



## Hepcidin gene expression induced in the developmental stages of fish upon exposure to Benzo[a]pyrene (BaP)

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### ABSTRACT

Hepcidin is known to be expressed in fish with bacterial challenge and iron overload. Here we first report the hepcidin expression induced in the developmental stages from embryo to fry of red sea bream (*Pagrus major*) and in juvenile black porgy (*Acanthopagrus schlegelii* B.) upon continuous waterborne exposure to BaP. The gene expression of CYP1A1 and IgL (immunoglobulin light chain) were both measured. Expression of the *Pagrus major* hepcidin gene (PM-hepc) was increased in post hatch fry at 24 h and 120 h exposure to BaP at concentrations of 0.1, 0.5 and 1.0 µg/l, respectively. The gene expression pattern was comparable to that of CYP1A1 but different from that of IgL. In addition, a high number of AS-hepc2 transcripts (*Acanthopagrus schlegelii* B. hepcidin gene) were detected in the liver upon exposure to 1.0 µg/l BaP. This study demonstrates that hepcidin gene expression is significantly induced in BaP-exposed red sea bream and black porgy.

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### 1. Introduction

Benzo[a]pyrene (BaP) exists widely and is therefore an important environmental contaminant, with carcinogenic and immunotoxic properties (White, 1986; Davila et al., 1996). It is known from mammalian studies that the immune system is sensitive to the effects of pollution and several in vivo and in vitro studies have indicated that BaP induces immune alterations in fishes (Faisal and Huggett, 1993; Holladay et al., 1998; Smith et al., 1999). A single BaP injection significantly compromises the immune function and the host resistance against infection of Japanese medaka (Carlson et al., 2002). The major molecular mechanism of BaP-induced immunotoxicity in fish appears to involve Aryl hydrocarbon receptor (AhR) induction of cytochrome P4501A1 (CYP1A1)-mediated metabolism of BaP (Carlson et al., 2002). The early life stages in fish development appear to be particularly sensitive to anthropogenic contaminants (Buhl and Hamilton, 1991). Furthermore, the embryonic, larval and juvenile stages of both marine and fresh-water species may be affected by polynuclear aromatic hydrocarbons (PAHs) (Goksøyr et al., 1991). Since the immune system is responsible for the defense of the host against infectious pathogens and any fluctuation in the immune response could potentially affect resistance of fish to diseases, measurements of immune status may predict fish health at-risk populations and indicate the impact of PAHs on fish farming.

Hepcidin was first found during studies of the antimicrobial peptides in human body fluids such as blood ultrafiltrate (Krause et al., 2000) and human urine (Park et al., 2001). The antimicrobial peptide hepcidin genes have been identified from various vertebrates including fish in recent years. Fish hepcidins possess antibacterial activity in vitro and their expression in the liver is dramatically induced following bacterial challenge (Shike et al., 2002) or induced by iron overload (Hu et al., 2007), indicating that besides their innate role in immune systems, some fish hepcidins exhibit functions other than antimicrobial activity. In a complex marine environment, whether hepcidin gene expression would be affected by environmental pollutants such as BaP has so far not been reported. In recent years, studies on gene expression responses in mammals and fishes exposed to environmental pollutants have been carried out, for example, some studies concerning on AhR-dependent or -independent 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-responsive genes (Puga et al., 2000; Frueh et al., 2001; Kurachi et al., 2002; Adachi et al., 2004; Volz et al., 2005). Organ-specific gene expression responses were revealed in male Japanese medaka (*Oryzias latipes*) exposed to TCDD (Volz et al., 2005). In addition, the higher transcript of a hepcidin-like precursor in medaka liver was detected based on cDNA array analysis, which was likely related to acute-phase responses associated with host defense and inflammation (Volz et al., 2005). Although profiling multiple gene expression has been reported in different fishes for identifying the toxic effects upon exposure to environmental contaminants (Larkin et al., 2003; Denslow et al., 2004; Filby et al., 2007), no study has so far focused on the interesting phenomenon that hepcidin mRNA expression could be induced upon exposure to

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environmental pollutants. This study aims to investigate the relationship between organic pollutant exposure and hepcidin gene expression in the developmental stages from embryo to fry of red sea bream and in juvenile black porgy. The study will lead us to further explore in future whether the gene expression alteration induced by BaP will affect the functions of fish hepcidin.

## 2. Materials and methods

### 2.1. Materials

BaP was purchased from Sigma (HPLC grade, USA) and was dissolved in acetone. All other chemicals were reagent grade and obtained from local commercial sources.

### 2.2. Experiment design

#### 2.2.1. Waterborne exposure of embryos, fry of red sea bream and juvenile black porgy to BaP

Fertilized eggs of the red sea bream (*Pagrus major*) were obtained from the Fisheries Research Institute of Fujian Province. At the early embryonic developmental stage, groups of approximately 2000 healthy embryos were exposed to acetone ( $V_{\text{acetone}}/V_{\text{seawater}} = 1/20,000$ ) or environmental relevant concentrations of BaP (0.1, 0.5, 1.0  $\mu\text{g/l}$ ) after 8 h post fertilization (hpf). Embryos were maintained in 60 l aquaria of sand-filtered inshore seawater with the following characteristics: dissolved oxygen about 8.0 mg/l; pH 7.6; and 30‰ salinity. Other experimental conditions involved: daily renewal of the test medium; a constant temperature of 20 °C; and 10:14 h light:dark conditions. The test comprised one control and the three toxicant concentrations, with three replicates of each concentration. The experiment was designed to reveal the hepcidin mRNA, CYP1A1 mRNA and IgL mRNA expression alterations in the developmental stages from embryo to fry of red sea bream. Briefly, after 48 h exposure to BaP post fertilization, continued to be exposed to BaP over the course of the experiment. Samples were taken at different times (8 hpf, 48 hpf, 24 h post hatch (hph), 72 and 120 hph), and approximately 100 embryos or fry per treatment were sampled, immediately frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$ . To assess whether expression of hepcidin, CYP1A1 and IgL mRNAs is induced by the BaP, controls (unexposed fish) were in parallel set up for each time point at which samples of exposed fish are taken.

Juvenile black porgy (50–70 g, body length 12–14 cm, male) were obtained from a fish farm (TongAn marine-culture farm) in Xiamen, Fujian province, China. Fish were reared in seawater tanks in the laboratory at room temperature ( $21 \pm 1\text{ }^{\circ}\text{C}$ ) for seven days for acclimatization prior to the start of the experiment, and fed with commercial pellets at 1% of body weight daily during this period. Healthy fish were chosen for the toxicological experiment. The experiment was designed as follows: the control group without adding BaP; BaP-exposed groups with a concentration of 1  $\mu\text{g/l}$ ; the solvent control ( $V_{\text{acetone}}/V_{\text{seawater}} = 1/20,000$ ). The test solution was renewed daily and during exposure period the fish were not fed. Samples were collected after 0, 6, 24 and 48 h exposure to BaP. Three fish from each group were taken at each exposure time point. Samples of liver, gill, spleen, kidney, stomach and intestine from each individual fish were separately collected. Samples were immediately frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$ .

#### 2.2.2. Quantitative real-time PCR analysis of gene expression of hepcidin, CYP1A1 and IgL

**2.2.2.1. Cloning of CYP1A1 cDNA.** The red sea bream CYP1A1 cDNA sequence (GenBank accession no. EU163982) and the black porgy CYP1A1 cDNA sequence (GenBank accession no. DQ898145) were

**Table 1**

Primers and TaqMan probes developed in this study.

Genes	Primers (Forward and Reverse)/Probes (5'-3')	Accession number
18S	F: ACAAGGGCAGGGACTTAATCA	AB259837
	R: TCCCATGAACGAGGAATTC	
	P: CGCGAGCTTATGACCCGCGC	
CYP1A1	F: GCCGATGGCAGCTTTGAC	EU163982
	R: GCACATGCCACAGATCACATT	
	P: CTTCCGCCACATTTGCGTCICCC	
Hepcidin	F: TGGAAAGATGCCGTATAACAACAGA	AY557619
	R: CCTAACATGATTGGATGTGGTGTC	
	P: CCCTGCTGGCTGTCGCTTTTGC	
IgL	F: CCAGAGGTCAGTACACAGTGACTCA	AY190706
	R: GTGGTAGCTGCTAAAATAAACGTTCT	
	P: CCCTGCTGGCTGTCGCTTTTGC	

both amplified from liver RNA with RT-PCR. Specific primers and probes for analyzing CYP1A1 mRNA expression in red sea bream and black porgy using quantitative real-time PCR were designed based on a 69 bp sequence (Table 1), and showed that more than 95.6% of the amino acids were identical between red sea bream CYP1A1 and black porgy CYP1A1 cDNA. The 5'-CTTCCGCCACATTGCTCTCCG-3' probe for hybridization was designed based on the red sea bream CYP1A1 cDNA sequence, which completely matched the corresponding sequence of black porgy.

**2.2.2.2. Cloning of hepcidin cDNA.** An AS-hepc2 (*Acanthopagrus schlegelii* B. hepcidin gene) cDNA sequence of black porgy was reported previously (Yang et al., 2007) and that of red sea bream (PM-hepc) was amplified in our laboratory and released on GenBank (GenBank accession no. AY557619). The AS-hepc2 gene was one of seven hepcidin variants previously identified in black porgy for which gene expression in different tissues of this animal challenged with bacteria was evaluated using real-time PCR. Specific primers and probes for analyzing hepcidin mRNA expression in red sea bream and black porgy using real-time PCR were designed based on a 93 bp sequence, and more than 95.6% of the amino acids were identical between PM-hepc and AS-hepc2 cDNA. The 5'-CCCTGCTGGCTGTCGCTTTTGC-3' probe for hybridization was designed based on the PM-hepc cDNA sequence, which showed completely identical amino acids to the corresponding sequence of AS-hepc2.

**2.2.2.3. Primers and probes designed for analyzing IgL transcripts.** A pair of primers and a probe were designed (Table 1) for real-time PCR in order to examine the IgL mRNA expression changes according to the IgL cDNA sequence of red sea bream released on GenBank (AY190706). Total RNA of embryos and fry of red sea bream were isolated from a pool of over 100 embryos or fry using the TRIzol method (Invitrogen), and reverse transcribed into cDNA using the One-Step TaKaRa Primescript™ RT Reagent Kit (Perfect Real-time, DRR037S, TaKaRa). The procedure followed as described in Yang et al. (2007).

**2.2.2.4. Quantitative real-time PCR analysis of gene expression.** To evaluate the influence of BaP exposure on the hepcidin mRNA expression of red sea bream and black porgy, a real-time PCR approach was developed. Real-time PCR assays were performed using

the Taqman probe methodology and an ABI 7500 System (Applied Biosystems). Gene-specific primers (Table 1) were designed with the Primer Express(r) Software v3.0 (Applied Biosystem). The housekeeping gene for 18S rRNA was used as the endogenous assay control and the 18S rRNA transcript maintained stable under the treatments analyzed by semi-quantitative PCR (data were not shown). The reactions were performed in a 25  $\mu$ l mixture containing 12.5  $\mu$ l Taqman Universal PCR Master Mix (ABI), 500 nM primers, 200 nM Probes and 1  $\mu$ l cDNA, and the real-time RT-PCR reactions were performed in triplicate for each cDNA sample. Thermocycling was conducted as follows: 50 °C for 2 min followed by 10 min incubation at 95 °C; then 40 temperature cycles (95 °C, 30 s; 60 °C, 60 s). The relative expression levels (fold induction) of the tested genes, were calculated using the relative expression software tool (ABI), based on the  $2^{-\Delta\Delta CT}$  relative response method (Livak and Schmittgen, 2001). Meanwhile, the CYP1A1 and Igl mRNA expressions were in parallel measured following the same procedure. The CYP1A1 mRNA expression was measured here as an indicator showing P4501A1 involved in BaP metabolism.

### 2.2.3. Measurement of accumulative concentrations in black porgy

The accumulative concentrations were measured in the liver of juvenile black porgy upon exposure to 1  $\mu$ g/l BaP and the procedure was performed following the method of Del Castillo et al. (1992).

### 2.2.4. Statistical analysis

Results are presented as mean  $\pm$  SD. Each data was analyzed from three fish for each time point and all experiments were performed three times. The levels of statistical significance were analyzed by one-way ANOVA using SPSS 11.5 version, and results were considered to be significant when  $p < 0.05$ .

## 3. Results

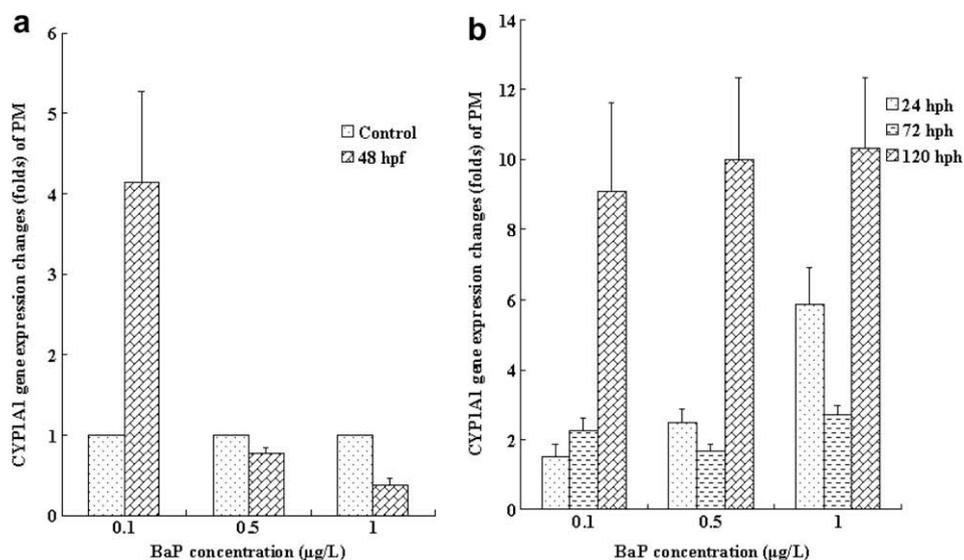
### 3.1. CYP1A1 mRNA expression in the developmental stages from embryo to fry of red sea bream exposed to BaP

As shown in Fig. 1a, CYP1A1 expression increased 4-fold over controls in embryos after a 48 h exposure to 0.1  $\mu$ g/l BaP. Increas-

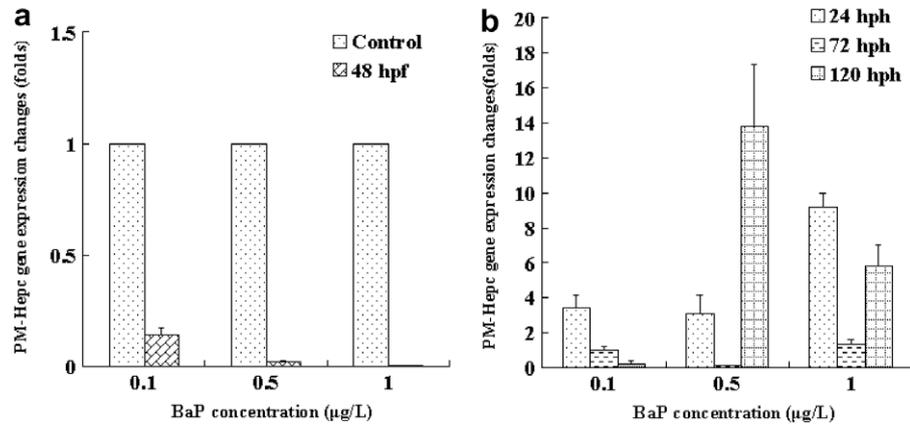
ing the dosage to 0.5 or 1.0  $\mu$ g/l did not cause any further increase. A slight rise of CYP1A1 expression level was found in the fry stage after 24 h exposure to all three doses of BaP and was significantly increased (by 6-fold) at the 1.0  $\mu$ g/l dose (Fig. 1b). However, no significant change was observed after 72 h exposure to 0.1 and 0.5  $\mu$ g/l BaP, while a slight alteration (<3-fold) was detected at the 1.0  $\mu$ g/l dose. The mRNA expression of CYP1A1 was induced markedly after 120 h exposure to all three doses of BaP. The increased level could reach 10-fold that of the control. The result demonstrated that, after 120 h, the CYP1A1 expression could be induced to a much higher level in the fry phase of red sea bream exposed to each of the doses tested compared to normal healthy fish (Fig. 1b). The control, albeit low, was also measurable in all the developmental stages examined.

### 3.2. Hepcidin mRNA expression in the developmental stages from embryo to fry of red sea bream exposed to BaP

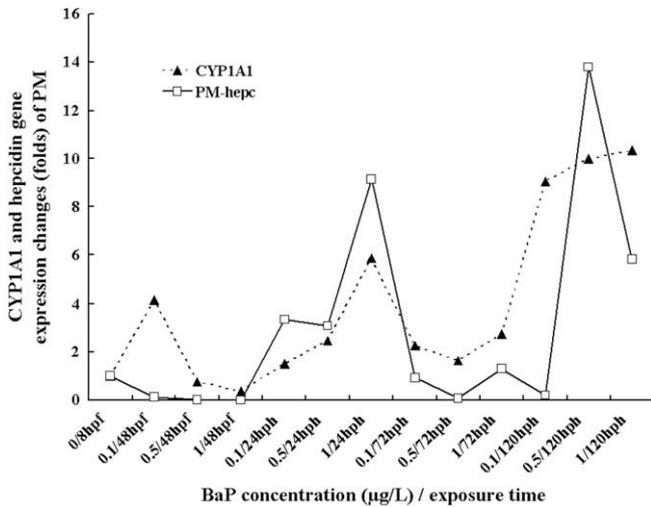
To determine whether hepcidin expression in red sea bream was induced upon exposure to BaP, expression of PM-hepc mRNA in the developmental stages from embryo to fry of red sea bream during different periods of exposure to BaP at three doses (0.1, 0.5 and 1.0  $\mu$ g/l) was determined by quantitative real-time PCR. As shown in Fig. 2b, PM-hepc transcripts were observed during the fry phase of red sea bream exposed to the three doses of BaP. A higher than 9-fold increase in PM-hepc expression was seen in fry after 24 h exposure to 1.0  $\mu$ g/l BaP, and roughly a 3-fold increase was detected after 24 h exposure to either 0.1 or 0.5  $\mu$ g/l BaP. A marked increase in PM-hepc mRNA expression was observed after 120 h sampling time; it was 14-fold the control at a dose of 0.5  $\mu$ g/l BaP and 6-fold higher than the control at a dose of 1.0  $\mu$ g/l BaP. However, no significant alteration in PM-hepc transcript expression was discerned in fry after 72 h exposure to the three experimental doses of BaP in comparison with the control. The PM-hepc expression pattern in the developmental stages from embryo to fry of red sea bream induced by BaP at the tested doses appear to be comparable to the CYP1A1 mRNA expression (Fig. 3). In addition, it was found that PM-hepc transcripts were decreased at 48 hpf when embryos were exposed to three concentrations of BaP (Fig. 2a). Correspondingly, CYP1A1 transcripts were also decreased (Fig. 3).



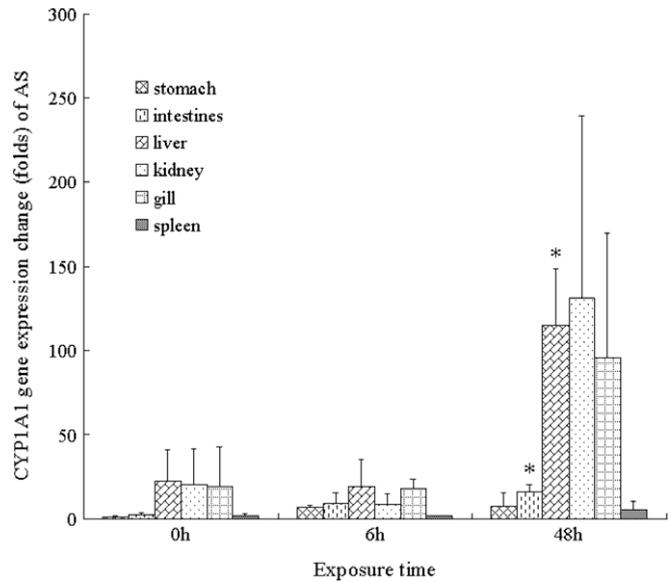
**Fig. 1.** Real-time PCR assay of CYP1A1 mRNA expression induced in the developmental stages from embryos to fry of red sea bream upon continuous waterborne exposure to BaP. (a) CYP1A1 mRNA expression change in embryos at 48 h post fertilization (hpf); The control (healthy embryos) without BaP exposure at 48 hpf is used as a calibrator for analysis of CYP1A1 mRNA expression and the bars represent the fold expression levels compared to the 18S gene. (b) CYP1A1 mRNA expression changes in fry at 24, 72 and 120 h post hatch (hph), respectively. The data was normalized corresponding to the control (unexposed fish) at each time of exposure, respectively. Error bars represent mean standard deviation.



**Fig. 2.** Real-time PCR assay of hepcidin mRNA expression induced in the developmental stages from embryo to fry of red sea bream upon continuous waterborne exposure to three concentrations of BaP. (a) PM-hepc mRNA expression change in embryos at 48 hpf; (b) PM-hepc mRNA expression changes in fry at 24, 72 and 120 hph, respectively. The control (healthy embryos) without BaP exposure at 48 hpf is used as a calibrator for analysis of PM-hepc mRNA expression and the bars represent the fold expression levels compared to the 18S gene. The data was normalized corresponding to the control (unexposed fish) at each time of exposure, respectively. Error bars represent mean standard deviation.



**Fig. 3.** Correspondence of the gene expression patterns between CYP 1A1 and hepcidin in the developmental stages from embryo to fry of red sea bream upon continuous waterborne exposure to three concentrations of BaP.



**Fig. 4.** Real-time PCR assay of CYP1A1 mRNA expression in various tissues (stomach, intestine, liver, kidney, gill and spleen) of juvenile black porgy upon continuous waterborne exposure to 1 µg/l BaP. The data was normalized corresponding to the control (unexposed fish) at each time of exposure, respectively. Error bars represent mean standard deviation.

### 3.3. IgL mRNA expression in the developmental stages from embryo to fry of red sea bream exposed to BaP

For comparison with the alteration of hepcidin transcript expression in red sea bream exposed to BaP, we set up a parallel trial to examine IgL mRNA expression change during the period of exposure to BaP in order to look for a difference in the gene expression pattern between an innate immune-related gene (hepcidin) and an adaptive immune-related gene (IgL). IgL mRNA expression alteration in the developmental stages from embryo to fry of red sea bream during the course of exposure to three BaP doses, 0.1, 0.5 and 1.0 µg/l, was analyzed using quantitative real-time PCR. IgL mRNA expression was not altered 48 hpf and showed no response in the fry 24 and 72 hph upon exposure to doses of 0.1, 0.5 and 1.0 µg/l BaP. An increase of IgL expression was detected only in fry after 120 h exposure. At the time, higher than roughly 50-fold and 20-fold IgL expression was seen in fry exposed to 0.1 and 0.5 µg/l BaP, respectively. Particularly, a high level of IgL mRNA expression (rising to 414-fold the control) was detected in fry 120 hph upon exposure to 1.0 µg/l BaP.

### 3.4. CYP1A1 mRNA and AS-hepc2 mRNA expression in multiple tissues of juvenile black porgy upon exposure to BaP

The levels of CYP1A1 mRNA expression in various tissues of juvenile black porgy were assessed using quantitative real-time PCR in BaP-exposed fish. Samples included liver, stomach, intestine, kidney, gill and spleen were individually collected from the tested fish after 0, 6 and 48 h exposure to a concentration of 1 µg/l BaP. No significant change of CYP1A1 expression was detected in the liver, kidney and gill after 6 h exposure to BaP compared with the control (Fig. 4). CYP1A1 transcript expression was markedly induced after 48 h and increased to a higher level in the liver, kidney and gill. The expression level for these tissues was increased to 115-fold, 131-fold and 96-fold the control level, respectively (Fig. 4). Additionally, the CYP1A1 mRNA expression was induced in the intestine at 6 h exposure to BaP, and signifi-

cantly increased at 48 h exposure (Fig. 4). Meanwhile, rise of CYP1A1 expression was also observed in the stomach after 6 and 48 h and in the spleen after 48 h exposure to BaP (Fig. 4).

Although the data for CYP1A1 expression analysis from the samples exposed to BaP at 24 h missed due to RNA contamination, this may not influence the assessment on BaP altering AS-hepc2 gene expression. As described above, the CYP1A1 mRNA expression was measured here as an indicator showing P4501A1 involved in BaP metabolism. Even though missing the result of CYP1A1 mRNA expression at 24 h exposure to BaP, the data at 6 and 48 h shown in Fig. 4 still clearly indicates BaP metabolism in juvenile black porgy.

AS-hepc2 mRNA expression in various tissues of juvenile black porgy was analyzed using real-time PCR after 0, 6, 24 and 48 h exposure to BaP. A high amount of AS-hepc2 transcripts was detected in the liver upon exposure to BaP at a dose of 1.0  $\mu\text{g/l}$ , the level increased to 350, 22, and 4838 -fold after 6, 24, and 48 h, respectively (Fig. 5). In particular, the hepatic AS-hepc2 expression level was highly induced (4838-fold) in juvenile black porgy exposed to BaP.

To look for whether there is a relationship between the hepatic accumulative BaP concentration and AS-hepc2 gene expression, a preliminary experiment was carried out to investigate a BaP-accumulating trend in the liver of juvenile black porgy during the period of exposure to BaP. The result showed that the accumulative BaP concentration in the liver changed with exposure time and reached its highest level 48 h after exposure to 1  $\mu\text{g/l}$  BaP (Fig. 6). Interestingly, the AS-hepc2 mRNA was highly expressed in the liver upon exposure to BaP at 48 h when BaP concentration in the liver was accumulating up to its highest level. It seems that AS-hepc2 mRNA expression increased with the hepatic accumulative BaP concentration and exposure time as observed in Fig. 5 and Fig. 6.

#### 4. Discussion

In this study, the effects of BaP on the gene expression of the antimicrobial peptide PM-hepc in red sea bream and AS-hepc2 in black porgy were investigated using quantitative real-time PCR. It was found that PM-hepc gene expression was markedly induced in the fry stage of red sea bream and AS-hepc2 in juvenile black porgy exposed to 0.5  $\mu\text{g/l}$  and 1.0  $\mu\text{g/l}$  BaP respectively. This is a very interesting observation to date on hepcidin expression in fishes exposed to BaP. In China, many commercial fish species,

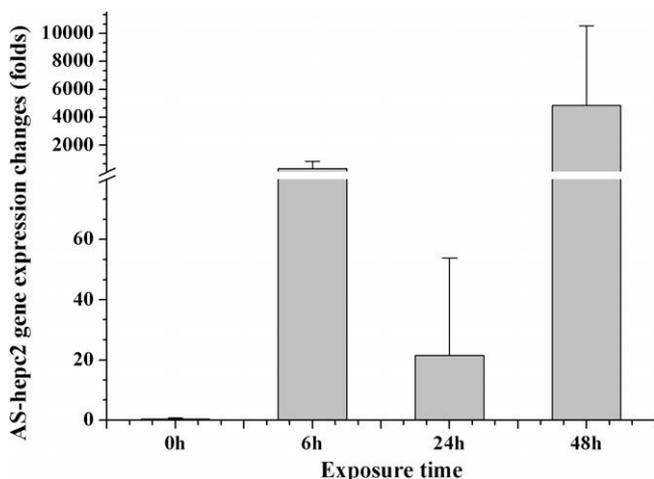


Fig. 5. AS-hepc2 mRNA expressions in liver juvenile black porgy upon continuous waterborne exposure to 1  $\mu\text{g/l}$  BaP analyzed using real-time PCR. Error bars represent mean standard deviation.

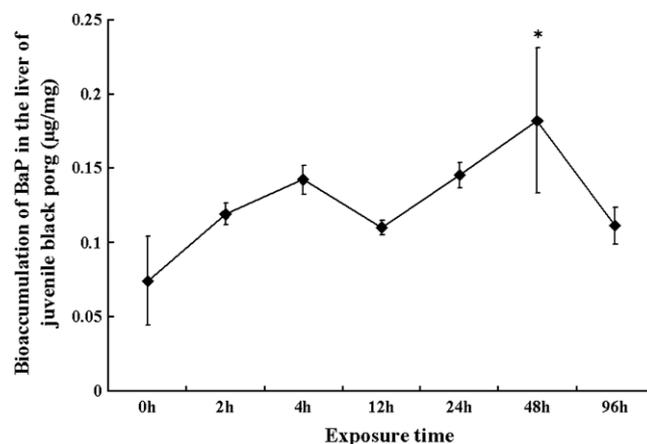


Fig. 6. Bioaccumulation of BaP in the liver of juvenile black porgy upon continuous waterborne exposure to 1  $\mu\text{g/l}$  BaP. Error bars represent mean standard deviation.

including red sea bream and black porgy, were raised in coastal waters, which probably have been polluted with various persistent organic pollutants such as PAHs for a long time (Maskaoui et al., 2002). These coastally raised fishes may be exposed to various microorganisms and toxic pollutants daily, but still maintain a sufficiently healthy status to be able to grow causes us to speculate whether some fish antimicrobial peptides such as hepcidin might function in the regulation of these environmental pollutants as it did in the regulation of bacterial challenge. However, at present, there is no direct evidence to support this presumption. To investigate this presumption, the induction of hepcidin expression was investigated in red sea bream and black porgy upon exposure to an experimental BaP. Our studies clearly demonstrated that the target hepcidin gene (PM-hepc and AS-hepc2) expression in BaP-exposed fishes was significantly induced in the developmental stages and the expression pattern was to some extent associated with P450 1A1 metabolism (Fig. 3).

The liver is considered as the main organ for CYP mediated biotransformation in fishes (Stegeman and Kloepper-Sams, 1987). Also, CYP1A induction has been measured in other tissues, including those contacting directly with the environment, such as gills (Miller et al., 1989), intestine (Van Veld et al., 1990), kidney (Wolkers et al., 1998), and so on. The change of CYP1A expression in fish tissues is frequently used as an indicator of exposure to PAHs or PCB contaminants (Stegeman et al., 2001; Miller et al., 2004). In our study, relative to acetone-treated fishes (solvent control), mean CYP1A1 transcript levels were roughly 115-fold, 131-fold and 96-fold higher in the liver, kidney and gill, respectively, after 48 h exposure to a concentration of 1.0  $\mu\text{g/l}$  BaP (Fig. 4), demonstrating that there were significant organ-dependent differences in CYP1A1 transcript levels at this sampling time and dose. Significantly higher levels of CYP1A1 transcript detected in exposed juvenile black porgy in this study supported liver and other tissues exposure to BaP. It is also noteworthy that the high induction of CYP1A1 in the kidney of juvenile black porgy was in accord with previous studies (Wolkers et al., 1998), suggesting that the kidney may possibly be involved in contaminant-related induction of CYP1A1. The role of the kidney as an extra-hepatic tissue in xenobiotic metabolism might contribute to the biotransformation of PAHs (Kennedy and Walsh, 1994; Van Veld et al., 1997). The interesting work is left to be further investigated. In comparison with the CYP1A1 expression results (Fig. 4), we found that when CYP1A1 mRNA expression was significantly induced after 48 h post-exposure, the expression level of AS-hepc2 mRNA was markedly increased at this time, showing a similar expression pattern between CYP1A1 and AS-hepc2 in the liver of black porgy exposed

to 1.0 µg/l BaP. However, unlike CYP1A1, no significant induction of hepcidin gene was observed in the other tissues tested (gill, intestine and stomach) after 6, 24 and 48 h exposure to 1.0 µg/l BaP (Fig. 5). It indicates that hepcidin gene is not widely expressed in tissues of black porgy upon exposure to BaP.

So far, knowledge regarding the effects of BaP on the immune system of fishes is still lacking. Considering that PAHs may alter both specific and non-specific immunity in fishes and mammals (Faisal and Huggett, 1993; Wolkers et al., 1998; Carlson et al., 2004), it would be worthwhile to understand the ability of the immune system in fishes to deal with challenges from the external environment pollutants. Studies have shown that most teleost fish immunoglobulins functionally resemble the mammalian immunoglobulin M (IgM), and it is important to response against various antigens (Uchida et al., 2000; Srisapoomee et al., 2004). BaP can significantly alters the levels of immunoglobulins in the mammalian immune system (Senthilnathan et al., 2006), but there is no related report in fish. Unlike hepcidin mRNA expression, the IgL gene showed no response during continuous exposure to BaP at three different concentrations, even up to 72 h post-exposure and the obvious expression change of IgL mRNA was only detected after 120 h exposure. The comparative result of gene expression patterns between hepcidin and IgL upon exposure to BaP suggested that the innate immune-related gene is more acute and sensitive to exposure to this pollutant than the adaptive immune-related gene, as previously mentioned (Luster et al., 1988). However, the present result does not exclude the possibility of a short-term passive immunization exerted by the IgL. Further studies will be required to see whether IgL mRNA expression induced by BaP would contribute to the specific humoral defence of offspring during early development in fishes.

Our studies demonstrated that the target hepcidin gene (PM-hepc and AS-hepc2) expression was markedly induced in the fry stage of red sea bream and in juvenile black porgy upon exposure to the typical environmental pollutant BaP at concentrations of 0.5 and 1.0 µg/l. To our knowledge, this finding has not been reported in previous studies on hepcidin expression in vivo. It is well known that hepcidin gene expression can increase to a high level in response to bacterial infection (Krause et al., 2000; Park et al., 2001; Shike et al., 2002), iron overload (Pigeon et al., 2001) and inflammation (Nemeth et al., 2003). Even more in Atlantic salmon and in black porgy, the hepcidin transcripts could be detected in tissues under normal culture conditions (Douglas et al., 2003; Yang et al., 2007). Until now, there is no evidence available on hepcidin gene expression induced in fish by BaP exposure. However, AhR-dependent or -independent TCDD-responsive genes have been demonstrated to change expression upon exposure to pollutants (Puga et al., 2000; Frueh et al., 2001; Kurachi et al., 2002; Adachi et al., 2004; Volz et al., 2005). Moreover, an antimicrobial peptide defensin gene level was induced highly, up to 9.76-fold of BaP-exposed *Mytilus edulis* (Brown et al., 2006). In addition, it is also noteworthy that the immune response related gene hepcidin precursor significantly higher transcribed in TCDD-exposed liver of Male Japanese Medaka by analysis of cDNA array (*Oryzias latipes*) (Volz et al., 2005). The present study demonstrated that BaP induced higher expression of the PM-hepc and AS-hepc2 transcripts in the developmental stages of red sea bream and in juvenile black porgy (Figs. 2 and 5) respectively, and also AS-hepc2 mRNA was highly expressed in the liver of juvenile black porgy exposed to 1.0 µg/l BaP (Fig. 5). These results prompted us to hypothesize that the highly enhanced expression of hepcidin induced by the environmental pollutant BaP might be a positive response related to an innate immune function as referred to one of many aspects of innate host defenses associated with an acute inflammation (Hancock and Diamond, 2000). This kind of response caused by exposure to BaP might be involved in immune- and/or inflamma-

tion-related pathways as described in TCDD-exposed medaka liver (Volz et al., 2005). In fishes, the innate immune response has been considered an essential component in host defense against pathogenic organisms due to an undeveloped adaptive immune response (Magnadottir, 2006). Current opinion tends towards a more complex view of the immune system as a sophisticated, complex, multilevel network of various defensive mechanisms (Danilova, 2006). The acute response of hepcidin to opposing exogenous pollutants may act as a signal to provide an early warning to the adaptive immune system of fishes to mount a response (Fearon and Locksley, 1996; Carroll and Prodeus, 1998) against invading pathogens or inner existing pathogenic microorganisms which might be robust enough to cause infection when the health of a BaP-exposed fish becomes worse. Taken together, the role of fish hepcidin might extend its functions to more than a key regulator of iron absorption and a mediator of inflammation (Ganz, 2003). It is known that the increased hepcidin expression induced by both bacterial challenge and iron overload were thought to be important in the host response to pathogens, however, whether the increased hepcidin expression induced by BaP is involved in the host defense against invading microorganism needs to be more clearly defined. A comparative study on hepcidin expression in bacteria-challenged fish exposed to BaP is ongoing following this work.

## 5. Conclusion

To our knowledge, this is the first report of PM-hepc expression significantly induced in the fry stage of red sea bream and AS-hepc2 in juvenile black porgy with exposure to the environmental pollutant BaP. This study suggested that fishes surviving in the complex marine environment might develop multi-functions to maintain their health and the antimicrobial peptide hepcidin might be one of the principal actors involved in innate immunity in responding to various exogenous factors, and thus to play their roles either directing the host acute- hase inflammatory response or participating in an antimicrobial defense mechanism. Further exploration of the role of fish hepcidin after exposure to BaP will be focus on whether increasing levels of hepcidin transcripts could exert an immune response against bacterial invading or infection that may provide new insights into the molecular mechanisms of hepcidin.

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