Marine Pollution Bulletin xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study

Jun Bo [a], Ling Cai [a,b], Jia-He Xu [a], Ke-Jian Wang [b,*], Doris W.T. Au [a,*]

[a] State Key Laboratory in Marine Pollution and Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong SAR, PR China
[b] State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen, Fujian 361005, PR China

ARTICLE INFO

Keywords:
Marine medaka
Hepcidin
Innate immune response
Immunotoxicology
Bacterial challenge

ABSTRACT

The objective of this study is to develop the marine medaka *Oryzias melastigma* as a potential marine fish model for innate immune and immunotoxicological studies. Hepcidin plays an important role in innate immune system. Two hepcidin genes (*OM-hep1* and *OM-hep2*) were identified and characterized in the *O. melastigma*, which were highly conserved with other reported hepcidins. During embryogenesis, significant elevation of *OM-hep1* and *OM-hep2* transcripts were coincided with liver development in the embryos. In adult medaka, differential tissue expressions of both hepcidin transcripts were evident: high in liver, moderate in spleen and low in non-immune tissues. After bacterial challenge, the two hepcidin mRNAs were rapidly and remarkably induced in liver and spleen, suggesting the two OM-hepcidins in *O. melastigma* play a complementary role in innate defense. Gender difference in time of induction and extent of the two hepcidin mRNAs elevation in infected *O. melastigma* should be considered in immunotoxicological studies.

1. Introduction

The marine medaka *Oryzias melastigma* has been increasingly shown a potential marine fish model for ecotoxicological studies (Kong et al., 2008; Shen et al., 2010; Merwe et al., 2011; Wang et al., 2011). The marine *O. melastigma* is phylogenetically closed to the freshwater counterpart, Japanese medaka *Oryzias latipes* (Kong et al., 2008; Au et al., 2009). The whole genome of *O. latipes* has been worked out (Kasahara et al., 2007), which offers significant advantage for using *O. melastigma* as a model for assessing multiple *in vivo* molecular responses to stresses in the marine environment.

Most fish diseases are related to poor environmental quality and many environmental pollutants are potential immunotoxicants (Robertson et al., 2009). Despite the freshwater *O. latipes* has been employed for immune-toxicological investigations (Carlson et al., 2002; Prophete et al., 2006), several studies in teleosts have shown that salinity potentiates the toxicity of various chemicals (Brecken-Folse et al., 1994; El-Alfy, 2002). A faster drinking rate in marine teleosts (hypoosmotic to seawater) may suggest a more rapid uptake of water soluble toxicants than the freshwater counterparts (which avoid drinking water due to hyperosmotic to the environment) (Evans, 1998). Erroneous conclusions could easily be drawn by direct extrapolation of freshwater toxicity data to predict saltwater toxicity in fish (Wheeler et al., 2002). As such, a marine fish model for assessing stresses induced immunotoxicity in the marine environment is urgently needed.

In teleosts, the innate immune system is particular important as the acquired immune system is not well developed. Fish innate immune system is the first line of host defense in opposing pathogenic organisms, which is essential to deal with any foreign materials until the adaptive immune system is ready and potent enough to take over (Whyte, 2007). Impairments of innate immune responses are useful to assess immunotoxicity of fish under various environmental stresses (Reynaud and Deschaux, 2006; Thilagam et al., 2009).

Hepcidin, also termed LEAP-1 (liver expressed antimicrobial peptide), is known to be an important antimicrobial peptide (AMP) in the innate immune system of mammals (Krause et al., 2000; Park et al., 2001). Hepcidin cDNA and genomic DNA organization have been determined in a dozen of fish species (Shike et al., 2002; Douglas et al., 2003; Hirono et al., 2005; Ren et al., 2006; Cuesta et al., 2008; Wang et al., 2009a). The wide existence of hepcidin genes in fishes, their inducible expression in bacteria or lipopolysaccharide (LPS) challenged fish and *in vitro* antimicrobial activity (Huang et al., 2007) have strongly supported that hepcidin is an important component in fish innate immune system.

In this study, we cloned and characterized the hepcidin genes (*OM-hep1* and *OM-hep2*) from *O. melastigma*. Capitalizing on the

* Corresponding authors. Tel.: +86 592 2184658; fax: +86 592 2180655 (K.-J. Wang), tel.: +852 3442 9710; fax: +852 3442 0522 (D.W.T. Au).
E-mail addresses: wkjian@xmu.edu.cn (K.-J. Wang), bhdwtau@cityu.edu.hk (D.W.T. Au).

0025-326X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2011.05.014

short generation time (2–3 months), high fecundity and distinct sexual dimorphism of this model fish, we investigated age, tissue and gender specific expressions of hepcidin genes in *O. melastigma*. The innate immune function of *OM-hep1* and *OM-hep2* was assessed by their *in vivo* expressions in marine medaka under bacterial challenge. The overall objective of this study is to demonstrate that *O. melastigma* can serve as a potential marine fish model for immunotoxicological studies.

2. Materials and methods

2.1. Marine medaka

The marine medaka *O. melastigma* were purchased from a commercial hatchery in Taiwan. The State Key Laboratory in Marine Pollution (City University of Hong Kong) has established a self-propagating population of *O. melastigma* for more than 30 generations. Standard operating procedures (SOPs) for large-scale culturing of *O. melastigma* were established. Fish were maintained in the laboratory in aerated 30‰ artificial seawater at 5.8 ± 0.2 mg O_2 L^{-1}, 28 ± 2 °C in a 14-h light:10-h dark cycle.

2.2.1. Preparation of Vibrio parahaemolyticus

Vibrio parahaemolyticus is a curved, rod-shaped, Gram-negative bacteria found in brackish saltwater, which caused great loss in aquaculture (Cai et al., 2006). *V. parahaemolyticus* (purchased from China General Microbiological Culture Collection Center, CGMCC, Beijing, China) was cultured in LB broth at 28 °C with shaking at 200 rpm overnight. The bacteria in culture was collected by centrifugation (3000*g* for 10 min at 4 °C) and suspended in sterile saline solution (0.65% NaCl) at a concentration of 2.85×10^8 colony forming units (cfu)/mL.

2.3. Determination of the cDNA and genomic DNA for OM-hep1 and OM-hep2

To isolate the full-length *OM-hep1* cDNA sequence of *O. melastigma*, 5′-RACE and 3′-RACE were performed. Specific primers for *OM-hep1* cDNA were designed according to the partial cDNA sequence (Genbank Accession No. AU180044) of *O. latipes*. The primers used for RACE were listed in Table 1, and the detailed procedure was similar to our previously described (Yang et al., 2007). The complete cDNA sequence of *OM-hep2* was obtained from our suppression subtractive hybridization (SSH) library of liver for the marine medaka *O. melastigma* challenged with *V. parahaemolyticus*.

Genomic DNA was isolated using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The primers used for genomic DNA amplification were listed in Table 1. The PCR product was purified and its sequence analyzed as described by Yang et al. (2007).

Table 1
The primer sequence for *OM-hep1* and *OM-hep2* used in this study.

Gene name	Primer sequence (Forward and Reverse) (5′ → 3′)	Purpose
OM-hep1	F: CCTCGTGGAAGACGCTGTATGAC	
	R: TCTGTGGAAGTAGAAGCTGGTGG	q-PCR and ISH
	F: CCGACACCTATGAGAGAGAAAACG	RACE and
	R: GTGGAGAGTAAGGAAAAACAAGCC	g-DNA amplification
OM-hep 2	F: CAAGATGCCAGTGACCTCGTG	
	R: ACACTCTCCACATCCACTCATCCCAC	q-PCR and ISH
	F: CAATCTGACTGCGAATCTTCTCCA	
	R: GCACACTCCACATCCACTCATCCT	g-DNA amplification
18S	F: CCTGCGGCTTAATTTGACCC	
	R: GACAAATCGCTCCACCAACT	q-PCR

2.4. Bioinformation analysis

The gene and deduced amino acid sequences were analyzed by ClustalX 1.83. Homology searches were performed using BLASTn and BLASTp by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The cleavage site for the signal peptide was predicted by the SignalP (http://www.cbs.dtu.dk/services/SignalP). The charge for the deduced peptide was calculated by the ProtParam tool (http://cn.expasy.org/tools/protparam.html).

2.5. In vivo expression of OM-hep1 and OM-hep2 in multiple tissues

2.5.1. Sampling of embryos

Staging of embryonic development in *O. melastigma* is similar to that of *O. latipes* (Iwamatsu, 2004). Three developmental stages of *O. melastigma* embryos were studied: (i) at 6 h post fertilization (hpf, from early morula stage to late blastula stage), (ii) 7 days post fertilization (dpf, the liver is well developed), and (iii) 10 dpf (just hatched larva). The pooled samples of 50 embryos (at 6 hph, 7 dph) or whole larva (10 dph) were frozen immediately in liquid nitrogen, and stored at −80 °C (50 embryos or larvae × 6 replicates, $n = 6$).

2.5.2. Bacterial challenge and sampling of fish

Sixty each male and female marine medaka at 5-month old were anesthetized with 0.02% tricaine methanesulfonate (MS-222, Sigma–Aldrich). Two microliters of stock bacterial suspension (5.69×10^5 cfu) for the bacterial challenge or equal volume of 0.65% NaCl for the control were administered into the peritoneal cavity, using 5 μL Hamilton syringe equipped with an ultra-fine needle under microscope. The selected sublethal dose of bacteria (5.69×10^5 cfu/fish) was based on the dose response in our preliminary experiment (data were not shown).

Six fish were collected at 0, 6, 12, 24 and 48 h post injection from the bacterial challenged and the vehicle control fish, individually. The tissues including the liver, spleen, brain, gonad (testis/ovary), gut and gill were isolated from each fish, then frozen immediately in liquid nitrogen and stored at −80 °C for total RNA extraction (each replicate were pooled from 2 fish, $n = 3$).

2.5.3. Quantitative real-time PCR (qPCR) analysis of hepcidin mRNA expression

Total RNA were extracted using the TRIzol method (Invitrogen), then reverse transcribed into cDNA using the One-Step TaKaRa Primescript™ RT Reagent Kit (TaKaRa). The qPCR analyses were carried out as previously described (Wang et al., 2009a). Briefly, qPCR assays were performed using the fluorescent dye Power SYBR Green PCR Master Mix and ABI 7500 System. Gene-specific primers (Table 1) were designed with the Primer Express(r) Software v3.0 (Applied Biosystem). 18S rRNA was used as the reference gene (Genbank Accession No. DQ105650). The relative expression levels (fold change) of the tested genes, were calculated using the relative expression software (ABI), based on the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6. In situ hybridization (ISH) for OM-hep1 mRNA and OM-hep2 mRNA detection

In situ hybridization (ISH) was performed for *OM-hep1* and *OM-hep2* and followed the procedures as previously described (Kong et al., 2008). Briefly, Adult male *O. melastigma* were fixed, dehydrated in graded serie of methanol, embedded in paraffin, serially

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014

sectioned (5 μm) on a rotary microtome (Leica RM2125, Germany), and mounted onto Superfrost® Plus slides (Menzel-Gläser, Germany). Hybridization was performed overnight with DIG-labeled probes (Table 1), followed by washing in a graded serie of SSC, 0.1% Tween 20 and incubated with anti-DIG antibody coupled to alkaline phosphatase (Roche Applied Science, Germany). Signals were detected using the NBT/BCIP substrate (Zymed, USA). Sections were counterstained with Nuclear Fast Red and then examined by light microscopy (Carl Zeiss, Germany).

2.7. Statistical analysis

All results were presented as mean ± standard deviation (SD). Prior to statistical analyses, data were log-transformed to normality if the data were not normally distributed. One-way ANOVA was used to test the null hypotheses there is no significant difference in expression of each hepcidin gene at different developmental stages/in different tissues of male or female marine medaka. If a significant difference ($p < 0.05$) was identified, pairwise comparison between groups was carried out using Tukey's post hoc test. Student's *t*-test was used to test if there is any difference in hepcidin gene expression between gender/before or after bacterial infection.

3. Results

3.1. Characterization of marine medaka hepcidin genes

Two hepcidin genes (*OM-hep1* and *OM-hep2*) were identified in *O. melastigma*. The full length of cDNA sequence of *OM-hep1* (Genbank Accession No. HM590747) and *OM-hep2* (Genbank Accession No. HM562669) was composed of 643 and 609 bp, encoding 90 and 89 amino acid residues, respectively (Fig. 1A and B). The open reading frame (ORF) of *OM-hep1* and *OM-hep2* consisted of three domains: signal peptide (24 aa), prodomain (40 aa) and mature peptide (26 aa for OM-hep1 and 25 aa for OM-hep2) (Table 2).

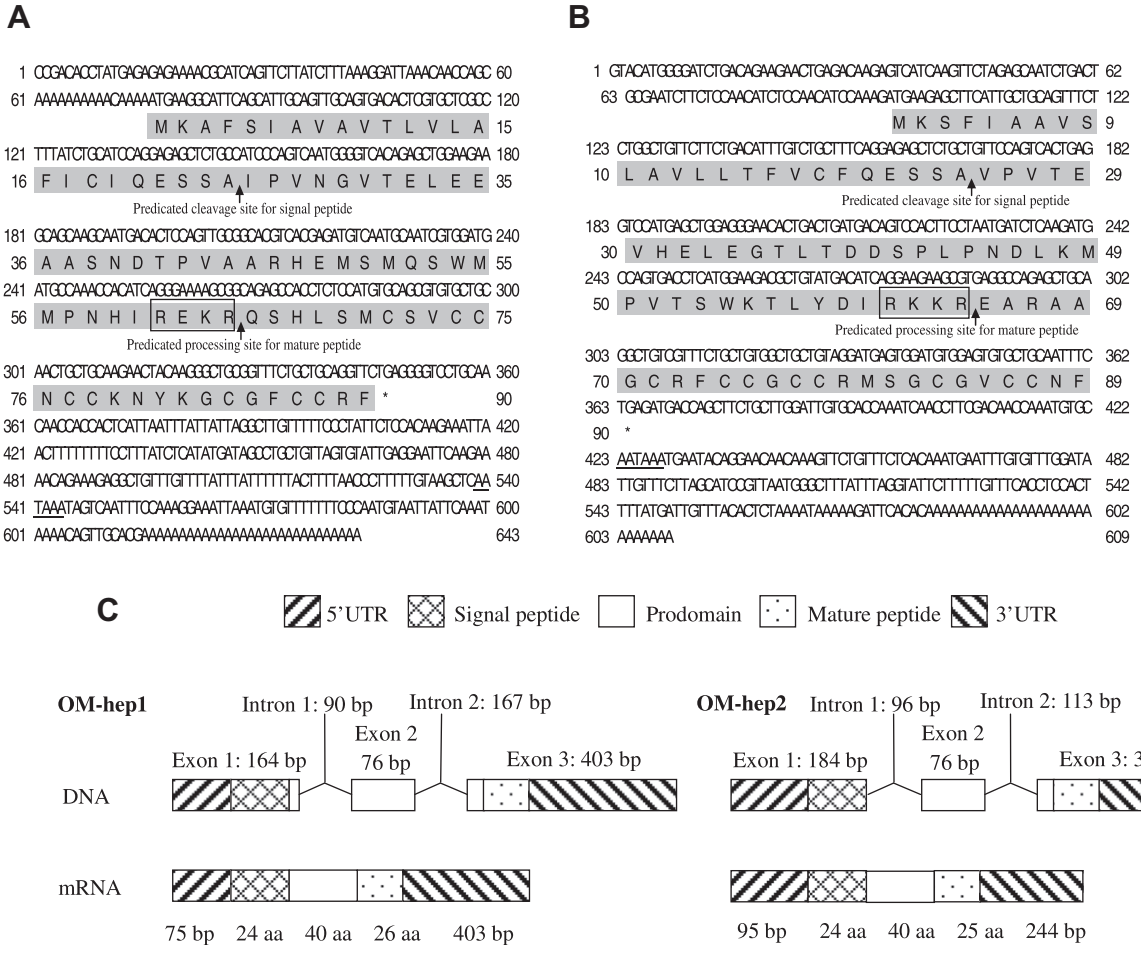

Fig. 1. The nucleotide sequences of cDNA, predicted amino acid sequences (A and B) and the genomic DNA organization of *OM-hep1* and *OM-hep2* (C). Polyadenylation signal (AATAAA) are underlined. The stop codon is indicated with an asterisk (*). Vertical arrows show the predicted cleavage sites for signal peptide and mature peptide, and the box indicates the putative motif RX(K/R)R for processing sites of mature peptide.

Table 2
Molecular characteristics of *OM-hep1* and *OM-hep2* genes.

Gene	cDNA	5′-UTR	3′-UTR	PI (aa)				Cysteines
				Whole	Signal peptide	Prodomain	Mature peptide	
OM-hep1	643	75	295	7.53 (90)	5.75 (24)	5.47 (40)	8.52 (26)	8
OM-hep2	609	95	244	7.48 (89)	5.75 (24)	5.61 (40)	8.25 (25)	8

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014

The genomic DNA sequence of *OM-hep1* (Genbank Accession No. HM990657) and *OM-hep2* (Genbank Accession No. HM990658) all consist of two introns and three exons (Fig. 1C). Exon 1 contained 5′-UTR, the signal peptide and part of prodomain sequence. Exon 2 encoded only a part of the prodomain, and its size (76 nt) was the same in the two hepcidin genes. Exon 3 included the final part of the prodomain, mature peptide and 3′-UTR.

Comparison of amino acid sequence between the two OM-hepcidins with other hepcidin proteins (piscine and mammals) was shown in Table 3. The amino acid sequence of OM-hep1 is highly conserved with the *O. latipes* hepcidin, with similarity and identity of amino acid sequence as high as 97% and 91%, respectively. Overall, OM-hep1 exhibited a high similarity (58–88%) and identity (50–82%) with other piscine, except for the zebrafish (Table 3). For OM-hep2, the amino acid sequence is less conserved between the two medaka species and generally exhibited a low similarity/identity with other piscine. Moreover, the two OM-hepcidins are generally low in identity and similarity with other mammals (23–30% for identity, 33–45% for similarity).

3.2. Multiple sequence alignment of marine medaka hepcidins

The multiple sequence alignment of deduced amino acid sequences for marine medaka hepcidins and some other known hepcidins was shown in Fig. 2. The identity and similarity of amino acid sequence between *OM-hep1* and *OM-hep2* was 43% and 63%, separately (Fig. 2A). Both of the OM hepcidin amino acid sequences contained 8 cysteines, which were highly conserved in most fish and mammals (Fig. 2B). The RX(K/R)R motif is highly conserved as most fish species.

3.3. Phylogenetic analysis of hepcidin

Phylogenetic analysis showed that all chosen hepcidins were clustered into two clades, mammals and teleost (Fig. 3). The two hepcidin genes of marine medaka were separately clustered into separate branches, *OM-hep1* was highly homologous with Japanese flounder 1, and *OM-hep2* was homologous with Japanese flounder 2.

3.4. Hepcidin gene expression in marine medaka

3.4.1. Hepcidin gene expression from early embryogenesis to hatching

OM-hep1 and *OM-hep2* mRNA expressions during embryogenesis were analyzed using qPCR (Fig. 4A and B). At 6 hpf early blastula stage, both the expression of *OM-hep1* and *OM-hep2* were low (0.9-fold and 1.5-fold, respectively). At 7 dpf before hatching, significant elevation of *OM-hep1* (173-fold) and *OM-hep2* (143-fold) expressions were observed in developing embryos ($p < 0.05$). At 10 dpf just after hatching, the expression level of *OM-hep1* continued to increase (602-fold) and that for *OM-hep2* increased to a lesser extent (62-fold) ($p < 0.05$).

3.4.2. Tissue-specific expression of OM-hepcidin in normal and bacterial challenged *O. melastigma*

Tissue-specific expression of *OM-hep1* and *OM-hep2* in normal fish and bacterial challenged fish was investigated using qPCR. In normal fish, the expressions of both OM-hepcidins transcripts were highly abundant in liver, followed by spleen, and relatively low in non-immune tissues: gonad (testis/ovary), brain, gut and gill (Fig. 4C and D). No gender difference was found for both *OM-hep1* and *OM-hep2* expressions in these tissues, except for *OM-hep2* in gill.

Four tissues (liver > spleen > brain > gill) with different intrinsic levels of hepcidins transcripts were studied for gender and tissue specific expressions of *OM-hep1* and *OM-hep2* in *O. melastigma* after bacterial challenge. At 6 h post bacterial treatment, a rapid induction of *OM-hep1 mRNA* was detected in liver of females (42-fold, $p < 0.05$) and males (21-fold, $p < 0.05$) (Fig. 5). At 12 h, both the liver and spleen of females (23.8-fold and 3-fold, respectively) and males (24.3-fold and 7-fold, respectively) exhibited significant induction of *OM-hep1 mRNA* ($p < 0.05$) (Fig. 5). At 24 h, *OM-hep1* mRNA expression was still strongly up-regulated in spleen of females ($p < 0.05$) (Fig. 5).

For *OM-hep2* mRNA, significant induction was detectable at 12 h post bacterial treatment in liver and spleen of females (19-fold and 7-fold, respectively) and males (22-fold and 8-fold, respectively) ($p < 0.05$) (Fig. 6). At 24 h, *OM-hep2 mRNA* expression was still strongly up-regulated in spleen of female ($p < 0.05$) (Fig. 6).

Table 3
Sequence identity and similarity between *OM-hep1*, *OM-hep2* and other hepcidin proteins. Size for cDNA, 5′UTR and 3′-UTR are indicated in bp. Whole peptide length is in aa. Isoelectric points for whole peptide, signal peptide, prodomain and mature peptide as well as the number of cysteines are shown.

Origin	Species (GenBank Accession No.)	OM-hep1		OM-hep2	
		%Identity	%Similarity	%Identity	%Similarity
Piscine	Japanese medaka (AU180044)	91	97	40	61
	Largemouth bass 1 (ACD13027)	82	88	46	64
	Smallmouth bass 1 (ACD13025)	82	87	44	63
	Turbot (AY994074)	73.3	83.3	42.2	61.1
	Japanese flounder 2 (BAE06233)	71	80	42	61
	Japan sea bass (AY604195)	59.3	64.8	55.1	69.7
	Hybrid striped bass (AF394245)	57.8	66.7	58.2	69.2
	Sea bass (DQ131605)	57.8	66.7	58.2	69.2
	Black porgy (AAU00798)	57.1	63.7	53.9	68.5
	Smallmouth bass 2 (ACD13026)	55	65	52	65
	Largemouth bass 2 (ACD13024)	53	64	54	65
	Red sea bream (AY994074)	52.7	60.4	50.5	63.7
	Japanese flounder 1 (BAE06234)	51	58	42	59
	Gilthead seabream (EF625900)	50	60	48.9	64.4
	Zebrafish 1 (P61516)	37	54	35	50
	Zebrafish 2 (Q7T273)	37	55	35	51
Mammals	House mouse 2 (NP_899080)	29	37	28	40
	Human (AAT74401)	28	39	30	42
	House mouse 1 (NP_115930)	28	39	27	41
	Pig (AAM77745)	26	45	27	40
	Norway rat (NP_445921)	23	33	29	40

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014

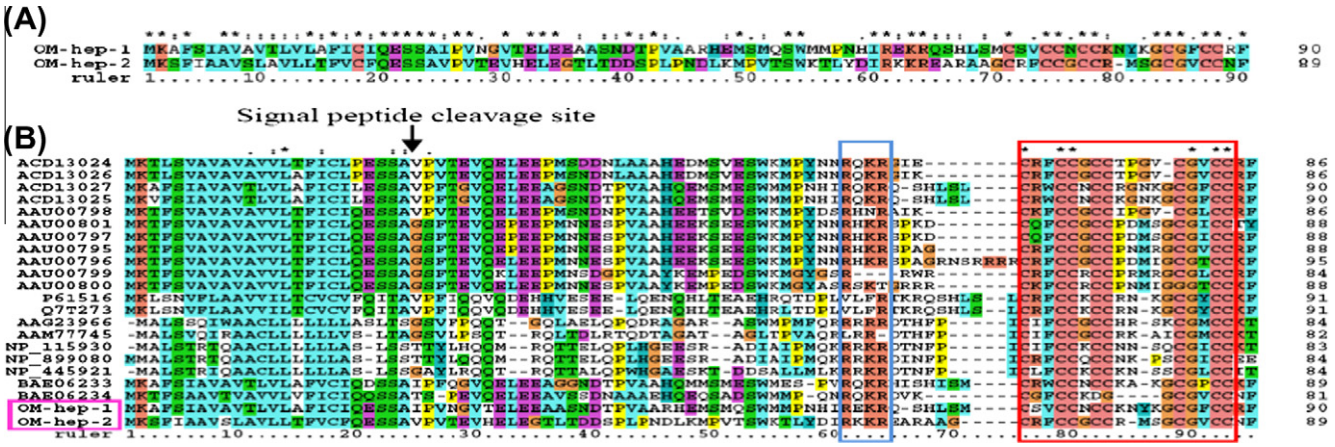

Fig. 2. Multiple sequence alignment of marine medaka hepcidins. (A) Sequence alignment of amino acid deduced from OM-hep-1 and OM-hep-2 and (B): Sequence alignment of amino acid deduced from *OM-hep-1* and *OM-hep-2* with some reported hepcidin sequences. '∗' Indicates positions which have a single, fully conserved residue; ':' indicates that one of the "strong" groups is fully conserved; '.' indicates that one of the "weaker" groups is fully conserved. The GenBank accession numbers are as follows: large mouth bass 1 (ACD13024), large mouth bass 2 (ACD13026), small mouth bass 1 (ACD13027), small mouth bass 2 (ACD13025), black porgy 1 (AAU00801), black porgy 2 (AAU00795), black porgy 3 (AAU00796), black porgy 4 (AAU00797), black porgy 5 (AAU00798), black porgy 6 (AAU00799), zebrafish 1 (P61516), zebrafish 2 (Q7T273), human (AAG23966), pig (AAM77745), house mouse 1 (NP_115930), house mouse 2 (NP_899080), Norway rat (NP_445921), Japanese flounder 1 (BAE06234), Japanese flounder 2 (BAE06233).

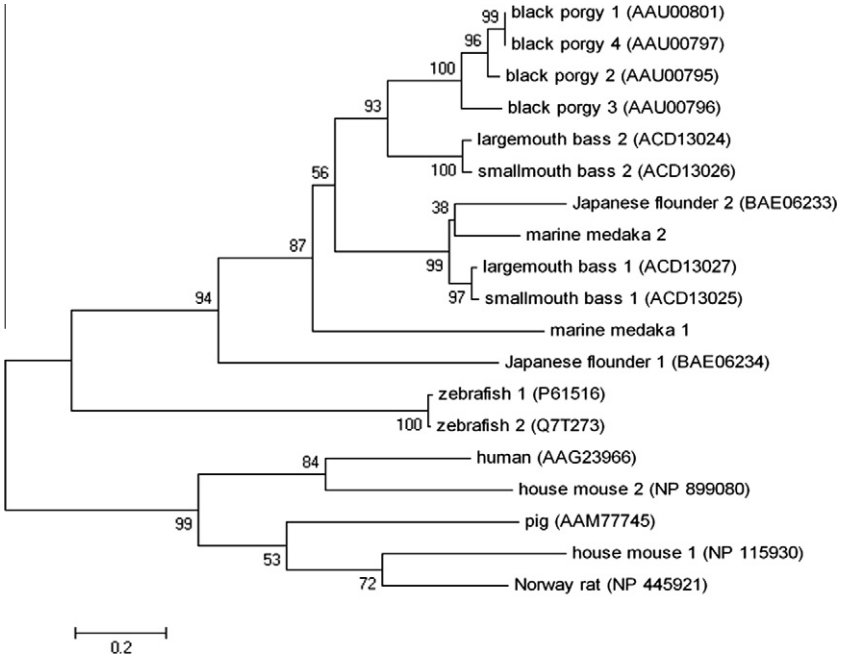

Fig. 3. Phylogenetic analysis of deduced amino acid sequences from OM-hep1 and 2 cDNA and other hepcidin precursor peptides with ClustalX1.83. Numbers next to the branches indicate bootstrap value from 1000 replicates.

At 48 h post bacterial challenge, no significant induction of the two hepcidins mRNAs was detected in liver and spleen of *O. melastigma* (Figs. 5 and 6). In both brain and gill of male and female fish, no significant change of *OM-hep1* and *OM-hep2* expressions was detected at all the time intervals studied (Figs. 5 and 6). Overall, both the hepcidin transcripts induction patterns were similar between the female and the male in all tissues studied, except for spleens at 24 h.

4. Expression and localization of *OM-hep1* and *OM-hep2* in hepatocyte

The relative expression and localization of *OM-hep1* and *OM-hep2* in liver of normal male was investigated by ISH. It was revealed that *OM-hep1* and *OM-hep2* expression was localized in both the nucleus and cytoplasm of hepatocytes (Fig. 7). High signal intensity of *OM-hep1* mRNA was found in nucleus and cytoplasm (Fig. 7B), and a moderate expression of *OM-hep2* was detected in nucleus and cytoplasm (Fig. 7C). No positive signal was observed in hepatocytes for the control group (using the sense probe) (Fig. 7A).

5. Discussion

Two cDNA sequence homologous hepcidin genes, *OM-hep1* and *OM-hep2*, were identified and characterized in the marine medaka *O. melastigma*. The existence of multiple hepcidin genes is not uncommon in fish, for instances, two hepcidin genes were found

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014

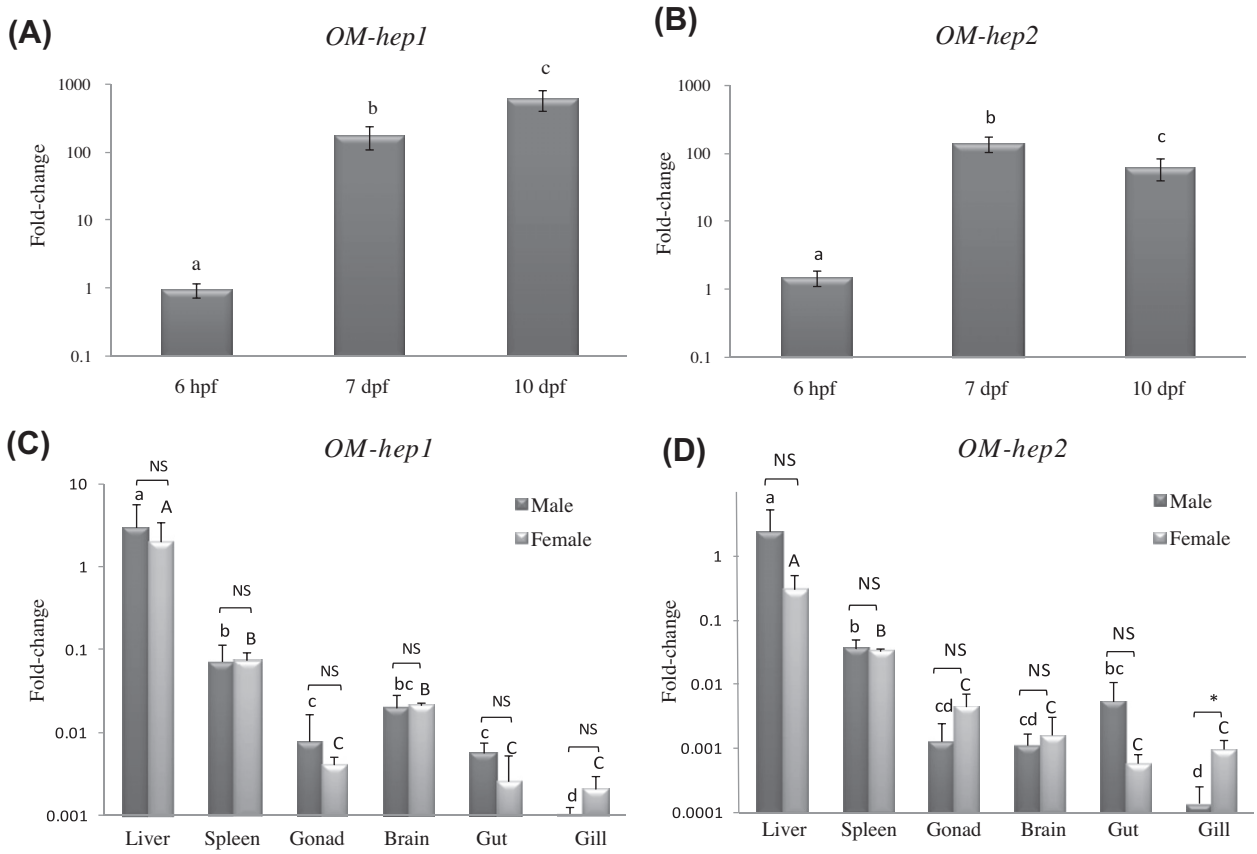

Fig. 4. Quantitative real time PCR analysis of *OM-hep1* and *OM-hep2* mRNA expression at different developmental stages (A and B) and in different organs of male and female medaka (5-month old) (C and D). The gene expression folds change were normalized to 18s rRNA. Data are expressed as mean ± SD (n = 6 for A, B; *n* = 3 for C and D). Different organs for the same gender marked with the same letter(s) are not significantly different from one another ($p > 0.05$). The same organ between gender marked with NS are not significantly different from one another, ∗ indicates statistically significant at $p < 0.05$.

in Japanese flounder, large-mouth bass and small-mouth bass (Hirono et al., 2005; Robertson et al., 2009), three in tilapia and rockbream (Huang et al., 2007), four in redbanded seabream (Martin-antonio et al., 2009), and seven in black porgy (Yang et al., 2007). The two hepcidin isoforms in *O. melastigma* could be differentially enhanced in liver and spleen after bacterial challenge (Figs. 5 and 6), suggesting a more advanced innate immune strategy for fish to survive in the complicated aquatic environment (such as diverse forms of pathogens and chemical contaminants).

The molecular characteristics of *OM-hep1* and *OM-hep2* (Table 2, Fig. 1) are typical of the other known vertebrate hepcidins. The C-terminus sequence for *OM-hep1* and *OM-hep2* was −CCRF and −CCRNF, correspondingly (Fig. 2B), and the motif (−CCR/KF) of C-terminal is conserved among most of the fish hepcidins. The N-terminal of the mature peptides for OM-hep1 and OM-hep2 contain the typical propeptide RX(K/R)R sequences (Fig. 2B) which are the characteristic processing site for propeptide convertases in fish (Shi and Camus, 2006).

In teleosts, the hepcidin amino acid sequence generally have a highly conserved signal peptide sequence with 24 amino acids in length, an acidic propiece of approximately 38–40 residues, and a mature peptide with 19–27 amino acids (Douglas et al., 2003). The mature peptide OM-hep1 and OM-hep2 contain 26 and 25 amino acids with a MW of 2925.4 and 2608.1 Da, respectively (Table 2), and such a small MW for mature peptide may offer an advantage for the host to respond rapidly to infection. Besides, the mature peptide of OM-hep1 and OM-hep2 also has eight cysteine residues, which is similar to the reported hepcidins in other fish species and mammals.

Liver is the key organ for the synthesis of defense molecules involved in innate immune function (Bayne et al., 2001). During embryogenesis, the expression levels of *OM-hep1* and *OM-hep2* increased dramatically (Fig. 4A), which coincides with the developmental process of liver in *O. melastigma*. The expression levels of hepcidin mRNAs in early stage blastula was very low (about 1-fold), which could be most likely by maternal transfer (Chen et al., 2007; Martin-Antonio et al., 2009). The embryonic liver was well developed from 7 dpf embryo to 10 dpf just hatched fry, and both the hepcidin transcripts were dramatically induced in the maturing embryos at 7 dpf (Fig. 4A). Despite a decline of *OM-hep2 mRNA* was observed in the just hatched fry, the expression level remained very high. The results imply that the two OM hepcidins may play an important role in innate defense at early life stages of *O. melastigma*.

Similar to other fish, the two hepcidins in adult *O. melastigma* were predominantly expressed in liver. The expression levels of hepcidins in spleen were significantly lower than that of the liver, but the levels were generally higher than the other non-immune organs (brain, testis, ovary, gut and gill). The results of ISH further showed that *OM-hep1* and *OM-hep2* transcripts were localized in nucleus and cytoplasm of hepatocytes, with the level of *OM-hep1* higher than that of *OM-hep2* (Fig. 7). The findings suggest that transcription and transport of hepcidins, in particular the *OM-hep1*, were active in hepatocytes under normal condition.

After bacterial challenge, the expression levels of *OM-hep1* and *OM-hep2* were rapidly induced in liver and spleen ($p < 0.05$), but no significant upregulation was detected in other non-immune organs (brain and gill) (Figs. 5 and 6). The induction of

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014

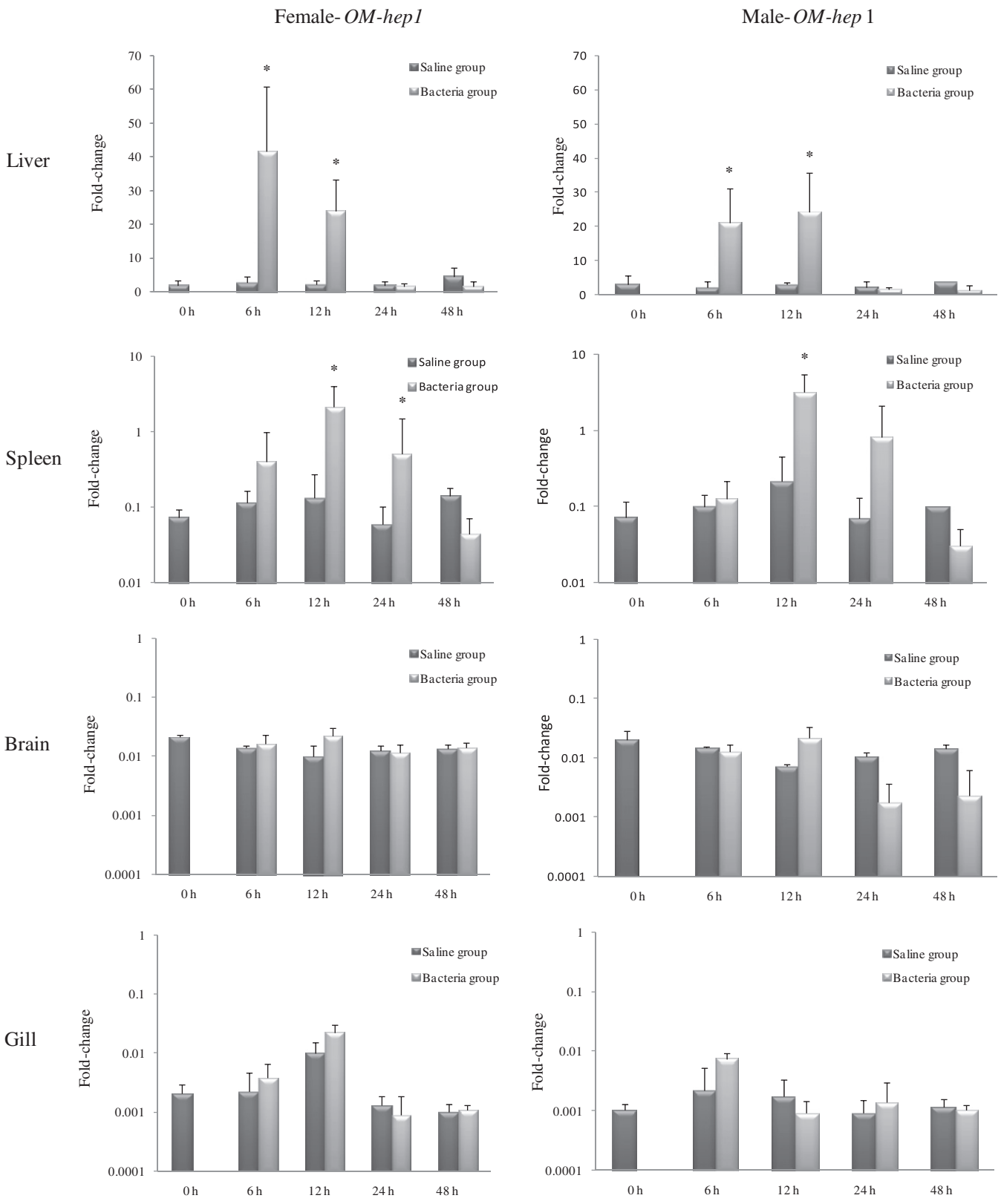

Fig. 5. Quantitative real time PCR analysis of the *OM-hep1* expression in different tissues of bacteria challenged female and male fish. The gene expression folds change were normalized to 18s rRNA. Data are expressed as mean ± SD (n = 3). Asterisks indicate significant difference between the saline group and the bacteria group at each time point ($p < 0.05$).

OM-hep1 mRNA was most rapid (within 6 h) and remarkable in livers of female and male fish, which was synchronized with a significant up-regulation of *OM-hep2* mRNA at 12 h. The rapidity and remarkable amplitude of hepatic *OM-hepcidins* induction patterns are in consistent with the acute phase response to infections observed in human and tilapia *Oreochromis mossambicus* (Lauth et al., 2005; Huang et al., 2007). In spleen, despite a relatively slower response time (at 12 h) and a lower induction level

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014

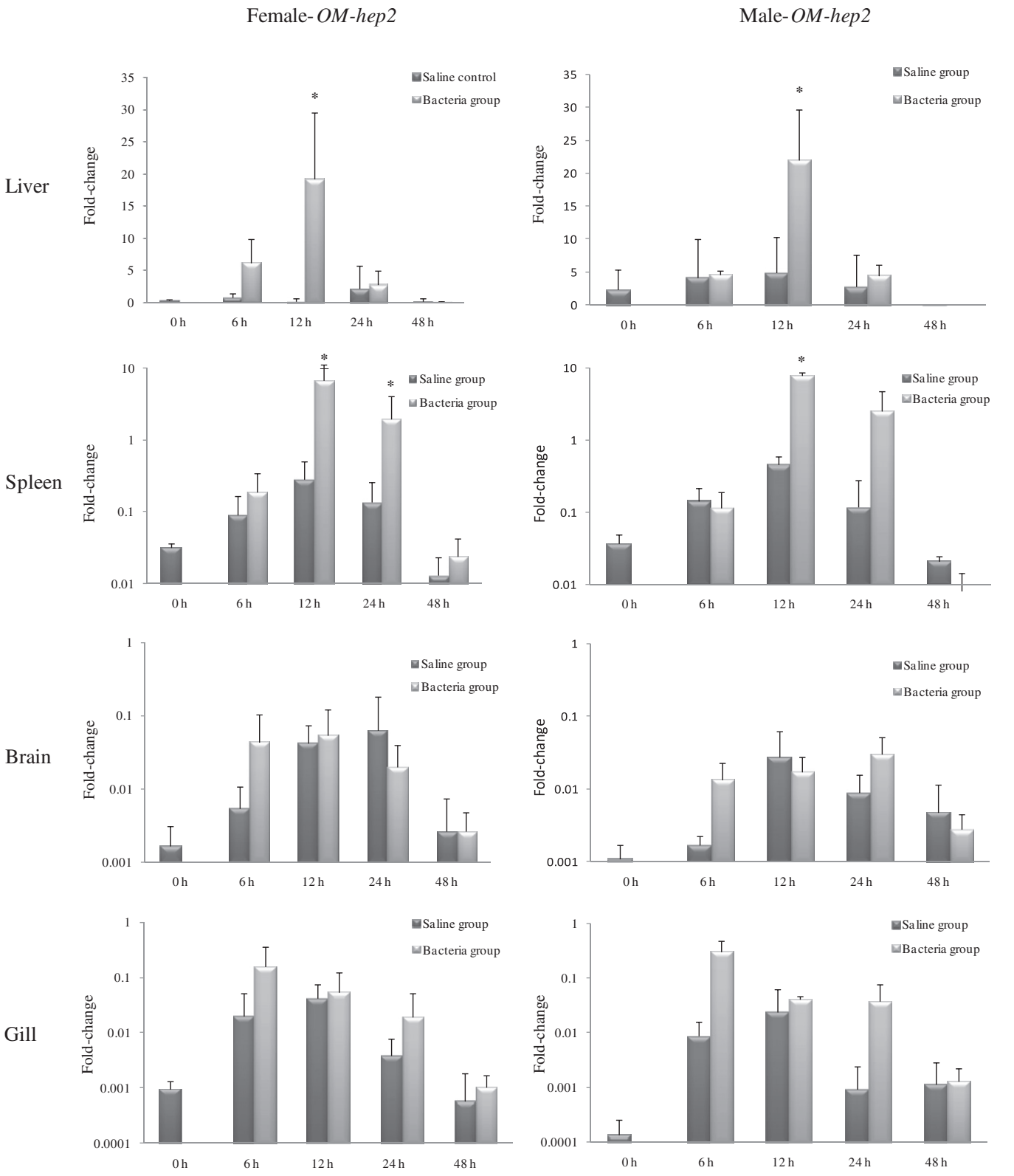

Fig. 6. Quantitative real time PCR analysis of the *OM-hep2* expression in different tissues of bacteria challenged female and male fish. The gene expression folds change were normalized to 18s rRNA. Data are expressed as mean ± SD ($n = 3$). Asterisks indicate significant difference between the saline group and the bacteria group at each time point ($p < 0.05$).

of *OM-hep1* mRNA and *OM-hep2* mRNA were observed, the induction responses were last till 24 h when that were already subsided in liver. This could be an important strategy for *O. melastigma* to sustain the innate immune response for protection of infected individuals. The time response of hepcidins in *O. melastigma* to bacterial challenge is in agreement with an earlier report of Kushner (1993) that hepcidin levels in plasma of humans may change markedly within 24 h.

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014

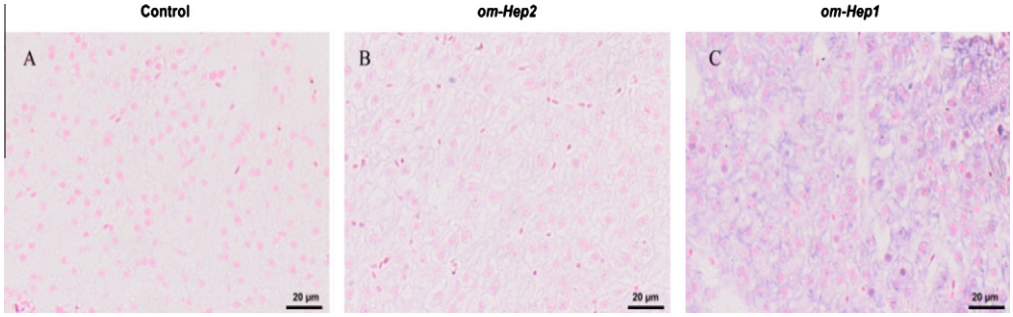

Fig. 7. Expression and localization of *OM-hep1* and *OM-hep2* mRNA in liver detected by *in situ* hybridation. Purple colour indicates positive signal of *OM-hep1* and *OM-hep2* mRNA expression. Negative control (the sense probe) (A), antisense probe for *OM-hep2* (B) and antisense probe for *OM-hep1* (C). Scale bar: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

No gender difference in endogenous expression of *OM-hepcidins* was found in all tissues of *O. melastigma* studied, except for *OM-hep2* expression in gill (females > males). The significance of such discrepancy remains to be elucidated. However, gender difference in transcriptional induction of hepcidins was detectable in bacterial challenged *O. melastigma* (Figs. 5 and 6). The induction response of *OM-hep1* in liver of females (42-fold) was much higher than that of the male fish (21-fold) at 6 h after bacterial challenge (Fig. 5). Moreover, upregulation of *OM-hep1* mRNA and *OM-hep2* mRNA in spleen could last longer in females (24 h) than in males (12 h). The findings imply gender difference in innate immune competence in *O. melastigma* (females > males).

Recent studies in fish have showed that TCDD (Volz et al., 2005), PCBs (Falciani et al., 2008), BaP (Wang et al., 2009b) and polluted sediments (Leaver et al., 2010) induced hepcidin gene expression in liver. Induction of hepcidin expression in non-microbial challenged fish may be useful to indicate exposure of fish to environmental contaminants that are potential immune-modulators. Gender difference should also be considered in future immunotoxicological studies when using hepcidin expressions as the endpoint parameter.

In summary, two hepcidin genes were cloned, identified and characterized for the marine medaka *O. melastigma*. The construct and function of *OM-hep1* and *OM-hep2* in *O. melastigma* showed high similarity with other known hepcidins in fish and mammals. A rapid induction of *OM-hepcidins* transcripts in liver and spleen of bacterial challenged *O. melastigma* suggest that the two hepcidins are involved in innate immunity and likely as an important effective component in response to invading microorganisms. Age-, tissue- and gender difference in expressions of *OM-hep1* and *OM-hep2* in normal and bacterial challenged *O. melastigma* were demonstrated. Overall, the present findings suggest that the small size marine medaka *O. melastigma* is of potential to serve as a marine fish model for innate immune and immunotoxicological studies.

Acknowledgements

This work was partially supported by the Area of Excellence Scheme under the University Grants Committee of the Hong Kong Special Administration Region, China (Project No. AoE/P-04/2004), the State Key Laboratory in Marine Pollution (CityU) and the Hong Kong-France Research Collaboration Grant (No. 9231003) to D. Au. The research was also supported by the Minjiang Scholar Program to K.-J. Wang (2009).

References

Au, D.W.T. et al., 2009. Japanese medaka: a new vertebrate model for studying telomere and telomerase biology. Comp. Biochem. Physiol. C 149, 161–167.

Bayne, C.J. et al., 2001. Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, *Oncorhynchus mykiss*, by means of suppression subtractive hybridization. Dev. Comp. Immunol. 25, 205–217.

Brecken-Folse, J.A. et al., 1994. Acute toxicity of 4-dinitrophenol, 2,4-dinitrophenol, terbufos and trichlorfan to grass shrimp (*Palaemontes* spp.) and sheepshead minnows (*Cyprinodon variegatus*) as affected by salinity and temperature. Environ. Toxicol. Chem. 13, 67–72.

Cai, L. et al., 2006. Isolation of *Vibrio parahaemolyticus* from abalone (*Haliotis diversicolor supertexta* L.) postlarvae associated with mass mortalities. Aquaculture 257, 161–166.

Carlson, E.A. et al., 2002. Exposure of Japanese medaka (*Oryzias latipes*) to benzo[a]pyrene suppresses immune function and host resistance against bacterial challenge. Aquat. Toxicol. 56, 289–301.

Chen, S.L. et al., 2007. Molecular cloning and expression analysis of a hepcidin microbial peptide gene from turbot (*Scophthalmus maximus*). Fish Shellfish Immunol. 22, 172–181.

Cuesta, A. et al., 2008. The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream. Mol. Immunol. 45, 2333–2342.

Douglas, S.E. et al., 2003. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. Dev. Comp. Immunol. 27, 589–601.

El-Alfy, A.T., 2002. Gender differences in the effect of salinity on aldicarb uptake, elimination, and *in vitro* metabolism in Japanese medaka, *Oryzias latipes*. Aquat. Toxicol. 61, 225–232.

Evans, D.H., 1998. The Physiology of Fishes. CRC press, Boca Raton, New York, pp. 157–176.

Falciani, F. et al., 2008. Hepatic transcriptomic profiles of European flounder (*Platichthys flesus*) from field sites and computational approaches to predict site from stress gene responses following exposure to model toxicants. Aquat. Toxicol. 90, 92–101.

Hirono, I. et al., 2005. Two different types of hepcidins from the Japanese flounder *Paralichthys olivaceus*. FEBS J. 272, 5257–5264.

Huang, P.H. et al., 2007. Three different hepcidins from tilapia, *Oreochromis mossambicus*: analysis of their expressions and biology functions. Mol. Immunol. 44, 1922–1934.

Iwamatsu, T., 2004. Stages of normal development in the medaka *Oryzias latipes*. Mech. Dev. 121, 605–618.

Kasahara, M. et al., 2007. The medaka draft genome and insights into vertebrate genome evolution. Nature 447, 714–719.

Kong, R.Y.C. et al., 2008. Development of a marine fish model for studying *in vivo* molecular responses in ecotoxicology. Aquat. Toxicol. 86, 131–141.

Krause, A. et al., 2000. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett. 480, 147–150.

Kushner, I., 1993. Regulation of the acute phase response by cytokines. Perspect. Bio. Med. 36, 611–622 Summer.

Lauth, X. et al., 2005. Bass hepcidin synthesis, solution structure, antimicrobial activities and synergism, and *in vivo* hepatic response to bacterial infections. J. Biol. Chem. 280, 9272–9282.

Leaver, M.J. et al., 2010. Hepatic gene expression in flounder chronically exposed to multiply polluted estuarine sediment: absence of classical exposure 'biomarker' signals and induction of inflammatory, innate immune and apoptotic pathways. Aquat. Toxicol. 96, 234–245.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Martin-Antonio, B. et al., 2009. Genomic characterization and gene expression analysis of four hepcidin genes in the redbanded seabream (*Pagrus auriga*). Fish Shellfish Immunol. 26, 483–491.

Merwe, J.P. et al., 2011. Bioaccumulation and maternal transfer of PBDE 47 in the marine medaka (*Oryzias melastigma*) following dietary exposure. Aquat. Toxicol. 103, 199–204.

Park, C.H. et al., 2001. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J. Biol. Chem. 276, 7806–7810.

Prophete, C. et al., 2006. Effects of elevated temperature and nickel pollution on the immune status of Japanese medaka. Fish Shellfish Immunol. 21, 325–334.

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014

Ren, H.L. et al., 2006. Cloning and organization analysis of a hepcidin-like gene and cDNA from Japan sea bass, *Lateolabrax japonicus*. Fish Shellfish Immunol. 21, 221–227.

Reynaud, S., Deschaux, P., 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review. Aquat. Toxicol. 77, 229–238.

Robertson, L.S. et al., 2009. Identification of centrarchid hepcidins and evidence that 17β-estradiol disrupts constitutive expression of hepcidin-1 and inducible expression of hepcidin-2 in largemouth bass (*Micropterus salmoides*). Fish Shellfish Immunol. 26, 898–907.

Shen, M. et al., 2010. Toxicity comparison between *Chattonella marina* and *Karenia brevis* using marine medaka (*Oryzias melastigma*): Evidence against the suspected ichthyotoxins of *Chattonella marina*. Chemosphere 80, 585–591.

Shi, J., Camus, A.C., 2006. Hepcidins in amphibians and fishes: antimicrobial peptides or iron-regulatory hormones? Dev. Comp. Immunol. 30, 746–755.

Shike, H. et al., 2002. Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. Eur. J. Biochem. 269, 2232–2237.

Thilagam, H. et al., 2009. Effect of 17β estradiol on the immunocompetence of Japanese sea bass *Lateolabrax japonicus*. Environ. Toxicol. Chem. 28, 1722–1731.

Volz, D.C. et al., 2005. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces organ-specific differential gene expression in male Japanese Medaka (*Oryzias latipes*). Toxicol. Sci. 85, 572–584.

Wang, K.J. et al., 2009a. Cloning and expression of a hepcidin gene from a marine fish (*Pseudosciaena crocea*) and the antimicrobial activity of its synthetic peptide. Peptides 30, 638–646.

Wang, K.J. et al., 2009b. Hepcidin gene expression induced in the developmental stages of fish upon exposure to benzo[a]pyrene (BaP). Mar. Environ. Res. 67, 159–165.

Wang, M. et al., 2011. Proteome profiles in medaka (*Oryzias melastigma*) liver and brain experimentally exposed to acute inorganic mercury. Aquat. Toxicol. 103, 129–139.

Wheeler, J.R. et al., 2002. Freshwater to saltwater toxicity extrapolation using species sensitivity distributions. Environ. Toxicol. Chem. 21, 2459–2467.

Whyte, S.K., 2007. The innate immune response of finfish – a review of current knowledge. Fish Shellfish Immunol. 23, 1127–1151.

Yang, M. et al., 2007. Genomic organization and tissue-specific expression analysis of hepcidin-like genes from black porgy (*Acanthopagrus schlegelii* B.). Fish Shellfish Immunol. 23, 1060–1071.

Please cite this article in press as: Bo, J., et al. The marine medaka *Oryzias melastigma* – A potential marine fish model for innate immune study. Mar. Pollut. Bull. (2011), doi:10.1016/j.marpolbul.2011.05.014