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Endocrine-disrupting chemicals impair the innate immune prophenoloxidase system in the intertidal mud crab, *Macrophthalmus japonicus*

Kiyun Park, Won-Seok Kim, Ihn-Sil Kwak*

Faculty of Marine Technology, Chonnam National University, Yeosu, 550-749, South Korea

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ABSTRACT

Endocrine-disrupting chemicals (EDCs), xenobiotics that interfere with endogenous hormone function, have been studied for their impacts in aquatic environments. However, there is limited information about the potentially hazardous impact of bisphenol A (BPA) and di-(2-ethylhexyl) phthalate (DEHP) on the marine environment. The aim of this study was to investigate the effects of BPA and DEHP on the immune response of the intertidal mud crab, *Macrophthalmus japonicus*. In order to examine immunological responses involving the prophenoloxidase (proPO) system, mRNA transcript and activity levels of six immune-related genes, including lipopolysaccharide and β-1,3-glucan-binding protein (LGBP), proPO, phenoloxidase (PO), peroxinectin (PE), serine protease inhibitor (Serpin), and trypsin (Tryp), were assessed in *M. japonicus* hepatopancreas and gills exposed to BPA or DEHP. Expression of immune genes generally decreased in *M. japonicus* hepatopancreas and gills exposed to all concentrations of BPA by days 4 and 7. However, at day 1, expression of Serpin and Tryp genes was significantly increased in *M. japonicus* hepatopancreas and gills exposed to BPA. For DEHP exposure, all genes, with the exception of Serpin, were significantly downregulated in *M. japonicus* gills. In the hepatopancreas, gene expression of PO, proPO, and LGBP increased at day 1, and then decreased by day 7, while mRNA expression of Serpin and Tryp exhibited up-regulation over all exposure periods. In addition, PE gene expression was upregulated in hepatopancreas at day 7 in a dose-dependent manner. Taken together, these results indicated that the crab immune responses were perturbed by exposure to BPA, and, in particular, DEHP.

1. Introduction

Environmental pollutants are a global concern, due to their potential effects of altering neurophysiological, biochemical, and behavioral parameters in many organisms [1]. In particular, endocrine-disrupting chemicals (EDCs) are harmful chemicals that can potentially interfere with the endocrine system in vertebrates [2] and invertebrates [3]. Bisphenol A (BPA) and di-(2-ethylhexyl) phthalate (DEHP) are currently the most-used raw materials in industry. They are used in the production of high-volume chemicals in a wide variety of manufactured and consumer products, such as food packaging materials, cloth, medical devices, and furnishings [4–6]. Due to their widespread use, many species have been exposed to various levels of BPA and DEHP. BPA was reported to reach a level of 320 ng L^{-1} in surface river water in the Netherlands, and 27.7 ng g^{-1} (dry weight) in estuarine sediment in China [7,8]. Furthermore, DEHP was reported to reach a level of 45.73 μg g^{-1} in the Yellow River, and 1.56 μg g^{-1} (dry weight) in Qiantang river sediment in China [9,10]. BPA and DEHP are some of the most representative polluting compounds, as they exhibit predominant effects on environmental and human health. As a result of their hazardous nature to the ecosystem, they have been included in the priority pollutants by the U.S. Environmental Protection Agency (USEPA) [11].

The endocrine disrupting effects of BPA and DEHP have been widely reported in aquatic animals, such as aquatic invertebrates, fish, and crabs [6,12,13]. Endocrine systems coordinate growth, reproduction, development, and physiological processes [14,15]. However, exposure to EDCs, such as BPA and DEHP, can alter reproductive processes, behavior, and immune responses. When exposed to 100 μg L^{-1} DEHP and BPA, chironomid larvae showed altered cellular development and changed expression of estrogen-related receptor [3,16]. In addition, some researchers reported that *Xenopus laevis* tadpoles and tilapia species (*Sarotherodon melanotheron*, *Tilapia guineensis*) showed degradation of reproductive function and feminization with EDC exposure

* Corresponding author. Faculty of Marine Technology, Chonnam National University, Chonnam, 550-749, South Korea.
E-mail addresses: iskwak@chonnam.ac.kr, inkwak@hotmail.com (I.-S. Kwak).

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[17,18]. Wen and Pan [19] reported benzo[a]pyrene caused reduction of 17β-estradiol and testosterone levels, and observed degeneration of the crustacean ovary.

The innate immune system is composed mainly of the prophenoloxidase (proPO) system, which is one of the most effective humoral defenses in crustacean invertebrates [20]. The activation of proPO system is triggered in response to the detection of specific pattern recognition proteins (PRPs), which recognize molecules displayed by the microorganisms, known as pathogen-associated molecular patterns (PAMPs) [21,22]. Different PRP types have been reported, including β-1,3-glucan binding protein (βGBP), lipopolysaccharide, β-1,3-glucan-binding protein (LGBP), peptidoglycan (PGBP), and gram-negative binding protein (GNBP) [23,24]. PRPs can trigger the serine proteinase cascade, which generates a pro-form activating enzyme. This leads to the synthesis of melanin, and reaction with an intermediate compound, invading microorganisms [25–28]. When proPO activation is knocked down, invertebrates become vulnerable to pathogens and viruses [29]. The proPO system consists of important proteins, including peroxinectin (PE), serine protease inhibitor (Serpin), and trypsin-like serine protease (Tryp). Crustacean proPO related genes have been identified in black tiger shrimp, *Penaeus monodon* [30], Pacific white shrimp *Ltipenaeus vannamei* [31], and Chinese mitten crab *Eriocheir sinensis* [32]. proPO activation by external stimuli, including pathogen challenges and high temperatures, has been identified in many crustacean species [33–35]. However, most proPO system studies have focused on pathogen and viral infection, and on the mechanisms of immunity in the vertebrate proPO system.

The intertidal mud crab (*Macrophthalmus japonicus*), a commercially important species, is distributed ubiquitously across the Indo-Pacific region, and dominantly in Korea and Japan [36,37]. The crab's distribution suggests a sensitive species response to intertidal environment changes [37,38]. Thus, *M. japonicus* inhabiting the sediment is a good indicator for monitoring the effects of concentrated chemicals in benthic environments. Furthermore, the crustacean gill and hepatopancreas have been reported as important components for the physiological processes, and for defense responses to external stress [39]. The tissue specific responses of *M. japonicus* can result from physiological stress exposure to low and high salinity [38,40]. Park et al. [37] observed changes to exoskeleton surface roughness, and expression of vital participation genes following exposure to the antifouling biocide Irgarol. In the present study, we evaluated the molecular processes involving the immunological responses of *M. japonicus* under BPA and DEHP stress conditions. To accomplish this, we investigated the survival rate, phenoloxidase (PO) activity, and proPO-related gene expression, including *Mj*-proPO, *Mj*-LGBP, *Mj*-PE, *Mj*-Serpin, and *Mj*-Tryp, in *M. japonicus* gills and hepatopancreas after BPA and DEHP exposure (Fig. 1).

2. Materials and methods

2.1. Test organisms

M. japonicus (width = 4.0 ± 0.5 cm; height = 3.5 ± 1.0 cm; weight = 8.0 ± 2.0 g) were collected from fish markets of Suncheon Bay in Korea. The organisms were transported to a laboratory, where they were cultured in natural seawater. The crabs were acclimatized to laboratory culture conditions, with aeration at 16.0 ± 1.5 °C for one day. They were fed approximately 180 mg of Tetramin (Tetra-Werke, Melle, Germany) daily. Experiments were conducted following the Chonnam National University Institutional Animal Care and Use Committee. All crabs were healthy, with both claws and appendages intact, and with no disease symptom.

2.2. Exposure experiment and survival experiments

BPA was purchased from Sigma-Aldrich (99.9% pure, USA) and DEHP solutions were prepared from the solid compound (99%, Junsei Chemical Co. Ltd., Japan). The concentration of stock solutions was 10 mg L^1 for BPA and DEHP, made by dissolving in analytical grade acetone (99%). The stock solutions were diluted with seawater to make test solutions of 1 μg L^{-1}, 10 μg L^{-1}, and 30 μg L^{-1}. In this study, crabs were divided into five conditions (1 μg L^{-1}, 10 μg L^{-1}, and 30 μg L^{-1} treatment groups, seawater and solvent controls; $n = 45$ for each condition), and animals in each condition category were then subsequently divided into two groups: one for survival rate ($n = 20$) and the other for mRNA expression analysis at various exposure times, after exposure to the three BPA and DEHP concentrations ($n = 25$). Experiments were performed in triplicate.

Survival rate was recorded every day until 7 days post BPA and DEHP exposure. Gills and hepatopancreas tissue were extracted at days 1, 4, and 7 from individuals in each treatment condition and from the control. Extracted tissues were frozen in liquid nitrogen after tissue sampling and were stored at −80 °C for further experiments. During experiments, salinity conditions, water temperature, dissolved oxygen, and solution concentration values were measured every day for all exposure and control animals.

2.3. Total RNA extraction and single-strand cDNA synthesis

The gills and hepatopancreas (30–35 mg $crab^{-1}$) of treatment and control group animals were homogenized in five volumes of TRIzol® reagent (Life Technologies, USA) with a tissue grinder. Total RNA was isolated according to the manufacturer's protocol. Genomic DNA was removed using Recombinant DNase I (RNase free) (Takara, Japan). Total RNAs were measured using a Nano-Drop 1000 (Thermo Fisher Scientific, USA), and the concentration was equalized using nuclease-free water. RNA integrity was checked by 1% agarose gel electrophoresis. The samples were stored at −80 °C. Single-stranded cDNA was synthesized from 1 μg of total RNA using an oligo dT primer (50 μM) for reverse transcription in 20 μL reactions (PrimeScript™ 1st strand cDNA synthesis kit, Takara) according to the protocol of Nikapitiya et al. [40].

2.4. PO activity assay

PO activities were evaluated with a modified method [41], assessing the transformation of L-3, 4-dihydroxyphenylalanine (L-DOPA D-9628, Sigma) to dopachrome at 490 nm. Briefly, 100 μL of the lysate supernatant was mixed with 50 μL of L-DOPA (3 mg mL^{-1}) dissolved in PBS. The control used the same volume of PBS. The formation of dopachrome was determined at 490 nm every 2 min over a period of 10 min using a Gene Quant 1300 spectrophotometer (GE Healthcare Bio-Science, UK). Enzyme activity was calculated by determining the increase in absorbance rate. One unit (U) was defined as $10^{-3} \times$ A490 min^{-1} per mg protein. The protein content was measured according to the BCA™ protein assay kit (Pierce, USA). The experiments were repeated three times.

2.5. Transcriptional analysis of M. japonicus candidate genes

Evaluation of the mRNA expression levels of five candidate genes, (*Mj*-proPO, *Mj*-LGBP, *Mj*-PE, *Mj*-Serpin, and *Mj*-Tryp), in the control and in the treated condition groups, was performed by quantitative real time polymerase chain reaction (qRT-PCR) analysis. Glyceraldehyde-3-phosphate dehydrogenase (*Mj-GAPDH*) was used as an internal control (the suitability of this gene as an endogenous control was tested [38]). The five candidate genes and *Mj-GAPDH* cDNA were amplified by qRT-PCR using the master mix (Bioneer, Korea) (Table 1 for primers sequences). Sequence information was identified from a transcript database [37]. qRT-PCR was performed using 5 μL of 30-fold diluted original cDNA, 10 μL of 2 × SYBR, 0.5 μL of each primer (10 μM) and 4.0 μL DEPC-treated water to a total volume of 20 μl. PCR conditions

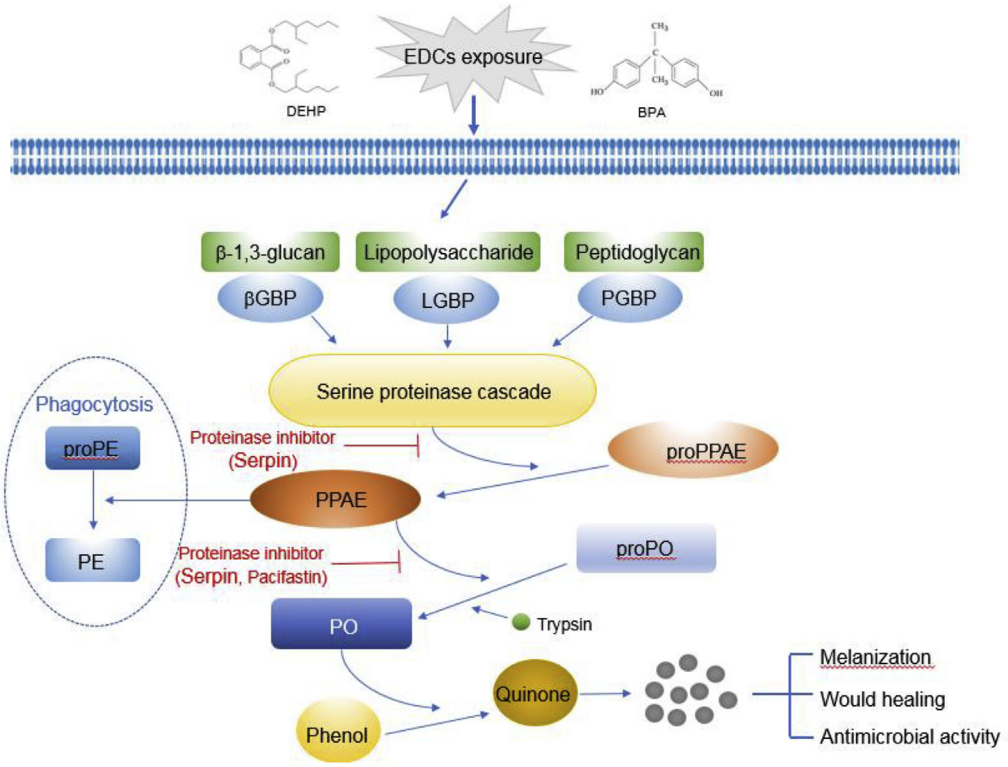

Fig. 1. Schematic diagram of the predicted EDC-induced prophenoloxidase (proPO)-activating system in *M. japonicus*, based on the following Refs. [22,23]. PE: Peroxinectin, proPE: Properoxinectin, PPAE: proPO-activating enzyme, proPPAE: proproPO-activating enzyme, PO: phenoloxidase, proPO: Prophenoloxidase.

were as follows: 95 °C for 3 min, 40 cycles each of 15 s at 95 °C, 35 s at 57 °C, and 20 s at 72 °C with a final step for melting curve analysis (from 67 °C to 95 °C, increment of 1 °C every 5 s). Amplification and detection of SYBR Green-labeled products were performed using an Exicycler™ 96 real time system software (version 3.54.8) (Bioneer, Korea). Relative gene expression levels were determined by normalization to *Mj-GAPDH* to account for differences in reverse transcriptase efficiency by the $2^{-\Delta\Delta ct}$ method [42].

2.6. Data analysis

SPSS 12.0 KO (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data are represented as means ± SD. Significant differences between the control and EDCs exposure groups were examined by one-way analysis of variance (ANOVA) combined with Tukey's multiple range test. Independent sample *t*-test was used to compare the statistical significance of mRNA expression between hepatopancreas and gills under different EDCs exposures. Differences with $P < 0.05$ were regarded as significant.

3. Results

3.1. Survival rates of M. japonicus after EDCs exposures

Fig. 2 shows the survival rate (%) of crabs for day 7 after exposure to various BPA and DEHP concentrations. *M. japonicus* exposed to 1 μg L^{-1} BPA began to die at day 1 (92.1%) and survival rate decreased by day 3 (86.8%) and was finally 73.7% at day 7 (Fig. 2A). In 10 μg L^{-1} BPA, *M. japonicus* also started to die from day 1 (97.4%); the survival rate declined at day 2 (94.7%) and showed 92.1% cumulative survival rate by day 7. Crabs exposed to 30 μg L^{-1} BPA started to die at day 1 (91.9%), and continually declined until day 7 (64.9%), and the survival rate was lower than 1 with 10 μg L^{-1} BPA at day 7.

M. japonicus exposed to 1 μg L^{-1} DEHP began to die at day 1 (97.1%), and the survival rate decreased at day 6 (88.6%) and was finally 85.7% by day 7 (Fig. 2B). *M. japonicus* exposed to 10 μg L^{-1} DEHP also began to die at day 1, and survival continually declined until day 7 (69.6%). In 30 μg L^{-1} DEHP, *M. japonicus* started to die at day 1 (70.0%), and continually declined until day 7 (25.0%). The DEHP

Table 1
Primer used in the real-time PCR amplification.

Gene Name	Primer sequence (5′-3′)	Amplification size (bp)	Efficiency E (%)	Accession number
Mj-LGBP 1F	AATGGCTTCTTCCCTGACGG	131	100.0	KJ653260
Mj-LGBP 2R	CTGATCTTGCCCTCACCCTG			
Mj-proPO F	CCTCTTCTTCACGACACTCAACTG	143	98.0	FJ215871
Mj-proPO R	TCACGAGATAACACAAAACGCC			
Mj-PX 3F	CTGACCACCATACACACGCT	98	90.0	KJ653262
Mj-PX 4R	TGGAACACTTGCTCGTCCTG			
Mj-Serpin 5F	TTTGGAACGTGGGAGTATGC	74	93.0	MH411109
Mj-Serpin 6R	TGCACATTGGGAATCGCATG			
Mj-Tryp 7F	CCTAGAGGTCGGGGTCAAGA	91	99.5	KJ653261
Mj-Tryp 8R	CCTATCCAGCTCGAGCAGTG			
Mj-GAPDH 9F	TGCTGATGCACCCATGTTTG	147	102.5	KJ653265
Mj-GAPDH 10R	AGGCCCTGGACAATCTCAAAG			

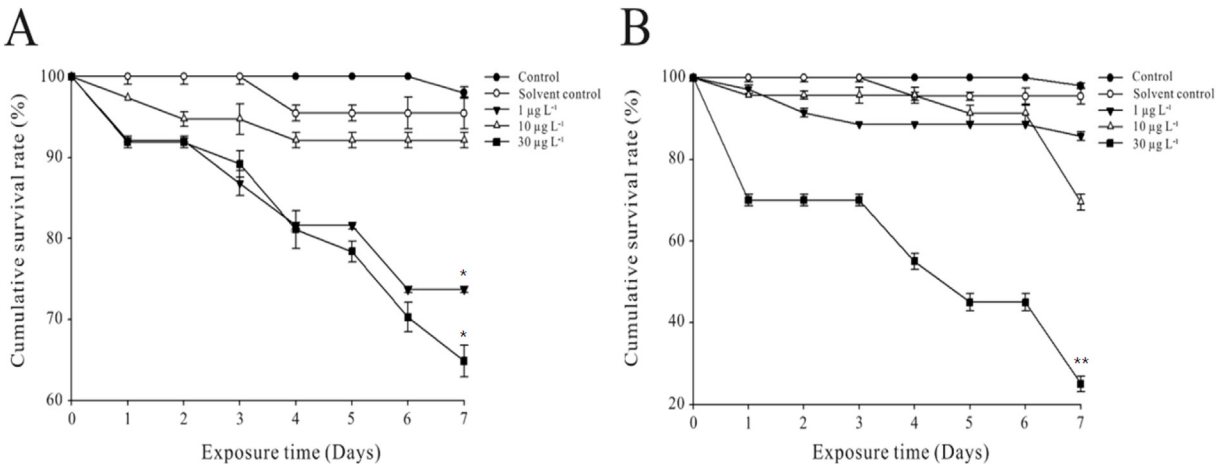

Fig. 2. Cumulative survival rate (%) of *M. japonicus* exposed to (A) BPA and (B) DEHP for 7 d. Each graph represents the mean value (n = 60) with the standard deviation (SD) ± . Differences between treated and non-treated samples (control) were considered to be significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

30 μg L^{-1} survival rate was lower than 1 μg L^{-1}, 10 μg L^{-1} DEHP of day 7. Control crabs that were not treated with BPA and DEHP had a 100% survival rate, which declined slightly by day 7 (98.0%). The solvent control crabs showed the highest survival rate (95.6% at day 7), which was similar to the survival rate of the control.

3.2. Expression of proPO related genes in M. japonicus gills exposed to BPA

The PO activity in *M. japonicus* gills was significantly inhibited at day 7 ($P < 0.05$), while it was slightly elevated at days 1 and 4 after exposure to 1 μg L^{-1} and 10 μg L^{-1} BPA, respectively (Fig. 3A). The level of *Mj*-proPO gene expression also increased at days 1 and 4, and then decreased at day 7, similar to the PO activity pattern (Fig. 3B). *Mj*-LGBP mRNA transcription showed up-regulation with 1 μg L^{-1} BPA exposure at day 4. At day 1, the gene expression of *Mj*-PE with 10 μg L^{-1} BPA exposure was significantly higher (6.3-fold) than the control. The levels of *Mj*-LGBP and *Mj*-PE expression at day 7 were low, compared to the level of the control, for all concentrations of BPA exposure (Fig. 3C and D). *Mj*-Serpin transcription at day 1 was observed to be at significantly higher levels than that of the control (Supplementary Table 1), and highest expression (45.5-fold) with 30 μg L^{-1} BPA. However, *Mj*-Serpin expression at day 4 decreased at 1 (0.2-fold), 10 (0.1-fold), and 30 (0.4-fold) μg L^{-1} BPA, compared to control levels (1.0-fold). Similarly, *Mj*-Serpin at day 7 showed lower expression levels than the control (Fig. 3E). In addition, the mRNA level of *Mj*-Tryp transcripts at day 1 showed high expression levels at 1 (4.9-fold), 10 (1.9-fold), and 30 (3.0-fold) μg L^{-1} BPA, compared to the control (Supplementary Table 1). At day 4 and 7, *Mj*-Tryp showed low expression at 1 (0.5-fold) and 10 (0.5-fold) μg L^{-1} BPA compared to control levels, but not with 30 μg L^{-1} BPA. *Mj*-Tryp transcription by day 7 had recovered to the control level with 10 and 30 μg L^{-1} BPA exposure conditions (Fig. 3F). The decreased expression of all proPO related genes was time-dependent in *M. japonicus* gills exposed to the relatively high concentration of 30 μg L^{-1} BPA.

3.3. Expression of proPO related genes in M. japonicus hepatopancreas exposed to BPA

Increased PO activity was observed in *M. japonicus* hepatopancreas at day 1. (Fig. 4A). In particular, at day 1, 1 μg L^{-1} BPA exposure significantly induced PO activity, compared to controls. However, the induced PO activity at all BPA concentrations was inhibited at day 7. After BPA exposure, the expression of *Mj*-proPO was significantly up-regulated in *M. japonicus* hepatopancreas at day 1, at 1 (4.8-fold), 10 (3.9-fold), and 30 (1.8-fold) μg L^{-1} BPA, compared to controls (Supplementary Table 1). Then, *Mj*-proPO mRNA expression was down-regulated at days 4 and 7 (Fig. 4B). Hepatopancreas exposed to 1 μg L^{-1} BPA at day 1 showed significantly increased levels of *Mj*-LGBP expression, compared with controls, 10 μg L^{-1} and 30 μg L^{-1} (Fig. 4C). *Mj*-LGBP gene expression showed a significant decrease at day 4 ($P < 0.05$) for all concentrations, and slightly recovered to the control levels by day 7. The level of *Mj*-PE gene expression was also upregulated in 1 μg L^{-1} BPA at day 1, and all expression of *Mj*-PE was inhibited at day 4, similar to *Mj*-LGBP (Fig. 4D). In addition, *Mj*-Serpin gene expression at day 1 showed significantly high expression levels after exposure to 1 μg L^{-1} (51.5-fold), 10 μg L^{-1} (158.9-fold), and 30 μg L^{-1} (154.1-fold) BPA ($P < 0.01$) (Supplementary Table 1). At day 4, *Mj*-Serpin transcription was observed at lower levels than that in the controls. At day 7, only 1 μg L^{-1} BPA exposure increased *Mj*-Serpin in *M. japonicus* hepatopancreas (Fig. 4E). The expression of *Mj*-Tryp transcript was elevated for all concentrations of BPA at day 1, inhibited at day 4, and then recovered to the control level at day 7 (Fig. 4F). The down-regulation of proPO-related gene expression was observed at day 4 after BPA exposure, but this was not the case for PO activity.

With BPA exposure, PO activity, and *Mj*-LGBP, *Mj*-Serpin and *Mj*-Tryp mRNA levels did not show significant differences between gills and hepatopancreas ($P > 0.05$). *Mj*-proPO and *Mj*-PE mRNA levels showed significant differences between gills and hepatopancreas tissues ($P < 0.001$). Two-way ANOVA results suggested a significant interaction between exposure time and BPA concentration in the PO activity of gills ($P < 0.001$), *Mj*-proPO of hepatopancreas ($P < 0.001$), *Mj*-LGBP of gills ($P = 0.001$) and *Mj*-Serpin of hepatopancreas ($P < 0.002$).

3.4. Expression of proPO related genes in M. japonicus gills exposed to DEHP

With DEHP exposure, PO activity was significantly inhibited at 30 μg L^{-1} DEHP at day 1, although this value was slightly increased at 1 and 10 μg L^{-1} DEHP. A significant decrease in PO activity was continually observed in *M. japonicus* gills exposed to all concentrations of DEHP at days 4 and 7 (Fig. 5A). The level of *Mj*-proPO gene expression was generally inhibited at all exposure times and concentrations of DEHP (Supplementary Table 2). After DEHP exposure, the pattern of *Mj*-proPO gene expression was decreased in a time-dependent manner (Fig. 5B). In addition, *Mj*-LGBP mRNA expression decreased at all concentrations of DEHP for all exposure times. In particular, *Mj*-LGBP gene expression was significantly down-regulated at day 1 (Fig. 5C). *Mj*-PE transcription levels on DEHP exposure in gills showed low expression at day 1, compared to the basal level of controls (Fig. 5D). At day 4, *Mj*-PE transcripts were observed to be similarly decreased at day 1.

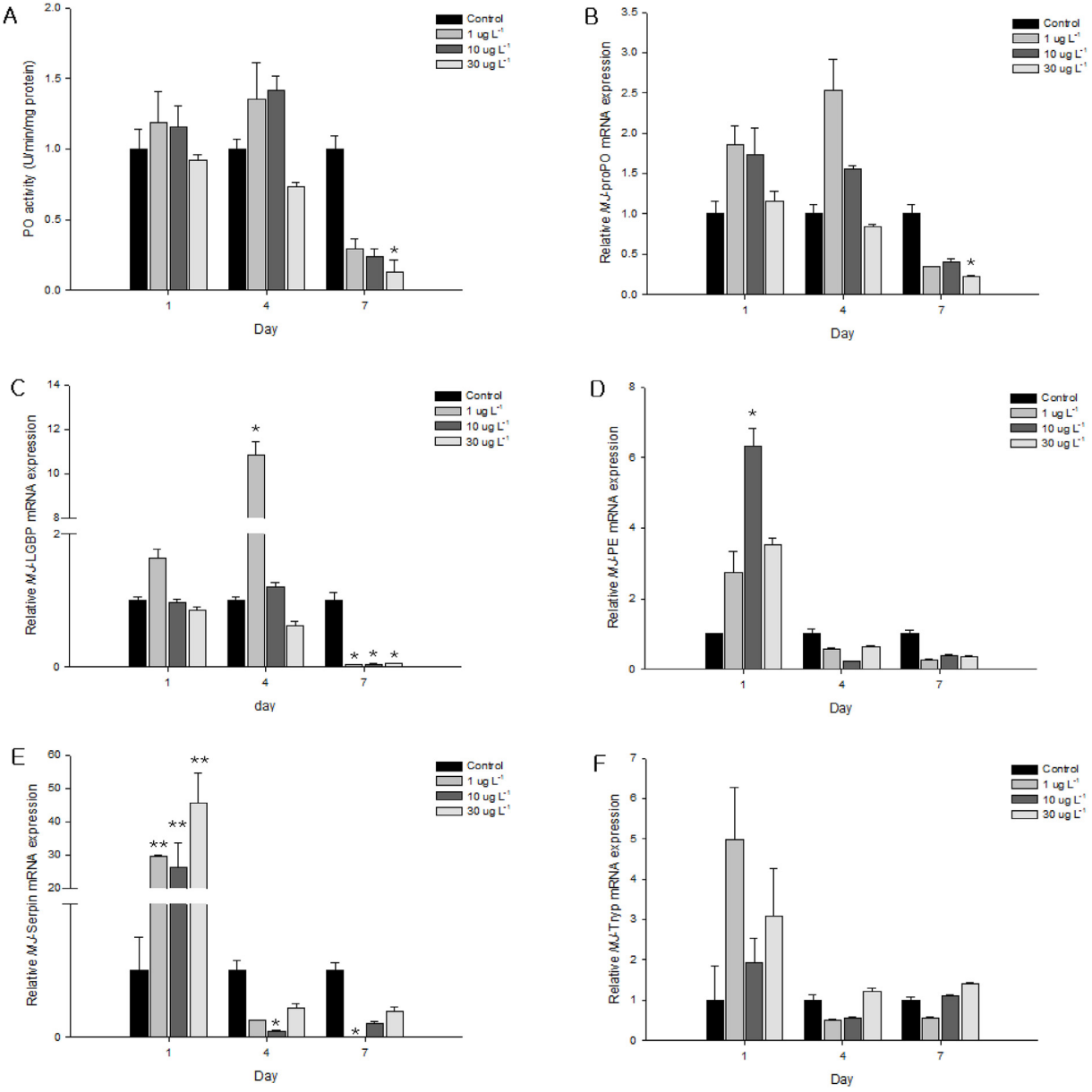

Fig. 3. Responses of six immune-related genes in *M. japonicus* gills exposed to 1, 10 and 30 μg L^{-1} BPA at days 1, 4 and 7. We analyzed (A) phenoloxidase (PO) activity (U/min/mg protein), and (B) relative mRNA expression of prophenoloxidase (*Mj*-proPO), (C) lipopolysaccharide and β-1,3-glucan-binding protein (*Mj*-LGBP), (D) peroxinectin (*Mj*-PE), (E) serine protease inhibitor (*Mj*-Serpin) and (F) trypsin (*Mj*-Tryp) genes. The values were normalized against GAPDH. Significance levels were $^{*}P < 0.05$ and $^{**}P < 0.01$, and values represent means ± SD.

Expression decreased in a dose-dependent manner. By day 7, the level of *Mj*-PE gene expression was also decreased in *M. japonicus* gills, whereas the expression of *Mj*-PE mRNA increased with 1 μg L^{-1} DEHP (2.0-fold) exposure. At day 1, *Mj*-Serpin transcription showed significantly high expression levels at all DEHP concentrations ($P < 0.01$) (Fig. 5E), with a similar expression pattern with BPA exposure at day 1 (Fig. 4E). At day 4, *Mj*-Serpin expression decreased gradually at 1 μg L^{-1} (0.8-fold), 10 μg L^{-1} (0.8-fold), and 30 μg L^{-1} (0.4-fold) DEHP. Furthermore, expression of *Mj*-Serpin at day 7 showed a low level, compared to controls, but not so 1 (39.8-fold) μg L^{-1} DEHP. *Mj*-Tryp mRNA expression generally was at similar levels, compared to controls, although *Mj*-Tryp was slightly decreased at 1 μg L^{-1} and 30 μg L^{-1} DEHP at 4 day. At day 1, *Mj*-Tryp transcription levels with 10 μg L^{-1} DEHP exposure were higher (1.9-fold) than control levels. Thus, the expression levels of *Mj*-Tryp generally exhibited no significant changes compared to controls. After DEHP exposure in gills, expression of proPO related genes, including PO, *Mj*-proPO, *Mj*-LGBP, and *Mj*-PE, was generally inhibited for all exposure times, which was not the case for *Mj*-Serpin and *Mj*-Tryp (Supplementary Table 2).

3.5. Expression of proPO related genes in M. japonicus hepatopancreas exposed to DEHP

In the hepatopancreas, PO activity was significantly elevated at all concentrations of DEHP at day 1. However, at days 4 and 7, PO activity generally showed at similar expression level compared to controls (Fig. 6A). The level of *Mj*-proPO gene expression increased at day 1, and gradually decreased at day 4. At day 7, *Mj*-proPO transcription level was down-regulated in *M. japonicus* hepatopancreas compared to control levels (Fig. 6B). In addition, the level of *Mj*-LGBP transcription at day 1 showed significantly higher levels at all concentrations of DEHP in comparison to controls ($P < 0.01$) (Fig. 6C and Supplementary

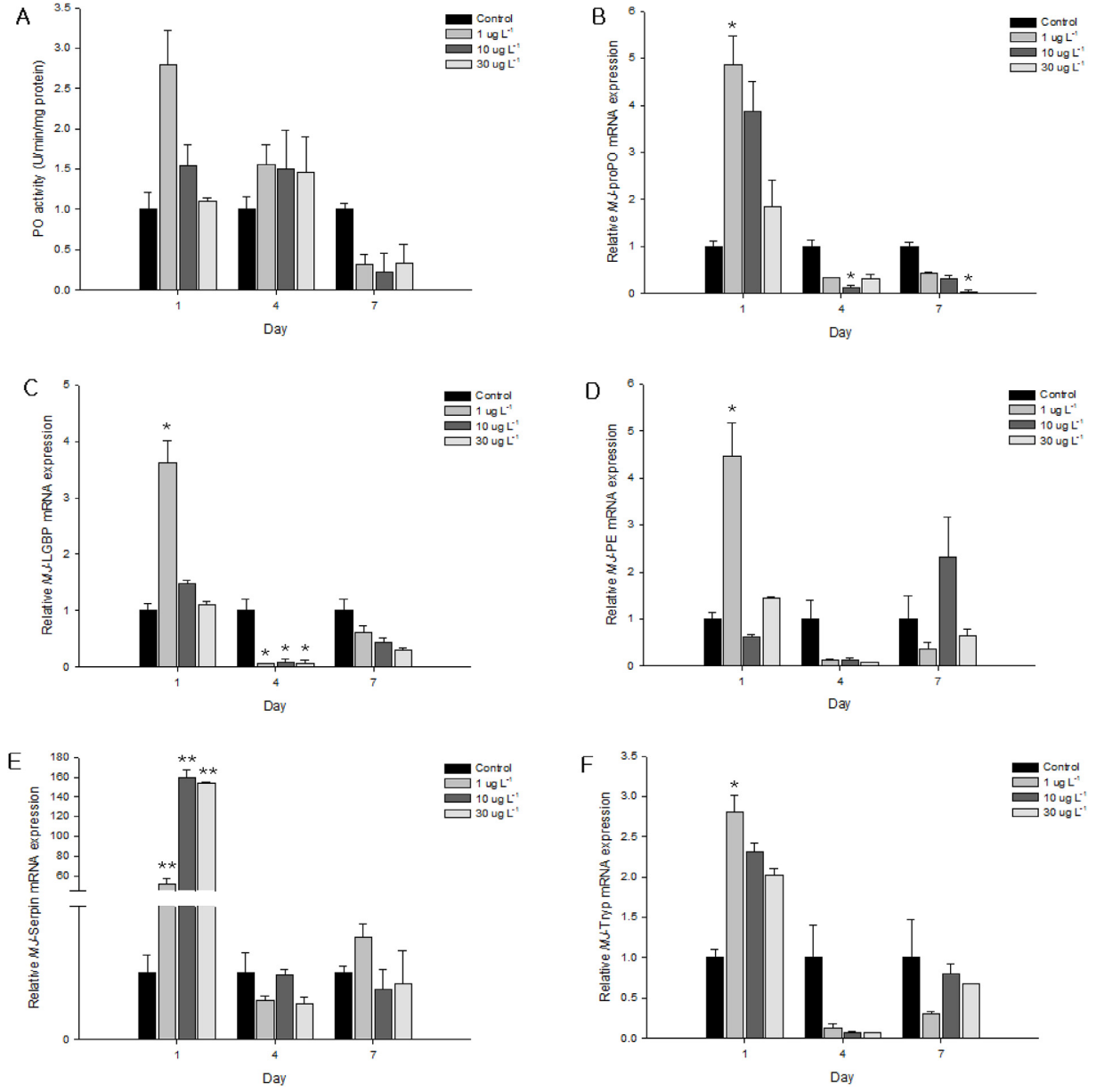

Fig. 4. Responses of six immune related genes in *M. japonicus* hepatopancreas exposed to 1, 10 and 30 μg L^{-1} BPA at days 1, 4 and 7. We analyzed (A) phenoloxidase (PO) activity (U/min/mg protein), and (B) relative mRNA expression of prophenoloxidase (*Mj*-proPO), (C) lipopolysaccharide and β-1,3-glucan-binding protein (*Mj*-LGBP), (D) peroxinectin (*Mj*-PE), (E) serine protease inhibitor (*Mj*-Serpin) and (F) trypsin (*Mj*-Tryp) genes. Values were normalized against GAPDH. Significance levels were $^{*}P < 0.05$ and $^{**}P < 0.01$, and values represent means ± SD.

Table 2). At day 4, 10 μg L^{-1} DEHP exposure induced higher level of *Mj*-LGBP than control, and 30 μg L^{-1} DEHP exposure induced lower level of *Mj*-LGBP than control. At day 7, *Mj*-LGBP gene expression decreased with all concentrations of DEHP (Fig. 6 C). The decreased pattern of *Mj*-proPO and *Mj*-LGBP gene expression was observed after exposure in a time-dependent manner. *Mj*-PE gene expression was generally downregulated compared to control levels, except at day 7 (Fig. 6D). At day 1 after DEHP exposure, *Mj*-PE transcription was decreased at 1 μg L^{-1} (0.3-fold), 10 μg L^{-1} (0.1-fold), and 30 μg L^{-1} (0.1-fold) DEHP, in comparison with the expression level of controls (1.0-fold) (Supplementary Table 2). Similar to day 1, the expression of *Mj*-PE decreased at day 4, compared to control levels. However, significant up-regulation of *Mj*-PE mRNA was observed in *M. japonicus* hepatopancreas at day 7, and the highest expression level was with 30 μg L^{-1} DEHP (17.2-fold). There was a trend of increasing *Mj*-PE gene expression in hepatopancreas in a time-dependent manner. At day 1, the level of *Mj*-Serpin gene transcription was significantly higher at all concentrations of DEHP, compared to control levels ($P < 0.01$) (Fig. 6E). *Mj*-Serpin gene expression also increased with all concentrations of DEHP at day 4. At day 7, the increasing pattern of *Mj*-Serpin expression was observed for 1 and 10 μg L^{-1} DEHP, but not with 30 μg L^{-1} DEHP. Thus, after DEHP exposure, *Mj*-Serpin gene expression was generally significantly elevated in *M. japonicus* hepatopancreas (Supplementary Table 2). In addition, DEHP exposure for 1 day significantly induced *Mj*-Tryp gene expression in *M. japonicus* hepatopancreas in a dose-dependent manner (Fig. 6F). At day 4, up-regulation of *Mj*-Tryp transcript levels was observed at 10 (9.7-fold) μg L^{-1} DEHP. At day 7, the level of *Mj*-Tryp gene transcription was significantly elevated at 1 μg L^{-1} (5.4-fold) and 30 μg L^{-1} (5.0-fold) DEHP, compared to controls.

With DEHP exposure, PO activity, and *Mj*-proPO, *Mj*-LGBP and *Mj*-PE mRNA levels did not show significant differences between gills and hepatopancreas ($P < 0.001$). *Mj*-Serpin and *Mj*-Tryp expression levels

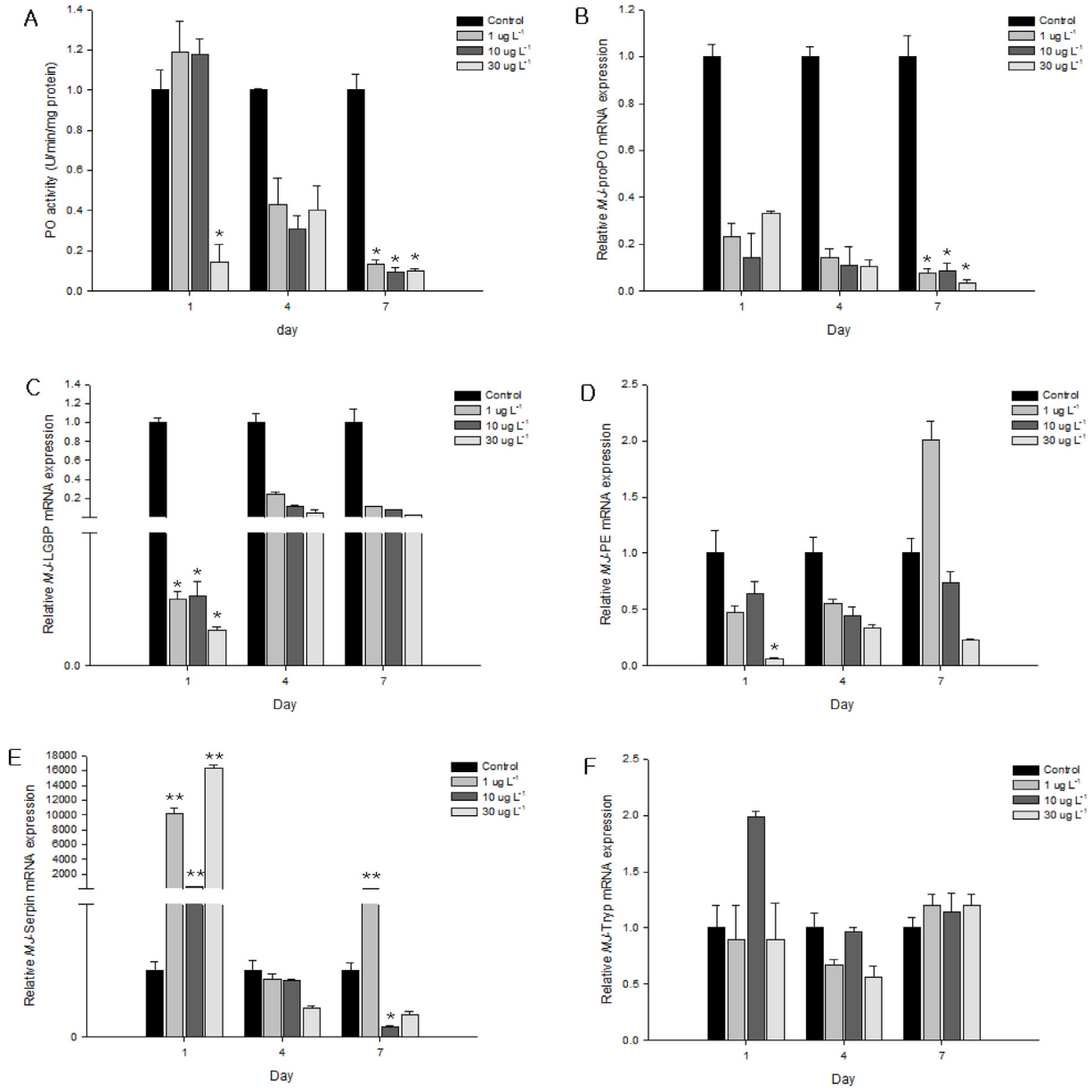

Fig. 5. Responses of six immune related genes in *M. japonicus* gills exposed to 1, 10 and 30 μg L^{-1} DEHP at days 1, 4 and 7. We analyzed (A) phenoloxidase (PO) activity (U/min/mg protein), and (B) relative mRNA expressions of prophenoloxidase (*Mj*-proPO), (C) lipopolysaccharide and β-1,3-glucan-binding protein (*Mj*-LGBP), (D) peroxinectin (*Mj*-PE), (E) serine protease inhibitor (*Mj*-Serpin) and (F) trypsin (*Mj*-Tryp) genes. The values were normalized against GAPDH. Significance levels were $^{*}P < 0.05$ and $^{**}P < 0.01$, and values represent means ± SD.

showed significant differences between gills and hepatopancreas tissues ($P > 0.05$). Two-way ANOVA results suggested a significant interaction between exposure time and DEHP concentration, in terms of the PO activity of hepatopancreas ($P < 0.001$), *Mj*-proPO of gills and hepatopancreas ($P < 0.002$), *Mj*-LGBP of hepatopancreas ($P < 0.001$), *Mj*-PE of hepatopancreas ($P < 0.001$), *Mj*-Serpin of gills and hepatopancreas ($P < 0.002$) and *Mj*-Tryp of hepatopancreas ($P < 0.001$).

4. Discussion

The innate immune system in invertebrates is vital to resist invading pathogens, because of the lack of an adaptive immune system [43]. The innate proPO system is primarily observed in invertebrates and is associated with a main role in immune recognition [44]. proPO-related genes have been reported as contributing to host defense against microbial and viral pathogens in crustaceans [33,45]. LGBP is a member of PRPs and plays an important function in the innate immune response of invertebrates. In previous studies, LGBP was shown to perform a role in an acute-phase condition, protecting crustaceans from viral infection and chemical exposure [26,45]. LGBP expression was observed in various tissues in crustaceans, including shrimp and crab, and was mainly detected in hemolymph and hepatopancreas [38,46]. In the present study, after EDC exposure, *Mj*-LGBP mRNA expression levels were generally observed in *M. japonicus* gills and hepatopancreas, and were down-regulated. The gills exhibit roles as the main interface between living organisms and the aquatic environment and are one of the first protective organs in contact with external stresses, such as salinity, bacteria, and toxic chemicals [37,40,47]. In contrast, Nikapitiya et al. [38] reported that LGBP expression in *M. japonicus,* after exposure to different salinity concentrations in gills, was up-regulated at day 7. This result suggests that differences in immune responses might depend on the stress source. Similarly, LGBP transcription in *L. vannamei* exposed

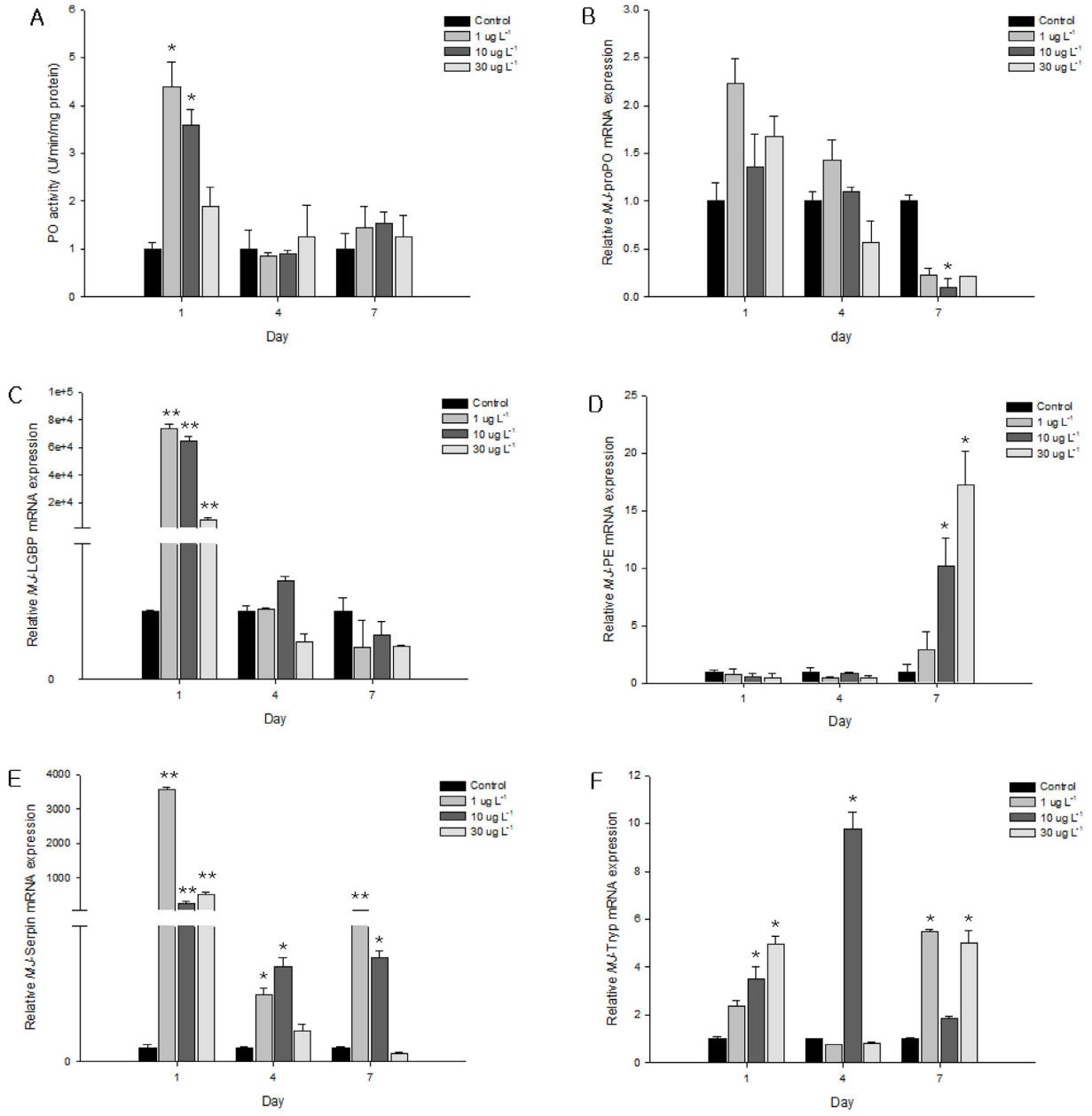

Fig. 6. Responses of six immune related genes in *M. japonicus* hepatopancreas exposed to 1, 10 and 30 μg L^{-1} DEHP at days 1, 4 and 7. We analyzed (A) phenoloxidase (PO) activity (U/min/mg protein), and (B) relative mRNA expression of prophenoloxidase (*Mj*-proPO), (C) lipopolysaccharide and β-1,3-glucan-binding protein (*Mj*-LGBP), (D) peroxinectin (*Mj*-PE), (E) serine protease inhibitor (*Mj*-Serpin) and (F) trypsin (*Mj*-Tryp) genes. Values were normalized against GAPDH. Significance levels were $^{*}P < 0.05$ and $^{**}P < 0.01$, and values represent means ± SD.

to *Vibrio anguillarum* was upregulated in hemocytes at 6 hpi, and returned to control levels at 12–24 hpi [48]. In the giant freshwater prawn (*Macrobrachium rosenbergii*), LGBP was significantly decreased in hemocytes exposed to high concentrations of trichlorfon for 24 h [26]. In addition, the expression of LGBP was highly induced in *M. rosenbergii* exposed to lower (22 °C) and higher (34 °C) temperatures, compared to a control group (28 °C) [25]. In particular, the mud crab *M. japonicus* exposed to DEHP in the present study possessed an immune-suppressive mechanism to inhibit the function of the proPO system. However, BPA seemed to induce increased expressions of proPO related genes at the initial 24 h, but decreased rapidly as exposure time increased. These results suggested that down-regulation of *Mj*-LGBP might result from the potential immune-suppressive effects induced by EDCs.

PE activation results in both peroxidase activity and cell adhesion and is induced concomitant with activation of the proPO system [49]. PE expression levels were upregulated in *E. sinensis* after being challenged with beads and lipopolysaccharide for 2 h [50], and high expression was observed at 6 h with *Vibrio alginolyticus* infection of *L. vannamei* [51]. Temperature induced stress-modulated immune processes in *M. rosenbergii*. PE gene expression increased in *M. rosenbergii* exposed to high temperatures [25]. Activities of PE are concomitantly induced and depend on the activated proPO system to play defense functions [52]. Transcriptional levels of male-associated PE were significantly increased by deltamethrin and azamethiphos [53]. These results suggest delousing drugs associated with modulation of PE in the Chilean salmon louse *Caligus rogercresseyi*. *Mj*-PE expression also exhibited a time-dependent increase with salinity changes [38]. Moreover, PE expression was inhibited by silencing the tumor necrosis factor receptor-associated factor 6 gene in the shrimp *Fenneropenaeus penicillatus* after *V. alginolyticus* injection. The inhibition of PE gene

expression is related to enhancement of bacterial and viral pathogen challenges [54]. In the present study, *Mj*-PE expression was induced at an early point after BPA exposure, and then levels of *Mj*-PE decreased generally at later exposure times. In contrast, DEHP exposure reduced transcriptional levels of *Mj*-PE at an early stage, and upregulated *Mj*-PE expression at later exposure time. Thus, EDC-induced stress modulated PE regulation in the *M. japonicus* proPO system differently following acute or chronic exposure.

proPO is a crucial enzyme in the crustacean innate immune system, and is involved in the melanization reaction [55]. PO is the terminal enzyme in the proPO mechanism, and activation of PO is triggered by several microbial polysaccharides [26]. PO activity decreased in the freshwater giant prawn, *M. rosenbergii*, exposed to norepinephrine. Norepinephrine induced dose-dependent inhibitory effects on PO activity in *M. rosenbergii* [56]. In addition, decreased PO activity was observed in *M. rosenbergii* exposed to trichlorfon and copper sulfate [57]. In the present study, decreased *Mj*-proPO, *Mj*-LGBP and *Mj*-PE expression in mud crabs exposed to EDCs might disrupt modulation of the proPO system, and result in decreased PO activity. The proPO system is a multi-functional one and is an energy-consuming mechanism to protect disruption to the immune balance [58]. In the present study, PO activity generally showed down-regulation in *M. japonicus* exposed to EDCs. The decrease in PO activity was clearly observed at late exposure times. These results suggested that EDC toxicity could be disruptive in immune defense process, as well as in endocrine systems. Thus, variations of the proPO-related genes and PO activity indicated that the proPO system plays key roles in defensive functions, and in stress recognition responses against EDCs, and might be involved in complicated immune responses to maintain immune balance disturbed by BPA and DEHP toxicity.

Serpins, important members of the serine protease inhibitor family, play an important role as mediators of immune responses by inactivating excessive protease activities [59]. Liu et al. [60] suggested that serpin might inhibit the transcriptional activities of clip-domain serine proteinases and proPO. In previous studies, the transcriptional pattern of serpin gene upregulation occurred at an early stage in *E. sinensis* and *L. vannamei* after microbial challenges [61,62]. The increased expression of serpin might be explained by the activity of proteases in innate immune responses, such as proPO activation and phagocytosis after bacterial challenge. A recent study reported that serpin is involved in critical roles of innate immune responses in the Chinese mitten crab *E. sinensis* against *Spiroplasma eriocheiris* pathogen [63]. The function of serpin, an important factor in immunity, has been reported as a negative regulator of excessive protease activities [64]. In addition, serpin negatively regulates proPO activation, and is involved in the production of antimicrobial peptides in Chinese oak silkworm, *Antheraea pernyi* [65]. The proPO process is impeded by serpin in the Pacific white shrimp *L. vannamei* [66]. These results suggest that serpin is an inhibitor in the proPO activating cascade. In the present study, the expression patterns of *Mj*-Serpin after BPA and DEHP exposure suggested that the serpin gene might participate in immune responses against external endocrine disruption toxicity. Furthermore, the *Mj*-Serpin expression results suggested that *Mj*-Serpin rapidly responds to EDC exposure and plays a role in immune protection, not only in microbial infection, but also in EDC chemical exposure stress.

Trypsin is a crucial member of the serine proteinase family and plays a critical role in physiological activities, such as hemolymph coagulation, melanin synthesis, dietary protein digestion, and hormone activation [67,68]. Serine proteinases have essential functions in the activation of the proteolytic cascade in proPO activation of the immune system [69]. Active and inactive precursor forms of PO are mediated by a cascade of clip domain serine proteinases, such as trypsin, which are important factors in the immune system of the giant freshwater prawn *M. rosenbergii* [70]. In the present study, the responses of *Mj*-Serpin and *Mj*-Tryp genes revealed up-regulation in *M. japonicus* hepatopancreas against DEHP toxicity stress. Many immune reactions in crustaceans are mediated by proteinase cascades, which are induced upstream by serine proteases, leading to melanization [71]. In previous studies, trypsin was significantly increased in the hepatopancreas after viral and bacterial challenges by 24 h in *F. chinensis* [72]. Trypsin and PO activities of the proPO-activating system are inhibited by alpha-2-macroglobulin protein in *L. vannamei* [73]. Thus, variations in *Mj*-Serpin and *Mj*-Tryp expression suggested that EDCs, such as BPA and DEHP, affect the proteinase cascade process associated with immune activation by disrupting the balance between proteinase inhibitors and serine proteinases.

We found that there are different response patterns, depending on the kind of EDCs, in the immune responses of the proPO activation system, although both BPA and DEHP are endocrine disruptors in the aquatic environment. The proPO related gene responses in *M. japonicus* gills and hepatopancreas to exposure to BPA generally resulted in up-regulation at the initial exposure period, and then down-regulation after relatively prolonged exposures. However, in gills exposed to DEHP, the responses of proPO related genes showed down-regulation from initial exposure to prolonged exposure times (but not to *Mj*-Serpin and *Mj*-Tryp). The present study suggested that there are different sensing responses to BPA and DEHP toxicity in the proPO immune system of the mud crab. In contrast to responses in the gill, in hepatopancreas, an important immune tissue, DEHP exposure induced up-regulation of *Mj*-LGBP on initial exposure, and up-regulation of *Mj*-PE after prolonged exposure periods. In addition, the responses of *Mj*-Serpin and *Mj*-Tryp genes involved up-regulation for all exposure periods. Thus, *M. japonicus* gills exposed to DEHP were not clearly involved in immediate immune defense responses and triggered immune suppression of the proPO activation system. *M. japonicus* hepatopancreas exposure to DEHP exhibited late immune responses by proPO related genes at day 7. These results suggested that DEHP exposure has more potential risk than BPA in the mud crab under long-term exposure conditions.

In summary, the activation changes of six proPO-related genes were examined in the immune responses of the mud crab *M. japonicus* to EDCs exposure. The decreased expression of *Mj*-proPO, *Mj*-LGBP and *Mj*-PE implied that immune-suppressive functions triggered by EDCs were involved in the activation of the proPO system in *M. japonicus*. In addition, variations in *Mj*-Serpin and *Mj*-Tryp transcript levels indicated that EDCs might affect the proteinase cascade mechanisms associated with defense and stress recognition responses in the crab immune system. Thus, exposure to EDCs, such as BPA and DEHP, induced immuno-suppressive mechanisms of the proPO system in the gill and hepatopancreas of the mud crab *M. japonicus*. Our results suggested that there are different sensing responses involving BPA and DEHP toxicity in the proPO immune system of the mud crab.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.025.

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