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Identification and characterization of three novel antimicrobial peptides from *Acipenser dabryanus*

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A B S T R A C T

Antimicrobial peptides (AMPs) play essential roles in the innate immune system to protect against a wide variety of pathogens in aquatic environments. In this study, three very important AMPs, cathelicidin, hepcidin and defensin, were identified in the critically endangered *Acipenser dabryanus*. The full-length cDNA sequences of these three AMPs were identified from transcriptome sequencing and the rapid amplification of cDNA ends (RACE) technique. Phylogenetic analysis showed that cathelicidin formed a clade with the other members of the cathelicidin family, and similar results were obtained for hepcidin. The *A. dabryanus* β-defensin belonged to the fish class 2 β-defensins. A tissue distribution study showed that the three AMP transcripts could be detected constitutively in various tissues. The highest expression levels of cathelicidin and hepcidin were found in the liver, while defensin was primarily expressed in the skin. Bacterial challenge *in vivo* revealed significant changes in the gene expression of the three AMPs at both mucosal sites and systemic sites. Striking upregulation of cathelicidin and hepcidin was observed in the skin at 12 h post-challenge, with increases of more than 7000-fold and 1000-fold, respectively, compared to the control, and the expression of defensin mRNA was remarkably elevated in the hindgut (by 230-fold at 6 h post-challenge). Moreover, according to the expression profiles of the AMPs post-challenge, we found that the mucosal immune response occurred earlier than the systemic immune response following bacterial infection. Our results suggest that these three novel AMPs may play important roles in the innate immune system of *A. dabryanus* to protect against invading pathogens, especially during the mucosal immune response.

1. Introduction

Antimicrobial peptides (AMPs) are considered part of the innate immune system in almost all living organisms [1]. As a major component of the aquatic fauna, fish possess a strong innate immune system and secrete different types of AMPs to defend against frequent infection by a broad spectrum of pathogenic bacteria [2]. To date, more than 2800 AMPs have been recorded in the Antimicrobial Sequences Database (http://aps.unmc.edu/AP/main.php). Generally, AMPs are small in size, have a positive charge and adopt an amphipathic structure which allow them to interact with negatively charged phospholipid head groups and hydrophobic fatty acid chains in microbial membranes [3,4]. As a potential alternative to antibiotics, AMPs have been isolated and characterized in diverse fish species. Thus far, the AMPs identified in fish generally include cathelicidins, β-defensins, hepcidins, liver-expressed antimicrobial peptides-2 (LEAP-2), the piscidin and pleurocidin families, and histone-derived peptides [5].

Cathelicidin is one of the major classes of mammalian AMPs and is well characterized in the literature [6]. Cathelicidins are a group of bipartite molecules with a highly conserved N-terminal cathelin domain and a highly divergent C-terminal antimicrobial domain [7]. Cathelicidins are stored in neutrophils and macrophages and can be released extracellularly upon leukocyte activation, and their expression can also be detected in epithelial cells [8]. In mammals, cathelicidins are involved in a wide range of biological responses: direct killing of microorganisms, chemotaxis and chemokine induction, the regulation of inflammatory responses, angiogenesis and wound healing [9–11]. Cathelicidins are well understood in mammals, but fish cathelicidins have been studied at only a rudimentary level. In fish, cathelicidins possess features similar to those of mammalian cathelicidins, such as the cathelin region, which contains four cysteine residues that form two disulphide bridges, as well as several other conserved motifs [12]. A few studies have suggested a role for cathelicidins in fish immunity due to their upregulated expression upon pathogen stimulation [13–15].

Hepcidin, which is also termed LEAP-1, is a cationic amphipathic peptide that is made in the liver, released into the plasma and excreted

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in the urine [16,17]. Hepcidin is a 20-25-residue peptide that contains four disulfide bonds, a distinctive cystine bridge structure that is unique among other antimicrobial peptides [16,18]. In mammals, hepcidin was first acknowledged for its antimicrobial activity and later recognized as a key regulator of intestinal iron absorption and iron recycling by macrophages [17,19,20]. In addition to its direct antimicrobial activity, hepcidin was also found to be markedly induced during infections and inflammation [19]. In fish, hepcidin genes have been identified, but information regarding their function and regulation is very limited.

Defensin and cathelicidin are two of the most well-studied AMPs in mammals. As a major family of antimicrobial peptides produced by epithelial cells and the respiratory and digestive systems, defensins act as an essential defensive substance on the front lines of the immune system [21]. Defensins from vertebrates are small in size (29–43 amino acids in the mature peptide), are cationic and have three intramolecular disulfide bonds mediated by six conserved cysteines [22]. There are three main subfamilies: the α-, the β- and the θ-defensins, based on cysteine pairing and the length of the peptide fragments between the cysteines [23]. In addition to their direct microbicidal activity, defensins can enhance the mobilization of dendritic cells and antigen-specific immune responses *in vivo*, facilitate antigen uptake, induce the maturation of dendritic cells and contribute to T lymphocyte mobilization [24]. To date, only β-defensins have been identified in fish, and homologous sequences of β-defensins have been identified in diverse fish species.

Most infections start at the mucosal epithelium in animals, and the mucosal immune system provides the first line of defence for the inner body surfaces [25]. In fish, the mucosal-associated lymphoid tissue (MALT) includes gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), and gill-associated lymphoid tissue (GIALT) [26]. Fish are in continuous contact with a wide variety of pathogenic microbes; thus, mucosal infections are very common in teleost fish and lead to both local mucosal and systemic immune responses [27]. A recent evolutionary hypothesis is that adaptive immunity may be driven mainly by the microbial colonization of mucosal surfaces [28–30]. If the mucosal barrier is broken down and an invading pathogen reaches the circulation, then an extensive network exists for the trapping of blood-borne substances in the head kidney and the spleen [31]. In fish, various cell types and proteins of both the innate and adaptive lineages that are present at these mucosal barriers can coordinate immune defence at both the local and systemic levels, and AMPs are one such class of proteins [32]. Thus, studies on the expression patterns of AMPs during pathogenic invasion in mucosal-associated tissues and systemic immune tissues will be very useful in understanding host-pathogen interactions and immune responses in the host.

Acipenser dabryanus, also called Dabry's sturgeon, is an endemic species restricted to the upper reaches of the Yangtze River System in China [33]. *A. dabryanus* is among the oldest and most primitive groups of existing fish in the world and represents the transition from cartilaginous fish to bony fish [34]. *A. dabryanus* was once an important economic fish in the upper reaches of the Yangtze River. However, natural resources of *A. dabryanus* have decreased dramatically due to anthropogenic impacts, including overfishing, mining and pollution, and there have been no reports of the natural capture of *A. dabryanus* in the upper Yangtze River since the 2000s [35,36]. Subsequently, *A. dabryanus* was listed as a critically endangered species by the International Union for Conservation of Nature (IUCN) (www.iucnredlist.org/details/231/0) and a first-class protected animal in China [37]. Thus, captive breeding efforts have started for this important species, and successful artificial breeding of *A. dabryanus* has helped to compensate for the lost natural resource. However, in recent years, the increasing occurrence of bacterial or viral diseases in *A. dabryanus* has been reported [38]. The study of AMPs and their potential function in *A. dabryanus* will help us to understand the innate immune response of sturgeon and help to prevent pathogenic bacterial invasion.

In the present study, we reported the isolation and characterization of three AMPs, cathelicidin, hepcidin and defensin, in Dabry's sturgeon; these AMPs were designated AdCATH, AdHEP and AdBD, respectively. Tissue distribution and gene expression analysis were performed after challenge with the sturgeon pathogen *Edwardsiella tarda*. Specifically, the different expression patterns of AMPs between the mucosal tissues of the skin, gill and hindgut and the systemic tissues of the head kidney, liver and spleen after bacterial challenge were studied. Our work can provide basic information on the possible roles of AMPs in the innate immune response of fish against bacterial infection.

2. Materials and methods

2.1. Fish specimens and bacterial preparation

All fish handling and experimental procedures were approved by the Animal Care and Use Committee of the Fishery Institute of the Sichuan Academy of Agricultural Sciences (20171208001A), and all animal collection and use protocols were carried out in accordance with the guidelines and regulations for the care and use of laboratory animals at the Fishery Institute of the Sichuan Academy of Agricultural Sciences.

A total of 60 healthy *A. dabryanus* (approximately five months from hatching) were collected for tissue distribution and bacterial challenge at the Fishery Institute of the Sichuan Academy of Agricultural Sciences. The *A. dabryanus* were reared in a constant flow-through circulating aerated freshwater system at 27 °C for two weeks. The fish were anaesthetized with tricaine methanesulfonate (MS222, Sigma, USA), and tissue fragments of the heart, liver, spleen, head kidney, gonad, muscle, hindgut, gill, skin, eye and brain were dissected and immediately placed into liquid nitrogen until totally frozen, after which the samples were stored at −80 °C until RNA isolation. A strain of *E. tarda* that was found to be pathogenic to Dabry's sturgeon [38] was previously isolated and identified from diseased *A. dabryanus* and stored in our laboratory.

2.2. Cloning the full-length AMPs

The partial sequences of cathelicidin, hepcidin and β-defensin from Dabry's sturgeon were identified from transcriptome sequencing using BLAST searches of the web servers of the National Center for Biotechnology Information (NCBI). The full-length cDNA sequences of the three AMPs were further obtained by rapid amplification of cDNA ends (RACE) PCR (Takara, Tokyo, Japan) using gene-specific primers (Table 1). The sequences were assembled with Vector NTI 10.3.0 (Invitrogen, Carlsbad, CA, USA) software to obtain the full-length cDNA of cathelicidin, hepcidin and β-defensin. Based on the assembled sequences, three pairs of primers (Table 1) were used to confirm the consistency of the sequences obtained by transcriptome unigene and RACE PCR. The PCR products were cloned into pEASY-T3 (TransGen, Beijing, China) for sequencing and BLAST analysis.

2.3. Bioinformatic analysis

The complete coding sequences of cathelicidin, hepcidin and β-defensin from Dabry's sturgeon were translated into amino acid sequences by Vector NTI 10.3.0. The deduced amino acid sequences of the three AMPs were subjected to phylogenetic analysis with other fish homologenes available from GenBank (Table 2); the sequences were aligned using ClustalX according to Multiple Sequence Alignment. The putative signal peptides were predicted using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree was constructed based on the deduced amino acid sequences using the neighbour-joining method in Mega version 6.0 based on the multiple sequence alignment by ClustalX. The reliability of the branching was assessed by bootstrap resampling (1000 pseudoreplicates).

Table 1
Primers designed for cloning full-length AMPs as well as for real-time PCR.

Gene name	Primer name	Sequence (5′-3′)	Application
cathelicidin	CATH-3′RACE GSP1	GTGACACAAGCATCCCTAACAAGCA	3′RACE
	CATH-3′RACE GSP2	AGTGAAGAGAGGCGGCAATGGAAGT	
hepcidin	HEP-3′RACE GSP1	GACCAGGATAGAGAACAACATCTTT	3′RACE
	HEP-3′RACE GSP2	TGCGGGAGAAAAGACAAAGCCACTT	
β-defensin	DE-3′RACE GSP1	GGAGATTCAGATGCTGTGTATTGCG	3′RACE
	DE-3′RACE GSP2	GCGTTTCTACAGAGCCTTCCTTGCC	
cathelicidin	CATH-5′RACE GSP1	CACTTTAACAGCAGTCCATTCTCCA	5′RACE
	CATH-5′RACE GSP2	CCGGCCAGCAACATAGTTTTCAAGT	
hepcidin	HEP-5′RACE GSP1	TAATGGGATGCTGTTGCTTGCTCGT	5′RACE
	HEP-5′RACE GSP2	GGACGATTAAAACGATAGCTGAGAT	
β-defensin	DE-5′RACE GSP1	GGTCCAAAATGTCACGTCAGCTTCC	5′RACE
	DE-5′RACE GSP2	TTTGTTGGTCTCCGTGCACCGTTTG	
cathelicidin	CATH-F	GATCCACAGTCTCAGCTCAG	cDNA amplification
	CATH-R	CAAGGTACGGGAGATCAAGCT	
hepcidin	HEP-F	GCACCTAAAGACGAACACAAC	cDNA amplification
	HEP-R	CCCACGCCTCTGGTTTC	
β-defensin	DE-F	GCACTTTACGCCAAAACAAATG	cDNA amplification
	DE-R	CTAATACCGCAATACACAGCATC	
cathelicidin	CATH-qF	ATGAGGACATACTTGAAAACTATGTTG	Real-time PCR
	CATH-qR	CTACATCAGGAGTCTCTCGAC	
hepcidin	HEP-qF	GGACACGAGCAAGCAACAGCATC	Real-time PCR
	HEP-qR	TACAGGCAGATCGGGAAGTGGC	
β-defensin	DE-qF	ATGGACAAACAAAAACGGACTATAC	Real-time PCR
	DE-qR	CTAATACCGCAATACACAGCATC	
β-actin	ACTIN-qF	GACCGAGGCACCCCTGAAC	Real-time PCR
	ACTIN-qR	GATGGGCACTGTGTGTGTGAC	

2.4. Tissue distribution

To examine the tissue distribution of cathelicidin, hepcidin and β-defensin in Dabry's sturgeon, five healthy Dabry's sturgeon specimens were sampled, and eleven tissues (heart, liver, spleen, head kidney, gonad, muscle, hindgut, gill, skin, eye and brain) were collected for RNA extraction. The expression level of AMP mRNA *in vivo* was determined by real-time RT-PCR using SYBR Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan) and the IQ Multicolour Detection System (Bio-Rad, Hercules, CA, USA). *β-actin* was chosen as a reference gene because of its stable expression in Dabry's sturgeon [39].

Real-time PCR was performed using a volume of 25 μl containing 5 μl of diluted cDNA template (20-fold dilution of the original template), 12.5 μl of 2 × SYBR Green Real-time PCR Master Mix, 1 μl of each target gene primer (10 μM), and 5.5 μl of water. The PCR cycling conditions 94 °C for 5 min, then 42 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; finally, the temperature was increased from 70 to 95 °C with a ramp up of 0.5 °C per 10 s to generate a melting curve and verify that only one PCR product was amplified. Each sample was analysed in triplicate. The $2^{-\Delta\Delta CT}$ method was used to analyse the expression levels of the AMP genes in different tissues [40]. All data were presented in terms of relative mRNA expression and expressed as the mean ± S.E.

2.5. Bacterial challenge

In the pathogenicity *in vivo* assay, we chose *E. tarda* (a pathogenic bacterium of Dabry's sturgeon) to study the expression of the AMPs following infection. Briefly, the bacteria were grown in Mueller-Hinton medium until they reached logarithmic phase. The bacterial stocks were centrifuged to discard the medium and washed with sterile water once and then diluted to approximately 1×10^7 colony forming units per millilitre. The fish were then challenged in a rectangular tank by immersion exposure with a freshly prepared culture of *E. tarda* for 2 h. Five fish were sacrificed at each time point, i.e., at 0, 6, 12 24 and 48 h after the bacteria were initially added. The mucosal-associated tissues (skin, gill and hindgut) and the systemic immunity-related tissues (spleen, liver and head kidney) were collected as described above and

Table 2
AMPs sequences used for multiple alignment and phylogenetic analysis.

Gene name	Species	Accession no.	Gene name	Species	Accession no.
cathelicidin	*Plecoglossus altivelis*	CBV36822.1	hepcidin	*Salmo salar*	XP_014025533.1
cathelicidin	*Salmo trutta fario*	ABW16873.1	hepcidin	*Hucho taimen*	ARD27131.1
cathelicidin	*Oncorhynchus mykiss*	NP_001117952.1	hepcidin	*Gobiocypris rarus*	AKL71657.1
cathelicidin	*Salvelinus fontinalis*	CAQ60111.1	hepcidin	*Oreochromis niloticus*	XP_003450578.1
cathelicidin	*Thymallus thymallus*	CAQ60112.1	hepcidin	*Oncorhynchus mykiss*	XP_021416707.1
cathelicidin	*Gadus ogac*	AIL25605.1	hepcidin	*Oryzias latipes*	XP_011483981.1
cathelicidin	*Gadus macrocephalus*	AIL25606.1	β-defensin	*Danio rerio*	NP_001075023.1
cathelicidin	*Gadus chalcogrammus*	AIL25607.1	β-defensin	*Ctenopharyngodon idella*	AQY18996.1
cathelicidin	*Boreogadus saida*	AIL25615.1	β-defensin	*Siniperca chuatsi*	ACO88907.1
cathelicidin	*Salmo salar*	XP_014046396.1	β-defensin	*Oryzias latipes*	XP_023817866.1
cathelicidin	*Gadus morhua*	ACE96051.1	β-defensin	*Nothobranchius guentheri*	AMR43367.1
cathelicidin	*Lepisosteus oculatus*	XP_015209760.1	β-defensin	*Oplegnathus fasciatus*	AJA33388.1
hepcidin	*Lepisosteus oculatus*	XP_006641712.1	β-defensin	*Chanodichthys ilishaeformis*	AOA33193.1
hepcidin	*Salvelinus alpinus*	XP_023844327.1	β-defensin	*Epinephelus coioides*	AFA41485.1
hepcidin	*Danio rerio*	NP_001276723.1	β-defensin	*Paramisgurnus dabryanus*	AGH10110.1
hepcidin	*Plecoglossus altivelis*	CBL59464.1	β-defensin	*Cyprinus carpio*	AGZ03658.1
hepcidin	*Pagrus auriga*	BAH03285.1			

kept in liquid nitrogen for RNA extraction. The resulting cDNA was stored at −20 °C for real-time PCR analysis. All fish survived throughout the experiment.

2.6. Data analysis

Data on AMP responses from tissue expression analysis after bacterial challenge were tested through one-way ANOVA using SPSS 22.0 software. Differences for which $p < 0.05$ were considered statistically significant, and differences for which $p < 0.01$ were considered highly significant.

3. Results

3.1. Molecular characterization of AMPs

The full cDNA sequence of cathelicidin was 2401 bp (GenBank Accession No. MK173026), including 179 bp of the 5′-untranslated region (UTR), 1715 bp of the 3′-UTR and 507 bp of the open reading frame (ORF) sequence. The full ORF sequence of cathelicidin encoded a putative 168-residue peptide. The signal peptide cleavage site was predicted between Ala^{22} and Glu^{23}. Then, a cathelin-like domain (CLD) was predicted to end at residue Ala^{130}-Arg^{131}, which is an elastase cleavage site for some mammalian cathelicidins [41]. The resulting putative mature cathelicidin had a theoretical p*I* of 7.76 and a molecular mass of 4183.43 Da.

The full cDNA sequence of hepcidin was 1103 bp (GenBank Accession No. MK173027), including 256 bp of the 5′-UTR, 577 bp of the 3′-UTR and 270 bp of the ORF sequence. The deduced amino acid sequence of hepcidin consisted of a 19-residue signal peptide, a 45-residue prodomain and a 25-residue predicted mature peptide. The signal peptide cleavage site was predicted between Ala^{19} and Ser^{20}. The RX(K/R)R motif typical of propeptide convertases [42] was present in AdHEP. Processed hepcidin contains 25 amino acids and was predicted to be positively charged. The putative mature AdHEP had a theoretical p*I* of 8.52 and a molecular mass of 2848.14 Da.

The β-defensin full cDNA sequence was 1040 bp (GenBank Accession No. MK173028), which contained 19 bp of the 5′-UTR, 823 bp of the 3′-UTR and 198 bp of the ORF sequence. The full ORF sequence of defensin consisted of a predicted 22-residue signal peptide and a 43-residue mature peptide. The putative mature AdBD had a theoretical p*I* of 8.66 and a molecular mass of 5131.30 Da.

3.2. Sequence alignment and phylogenetic analysis of AMPs

Sequence alignment of the AdCATH peptide with other reported cathelicidins in ten fish species was performed at the amino acid level (Fig. 1). The results revealed the following consensus between AdCATH and other fish species: *Lepisosteus oculatus* (44.3%), *Thymallus thymallus* (37.6%), *Salmo salar* (33.3%), *Salvelinus fontinalis* (33.0%), *Oncorhynchus mykiss* (32.9%), *Gadus ogac* (28.4%), *G. macrocephalus* (26.8%), *G. chalcogrammus* (25.7%), *G. morhua* (25.2%) and *Boreogadus saida* (24.3%). Multiple sequence alignment of AdCATH with other known cathelicidins showed that it contained a motif of four conserved cysteines, namely, Cys 77, Cys 88, Cys 99 and Cys 119, within the mature peptide (Fig. 1).

The translated amino acid sequence of the AdHEP gene was compared to those of ten known fish hepcidins by multiple alignment analysis (Fig. 1). The results revealed the following consensus between AdHEP and other fish species: *L. oculatus* (62.2%), *Plecoglossus altivelis* (47.8%), *S. alpinus* (47.8%), *O. mykiss* (47.8%), *Oreochromis niloticus* (47.3%), *Hucho taimen* (46.7%), *S. salar* (46.7%), *Pagrus auriga* (46.2%), *Oryzias latipes* (45.1%) and *Danio rerio* (44.6%). Similar to other known fish hepcidins, eight conserved cysteines were located within the mature peptide of AdHEP, namely, Cys 71, Cys 74, Cys 75, Cys 77, Cys 78, Cys 83, Cys 86 and Cys 87.

The deduced amino acid sequence of AdBD was aligned with those of β-defensins from other species, including ten fish β-defensins identified in previous studies. The amino acid sequence deduced from the Dabry's sturgeon β-defensin cDNA had the highest similarity to the sequence from *D. rerio* (62.1%), followed by *Epinephelus coioides* (60.6%), *Siniperca chuatsi* (59.1%), *Cyprinus carpio* L (58.2%), *Nothobranchius guentheri* (56.1%), *Oplegnathus fasciatus* (56.1%), *Ctenopharyngodon idella* (55.9%), *Paramisgurnus dabryanus* (54.5%), *Chanodichthys ilishaeformis* (52.1%) and *O. latipes* (44.4%). The signature for the β-defensins is the presence of six conserved cysteines, which were detected in AdBD at positions 32, 38, 42, 54, 60 and 61 (Fig. 1).

To evaluate the evolutionary relationship between AMPs from Dabry's sturgeon and AMPs from other species, the amino acid sequences were used to construct a phylogenetic tree based on the neighbour-joining method. As shown in Fig. 2, AdCATH clustered with other fish cathelicidins, with affinity to *L. oculatus* cathelicidin supported by a significant bootstrap value (99). The Dabry's sturgeon defensin sequence appeared to be most closely related to the *D. rerio* and *C. idella* groups. AdHEP and other fish hepcidins grouped together to form a distinct cluster.

3.3. Expression of AMPs in different tissues

Constitutive expression of AMPs was detected by SYBR green real-time quantitative RT-PCR in various tissues from five healthy Dabry's sturgeon specimens. As shown in Fig. 3, *AdCATH* mRNA was found to be constitutively expressed at different levels in eleven tissues. Expression was very high in the liver, gonad, and muscle and was also detected at relatively high levels in the brain, eye and skin. Low-level constitutive expression of *AdCATH* was observed in the head kidney, heart, hindgut, spleen and gill. Expression of *AdHEP* was detected in all the assayed tissues. The highest expression was found in the liver, while the remaining tissues all had a relatively low expression level. *AdBD* was also widely expressed in all tested tissues, with the highest gene expression detected in the skin, followed by the liver and eye. *AdBD* was also detected at relatively high levels in the hindgut, brain, muscle and heart. By contrast, a relatively lower transcription of defensin was found in the gill, head kidney, gonad and spleen.

3.4. Temporal expression of AMPs after microbial challenge

Skin, gill, hindgut, spleen, head kidney and liver samples collected at different times after challenge with *E. tarda* (a bacterium pathogenic to Dabry's sturgeon) were subjected to real-time PCR. AMPs were constitutively expressed in all the tissues examined after bacterial challenge, and diverse expression patterns in different tissues were observed. Remarkable upregulation of *AdCATH* was observed in the skin at 12 h post-challenge (Fig. 4A). The mRNA levels of *AdCATH* in the skin increased by approximately 0.83-, 7366.41-, 16.79- and 0.67-fold at 6, 12, 24 and 48 h post-challenge, respectively. In the other two mucosal-associated tissues, the respective mRNA levels of *AdCATH* at 6, 12, 24 and 48 h post-challenge increased by 0.84-, 0.61-, 2.90- and 0.80-fold in the gill and 0.71-, 0.83-, 7.83- and 54.57-fold in the hindgut. In systemic tissues, the expression of *AdCATH* started to increase at 24 h post-challenge in the spleen and decreased dramatically at 48 h post-challenge (Fig. 4B). In the liver and head kidney, the expression level of *AdCATH* did not change until 48 h post-challenge (Fig. 4B).

Striking upregulation of *AdHEP* was also observed in the skin at 12 h post-challenge (Fig. 4C). The mRNA levels of *AdHEP* in the skin increased by approximately 1.38-, 1936.74-, 1.32- and 1.04-fold at 6, 12, 24 and 48 h post-challenge, respectively. The respective mRNA levels of *AdHEP* at 6, 12, 24 and 48 h post-challenge increased by 0.25-, 0.18-, 1.26- and 1.49-fold in the gill and by 2.89-, 2.71-, 196.76- and 180.33-fold in the hindgut. The expression level of *AdHEP* increased gradually

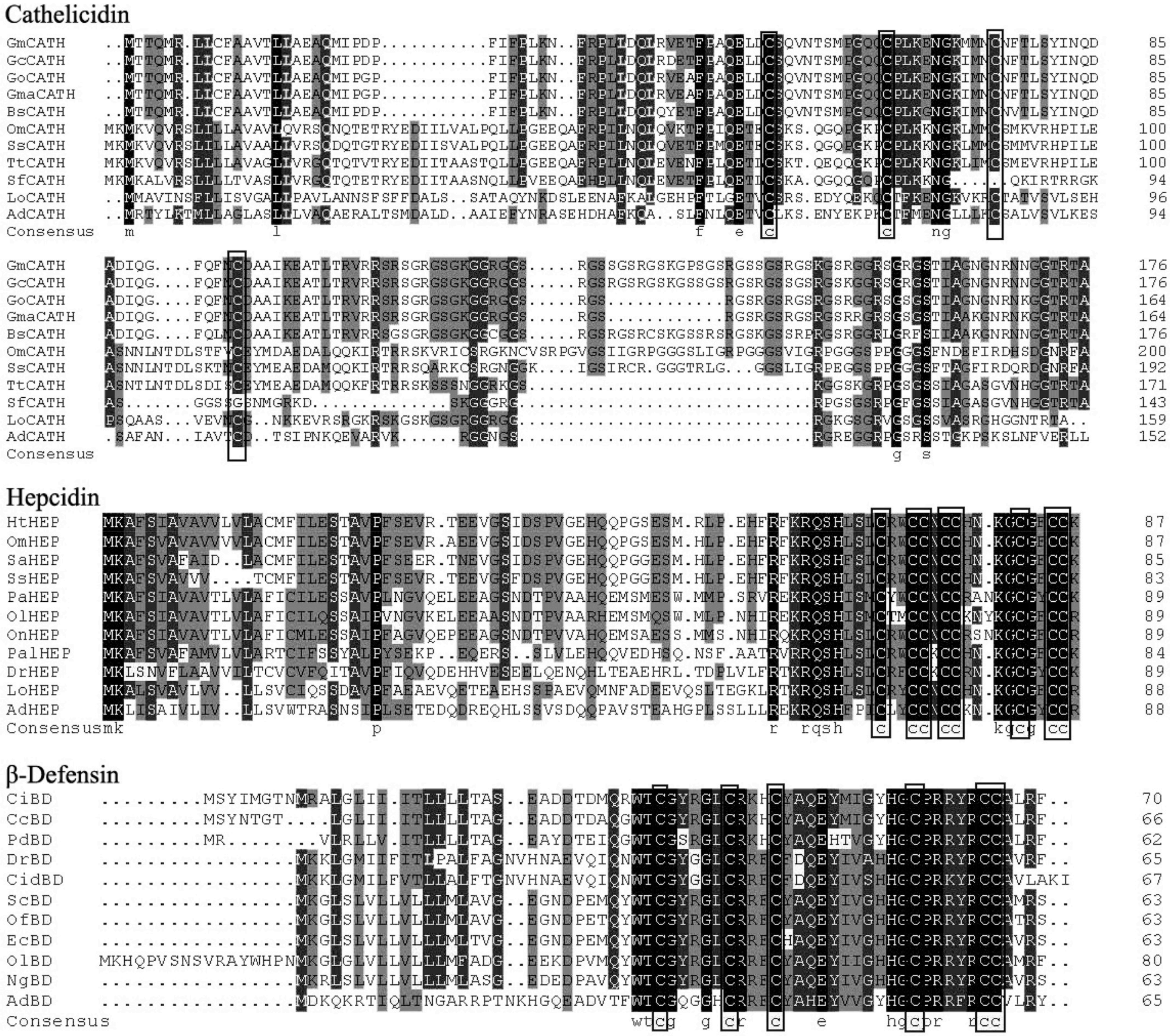

Fig. 1. Multiple sequence alignment of AMPs from *A. dabryanus* and other fish species. The conserved cysteine residues of cathelicidins, hepcidins and β-defensins were shown in box. GmCATH (*Gadus morhua* cathelicidin), GcCATH (*Gadus chalcogrammus* cathelicidin), GoCATH (*Gadus ogac* cathelicidin), GmaCATH (*Gadus macrocephalus* cathelicidin), BsCATH (*Boreogadus saida* cathelicidin), OmCATH (*Oncorhynchus mykiss* cathelicidin), SsCATH (*Salmo salar* cathelicidin), TtCATH (*Thymallus thymallus* cathelicidin), SfCATH (*Salvelinus fontinalis* cathelicidin), LoCATH (*Lepisosteus oculatus* cathelicidin), AdCATH (*Acipenser dabryanus* cathelicidin), HtHEP (*Hucho taimen* hepcidin), OmHEP (*Oncorhynchus mykiss* hepcidin), SaHEP (*Salvelinus alpinus* hepcidin), SsHEP (*Salmo salar* hepcidin), PaHEP (*Pagrus auriga* hepcidin), OlHEP (*Oryzias latipes* hepcidin), OnHEP (*Oreochromis niloticus* hepcidin), PalHEP (*Plecoglossus altivelis* hepcidin), DrHEP (*Danio rerio* hepcidin), LoHEP (*Lepisosteus oculatus* hepcidin), AdHEP (*Acipenser dabryanus* hepcidin), CiBD (*Chanodichthys ilishaeformis* β-defensin), CcBD (*Cyprinus carpio* β-defensin), PdBD (*Paramisgurnus dabryanus* β-defensin), DrBD (*Danio rerio* β-defensin), CidBD (*Ctenopharyngodon idella* β-defensin), ScBD (*Siniperca chuatsi* β-defensin), OfBD (*Oplegnathus fasciatus* β-defensin), EcBD (*Epinephelus coioides* β-defensin), OlBD (*Oryzias latipes* β-defensin), NgBD (*Nothobranchius guentheri* β-defensin), AdBD (*Acipenser dabryanus* β-defensin).

in the spleen (0.96-, 2.58-, 8.79- and 27.88-fold after 6, 12, 24, and 48 h, respectively, compared to 0 h post-challenge). In the liver and head kidney, the expression level of *AdHEP* did not change significantly until 48 h post-challenge (Fig. 4D).

Compared to those of *AdCATH* and *AdHEP*, the expression profile of *AdBD* after challenge with pathogens showed some unique characteristics. The mRNA levels of *AdBD* in the spleen and head kidney decreased at 12 and 24 h post-challenge (although the difference was not significant) and then increased significantly at 48 h post-challenge compared to those at 0 h post-challenge (Fig. 4F). The transcription level of defensin in the liver did not change significantly throughout the entire stimulation period (Fig. 4F). In mucosal-associated tissues, the mRNA levels in the hindgut increased 230-fold at 6 h post-challenge and decreased at 12 h post-challenge (Fig. 4E). At 24 h post-challenge, the expression level of defensin in the hindgut reached another peak, with 161-fold higher expression than that at 0 h post-challenge, and then decreased again at 48 h post-challenge (Fig. 4E). In the skin, the transcription level was upregulated at 12 h and 24 h post-challenge, with an approximately 8-fold change, and then returned to the resting level at 48 h post-challenge (Fig. 4E).

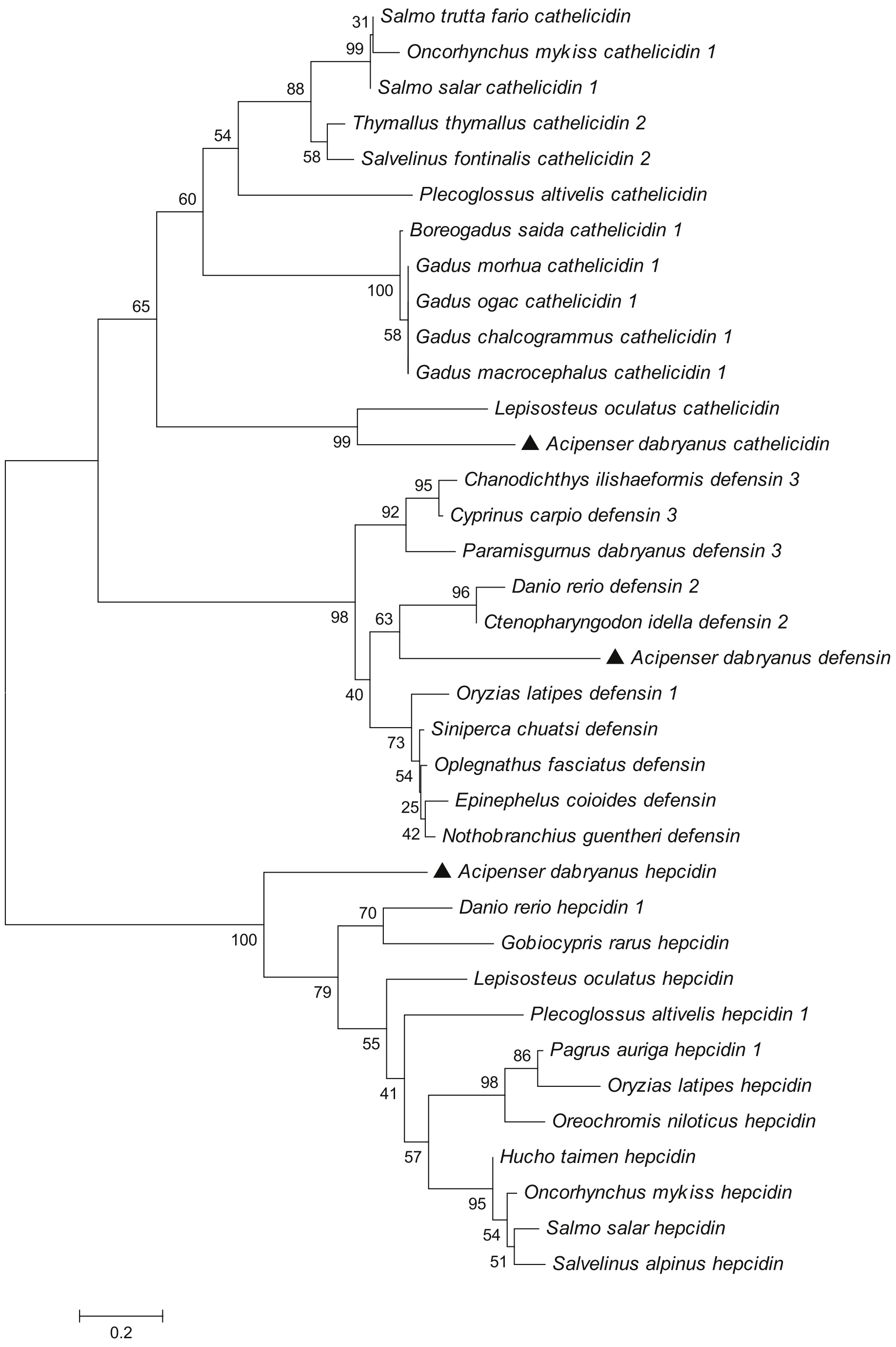

Fig. 2. Phylogenetic tree based on deduced amino acid sequences for AdCATH, AdHEP and AdBD and their homologues from fish. The phylogenic tree was constructed with the neighbour-joining method based on the multiple sequence alignment of ClustalX. The scale bar corresponds to 0.2 estimated amino-acid substitutions per site. "▲" represents AMPs from *A. dabryanus*.

Overall, the response of AMPs to bacterial challenge was slightly faster in mucosal-associated tissues than in systemic immunity-related tissues.

4. Discussion

The aquatic environment presents frequent and high levels of exposure to a large number of various pathogens. Fish lack a well-developed adaptive immune system, so a strong innate immune system that acts as the first line of defence is essential [2]. To fight against a broad spectrum of pathogens in aquatic environments, innate immune factors play major roles in the innate immune system and protect against a wide variety of pathogenic infections. In this study, we identified three very important AMPs, cathelicidin, hepcidin and defensin, in Dabry's sturgeon. To the best of our knowledge, these three AMPs were the first time to be identified and functionally analysed in sturgeons.

AdCATH shares common features with other fish cathelicidins; it is small in size (38-residue mature peptide) and cationic, and it contains four cysteines in the cathelin-like domain that form two intramolecular disulphide bonds, as well as the conserved glycine- and serine-rich residues in the C-terminal domain, which can be seen in previous described fish cathelicidins [14,15]. Similar to other known fish cathelicidins, AdCATH contains a clear N-terminal signal peptide, which

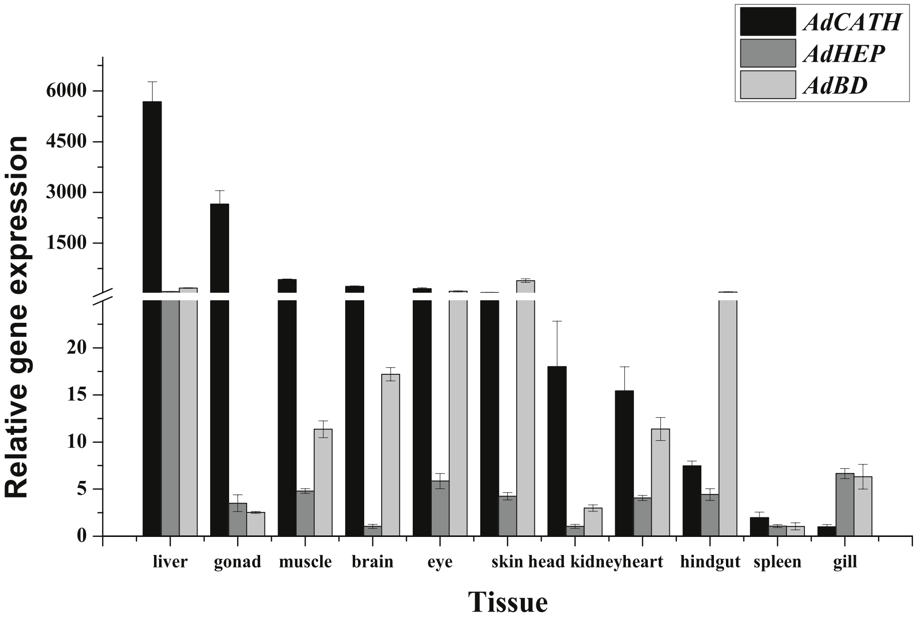

Fig. 3. Distribution of three AMPs in different tissues of *A. dabryanus*. The data were presented as the mean of measurements acquired from five fish. Error bars indicated the standard error.

suggests that it is secreted. Compared to mammalian cathelicidins, fish cathelicidins have a shorter signal peptide (22–26 aa in fish versus 29 to 30 aa in mammals). AdHEP contains eight concerved cysteines in the mature peptide, which are proposed to form four disulphide bonds in the following linkage pattern: 1–4, 2–8, 3–7, and 5–6 [17,43]. As a key characteristic feature of most hepcidins, the four disulphide bond structures are known to be associated with the antimicrobial activity of the protein; therefore, it can be predicted that AdHEP may also have this immunological function [16]. The tetrapeptide RXKR is highly conserved in front of the prodomain cleavage site among most fish species. The AdHEP is predicted to be processed by peptidases after the sequence REKR based on multiple alignments and sequences of isolated mammalian [16] and fish [44,45] hepcidins. However, the cleavage site of AdHEP needs to be further experimentally confirmed. Fish β-defensins are generally classified into four subgroups based on homology comparison and phylogenetic analysis [46,47]. Sequence analysis revealed that AdBD contained a PRRXR motif between the fourth and fifth cysteine residues, which is a conserved organization in other fish β-defensin 2 genes. Another feature of fish defensin 2 is that the first two cysteines are separated by five residues, which differs from fish β-defensins 1, 3, and 4, which are separated by six residues. The results demonstrated that the defensin gene from Dabry's sturgeon is a member of the fish β-defensin 2 family.

However, the amino acid sequences of the three AMPs from Dabry's sturgeon and other fish AMPs showed a rather low similarity, especially for AdCATH. The highest consensus was found for *L. oculatus* (44.3%), whereas other fish cathelicidins showed no more than 40% similarity with AdCATH. This result is not surprising since no other corresponding AMPs have yet been identified in the genus *Acipenser*. Phylogenetic analysis of the AMP family indicated that the deduced amino acid sequence from Dabry's sturgeon cathelicidin was in a branch with the published *L. oculatus* cathelicidin peptide, and AdHEP and AdBD were in a relatively independent position from other fish homologues (Fig. 2). These results all revealed a relatively weak relationship between sturgeon and the identified species and suggested that sturgeon might occupy a special evolutionary position.

Analysis of the sites of AMP expression in Dabry's sturgeon revealed widespread constitutive expression. These genes were positive at both mucosal sites (skin, gut, and gill) and systemic sites (spleen, head kidney, and liver). We found the highest expression of both *AdCATH* and *AdHEP* in the liver, which is consistent with previous studies that also showed high expression of fish cathelicidins or hepcidins in the liver [14,48–50]. Liver is one of the main immunocompetent tissues in teleosts [51] and the high expression of AMPs in liver indicates their important function in the sturgeon immune system. *AdBD* is highly expressed in the skin of Dabry's sturgeon, similar to results reported for channel catfish [52]. In humans, β-defensins were also found to be mainly expressed in the mucosal epithelium, including the skin [53–55]. Skin is a major mucosal tissue that forms the first line of defence against invasion by external pathogens, especially in scaleless fish, such as Dabry's sturgeon. In addition, a high expression level of *AdBD* in the eye was found in Dabry's sturgeon. Interestingly, two previous studies in medaka and loach also found that the eye was the major site for defensin expression [47,56]. In humans, β-defensin was reported to play an important role in establishing a corneal epithelial cell barrier against *Pseudomonas aeruginosa* during dry-eye syndrome [57,58], indicating the potential importance of defensin in preventing infection of the eye.

To understand the effects of bacterial infection on AMPs expression, *E. tarda* were used as stimulants in *in vivo* experiments. Both *AdCATH* and *AdHEP* were universally upregulated in response to pathogens in most of the tissues we examined, which was consistent with previous bacterial challenge results in other fish [13–15,48–50]. This upregulation was most profound in the skin at 12 h post-challenge, with an increase of over 1000-fold compared to the healthy control, suggesting a role of the AMPs in the initial immune response. However, the expression level of *AdCATH* and *AdHEP* in the skin at 24 and 48 h post-challenge did not differ significantly from that of the control groups, which may be associated with the altered proliferation of immune cells. A similar expression profile was also observed in a previous stimulation study of cathelicidin in other species, such as ayu [14]. The expression level of *AdCATH* and *AdHEP* at systemic sites generally started to increase at 48 h post-challenge. Such regulatory effect was also observed in common carp, which reported that the mucosal immune response performed faster than systemic immune response following bacterial infection [49]. The different responding times suggested that mucosal immune-related tissues, especially the skin, acted earlier than systemic immune sites in response to bacterial pathogens and that mucous layer might form an initial line of defence that protects epithelial tissues against bacterial infection. This expression profile implied that *AdCATH*

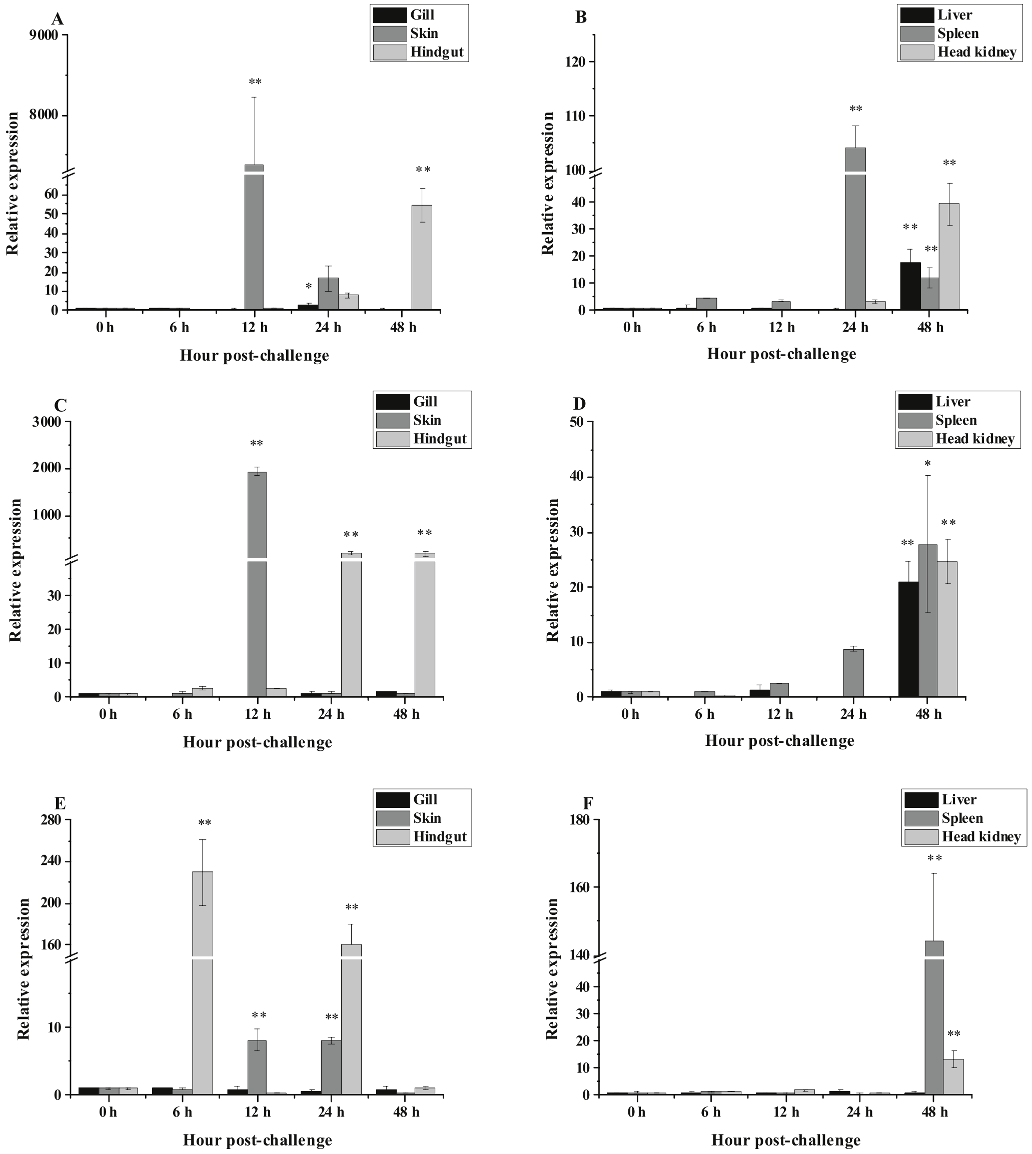

Fig. 4. Relative expression of AMPs mRNA in mucosal sites (A, C, E) and systemic sites (B, D, F) after infection with *E. tarda*. (A, B) *AdCATH*; (C, D) *AdHEP*; (E, F) *AdBD*. Statistically significant and highly significant upregulated expression (relative to the data for 0 h) is denoted with "*" ($p < 0.05$) and "**" ($p < 0.01$), respectively.

and *AdHEP* could be an important part of the mucosal immune system in Dabry's sturgeon to protect against bacterial infection.

Compared to the other two AMPs in Dabry's sturgeon, *AdBD* exhibited some diverse expression patterns post-*E. tarda* infection. Interestingly, in systemic tissues (spleen and head kidney), the mRNA level of defensin was downregulated early post-challenge and then increased at 48 h post-challenge. Previous studies have reported that some bovine β-defensins are mainly expressed in monocytes and macrophages, which might release some messages to induce the synthesis of β-defensin [59,60], and such immune cells in the systemic immune tissues may quickly migrate to sites of infected tissue post-streptococcal infection, leading to a decreased expression level of defensin in these tissues during early infection. A similar expression profile was observed in *O. niloticus*, in which the expression of β-defensin decreased in the spleen at 24 h post-challenge with *Streptococcus agalactiae* [61]. Surprisingly, as an immune tissue, the expression level of β-defensin in the liver did not change significantly at any time post-infection. Previous studies have also found upregulated defensin levels after bacterial challenge in examined tissues, except for the liver [61,62]. As systemic tissues of fish, the differential expression patterns of β-defensin among the liver, spleen and head kidney and the function of the liver in the immune system need further study. Similar to hepcidin and cathelicidin, the early responses of defensin in sturgeon were more dependent on the stimulus in mucosal tissue than in systemic tissues. Among all examined tissues, the expression of β-defensin mRNA was most elevated in the hindgut (by 230-fold at 6 h post-challenge). These results indicated that β-defensin might participate in the mucosal immune response against invading pathogens, especially in the hindgut of sturgeon.

In this study, it is interesting to notice that all three AMPs in the gill failed to respond to *E. tarda* infection at most of the time points after challenge. The expression levels of these three AMPs in the gill of healthy Dabry's sturgeon were all relatively low. It is not known why *E. tarda* exposure resulted in differential inducibility of AMPs. It is possible that the expression levels of AMPs in the gill in these animals were maximal at the time of challenge. Alternatively, different regulatory mechanisms for AMPs between the gill and the other evaluated tissues may explain this finding.

5. Conclusion

Antimicrobial peptides are important part of the innate immune system, which play essential roles in protecting against a wide variety of pathogens in aquatic environments. The current study is the first identification of three very important AMPs, cathelicidin, hepcidin and defensin from *A. dabryanus* and the first analysis of their phylogenetic relation and expression pattern responding to bacterial challenge. The *A. dabryanus* cathelicidin, hepcidin and defensin transcripts were expressed at different levels in various tissues and the highest expression levels of them were found in immune-related sites. Challenge of *A. dabryanus* with the pathogenic bacterium *E. tarda* significantly upregulated AMPs expression. The mucosal sites generally acted earlier and stronger than systemic sites towards bacterial infection. These results demonstrated that *A. dabryanus* AMPs might play an important role in the innate immune response, especially during mucosal immune response against bacterial pathogen.

Declarations of interest

None.

Acknowledgments

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