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Immunology

Expression of ayu antimicrobial peptide genes after LPS stimulation

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ABSTRACT. *Plecoglossus altivelis* (ayu) is one of the most important fish species in the Japanese islands and in internal fish hatcheries. Living in open aquatic environments exposes fish to many pathogens. Therefore, they require rapid and strong immune defenses. We investigated *in vivo* the direct association between the ayu innate immune response, represented by the relative transcription of genes encoding the cathelicidin and hepcidin antimicrobial peptides, and lipopolysaccharide (LPS), a conventional pathogen-associated molecular patterns (PAMPs) of Gram-negative bacteria. Different concentrations of LPS (1, 10 and 100 μg/fish) were injected intraperitoneally into young (sexually immature) and adult (fully sexually mature) ayu. The relative expression of the antimicrobial peptide genes was measured 6 hr, 24 hr and 1 week after stimulation with LPS. We found a direct association between the expression of the antimicrobial peptide genes investigated and LPS stimulation. This relationship was time-, dose- and age-dependent. Further research is required to determine the cell-specific transcriptional regulation and posttranscriptional regulation of these antimicrobial peptides.

KEY WORDS: antimicrobial peptide, ayu, innate immunity, LPS, mRNA expression

Plecoglossus altivelis (ayu), is among the most important fish of freshwater fisheries. It is produced from artificial fry, which are cultured and released into the rivers of Asian countries, including Japan, China and Korea [60]. Because of its economic importance and importance in sport fishing, interest in ayu farming has increased rapidly over the past 10 years [47]. A few vertebrates are semelparous, with a single breeding effort before they die. These include lampreys (*Petromyzon* and *Lampetra* spp.), eels (*Anguilla* spp.), Pacific salmon (*Oncorhynchus* sp.) [19] and a group of dasyurid marsupials [6]. Others are amphidromous, migrating between the sea and rivers, and these include salmonid fish. However, the ayu is a short-lived fish (a life cycle of ~1 year) with the characteristics of both amphidromous and semelparous fish species.

To protect themselves from a wide variety of microbes, including bacteria, viruses, fungi, parasites, water molds and yeasts [66], fish must have a good defense mechanism. Mammals and chicken, for example, have a germinal center [25], so they have a secondary adaptive immune response. However, fish have no germinal center [23]. Invertebrates and lower vertebrates predominantly depend on innate immune defenses to protect them from foreign invaders that cause infections and disease [14, 22]. Ayu lives at cool to cold water temperatures and migrates between the sea and rivers throughout its life cycle. It is poikilothermic and has a limited antibody repertoire, affinity maturation, poor memory and relatively slow lymphocytic proliferation [21, 51, 94]. Fish mainly depend on their innate immune systems for extended periods of time, beginning in the early stages of embryogenesis [15, 72, 88]. The fish antigen-specific immune response is also temperature dependent [1, 52] and is markedly affected by low temperatures [51]. Therefore, their innate immune defenses play important roles under suboptimal environmental conditions [89]. Fish develop very potent nonspecific immune defenses against a broad spectrum of microbes [86]. These mechanisms are constitutive and responsive under basal conditions, and are also inducible by external molecules. Under both conditions (constitutive and inducible), they react very rapidly [10]. A major component of this nonspecific defense mechanism in fish is the antimicrobial peptides, which play critical roles in the immune defenses of all fish [28, 83, 87, 98, 99], despite differences in the immune system elements of different fish species or even within the same species [52, 84, 94]. Antimicrobial peptides hold a position of primary importance in fish defenses and have therefore attracted the attention of researchers in this field [51, 52]. They are a major component of the fish innate immune response to a wide range of opportunistic pathogens, including bacteria, viruses, fungi, tumor cells, parasites and yeast [13, 27, 46, 70, 91, 100] either by direct antimicrobial action [6, 27, 46], by triggering an inflammatory reaction [7, 15, 64] or through a broad range of immunomodulatory functions [35, 85]. Fish are a great source of these peptides and express all the major classes of antimicrobial peptides, including defensins, cathelicidins, hepcidins,

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histone-derived peptides and a fish-specific class of the cecropin family, called piscidins [33, 56, 78, 96]. Fish cathelicidins have been identified in the rainbow trout (*O. mykiss*), Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*), brook trout (*Salvelinus fontinalis*), European grayling (*Thymallus thymallus*), hagfish (*Myxine glutinosa*), Arctic char (*S. alpinus*), chinook salmon (*O. tshawytscha*) and Atlantic cod (*Gadus morhua*) [11, 74]. The cathelicidin identified in ayu has a 20-amino-acid signal peptide, a conserved cathelin domain of 110 amino acids, and a mature antimicrobial peptide of 61 amino acids [50].

Hepcidin is a small cysteine-rich protein with antimicrobial activity (encoded by the *LEAP-1* and *LEAP-2* genes) that is expressed in the liver, as its name implies. It was first discovered in humans by two independent groups at the same time, in blood ultrafiltrate and urine [39, 40, 65]. Immunofluorescence studies have shown that the hepcidin precursor protein mainly localizes in the hepatocytes around the portal triads because increased hepcidin immunoreactivity is detectable at the basolateral membranes of periportal hepatocytes in both mammals [34, 39, 41, 59, 65] and fish [2, 3, 15, 20, 31, 33, 38, 50, 71, 79, 96]. Hepcidin cDNA and genomic DNA have been identified in many fish species from several orders, including Perciformes, Cypriniformes, Siluriformes, Gadiformes and Salmoniformes; from freshwater fish, saltwater fish and diadromous fish; and from fish living in extreme habitats [45]. Hepcidin has also been detected in ayu [12, 48]. A direct relationship between infection and hepcidin has been established in several fish species. The expression of fish hepcidin is induced in response to immune stimuli or direct infection, and its induction has been observed after challenge with infection, inflammation, vaccination, viruses, bacteria, bacterial components (lipopolysaccharides, LPSs), dextran and poly I:C (a double-stranded RNA molecule) [18, 45, 57, 101]. The ayu expresses both the hepcidin *LEAP-1* gene, which encodes the Q-S-H-L-S-L sequence [12], and the *LEAP-2B* gene, which does not encode Q-S-H-L-S-L [48]. In other studies, the antimicrobial activity of LEAP-1 against bacteria, fungi, viruses and protozoans has been identified in various fish species both *in vivo* and *in vitro* [55, 69, 79, 97].

LPS is the major component of the outer membrane of Gram-negative bacteria. It is composed of a core polysaccharide, an *O*-polysaccharide of variable length, and a lipid portion called "lipid A", which is responsible for the activation of the innate immune response in mammals and confers the molecule's endotoxic properties [68, 78].

The process of antimicrobial peptide production and the molecular mechanism regulating antimicrobial peptides are poorly understood. Although several studies of the regulation of antimicrobial peptide expression have been published, their results are conflicting [36].

The aim of our study was to determine for the first time the direct association between the expression of antimicrobial peptide (cathelicidin and hepcidin) genes and different concentrations of LPS during the different life stages of the ayu *in vivo*.

Most of the previous researches had been focused on identification and characterization of the genes encoding antimicrobial peptides (AMPs) from fish. However, we focused on the expression of the genes encoding ayu AMPs in response to LPS *in vivo* to clarify its antimicrobial activity in the different life stages of the ayu. From the results should indicate whether this particular antimicrobial peptides are involved in the fish's immune response against bacterial infections and, if so, the extent to which it is involved.

MATERIALS AND METHODS

Fish

Ayu (n=130) was purchased from Takahashigawa Fisheries Cooperative Association (Okayama, Japan). During the season from April 1 to the end of October, the fish were kept in 500 *l* plastic water tanks at 20 ± 2°C and fed regularly three times a day with a commercial pelleted ayu feed. All fish were treated in accordance with the Guidelines for Animal Experiments of Hiroshima University. The fish were divided according to age. Group 1 contained young (sexually immature) fish; internal examination confirmed the complete absence of sexual organs. Their average bodyweight was 8–9 g, and their average body length was 5–6 cm. Group 2 contained mature adult (sexually mature) fish; internal examination just before spawning confirmed fully ripened sexual organs in both sexes). Their average bodyweight was 40–60 g, and their average body length was 15–20 cm. Each group was subdivided into four subgroups treated with phosphate-buffered saline (PBS), 1 μg/fish LPS, 10 μg/fish LPS or 100 μg/fish LPS. Each subgroup contained 15 fish.

LPS

A solution of LPS from *Escherichia coli* 0127:BB (BioXtra; Sigma-Aldrich, St. Louis, MO, U.S.A.) was prepared in sterile PBS, and used for intraperitoneal injection.

Sample collection

The liver tissues of five fish from each subgroup were dissected at 6 hr, 24 hr or 1 week after stimulation. Five fish not injected with LPS were used to determine the constitutive mRNA expression of hepcidin (*LEAP-1*) and cathelicidin. The tissues were immersed immediately in liquid nitrogen and stored at −80°C until analysis. These steps were performed twice during the period of the experiment, in mid April and mid October, representing the two stages of the ayu life cycle.

Total RNA extraction and cDNA synthesis

Liver tissues (50 mg [young fish] −100 mg [sexual mature]) from five non-injected healthy fish were used for total RNA extraction. Total RNA was extracted with TRIzol Reagent (Ambion, Life Technologies, Carlsbad, CA, U.S.A.) and treated with DNase I (Takara Bio Inc., Kusatsu, Japan) to remove the genomic DNA. First-strand cDNA was synthesized from total RNA (1 μg)

with SuperScript IV Reverse Transcriptase (Invitrogen, Life Technologies) with oligo (dT_{20}) primer (Life Technologies), according to the manufacturer's instructions. The same process was repeated for each subgroup (five samples were transcribed to cDNA after total RNA extraction in each period: 6 hr, 24 hr and 1 week).

Amplification with semi-quantitative RT–PCR

The sequence of the AMPs from ayu was obtained based on information stored at Gene Bank (U.S.A.). cDNA sequence database accession number of cathelicidin, hepcidin and β-actin (*actb*) was FR667573.1, AB020884.1 and AB020884.1, respectively. The Primer-Blast was used to design the primers included the Primer3Plus software (http://www.bioinformatics.nl/ogibin/primer3plus/primer3plus-cgi), and the primer sequences were checked with a Blast search. The primer sequences were: cathelicidin forward primer, ATGAATTCGGAAGTATTGTCGTGCAAC; cathelicidin reverse primer, ATGAATTCCTCCTCCTCATCCTTATCTTT; hepcidin forward primer, AGCAGCTATGCTTTGCCCTA; hepcidin reverse primer, GCACCAACGACACAGAGAGA; *actb* forward primer, CCGACTACCTGATGAAGATCCTGACAGAG; and *actb* reverse primer, GGTGGTCTCGTGAATACCGCAAGACTC. The ayu housekeeping gene encoding β-actin (*actb*) was also amplified to assess the quality of the cDNA and to normalize the expression of the genes. Twenty-five μl of the PCR reactions contained Ex Taq® DNA Polymerase (Takara Biotechnology), 1 μl of template cDNA and 0.64 μl of the forward and reverse primers. The PCR products were isolated with gel electrophoresis on 2% agarose gel (Agrose LO_3; Takara Biotechnology), stained with ethidium bromide, and visualized with a UV transilluminator (UVP BioDoc-It Imaging System, Upland, CA, U.S.A.). The PCR cycle number was 35. It is the optimum cycle number in the logarithmic phase.

Normalization and estimation of mRNA expression

The ayu housekeeping gene encoding β-actin (*actb*) was amplified to assess the quality of the cDNA and to normalize the expression of the genes. To normalize the amount of total mRNA in each total RNA sample, the intensity of the β-actin cDNA in each sample was measured with the ImageJ software. The amount of total RNA was then adjusted to produce the same intensity as β-actin in one of the PBS-injected samples. The mRNA expression of the antimicrobial peptides was determined in these adjusted total RNA samples. The same software was used to measure the relative expression levels of the antimicrobial peptide genes.

The data were analyzed with one-way analysis of variance (ANOVA). A P value <0.05 indicated a significant change in mRNA expression.

RESULTS

The relative transcription of cathelicidin was analyzed in 120 ayu. These were divided into two main groups based on age. The first group (group 1) was divided into four treatment subgroups, injected intraperitoneally with PBS or LPS (1, 10, or 100 μg/fish). The samples were collected at 6 hr, 24 hr and 1 week after injection. A significant difference (P<0.05) was observed at 6 hr (Fig. 1A) after the stimulation of group 1 (young fish) with 10 μg/fish LPS (P=0.028). However, the subgroups stimulated with the other concentrations of LPS showed non-significant differences (P=0.061 at 1 μg/fish LPS; and P=0.055 at 100 μg/fish LPS). Cathelicidin expression differed significantly from the control (P=0.026) 24 hr after the ayu were stimulated with 1 μg/fish LPS. However, cathelicidin expression did not differ from that in the PBS-treated control group (P=0.155 at 10 μg/fish LPS; and P=0.088 at 100 μg/fish LPS; Fig. 1B). One week after stimulation, LPS-treated subgroups showed non-significant difference compared from the control (P=0.618 at 1 μg/fish LPS; P=0.590 at 10 μg/fish LPS; and P=0.129 at 100 μg/fish LPS; Fig. 1C). Group 2 (sexually mature fish) was divided into four subgroups, injected intraperitoneally with PBS or LPS (1, 10 or 100 μg/fish). After 6 hr, the transcription of cathelicidin did not differ significantly in any LPS-treated subgroup from that in the PBS-treated subgroup (Fig. 2A). This was also true at 24 hr after stimulation (Fig. 2B) and at 1 week after stimulation (Fig. 2C).

The relative transcription of hepcidin was also analyzed in the same 120 cDNA samples used to analyze cathelicidin expression. In group 1, the expression of hepcidin differed significantly in the LPS-treated subgroups from that in the control subgroup at 6 hr after stimulation with different concentrations of LPS (P=0.0027 at 1 μg/fish LPS; P=0.0026 at 10 μg/fish LPS; and P=0.0031 at 100 μg/fish LPS; Fig. 3A). However, at 24 hr and 1 week after LPS stimulation with LPS, the expression of hepcidin did not differ significantly between the treated and control groups (Fig. 3B and 3C). In group 2, there was no significance difference between any of the LPS-treated subgroups and the control group 6 hr after stimulation (Fig. 4A). However, 24 hr after LPS stimulation, the expression of hepcidin decreased significantly from that of the control when the ayu were stimulated with 10 μg/fish LPS (P=0.038) or 100 μg/fish (P=0.0338). However, 1 μg/fish LPS caused no significant difference in hepcidin expression (P=0.71; Fig. 4B). One week after LPS stimulation, hepcidin expression only differed significantly in fish treated with 100 μg/fish LPS (P=0.012), and no other LPS concentration significantly affected its expression (Fig. 4C).

The relative transcription of the cathelicidin gene when PBS was injected into young and sexually mature ayu (15 fish in each group) was plotted with the methods described above. Cathelicidin gene expression decreased slightly at 6 hr, 24 hr and 1 week after the injection of PBS in both groups 1 and 2 (Fig. 5A). Cathelicidin gene expression in the young fish (group 1) was higher than in the sexually mature fish (group 2). Hepcidin gene expression was measured in the same fish and was slightly lower at 6 hr than at 0 hr. In group 1, hepcidin expression differed significantly at 1 week from that at 0 hr. When hepcidin gene expression was compared between the young ayu (group 1) and sexually mature ayu (group 2), it was more strongly expressed in the sexually mature fish (Fig. 5B).

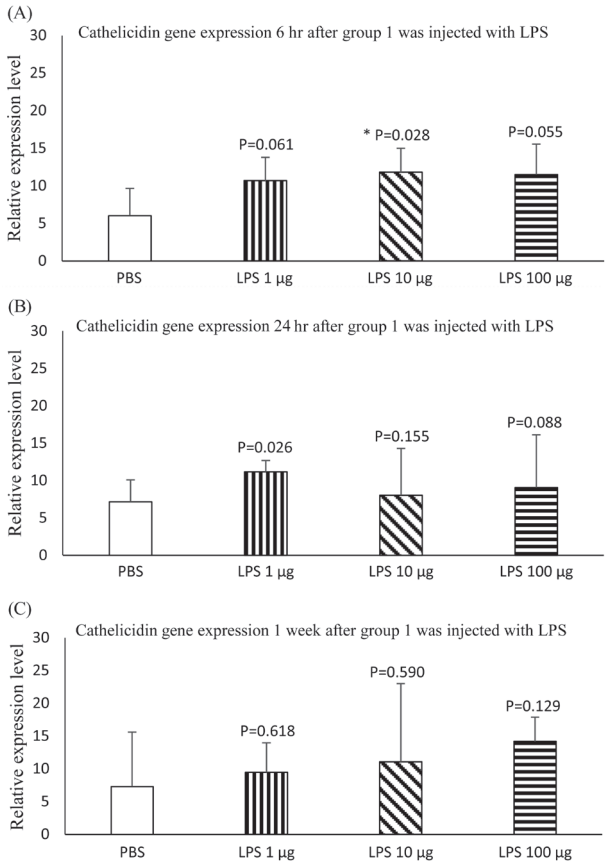

Fig. 1. Relative expression of the cathelicidin gene was measured with semiquantitative RT–PCR and the ImageJ software. Expression levels were normalized to β-actin gene expression. Each bar represents the mean ± SD (n=5). *$P \leq 0.05$ compared with PBS treatment. (A) 6 hr after group 1 was injected with PBS or LPS (1, 10 or 100 μg/fish); (B) 24 hr after group 1 was injected with PBS or LPS (1, 10 or 100 μg/fish); and (C) 1 week after group 1 was injected with PBS or LPS (1, 10 or 100 μg/fish). In Fig. 1C, there is no significant difference between any columns.

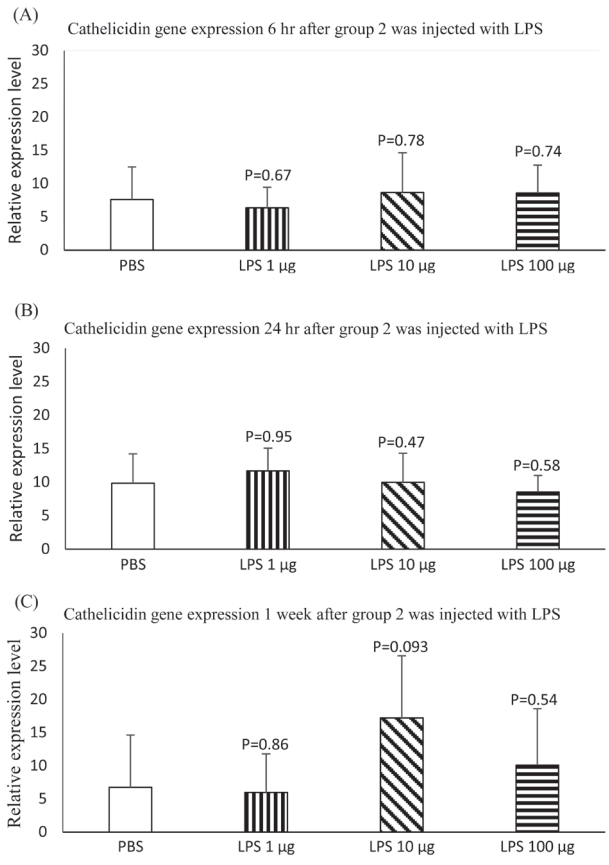

Fig. 2. Relative expression of the cathelicidin gene was measured with semiquantitative RT–PCR. Each bar represents the mean ± SD (n=5). *$P \leq 0.05$ compared with PBS treatment. (A) 6 hr after group 2 was injected with PBS or LPS (1, 10 or 100 μg/fish); (B) 24 hr after group 2 was injected with PBS or LPS (1, 10 or 100 μg/fish); and (C) 1 week after group 2 was injected with PBS or LPS (1, 10 or 100 μg/fish). There were no significant differences between any columns.

DISCUSSION

Cathelicidin transcripts are induced by pathogens and pathogen components, such as LPS, in a time- and species- dependent manner [8, 54]. We observed the differential regulation of ayu cathelicidin transcripts in response to different concentrations of LPS in different stages of the fish life cycle. In group 1 (sexually immature fish), cathelicidin expression was significantly upregulated 6 hr after stimulation with 10 μg/fish LPS. These observations were predictable, because young fish mainly rely on innate immune mechanisms during the first weeks or months of their development [72]. However, it suggests that antimicrobial peptides are constitutively expressed or alternatively, that their expression is induced by receptor stimulation, causing the upregulation of the antimicrobial peptide gene, or indirectly when a stimulus leads to the synthesis of proteins that then induce the transcription of antimicrobial peptide genes [16, 43]. However, the high concentrations of LPS required for such stimulation (1–100 μg/fish) may explain the resistance of ayu to LPS. It was noted also by Berczi *et al.* in 1966 [4], who reported that fish and amphibians are resistant to the toxic effects of LPS. This resistance to LPS may be attributable to a lack of toll-like receptors (TLRs) in some fish species. However, other studies have demonstrated the presence of two TLR4 orthologues in the zebrafish (Cypriniformes), an ancient teleost fish, but its absence in the more evolutionarily advanced puffer fish (*Tetradon* and *Fugu*, both Tetraodontiformes) [75]. There is still no evidence that the ayu contains TLR genes, so further research is required. Cathelicidin mRNA expression in the fish of group 1 treated with 10 μg/fish or 100 μg/fish LPS did not differ significantly from the control 24 hr after stimulation, although those two concentrations caused a rapid albeit temporary increase in cathelicidin transcription at 6 hr. This may indicate a time-dependent response. The delayed upregulation of cathelicidin expression by 1 μg/fish LPS (after 24 hr) may be attributable to the low dose injected, which only elicited a response after it had accumulated [8, 11]. One week after stimulation,

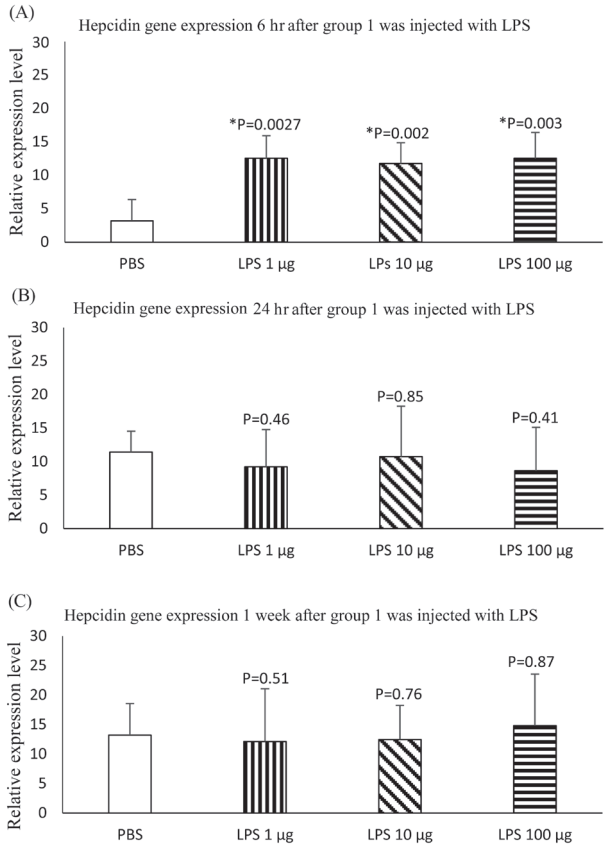

Fig. 3. Relative gene expression of hepcidin was measured with semiquantitative RT–PCR. Each bar represents the mean ± SD (n=5). *$P \leq 0.05$ compared with PBS treatment. (A) 6 hr after group 1 was injected with PBS or LPS (1, 10 or 100 μg/fish); (B) 24 hr after group 1 was injected with PBS or LPS (1, 10 or 100 μg/fish); and (C) 1 week after group 1 was injected with PBS or LPS (1, 10 or 100 μg/fish).

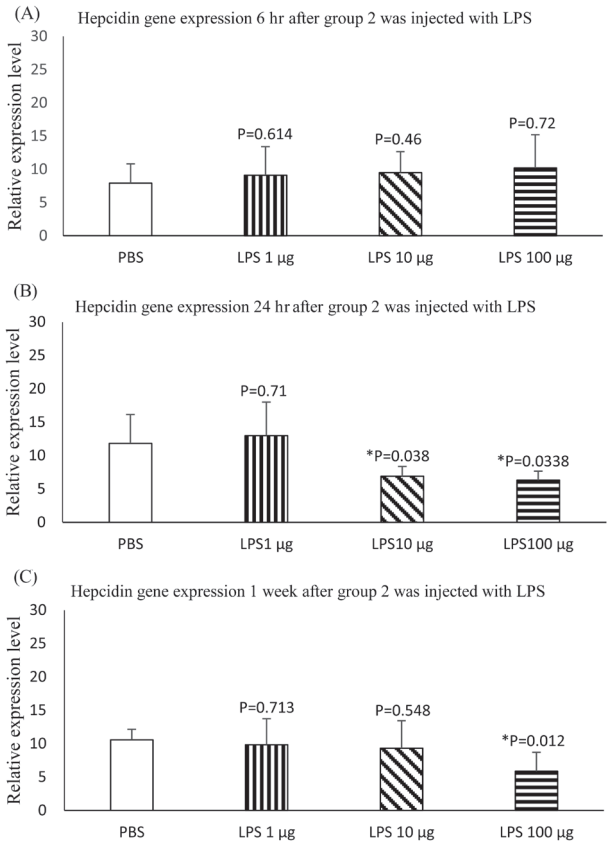

Fig. 4. Relative gene expression of hepcidin was measured with semiquantitative RT–PCR. Each bar represents the mean ± SD (n=5). *$P \leq 0.05$ compared with PBS treatment. (A) 6 hr after group 2 was injected with PBS or LPS (1, 10 or 100 μg/fish); (B) 24 hr after group 2 was injected with PBS or LPS (1, 10 or 100 μg/fish); and (C) 1 week after group 2 was injected with PBS or LPS (1, 10 or 100 μg/fish).

the fish in group 1 showed noticeable but non-significant up regulation of cathelicidin expression, and an increased in standard deviation within the same subgroup, indicating an increase in the variance of the response between the individuals of the same subgroup. This delayed response may be attributable to a negative feedback loop in which the cells protect themselves from high concentrations of antimicrobial peptides, which may damage it [8, 77]. The fish in group 2 (mature fish, 100% sexually mature) showed non-significant changes in cathelicidin expression in response to different concentrations of LPS at 6 hr, 24 hr and 1 week after stimulation. These changes may be age dependent, as observed by Menard *et al.* in 2008 [58]. They reported high constitutive expression of cathelin-related antimicrobial peptide (CRAMP) in the small-intestinal epithelia of neonatal mice, a site at which cathelicidin is not normally expressed in the adult. Peptide expression was limited to the first 2 weeks after birth and gradually disappeared with stem-cell proliferation and epithelial-cell migration. However, the expression of cathelicidin in adult fish is highly variable. Until now, the ayu was thought to have a single cathelicidin gene and appears to express a cathelicidin-1-type peptide [37]. The gene has the four exon/three intron genomic structure of mammalian cathelicidin [11, 53].

Many factors influence the regulation of hepcidin transcription throughout the life stages of the ayu, including age, reproduction stress and the strength of the stimulus [20, 24, 93]. In this study, we clarified the response of hepcidin mRNA expression after challenge with different doses of LPS (injected intraperitoneally) in different life stages of the ayu. The sexually immature fish showed significantly upregulated hepcidin expression 6 hr after stimulation with 1 μg/fish, 10 μg/fish or 100 μg/fish LPS, confirming the involvement of hepcidin in the fish's immune defenses against microbes, because hepcidin plays an important role in the immune response to microbial agents, such as viruses and bacteria [55, 69, 79]. This early and rapid increase in hepcidin depended predominantly on the age of the fish. Our observations are also largely consistent with other observations of the Japanese flounder. The expression of the *Hep-JF1* and *Hep-JF2* genes in the flounder liver was strongly enhanced by 4 and 10 injections of LPS, respectively. The response to stimulation was higher at 6 hr than at 3 hr after LPS injection [31]. The transcription of these genes also increased 2–3-fold 3 hr after LPS injection or challenge with *Vibrio anguillarum* [15]. The upregulation of hepcidin expression after the *in vivo* injection of LPS or bacterial challenge has also been observed in the mouse [67], catfish [3], black

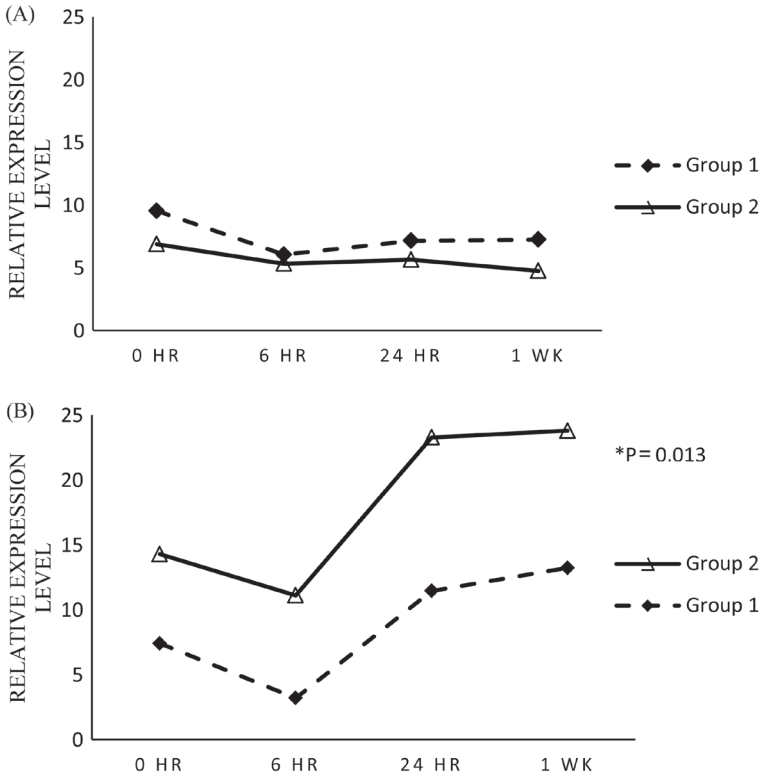

Fig. 5. (A) Relative expression of the cathelicidin gene was measured with semiquantitative RT–PCR. Expression levels were normalized to β-actin gene expression at 0, 6, 24 hr and 1 week. Each point represents a mean ± SD ($P \leq 0.05$) compared with expression at 0 hr within the same group. There was no significant difference at any time point. (B). Relative expression of the hepcidin gene was measured with semiquantitative RT–PCR. Expression levels were normalized to β-actin gene expression at 0, 6, 24 hr and 1 week. Each point represents a mean ± SD ($P \leq 0.05$) compared with the expression at 0 hr within the same group.

porgy [96] and tilapia [33], but the manner of regulation differs according to the species. Group 1 showed no significant change in hepcidin expression at either 24 hr or 1 week after stimulation with LPS. The results of this study imply a direct relationship between fish hepcidin transcripts and infection or inflammation, based on the upregulated hepcidin mRNA expression in the livers of young ayu. These observations agree well with a report that hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) showed an increase in hepcidin mRNA expression of about 4,500-fold relative to the control level after experimental infection with the fish pathogen, *Streptococcus iniae* [79]. The expression of hepcidin mRNA in the liver of the sea bass (*Dicentarchus labrax*) increased in response to bacterial infection, despite the anemia observed in the infected group [71]. LPS probably acts on macrophages, including hepatic Kupffer cells, to induce the production of interleukin 6 (IL-6), and this cytokine in turn induces the production of hepcidin in hepatocytes [61].

The fish in group 2 (fully sexually mature adult fish just before spawning) showed no significant change in hepcidin expression 6 hr after stimulation with 1, 10 or 100 μg/fish LPS. However, its expression was significantly down regulated 24 hr after stimulation with 10 μg/fish or 100 μg/fish LPS. This down regulation persisted for 1 week after stimulation with 100 μg/fish LPS. This temporal delay in the down regulation of hepcidin expression may be regulated by several factors, including age and reproductive stress, in a time- and dose-dependent manner, and by the characteristic semelparity of the ayu. Some fish species stop feeding several weeks or months before spawning, and the reproductive system develops at the expense of body tissues [44]. To increase their reproductive success, individuals may be forced to divert resources from traits, such as immunity to their reproductive effort [30, 49, 62, 76].

During our study, the fish were fed normal amounts throughout the experimental period, because we tried to reduce the stress of natural starvation on the ayu. Immunosuppression is expected to occur during this period of sexual maturity, especially with the increases in stress-induced steroid hormones and sex hormones, which are potent immunomodulators in fish [29]. Their effects on immunosuppression in mammalian species are well documented [26]. For example, in fish (*Barbatula barbatula*), parasite loads show parallel seasonal changes with changes in the gonads. A similar pattern was observed in *Rutilus rutilus*, where the prevalence and abundance of *Gyrodactylid monogenean* parasites also peak in the spring, during the spawning period [80]. Cortisol, the major stress hormone in teleost fish, has a predominantly suppressive effect on the immune defenses [17, 63, 95]. For example, it directly causes the programmed cell death of leukocytes [73]. When injected experimentally into the grass carp (*Ctenopharyngodon idella*), cortisol reduced the phagocytosis of the head kidney macrophages, the relative mass of the spleen, lysozyme activity in the serum

and resistance to *Aeromonas hydrophila* [90]. These fact are consistent with the downregulation of hepcidin in the sexually mature ayu before spawning and the stress of reproduction. The main androgen in teleost fish is 11-ketotestosterone [5]. A study in 1995 characterized the receptors that respond to testosterone in *O. mykiss* leukocytes and suggested that they are an important link in testosterone-mediated immunosuppression [81]. Since then, several studies have confirmed that testosterone exerts suppressive effects on both the innate and specific immune defenses in salmonids [9, 32, 82]. In a recent study, 11-ketotestosterone was shown to suppress the immune functions and increase oxidative stress in the three-spined stickleback (*Gasterosteus aculeatus*) [42]. Similar results were obtained in *Cyprinus carpio*, in which 11-ketotestosterone (among other steroids) suppressed phagocytosis and the production of the superoxide anion and nitric oxide in kidney macrophages [92].

In conclusion, the transcription of ayu antimicrobial peptide genes is regulated *in vivo* in a time-, dose- and age-dependent manner when different concentrations of LPS are injected intraperitoneally into ayu of different ages.

Further research is required to determine the epithelial cell specificity and posttranscriptional regulation of hepcidin as a bactericidal agent at local sites of infection.

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