESULT AND DISCUSSION

Recently, significant attention has been paid to the presence of di-octyl phthalate (DOP) in marine ecological systems and its effects on aquatic organisms. Some studies have shown that marine species are exposed to DOP in their natural habitat (Thurén, 1986; Tan, 1995). In higher animals, DOP disrupts the oxidative balance by increasing the production of nitric oxide (NO) and reactive oxygen species (ROS), leading to lipid oxidation, DNA damage, and activation of the ERK/NFkB signaling pathway (Ghosh et al., 2010). In crustaceans such as the giant freshwater prawn (*Macrobrachium rosenbergii*), DOP mediates the immune responses of hemocytes by inhibiting O^{2-} generation (Sung et al., 2003). To better understand the toxic effects of DOP on *T. granosa*, we evaluated the expression of six genes involved in the immune response using qRT-PCR.

3.1 Small heat shock proteins

Heat shock proteins (HSPs) are a class of functionally related proteins found in virtually all living organisms. They are divided into several families, including HSP90, HSP70, HSP60, HSP40, and small HSP (sHSP) according to their molecular size (Mayer, 1997; Santoro, 2000). Stressors, including DOP, that cause oxidative stress also affect the expression of HSPs (Wood et al., 1997; Lee et al., 2006). The sHSPs are small stress-induced proteins that function to prevent stress-induced cell damage by binding and maintaining the denatured proteins in a folding-competent state. A growing body of evidence indicates that increased expression of sHSPs in different cell types can increase tolerance to a variety of stresses, including heat, salt, drugs, viral infection, and oxidants (Jaya et al., 2009).

In our study, clam sHSP was up-regulated at all DOP dosage levels (Fig.1) after 7 d exposure to DOP. Indeed, we observed a 7.60-fold increase in expression when clams were exposed to 31.2 mg/L DOP. This is similar to the effect observed following exposure to other chemicals and heavy metals for 10 d (Comporti, 2002; Lesser, 2006). We speculate that the increase is a result of disturbances in the normal redox state and oxidative stress. Interestingly, the effects of DOP on the expression of sHSP were negligible by day 14.

3.2 Tissue inhibitor of metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) were originally characterized as inhibitors of matrix metalloproteinases (MMPs), but are also multifunctional proteins that are vital to the regulation of ECM (extracellular matrix) metabolism. The ECM plays a role in the defense against pathogens and viral infection. Because of their role in regulating ECM metabolism, TIMPs are also involved in the defense against infection by pathogens (Lepore et al., 1996; Okamoto et al., 1997; Miyoshi and Shinoda, 2000). The expression of TIMP is induced by shell damage or the pollutants in the aquatic environment (Montagnani et al., 2007).

To determine the effect of DOP on TIMP expression, we conducted a time-course analysis on expression levels of the gene in clams following exposure to different concentrations of DOP. The mRNA expression of TIMP in *T. granosa* was up-regulated significantly after the clams were stimulated by DOP, reaching a peak (24.7-fold compared with the control group) at day 7 at the highest concentration of DOP (31.2 mg/L) (Fig.2). The relative expression of TIMP exhibited the same pattern as sHSP—expression increased significantly after 7 d exposure and was highest in the group exposed to 31.2 mg/L DOP. Similarly, TIMP expression in the three experimental groups was not different from the control group after 14 d. Because the effects of DOP exposure on TIMP expression are poorly understood, we compared our findings with the response of TIMP to bacterial challenge (Okamoto et al., 1997; Montagnani et al., 2007). It was suggested that TIMP appears to play an important role in the immune regulation of clams exposed to short-term stressors, including DOP. Organisms can increase TIMP mRNA expression to

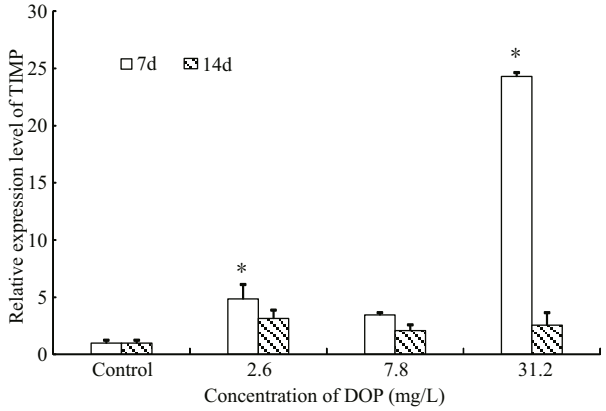

Fig.2 Mean±SD (*n*=5) expression of TIMP in the viscera of *T. granosa* after exposure to DOP for 7 and 14 days

Significant differences between the means of expression level were analyzed by one-way ANOVA followed by Tukey's test. The symbols * and ** indicate $P<0.05$ and $P<0.01$, respectively.

inhibit proteases that participate in ECM destruction and inactivate protease inhibitors and activate the latent form of MMP.

3.3 Tg-ferritin and SOD

Ferritin is a ubiquitous and highly conserved iron-binding protein and the principal protein for iron storage and metabolism. Additionally, ferritin has a protective role against oxidative damage (Zheng et al., 2010). Oxidative stress is an important component of the stress response in marine organisms (Vaughan, 1997; Livingstone, 2003). Bivalve mollusks produce ROS following exposure to heavy metals, high temperatures, and other environmental disturbances (Abele et al., 2002). Generally, ferritin is thought of as a "protective" protein that functions to protect cells from oxidative stress (Larade and Storey, 2004). Additionally, antioxidant enzymes also play important roles in eliminating ROS. In this process, superoxide dismutase (SOD) is a key enzyme that catalyzes the dismutative reaction of O^{2-} and transforms it into hydrogen peroxide and oxygen (Lee et al., 2007; Li et al., 2008).

The changes in expression of Tg-ferritin and SOD mRNA were similar, with up-regulation occurring in clams exposed to 2.6 and 7.8 mg/L DOP (Fig.3). At day 14, ferritin and SOD expression had increased 5.43-fold and 2.16-fold, respectively, relative to the control. At the highest concentration of DOP (31.2 mg/L), Tg-ferritin and SOD were down-regulated. There is evidence that hosts can differentiate the hazard and adopt different strategies to control ROS at a sustainable level by regulating the expression or activities of antioxidant enzymes. We speculate

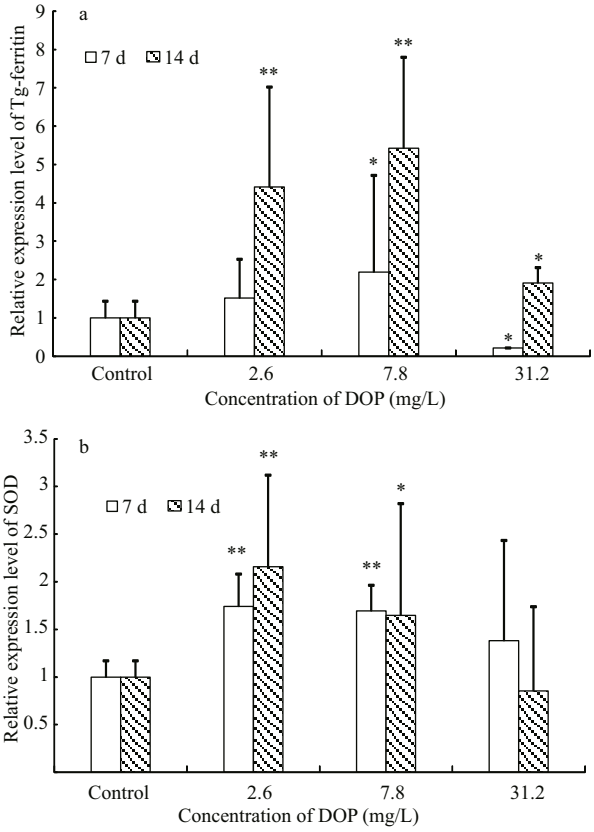

Fig.3 Mean±SD (*n*=5) expression of ferritin (a) and SOD (b) in the viscera of *T. granosa* after exposure to DOP for 7 and 14 days

Significant differences between the means of expression level were analyzed by one-way ANOVA followed by Tukey's test. The symbols * and ** indicate $P<0.05$ and $P<0.01$, respectively.

that Tg-ferritin and SOD are acute response proteins involved in the response to DOP exposure in *T. granosa* and that both proteins regulate the immune response of the host via the same immunologic mechanism.

3.4 Metallothionein

Metallothionein (MT) exists in most organisms and represents a group of cysteine-rich, low-molecular-weight intracellular proteins that act to bind and detoxify heavy metal ions (Kägi, 1991). MTs play important roles in the detoxification of essential metals or non-essential metals and they have been used as biomarkers for monitoring heavy metal pollution in many marine organisms, including fish, crabs, and shellfish (Coyle et al., 2002; Berthet et al., 2005). MT expression has been implicated as a transient response to virtually any form of injury or stress, providing a cytoprotective mechanism against the potential damaging effects of oxygen-derived free radicals through scavenging of reactive metal ions

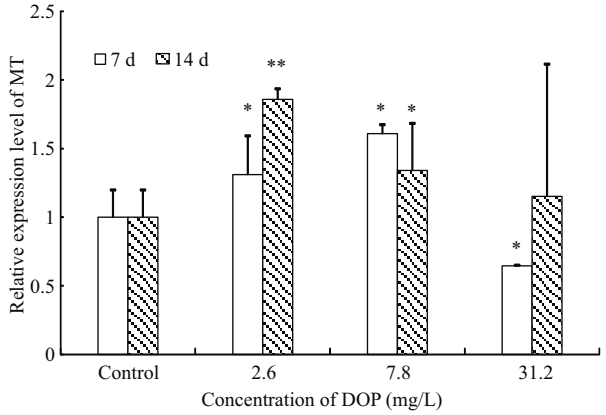

Fig.4 Mean±SD (*n*=5) expression of MT in the viscera of *T. granosa* after exposure to DOP for 7 and 14 days

Significant differences between the means of expression level were analyzed by one-way ANOVA followed by Tukey's test. The symbols * and ** indicate $P<0.05$ and $P<0.01$, respectively.

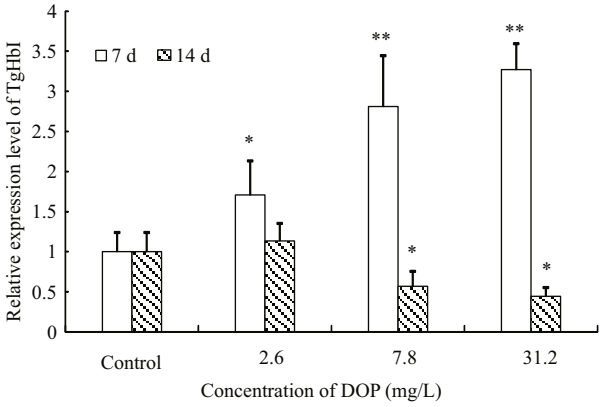

Fig.5 Mean±SD (*n*=5) expression of Tg-HbI in the viscera of *T. granosa* after exposure to DOP for 7 and 14 days

Significant differences between the means of expression level were analyzed by one-way ANOVA followed by Tukey's test. The symbols * and ** indicate $P<0.05$ and $P<0.01$, respectively.

(Ghosh et al., 2010).

The relative expression of MT was up-regulated then down-regulated as the concentration of DOP increased. In this study, 1.60-fold increase was detected at 7.8 mg/L on days 7, while 1.86-fold increase at 2.6 mg/L on days 14 (Fig.4). This pattern of MT expression is consistent with prior reports (Maity et al., 2011). We speculate that DOP exposure induces *T. granosa* to produce a poison stimulatory effect to induce MT gene expression. When the concentration of MT is sufficiently high to allow for DOP detoxification, MT gene expression was inhibited and exhibited a clear downward trend. The decrease in MT expression may be caused by the induction of other immune protective genes and/or increased tolerance through a different detoxification and adaptive mechanism (Posthuma and van Straalen, 1993).

3.5 Hemoglobin

Hemoglobin (Hb) is the iron-containing oxygen transport metalloprotein in the red blood cells of vertebrates and some invertebrates (Hardison, 1998), including some mollusks. In addition to the function of carrying oxygen, Hbs have been implicated in a number of processes, depending on the Hb types and the species (Coates, 1975; Terwilliger, 1998; Wajcman and Kiger, 2002). In the bivalve mollusk genus *Scapharca*, two distinct types of Hb, dimeric (HbI) and tetrameric (HbII), were found (Chiancone et al., 1981; Gambacurta et al., 2000). Bao et al. (2011) designated the HbI gene from the bloody clam *T. granosa* as Tg-HbI.

In our experiments, Tg-HbI exhibited two distinct expression patterns at two time points (Fig.5). At day 7, Tg-HbI expression was significantly up-regulated and positively correlated with the concentration of DOP, with the peak expression level detected at 31.2 mg/L DOP with a 3.27-fold increase compared with the untreated samples. At day 14, Tg-HbI expression was significantly down-regulated and reached a level that was approximately 0.44-fold that of the untreated samples at the highest concentration of DOP (31.2 mg/L). This expression pattern suggests that Hb is an inducible protein that is sensitive to the timing of DOP exposure. Previous studies have shown that Hb can be directly activated by microbial proteases and enhanced by pathogen-associated molecules (Bao et al., 2011). We speculate that the different patterns of Tg-HbI expression upon DOP exposure are related to ROS production and its detoxification. However, to better understand the role of Tg-HbI during DOP exposure, the molecular mechanism and the changes in Tg-HbI activity at the protein level behind this interaction need to be investigated further.

4 CONCLUSION

Our results suggest that the expressions of the immune genes Tg-ferritin, SOD, and MT are affected by exposure to DOP for up to 14 d in *T. granosa*. Conversely, the effects of DOP on other genes, particularly sHSP and TIMP, were limited to the first 7 d of exposure.

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