Fish & Shellfish Immunology 56 (2016) 239–247

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Cloning and expression of two hepcidin genes in the mudskipper (*Boleophthalmus pectinirostris*) provides insights into their roles in male reproductive immunity

Zhe Li [a], Wan Shu Hong [a, b, c], Heng Tong Qiu [a], Yu Ting Zhang [a], Ming Shu Yang [a], Xin Xin You [d], Shi Xi Chen [a, b, c, *]

[a] State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences Xiamen University, Xiamen, 361102, China
[b] State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen University, Xiamen, 361102, China
[c] Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources (Xiamen University), Xiamen, 361102, China
[d] Shenzhen Key Laboratory of Marine Genomics, Marine and Fisheries Institute, BGI-Shenzhen, Shenzhen, 518083, China

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form
14 July 2016
Accepted 14 July 2016
Available online 16 July 2016

Keywords:
Hepcidin
Antimicrobial peptide
Boleophthalmus pectinirostris
Reproductive immunity

ABSTRACT

The mudskipper *Boleophthalmus pectinirostris* is a burrow-dwelling fish inhabiting intertidal mudflats. During the spawning season, in a spawning chamber located at the center of their burrow, a pair of male and female fish mate and fertilized eggs adheres onto the inner walls and ceiling with filamentous attachments. During 5 days of incubation, the fertilized eggs are kept clean and hatch with a very high hatching rate under the natural conditions filled with microorganisms. This suggests that the male and/or female reproductive tract may synthesize antimicrobial substances to offer protection against microorganisms that may be deleterious to fertility. To study the antimicrobial strategy of this fish in the spawning season, we first cloned the two *hepcidin* isoforms from *B. pectinirostris*, and designated them as *Hepcidin-1* and *Hepcidin*-2 based on phylogenetic analyses. Both of these *hepcidin* isoforms were highly expressed in the liver, but only *Hepcidin-1* showed significant change in response to iron overload. Interestingly, these two *hepcidin* isoforms were expressed in male reproductive tracts, i.e. the testes and seminal vesicles. The monthly expression pattern indicated that *Hepcidin-1* transcript levels showed a peak point only in March (before spawning) in the seminal vesicle, while *Hepcidin-2* transcript levels were correlated with male reproductive status and reached their highest level in May (the peak spawning period). Under experimental conditions, the expression of these two *hepcidin* isoforms showed no response to iron overload in the male gonad. However, after lipopolysaccharide injection, the *Hepcidin-1* transcript level was significantly up-regulated in the testes and seminal vesicle 6 h post injection, while *Hepcidin-2* transcript levels exhibited a clear time-course dependent upregulation pattern and reached the highest levels 24 h post injection. More interestingly, after injection with LHRH-A_3, the expression of *Hepcidin-2* was significantly up-regulated in both testes and seminal vesicle. Results from *in situ* hybridization showed that *Hepcidin*-2 was expressed in the Leydig cells of the testes and in the epithelium of the seminal vesicle. Taken together, the results from our study indicated that these two *hepcidin* isoforms in the mudskipper may have different functions: Hepcidin-1 may play a dual role in both iron metabolism regulation in the liver and a short antimicrobial response in male reproductive tracts, while Hepcidin-2 is more specialized in reproductive immunity in male reproductive tracts.

1. Introduction

The male and female reproductive tracts are in contact with the external environment and are therefore potential ports of entry for microorganisms/pathogens that may be deleterious. The ability of the gonads or other components in the reproductive tracts to react to a microbial attack has been explored mainly in mammalian models [1] and insect species [2], and only a few studies have investigated the reproductive immunity in aquatic organisms [3,4].

The mudskipper *Boleophthalmus pectinirostris* (Linnaeus 1758) is

* Corresponding author. Address. College of Ocean and Earth Sciences, Xiamen University, Xiamen, Fujian Province, 361102, China.
E-mail address: chenshixi@xmu.edu.cn (S.X. Chen).

http://dx.doi.org/10.1016/j.fsi.2016.07.025
1050-4648/

distributed throughout the intertidal regions of China, Korea and Japan [5,6]. The spawning season of this species is from May to September with a peak spawning period in May in the coastal water of Fujian Province, China. Our previous studies indicate that, as a burrow-dwelling fish inhabiting intertidal mudflats, the mudskipper is an exceptional model among fishes for its behavior and numerous physiological and morphological specializations adapted for an amphibious life [7–13]. During the spawning season, the vertical tunnel at the center of the burrow is dilated to form a spawning chamber, in which a pair of male and female fish mate and lay eggs on the inner walls and ceiling with filamentous attachments. During 5 days of incubation, the fertilized eggs are kept clean and hatch with a very high hatching rate under the natural conditions filled with microorganisms [12]. This observation indicated that male and/or female reproductive tracts may synthesize antimicrobial substances, which may be released during spawning in order to offer protection against microorganisms that may be deleterious to fertility.

Hepcidin, a cysteine-rich cationic antimicrobial peptide (HAMP), was first found in human plasma ultrafiltrate and urine [14,15]. Then it was identified in mammals, birds, frogs and fish [16]. In mammals, the study of hepcidin focused on its function in iron regulation [17], while in fish much research shows that it has dual functions: iron regulation and antimicrobial activity [18]. *Hepcidin* has been detected mostly in the liver but also in a variety of tissues such as spleen, intestine, head kidney, muscle, brain, gill, skin and stomach. To our surprise, in our primary study, two expressed genes closely related to *Hepcidin* were identified in transcriptome data (data not shown) of the *B. pectinirostris* seminal vesicle, a paired structure situated posterior to the testes along the whole length of the nontesticular part of the sperm ducts [19]. So far, little is known about the potential functions of hepcidin in the reproductive immunity of teleosts. Therefore, the present study was aimed to understand the biological roles of the two hepcidin genes, particularly with regard to their antimicrobial function in the male reproductive organ in *B. pectinirostris*.

2. Materials and methods

2.1. Experimental fish and sampling

All the fish used in this study were purchased from a seafood market in Xiamen, Fujian, China. In the market, the fish were maintained in plastic boxes with 1.5 cm deep seawater at a salinity about 15 ppt and with aeration. In order to maintain the best activity of the mudskipper, we purchased fish that were caught from the intertidal mudflat one day before. Before sampling, the fish were anesthetized with 0.01% MS222 (Sigma-Aldrich, St. Louis, MO, US), and body length and body weight (BW) were measured (110–150 mm and 19.5–50.0 g). All experiment protocols were reviewed and approved by the Institute of Animal Care and Use Committee of Xiamen University.

2.2. Cloning Hepcidin-1 and Hepcidin-2

B. pectinirostris seminal vesicle was used for cloning the *Hepcidin-1* and *Hepcidin-2* cDNA. Total RNA was extracted using the RNAzol reagent (MRC, Cincinnati, OH, USA) and reverse transcribed into first strand cDNA using the ReverAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's instructions. The primers were designed based on the sequences from the transcriptome of the *B. pectinirostris* seminal vesicle. The PCR amplification was carried out in a 20 μL volume using recombinant Taq™ DNA polymerase (TaKaRa, Japan). The PCR reaction was performed under the following cycling conditions: 1 cycle of denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30s, 50 °C for 30s, and 72 °C for 45s, followed by a 10 min extension at 72 °C. The PCR products with expected size were extracted from agarose gel following electrophoresis, sub-cloned into vector pTZ57 R/T (InsTAclone™ PCR Cloning Kit, Fermentas, Canada), and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI, USA). Several positive clones were selected and sequenced by Invitrogen Ltd. (Guangzhou, China).

2.3. Sequence and phylogenetic analyses

After obtaining the cDNAs for the *B. pectinirostris Hepcidin-1* and *Hepcidin-2*, the deduced amino acid sequences were obtained using the ExPASy Translate Tool (http://www.expasy.ch/tools/dna.html). The cleavage sites for the signal peptides were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP) [20]. The charges for the deduced mature peptides of *Hepcidin-1* and *Hepcidin-2* were calculated using the ProtParam tool (http://cn.expasy.org/tools/protparam.html) [21]. A homology search was performed using the BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The alignment and the neighbor-joining (NJ) phylogenetic tree of the *Hepcidin-1* and *Hepcidin-2* that had been published were performed using the Megalign program of the Lasergene software package (DNASTAR Inc., Madison, WI, USA) and the Clustal V method. GenBank accession numbers for alignment of amino acids and phylogenetic trees are as follows: Hepcidin *Scophthalmus maximus* (AAX92670.1); Hepcidin *Oryzias melastigma* (AEG78327.1); Hepcidin 1 *Paralichthys olivaceus* (BAE06232.1); Hepcidin 2 *Paralichthys olivaceus* (BAE06233.1); Hepcidin 1 *Micropterus salmoides* (ACD13023.1); Hepcidin 1 *Micropterus dolomieu* (ACD13025.1); Hepcidin 1 *Pagrus auriga* (BAH03285.1); Hepcidin 1 *Salmo salar* (NP_001134321.1); Hepcidin *Lateolabrax japonicus* (AAT09138.1); Hepcidin *Channa maculata* (AFN73128.1); Hepcidin 1 *Miichthys miiuy* (AEK98541.1); Hepcidin 2 *Miichthys miiuy* (AEK98542.1); Hepcidin *Cynoglossus semilaevis* (AFK93414.1); Hepcidin 1 *Solea senegalensis* (BAG69595.1); Hepcidin 5 *Acanthopagrus schlegelii* (AAU00798.1); Hepcidin 1 *Sebastes schlegelii* (ACD80120.1); Hepcidin 4 *Acanthopagrus schlegelii* (AAU00797.1); Hepcidin 1 *Acanthopagrus schlegelii* (AAU00801.1); Hepcidin 3 *Acanthopagrus schlegelii* (AAU00796.2) Hepcidin 3 *Pagrus auriga* (BAH03287.1); Hepcidin 6 *Acanthopagrus schlegelii* (AAU00799.1); Hepcidin *Gadus morhua* (ACA42770.1); Hepcidin *Pagrus major* (AAR28077.1); Hepcidin 2 *Pagrus auriga* (BAH03290.1); Hepcidin 4 *Pagrus auriga* (BAH03292.1); Hepcidin 2 *Micropterus dolomieu* (ACD13026.1); Hepcidin 2 *Micropterus salmoides* (ACD13024.1); Hepcidin 2 *Acanthopagrus schlegelii* (AAU00795.1); Hepcidin *Sparus aurata* (CAO78619.1); Hepcidin 2 *Scophthalmus maximus* (AFE88614.1); Hepcidin *Siniperca chuatsi* (AKA66314.1); Hepcidin 7 *Acanthopagrus schlegelii* (AAU00800.1); Hepcidin *Schizothorax richardsonii* (AHB79194.1); Hepcidin *Ictalurus punctatus* (ABA43709.1); Hepcidin *Danio rerio* (AAI63916.1); Hepcidin *Morone chrysops* (AAM28440.1); Hepcidin *Oncorhynchus mykiss* (AAG30029.1); Hepcidin 1 *Xenopus tropicalis* (ABL75283.1); Hepcidin 2 *Xenopus tropicalis* (ABL75284.1); Hepcidin *Homo sapiens* (NP_066998.1); Hepcidin 1 *Mus musculus* (NP_115930.1); Hepcidin2 *Mus musculus* (NP_899080.1); Hepcidin *Rattus norvegicus* (NP_445921.1); Hepcidin 1 *Boleophthalmus pectinirostris* (KU665296.1) Hepcidin 2 *Boleophthalmus pectinirostris* (KU665297.1).

2.4. Real-time quantitative PCR (qPCR)

The qPCR was performed following MIQE guidelines [22]. Specific primers for detecting target genes (Table 1) were designed and examined for their specificity and amplification efficiency on serial

dilutions of respective target gene plasmid DNA (1×10^3–1×10^8 copies/μL). The melting curve analysis displayed a single peak, and the PCR efficiency was 99.5% for *Hepcidin-1*, 98.6% for *Hepcidin-2* and 99.6% for *β-actin* (data not shown). All qPCRs were conducted on an ABI 7500 fast real-time PCR System with a 20 μL reaction system using the SYBR® Select Master Mix (Applied Biosystems, USA) which was a mixture containing SYBR® GreenER™ Dye and ROX™ dye as a negative reference. The thermal cycling condition was: 1 cycle of 95 °C for 2 min, and 40 cycles of 95 °C for 30s, 60 °C for 30s and 72 °C for 30s. No-template and no-reverse transcription reactions were used as the negative control. The quantification cycles (Cq) values were analyzed in an ABI 7500 fast Real-time PCR System version 2.0.5 (Applied Biosystems, USA). The relative abundance of mRNA for target genes was determined using the comparative Cq method [23] with the *β-actin* gene as the internal control, and there was no significant difference among the groups under different experimental conditions. For absolute quantification, a standard curve was generated using the plasmid ligated *Hepcidin-1* and *Hepdcidin-2* gene sequences (1×10^3-1×10^8 copies/μL).

2.5. Tissue distribution of Hepcidin-1 and Hepcidin-2

To examine the tissue distribution of *Hepcidin-1* and *Hepcidin-2* mRNAs, liver, heart, intestine, kidney, spleen, gill, ovary, testes, and seminal vesicle samples were collected in May (peak spawning season) [7,10,12]. All the samples collected were immediately dipped into liquid nitrogen and stored at −80 °C. Total RNA extraction and cDNA synthesis of these organs were conducted as described in Section 2.2. qPCR was performed as described in Section 2.4.

2.6. Monthly expression of Hepcidin-1 and Hepcidin-2 in the testes and seminal vesicle

Before the spawning period (from January to April) and during the peak spawning period (May and June) [7,10,12], six male fish were purchased monthly from the same seafood market mentioned in Section 2.1, and anesthetized and humanely decapitated after being transported to the laboratory without further acclimation to laboratory conditions. The testes and seminal vesicle were collected, weighed and immediately dipped into liquid nitrogen and stored at −80 °C. The gonado-somatic index (GSI) was calculated as GSI (%) = [gonad weight (g)/total body weight (g)] × 100%. Total RNA extraction and cDNA synthesis of these organs were conducted as described in Section 2.2. qPCR was performed as described in Section 2.4.

2.7. In vivo stimulation by iron overload, lipopolysaccharide (LPS) and luteinizing hormone releasing hormone A_3 (LHRH-A_3)

As a burrow-dwelling fish inhabiting estuarine intertidal mudflats with low salinity seawater, the mudskipper hides in the mud burrow during flood tide, and leaves the burrow and slides on the mud surface to feed on benthic diatoms during ebb tide. In order to mimic the environmental conditions, we kept the fish in plastic boxes (length × width × height, 42 × 31 × 15 cm) at a density of six fish/box with aerated seawater (salinity 15 ppt) and at 28–28.5 °C. In addition, in order to keep the dorsal body and eyes exposed to air, the depth of the seawater was 1.5 cm. The plastic box was covered with shelter in order to avoid disturbance from people during the two day acclimation process. For the iron overload experiment, male fish were randomly divided into two groups (six individuals/group) and each fish was intraperitoneally injected with dextran-iron (Sigma, USA) dissolved in sterile 200 μL PBS at the dose of 0.2 μg per fish or with 200 μL sterile PBS in the control. At 24 h post injection (hpi), liver, testes and seminal vesicle were surgically sampled, frozen immediately in liquid nitrogen and stored at −80 °C. For the LPS challenge experiment, male fish were randomly divided into seven groups (six individuals/group) and each fish was intraperitoneally injected with LPS (Sigma, *E. coli* 0127:B8) dissolved in sterile 200 μL PBS at the dose of 0.5 μg/g BW in the treatment groups or with 200 μL sterile PBS in the control. At 0, 6, 12, 24 hpi, testes and seminal vesicle were surgically sampled, frozen immediately in liquid nitrogen and stored at −80 °C. For the LHRH-A_3 stimulation experiment, male fish were randomly divided into two groups (six individuals/group) and each fish was intraperitoneally injected with LHRH-A_3 (Ningbo Shansheng Pharmaceutical Co., China) dissolved in sterile 200 μL PBS at the dose of 1 μg per fish or with 200 μL sterile PBS in the control. At 24 hpi, testes and seminal vesicle were surgically sampled, frozen immediately in liquid nitrogen and stored at −80 °C. Total RNA extraction and cDNA synthesis of these organs were conducted as described in Section 2.2. qPCR was performed as described in Section 2.4.

2.8. Cellular localization of Hepcidin-2 expression in the testes and seminal vesicle

The localization of *Hepcidin-2* mRNA expression in the testes and seminal vesicle of *B. pectinirostris* was investigated using *in situ* hybridization (ISH) as described previously [24]. In brief, *B.*

Table 1
Primers used for cloning and gene expression analyses.

Gene name	Primer name	Primer sequence (5′–3′)
Cloning		
Hepcidin-1	Forward	CACTCTGAGCCCAACTCACA
	Reverse	GCAAGTTCTGACCATGTGCAC
Hepcidin-2	Forward	AAAGGTCTTTGTTAGGTGATGGA
	Reverse	GGACTTGTGGATTTGGTTGG
Real-time PCR assay		
Hepcidin-1	Forward	ACTCGTGCTGGCCTTTGTTTGC
	Reverse	GGACAGGTGGCTCTGGCGTTT
Hepcidin-2	Forward	ACTGGAATGTCCACGTCCTC
	Reverse	CCAACCAAATCCACAAGTCC
β-actin	Forward	GGGAGTGATGGTTGGTATGG
	Reverse	CACAATACCGTGCTCAATGG
In situ hybridizationprobe		
Hepcidin-2	Forward	GGGCGGGTGTTATTAACCCTCACTAAAGGGATGAAGACTGTGAGAGTGGC
	Reverse	CCGGGGGGTGTAATACGACTCACTATAGGGCAGAGTCCACAGACTCCAGG

Primers used for generating the probe of *in situ* hybridization contain the T3 or T7 polymerase promoter sequence (underlined) at their 5′-end.

pectinirostris Hepcidin-2 specific PCR products were generated with ISH primers (Table 1), which contained the T3 and T7 RNA polymerase promoter sequence attached at their 5′ ends. The PCR product obtained was gel purified and served as a template for digoxigenin-labeled cRNA probe synthesis (Roche, Swiss). Freshly collected testes and seminal vesicle samples were fixed in 4%w/v paraformaldehyde in PBS pH 7.4 overnight at 4 °C, followed by immersion in 25% w/v sucrose in PBS at 4 °C until they sank, and they were then embedded in embedding medium (optimal cutting temperature, Tissue-Tek™, Sakura, USA) by freezing in liquid nitrogen. ISH was performed on 10-μm-thick cryosections, and probe was added in a final concentration of 900 ng/mL.

2.9. Statistical analysis

All data were presented as the mean ± the standard error of the mean (SEM). The values were subjected to analysis using one-way ANOVA followed by Tukey's post hoc test to assess statistically significant differences among the individual groups. Student's t-test was also conducted to determine any significant differences between two groups. The analyses were performed using the GraphPad Prism 5 software package (GraphPad Software, San Diego, CA).

3. Results

3.1. Molecular characterization and sequence analyses of Hepcidin-1 and Hepcidin-2

Using the specific primers designed based on the transcriptome data of the *B. pectinirostris* seminal vesicle we obtained partial coding sequences of *Hepcidin-1* and *Hepcidin-2*. The sequence of *Hepcidin-1* (GenBank accession number KU665296.1) contained an open reading frame of 267 bp, encoding a polypeptide of 88 amino acids (aa). A potential cleavage site for the signal peptide was predicted between Val22 and Leu23 and a motif for the propeptide cleavage was identified between Arg62 and Gln63 (Fig. 1). Thus Hepcidin-1 prepropeptide consisted of a 22-aa signal peptide, a 40-aa prodomain and a 26-aa mature peptide. The mature peptide had a predicted molecular weight (m.w.) of 2925 Da and an isoelectric point of 8.67. The *Hepcidin-2* gene sequence (GenBank accession number KU665297.1) was similar to *Hepcidin-1*. It contained an open reading frame of 267 bp, encoding a polypeptide of 88-aa. A potential cleavage site for the signal peptide was predicted between Val24 and Gln25 and a motif for the propeptide cleavage was identified between Arg66 and Glu67. Thus, Hepcidin-2 prepropeptide consisted of a 24-aa signal peptide, a 42-aa prodomain and a 22-aa mature peptide. The mature peptide had a predicted m.w. of 2301 Da and an isoelectric point of 8.53. The alignment of *B. pectinirostris* Hepcidin-1 and Hepcidin-2 with selected vertebrate preprohepcidins showed that in the C-terminus region of these hepcidins, there were eight cysteine residues which were conserved in vertebrates (Fig. 1). Furthermore, in comparison with Hepcidin-2, Hepcidin-1 showed a hypothetical iron regulatory sequence Q-S/I-H-L/I-S/A in the N-terminus region of the mature peptide. The deduced amino acid sequence of *B. pectinirostris* Hepcidin-1 and Hepcidin-2 showed high identity with *Micropterus salmoides* hepcidin 1 (72.7%) and *Lateolabrax japonicus* hepcidin (53.5%), respectively. During the preparation of this manuscript, the complete mRNA and genomic gene sequences for both hepcidin-1 and hepcidin-2 isoforms from the same species *B. pectinirostris* have been available with the accession numbers KM924542, KM924543, KM977566 and KM977567. Their nucleotide and amino acid sequences of open reading frame are identical with those presented in this manuscript.

3.2. Phylogenetic analysis

The phylogenetic tree was constructed using the NJ method on the basis of deduced amino acid sequences of hepcidin prepropeptide of vertebrate hepcidins (Fig. 2). The NJ tree showed that hepcidin-1 and hepcidin-2 of *B. pectinirostris* and other fish hepcidins were grouped together to form a teleost clade that was a sister clade of non-teleost (mammal and amphibian) clusters. In the teleosts clade, there were two subclades named as HAMP 1 and HAMP 2. The hepcidin-1 and hepcidin-2 of *B. pectinirostris* were clustered into HAMP 1 and HAMP 2, respectively. In the HAMP 1 subclade, teleost species belonged to acanthopterygii, atherinomorphae, ostariophysi and protacanthopterygii. However, only teleost species belonging to acanthopterygii presented in HAMP 2 subclade. This tree indicated that several isoforms of hepcidin are common in the acanthopterygii fish. The hypothetical iron regulatory sequence Q-S/I-H-L/I-S/A was found in all mature peptiedes of HAMP 1 group, inculding *B. pectinirostris* hepcidin-1, but not found in any of mature peptides belonging to HAMP 2 group, inculding *B. pectinirostris* hepcidin-2 (Fig. 2).

3.3. Tissue distribution of Hepcidin-1 and Hepcidin-2 mRNA

The expression of *Hepcidin-1* and *Hepcidin-2* genes in different tissues of adult *B. pectinirostris* was analyzed using absolute quantification qPCR. The expression level of *Hepcidin-1* in the liver was significantly higher than those in the spleen, kidney, seminal vesicle and testes (Fig. 3). In contrast to *Hepcidin-1*, *Hepcidin-2* was detectable in a wide array of tissues, but significantly higher expression levels of *Hepcidin-2* were found in the liver, spleen and testes than those in other tissues.

3.4. Monthly expression patterns of Hepcidin-1 and Hepcidin-2 in the testes and seminal vesicle

The GSI values of male fish began to increase in April and reached the significantly highest level in May, maintaining a similar level in June (Fig. 4 A). The expression of *Hepcidin-1* and *Hepcidin-2* mRNA in the testes of *B. pectinirostris* (Fig. 4 B) and the seminal vesicle (Fig. 4 C) from January to June was analyzed using relative abundance qPCR. The *Hepcidin-1* transcription levels were not significantly different between each month in the testes from January to June, while in the seminal vesicle *Hepcidin-1* transcription levels showed a peak in March, which was significantly higher than in any other month. In both the testes and seminal vesicle, the highest expression levels of *Hepcidin-2* were observed during the peak spawning period (May), and they were significantly higher than those in any other month.

3.5. Expression of Hepcidin-1 and Hepcidin-2 genes in response to iron overload, LPS challenge and LHRH-A_3 stimulation

Considering that the function of hepcidin as a key regulator of iron homeostasis is conserved in vertebrates, we first examined the expressions of both *Hepcidin-1* and *Hepcidin-2* in response to iron overload in the liver, testes and seminal vesicle. At 24 hpi, the expression of *Hepcidin-1* was significantly up-regulated in the liver, but the expression of *Hepcidin-2* was not different from that of the control in the liver (Fig. 5 A); while the expressions of *Hepcidin-1* and *Hepcidin-2* were not different from that of the control in the testes (Fig. 5 B) and seminal vesicle (Fig. 5 C). Because our study aimed to investigate the potential immune function of hepcidin in male reproductive tracts, we further examined the expressions of both *Hepcidin-1* and *Hepcidin-2* in response to LPS challenge in the testes and seminal vesicle at 0, 6, 12, and 24 hpi. In both testes and

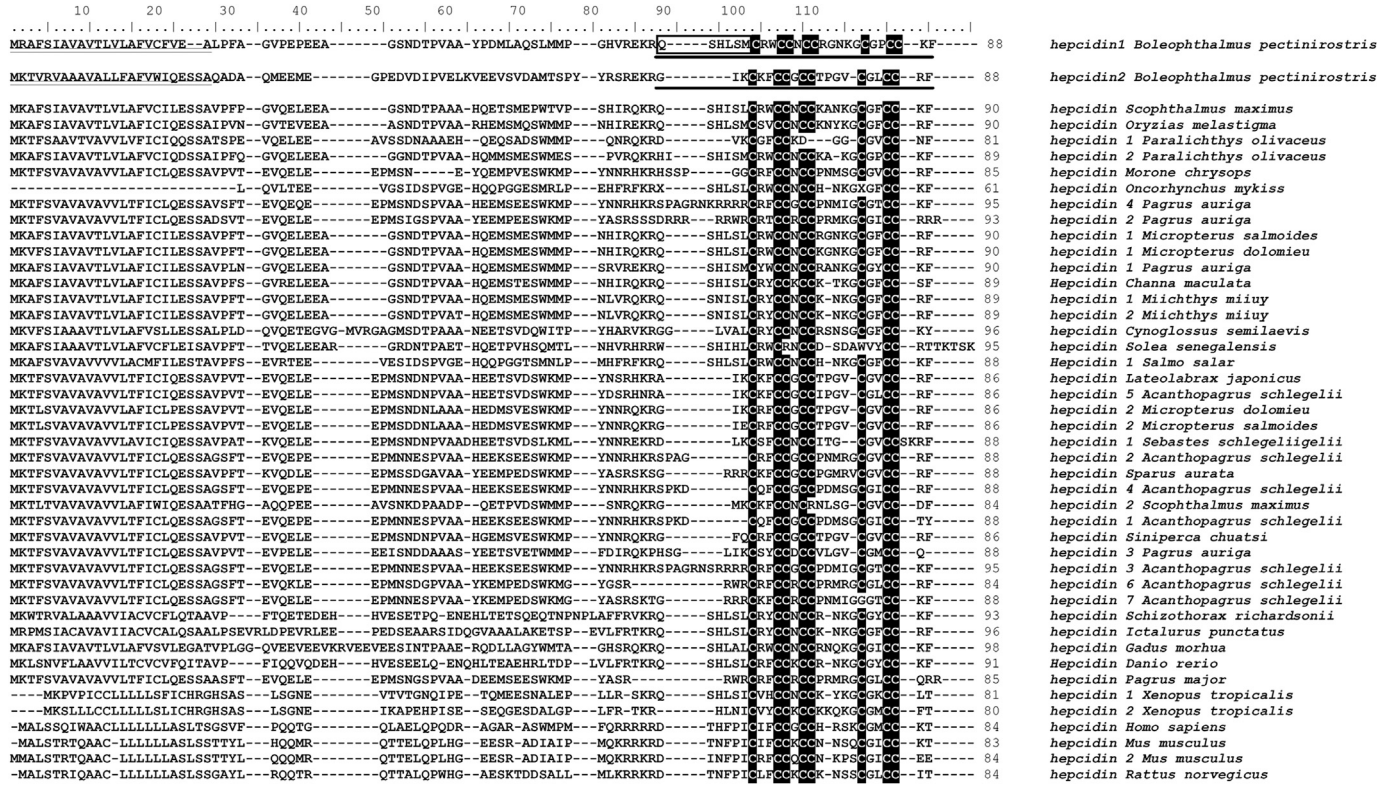

Fig. 1. Deduced amino acid sequence of *B. pectinirostris* hepcidins and their amino acid alignment with hepcidin peptides from other vertebrates. The sequence segments with double underline were signal peptide, and the sequences with heavy underline were mature peptide. Eight cysteine residues of the mature peptide were in black shadow. Black windows indicated the Q-S/I-H-L/I-S/A motif of the mature peptides.

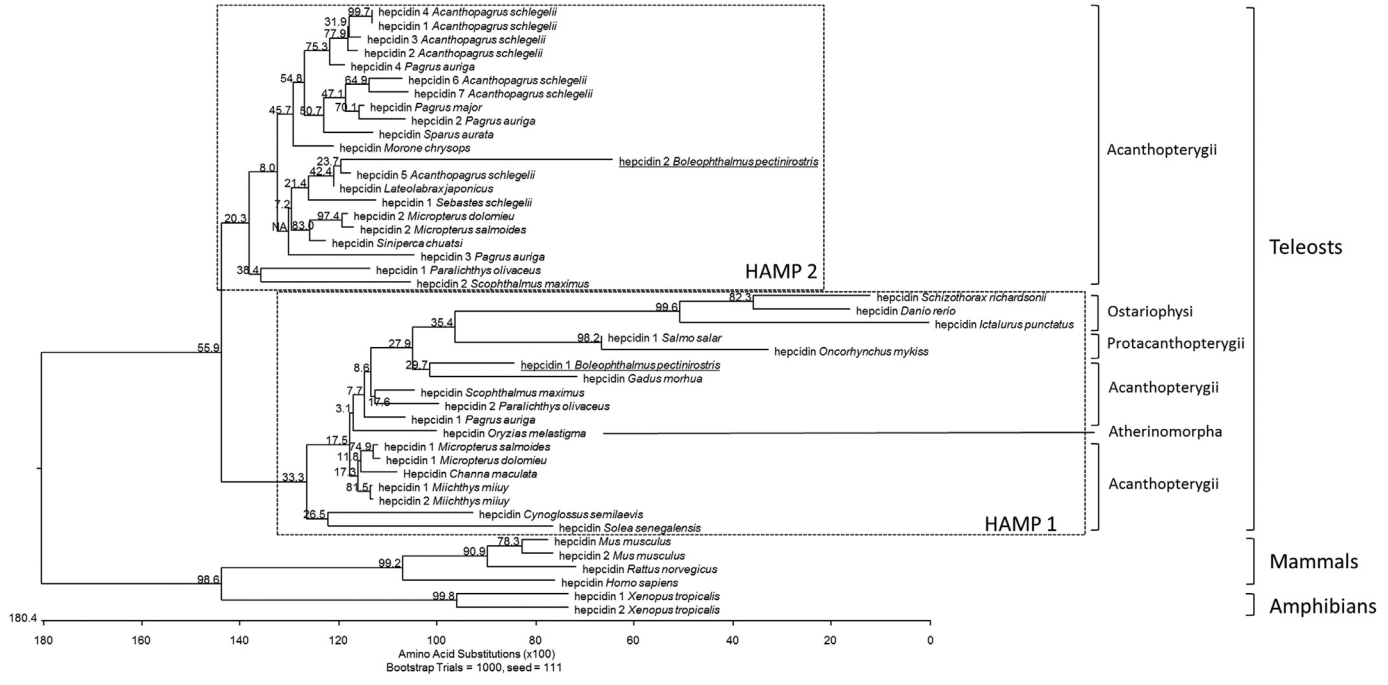

Fig. 2. Phylogenetic tree of hepcidin amino acid sequences. The tree was generated using the method of Neighbor-Joining. Numbers above the nodes represented bootstrap percentages of 1000 replicates. The GenBank accession numbers or references for these hepcidin sequences are shown in Section 2.3. HAMP 1 is short for hepcidin-1, and HAMP 2 for hepcidin-2.

seminal vesicle, a significant upregulation of *Hepcidin-1* transcripts levels was only observed at 6 hpi (Fig. 6 A, B); In contrast, the *Hepcidin-2* expression levels exhibited a clear time-course dependent upregulation pattern (Fig. 6 C, D). Considering the monthly

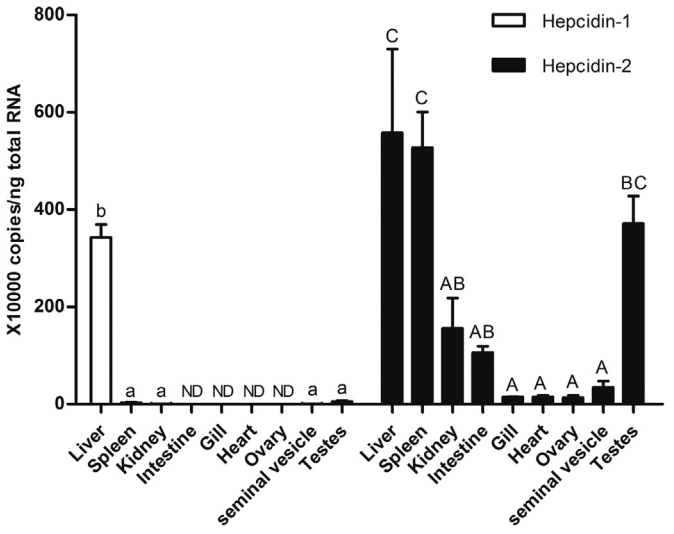

Fig. 3. Amounts of *Hepcidin-1* and *Hepcidin-2* mRNA in different tissues (liver, spleen, kidney, intestine, gill, heart, ovary, seminal vesicle and testes) of *B. pectinirostris* in May. Data are expressed as the mean ± SEM. ND, not detectable. Bars marked with different letters are significantly different from each other ($p < 0.05$).

expression pattern of *Hepcidin-2* was correlated with male reproductive status, we further examined the effect of LHRH-A_3 on the expression of *Hepcidin-2* transcripts. The results showed that the expression of *Hepcidin-2* transcripts showed significantly up-regulation in both testes and seminal vesicle after injection with LHRH-A_3 for 24 h (Fig. 7).

3.6. Cellular localization of Hepcidin-2 mRNA expression in the testes and seminal vesicle

Because results from tissue distribution, monthly expression, responses to LPS challenges and LHRH-A3 stimulation suggested that Hepcidin-2 may play a specialized role in male reproductive immunity, we further identified the cell types expressing the *B. pectinirostris Hepcidin-2* mRNA in the testes and seminal vesicle using ISH. In the testes strong ISH signals of *Hepcidin-2* were detected in the Leydig cells (Fig. 8 A, B); while in the seminal vesicle (Fig. 8 C, D) strong *Hepcidin-2* signals were detected in the epithelial cells of the cysts. No ISH signal was observed when sections were hybridized with the cRNA *Hepcidin-2* probe (data not shown).

4. Discussion and conclusions

Many researchers found that most fishes, especially in the Perciformes and Pleuronectiformes, have more than two hepcidin isoforms rather than in mammals, which only have a single hepcidin gene, except for the mouse [26]. Previous studies in *Dicentrarchus labrax*, *Acanthopagrus schlegelii* and other fishes show that different hepcidin isoforms have different functions [27,28]. In the present study, we cloned two *hepcidin* isoforms from *B. pectinirostris*. The alignment of the putative hepcidin protein sequences from *B. pectinirostris* showed that there were eight cysteine residues in the C-terminus region of these two hepcidins. These cysteine residues are conserved in most vertebrates [29,30], and the four disulfide bonds they form in the mature peptide are essential for the peptide to conform the characteristic antiparallel β-sheet which is important for their antimicrobial activity [29,31]. Phylogenetic analysis shows that these two types of hepcidin isoforms are divided into two branches: HAMP 1 and HAMP 2 [25,32]. The hypothetical iron regulatory sequence Q-S/I-H-L/I-S/A was missing in the N-terminus of all the peptides of the HAMP 2 cluster. Taken together, the results from phylogenetic and amino acid sequence analyses suggested that these two hepcidin isoforms in *B. pectinirostris* may have different functions.

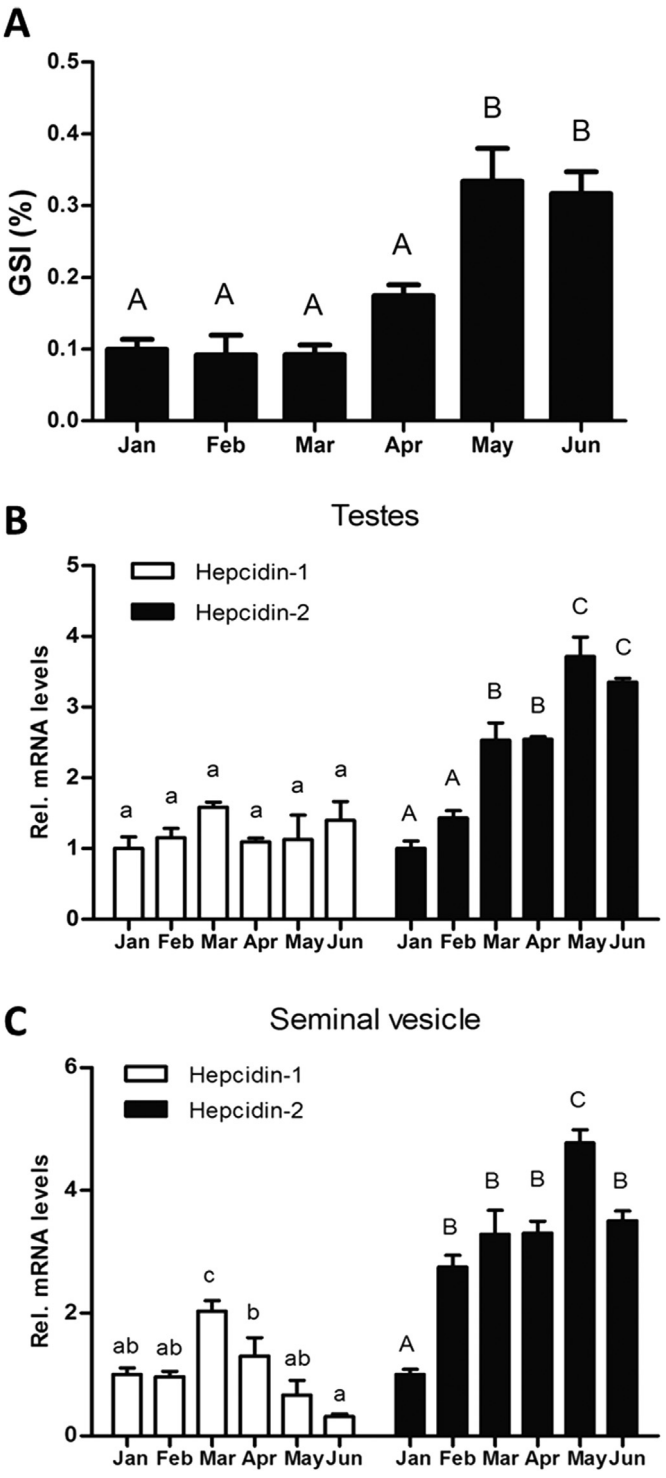

Fig. 4. The patterns of GSI value (A), relative abundance in the *Hepcidin-1* and *Hepcidin-2* mRNA in the testes (B) and the seminal vesicle (C) of male *B. pectinirostris* collected from January to June. Data are expressed as the mean ± SEM. Bars marked with different letters are significantly different from each other ($p < 0.05$).

As in many fish species and other vertebrates, in our research we found that *Hepcidin-1* was predominately expressed in the liver.

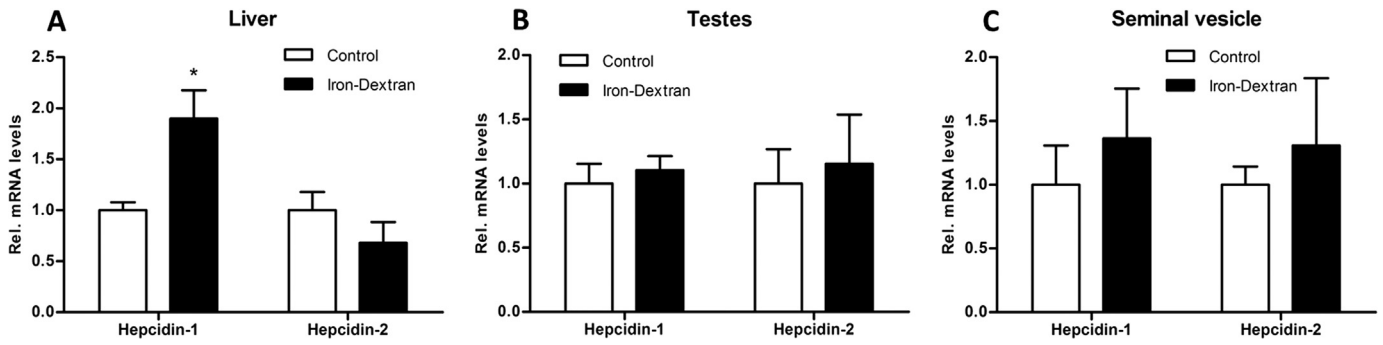

Fig. 5. The relative abundance of the *Hepcidin-1* and *Hepcidin-2* mRNA of *B. pectinirostris* in liver (A), testes (B) and seminal vesicle (C) after injection with iron-dextran. Data are expressed as the mean ± SEM. Bars marked with "*" are significantly different from each other ($p < 0.05$).

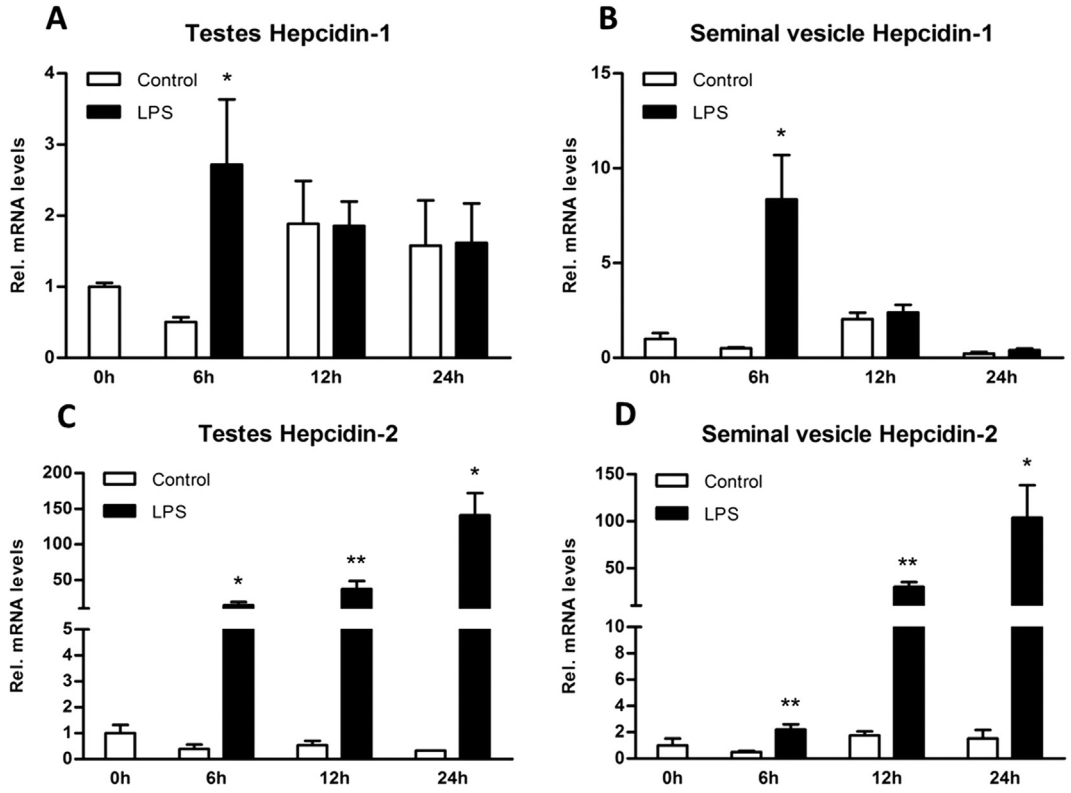

Fig. 6. The relative abundance of the *Hepcidin-1* and *Hepcidin-2* mRNA of *B. pectinirostris* in testes (A, C) and seminal vesicle (B, D) after injection with LPS. Data are expressed as the mean ± SEM. Bars marked with "*" ($p < 0.05$) "**" ($p < 0.01$) are significantly different from their respective controls.

Therefore, it was not surprising to find that, after the iron dextran injection, the expression of *Hepcidin-1* was significantly up-regulated in the liver, which is the major organ in the control of systemic iron metabolism. Besides its function as a key regulator of iron homeostasis, the role of hepcidin as an antimicrobial peptide (AMP) has been explored mainly in fish. Up-regulation of *hepcidin* is observed in many teleost species during injection with bacterial pathogen or LPS [30]. One interesting observation in our study was that *Hepcidin-1* was expressed in the testes and seminal vesicle and, therefore, we further examined the expression levels of *Hepcidin-1* transcripts in the male reproductive system after LPS injection. It was very interesting to find that after injection with LPS for 6 h, the expression of *Hepcidin-1* was significantly up-regulated in the testes and seminal vesicle, but the expression pattern of *Hepcidin-1* in the testes did not exhibit significant variation before and during peak spawning periods. However, in the seminal vesicle a significant high expression level of *Hepcidin-1* was observed in March. The reason for this phenomenon is still unclear and more work is needed. Taken together, our results indicated that Hepcidin-1 may play a dual role in both iron metabolism regulation in the liver and a short antimicrobial response in the seminal vesicle.

In contrast with *Hepcidin-1*, *Hepcidin-2* has a broad expression pattern. Although *Hepcidin-2* was also highly expressed in the liver, its expression levels in this tissue showed no significant changes in response to iron overload, which may suggest that iron homeostasis is not the major function of Hepcidin-2. It was very interesting to find that *Hepcidin-2* was highly expressed in both the testes and the seminal vesicle. So far, only a few studies have reported that *hepcidin* is expressed in gonads [33,34]. However, little is known about the function of hepcidin in the gonad. In a mammalian model, the testicular innate antiviral and antimicrobial

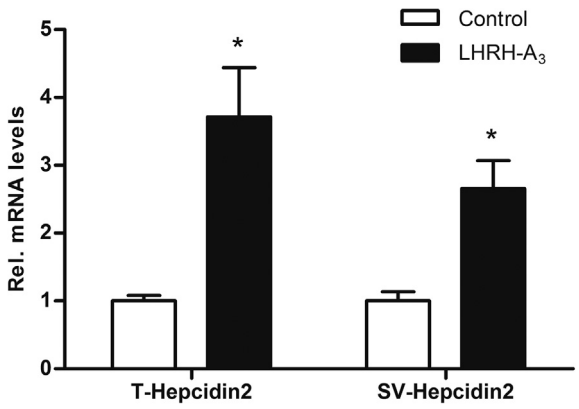

Fig. 7. The relative abundance of the *Hepcidin-2* mRNA of *B. pectinirostris* in the testes and the seminal vesicle after injection with LHRH-A_3. T is short for testes and SV is short for seminal vesicle. Data are expressed as the mean ± SEM. Bars marked with "*" are significantly different from their respective controls ($p < 0.05$).

defenses are demonstrated based on a series of studies on the expression and regulation of interferons, antiviral proteins and defensins [35]. Therefore, in the *B. pectinirostris* testis, Hepcidin-2 may have a local innate immune function to resist the invasion of pathogens.

In order to better understand the potential function of Hepcidin-2 in the male reproductive tract, we further examined the monthly expression pattern of *Hepcidin-2* before and during peak spawning periods. The results showed that, in both the testes and seminal vesicle, the highest expression of *Hepcidin-2* was observed in May, i.e. the peak spawning period. At this time, both the testes and seminal vesicle contain mainly spermatozoa [36]. Seminal vesicles, also described as testicular glands, testicular blind pouches, or sperm duct glands, have been reported in Gobiidae, Siluridae and Blennidae. The organ is reported to have various functions such as storage of spermatozoa, production of steroids and enhancement of sperm motility and fertilization efficiency [37], but its potential immune function remains unclear. Studies in insect models demonstrate that diverse AMPs synthesized in the male accessory glands [2] as well as in the ejaculatory duct [38] can be transferred into the female during mating and protect the oviduct and the gametes from microbial infection and other stressors. In humans, the amount of human β-defensin 1 (a small secretory AMP) in sperm from infertile men exhibiting either leukocytospermia or asthenozoospermia (both of which are associated with reduced motility and reduced bactericidal activity in sperm) is much lower compared to that in normal fertile sperm [39]. Taken together, it is possible that, during mating, the Hepcidin-2 secreted by seminal vesicles would be released with the semen, and this substance could protect both the sperm and eggs from the microorganisms in the mud burrow, facilitating fertilization and incubation of the eggs.

We further investigated the expression of *Hepcidin-2* in the testes and the seminal vesicle in response to LPS challenge and iron overloading. The results showed that the expression levels of *Hepcidin-2* in the testes and seminal vesicle increased dramatically in response to LPS but not to iron overload, which may suggest that this protein may act as an innate immune factor, and participate in

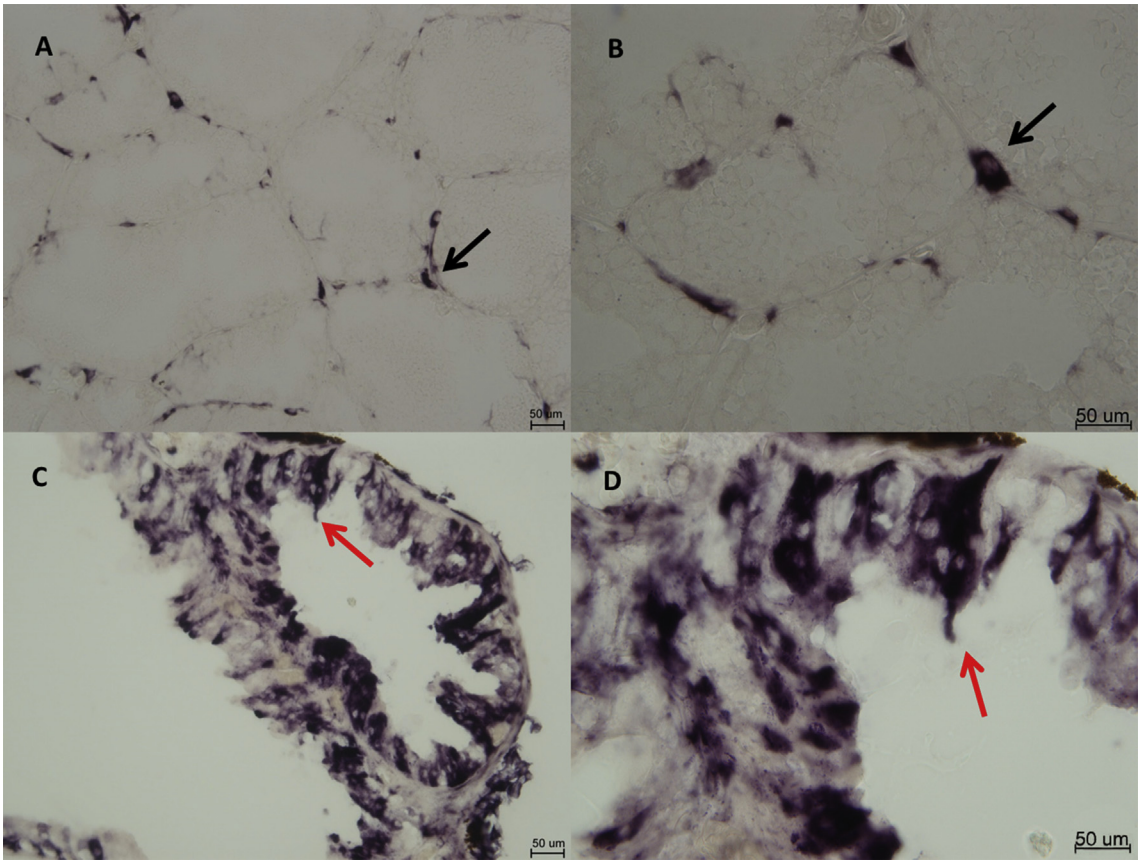

Fig. 8. Cellular localization of *Hepcidin-2* mRNA in the testes (A, B) and the seminal vesicle (C, D) using *in situ* hybridization. Black arrows indicate Leydig cells. Red arrows indicate epithelial cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

male reproductive immunology. A surprise finding from the present study is that LHRH-A_3 induced the up-regulation of *Hepcidin-2* in both the testes and seminal vesicle. LHRH-A_3 is a synthetic polypeptide, which stimulates mature animals to release the gonadotrophins (follicle stimulating hormone and luteinizing hormone), and is widely used in fish to induce maturation and breeding. The ISH results from our study indicated that *Hepcidin-2* was expressed in the Leydig cells in the testes and the epithelium of the seminal vesicle of *B. pectinirostris*. In some fish, both FSH and LH receptors are located in Leydig cells [38,40]. Therefore, it is possible that *Hepcidin-2* in the male reproductive tracts is partly regulated by gonadotropins, most likely the LH, which reaches the highest levels during peak spawning period in many fish species [41].

In conclusion, we cloned two Hepcidin genes from *B. pectinirostris*. The results from our study suggested that these two hepcidin genes may have had different functions: Hepcidin-1 played a dual role in both iron metabolism regulation in the liver and a short antimicrobial response in the gonad, while Hepcidin-2 was more specialized in the immune defense in *B. pectinirostris*. One novel finding from our study was that Hepcidin-2 in male reproductive tracts exhibited the highest expression levels during the peak spawning period, and was up-regulated by LPS and LHRH-A_3. This study, for the first time, indicated that hepcidin may participate in the male reproductive immunology of fish.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (No. 40976095), Xiamen University President Research Award (2013121044) and Xiamen Southern Oceanographic Center (No. 13GST001NF13 and No. 14GZY019NF19). Professor John Hodgkiss is thanked for editing the English.

References

[1] E. Com, F. Bourgeon, B. Evrard, T. Ganz, D. Colleu, B. Jegou, C. Pineau, Expression of antimicrobial defensins in the male reproductive tract of rats, mice, and humans, Biol. Reprod. 68 (2003) 95–104.
[2] D. Wei, C.B. Tian, S.H. Liu, T. Wang, G. Smagghe, F.X. Jia, W. Dou, J.J. Wang, Transcriptome analysis to identify genes for peptides and proteins involved in immunity and reproduction from male accessory glands and ejaculatory duct of *Bactrocera dorsalis*, Peptides (2015), http://dx.doi.org/10.1016/j.peptides.2015.08.007.
[3] K.J. Wang, W.S. Huang, M. Y, H.Y. Chen, J. Bo, S.J. Li, G.Z. Wang, A male-specific expression gene, encodes a novel anionic antimicrobial peptide, scygonadin, in *Scylla serrata*, Mol. Immunol. 44 (2007) 1961–1968.
[4] J.Y. Jin, L. Zhou, Y. Wang, Z. Li, J.G. Zhao, Q.Y. Zhang, J.F. Gui, Antibacterial and Antiviral Roles of a fish β-defensin expressed both in pituitary and testis, PLoS One 5 (2010) e12883.
[5] D.A. Clayton, Mudskippers. Oceanography and Marine Biology: an Annual Review, 1993.
[6] W. Chen, W.S. Hong, S.X. Chen, Q. Wang, Q.Y. Zhang, Population genetic structure and demographic history of the mudskipper *Boleophthalmus pectinirostris* on the northwestern Pacific coast, Environ. Biol. Fish. 98 (2015) 845–856.
[7] L.Y. Hong, W.S. Hong, W.B. Zhu, Q. Shi, X.X. You, S.X. Chen, Cloning and expression of melatonin receptors in the mudskipper *Boleophthalmus pectinirostris*: their role in synchronizing its semilunar spawning rhythm, Gen. Comp. Endocr. 195 (2014) 138–150.
[8] R.X. Wu, W.S. Hong, Q.Y. Zhang, S.X. Chen, Comparative enzyme activities of the intestinal brush border membranes of the herbivorous mudskipper *Boleophthalmus pectinirostris* and the carnivorous Chinese black sleeper *Bostrichthys sinensis*, J. Appl. Ichthyol. 25 (2009) 571–575.
[9] S.X. Chen, W.S. Hong, Y.Q. Su, Q.Y. Zhang, Microhabitat selection in the early juvenile mudskipper Boleophthalmus pectinirostris (L.), J. Fish. Biol. 72 (2008) 585–593.
[10] Q. Wang, W.S. Hong, S.X. Chen, Q.Y. Zhang, Variation with semilunar periodicity of plasma steroid hormone production in the mudskipper *Boleophthalmus pectinirostris*, Gen. Comp. Endocr. 155 (2008) 821–826.
[11] S.X. Chen, W.S. Hong, Q.Y. Zhang, Y.Q. Su, Why does the mudskipper *Boleophthalmus pectinirostris* form territories in farming ponds? J. Mar. Biol. Assoc. U. K. 87 (2007) 615–619.
[12] W.S. Hong, S.X. Chen, Q.Y. Zhang, W. Qiong, Reproductive ecology of the mudskipper *Bolephthalmus pectinirostris*, Acta Oceanol. Sin. 26 (2007) 72–81.
[13] S.X. Chen, W.S. Hong, Q.Y. Zhang, R.X. Wu, Q. Wang, Rates of oxygen consumption and tolerance of hypoxia and desiccation in Chinese black sleeper (*Bostrichthys sinensis*) and mudskipper (*Boleophthalmus pectinirostris*) embryos, Acta Oceanol. Sin. 25 (2006) 91–98.
[14] A. Krause, S. Neitz, LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity, Fed. Eur. Biochem. Soc. 480 (2000) 147–150.
[15] C.H. Park, E.V. Valore, A.J. Waring, T. Ganz, Hepcidin, a urinary antimicrobial peptide synthesized in the liver, J. Biol. Chem. 276 (2001) 7806–7810.
[16] W. Lin, S. Liu, L. Hu, S. Zhang, Characterization and bioactivity of hepcidin-2 in zebrafish: dependence of antibacterial activity upon disulfide bridges, Peptides 57 (2014) 36–42.
[17] T. Ganz, E. Nemeth, Hepcidin and iron homeostasis, BBA Mol Cell Res. (2012) 1434–1443.
[18] J.V. Neves, C. Caldas, I. Vieira, M.F. Ramos, P.N. Rodrigues, Multiple hepcidins in a teleost fish, *Dicentrarchus labrax*: different hepcidins for different roles, J. Immunol. 195 (2015) 2696–2709.
[19] W.S. Hong, S.X. Chen, Q.Y. Zhang, X.L. Ma, Z.M. Ni, Morphology and structure of the seminal vesicle in male Boleophtalmus pectinirostris, J. Fish. Sci. China 11 (2004) 396–403.
[20] H. Nielsen, J. Engelbrecht, S. Brunak, G. Von Heijne, Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites, Protein Eng. 10 (1997) 1–6.
[21] B. Bjellqvist, G.J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J.C. Sanchez, S. Frutiger, D. Hochstrasser, The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences, Electrophoresis 14 (1993) 1023–1031.
[22] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2009) 611–622.
[23] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative CT method, Nat. Protoc. 3 (2008) 1101–1108.
[24] S.X. Chen, J. Bogerd, A. García-López, H. de Jonge, P.P. de Waal, W.S. Hong, R.W. Schulz, Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor, Biol. Reprod. 82 (2010) 171–181.
[25] Y. Wang, X. Liu, L. Ma, Y. Yu, H. Yu, S. Mohammed, G. Chu, L. Mu, Q. Zhang, Identification and characterization of a hepcidin from half-smooth tongue sole *Cynoglossus semilaevis*, Fish. Shellfish Immun. 33 (2012) 213–219.
[26] D.Q. Lou, G. Nicolas, J.C. Lesbordes, L. Viatte, G. Grimber, M.F. Szajnert, A. Kahn, S. Vaulont, Functional differences between hepcidin 1 and 2 in transgenic mice, Blood 103 (2004) 2816–2821.
[27] P.N. Rodrigues, S. Vazquez-Dorado, J.V. Neves, J.M. Wilson, Dual function of fish hepcidin: response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*), Dev. Comp. Immunol. 30 (2006) 1156–1167.
[28] M. Yang, K.J. Wang, J.H. Chen, H.D. Qu, S.J. Li, Genomic organization and tissue-specific expression analysis of hepcidin-like genes from black porgy (*Acanthopagrus schlegelii* B), Fish. Shellfish Immun. 23 (2007) 1060–1071.
[29] C.A. Alvarez, F. Guzman, C. Cardenas, S.H. Marshall, L. Mercado, Antimicrobial activity of trout hepcidin, Fish. Shellfish Immun. 41 (2014) 93–101.
[30] L.C. Gong, H. Wang, L. Deng, Molecular characterization, phylogeny and expression of a hepcidin gene in the blotched snakehead *Channa maculata*, Dev. Comp. Immunol. 44 (2014) 1–11.
[31] J.P. Powers, R.E. Hancock, The relationship between peptide structure and antibacterial activity, Peptides 24 (2003) 1681–1691.
[32] K.B. Hilton, L.A. Lambert, Molecular evolution and characterization of hepcidin gene products in vertebrates, Gene 415 (2008) 40–48.
[33] Y.K. Nam, Y.S. Cho, S.Y. Lee, B.S. Kim, D.S. Kim, Molecular characterization of hepcidin gene from mud loach (*Misgurnus mizolepis*; Cypriniformes), Fish. Shellfish Immun. 31 (2011) 1251–1258.
[34] H. Li, F.M. Zhang, H.Y. Guo, Y.Y. Zhu, J.D. Yuan, G.W. Yang, L.G. An, Molecular characterization of hepcidin gene in common carp (*Cyprinus carpio* L.) and its expression pattern responding to bacterial challenge, Fish. Shellfish Immun. 35 (2013) 1030–1038.
[35] S. Zhao, W. Zhu, S. Xue, D. Han, Testicular defense systems: immune privilege and innate immunity, Cell Mol. Immun. 11 (2014) 428–437.
[36] J. Luo, F.J. Cao, C.W. Liu, Observation on the over development of *Boleophthalmus pectinirostris*, Acta Hydrobiol. Sin. 34 (2010) 418–425.
[37] F. Lahnsteiner, B. Berger, T. Weismann, R.A. Patzner, Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism, Fish Physiol Biochem. 15 (1996) 167–179.
[38] T. Ohta, H. Miyake, C. Miura, H. Kamei, K. Aida, T. Miura, Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese Eel, Anguilla japonica, Biol. Reproduction 77 (2007) 970–977.
[39] R. Diao, K.L. Fok, H. Chen, M.K. Yu, Y. Duan, C.M. Chuang, Z. Li, H. Wu, Z. Li, H. Zhang, Z. Ji, W. Zhen, C.F. Ng, Y. Gui, Z. Cai, H.C. Chan, Deficient human beta-defensin 1 underlies male infertility associated with poor sperm motility and genital tract infection, Sci. Transl. Med. 6 (2014) 249ra108.
[40] A. Garcia-Lopez, J. Bogerd, J.C.M. Granneman, W. van Dijk, J.M. Trant, G.L. Taranger, R.W. Schulz, Leydig cells express follicle-stimulating hormone receptors in african catfish, Endocrinology 150 (2009) 357–365.
[41] R.W. Schulz, L.R. de Franca, J.J. Lareyre, F. Legac, H. Chiarini-Garcia, R.H. Nobrega, T. Miura, Spermatogenesis in fish, Gen. Comp. Endocrinol. 165 (2010) 390–411.