Isolation of Two Hepcidin Paralogs, *Hamp1* and *Hamp2*, from a Euryhaline Javanese Ricefish (*Oryzias javanicus*: Beloniformes)

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Two hepcidin paralogs (*ojhamp1* and *ojhamp2*) were isolated and characterized from a euryhaline Javanese ricefish (*Oryzias javanicus*: Beloniformes). The *ojhamp1* cDNA encoded 90 or 91 amino acids (aa) of a typical HAMP1 preproprotein. This preproprotein is believed to cleave and yield the 66 or 67 aa-proprotein, followed by the 26 aa-mature peptide, composed of 8 conserved cysteine residues and the QSHL amino terminal motif. The *ojhamp2* cDNA encoded 89 aa of HAMP2 preproprotein, cleaved to yield a 65 aa proprotein, and subsequently the 25 aa-mature peptide. The mature OJHAMP1 possessed a cationic isoelectric point (pI), whereas OJHAMP2 had an anionic charge. At the genomic level, both *ojhamp1* and *ojhamp2* share a conserved tripartite structure (three exons interrupted by two introns) with other vertebrate hepcidin genes. However, the *ojhamp1* was shown to exist as two distinct mRNA species, encoding 90 or 91 aa, due to alternative splicing at the junction site between intron I and exon II. Both *ojhamp1* and *ojhamp2* transcripts were detected in a wide range of tissue types with varying levels of basal expression, although the highest expression was observed in the liver for both isoforms. Transcriptional response to bacterial challenge using *Edwardsiella tarda* showed that *ojhamp1* was moderately upregulated in the liver but remained unchanged in the kidney. However, the *ojhamp2* was significantly suppressed in both the kidney and liver, suggesting a potential diversification between the two paralogs.

Key words: *Oryzias javanicus*, Gene structure, *Hamp1*, *Hamp2*, mRNA expression

Introduction

Fish, the lowest vertebrate group, typically possess a weaker acquired immune response as compared to mammals. Therefore, the innate immune response to infectious invaders is generally agreed to be more vital to fish than mammals (Shi and Camus, 2006; Magnadottir, 2010). Antimicrobial peptides (AMPs) play crucial roles as the first line of defense against invading microbes, and diverse AMPs with different structures and charges are involved in the defense pathway in an interactive or coordinated fashion (Cuesta et al., 2008; Rajanbabu and Chen, 2011).

Hepcidin, a recently discovered AMP, is the first identified from human urine (Park et al., 2001). Similar to a number of naturally occurring AMPs, hepcidins are cysteine-rich and form multiple disulfide bonds in the β-sheet structures (Shi and Camus, 2006). Many previous studies have shown

that hepcidin is central in the cross talk between innate immunity and iron homeostasis in vertebrates, although the primary role of mammalian hepcidins is now believed to be hormonal, serving as a negative regulator of iron homeostasis (Vyoral and Petrák, 2005; Hugman, 2006; Atanasiu et al., 2007). The function of mammalian hepcidin is believed to be conserved in fish. Previous studies support this hypothesis, showing that fish hepcidin peptides (both natural and synthetic versions) have potent antimicrobial activities (Lauth et al., 2005; Huang et al., 2007); the transcription of fish hepcidin genes are rapidly activated by experimental challenges using live bacteria, lipopolysaccharides (LPS), poly I:C, and viral suspensions (Shike et al., 2002; Douglas et al., 2003a; Hirono et al., 2005; Chiou et al., 2007); and fish hepcidins are significantly stimulated by iron overload and can regulate mammalian ferroportin, a known iron exporter in enterocytes and macrophages (Huang et al., 2007; De Domenico et al., 2008; Cho et al., 2009).

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Unlike most mammals, having only one genomic copy of hepcidin (*hamp1*), many fish species (especially those belonging to the superorder Acanthopterygii) possess multiple *hamp* gene copies (at least two, *hamp1* and *hamp2*) in which the fish *hamp1* is thought to be an ortholog of the mammalian *hamp*, while fish *hamp2* is believed to play a central role in supporting innate immunity of the fish (Hilton and Lambert, 2008). Moreover, certain species (especially belonging to Perciformes or Pleuronectiformes) have multiple diversified *hamp2* copies by way of gene duplication, which is the result of positive Darwinian selection driven by a host-pathogen interaction (Padhi and Verghese, 2007). Based on this finding, one can reasonably assume that fish hepcidins experienced a different evolutionary path from that of mammals. Consequently, this suggests that isoform-specific responses or diversification of hepcidin function should be considered when evaluating fish species. The differential regulation of *hamp1* and *hamp2* in fish species responding to different biological stimulations remains poorly characterized. Also, hormonal or antimicrobial functions of hepcidin isoforms, as well as tissue distribution patterns, are variable among different fish species (Douglas et al., 2003a; Huang et al., 2007; Cho et al., 2009).

The Javanese ricefish, or medaka (*Oryzias javanicus*: Beloniformes), is a small, egg-laying fish species, similar to Japanese medaka (*Oryzias latipes*), a popular model organism for vertebrate genetic and genomic research. Like other *Oryzias* species, these species possess numerous advantages for use in biological experiments, such as efficient laboratory rearing, transparent embryos, and year-round spawning under controlled conditions (Wittbrodt et al., 2002; Song et al., 2010). Also, because of its ability to efficiently osmoregulate, this euryhaline species would be a useful model system to study the interrelationship between environmental salinities and innate immune function of the brackish fishes. Based on our long-term goal to develop *O. javanicus* as a model fish system to study the functional genomics of AMP-mediated immunity, the objective of this study was to isolate and characterize two hepcidin paralogs, *hamp1* and *hamp2*, from *O. javanicus*.

Materials and Methods

Fish specimen and rearing conditions

Fish specimens used in this study were laboratory-propagated at the Institute of Marine Living Modified Organisms (IMLMO, PKNU). The conditions for general fish culture have been described previously by Cho et al. (2010) and Song et al. (2010). Fish were fed with *Artemia* nauplii (INVE Aquaculture Inc., Salt Lake City, UT, USA) and artificial diets of flounder larvae (150-500 μm in diameter, 50% crude protein; Woosung Feed Corp., Daejeon, Korea) on an *ad libitum* basis. Water temperature was maintained at $25 \pm 1°C$, and dissolved oxygen was maintained at 5 ± 1 ppm throughout the experiment. The daily water exchange rate was approximately 20%.

Nucleic acid preparation and cDNA library construction

Genomic DNA was isolated from fin tissue using conventional sodium dodecyl sulfate/proteinase K digestion, followed by phenol/chloroform extraction and ethanol precipitation. Total RNAs from various tissues were purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including the DNase treatment step. The cDNA library was constructed from total RNA purified from whole body *O. javanicus* juveniles using the Lambda Zap cDNA Synthesis and Packaging Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Plasmid DNA pools were directly prepared from the *Escherichia coli* SOLR cells (Stratagene) infected with the excised phagemid stock of the *O. javanicus* cDNA library to prepare the template for vectorette PCR isolation of the hepcidin cDNA copies.

Isolation of hepcidin genomic and cDNA genes

Based on sequence information from previously characterized *hamp1* and *hamp2* orthologs obtained from BLAST and/or text searches against the NCBI GenBank (http://ncbi.nlm.nih.gov/) and Ensembl genome databases (http://www.ensembl.org/index.html), two pairs of degenerate primers (OJHAMP1 gFW/gRV and OJHAMP2 gFW/gRV) were designated to PCR-amplify the *O. javanicus hamp1* and *hamp2* genes, respectively. Information on the primers and thermal cycling conditions used in this study are listed in Table 1. Using two sets of PCR primers, PCR was performed independently to isolate putative *hamp1* and *hamp2* fragments from *O. javanicus* genomic DNA. Six randomly chosen PCR clones for the putative *O. javanicus hamp1* or *hamp2* genes were sequenced in both directions. The raw sequence data were edited manually using the sequence editing software Sequencher (Gene Codes Corp., Ann Arbor, MI, USA). Based on the genomic gene sequences, cDNA sequences for *hamp1* and

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')	Thermal cycling conditions	Purpose
OJHAMP1 gFW	TGGYCMGACACCTATGAGA		
OJHAMP1 gRV	CTATCACAYKAYAGACTGWGRT	35 cycles at 94°C for 30 s, 58°C for 30 s	Isolation of genomic *ojhamp1, 2*
OJHAMP2 gFW	TGAGGAAACAGCTWCTAGMAR	and 72°C for 1 min	sequences
OJHAMP2 gRV	MTGAGRTRMAGATYTGGTGGA		
OJHAMP1 cFW	TGGTCAGACACCTATGAGAG	35 cycles at 94°C for 30 s, 58°C for 30 s	Isolation of *ojhamp1, 2* cDNA
OJHAMP2 cFW	TATCTGCAGAGTCGGCATGT	and 72°C for 1 min	fragments using vectorette PCR
OJ18S RV	CAAGAATTTCACCTCTAGCGGC	-	Preparation of normalized control
qOJHAMP1 1F	TTGCAGTTGCAGTGACACTC	45 cycles (real time RT-PCR) or 32	
qOJHAMP1 1R	CAGAAACCGCAGCCCTTGTA	cycles (semi-quantitative RT-PCR) at	Real-time RT-PCR assay of
qOJHAMP2 1F	CTCTGGCTGTTCTTCTGAC	94°C for 20 s, 58°C for 20 s and 72°C	*ojhamp1, 2* transcripts
qOJHAMP2 1R	CGACTGAGATACAGATCTGG	for 20 s	
qOJ18S 1F	TCCAGCTCCAATAGCGTATC	45 cycles (real time RT-PCR) or 20 cycles (semi-quantitative RT-PCR) at	Real time RT-PCR of 18S rRNA
qOJ18S 1R	AGAACCGGAGTCCTATTCCA	94°C for 20 s, 58°C for 20 s and 72°C for 20 s	(normalization control)

Each thermal cycling included a predenaturation step at 94°C for 2 min.

hamp2 were isolated using vectorette PCR. Two gene-specific forward primers (OJHAMP1 cFW and OJHAMP2 cFW) were included in the vectorette PCR reactions, respectively, to isolate *hamp1* and *hamp2*. The T7 primer was used as a reverse vector primer, of which the binding site was located at one end of multiple cloning site in the phagemid vector, pBluescript SK(-), which was used to construct the *O. javanicus* cDNA library. Twelve clones for the putative *O. javanicus hamp1* and *hamp2* genes were sequenced to identify the cDNA copies corresponding to their genomic counterparts.

Bioinformatic sequence analysis

Sequence homology of *O. javanicus* HAMP1 and HAMP2 was compared with the orthologs from representative teleosts using a BLAST search against the NCBI GenBank database (http://ncbi.nlm.nih. gov/Blast.cgi). The putative cleavage sites for the preprotein and proprotein of the *O. javanicus* HAMP1 and HAMP2 were predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/ SignalP/) and the ProP 1.0 server (http://www.cbs. dtu.dk/services/ProP/), respectively. Theoretical isoelectric point (pI) values for different regions of HAMP1 and HAMP2 were estimated using the ExPASy ProtParam tool (http://www.expasy.org/ tools/protparam.html). Multiple sequence alignment of mature or premature hepcidin proteins of *O. javanicus* HAMP1 and HAMP2, along with vertebrate orthologs, were generated using ClustalW (http://align.genome.jp/). Gene structures (exon-intron organization) of the *O. javanicus* hepcidin

isoforms were compared with previously characterized vertebrate hepcidin genes (for species and accession codes, see Figs. 3 and 4).

Tissue distribution assay of hepcidin transcripts

Basal expression patterns of *O. javanicus hamp1* and *hamp2* mRNA in adult tissues and semiquantitative reverse transcription (RT)-PCR assays were performed. Total RNA was prepared from the brain, eye, fin, gill, intestine, kidney, liver, muscle, ovary, and testis, as described above. Two micrograms of purified total RNA was reverse transcribed using the Omniscript RT Kit (Qiagen) according to the manufacturer's instruction. To prepare the normalization control, an 18S rRNA reverse primer (OJ18S RV) was included in the RT reaction, as previously described by Cho et al. (2009). The *O. javanicus hamp1* transcripts were amplified by RT-PCR using the *O. javanicus hamp1* transcript-specific primers qOJHAMP1 1F/1R (amplicon=244, 247 bp). The *O. javanicus hamp2* transcripts were amplified using the qOJHAMP2 1F/1R primers (amplicon=286 bp). The normalization control 18S rRNA was amplified by using the qOJ18S 1F/1R primers (amplicon=253 bp). Amplified product was separated on an agarose gel (1.5%), visualized with ethidium bromide) staining, and assessed by Quantity-One™ implemented in VersaDoc 4000 (Bio-Rad, Hercules, CA, USA). Relative expression levels of *hamp1* and *hamp2* mRNAs across tissues were assessed based on normalization against specific 18S rRNA levels in each tissue. Assays were performed independently in triplicate.

Bacterial challenge and mRNA expression assay

To examine differential modulation of *hamp1* and *hamp2* in response to immune challenge, an experimental challenge was performed using the Gram-negative pathogenic bacterium *Edwardsiella tarda* (FSW910410). Healthy adult *O. javanicus* individuals (*n*=12; six females and six males) were immersed in a bacterial suspension (1×10^6 cells/L) contained in a 5 L water bath for 5 h. The bacterial suspension was prepared by diluting freshly grown *E. tarda* culture (OD_{600nm}=1.0) in 5 ppt water by using synthetic sea salt (Kent Marine Inc., Franklin, WI, USA). After the immersion treatment, the fish were washed three times with a large volume (>20 L) of clean brackish (5 ppt) water (5 min per wash) and then immediately transferred to the water recirculating tank (30 L). A non-challenged control group was also treated identically, except for not being exposed to *E. tarda*. At 24 and 48 h post-transfer to the recirculating tanks, six individuals (three females and three males) were randomly chosen from each tank for gene expression assay. From each individual, the liver and kidney were surgically removed and pooled from the six individuals prior to RNA extraction. Total RNA samples and cDNA templates were prepared as described above. The real-time RT-PCR assay was performed using two gene-specific primer pairs that were used for the tissue distribution assay. Amplification was carried out using the 2× SYBR Supermix (Bio-Rad) and iCycler Real-Time Optic Module (Bio-Rad). The normalization control 18S rRNA was also amplified using two PCR primers, as described above. Based on the standard curves for both hepcidins and the 18S rRNA control, the fold changes of *hamp1* and *hamp2* during bacterial challenge were estimated based on the comparative Ct method in duplicate (Schmittgen and Livak, 2008).

Statistics

Differences in the basal expression levels of hepcidin transcripts across tissues were assessed by ANOVA, followed by Duncan's multiple range test. Transcriptional modulation in response to bacterial challenge was expressed as the fold change of hepcidin transcripts in bacteria-challenged groups compared to non-challenged controls. Differences in expression levels between challenged and non-challenged groups were assessed using Student's *t*-test. Statistical analysis was carried out the SPSS software version 10.1.3 (SPSS Inc., Chicago, IL, USA) and the difference was considered to be significant when $P < 0.05$.

Results

Characteristics of *O. javanicus hamp1* sequences

Based on the genomic PCR isolation, the *O. javanicus hamp1* gene (*ojhamp1*) had a tripartite structure consisting of three exons interrupted by two introns (Fig. 1) (GenBank accession no. JF419525). The *ojhamp1* clones possessed variable repeats of poly(A)+ and poly(C)+ in the 5′-untranslated region (UTR) and intron II, respectively. At the mRNA level, the two were homogenous but encode different amino acids. *O. javanicus hamp1* cDNA (*ojhamp1-1*) matched the predicted sequence of the genomic *ojhamp1*. The representative *ojhamp1-1* cDNA consisted of 270 bp of an open reading frame (ORF) encoding 90 aa. In the 3′-UTR, two putative polyadenylation signals (AATAAA) were found either 18 or 90 bp before the poly(A)+ tail. The *ojhamp1-2* cDNA had an identical ORF sequence to *ojhamp1-1*, except for three additional nucleotides (CAG) encoding glutamine (Gln). In both the 5′- and 3′-UTRs, the sequence homology between *ojhamp1-1* and *ojhamp1-2* was greater than 95%. The *ojhamp1-2* transcripts had some sequence variation in the 3′-UTR, including the mRNA species, with different 3′-UTR lengths processed by one of the two polyadenylation signals. Consequently, the deduced polypeptide sequence of the OJHAMP1 preproprotein consisted of 90 or 91 amino acids, in which two cleavage sites for processing the proprotein and mature peptides were predicted at Ala^{24}/Ile^{25} and Arg^{64-65}/Gln^{65-66}, respectively. The whole prepro-protein and proprotein of OJHAMP1 were predicted to have moderate cationic charges (pI=7.53 and 7.73, respectively), while the final form of the mature OJHAMP1 peptide had a higher cationic charge (pI=8.52). The mature OJHAMP1 had eight Cys residues (8/26; 30.8%) at conserved positions.

Characteristics of the *O. javanicus hamp2* sequences

The PCR-isolated *O. javanicus hamp2* gene (*ojhamp2*) had a conserved structure, typical of vertebrate *hamp* genes (Fig. 2). The lengths of the three exons (87, 78, and 105 bp for exon I-III, respectively) and two introns (78 and 122 bp for introns I and II, respectively) were not significantly different from those of *ojhamp1* (GenBank accession no. JF419526). The exon sequences at the genomic level were similar to the *ojhamp2* cDNA counterpart. The *ojhamp2* cDNA possessed 132 bp of the 5′-UTR, 267 bp of a single ORF encoding 89 aa and 106 bp of the 3′-UTR, excluding the poly(A)+ tail. A consensus polyadenylation signal (AATAAA) was found 25 bp

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                                                                        (A+)17-21
tggtcagacacctatgagagatcagctcttatctttaaaggattaaagcaaaaaaaaaaaaaaaaaaaATGAAGGCA
                                                                          M  K  A
TTCAGCATTGCAGTTGCAGTGACACTCGTGCTCGCCTTTATTTGCATCCAGGAGAGCTCTGCCATCCCAGTCAAT
 F  S  I  A  V  A  V  T  L  V  L  A  F  I  C  I  Q  E  S  S  A  I  P  V  N
                    Signal peptide (24 aa; pI=5.75)
GGGgtaaggatttcacttaaacttatcatttccttattaaccatataatgttttaagatgcgcctcctaaatgtct
 G
gcaactcgttttagCAGGTCACAGAGCTGGAAGAAGCAGCAAGCAATGACACTCCAGTTGCGGCACGTCACGAGAT
           (Q)  V  T  E  L  E  E  A  A  S  N  D  T  P  V  A  A  R  H  E  M
                       Proregion (40 or 41 aa; pI=5.47)
GTCAATGCAATCGTGGATGgtatgttcagtcaactgaatgaattaagtcaattaccagcagcaaattaacattca
 S  M  Q  S  W  M
                                     (C+)13-16
agtggaggagttttcccccagcgggggcatgccgagcccccccccccccgtgctgcggaaaggcttgaaatgct
cagacatgacaccttgtgcacttgtgttgtctctcatgcagATGCCAAACCACATCAGGGAAAAGCGGCAGAGCC
                                            M  P  N  H  I  R  E  K  R  Q  S  H
ACCTCTCCATGTGCAGCGTGTGCTGCAACTGCTGCAAGAACTACAAGGGCTGCGGTTTCTGCTGCAGGTTCtgag
 L  S  M  C  S  V  C  C  N  C  C  K  N  Y  K  G  C  G  F  C  C  R  F  *
                  Mature peptide (26 aa; pI=8.52)
gggtcctgcgacaaccaccacacattaatttattattaggcttgttttccatactttccacaagaaattcactt
                                            Ins/del
ttttttccctctttatctcagtctgtcatgtgatacttttattgtaatgaggaactcaagaaaacagataaaggct
                                      ggata
gttggtttta ttattttctactct ttttgtaagatcaataaattgtcaatttccaaaggaaattaaatgaatga
                                                           ↑
                                                      poly(A)+ tail
attaaatgtgtttttttttcaatgtaattattcaaataaaacagttgtatgg
                                              ↑
                                         poly(A)+ tail
```
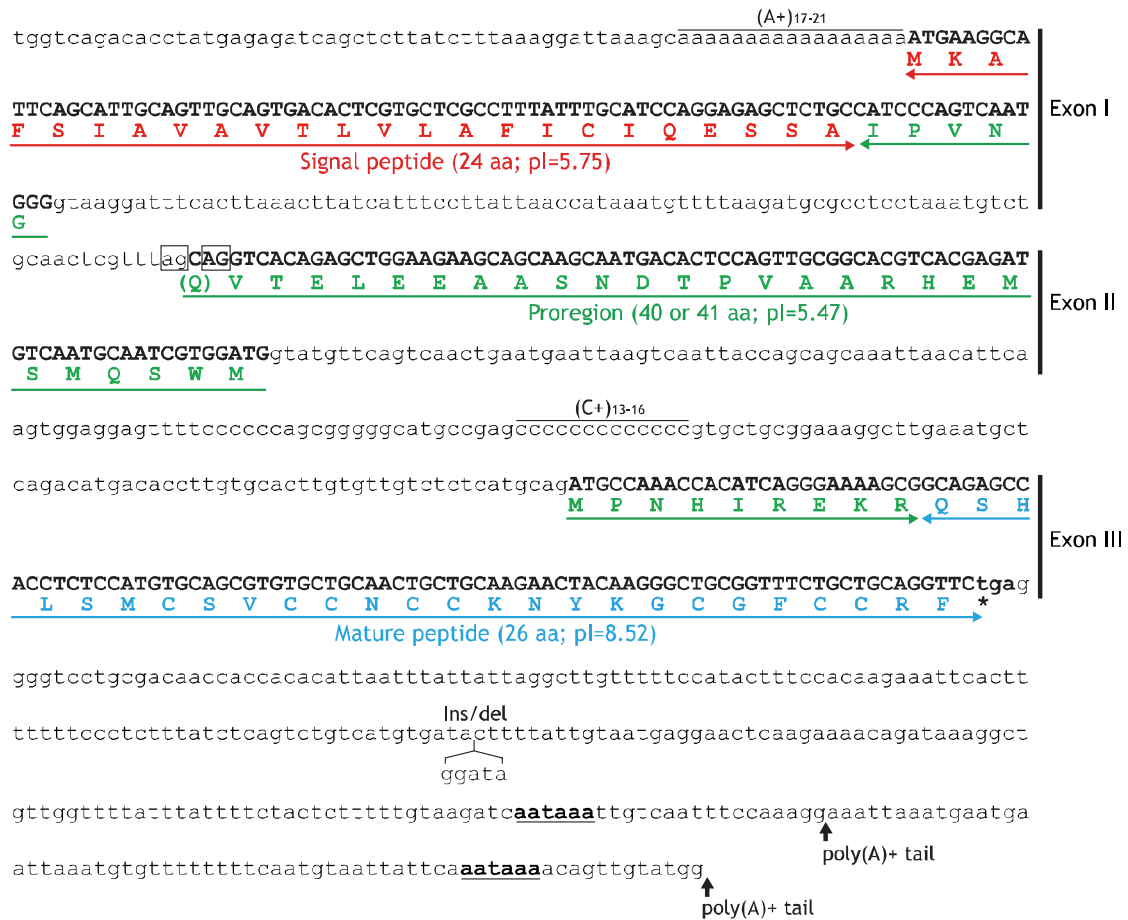
Fig. 1. Nucleotide and deduced amino acid sequences of *Oryzias javanicus* hepcidin isoform *hamp1*. Coding region is indicated by bold uppercase letters while non-coding region by lower case letters. Stop codon (tga) is indicated by an asterisk. Deduced amino acid sequence in the singlet code is provided below the nucleotide sequence. Amino acid number and theoretical isoelectric point (pI) value for each of three regions (signal peptide, proregion and mature peptide) are also indicated. The variable repetitive numbers for adenines (in the 5′-UTR) and cytosines (in the intron II) are observed. Two adenine-guanine sequences in the junction site between intron I and exon II are boxed, which is a potential target site for alternative splicing to produce 90 or 91 aa-encoded mRNA species. The Gln missed in the 90 aa-encoded mRNA is noted in the parenthesis. In the 3′-UTR, an insertion/deletion of five nucleotides (ggata) was found among different cDNA clones, and mRNAs processed with two different polyadenylation signals (**aataaa**; underlined and bolded) were also detected. The sites observed for the poly(A)+ tailing are indicated by vertical arrows. UTR, untranslated region.

before the poly(A)+ tail. The amino acid sequence of the OJHAMP2 polypeptide (the whole preproprotein) was predicted to have a negative overall charge (pI=5.70). The preproprotein OJHAMP2 had two potential cleavage sites: one between Ala[24] and Val[25] (cleavage of the signal peptide), and the other between Arg[64] and Glu[65] (conversion of the proprotein to the mature peptide). The pI values for the three regions were 5.75 (for the signal peptide; 24 aa), 5.64 (proregion; 40 aa), and 6.03 (mature peptide; 25 aa). The OJHAMP2 mature peptide also had eight cysteine residues.

Multiple sequence alignment of mature peptides with their representative orthologs

The mature peptide sequence of OJHAMP1 was aligned with 40 orthologs compiled from nine orders (Fig. 3). Fish HAMP1s possessed either 25 or 26 aa, displaying a cationic pI range from 7.73 to 8.94 (Fig. 3). They shared a relatively high degree of sequence homology, with the eight conserved Cys residues in all the active sequences, except one isoform from Atlantic salmon *Salmo salar* having only seven Cys. Besides these conserved Cys residues, the teleostean HAMP1s possessed four more conserved amino acid

tgaccaaacagcttctagcaggagtatataatatctgcagagtcggcatgttccagcatctgacagaagaactgag

acaagagtcatcaagttcaagactgatctgactgagaatcttctccaacatccaaag**ATGAAGAGCTTCATTGCT**
                                                           M   K   S   F   I   A
                                                           ←———————————————————
GCAGTTTCTCTGGCTGTTCTTCTGACATTTGTCTGCTTTCAGGAGAGCTCTGCTGTTCCAGTCACTGAGgtaaga
A   V   S   L   A   V   L   L   T   F   V   C   F   Q   E   S   S   A | V   P   V   T   E
———   ←———————————
                    Signal peptide (24 aa; pI=5.75) | Exon I

agttcattgaaagaattctgtccttcattcatgattc-gtcgattttattgaacggatgaaatcctctgaaag**GTC**
                                                                            V
                                                                            ——
CATGAGCTGGAGGGAACACTGACTGATGACAGTCCACTTCCTGCTGAGCTCAAGATGCCAGTGACCTCATGGAAG | Exon II
H   E   L   E   G   T   L   T   D   D   S   P   L   P   A   E   L   K   M   P   V   T   S   W   K
———
                         Proregion (40 aa; pI=5.64)

gtgagttcacctcaacaagtcaatgaaagagagttatattgtctctatttatgatagtgtaagagtgttaggttt

ataactcatcaagctgcagatgatcagtgtcctgttgtgtgtctcag**ACGCTGTATGACATCAGGAGGAAGCGTG**
                                                T   L   Y   D   I   R   R   K   R | E
                                                →——————————————————————————————— | Exon III
AGGCCAGCGCTGCAGGCTGTCGTTTCTGCTGTGGCTGCTGTGGGATGAGTGGATGTGGAGTGTGCTGTCCTTTCt
A   S   A   A   G   C   R   F   C   C   G   C   C   G   M   S   G   C   G   V   C   C   P   F | *
→———
                    Mature peptide (25 aa; pI=6.03)

gacatgtgctgtttcaacttccaccagatctgtatctcagtcgtttgtcaaacgaatgtacaatatatgaatgca

ttcac**aataaa**gatctgtttgtcacaatttt
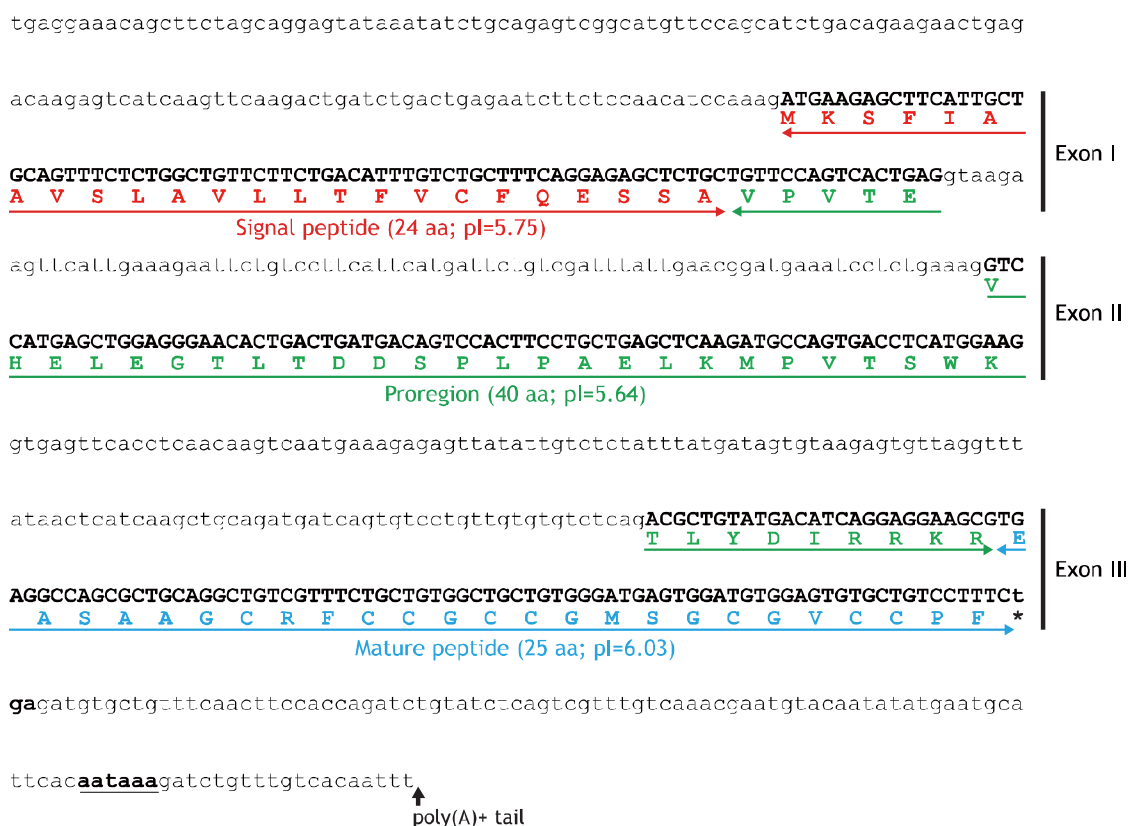   ↑
                       poly(A)+ tail

Fig. 2. Nucleotide and deduced amino acid sequences of *Oryzias javanicus* hepcidin isoform *hamp2*. Coding region is indicated by bold uppercase letters while non-coding region by lower case letters. Stop codon (tga) is indicated by an asterisk. Deduced amino acid sequence in the singlet code is provided below the nucleotide sequence. Amino acid number and theoretical isoelectric point (pI) value for each of three regions (signal peptide, proregion and mature peptide) are also indicated. A putative polyadenylation signal (aataaa) is underlined and bolded, while the site for poly(A)+ tailing is indicated by vertical arrow.

residues at positions 3 (His), 20 (Gly), 22 (Gly), and 27 (Phe) (alignment positions including gaps in Fig. 3). Moreover, the sequence identities across the species were roughly in agreement with the known taxonomic appraisal of the species, although we did not reconstruct the molecular phylogeny in detail. As shown in the alignment, teleostean HAMP1 represented a well conserved N-terminal motif (QSHL/I). Exceptions were the ISHI signals found in a few species belonging to Pleuronectiformes and the QIHL observed in one of the three isoforms recovered from Atlantic salmon. However, these substitutions did not change the pI values of the first 5 aa essential for hormonal function of HAMP1. All the HAMP1s had identical pI values (6.74) in this region.

Alignment of the major HAMP2 sequences (only HAMP2s having eight clearly conserved Cys residues, aligned in Fig. 4) revealed that teleostean HAMP2s were more variable and heterogeneous than HAMP1s

in both the length and charge of the mature peptide (Fig. 4). The HAMP2 sequences recovered from species belonging to the superorder Acanthopterygii and many of species from Perciformes possess multiple HAMP2 copies. The fish HAMP2 mature peptides are predicted to have amino acid lengths ranging from 21 aa (empirically identified from the bass species *Morone chrysops* in Fig. 4) to 26 aa. Unlike fish HAMP1s, which have a conserved length in the N-terminal amino acids prior to the first conserved cysteine, the corresponding HAMP2 region varied in length (1-6 aa). The pI values of the HAMP2 mature sequences were also variable, ranging from negative (the most anionic value was 4.21, estimated in one isoform from *Acanthopagrus schlegelii*: Perciformes) to positive (the most cationic value was 8.97, observed in several perciform species). Moreover, the pI values of the first five N-terminal amino acids were highly variable among HAMP2s. The lowest values (3.80-4.00) were

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O. javanicus    (JF419525)            QSHLSMCSVCCNCCK-NYKGCGFCCRF : 26/8.52  Beloniformes
O. latipes      (FM166049)            ...I...TM......-............ : 26/8.52
F. heteroclitus (CN990077)            .....L.RY..T...-.-.......... : 25/8.75  Cyprinodontiformes
G. aculeatus    (ENSGACP00000016484)  .......RW..K..RSY-....Y..K.. : 26/8.92  Gasterosteiformes
S. maximus      (AAX92670)            ...T.L.RW......A.-.......K.. : 26/8.75
S. maximus      (CAJ34592)            ...I.L.RW......AY-.......... : 26/8.75
P. americanus   (AWC13026)            I..I.L.RW......A.-......K.. : 26/8.75   Pleuronectiformes
P. flesus       (DV568154)            I..I.L.RW......A.-......K.. : 26/8.75
P. olivaceus    (AAT01563)            I..I...RW......A--....P..K.. : 25/8.75
C. mirabilis    (EB648659)            .....L.RW..K..RGY-.......K.. : 26/8.94
L. dearborni    (ABY84843)            .....L.RW.....RAY-.......K.. : 26/8.75
P. scotti       (ABY84821)            .....L.RW......G.-..........K.. : 26/8.76
D. mawsoni      (ABY84824)            .....L.RW......G.-.......... : 26/8.76
E. maclovinus   (ABY84822)            .....L.RW......G.-.......... : 26/8.76
N. angustata    (ABY84825)            .....L.RW......G.-.......... : 26/8.76
P. fluviatilis  (DT901803)            .....L.RW.....RA.-.......K.. : 26/8.76  Perciformes
D. labrax       (DV216833)            .....L.RW.....RG.-.......K.. : 26/8.76
L. crocea       (ABC18307)            .....L.RW......S.-.......... : 26/8.76
M. salmoides    (ACD13023)            .....L.RW.....RG.-.......... : 26/8.78
M. dolomieu     (ACD13025)            .....L.RW......G.-.......... : 26/8.76
S. aurata       (CB177060)            ...I...YY.....RA.-....Y..K.. : 26/8.50
L. mormyrus     (EB507090)            ...I...YY.....RA.-....Y..K.. : 26/8.50
P. auriga       (BAH03285)            ...I...YW.....RA.-....Y..K.. : 26/8.51
S. quinqueradiata (AU312565)          .......RW.....TA.-.......... : 26/8.54
O. niloticus    (TH2-3)               .....L.RW.....RS.-.......... : 26/8.78
D. rerio        (AAR18592)            .....L.RF..K..R-.-....Y..K.. : 25/8.74
H. nobilis      (ACO51156)            .....L.RY.....R-.-....Y..K.. : 25/8.74
P. sarana       (CAZ68137)            .....L.RY.....R-.-....Y..K.. : 25/8.74  Cypriniformes
P. promelas     (DT343090)            .....L.RY.....R-.-....Y..K.. : 25/8.74
I. punctatus    (ABA43709)            .....L.RY.......-.-.......K.. : 25/8.75
T. furcatus     (AAX39714)            .....L.RY.......-.-.......... : 25/8.75  Siluriformes
P. fulvidraco   (ABX46065)            .....L.RY.......-.-.......... : 25/8.75
G. morhua       (ACA42770)            ....AL.RW.....R-.O....I..K.. : 26/8.76  Gadiformes
G. morhua       (ACA42769)            ....AL.RW.....R-.Q.......K.. : 26/8.76
O. mykiss       (ADU85830)            .....L.RW.....H-.-.......K.. : 25/8.53
O. tshawytscha  (EL553997)            .....L.RW.....H-.-.......K.. : 25/8.53
S. salar        (ACI69335)            .....L.RW.R...H-.-.......K.. : 25/8.85  Salmoniformes
S. salar        (NP_001134321)        .....L.RW.....H-.-.......K.. : 25/8.53
S. salar        (AAO85553)            .-...L.GI.....H-.-T......K.. : 25/7.73
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Right side bracket labels:
Beloniformes
Cyprinodontiformes
Gasterosteiformes
Pleuronectiformes — Acanthopterygii
Perciformes
Cypriniformes — Ostariophysi
Siluriformes
Gadiformes — Paracanthopterygii
Salmoniformes — Protacanthopterygii

Header: aa pl

Fig. 3. Multiple alignment of *Oryzias javanicus* mature HAMP1 peptide along with its teleostean orthologs. Seven clearly conserved Cys residues are noted by asterisks on the top while four conserved non-cysteine residues by open circle. One Cys residue conserved in most species except one isoform from Atlantic salmon (*S. salar*) was indicated by a closed square on the top. Predicted amino acid number (aa) and theoretical isoelectric point (pI) value are provided at the end of each sequence. For the accession code for *O. niloticus* (TH2-3), refer to Huang et al. (2007).

estimated in the two *Oryzias* species, whereas several perciform species from Notothenioidei represented an extremely positive charge (10.06) in this region.

Basal expression patterns of *hamp1* and *hamp2* in adult tissues

Using the RT-PCR assay, *ojhamp1* and *ojhamp2* mRNAs were evaluated in eight somatic and two gonadic tissues (Fig. 5). Basal expression levels of *ojhamp1* transcripts were highest in the liver (*P<0.05*), although many other tissues had detectable levels of *ojhamp1* transcripts. The ovary, testis, and kidney showed the next highest expression level, after the liver. Brain, gill, and intestine showed a moderate level of *ojhamp1* mRNA, and eye tissue exhibited very low levels of expression (*P<0.05*). However, in fin and muscle, *ojhamp1* transcripts were not detected. The tissue distribution pattern of *ojhamp2* transcript was slightly more ubiquitous than that of *ojhamp1*. The *ojhamp2* transcripts were

detected in all the tissues examined. Similar to *ojhamp1*, the expression of *ojhamp2* was highest in the liver (*P<0.05*). The expression levels of *ojhamp2* in the ovary and testis were comparable to that of the liver. Again, expression of *ojhamp2* was the lowest in the fin and muscle (*P<0.05*).

Differential expression of *ojhamp1* and *ojhamp2* resulting from bacterial challenge

After bacterial challenge with *E. tarda*, the two hepcidin isoforms had different modulation patterns in the kidney and liver (Fig. 6). Expression of *ojhamp1* in the kidney was slightly downregulated, but not significantly at either detection time (24 and 48 h post-challenge; HPC) (*P>0.05*). However, hepatic *ojhamp1* transcripts were upregulated 1.5-fold at 24 HPC (*P<0.05*) and 2-fold at 48 HPC (*P<0.05*) compared to the nonexposed control. The *ojhamp2* transcripts, however, were significantly downregulated in response to bacterial challenge in

```
                                                   *  ** **      *  **        aa/pI-w/pI-n
O. javanicus    (JF419526)            RRKREASAACCRFCCGCCC-MSCCGVCC--PF- : 25/6.03/4.00    ⎤ Beloniformes
O. latipes      (HS106028)            .N..D..............-.........--..- : 25/5.79/3.80   ⎦
T. rubripes     (NEWSINFRUP000000179783) .I...--.PKR.....N..PR.V...T..--K.- : 24/8.96/9.50 ⎤ Tetraodontiformes
T. nigroviridis (CAG07920)            .H..--.P..........PR.R.......--K.- : 24/8.77/5.24   ⎦
P. major        (AAS66305)            .F..--.P..........PN.T.......-R.- : 24/8.18/5.24    ⎤
L. mormyrus     (EB508954)            .H..--.P..........PGVV.......--R.- : 24/8.18/5.24   |
A. schlegelii   (AAU00795)            .H..--.P..........PN.R.......--R.- : 24/8.53/5.24   |
A. schlegelii   (AAU00797)            .I...--.PKD.O......PD.....I..--R.- : 24/5.66/5.55   | Perciformes
A. schlegelii   (AAU00801)            .H..--.PKD.O......PD.....I..TY--- : 24/4.21/5.55    |
L. japonicus    (AAS55063)            .H..--.P.D........TDV........--R.- : 24/5.66/5.55   |
D. labrax       (AAZ85124)            .H..HS.PG......N..PN.........--R.- : 26/8.23/6.74   |
M. chrysops     (AAM28440)            .H..HS.PG......N..PN.........--R.- : 26/8.23/6.74   |
                                                                          (21/8.23/8.07)  ⎦
G. aculeatus    (EG588539)            .E..---GIK.K......-TPGV..L...--R.- : 22/8.53/9.31   ⎤ Gasterosteiformes
P. americanus   (AW013287)            .Q..---GFK.K......-RAGV..L...--R.- : 22/8.75/9.31   ⎤
P. flesus       (DV569520)            .Q..---SFK.K......-RAGV..L...--R.- : 22/8.76/9.30   |
S. maximus      (AI65387)             .H..---.IK.K......-TPGV......--R.- : 22/8.53/9.32   | Pleuronectiformes
H. hippoglossus (CF931737)            .Q..---GFK.K......-RPGV..L...--R.- : 22/8.76/9.31   ⎦
O. niloticus    (ABD46831)            .H..---GIK........-TPGI......--R.- : 22/8.54/9.51   ⎤
G. mirabilis    (AW783824)            .E..---GIK.K......-TPGV......--R.- : 22/8.53/9.31   |
M. salmoides    (ACD13024)            .O..---GTR........-TPGV......--R.- : 22/7.70/5.99   |
M. dolomieu     (ACD13030)            .Q..---GIK........-TPGV......--R.- : 22/8.54/9.51   |
J. scabass      (AAT09138)            .H..---.IK.K......-TPGV......--R.- : 22/8.53/9.32   |
A. schlegelii   (AAU00798)            .HN.---.IK.K......-IPGV..L...--R.- : 22/8.53/9.32   |
S. chuatsi      (ACO88905)            .O..---GFO........-TPGV......--R.- : 22/8.23/8.25   | Perciformes
P. fluviatilis  (ABR04075)            .H..---GFK..V.....-TPGV..L...--R.- : 22/8.54/9.51   |
P. fluviatilis  (DV671272)            .H..---GFK........-TPGV..L...--R.- : 22/8.54/9.51   |
O. fasciatus    (ACF49401)            .Q..---GFK.K......-RRGV..L...--R.- : 22/8.97/9.31   |
O. fasciatus    (ACF49398)            .O..---GFK.K......-GRGV..L...--R.- : 22/8.76/9.31   |
N. angustata    (ABY84831)            .E..---NSK..V..N..SFHNA..L...--D.- : 23/7.71/9.51   |
C. aceratus     (ABY84828)            ..R.----RK.K...N..--SNI.QT..TRR.- : 22/8.97/10.06   |
D. mawsoni      (ABY84829)            ..R.----RK.K...N..--SNI.QT..TRR.- : 22/8.97/10.06   |
D. mawsoni      (ABY84830)            ..R.----RK.K...N..--SNI.QT..TRRL- : 22/8.97/10.06   |
E. maclovinus   (ABY84826)            ..R.----RK.........-NPGI.QT..TKA.C : 24/8.76/10.06   ⎦
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Fig. 4. Multiple alignment of *Oryzias javanicus* mature HAMP2 peptide along with its teleostean orthologs in a quantitative mode. Eight clearly conserved Cys residues are noted by asterisks on the top. Putative RXKR or RRXR cleavage sites are also shown and boxed based on the prediction using the ProP Server. Cleavage sites based on the bioinformatic prediction or experimental isolation (from the hybrid bass *M. chrysops*) are indicated by vertical arrows. Predicted amino acid number (aa) and theoretical isoelectric point (pI) values for whole predicted mature peptide (pI-w) and the five N-terminal amino acids (pI-n) are provided at the end of each sequence.

both tissues. The *ojhamp2* transcripts in the kidney were suppressed 5-fold at 24 HPC, and then partially recovered, as compared to the control level (*P*<0.05). Hepatic *ojhamp2* transcripts were markedly suppressed 20-fold at 48 HPC (*P*<0.05).

Discussion

Teleosts, which belong to Acanthopterygii, are thought to possess at least two copies of *hamp* genes. In this study, we isolated and characterized the genetic determinants for two paralog *hamp* genes, *hamp1* and *hamp2*, from a beloniform species, *O. javanicus* (*ojhamp1* and *ojhamp2*). Both *ojhamp1* and *ojhamp2* had a conserved tripartite genomic structure, similar to all the vertebrate *hamp* genes characterized so far (Douglas et al., 2003a; Cuesta et al., 2008; Hilton and Lambert, 2008; Wang et al., 2009). However, at the mRNA level, *ojhamp1* was highly

variable. First, the *ojhamp1* mRNA could undergo processing, yielding different 3′-UTR lengths due to the presence of the two functional polyadenylation signals. Second, the *ojhamp1* could be transcribed into two different mRNA variants, encoding either 90 or 91 aa in the preprotein, which may have been caused by alternative splicing at the junction site between intron I and exon II (…ttt**ag**C**AG**GTC…). As a result, the splicing machinery may differentiate between the nucleotide sequence CAG as either the first codon for Gln (*i.e.*, 91 aa protein; *ojhamp1-2* type) or an intronic **AG** acceptor site (*i.e.*, 90 aa-protein; *ojhamp1-1* type). However, the biological consequences of these alternatively spliced forms are not yet understood. Alternatively, partially spliced transcripts of the immune gene have been previously reported in carp interleukin (Engelsma et al., 2001) and mouse tumor necrosis factor genes (Reddy et al., 2001) under stimulated conditions. More importantly,
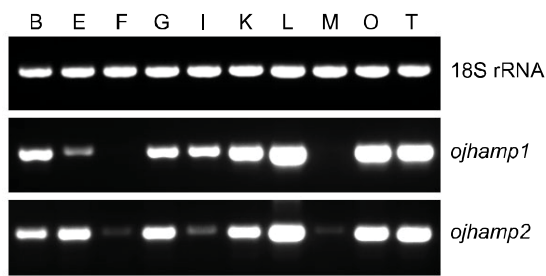
Fig. 5. Representative reverse transcription-PCR gels showing the tissue distribution of *Oryzias javanicus hamp1* and *hamp2* transcripts. Abbreviations for tissues are brain (B), eye (E), fin (F), gill (G), intestine (I), kidney (K), liver (L), muscle (M), ovary (O) and testis (T). A gel for 18S rRNA normalization control is also shown on the top.

a similar alternative splicing pattern has been reported in hepcidin isoforms from the rockbream *Oplegnathus fasciatus*. Notably, the site (between intron I and exon II) targeted for the alternative splicing in the rockbream hepcidin was the same as that in *ojhamp1* (Cho et al., 2009), and furthermore, the amino acid (Gln) neglected during the alternative recognition was the same in the two hepcidin genes. This suggests that many other *hamp* genes with similar junction sequences could be subjected to alternative splicing, producing variant *hamp* transcripts. Currently, we have not evaluated the effects of alternative splicing on the structure and function of the hepcidins at either the mRNA or proprotein levels, since the presence or absence of Gln (uncharged and polar) did not alter the pI or the protein configuration, as assessed by *in silico* prediction (data not shown).

Based on the multiple sequence alignments of the mature hepcidin peptides, fish HAMP1s have highly conserved characteristics in their amino acid lengths, as well as in the pI values. However, in contrast to the HAMP1 group, the teleost HAMP2s are highly variable in these parameters across species and isoforms. Previous studies have claimed that fish *hamp1*s are orthologs of the human counterpart (*hamp*), and hence fish HAMP1 should function as a negative regulator of iron homeostasis, similar to mammalian HAMPs. The fish *hamp2*s, however, have been acquired through a different pathway than that of mammalian *hamp* genes (Rodrigues et al., 2006; Hilton and Lambert, 2008). This claim is based not only on the chromosomal synteny between mammalian *hamp* and fish *hamp1*, but also the observation that most fish HAMP1s (mature form) display an anionic charge in the N-terminal region of
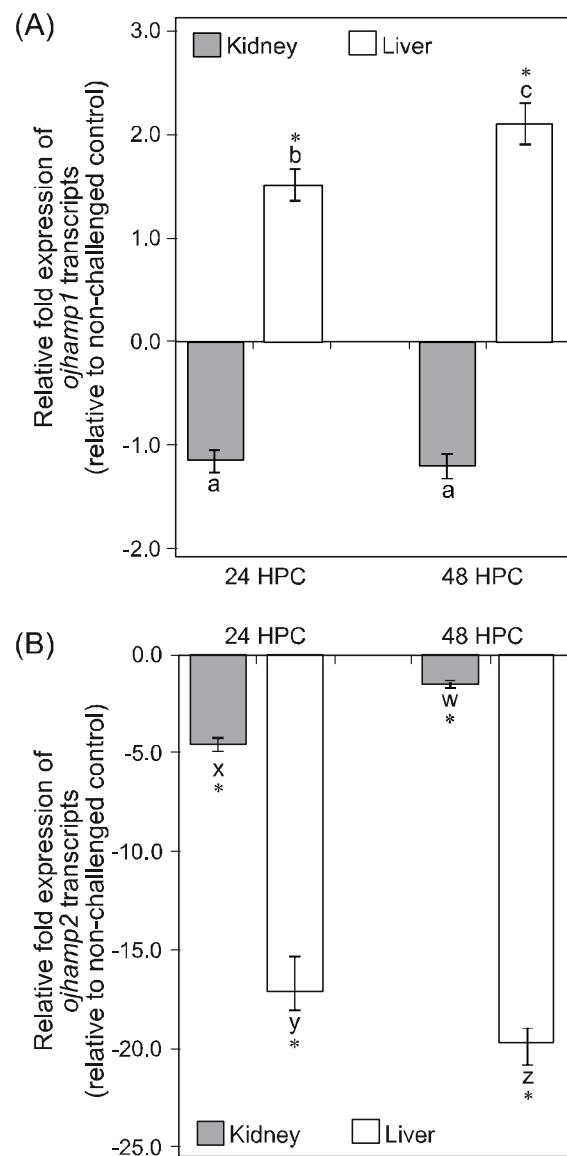

Fig. 6. Differential expression of *Oryzias javanicus hamp1* (A) and *hamp2* (B) in the liver and kidney in response to the bacterial challenge using *Edwardsiella tarda*. Real-time RT-PCR assay was performed at 24 or 48 h post challenge (HPC) and the gene modulations are defined as fold changes of transcript levels relative to non-challenged control. Mean±SDs with different letters (a-c or w-z) are significantly different based on ANOVA ($P<0.05$), while the significant up- or down-regulations from the control level are noted by asterisks based on the student's *t*-test ($P<0.05$).

the mature peptide, similar to mammalian HAMPs. The negatively charged N-terminal region (usually 5 aa prior to the first conserved Cys) is known to be essential for its interaction with ferroportin (Nemeth

et al., 2006). The QSHLS motif of the zebrafish (*Danio rerio*) HAMP1 is known to internalize ferroportin molecules, similar to the mammalian motif DTHEP (Fraenkel et al., 2005). The OJHAMP1, described in this study, represents a clearly conserved signature in the N-terminal region, and it shared an identical anionic pI value with all other fish HAMP1 orthologs, suggesting that the dual function of mammalian HAMP1s should be conserved in the broad teleostean taxa. In contrast to HAMP1, the fish HAMP2s were considerably heterogeneous in their structures and charges. Recent molecular phylogeny has argued that, unlike *hamp1*, which likely exists in every fish species, the fish *hamp2* copies are recovered only from Acanthopterygii and could play a primary role in the immune response (*e.g.*, antimicrobial function) rather than iron regulation (Hilton and Lambert, 2008). This argument was based on the cationic N-terminal charge of fish HAMP2 mature peptides, which was different from fish HAMP1s and mammalian HAMPs. However, this study suggests that this argument should be carefully validated with various fish HAMP2 isoforms. The OJHAMP2 isolated from this study displays a highly anionic charge in the predicted N-terminal region (see also the neighbors of OJHAMP2 in Fig. 4). Furthermore, based on bioinformatic predictions, the overall charge of mature OJHAMP2 is not 'cationic,' suggesting that extensive expression analyses may be required to determine whether pI-dependent functional diversification exists among fish HAMP2 peptides. Experimental purification of the active peptide, rather than prediction, should be completed to provide direct evidence to support this concept because one example of an isolated HAMP2 from the hybrid bass (see MCH in Fig. 4) was shown to be shorter (only 21 aa) than predicted (26 aa) (Shike et al., 2002; Lauth et al., 2005). Also, human hepcidin proprotein is known to undergo some processing to yield mature HAMP with different lengths (Valore and Ganz, 2008).

Tissue distribution patterns of fish hepcidin transcripts are known to be species-specific, unlike the liver-exclusive or predominant patterns found in most mammalian species. In this study, both *ojhamp1* and *ojhamp2* transcripts had a wide tissue distribution. Although the liver had the highest level of both isoforms, other tissues (such as the gonads and kidney) had comparable amounts of the hepcidin transcript. This finding supports previous reports in many other species, including channel catfish *Ictalurus punctatus*, black porgy *A. schlegelii*, red sea bream *Chrysophrys major,* turbot *Scophthalmus*

maximus, gilthead seabream *Sparus aurata*, and redbanded seabream *Pagrus auriga* (Bao et al., 2005; Chen et al., 2005, 2007; Yang et al., 2007; Cuesta et al., 2008; Martin-Antonio et al., 2009). In contrast, our finding is significantly different from the highly liver-predominant expression observed in winter flounder *Pseudopleuronectes americanus*, sea bass *Dicentrarchus labrax*, and orange-spotted grouper *Epinephelus coioides* (Douglas et al., 2003b; Rodrigues et al., 2006; Yin et al., 2006).

During immersion treatment with *E. tarda*, the two isoforms, *ojhamp1* and *ojhamp2*, revealed different transcriptional responses to bacterial challenge. The *ojhamp1* was not significantly responsive to bacterial challenge in the kidney, yet was moderately upregulated in the liver. Moreover, *ojhamp2* was consistently downregulated 20-fold in the liver at 48 HPC during the same bacterial challenge. Such an opposing isoform-dependent regulation in a given tissue (*i.e.*, the liver in this study) has not been reported so far, and the mechanism for this pheno-menon requires further characterization. Many post-mortem evaluations on the upregulation of hepcidin transcripts in fish during bacterial challenges have been based on direct injection (often intraperitoneal or intramuscular) of bacteria (Chen et al., 2007; Cuesta et al., 2008; Yang et al., 2011), while only a few studies have used the immersion technique (Yang et al., 2007). For this reason, performing experi-mental challenges with a series of bacterial cell numbers may be valuable in future studies to examine the 'bacterial burden'-dependent regulation of *ojhamp1* and *ojhamp2*. A previous study on zebrafish indicated that only clinically affected individuals, after bacterial challenge, exhibited a significant elevation of hepcidin transcripts, while healthy fish did not respond (Shike et al., 2004). In addition, redbanded seabream (*P. auriga*) hepcidin isoforms are significantly downregulated in the spleen by immune challenge using LPS (Martin-Antonio et al., 2009).

In summary, two hepcidin paralogs (*ojhamp1* and *ojhamp2*) were isolated and characterized from the Javanese ricefish *O. javanicus*, a potential model fish species. Both *O. javanicus hamp* genes have a conserved tripartite gene structure, typical of vertebrate hepcidin genes. However, *ojhamp1* could be transcribed into two mRNA species encoding 90 or 91 aa, potentially through an alternative splicing mechanism, whereas *ojhamp2* consistently produced a single mRNA encoding 89 aa. Transcripts of both isoforms were expressed in a wide array of adult tissue types, with the highest expression level in the

liver. Upon bacterial challenge using *E. tarda*, the two isoforms exhibited different patterns of transcriptional responses, including significant downregulation, no apparent alteration, or moderate upregulation.

Acknowledgments

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