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Identification, characterization and expression in response to *Aeromonas hydrophila* challenge of five interferon regulatory factors in *Megalobrama amblycephala*

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ABSTRACT

Interferon regulatory factor (Irf) family represents one of the most important transcription factor families, with multiple biological roles. In this study, we characterized five Irf family members (*irf4a, irf4b, irf6, irf8 and irf10*) in *Megalobrama amblycephala* at the cDNA and (predicted) amino acid levels, analyzed them phylogenetically, and developed gene-specific primers for qPCR analysis. All five *irfs* were constitutively expressed in all examined tissues, but their transcription was significantly higher in lymphoid organs and tissues, such as kidney, spleen and intestine. Exceptions were *irf8*, which was expressed at a high level in heart and brain tissues, and *irf6*, expressed at low levels in most tissues. After a bacterial immune challenge with *Aeromonas hydrophila,* the expression of *irfs* in liver was up-regulated: *mairf4a* 8.12-fold, *mairf4b* 29.9-fold, *mairf6* 1.38-fold and *mairf10* 1.65-fold (*mairf8* was an exception: 0.07-fold). In spleen, kidney, intestine and gills, transcript levels of studied *irfs* increased only at specific time-points. The results suggested that irfs are involved in the immune response to bacterial infection in *M. amblycephala*, which will help elucidate the biological functions of irfs in the immune system of teleost fish.

1. Introduction

Interferon regulatory factors (Irfs) were originally identified as proteins binding to DNA elements, serving as transcription regulators of interferon expression [1]. Over the last few decades, they have been the focus of many immunological and medical studies [2,3], which revealed that they have diversified functions in immune responses, hematopoietic differentiation and immune modulation [4]. They are found in the cytoplasm or nuclei of various types of cells and play an important role in anti-viral defense, innate and adaptive immunity, cell development, and oncogenesis [5]. Since the identification of the first Irf (Irf1), a total of eleven family members have been identified in vertebrates [6,7]. These irfs are currently classified into the following four subfamilies: Irf1 (Irf1, 2 and 11), Irf3 (Irf3 and 7), Irf4 (Irf4, 8, 9 and 10), and Irf5 (Irf5 and 6) [8]. Originally, studies have largely focused on functions of Irfs in mammals [9], but in recent years Irfs are increasingly investigated in other classes of vertebrates. In fish, a few Irf family members, such as Irf3, Irf4a, Irf4b and Irf7, have been identified in some aquacultured species [10,11], eleven were identified in zebrafish [12], and thirteen in grass carp [13].

These transcription factors have a unique 'tryptophan cluster' DNA-binding domain (DBD) [14], which possesses five conserved tryptophan repeats and is responsible for binding to the IFN-regulatory factor element present in the IFN-β promoter [15,16]. Except Irf1 and Irf2, they all have an IRF-associated domain (IAD) at the C terminus, which is responsible for mediating protein-protein interactions of homodimers or heterodimers with other Irfs or other transcription factors [17]. Some Irfs also have a repression domain or a nuclear-localization signal [18].

Putative *irf1* to *irf11* homologues have been found in zebrafish [12], but their functions in teleost fish remain poorly understood. The expression of *irf4* and *irf8* is restricted to hematopoietic cells of both lymphoid and myeloid lineages [19]. Whereas the functions of Irf8 in the myeloid lineage have been described in detail [20,21], functions of Irf4 in the myeloid lineage are not well characterized. In early reports, the expression of *irf4* was observed only in lymphocytes, but later studies found that it is also expressed in macrophages [22]. It was reported that the stimulation of macrophages with LPS induces translocation of Irf4 from the cytosol to the nucleus [23]. Irf4 has critical roles

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in the development and function of B and T cells [24]. Functional characterization of Irf6 is also incomplete, but it is known that mutations in this gene can lead to the autosomal dominant Van der Woude syndrome orpopliteal pterygium syndrome in mammals [25]. However, Irf6 appears to have some unique functions in fish: whereas in mammals it does not affect the production of interferons, fish Irf6 is a positive regulator of interferon transcription [26]. Functionally, Irf10 is similar to Irf1, which regulates the expression of guanylate-binding protein (GBP) and major histocompatibility complex (MHC) class I or II, but in chicken, Irf10 may selectively regulate type II over type I IFN target genes. Therefore, Irf10 might also have unique functions in the immune responses of fish [27,28].

In the cultivation of *Megalobrama amblycephala* (blunt snout bream), the most harmful bacterial infections are caused by *Aeromonas hydrophila,* which often causes grave extraintestinal infections and enteritis [29]. Identification of immunity-related genes shall improve our understanding of the fundamentals of immune responses to infection by *A. hydrophila* in fish. Therefore, in this study we cloned five *irf* family genes from the genome of *M. amblycephala*, characterized the structure of these genes and their deduced amino acid sequences, and investigated their constitutive expression in different tissues, as well as their expression after *A. hydrophila* infection.

2. Materials and methods

2.1. Fish and bacterial challenge

Over 100 juvenile *M. amblycephala* specimens (weight: 100 ± 10g) were obtained from the Tuanfeng fish farm (Hubei province) and kept in circulating freshwater system for one to two weeks in the Huazhong Agricultural University (Wuhan, Hubei, China). The fish were fed twice daily, at 8:30am and 6:30pm. The amount of feed was regularly adjusted according to the amount of uneaten feed, but generally 2.0–3.0% of the body weight fish. Water temperature, oxygen content, nitrites and total ammonia nitrogen (TAN = NH3-N + NH4+-N) in the tanks were controlled: 25 ± 2 °C, > 8 mg O_2/L, < 0.1 mg NO_2-/L, < 0.1 mg TAN/L, respectively. All animal experimental protocols were performed following the guidance of the Animal Welfare and Experimental Animal Regulation of the Hubei Province.

Aeromonas hydrophila colonies were isolated in our laboratory, and the experimentally determined median lethal concentration (LD50) was 1×10^7 CFU/mL. After the acclimation period, healthy fish were selected for bacterial challenge: the fish were divided into control (n = 30), trial (n = 50) and blank (n = 30) groups. Each fish in the trial group received an intraperitoneal injection of 0.1 mL 1×10^7 CFU/mL of bacterial suspension, while the control group fish were injected 0.1 mL of sterile phosphate buffer saline (PBS, pH 7.4), and fish in the blank group were untreated. After treatment, all the fish were returned to tanks. At 6, 12, 24, 48, 72 and 120 h after the injection (hpi), three specimens were collected from each group (at each time-point), euthanized using 300 mg/L methanesulfonate (MS222; Sigma-Aldrich, USA). The liver, spleen, kidney, intestine, and gill tissues were collected immediately after dissection, flash-frozen in liquid nitrogen, and stored at −80 °C. To determine constitutive expression in a broader range of tissues, four additional tissues, blood, heart, muscle and skin, were also collected from three specimens from the blank group in the same way. Blood samples were collected from caudal veins using 1 mL sterile injections and preserved in RNAiso Plus (TaKaRa, Dalian, China).

2.2. Identification and cloning of irf genes

BLASTN and TBLASTX programs were employed to use zebrafish and grass carp *irf* homologs to search the whole genome [30] and transcriptome [31,32] of *M. amblycephala* to identify homologous genes. To confirm the genes predicted by genome and transcriptome searches, we designed primers to amplify these sequences (Table 1).

Table 1
Primers used for PCR analyses in this study.

Primer name	Sequence (5′-3′)	Target gene	
Irf4a-F	AGGAAGTCACCACCAACAGC	*Irf4a*	q-pcr
Irf4a-R	GGTATGGGAAGGGAAAGAGC		
Irf4b-F	GAACTGCAAACTGCTGGACA	*Irf4b*	
Irf4b-R	CTTCTTGGCCTTTGAGCATC		
Irf6-F	TGGAGACCGGGAAGTATCAG	*Irf6*	
Irf6-R	TCATGGGAACCTCTTTGGTC		
Irf8-F	TGAATACATGGGCATCCTGA	*Irf8*	
Irf8-R	CAGCATTGTGAGATCCTTGC		
Irf10-F	ACCTGGAGAAAGGTGTGCTG	*Irf10*	
Irf10-R	TCTGTCTGTGCTCTGCCAAT		
β-actin-F	ACCCACACCGTGCCCATCTA	*β-actin*	
β-actin-R	CGGACAATTTCTCTTTCGGCTG		
Irf4a-F	ATGAACTTAGATGGGGACTGCA	*Irf4a*	Gene
Irf4a-R	TTACTCTTGCAAGTGTTGGATGC		
Irf4b-F	ATGTGTTCGGATGAAGAGCGC	*Irf4b*	
Irf4b-R	TTATGGTGATGCGTAATGACTCT		
Irf6-F	ATGTCGTCTCATCCGCGTC	*Irf6*	
Irf6-R	TCACTGCGTCTGCAGGGC		
Irf8-F	ATGAATCCGGGTGGCCG	*Irf8*	
Irf8-R	TCAGACTGGAATTGGCAGGT		
Irf10-F	ATGGAAGACAGGTCGAGGCA	*Irf10*	
Irf10-R	CTAATCATTGGTTTTCCTGTGTGG		

These PCR products were ligated into PMD-18T vector (Takara, China) for sequencing. Full-length sequences of the *irfs* were assembled using SeqMan software.

2.3. Expression analysis of irfs

The liver, spleen kidney, intestines, gills, blood, heart and brain were collected to determine the basal (or constitutive) expression of *irfs*. The expression of *irfs* after the bacterial challenge was studied only in the liver, spleen, kidney, intestine and gills. Quantitative real-time PCR (qPCR) was performed in a LightCycler®480 II (Roche Diagnostics GmbH, USA). Primers (Table 1) were designed using Primer Premier 5 software (Premier Biosoft, USA) and synthesized by the Sangon Biotech (Shanghai). The primers amplified fragments that spanned introns. qPCR was conducted in a 20 μL reaction volume, containing 10 μL of LightCycler® 480 SYBR Green I Master, 6 μL ddH_2O, 1 μL of forward and reverse primer (each), and 2 μL of cDNA template (5-fold diluted). Reactions were performed in triplicate for each sample. *β-actin* was chosen as the internal reference gene on the basis of zebrafish studies [33] and our previous *M. amblycephala* studies [34,35], where *β-actin* was consistently shown to be the most stable reference gene. Gene expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method [36]. Data were calculated as mean ± SD using Microsoft Excel and IBM SPSS 19.0 (SPSS Inc., USA). The significance of differences among samples were analyzed by one-way analysis of variance (ANOVA) using LSD (Least Significant Difference), with $P < 0.05$ chosen as the statistical significance threshold.

2.4. Sequence analyses and phylogenetic analysis

Similarity with other *irf* homologs available in the GenBank was determined using BLASTX and BLASTP tools available from the NCBI (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi/). Putative amino acid sequences were predicted using NCBI's ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Nuclear Localization Sequence (NLS) prediction was performed using PSORTII web server (http://psort.hgc.jp/form2.html). Physicochemical properties of the putative polypeptides were determined by Expasy's ProtParam prediction server (http://web.expasy.org/protparam/). The signal peptide sequences were identified by SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP-3.0/). Secondary and tertiary structures of protein domains

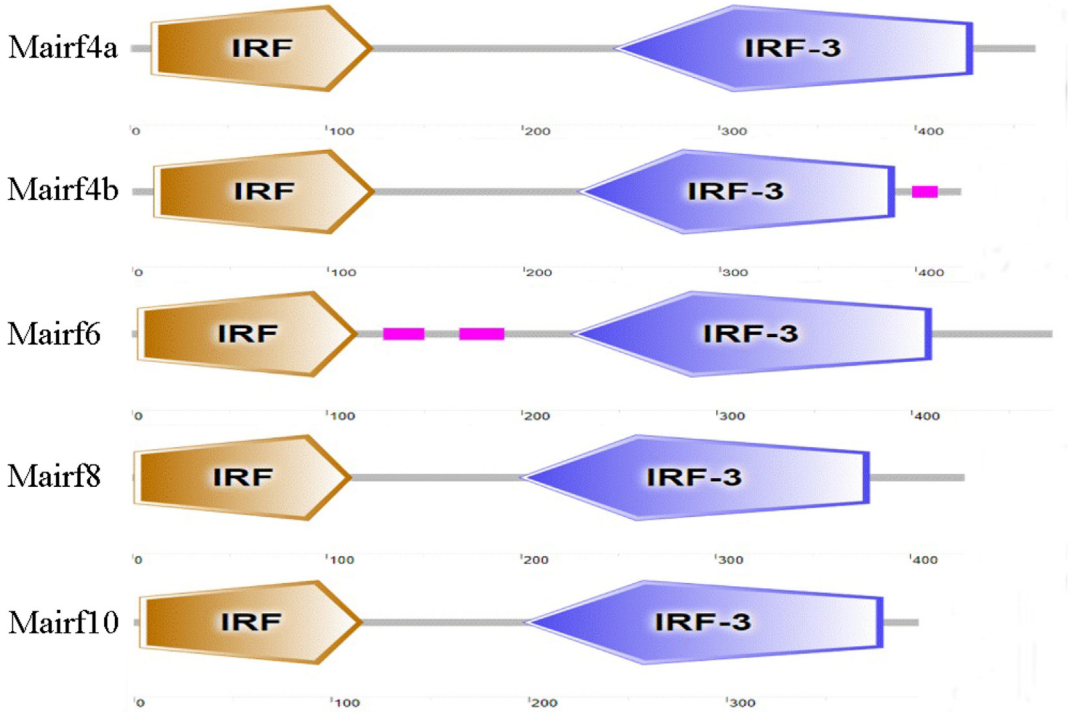

Fig. 1. Domain architecture of the IRFs of *M. amblycephala*: IRF domain (light brown pentagon), low-complexity region (pink square) and IRF-3 domain (blue pentagon). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were predicted using NPS@ (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html), SMART, SWISS-MODEL and NetPhos web tools. Transmembrane domains were identified using TMpred server (https://embnet.vital-it.ch/software/TMPRED_form.html/). Multiple sequence alignments of polypeptide sequences were performed by BioEdit. Phylogenetic tree of the predicted amino acid sequences was generated using the Neighbor-Joining method in Mega 7.0 program.

3. Results

3.1. Identification and comparative analyses of mairfs

Five interferon regulatory factor genes were identified in the transcriptome of *M. amblycephala*: *mairf4a* (MH998234), *mairf4b* (MH998235), *mairf6* (MH998236), *mairf8* (MH998237) and *mairf10* (MH998238) (Fig. S2). To corroborate the identification of these *mairfs*, a phylogenetic analysis with vertebrate homologues was conducted, with all five genes clustering within their corresponding families (Table S5 and Fig. 3). Among the studied bony fish homologues, genes (nucleotides) exhibited the highest sequence similarity to grass carp (98–99%) and zebrafish (79–88%) *irf* orthologs (Table S1). The N-terminal sequence similarity was higher than that of the full-length sequence, from 90% to 100% (Table S1). Amino acid sequence similarity between the full-length Mairf proteins and teleost and mammalian Irf orthologs was also very high: from 96% to 100% (Table S2). Among the different *irf* family members, sequence similarity was low, ranging from 30% to 56% (Table S3). The highest similarity was found between *mairf4a* and *mairf4b* paralogs. Except *mairf6* (seven exons), other genes all have eight exons.

3.2. Protein comparison and phylogenetic analysis

Domain architecture differed among the inferred MaIrf peptides: Mairf4a had one putative vacuolar targeting motif; maIrf4b had one NLS; MaIrf6 had one cleavage site for mitochondrial presequence and two NLSs; MaIrf8 had one cleavage site for mitochondrial preseq, one NLS and an IRF family signature motif (DNA-binding motifs, which can be useful to distinguish between nuclear proteins); and MaIrf10 had one NLS, two ER Membrane Retention Signals (XXRR-like motif in the N-terminus: EDRS, and KKXX-like motif in the C-terminus: RKTN), and one IRF family signature (DNA-binding) motif. Nucleus was the predicted locality for all proteins, and no signal peptides were predicted. Only MaIrf8 may have a transmembrane domain (the remaining MaIrfs probably do not have one). Secondary and tertiary structures of protein domains are shown in Table S4, Figs. 1 and 2.

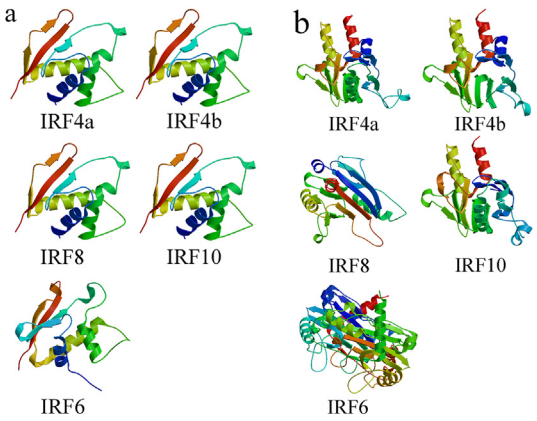

Fig. 2. Tertiary structure of (a) protein DNA-binding domains (DBD); and (b) IRF-associated domains (IAD); (c) amino acid sequences of the five irfs in the N- to C-terminal direction, where alpha helices are highlighted in purple and beta sheets in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Constitutive expression of mairfs

We used qPCR to investigate the expression of *mairf* genes in liver, spleen, kidney, intestine, gills, blood, heart, muscle and brain. *mairf*

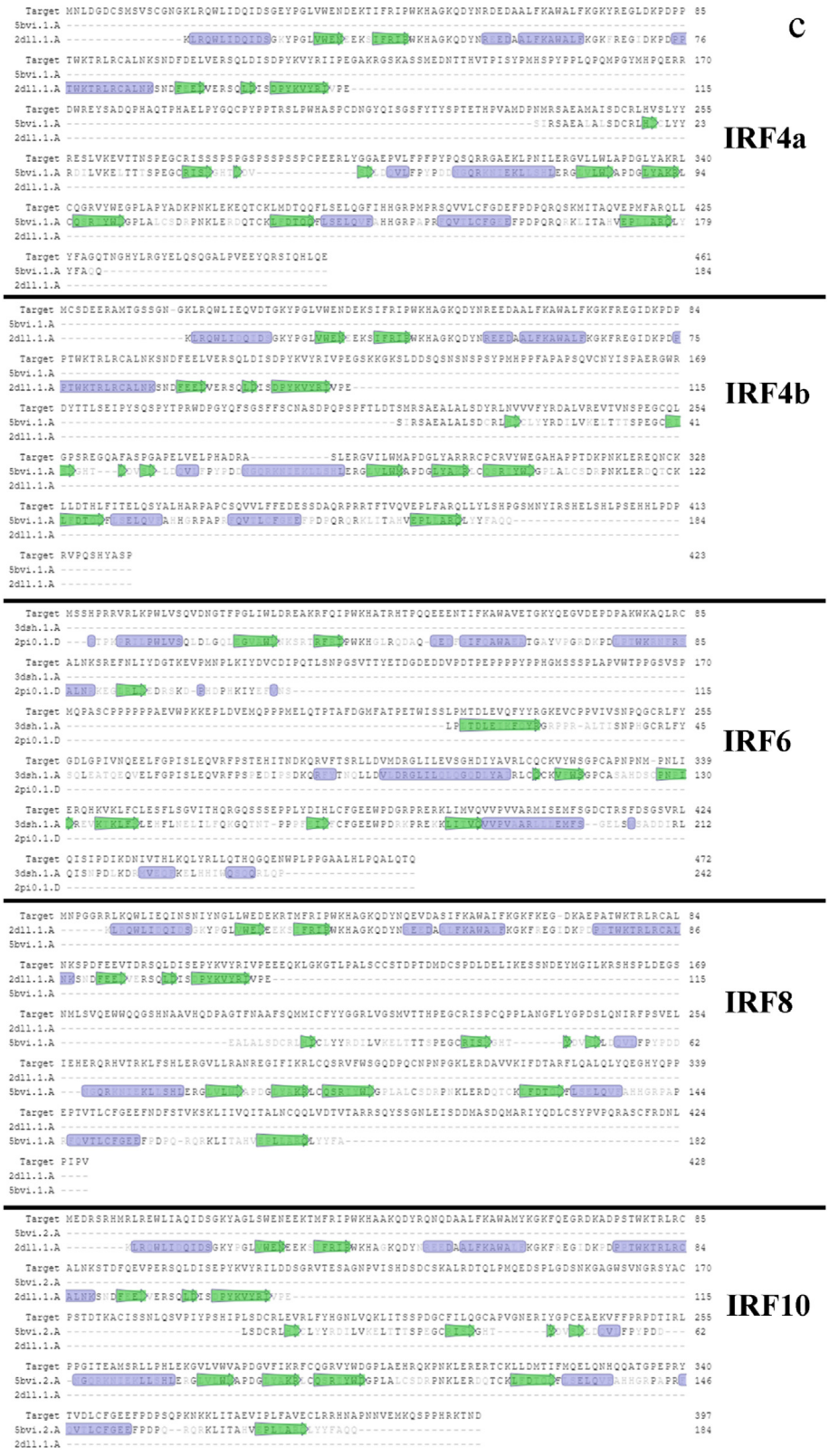

Fig. 2. (*continued*)

mRNAs were detected in all nine different tissues (Fig. 4), which indicates that *mairfs* are constitutively expressed in all examined tissues of healthy *M. amblycephala*. However, levels of expression were different among different *irfs*. *Mairf6* was highly expressed in liver, intestine and gills (1083-, 3295-, and 1297-fold, $P < 0.05$). The highest level of expression of *mairf10* and *mairf4b* was observed in blood and gills respectively. In contrast, the expression of *mairf4a* and *mairf8* was highest in the heart tissue.

3.4. Expression of mairfs upon experimental bacterial infection

To examine the expression patterns of the *mairfs* upon bacterial

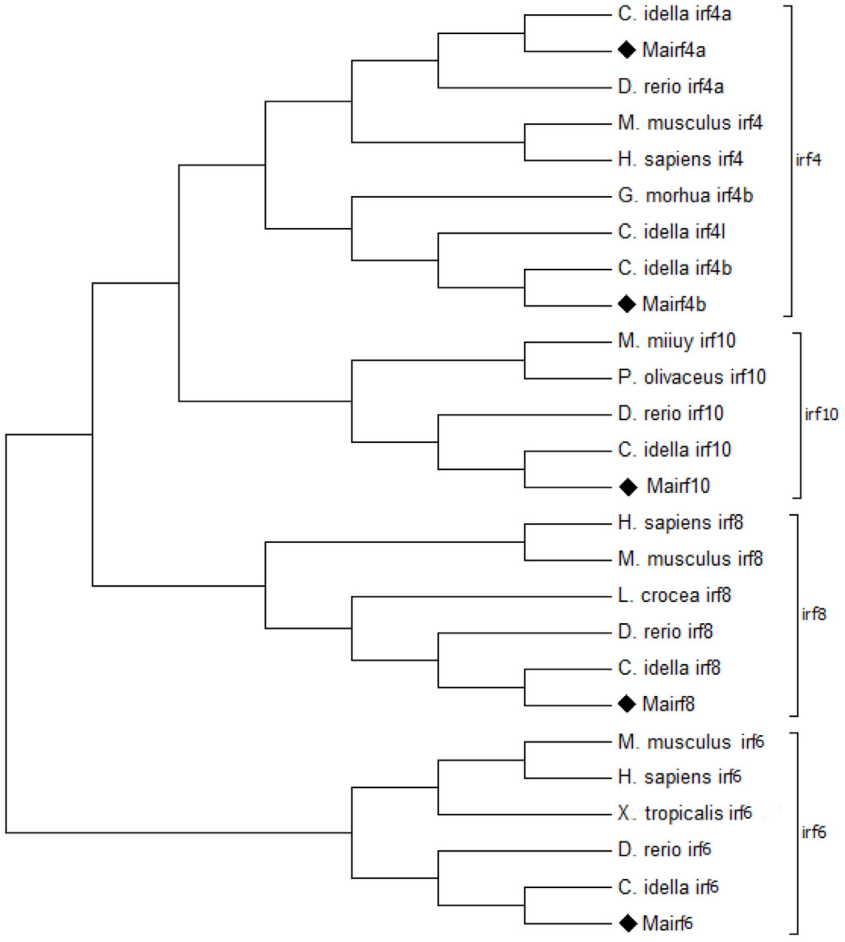

Fig. 3. Phylogenetic analysis of IRF family members in vertebrates. The neighbor-joining phylogram was constructed with MEGA 7.0. GenBank accession numbers are available in Table S3. The studied five blunt snout bream irfs are highlighted by black rhombuses.

infection, the blunt snout bream was challenged experimentally with the bacterial pathogen *A. hydrophila*. The expression of *mairfs* was determined by qPCR at 6, 12, 24, 48, 72 and 120 h post-bacterial infection in liver, spleen, kidney, intestine, and gills of the infected fish (Fig. 5). Expression patterns of the studied *mairfs* were time- and tissue-dependent. Specifically, expression of *mairf4a*, *mairf4b*, *mairf6* and *mairf10* increased in all five examined tissues after the infection with *A. hydrophila*, but peaks occurred at different time-points after the infection. For *mairf4a*, a particularly high expression was observed in kidney; peak expression in liver occurred at 6hpi (8.12- fold); two peaks were observed in spleen, at 6hpi (4.48-fold) and 120hpi (11.65-fold); variable expression was observed in kidney, with major peaks at 6hpi (16.09-fold) and 120hpi (12.42-fold); expression in the intestine was also variable, with a peak at 12hpi (4.04-fold); and in gills an initial down-regulation was followed by a slight upregulation in the later phases, at 48 and 72hpi (3.04-fold). *Mairf4b* was particularly highly expressed in liver, with up-regulation at all studied time-points, peaking at 12hpi (29.96-fold); in spleen and intestine, the expression of this gene was relatively stable until the last studied time-point, 120hpi, when a slight up-regulation was observed; a similar pattern was observed in gills, but the up-regulation was observed earlier, at 48hpi; in kidney, the expression was up-regulated at first three time-points, peaking at 12hpi, and then reverted to the normal level. The expression of *mairf6* was particularly high in the intestine, where an initial down-regulation was followed by a strong up-regulation at 24hpi, peaking at 48hpi (7.19-fold); in liver and spleen, an early up-regulation (6hpi) was followed by down-regulation; and in kidney and gills, up-regulation was observed only at 12hpi. The expression of *mairf10* was down-regulated at 6hpi in all tissues except spleen; maximum expression occurred at 24hpi in liver, spleen and kidney (1.66-fold, 2.30-fold and 2.79-fold respectively), and at 48hpi in intestine (3.75-fold) and gills (1.48-fold). As for *mairf8*, in liver the expression was down-regulated at all time-points, in spleen it exhibited two peaks, at 6hpi (2.12-fold) and 120hpi (3.93-fold); in kidney, an initial down-regulation at 6hpi was followed by a strong up-regulation at 12hpi (2.4-fold), and then again down-regulation at later time-points; expression in the intestine exhibited somewhat similar pattern, with the initial down-regulation at 6hpi (0.07-fold) followed by a gradual increase in the expression, peaking at 48hpi; in gills, the expression exhibited two peaks, at 6hpi (1.36-fold) and 72hpi (1.35-fold).

4. Discussion

The IFN system plays pivotal roles in defense against bacterial infections. Irfs are key regulators that activate the IFN system in response to a pathogen invasion. Irfs, as transcription mediators, also play important roles in apoptosis and cell growth. The number of *irfs* varies in different species, which may be a result of gene duplication, or chromosome rearrangement and gene shuffling; and this variation is particularly pronounced in teleost fishes [6]. For example, *irf10* was not found in mammals (humans and mice), but it has been detected in many teleost fishes. To extend the understanding of *irfs* in teleost fish, we identified and characterized the *irfs* in blunt snout bream, and studied their expression, both constitutive and after an immune challenge.

All of the MaIrfs had similar structure, with two conserved IRF domains, the N-terminus DBD and a C-terminus IAD [37]. The DBD is

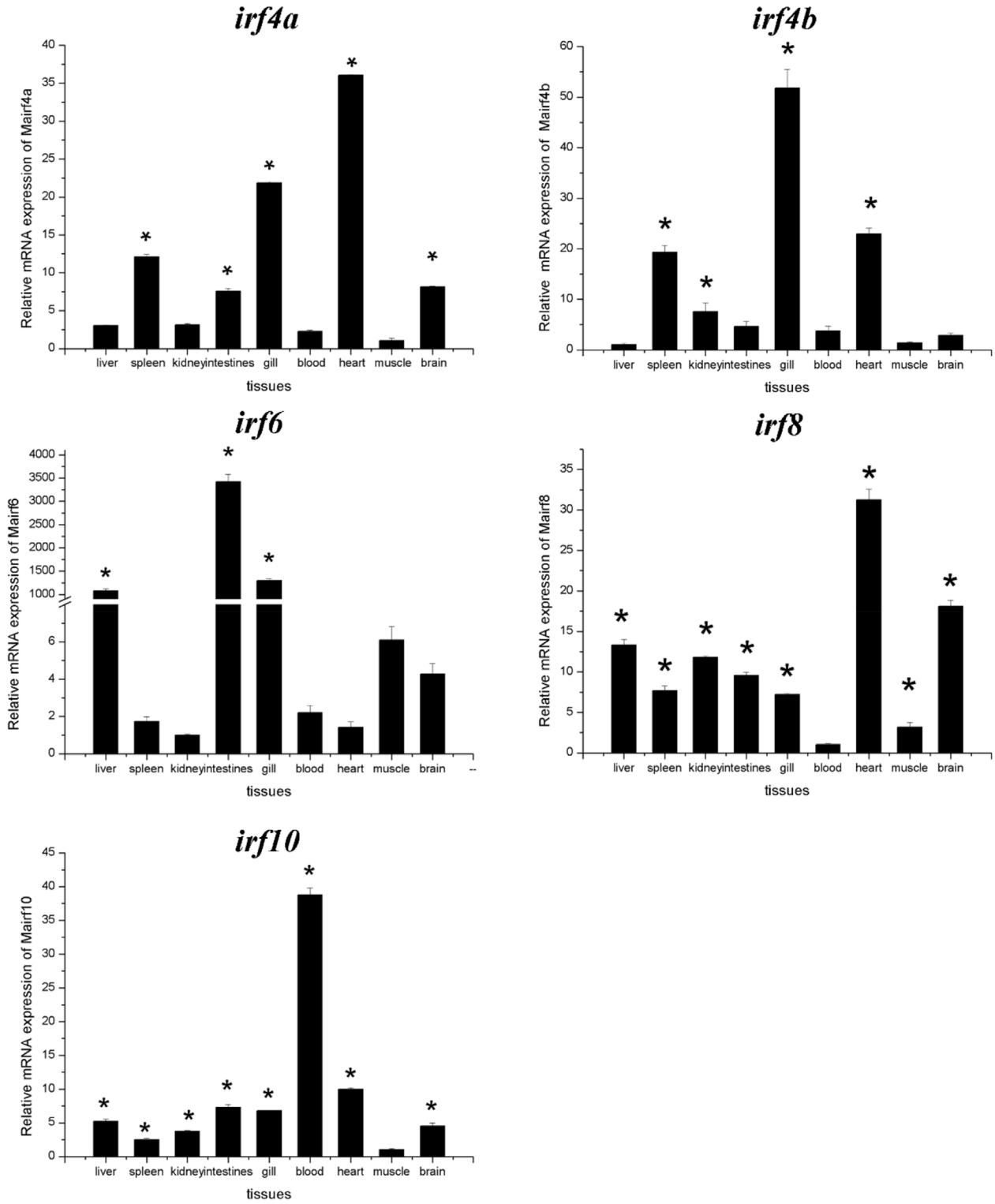

Fig. 4. Constitutive expression of five IRFs (mRNA) in nine tissues of blunt snout bream. The expression was detected by qPCR and normalized to *β-actin*. Vertical bars represent mean ± SD of three technical replicates, and asterisks "*" above the bars represent statistically significant differences from the control samples at the $P < 0.05$ threshold.

typical for all Irf family members and characterized by a series of relatively well-conserved tryptophan-rich repeats forming a helix-turn-helix structure, with the function of binding to the ISRE/IRF-E consensus sequence in target promoters [38]. Similar to other vertebrates, the five studied MaIrfs possess conserved tryptophan residue repeat motifs (a characteristic repeat of five tryptophan residues spaced by 10–18 aa [39]) in the DBD, and association modules for the C-terminal region. All five MaIrfs possessed five tryptophan residues. The highest level of conservation was found for the DBD, indicating that the mode of action of these molecules is likely to be evolutionarily conserved, and that this domain plays a key functional role. Another conserved domain, IAD, is essential the interaction with both homologous and heterologous Irfs, and association with other transcription factors. These two conserved domains were found in all five MaIrfs, which indirectly

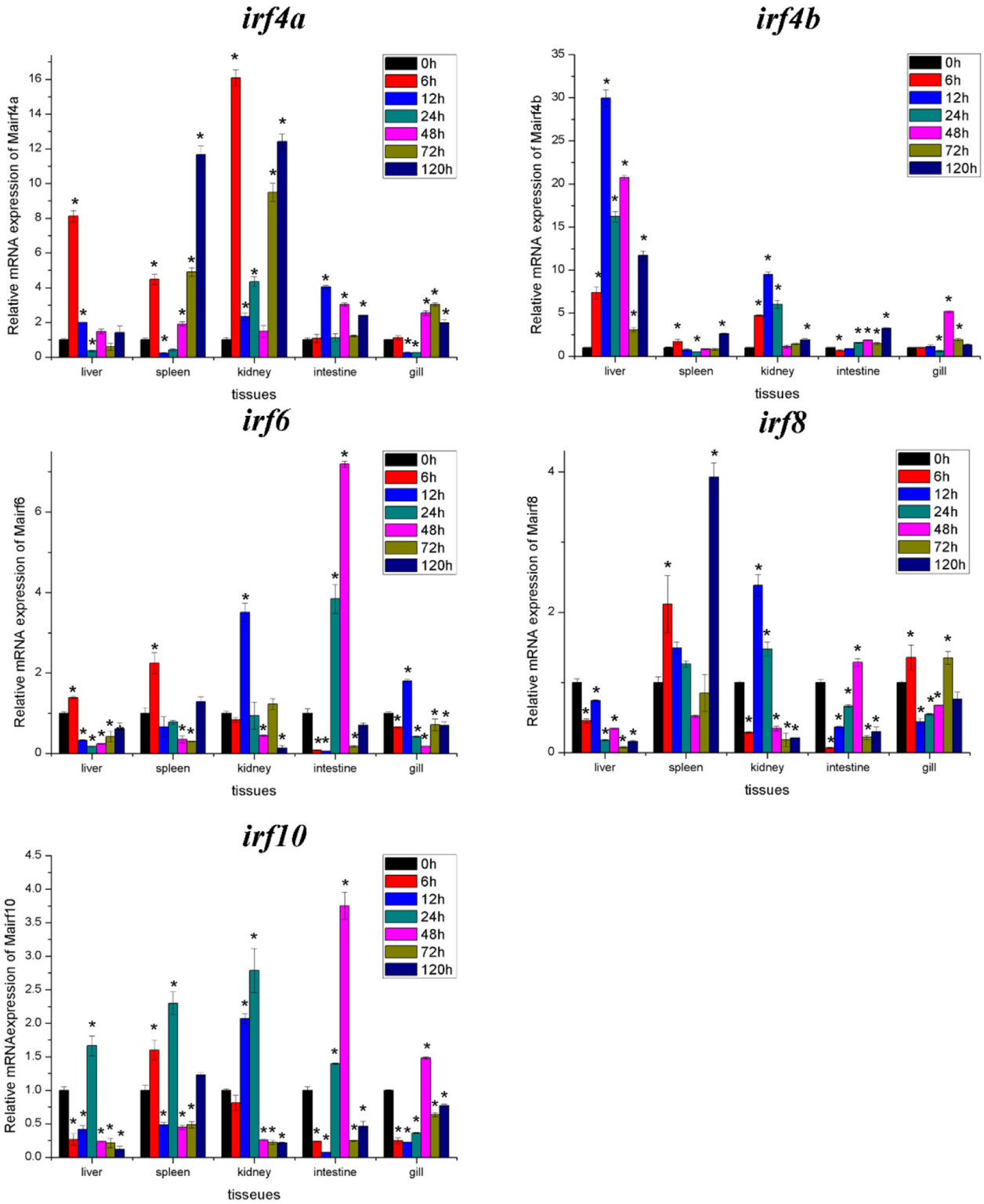

Fig. 5. Transcript expression responses of five IRF family members to *Aeromonas hydrophila* challenge in five tissues of blunt snout bream. The expression was detected by qPCR and normalized to *β-actin*. Vertical bars represent mean ± SD of three technical replicates, and asterisks "*" above the bars represent statistically significant differences from the control samples at the $P < 0.05$ threshold.

corroborates their functionality, as IRF-protein interactions are crucial for the regulation of immune system-related genes [40]. Those proteins all had an interferon-regulatory factor 3 superfamily domain; this chain with hetero-dimeric structure also contains a shorter chain named CREB-binding protein. These two subunits make up the DRAF1 (double-stranded RNA-activated factor 1). Viral dsRNA produced during viral transcription or replication causes the activation of DRAF1 [41]. Multiple alignments also revealed that the functions of MaIrfs might be conserved throughout the vertebrates. Phylogenetic analysis of predicted Irf amino acid sequences in the blunt snout bream and other vertebrates indicates that the evolutionary origin of subfamilies predates the diversification of fish and tetrapods, which in turn indicates

that Irfs in fish may possess similar roles as their counterparts in mammals [19,42].

Tissue expression analysis showed that *mairfs* were constitutively expressed in all studied tissues, but the level of expression varied among the tissues. They were especially strongly expressed in hematopoietic tissues (kidney), lymphoid tissues (gills, intestines and spleen), and blood, all of which are important immune tissues in blunt snout bream. Constitutive expression was also observed in various tissues of other teleosts, such as mandarin fish [43], yellow croaker [44], rainbow trout [19], Atlantic cod [10] and Japanese flounder [27,45]. Similar to zebrafish, *irf4a* and *irf4b* were expressed predominantly in lympho-myeloid tissues, and *irf4b* more highly than *irf4a* [43,46]. In spleen, kidney and gills, the expression of *mairf4b* was higher than that of *mairf4a*. Relatively high levels of *mairf8* in the brain suggest a potential role in both immune and non-immune tissues. In tongue sole, *irf4a* expression (mRNA) was low in heart, *irf4b* expression was low in gills, *irf6* expression was high in muscle, and the expression of *irf8* in heart was relatively low [8]. In mandarin fish, the highest expression of *irf10* was observed in the muscle [43]. In zebrafish, *irf4a* and *irf4b* were highly expressed in spleen and kidney [46]. All these results are different from our observations in blunt snout bream, which suggests that *irf* expression in fish exhibits temporal variations, and that it may be species-specific.

Irfs play important roles in antiviral and antibacterial immunity. Previous studies showed that *irfs* were significantly upregulated after stimulation with poly I:C and LPS, which is the major component of the outer membrane of gram-negative bacteria and the major bacterial mediator of immunopathogenesis [47]. The importance of LPS-induced *irf4/8* expression in myeloid cells is highlighted by the functional importance of each gene in the regulation of pro-inflammatory cytokine expression in LPS-stimulated macrophages and dendritic cells [19,48]. In mammals, Irf4 was identified as a is key hematopoietic-specific transcription factor critical for lymphoid and myeloid lineage development and function, and its expression is thus restricted to immune cells such as T cells, B cells, macrophages, and dendritic cells (DCs) [18,48]. Studies in mammals demonstrated that *irf4* is up-regulated by LPS in purified B/T cells, macrophages, and dendritic cells [49]. *Mairf4a* mRNA expression level was significantly up-regulated at 6 hpi with *A. hydrophila* in liver and kidney, whereas the expression level of *mairf4b* was significantly up-regulated at 12 hpi in liver and kidney. Expressions of *mairf4a* and *mairf4b* were particularly variable in spleen and liver respectively. In the spleen of Atlantic cod, *irf4b* was responsive to stimulation with *Aeromonas salmonicida*, while *irf4a* was not. In rock bream, *irf4* transcript level was significantly down-regulated after a challenge with LPS, *Edwardsiella tarda* and *Streptococcus iniae* [37]. Both pathogens caused a decrease in *irf4* expression in spleen and head kidney at 3hpi, followed by an increase at 12hpi, and then another decrease at the final (48hpi) time point. This suggests that *mairf4b* may share some similarity in function with the putative orthologues in rock bream (*irf4*) and cod (*irf4b*). In zebrafish, two *irf4* paralogues, *irf4a* and *irf4b*, exhibited distinct expression patterns. These results suggest that irf4 paralogs might play different roles in the immune system.

In mammals, Irf6 is as a crucial transcriptional regulator of keratinocyte differentiation and controls the switch from proliferation to differentiation by activating differentiation-associated genes. As opposed to the mammalian *irf6*, which is unaffected by the IFN expression, in fish, *irf6* is a positive regulator of the IFN transcription [26]. However, studies in zebrafish indicate that functions of Irf6, specifically the regulation patterns of toll-like receptors (TLRs) and retinoic acid-inducible gene-I-like receptor (RLR) sensors, might be different between fish and mammals [26]. We found that the bacterial challenge induced the expression of *mairf6* in all studied tissues of blunt snout bream. Congruent results were reported in half-smooth tongue sole [8], where *irf6* was significantly up-regulated in all three tissues (liver, spleen and head kidney) after a bacterial infection. This result corroborates that further studies are needed to explore the functional idiosyncrasies of Irfs in fish.

Among the nine members of the mammalian *irf* family, *irf4* and *irf8* share the highest similarity, and they are predominantly expressed in lymphocytes, macrophages, and dendritic cells [50]. Along with TRAF6, mammalian Irf8 modulates TLR signaling, and may contribute to the cross-talk between IFN-γ and TLR signaling pathways as well [51]. In zebrafish, Irf8 is a critical determinant for neutrophil versus macrophage fate choice during its primitive myelopoiesis; expression of *irf8* promotes the formation of macrophages and suppresses that of neutrophils, whereas a knockdown of *irf8* leads to a depletion of macrophages with a concomitant expansion of neutrophil population [20]. Our results, significantly up-regulated *mairf8* expression after bacterial challenge in kidney and spleen, are similar to those reported in rock bream [37], where the *irf8* mRNA expression was significantly up-regulated at 3 hpi in head kidney and spleen after stimulation with LPS. In half-smooth tongue sole, significant upregulation of *irf8* was detected after infection with an intracellular bacterial pathogen *E. tarda* [8]. In turbot, *irf8* was up-regulated after stimulation by both poly I:C and turbot reddish body iridovirus, but the induction by poly I:C was faster and stronger [39]. In large yellow croaker, there was clear upregulation of *irf8* after a *V. anguillarum* challenge, but in trout splenocytes, the expression of *rtirf8* did not respond to LPS stimulation. Our study showed that, after the bacterial challenge, the spleen exhibited the highest and earliest up-regulation of *mairf8*, which suggests that this organ is important in immune responses and that it rapidly comes into contact with injected stimuli.

irf10 mRNA was particularly strongly constitutively expressed in hematopoietic tissues, such as kidney, and lymphoid tissues, such as gills, heart, intestines, spleen and blood. After the *A. hydrophila* challenge, *mairf10* was up-regulated in five studied tissues. This corresponds well to the expression pattern, both constitutive and after LPS stimulation, of *irf10* in Japanese flounder (*Poirf10*) [27]. In chicken, *irf10* mRNA was also detected in hematopoietic tissues; after treatment with IFNs, *irf10* could induce the expression of GBP and MHC class I, whereas the expression of *irf10* was induced by v-Rel and Irf4 synergistically [28]. Similarly, GBP is also regulated by mammalian Irf1, but not by chicken Irf1, which indicates that it might be functionally supplemented by Irf10 in chicken [52].

*S*uppression of transcription in early post-challenge phases, here observed for some genes (*mairf8* and *mairf10*) in some tissues (liver), was also observed in cod after a challenge with live pathogens, and is believed to be associated with the immune-response suppression capability of some pathogens by evasion mechanism(s), which primarily interfere and block the IFN-signaling cascade [37]. Regardless of this, along with the results reported in Japanese flounder, where the expression of *irf10* was up-regulated by LPS in peripheral blood lymphocytes [27], and the Atlantic cod, where the expression of both *irf10* splice variants was responsive to *Aeromonas salmonicida* [10], this suggests that *irf10* is likely to play an important role in immune responses in fish.

In summary, we identified and characterized five *irfs* in blunt snout bream, and studied their expression patterns. Expression of *mairfs* was significantly up-regulated after a bacterial infection in liver, spleen, kidney, intestine and gill. This indicates that *mairfs* are likely to be involved in the type I IFN immune responses and play an important part in inflammatory response. These data shall provide the basis for future studies of immune responses to pathogen infection in teleost fish.

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Appendix A. Supplementary data

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