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Ontogeny and modulation after PAMPs stimulation of β-defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*)

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ABSTRACT

Antimicrobial peptides (AMPs), components of innate immunity, play an important role in protecting fish. In this study we report the molecular cloning of full open reading frames and characterization of expression of three AMP genes (*β-defensin* (*defb*), *hepcidin* (*hep2*), *piscidin* (*pisc*) in meagre (*Argyrosomus regius*). A phylogenetic analysis of the expressed sequences obtained shows the *defensin* isoform forms a clade with the other members of the beta class of this family, *hepcidin* corresponds to *hepcidin 2*, and *piscidin* corresponds to class I of its respective family. Gene expression profiles of AMPs was investigated, by means of quantification of mRNA in nine development stages, from 8 days post-hatching (dph) to accomplishment of juvenile form (120 dph). During development it was demonstrated *defb, hep2, pisc* were expressed in all stages of larval development and in juvenile tissues (kidney, spleen gut and gill). Moreover, expression patterns suggest the expression levels of theses AMPs are influenced by live prey (rotifer, *Artemia*) and first intake of commercial diet. Induction experiments *in vivo* (24 h) and *in vitro* (4, 12, 24 h) with PAMPs (LPS, poly (I:C), β-glucan) revealed significant changes in gene expression of the three AMP genes, in kidney, spleen, gut and gill. However, expression profiles differed in magnitude and time course response. *defb* expression shows a similar trend *in vivo* and *in vitro* in kidney at 24 h after LPS and β-glucan stimulation. The *hep2* expression levels were up-regulated upon β-glucan challenge *in vivo*, more in gut and gills than kidney, while *in vitro hep2* expression was up-regulated in kidney cells by LPS, poly (I:C), β-glucan (4 h). *pisc* expression was up-regulated in kidney cells, splenocytes by β-glucan, but in gill cells by poly (I:C) and β-glucan *in vivo*. However, *pisc* expression was upregulated in kidney cells by β-glucan and gill cells by LPS at 4 post-stimulation *in vitro*. These data suggest that AMPs play an important role in defense against pathogens, with each AMP having differing efficacies against specific types of microorganisms, although follow-up studies focusing on the biological activities in fish are needed.

1. Introduction

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune response and recognized as a critical first-line defense against many pathogens. These innate effector molecules are present in both vertebrate and invertebrate life forms and play an important role in protection against a broad spectrum of pathogens including those of parasite, bacterial, fungal and viral aetiology [1] by molecular mechanisms of cellular disruption [2]. Fish is continuously fights against pathogens by secreting a wide range of AMPs [3] They are typically present in leukocytes (mast cells, neutrophils), mucus cells (goblet and rodlet cells), cells lining epithelial surfaces and in gill, skin intestine and other tissues [4–7].

As a broad category of innate immune effector molecules, AMPs are divided into different families. Several studies have found peptides in a wide variety of species belonging to the defensin, parasin, cathelicidin and hepcidin families, as well as piscidin, a family unique to teleost fish [8]. Many of the peptides identified have antibiotic activity as well as immunomodulatory functions.

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There has also been a recent effort to study the potential of these peptides for applications in human medicine, and animal welfare [3].

Fish AMPs are up-regulated in response to pathogen and appear to have direct broad-spectrum antimicrobial activity towards both human and fish pathogens. However, the regulation the AMPs transcripts in tissues in response to pathogen challenge is species and gene specific [9]. A major pathway of AMP production is via the recognition of pathogen associated molecular patterns (PAMPs) through the pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), triggering an intracellular signaling cascade involving MyD88, TRAF6, IRAK1 and IKK. This signal promotes the activation and translocation of NF-kB to the nucleus where it transcriptionally activates AMPs and other effector molecules.

Defensins are a family of small cationic, amphipathic, cysteine-rich AMPs found in plants, fungi, invertebrates, and vertebrates, including fish. These AMPs are involved in host-microbiota interactions, immunomodulation, and provide strong links between innate and adaptive immunity. In vertebrates three different Defensin subfamilies exist (α-, β- and θ-defensins) that differ on the basis of the disulfide bridges formed by the pairing and positioning of their six conserved cysteine residues [4,10]. However, α-defensins are only found in mammals whilst θ-defensins exist only in non-human primates [11]. In fish, β-defensin paralogue sequences have been isolated in zebrafish (*Danio rerio*), fugu (*Takifugu rubriges*), tetraodon (*Tetraodon nigroviridis*) and medaka (*Oryziaas latipes*) [12]. A recent study has also identified a novel group of anionic-defensins (fBDI-1 to −5) in flounder *Paralichthys olivaceus* that are expressed during the larval period [10]. Constitutive β-defensin expression varies between species and depends on the tissue type. Their biological functions have been investigated in relatively few fish species and have broad-spectrum antimicrobial, antiviral and chemotactic ability, as reported for gilthead seabream (*Sparus aurata*) and Atlantic cod (*Gadus morhua*) [13,14]. Remarkably, Defensins have also been implicated in some functions related to the reproductive system in orange-spotted grouper (*Epinephelus coioides*) [15].

Hepcidins are another family of cysteine-rich peptides, structurally stabilised by disulphide bridges. They were first identified in human liver [16] and named liver-expressed AMPs (LEAP-1 and LEAP-2). The biological functions of mammalian Hepcidin include antimicrobial activity and regulation of iron metabolism. Hepcidin (Hep) has been extensively documented in many species of fish [8], where it has been shown to respond to bacterial infections and to function in antimicrobial activity. Whilst the highest expression level of hepcidin may be in the liver [17–19], as in mammals, in some fish species [20] these peptides are also expressed in other organs cardiac stomach, esophagus [17] heart, gill, spleen, kidney, peripheral blood leucocytes [18] dependent upon species. In mammals, two hepcidin isoforms are known. However, many teleosts (e.g., Perciform and Pleuronectiform fishes) possess up to seven isoforms of *hep*, as seen in winter flounder (*Pleuronectes americanus*) [17], rockbream (*Oplegnathus fasciatus*) [21] and red-banded seabream (*Pagrus auriga*) [22]. The presence of diverse isoforms might reflect positive Darwinian selection during evolution, as a consequence of different environments and host-pathogen interactions [23].

Piscidins are AMPs that have cationic properties and an amphipathic α-helical structure. A total of 9 piscidins have now been identified in striped bass (*Morone saxatilis*) and 8 orthologous forms have been detected in hybrid striped bass [24]. They represent an evolutionarily conserved family of peptides unique to teleost fish and were first identified in the winter flounder with subsequent studies in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) wherein the name was acquired [5,25]. They are widely active against Gram-positive and negative bacterial species, such as *Streptococcus, Pseudomonas, Bacillus* and *Vibrio* species [8], as well as being anti-fungal and anti-parasitic [26]. The primary mode of action is based on permeabilization of the plasma membrane of the pathogen. The immune-modulatory capacity of piscidins is another feature that has been widely assessed as they are able to modulate the expression of primarily pro-inflammatory genes as well as other immune-related genes, such as IL-1β, IL-10, TNF-α, NF-kb, NOS2, Myd88, TLR1, TLR3 and TLR4a through mechanisms involving a release of inhibition of NF-κB by down-regulation of IKB and, down-regulation of some anti-inflammatory signals such as IL-10, cAMP cascades and also indirect reduction of expression of inflammatory cytokines by elimination of pathogens [27,28]. Similarly, a study in orange-spotted grouper showed the immune-modulatory capacity of the *epinecidin-1* (*epi*) gene by electrotransfer of the *epi* gene in the absence of bacterial infection, increases in the levels of transcripts encoding MYD88, TNF-a, TNF2, and NACHT [29–31]. Studies based on peptide structure, phylogenetic analysis, gene expression and antimicrobial activity have demonstrated that there are three different groups of Piscidin each of which may have different functions.

Meagre (*Argyrosomus regius*) is considered a major candidate species for large-scale fish farming in the Mediterranean and many regions of Europe, due to its favorable characteristics related to fast growth rate and flesh quality. However, captive rearing of fish requires a well-developed larval rearing protocol and several factors affect the quality and survival rate of fish larvae, such as genetic background of the stock, diet, and the environmental conditions in which they are reared. This type of commercial growing necessitates intensive rearing in which crowding can augment skin abrasion/damage and lead to increased stress, facilitating transmission of infectious disease [32]. This study aims to elucidate one aspect of the innate response likely to be crucial in combating infectious bacterial diseases, namely some family members of AMPs that are present, and their level of expression in specific tissues. Target tissues were chosen for their significance as portals of entry of pathogens and their function in hematopoiesis, mucosal tissues of gills and intestine, and systemic tissues of kidney and spleen, respectively. We have characterized a *defb, hep* and *pisc* gene in meagre and describe their expression patterns during larval growout to evaluate when in development these peptides appear. In addition, we have examined how their expression is modulated by a variety of PAMPs, both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Larvae production, and sample collection to determine AMP ontogeny

Larvae used in the study were provided from meagre broodstock held in IRTA San Carlos de la Rapita, Spain. The broodstock were maintained under controlled simulated natural water temperature and photoperiod using recirculation system (IRTAmar®). Breeders were fed frozen sardines (Clupeidae, fishmarket, Sant Carlos de la Rápita, Tarragona, Spain), frozen Patagonian squid (*Loligo gahi)* (from Falkland Islands, Congelados Marcos, Tarragona, Spain) and a broodstock diet (Vitalis Repro, Skretting, Burgos, Spain). During the natural reproductive period (April–June), mature fish were selected based on oocyte size (<550 μm) and ease to extract sperm. Pairs of mature fish (21.2 ± 3.7 kg females and 16.1 ± 2.6 kg males) were hormonally induced (15 μg/kg of des-Gly10, [D-Ala6]-gonadotropin-releasing hormone ethylamide, Sigma, Spain) to spawn spontaneously in 10,000 L tanks and spawned eggs were collected with a passive egg collector placed in the outflow of the tank. Egg number was estimated by counting the total number of

eggs in three sub-samples of 10 mL and fertilization by examining the development of 100 eggs. Batches of 50,000 eggs were incubated (18–19 °C) in 35 L mesh (300 μm mesh) bottomed incubators with aeration and gentle air-lift water exchange that were placed in 2000 L tanks. Hatching rate was determined by estimating the number of larvae (three 100 mL sub-samples) obtained from the stocked eggs.

Larvae were transferred and distributed into two 1.5 m^3 tanks under a mesocosm system. Water temperature was maintained at 20 °C. From two days post-hatching (dph), larvae were fed enriched rotifers until 11 dph. Freshly enriched *Artemia* metanauplii were introduced at 9 dph until 31 dph and a formulated artificial diet was incorporated at 21 dph until the end of the experiment (Fig. 1a). Samples of larvae were collected from post hatch until juvenile for growth measurements. Random samples of larvae and juvenile were taken at 8, 15, 29, 40, 43, 60, 85, 96, and 120 dph for each sample point collected in an Eppendorf tube on ice containing RNAlater™ (Ambion, Austin, Texas), then preserved at −80 °C until RNA extraction. Time points for analysis were chosen as proxies for the specific periods of change in rearing practices; primarily changes in diet, but also rapid changes in organogenesis, all of which increase stress and enhance susceptibly to diseases. The fish were euthanized using a high concentration of MS222 (1 g/L) (Aldrich, E10521) prior to sample collection. Larger larvae (post-29 dph) had excess tissue trimmed to reduce signal dilution from non-target tissues; the anterior section from the gills onwards, and the posterior part from the anus to the tail. The samples were collected from each time point for the study of AMP gene expression. At days

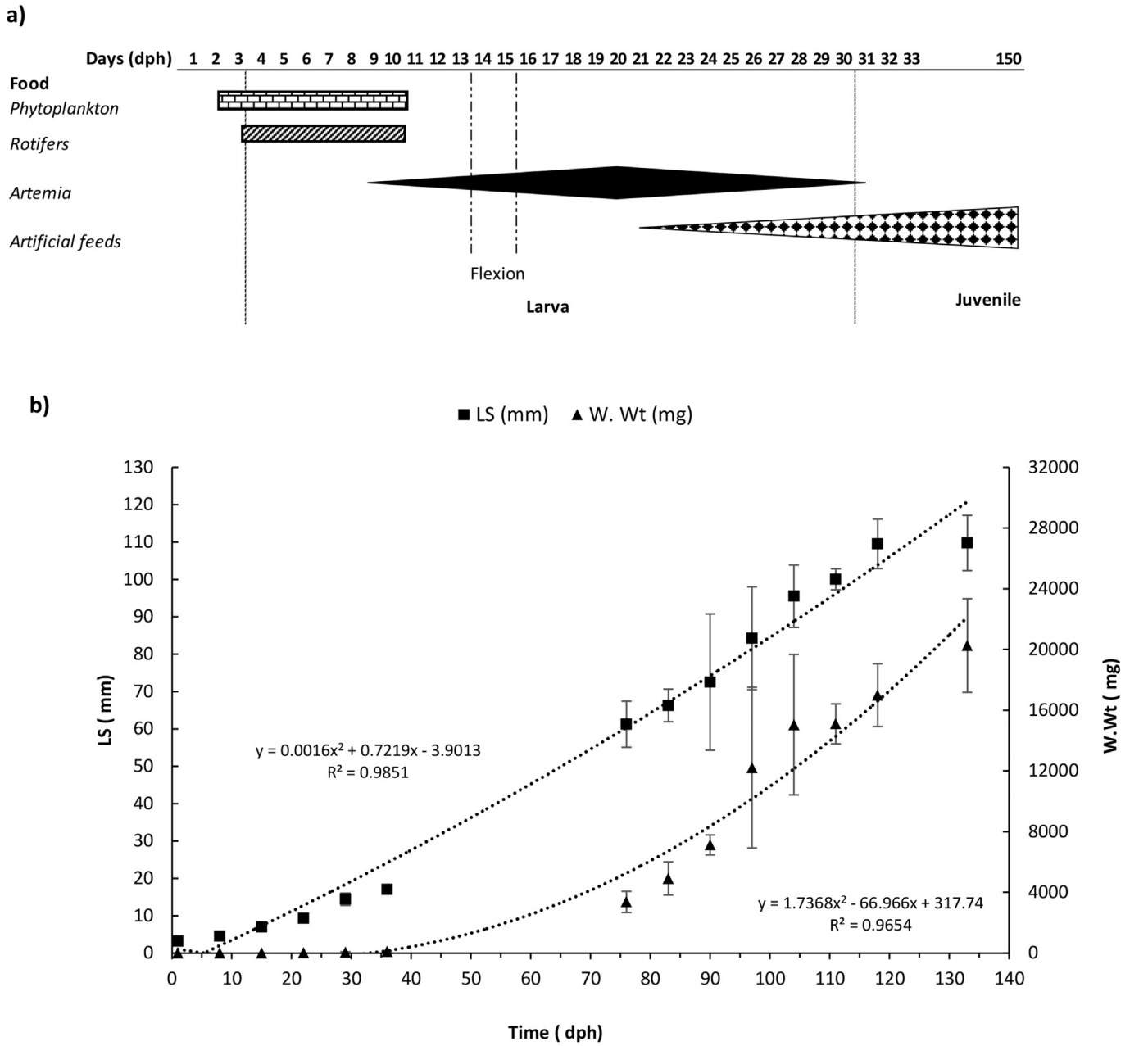

Fig. 1. Growth performance of meagre (*Argyrosomus regius*) larvae. a) The type and relative amount of different feed items offered during rearing are shown as an increase or decrease in thickness of the bar representing rotifers, *Artemia* metanauplii and artificial feeds, respectively. b) Total standard length and wet weight (mean ± SD) during larva rearing, in relation to time (days post-hatching). The regression lines describe the best-fit for growth and wet weight curves of meagre larvae.

85, 96, and 120 individual tissues (gill, kidney, spleen, and intestine) were excised aseptically.

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. RNA concentration and purity was determined by spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at 260 and 280 nm. The quality of extracted RNA was verified with visualization of the 28S and 18S ribosomal RNA bands by agarose gel electrophoresis. For preparation of cDNA total RNA was treated with DNase 1, Amplification Grade (AMPD1–1 KT, Sigma–Aldrich, Broendby, Denmark), according to manufacturer's instructions to remove possible contaminating genomic DNA. Total RNA was reverse transcribed in a 20 μL reaction volume containing 2 μg total RNA using the ThermoScript ™ Reverse Transcriptase (Invitrogen) with oligo (dT) $_{(12\text{-}18)}$ (0.5 μg/μL) and random hexamer primers (50 ng/μL) 10X RT buffer [200 mM Tris-HCl (pH8.4), 500 mM KCl] 1.5 mM $MgCl_2$, 800 mM dNTP mix, RNase inhibitor, SuperScript ™ II RT, followed by RNAse H (Invitrogen) treatment. Reverse transcription reactions were prepared, which were placed in a thermocycler (Mastercycle® nexus GSX1, Eppendorf AG, Hamburg, Germany) and run according to manufacturer's instruction. Negative controls (no RT enzyme) were included to confirm absence of genomic DNA contamination. The samples were then diluted 1:20 in molecular biology grade water and stored at −20 °C.

2.3. Gene isolation

Meagre were sampled for collecting data on specific growth rate of the chronological samples. The samples were collected in RNAlater at each time point, for extraction of RNA to be used in gene expression analysis. Sequences of target genes from the online database Genbank were chosen from extant marine teleost species that were available, such as large yellow croaker (*Larimichthys crocea*), mandarin fish (*Synchiropus splendidus*), Humphead snapper (*Lutjanus sanguineus*), European seabass (*Dicentrarchus labrax*), orange spotted grouper, but also tilapia (*Oreochromis* spp). The sequences were aligned, using CLUSTAL W incorporated in the package BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), for designing degenerate/consensus primers for amplification from cDNA of tissues. The fragments amplified were separated by gel electrophoresis and resulting bands of the expected length were excised, purified (QIAQuick PCR purification kit, Qiagen) and sequenced (Sistemas Genomicos, Valencia, Spain). The transcript sequences thus obtained were used as templates to design primers for specific q-PCR assays.

For some target genes, there are suspected to be paralogs that could create “noise” for the gene expression analysis if the gene specific primers targeted only the most conserved regions. To avoid this 5′-3′ RACE reactions were performed to obtain the full open reading frame using the method described by Zou et al. [33]. The RACE reactions followed the initial fragment isolation using degenerate-primed PCR with the primers: Pisc F - TGGTTGTTCTCATGGCTGAAC, Pisc R - GGTCA TAAGAAAGTGAACGT, Hep F - ATGAAGACATTCAGTGTTGC, Hep R-CAGCAACCGCAGCAAA, Defb F - GTGCTTCTCCTGATGCTCGC, and Defb R - CTGTATCTTCGAGGGCAAC.

2.4. Phylogenetic analysis

Confirmation of the relation of the AMP sequences obtained to extant marine teleost sequences was performed using a phylogenetic analysis. Evolutionary analyses were conducted in MEGA5 [34] utilizing only the coding portion of the open reading frames of each AMP. The nucleotide substitution model used was chosen utilizing the tool incorporated in MEGA5. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [35]. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise, the BIONJ method with MCL distance matrix was used. The analysis involved 33 nucleotide sequences. All codon positions were included in the analysis, however, positions containing gaps and missing data were eliminated. There were a total of 114 positions in the final dataset.

2.5. PAM stimulation

To investigate the effect of PAMP stimulation on the expression of *defb, hep* and *pisc*, 38 healthy juvenile individuals (30–40 g each) of meagre were held in a recirculating water tank (100 L) with seawater at 20 °C. The fish were fed with a commercial diet twice a day (9:00 h and 16: 00 h) for two weeks to acclimate them to the indoor culture environment. For primary cell culture and collection of samples, fish were killed after anesthesia with 50 mg/L MS-222.

2.5.1. AMPs expression after in vivo stimulation

Fish were injected intraperitoneally with 100 μL PBS containing 100 μg poly (I: C) (3.3 mg/kg) (Sigma, UK. P1530), 400 μg LPS (13.3 mg/kg) (Sigma, UK. L3129) or 100 μg β-glucan (3.3 mg/kg) (Sigma, UK. 89862). The control animals were injected with PBS only. After 24 h, the individuals were dissected and tissues (kidney, spleen, gut and gill) sampled. Total RNA was isolated and cDNA was prepared as described above. The mRNA expression level of *defb, pisc* and *hep* were determined by q-PCR. Relative expression was normalized to GAPDH expression and calculated as arbitrary units and converted to a proportion relative to the PBS control samples.

2.5.2. AMPs expression after in vitro stimulation

Tissues (kidney, spleen, gill, intestine) from apparently healthy fish were passed through a 100 μm nylon mesh cell strainer (SefarNytal PA-13xxx/100, Spain) in Leibovitz L15 medium (Gibco) containing penicillin/streptomycin (Gibco, #15140-122) at 1:1000 and 2% foetal calf serum (Gibco, #10270-098). The resulting cell suspension was collected and centrifuged at 400 × g for 10 min. The supernatant was removed and replaced with 10 mL of the previously described L15 media. The cell suspension was again centrifuged and supernatants removed and replaced with 30 mL of media. Cells were distributed to 12 well microtiter plates in 5 mL aliquots. Wells were stimulated using LPS (Sigma, #L3129-100 MG) at 50 μg/mL, poly (I:C) (Sigma, #P1530-25 MG) at 100 μg/mL, and β-glucan (Sigma, #89862-1G-F) at 50 μg/mL in triplicate. Control samples included 250 μL of PBS. Four, 12 and 24 h after stimulation the cells were harvested and centrifuged at 400 × g for 10 min, the supernatant discarded, and the pellet suspended in RNAlater. Total RNA was isolated and cDNA was prepared as described above.

2.6. Real-time q-PCR (RT-qPCR)

The q-PCR reactions for AMP gene expression were carried out in duplicate on a LightCycler® 480 Real-Time (Applied Biosystems, Roche). A master mix was prepared from: 6 μL SYBR Green Supermix (Life Technologies), 10 μM of each primer, diluted in molecular biology grade water (Sigma) and 4 μL of cDNA in a final volume of 10 μL. The real-time q-PCR cycling was carried out as follows: 10 min at 95 °C, 40 cycles of 95 °C for 25 s, followed by an annealing step of approximately 59 °C for 25 s (annealing temperatures were

adjusted for each specific primer pair), followed by 72 °C for 15 s, with a final melt curve stage of 0.5 °C increments from 75 °C to 95 °C. Each sample on the q-PCR plate had two technical replicates. Primer sequences used and amplicon lengths for each assay are shown (Table 1). The specificity of the primers was checked by running a q-PCR and confirming that only one melt peak was produced and also by running a subsample on an agarose gel (2%) to confirm the presence of a single band of the expected size. The efficiency of amplification (E %) of each primer pair was assessed from five serial ten-fold dilutions of cDNA from individual tissues, then calculated following the equation: $E\% = 10^{(-1/\text{slope})} - 1$ where the "slope" is that calculated from the regression line of the standard curve. Efficiencies of the gene expression assays ranged from 101.95% to 104.3% (102.85 ± 1.27) (Table 1). The absence of primer dimer formation in the NTC was also confirmed. Relative expression of genes from larvae, and each tissue from juveniles (ontogeny study), was normalized using the three endogenous controls glyceraldehyde phosphate dehydrogenase (GAPDH), beta-actin (β-actin), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) as determined using geNORM (http://www.primerdesign.co.uk/products/9461-genormplus-kits/), while the relative quantification at 120 dph, showing the least expression, was used as the calibrator.

For analyzing the relative expression *in vivo* and *in vitro* after PAMP stimulation, data was normalized to GAPDH expression, as this endogenous control gene showed less variability under the conditions tested. After normalization to this endogenous control as arbitrary units, results were converted to a proportion relative to the control group (PBS injected fish).

2.7. Statistical analysis

All data were checked for homogeneity of variances using a Levene's test by univariate analysis in a general linear model, based on a Tukey HSD post-hoc test, with a sample size of $n = 10$ (larva) $n = 8$ (juvenile) to determine differences between time points ($P \leq 0.05$). The Kruskal-Wallis test was used for analyzing the expression for the *in vivo* ($n = 8$) and *in vitro* ($n = 6$) samples after PAMP stimulation, using the statistical software package SPSS 20.0 (SPSS Inc., US).

3. Results

3.1. Gene isolation

Specific gene products were obtained using degenerate or consensus-primed PCR assays. All sequences were analyzed using the BLAST utility via the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nig.gov/) to confirm their identity as proper orthologs of the intended target gene. Sequencing followed by BLAST analysis provided presumptive identification of AMP transcripts for *defb, hep* and *pisc*, reported for the first time in this species. Sequences were also obtained for GAPDH, β-actin and HPRT as endogenous control genes for gene expression assays for which sequences have been provided in GenBank under the following accession numbers MF186586, MF186587 and MF186588, respectively. The AMP sequences have been uploaded to GenBank under the following accession numbers and length the fragment *defb* = MF074072 (195 bp), *hep2* = MF074074 (255 bp) and *pisc* = MF074073 (216 bp).

3.2. Phylogenetic analysis

To verify the relationship of meagre AMPs to known AMP genes, the *A. regius* sequences were used to construct a phylogenetic tree with extant marine teleost sequences of AMPs (when available) (Fig. 2). Results indicate the meagre hepcidin gene is more similar to that of *hep 2* from *Pseudosciaena crocea* and species of the moronidae family (*Dicentrarchus labrax* and *M. chrysops*). The *defb* sequences appear to be most closely related to mugilidae (*Liza haematocheila*). Meagre *pisc* was included in the class I clade of piscidins according to the scheme of Salger et al. [24], with affinity to *Larimichthys crocea* piscidin supported by a significant bootstrap value (94).

3.3. Gene expression analysis

3.3.1. Ontogeny of AMPs

In the present study, AMP expression during larval development has been assessed. Meagre growth was described by the equation LS = 0.0016 dph + 0.7219 dph - 3.9013 ($R^2 = 0.9851$) and wet weight by the equation W.Wt = 1.7368 dph - 66.966 dph + 317.74 ($R^2 = 0.9654$) between 0 and 133 dph of the experimental rearing (Fig. 1b).

The expression of *defb* exhibited marked differences between the larval and juvenile stage, with a statistically significant increase in expression during transition from *Artemia* to standard commercial dry pellet feed at 29 dph, followed by a gradual decrease until 60 dph. Expression patterns of *defb* transcripts in individual tissues show significantly different expression profiles from 85 dph to 120 dph. The highest expression was seen in kidney compared to other tissues tested, however, in spleen there was no difference in expression over time. (Fig. 3a).

The *hep2* expression in larvae remained markedly low from day 8 to day 15, where the expression was increase at day 29, when the switch from feeding on *Artemia* to artificial feed is nearly complete. In the analysis of separate immune tissues a gradual increase of expression was observed over time (85, 96, or 120 dph) in spleen, gill and gut. Although the trend of increase in expression over time is similar in all three of these tissues, a difference in mRNA level was only statistically significant in gut, where an increase was seen at 96 to 120 dph. However, *hep2* gene expression was highest in kidney compared with the other tissues (gill, gut, spleen) (Fig. 3b).

The *pisc* transcripts showed a pattern of expression markedly different across developmental stages. The *pisc* gene expression remained low from 8 to day 29, where the expression increased at

Table 1
Primers used for gene expression analysis by real-time q-PCR, including the amplicon size and primer sequences. Abbreviations: *gapdh*; glyceraldehyde 3-phosphate dehydrogenase; *β-actin*, beta-actin; *hprt*, hypoxantine-guanine phosphoribosyltransferase; *defb*, β-defensin; *hep*, hepcidin; *pisc*, piscidin.

	Genes	E (%)	Size (bp)	Forward primer (5′- > 3′)	Reverse primer (5′- > 3′)
Reference genes	*gapdh*	100.00	109	CCAGTACGTGGTGGAGTCCACTG	AGCGTCAGCGGTGGGTGCAGAG
	β-actin	100.05	212	TGGGGGAGCAATGATCTTGATCTTCA	AGCCCTCTTTCCTCGGTATGGAGTC
	hprt	100.95	137	CATGGACTCATCTTGGACAGGACAGA	GCCTTGATGTAGTCCAGCAGGTC
Amp genes	*defb*	102.30	138	GGGAACGAAGATCCAGAGATGCAGTATTGGAC	CTAAGACCTCACAGCACAGCACCTG
	hep	104.30	140	CCGTCATGCTCGCCTTCG	CTCACGCATGTAATACGGAATCTTGCATG
	pisc	101.95	111	CAATGATCCATGGGCTTATCC	TTCAGTCTCGCCATTGAAGC

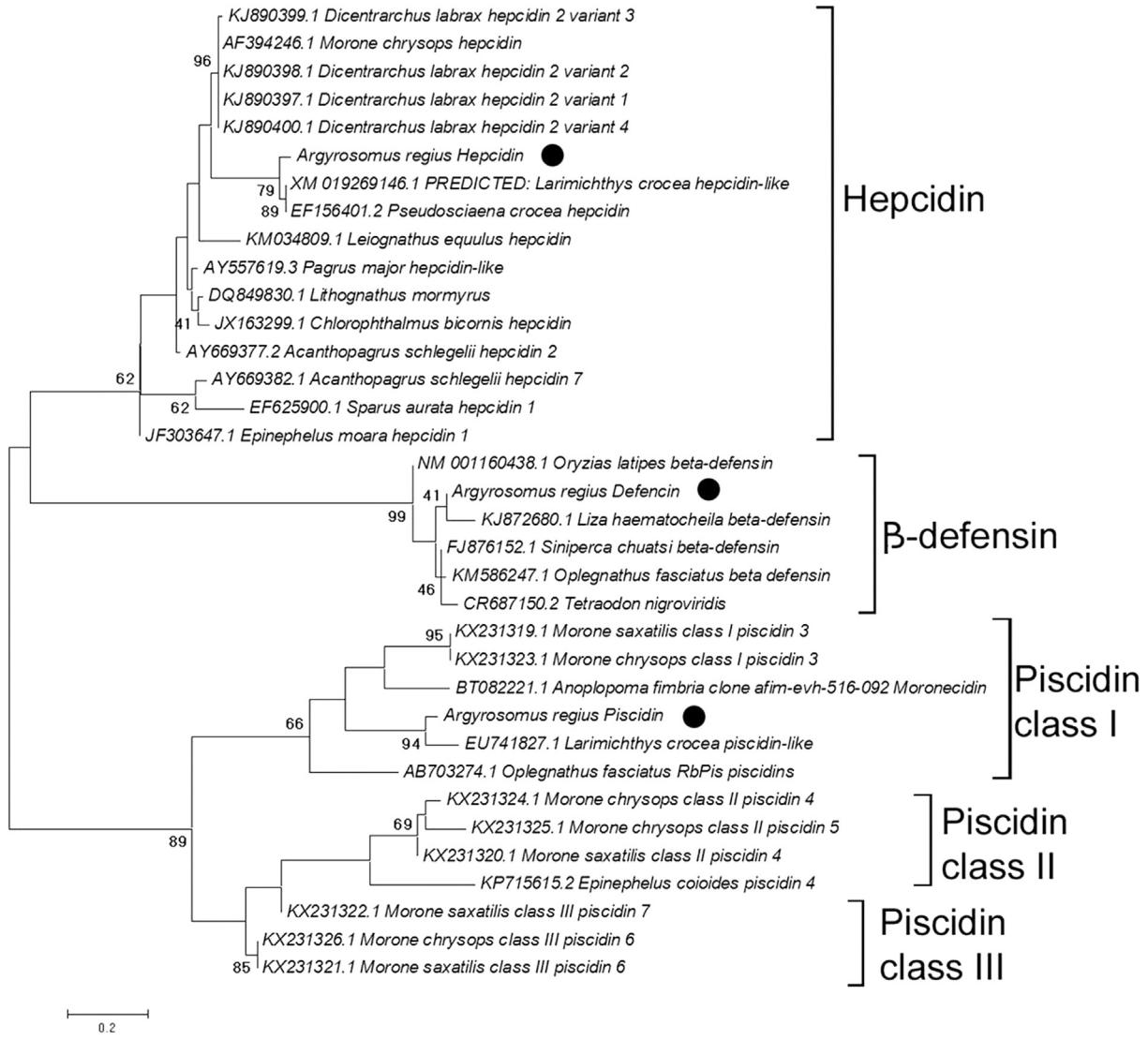

Fig. 2. Phylogenetic relationship among *defb, hep2* and *pisc* isolated from *A. regius* obtained by the Maximum Likelihood method. The tree with the highest log likelihood (−1611.2745) is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are indicated at nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

day 40, when the larvae feed includes only artificial feed, followed by a gradual decrease thereafter. In isolated tissues, high *pisc* expression was detected in gills, but similar patterns of expression were found in kidney and spleen at 85, 96, 120 dph. However, gut tissue had consistently low levels of *pisc* expression during the time points examined (Fig. 3c).

3.3.2. Response of meagre AMPs genes to in vivo PAMP stimulation

After injection of fish with PAMPs, the expression levels of *defb*, *hep2* and *pisc* were examined by q-PCR at 24 h post-stimulation. The *defb* transcription was up-regulated in kidney post-injection with LPS and β-glucan while a significant down-regulation was seen in spleen, gut and gills following LPS injection, and in spleen following poly (I:C) injection (Fig. 4a). In contrast, *hep2* was significantly up-regulated in gut and gills by β-glucan, and in gills by LPS (Fig. 4b). Lastly, the transcription of *pisc* showed significant up-regulation in kidney, spleen and gills by β-glucan treatment, and there was also significant up-regulation in gills by poly (I:C) stimulation (Fig. 4c).

3.3.3. Response of meagre AMPs genes to in vitro PAMP stimulation

After stimulation of isolated leucocytes *in vitro* with PAMPs, the kinetics of changes in expression levels of *defb, hep2* and *pisc* were examined by q-PCR. No change in expression of *defb* gene was found in the stimulated kidney cells at 4 h and 12 h, but at 24 h there was significant up-regulation ($P \leq 0.05$) using poly (I:C), LPS and β-glucan, greater than 20-fold in the latter case. With splenocytes no changes were seen at 4 h or 24 h, but at 12 h a small decline in the level was detected after stimulation with poly (I:C), LPS and β-glucan. The mRNA expression of *defb* in gill cells was significantly up-regulated at 4 h after stimulation by poly (I:C), but thereafter it showed a decline at 12 h - 24 h. Similarly, *defb* was up-regulated with LPS stimulation at 12 h, but declined at 24 h β-glucan stimulation resulted in down-regulation from 12 h onwards. Lastly, the mRNA expression of *defb* in intestinal cells was significantly up-regulated ($P \leq 0.05$) at 4 h and 12 h post-stimulation with poly (I:C), LPS and β-glucan, however an up-regulation at 24 h was only seen with LPS and β-glucan stimulation (Fig. 5a).

For *hep2*, transcription in kidney cells was significantly up-regulated at 4 h post-stimulation with poly (I:C), LPS and β-

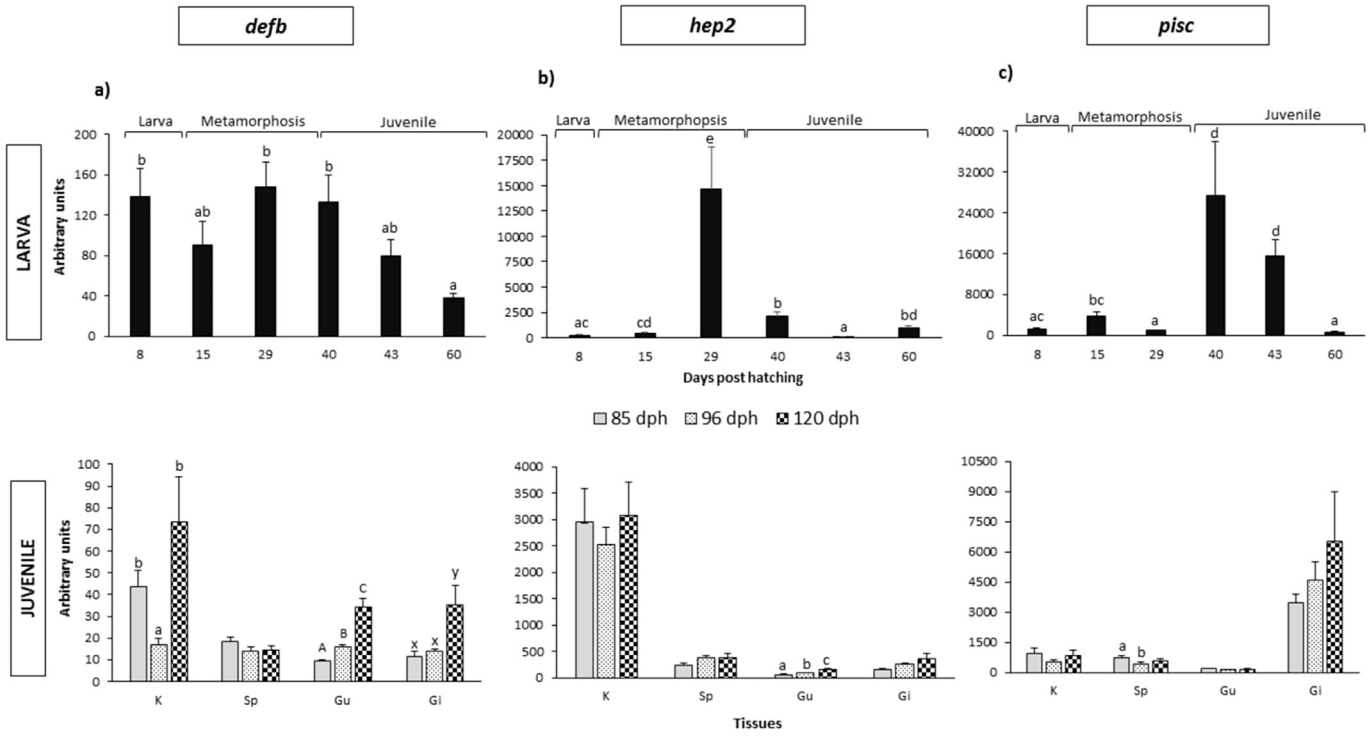

Fig. 3. Temporal change in gene expression levels (relative quantification), from larvae, juveniles and tissues. from 8 to 120 dph, of *A. regius* transcripts coding for *defb* (a), *hep2* (b) and *pisc* (c). Relative expression of *defb*, *hep2* and *pisc* transcripts were normalized using an arithmetic mean of three housekeeping genes: GAPDH, HPRT and β-actin. Transcriptional fold changes of *defb*, *hep2* and *pisc* at different time points were calculated by comparison to each previous timepoint. Different letters above the bars indicate significant differences among different time points (Tukey's test, $P \leq 0.05$). Results are expressed as the mean ± SEM. The biological samples were for larva ($n = 10$) and tissues, ($n = 8$) of gene expression. K = kidney, Sp = spleen, Gu = gut, Gi = gill.

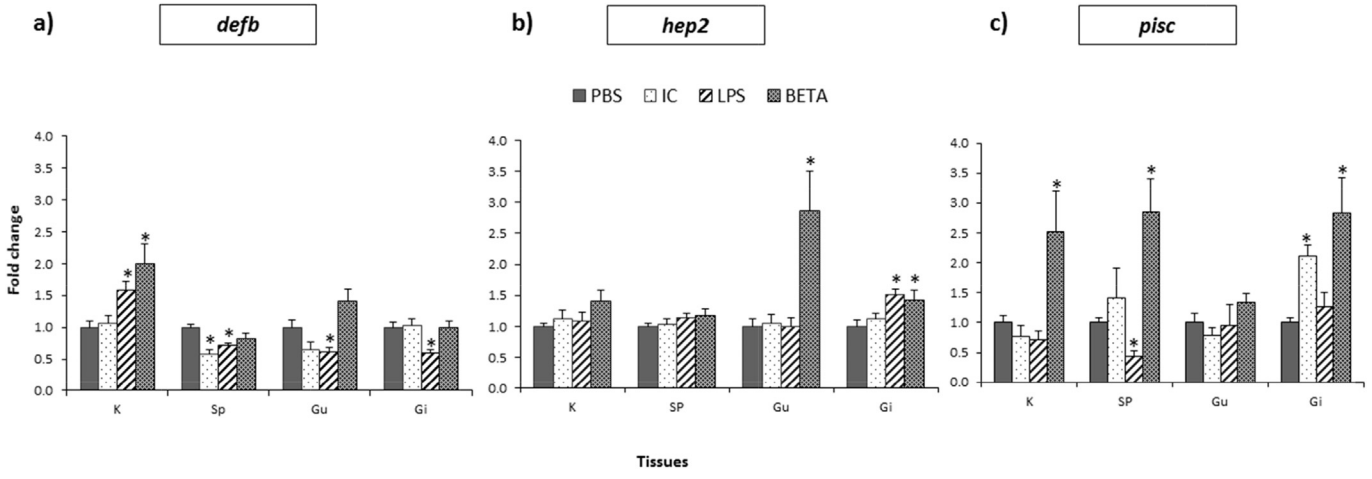

Fig. 4. Relative expression of *defb* (a), *hep2* (b) and *pisc* (c) in different tissues. Fish ($n = 8$) were injected intraperitoneally with poly (I:C), LPS and β-glucan and sampled at 24 h post-injection. GAPDH, was employed as an internal reference. Asterisks (*) mark significant differences between stimulated and control groups (Kruskal Wallis test, $P \leq 0.05$). Data are means ± SEM. K = kidney, Sp = spleen, Gu = gut, Gi = gill.

glucan and, thereafter, it showed a drastic decline at 24 h in the presence of these three PAMPs. In splenocytes only at 24 h after stimulation by PAMPs was a significant effect seen, with down-regulation apparent for all three PAMPs. In gill cells, the gene transcripts were rapidly and significantly up-regulated at 4 h after stimulation by poly (I:C), but showed a decline at 12 h, and later returned to basal levels at 24 h. In intestinal cells, the gene transcripts were significantly up-regulated at 4 h after LPS and β-glucan treatment, had declined at 12 h, but were again enhanced at 24 h after LPS stimulation (Fig. 5b).

A significant up-regulation of *pisc* expression was seen in kidney at 4 h and 12 h after stimulation by β-glucan and at 24 h after stimulation using poly (I:C). In splenocytes, up-regulated was seen at 24 h after stimulation by LPS. The gills cells displayed a higher expression level at 4 h and 12 h after stimulation with LPS, and at 4 h with β-glucan, compared with the control. Significant down-regulation of the expression of *pisc* transcripts was observed in the intestine, but only at 12 h post-stimulation with the PAMPs (Fig. 5c).

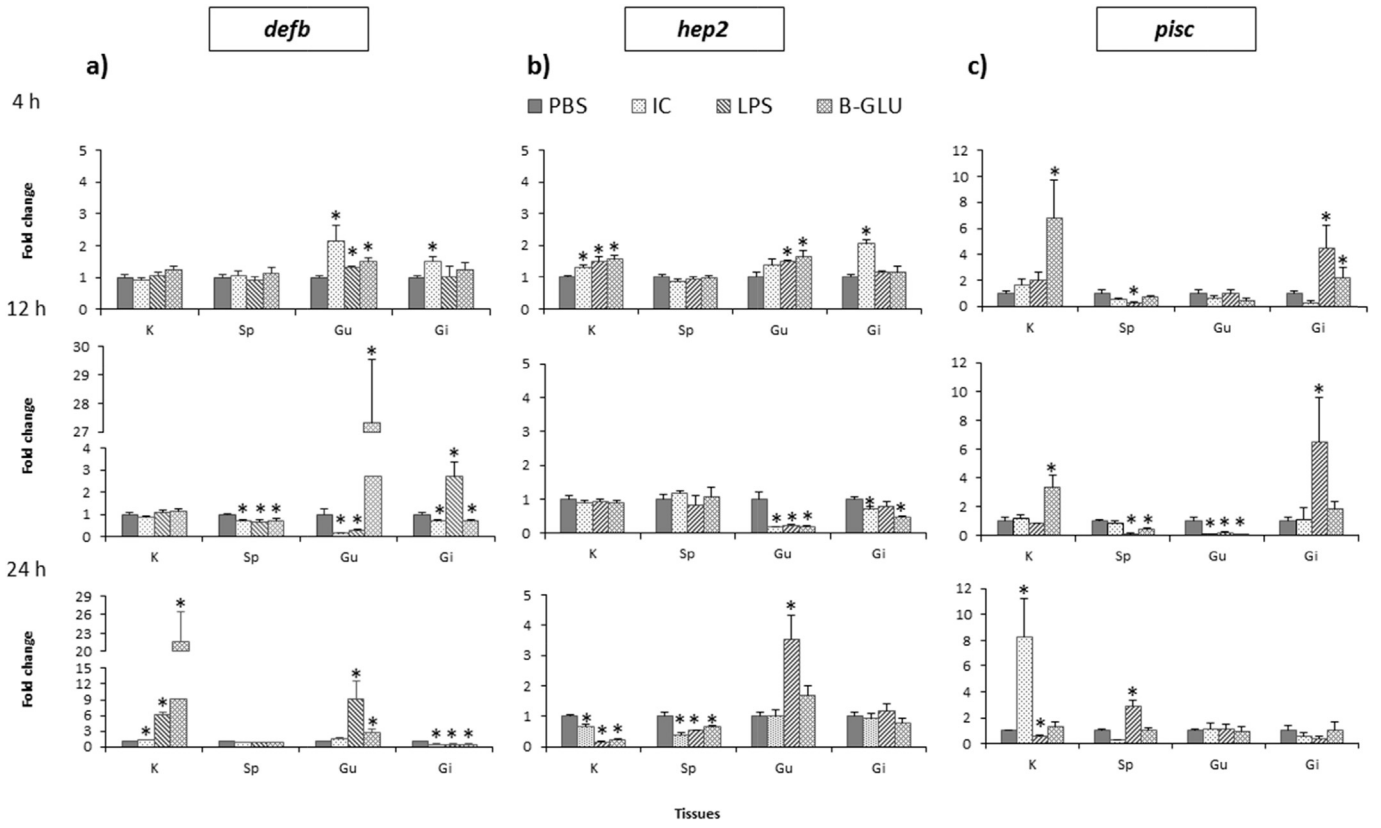

Fig. 5. Expression patterns of *defb* (a), *hep2* (b) and *pisc* (c) mRNA after PAMP stimulation of cell suspensions isolated from different meagre (*Argyrosomus regius*) tissues, extracted from healthy fish ($n = 6$). The cells were stimulated with poly (I:C), LPS and β-glucan, and sampled at different time points post-stimulation (4, 12 and 24 h), for RNA extraction and q-PCR. GAPDH, was employed as an internal reference. Asterisks (*) mark significant differences between stimulated and control groups (Kruskal Wallis test, $P \leq 0.05$). Data are means ± SEM. K = kidney, Sp = spleen, Gu = gut, Gi = gill.

4. Discussion

Antimicrobial peptides are a diverse group of small peptides that exhibit broad-spectrum antimicrobial activity towards pathogens (parasites, bacteria, viruses and fungi). They function as a part of the primary defense within the innate immune system protecting the host from attack by foreign particles and organisms in a non-specific manner [36]. Fish are free-living organisms from the initial stages of development and exposed to numerous pathogens before adaptive immunity has fully developed. It is known that some aspects of teleost immunological capacity are limited in early development and they rely on innate mechanisms such as AMPs for survival. This is the first description of the temporal appearance and flux of expression during larval development of representative members of three AMP families of immune effectors from *A. regius*, including Defensin, Hepcidin, and Piscidin. A phylogenetic analysis of the expressed sequences obtained (Fig. 2) shows that among the transcripts from *A. regius*, the *defensin* isoform forms a clade with the other members of the beta class of this family, *hepcidin* corresponds to *hepcidin* 2, and *pisc* corresponds to class I of its respective family.

The phylogenetic tree reveals that the isoform of *def* identified from meagre is *defb*, although no homolog from *L. crocea* was found in the GenBank database so the formation of a clade with *defb* from *Liza haematocheila* is likely an artifact of the database content. During growout the ontogeny of the expression of the identified AMPs from meagre was evaluated. In this study, *defb* was found expressed in all stages of larval development and in juvenile tissues examined in meagre. In larva, expression levels were identified after complete absorption of the yolk sac and were found to be significantly up-regulated around the time of *Artemia* feeding though decreased after weaning. This increased expression after the start of the exogenous feeding may reflect a need for efficient defense mechanisms against potential pathogens that can enter with the live feed or simply up-regulation due to PAMP stimulation from commensal organisms in the feed. The *defb* expression at early developmental stages has been demonstrated in vertebrates and invertebrate [4]. Two β-defensin genes were identified in snout bream (*Megalobrama amblycephala*) during early development of embryos and fry [37]. Investigation of the multiple DefB isoforms were found in olive flounder (*Paralichthys olivaceus*) after hatching, revealed a constitutive *FBD1* mRNA expression before and after hatching at 1–35 dph [10]. While only one isoform was identified in the current study, it also exhibited constitutive expression as seen with *FBD1* mRNA. In common sole (*Solea solea*) *defb* transcripts showed high transcription level across all larval stages [38]. In this context, expression patterns observed in this study might suggest that this peptide is implicated in innate conditioning of the early larval stages, preventing pathogenic assault, and conditioning of the gut microbiota [39].

The β-defensin gene expression in teleosts has been assessed in tissues from healthy fish, but the levels and patterns of expression varied for individual defensin genes [13,37]. In the present study with meagre, analysis of the isoform obtained revealed expression remained somewhat constitutive, but was highly expressed in kidney with lower levels in gill, gut, and spleen at 120 dph. The decrease in expression seen between 29 dph and 60 dph may be due to the expression signal being diluted as the larvae grew, as the

abundance of *def*-positive cells likely did not increase in the same proportion as the general growth and increase in musculature. However, the isoform under study can influence the expression pattern as well as the host species. In addition to viable microbial pathogens, the components of a fish's diet can act to regulate *defb* expression. In common carp, *BD1* and *BD2* in gills and *BD1* in skin were found significantly higher upon addition of β-glucan to the food [40]. As studies with Gilthead seabream fed microalgae (*Nannochloropsis gaditana,* and *Tetraselmis chuii*) show, an increase in the expression level of *defb* in head-kidney and gut can be accomplished via dietary supplement [41]. On the basis of the results obtained in this study (Fig. 3), further dietary studies to investigate how change of food from live prey (*Artemia*) to commercial feed may influence AMPs expression in larvae.

The potential to modulate the expression level of the AMPs (*defb, hep 2, pisc*) was also analyzed after *in vivo* and *in vitro* immune activation by PAMPs (LPS, poly (I:C), β-glucan). The baseline expression for all three types of AMPs was lowest in isolated gut tissue. This has biological sense when considering the importance of maintaining a healthy gut microbiota. A leaky promoter or slight defects in the secretion systems could have disastrous consequences for the health of the host [42]. Maintaining a tight regulation of control would help to avoid incurring inadvertent damage to the gut microbiota, therefore the control in the gut is more likely to be bi-model rather than multi-model [42]. Our results showed that the expression of *defb* was significantly up-regulated in the kidney after injection with LPS and β-glucan at 24 h. This result is in accordance with the idea that the kidney is an important hematopoietic organ and filters the blood where strong up-regulation of *defb* can be expected as a prophylactic measure to ensure pathogen elimination. Similar results have been seen in Atlantic cod, where *defb* is up-regulated in the kidney 48 h post-challenge with *Vibrio anguillarum* [43], and in the kidney in olive flounder 1 h after infection with *Edwardsiella tarda* [10]. Using cells isolated from individual tissues our results in meagre also show up-regulation of AMPs following PAMPs stimulation. The expression of *defb* was increased in kidney and gut cells at 4 and 24 h after stimulation by β-glucan, LPS and poly (I:C). These results suggest early responses (4–12 h) of *defb* in meagre are more dependent on the stimulus in mucosal tissue than in systemic tissues where significant differences were not observed. Although, later response (24 h) in the kidney were more pronounced. Interestingly, similar trends were found *in vivo* and *in vitro* with regard to *defb* expression in kidney at 24 h after LPS and β-glucan stimulation, possibly reflecting a large population of antigen presenting cells responsive to PAMPs in this tissue.

The phylogenetic analysis also reveals that meagre *hep2* and *pisc* were more similar to equivalent molecules in *Larimichthys crocea* as expected, since this is taxonomically a quite similar species of fish. Hepcidin is highly conserved from fish to humans [44,45]. In fish, it plays a major role in the innate immune response and iron regulation. In the present findings, meagre *hep2* gene expression patterns during larval development showed high expression levels between 8 dph and 15 dph, then increased significantly at 29 dph at the end of metamorphosis. Similar results were observed in *Solea solea* where *SsoHAMP* expression levels increased from 8 dph [38], as well as in Atlantic cod [46] and half -smooth tongue sole [47]. However, in the redbanded seabream and in *Pseudopleuronectes americanus hep* isoforms were found to peak earlier between 5 and 15 dph [17,22], whilst in Medium carp (*Puntius sarana*) a *hep* homologue had stable expression from 6 h post-fertilization onwards [48]. During these timings, numerous changes occur in embryogenesis and early larval development. Elevated expression at this time likely indicates a preparation of the larvae for dealing with a pathogen-laden environment after hatching, as well as conditioning the gut mucosa by stabilization of the normal commensal microbiota that prevent colonization of the gut by opportunistic pathogens through spatial exclusion [39]. Physical barriers of the innate immune response are not sufficient by themselves and when these barriers should be insufficient, additional mechanisms, such as the production of AMPs, are required. From the moment of hatching the fish larvae needs adequate defense mechanisms, with the commencement of external feeding potentially exposing larvae to viable biological agents contaminating the feed.

In this study, *hep2* was expressed in all tissues examined, and highly abundant in kidney followed by spleen and gills. Earlier studies from other species, have shown that *hep* transcripts are usually observed at a higher level in the liver in fish, although they can also be detected in other tissues [43–46]. Indeed, the expression patterns between different fish *hep* is highly divergent and may be related to the specific isoform(s) examined [18].

The *in vivo* challenge results indicated, that *hep2* was also found to be up-regulated upon β-glucan stimulation, but in gut and gills rather than kidney. Further, in gills *hep2* was significantly up-regulated by LPS stimulation. Studies in fish species of Hep protein have shown multiple isoforms exist and these are classified into two groups: Hamp type 1 and Hamp type 2. One Hep isoform has iron regulatory roles sharing a high degree of homology with mammalian counterparts, and the second Hep copy has retained only the antimicrobial role [9,49]. Phylogenetic analysis in this study has shown the isoform identified in meagre is more similar to Hamp 2 and likely does not retain the iron regulatory functions. In this study the responsiveness of this isoform has only been demonstrated with the *in vitro* and *in vivo* PAMP stimulation indicating its antimicrobial functions. Additionally, there may exist other *hep* isoforms with different tissue-specific expression patterns related to distinct tissue-specific regulation via their promoters [9]. Indeed, different *hep* isoforms in a single species can have different levels of expression within the same tissue, and can be affected by different stimuli [21,22,50]. For example, in the olive flounder where multiple isoforms exist, Hep-JF1 was induced in a wide array of tissues (including liver, gill, kidney and spleen) by LPS, but another variant Hep-JF2 was unaffected [18]. However, whilst LPS is well known to modulate *hep* expression in teleost fish, in large yellow croaker (*Pseudosciaena crocea*) where spleen, heart, stomach, kidney, and liver tissues were examined, LPS stimulation significantly enhanced expression only in spleen, heart and stomach [20].

The in vitro challenge results indicated, *hep2* expression was up-regulated in kidney and gut cells at 4 h post-stimulation by β-glucan and LPS, and at the same time in kidney after poly (I:C) treatment (4 h), with later (24 h) stimulation of expression in intestinal cells by LPS. However, the changes were relatively small as compared to what might be expected in hepatocytes which were not analyzed in this study. The *hep* expression has been shown to be up-regulated *in vitro* by several PAMPs in other species [51]. Also in cell culture, in rainbow trout, where *hep* mRNA level was increased in a macrophage (RTS11) cell line treated with poly (I:C) [52]. In tilapia, Hep2 and Hep3 (TH2-3) showed inhibition of proliferation and migration of a tumor cell line (HT1080 cells) in a concentration-dependent manner [53].

Class I Piscidin has been shown to be more effective against prokaryotes and ciliated protozoan infections [24], as compared to class II and class III. Although in this study functional analyses have not been evaluated, on the basis of q-PCR results, meagre class I Piscidin showed up-regulation with all three PAMPs both *in vivo* and *in vitro*, which is suggestive of it being more responsive to a broader range of stimuli. Piscidin is generally expressed early during fish development and increases in expression over time. Our

results showed that *pisc* transcripts were detected in 8 dph larvae, with maximum levels seen at 40 dph when they have completed the transition to juveniles. There was a decrease after 40 dph, but as discussed above for *defb*, this decrease may in fact be related to a dilution effect due to rapid growth after the end of metamorphosis. In more developed juveniles where individual tissues were examined, *pisc* transcripts were detected at high levels in the gill, with lower expression in the kidney and spleen, and lowest expression in the gut. In studies of striped bass, Piscidin was observed by immunofluorescent labelling in post-vitellogenic oocytes, embryos or larvae from 14 dph [54]. European seabass Piscidin 3 was detected in cytoplasmic granules of inflammatory cells, and mast cells [55]. Until recently, piscidins had only been found in fish species from the superorder Acanthopterygii, but there has recently been *pisc* genes identified in Atlantic cod [56], where expression is seen in more diverse tissues including liver, blood, gall bladder, pyloric caeca, stomach, rectum, muscle, heart, and brain [56–58], as well as in mast cells and rodlet cells of the skin, gill and intestine [59].

In the present study *in vivo* stimulation also modulated *pisc* expression, with the highest level of expression found in kidney, spleen and gills at 24 h after injection with β-glucan, and in gills after poly (I:C) treatment. In other fish species *pisc* genes can be induced by a variety of stimuli such as LPS [60,61], parasite antigens, poly (I:C) [62] and β-glucan as a representative PAMP from fungi [58]. For example, in Mandarin fish (*Siniperca chuatsi*) a significant up-regulation of *pisc* was seen in most tissues, except the liver, after stimulation with LPS [63], while in large yellow croaker, *pisc* expression was up-regulated in response to the ectoparasite, *Cryptocaryon irritans* [26]. Furthermore, in hybrid tilapia (*Oreochromis* spp.) Piscidin showed a correlate with lower mortality after injection with *V. vulnificus* [64]. Regarding *pisc* expression *in vitro*, this was up-regulated in kidney cells by β-glucan and in gill cells by LPS at 4 and 12 h post-stimulation. A late induction in spleen and kidney cells was also seen in response to LPS and poly (I:C) at 24 h post-stimulation. Interestingly, *pisc* expression *in vitro* (4–12 h) and *in vivo* (24 h) showed up-regulation in the same tissues from whole animals and isolated cells (kidney and gills) by β-glucan stimulation. These results demonstrate that timing of induction from each stimulant has tissue-specific differences, although the *pisc* isoform identified can be induced by all three types of PAMPs.

In conclusion, the current study is the first identification of AMPs from meagre and demonstrates their expression modulation during development and in response to PAMPs. Further studies will be needed to elucidate whether additional isoforms exist and the antimicrobial activities of the peptide proteins. The *A. regius defb, hep2,* and *pisc* transcripts were expressed at different levels in early developmental stages and tissues of juvenile meagre, and the data hint that expression is regulated or influenced by dietary factors such as switching to live feed, or weaning onto artificial diets. This may allow larval feed composition for *A. regius* larvae to be manipulated to augment AMP gene expression to improve antimicrobial defenses as has been seen for *defb* expression in common carp [40] and gilthead seabream [41]. This work provides useful information for future investigations of the innate defense mechanisms in early development of meagre and studies focusing on the biological activities of these antimicrobial peptides for this fish species.

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References

[1] H. Jenssen, P. Hamill, R.E. Hancock, Peptide antimicrobial agents, Clin. Microbiol. Rev. 19 (2006) 491–511, http://dx.doi.org/10.1128/CMR.00056-05.
[2] V.J. Smith, A.P. Desbois, E.A. Dyrynda, Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae, Mar. Drugs 8 (2010) 1213–1262, http://dx.doi.org/10.3390/md8041213.
[3] V. Rajanbabu, J.-Y. Chen, Applications of antimicrobial peptides from fish and perspectives for the future, Peptides 32 (2011) 415–420, http://dx.doi.org/10.1016/j.peptides.2010.11.005.
[4] E. Casadei, T. Wang, J. Zou, J.L. González Vecino, S. Wadsworth, C.J. Secombes, Characterization of three novel β-defensin antimicrobial peptides in rainbow trout (*Oncorhynchus mykiss*), Mol. Immunol. 46 (2009) 3358–3366, http://dx.doi.org/10.1016/j.molimm.2009.07.018.
[5] U. Silphaduang, E.J. Noga, Antimicrobials: peptide antibiotics in mast cells of fish, Nature 414 (2001) 268–269, http://dx.doi.org/10.1016/j.peptides.2010.11.005.
[6] H.M. Murray, J.W. Gallant, S.E. Douglas, Cellular localization of pleurocidin gene expression and synthesis in winter flounder gill using immunohistochemistry and in situ hybridization, Cell Tissue Res. 312 (2003) 197–202, http://dx.doi.org/10.1007/s00441-003-0723-3.
[7] G. Salerno, N. Parrinello, P. Roch, M. Cammarata, cDNA sequence and tissue expression of an antimicrobial peptide, dicentracin; a new component of the moronecidin family isolated from head kidney leukocytes of sea bass, *Dicentrarchus labrax*, Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 146 (2007) 521–529, http://dx.doi.org/10.1016/j.cbpb.2006.12.007.
[8] A.J. Masso-Silva, G. Diamond, Antimicrobial peptides from fish, Pharmaceuticals 7 (2014), http://dx.doi.org/10.3390/ph7030265.
[9] B.A. Katzenback, Antimicrobial peptides as mediators of innate immunity in teleosts, Biol. (Basel) 4 (2015) 607–639, http://dx.doi.org/10.3390/biology4040607.
[10] B.-H. Nam, J.-Y. Moon, Y.-O. Kim, H.J. Kong, W.-J. Kim, S.-J. Lee, K.-K. Kim, Multiple β-defensin isoforms identified in early developmental stages of the teleost *Paralichthys olivaceus*, Fish. Shellfish Immunol. 28 (2010) 267–274, http://dx.doi.org/10.1016/j.fsi.2009.11.004.
[11] Y.Q. Tang, J. Yuan, G. Osapay, K. Osapay, D. Tran, C.J. Miller, A.J. Ouellette, M.E. Selsted, A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins, Science 286 (1999) 498–502, http://dx.doi.org/10.1126/science.286.5439.498.
[12] J. Zou, C. Mercier, A. Koussounadis, C. Secombes, Discovery of multiple beta-defensin like homologues in teleost fish, Mol. Immunol. 44 (2007) 638–647, http://dx.doi.org/10.1016/j.molimm.2006.01.012.
[13] A. Cuesta, J. Meseguer, M.Á. Esteban, Molecular and functional characterization of the gilthead seabream β-defensin demonstrate its chemotactic and antimicrobial activity, Mol. Immunol. 48 (2011) 1432–1438, http://dx.doi.org/10.1016/j.molimm.2011.03.022.
[14] A. Falco, V. Chico, L. Marroqui, L. Perez, J.M. Coll, A. Estepa, Expression and antiviral activity of a β-defensin-like peptide identified in the rainbow trout (*Oncorhynchus mykiss*) EST sequences, Mol. Immunol. 45 (2008) 757–765, http://dx.doi.org/10.1016/j.molimm.2007.06.358.
[15] J.-Y. Jin, L. Zhou, Y. Wang, Z. Li, J.-G. Zhao, Q.-Y. Zhang, J.-F. Gui, Antibacterial and antiviral roles of a fish β-defensin expressed both in pituitary and testis, PLoS One 5 (2010), http://dx.doi.org/10.1371/journal.pone.0012883 e12883.
[16] A. Krause, S. Neitz, H.-J. Mägert, A. Schulz, W.-G. Forssmann, P. Schulz-Knappe, K. Adermann, LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity, FEBS Lett. 480 (2000) 147–150, http://dx.doi.org/10.1016/S0014-5793(00)01920-7.
[17] S.E. Douglas, J.W. Gallant, R.S. Liebscher, A. Dacanay, S.C.M. Tsoi, Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish, Dev. Comp. Immunol. 27 (2003) 589–601, http://dx.doi.org/10.1016/S0145-305X(03)00036-3.
[18] A.T. Hirono I, J. Hwang, Y. Ono, T. Kurobe, T. Ohira, R. Nozaki, Two different types of hepcidins from the Japanese flounder *Paralichthys olivaceus*, FEBS J. 272 (2005) 5257–5264, http://dx.doi.org/10.1111/j.1742-4658.2005.04922.x.
[19] H. Shike, C. Shimizu, X. Lauth, J.C. Burns, Organization and expression analysis of the zebrafish hepcidin gene, an antimicrobial peptide gene conserved among vertebrates, Dev. Comp. Immunol. 28 (2004) 747–754, http://dx.doi.org/10.1016/j.dci.2003.11.009.
[20] K.-J. Wang, J.-J. Cai, L. Cai, H.-D. Qu, M. Yang, M. Zhang, Cloning and expression of a hepcidin gene from a marine fish (*Pseudosciaena crocea*) and the antimicrobial activity of its synthetic peptide, Peptides 30 (2009) 638–646, http://dx.doi.org/10.1016/j.peptides.2008.12.014.
[21] Y.S. Cho, S.Y. Lee, K.H. Kim, S.K. Kim, D.S. Kim, Y.K. Nam, Gene structure and differential modulation of multiple rockbream (*Oplegnathus fasciatus*) hepcidin isoforms resulting from different biological stimulations, Dev. Comp. Immunol. 33 (2009) 46–58, http://dx.doi.org/10.1016/j.dci.2008.07.009.
[22] B. Martin-Antonio, R.M. Jiménez-Cantizano, E. Salas-Leiton, C. Infante, M. Manchado, Genomic characterization and gene expression analysis of four

hepcidin genes in the redbanded seabream (*Pagrus auriga*), Fish. Shellfish Immunol. 26 (2009) 483–491, http://dx.doi.org/10.1016/j.fsi.2009.01.012.
[23] A. Padhi, B. Verghese, Evidence for positive Darwinian selection on the hepcidin gene of Perciform and Pleuronectiform fishes, Mol. Divers 11 (2007) 119–130, http://dx.doi.org/10.1007/s11030-007-9066-4.
[24] S.A. Salger, K.R. Cassady, B.J. Reading, E.J. Noga, A diverse family of host-defense peptides (piscidins) exhibit specialized anti-bacterial and Anti-Protozoal activities in fishes, PLoS One 11 (2016), http://dx.doi.org/10.1371/journal.pone.0159423 e0159423.
[25] A.M. Cole, P. Weis, G. Diamond, Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder, J. Biol. Chem. 272 (1997) 12008–12013.
[26] S.-F. Niu, Y. Jin, X. Xu, Y. Qiao, Y. Wu, Y. Mao, Y.-Q. Su, J. Wang, Characterization of a novel piscidin-like antimicrobial peptide from *Pseudosciaena crocea* and its immune response to *Cryptocaryon irritans*, Fish. Shellfish Immunol. 35 (2013) 513–524, http://dx.doi.org/10.1016/j.fsi.2013.05.007.
[27] W.-C. Lin, H.-Y. Chang, J.-Y. Chen, Electrotransfer of the tilapia piscidin 3 and tilapia piscidin 4 genes into skeletal muscle enhances the antibacterial and immunomodulatory functions of *Oreochromis niloticus*, Fish. Shellfish Immunol. 50 (2016) 200–209, http://dx.doi.org/10.1016/j.fsi.2016.01.034.
[28] Y. Jing, L.U. Xin-Jiang, C. Fang-Chao, C. Jiong, Molecular characterization and functional analysis of a piscidin gene in large yellow croaker (*Larimichthys crocea*), Zool Res. 37 (2016) 347–355.
[29] C.-Y. Pan, T.-C. Huang, Y.-D. Wang, Y.-C. Yeh, C.-F. Hui, J.-Y. Chen, Oral administration of recombinant epinecidin-1 protected grouper (*Epinephelus coioides*) and zebrafish (*Danio rerio*) from *Vibrio vulnificus* infection and enhanced immune-related gene expressions, Fish. Shellfish Immunol. 32 (2012) 947–957, http://dx.doi.org/10.1016/j.fsi.2012.01.023.
[30] C.-Y. Pan, J.-L. Wu, C.-F. Hui, C.-H. Lin, J.-Y. Chen, Insights into the antibacterial and immunomodulatory functions of the antimicrobial peptide, epinecidin-1, against *Vibrio vulnificus* infection in zebrafish, Fish. Shellfish Immunol. 31 (2011) 1019–1025, http://dx.doi.org/10.1016/j.fsi.2011.09.001.
[31] S.-B. Lin, T.-W. Fan, J.-L. Wu, C.-F. Hui, J.-Y. Chen, Immune response and inhibition of bacterial growth by electrotransfer of plasmid DNA containing the antimicrobial peptide, epinecidin-1, into zebrafish muscle, Fish. Shellfish Immunol. 26 (2009) 451–458, http://dx.doi.org/10.1016/j.fsi.2009.01.008.
[32] C.A. Shoemaker, J.J. Evans, P.H. Klesius, Density and dose: factors affecting mortality of Streptococcus iniae infected tilapia (*Oreochromis niloticus*), Aquaculture 188 (2000) 229–235, http://dx.doi.org/10.1016/S0044-8486(00)00346-X.
[33] J. Zou, S. Bird, R. Minter, J. Horton, C. Cunningham, C.J. Secombes, Molecular cloning of the gene for interleukin-1β from *Xenopus laevis* and analysis of expression in *vivo* and in *vitro*, Immunogenetics 51 (2000) 332–338.
[34] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol. Biol. Evol. 28 (2011) 2731–2739, http://dx.doi.org/10.1093/molbev/msr121.
[35] T.H. Jukes, C.R. Cantor, Evolution of protein molecules, Mamm. Protein Metab. 3 (1969) 132.
[36] J.-G. Zhao, L. Zhou, J.-Y. Jin, Z. Zhao, J. Lan, Y.-B. Zhang, Q.-Y. Zhang, J.-F. Gui, Antimicrobial activity-specific to Gram-negative bacteria and immune modulation-mediated NF-κB and Sp1 of a medaka β-defensin, Dev. Comp. Immunol. 33 (2009) 624–637, http://dx.doi.org/10.1016/j.dci.2008.11.006.
[37] T. Liang, D.-D. Wang, G.-R. Zhang, K.-J. Wei, W.-M. Wang, G.-W. Zou, Molecular cloning and expression analysis of two β-defensin genes in the blunt snout bream (*Megalobrama amblycephala*), Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 166 (2013) 91–98, http://dx.doi.org/10.1016/j.cbpb.2013.07.006.
[38] S. Ferraresso, A. Bonaldo, L. Parma, F. Buonocore, G. Scapigliati, P.P. Gatta, L. Bargelloni, Ontogenetic onset of immune-relevant genes in the common sole (*Solea solea*), Fish. Shellfish Immunol. 57 (2016) 278–292, http://dx.doi.org/10.1016/j.fsi.2016.08.044.
[39] M.J. Ostaff, E.F. Stange, J. Wehkamp, Antimicrobial peptides and gut microbiota in homeostasis and pathology, EMBO Mol. Med. 5 (2013) 1465–1483, http://dx.doi.org/10.1002/emmm.201201773.
[40] M. van der Marel, M. Adamek, S.F. Gonzalez, P. Frost, J.H.W.M. Rombout, G.F. Wiegertjes, H.F.J. Savelkoul, D. Steinhagen, Molecular cloning and expression of two β-defensin and two mucin genes in common carp (*Cyprinus carpio L.*) and their up-regulation after β-glucan feeding, Fish. Shellfish Immunol. 32 (2012) 494–501, http://dx.doi.org/10.1016/j.fsi.2011.12.008.
[41] R. Cerezuela, F.A. Guardiola, J. Meseguer, M.A. Esteban, Enrichment of gilthead seabream (*Sparus aurata L.*) diet with microalgae: effects on the immune system, Fish Physiol. Biochem. 38 (2012) 1729–1739, http://dx.doi.org/10.1007/s10695-012-9670-9.
[42] L. Huang, Z. Yuan, P. Liu, T. Zhou, Effects of promoter leakage on dynamics of gene expression, BMC Syst. Biol. 9 (2015) 16, http://dx.doi.org/10.1186/s12918-015-0157-z.
[43] J. Ruangsri, Y. Kitani, V. Kiron, J. Lokesh, M.F. Brinchmann, B.O. Karlsen, J.M.O. Fernandes, A novel beta-defensin antimicrobial peptide in Atlantic cod with stimulatory effect on phagocytic activity, PLoS One 8 (2013), http://dx.doi.org/10.1371/journal.pone.0062302 e62302.
[44] J. Shi, A.C. Camus, Hepcidins in amphibians and fishes: antimicrobial peptides or iron-regulatory hormones? Dev. Comp. Immunol. 30 (2006) 746–755, http://dx.doi.org/10.1016/j.dci.2005.10.009.
[45] K.B. Hilton, L.A. Lambert, Molecular evolution and characterization of hepcidin gene products in vertebrates, Gene 415 (2008) 40–48, http://dx.doi.org/10.1016/j.gene.2008.02.016.
[46] M. Seppola, H. Johnsen, S. Mennen, B. Myrnes, H. Tveiten, Maternal transfer and transcriptional onset of immune genes during ontogenesis in Atlantic cod, Dev. Comp. Immunol. 33 (2009) 1205–1211, http://dx.doi.org/10.1016/j.dci.2009.06.013.
[47] Y. Wang, X. Liu, L. Ma, Y. Yu, H. Yu, S. Mohammed, G. Chu, L. Mu, Q. Zhang, Identification and characterization of a hepcidin from half-smooth tongue sole *Cynoglossus semilaevis*, Fish. Shellfish Immunol. 33 (2012) 213–219, http://dx.doi.org/10.1016/j.fsi.2012.04.011.
[48] A. Das, A. Mohapatra, P.K. Sahoo, Cloning and characterization of antimicrobial peptide, hepcidin in medium carp, *Puntius sarana*, Int. J. Pept. Res. Ther. 21 (2015) 139–147, http://dx.doi.org/10.1007/s10989-014-9438-4.
[49] J.V. Neves, C. Caldas, I. Vieira, M.F. Ramos, P.N.S. Rodrigues, Multiple hepcidins in a teleost fish, *Dicentrarchus labrax*: different hepcidins for different roles, J. Immunol. 195 (2015) 2696–2709.
[50] Y.-O. Kim, E.-M. Park, B.-H. Nam, H.J. Kong, W.-J. Kim, S.-J. Lee, Identification and molecular characterization of two hepcidin genes from black rockfish (*Sebastes schlegelii*), Mol. Cell. Biochem. 315 (2008) 131, http://dx.doi.org/10.1007/s11010-008-9796-3.
[51] A. Cuesta, R. Cerezuela, M.Á. Esteban, J. Meseguer, In *vivo* actions of melatonin on the innate immune parameters in the teleost fish gilthead seabream, J. Pineal Res. 45 (2008) 70–78, http://dx.doi.org/10.1111/j.1600-079X.2008.00557.x.
[52] P.P. Chiou, C.-M. Lin, N.C. Bols, T.T. Chen, Characterization of virus/double-stranded RNA-dependent induction of antimicrobial peptide hepcidin in trout macrophages, Dev. Comp. Immunol. 31 (2007) 1297–1309, http://dx.doi.org/10.1016/j.dci.2007.03.009.
[53] J.-Y. Chen, W.-J. Lin, T.-L. Lin, A fish antimicrobial peptide, tilapia hepcidin TH2-3, shows potent antitumor activity against human fibrosarcoma cells, Peptides 30 (2009) 1636–1642, http://dx.doi.org/10.1016/j.peptides.2009.06.009.
[54] S.A. Salger, B.J. Reading, E.J. Noga, Tissue localization of piscidin host-defense peptides during striped bass (*Morone saxatilis*) development, Fish. Shellfish Immunol. 61 (2017) 173–180, http://dx.doi.org/10.1016/j.fsi.2016.12.034.
[55] B.S. Dezfuli, F. Pironi, L. Giari, E.J. Noga, Immunocytochemical localization of piscidin in mast cells of infected seabass gill, Fish. Shellfish Immunol. 28 (2010) 476–482, http://dx.doi.org/10.1016/j.fsi.2009.12.012.
[56] J. Ruangsri, S.A. Salger, C.M.A. Caipang, V. Kiron, J.M.O. Fernandes, Differential expression and biological activity of two piscidin paralogues and a novel splice variant in Atlantic cod (*Gadus morhua L.*), Fish. Shellfish Immunol. 32 (2012) 396–406, http://dx.doi.org/10.1016/j.fsi.2011.11.022.
[57] Q.-J. Zhou, Y.-Q. Su, S.-F. Niu, M. Liu, Y. Qiao, J. Wang, Discovery and molecular cloning of piscidin-5-like gene from the large yellow croaker (*Larimichthys crocea*), Fish. Shellfish Immunol. 41 (2014) 417–420, http://dx.doi.org/10.1016/j.fsi.2014.09.023.
[58] Y. Jing, L.U. Xin-Jiang, C. Fang-Chao, C. Jiong, Molecular characterization and functional analysis of a piscidin gene in large yellow croaker (*Larimichthys crocea*), Zool. Res. 37 (2016) 347–355, http://dx.doi.org/10.13918/j.issn.2095-8137.2016.6.347.
[59] U. Silphaduang, A. Colorni, E.J. Noga, Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish, Dis. Aquat. Organ 72 (2006) 241–252, http://dx.doi.org/10.3354/dao072241.
[60] E.J. Noga, U. Silphaduang, N.G. Park, J.K. Seo, J. Stephenson, S. Kozlowicz, Piscidin 4, a novel member of the piscidin family of antimicrobial peptides, Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 152 (2009) 299–305, http://dx.doi.org/10.1016/j.peptides.2010.11.005.
[61] J.-S. Bae, S.H. Shim, S.D. Hwang, M.-A. Park, B.-Y. Jee, C.-M. An, Y.-O. Kim, J.-W. Kim, C.-I. Park, Expression analysis and biological activity of moronecidin from rock bream, *Oplegnathus fasciatus*, Fish. Shellfish Immunol. 40 (2014) 345–353, http://dx.doi.org/10.1016/j.fsi.2014.07.023.
[62] V.G. Chinchar, L. Bryan, U. Silphadaung, E. Noga, D. Wade, L. Rollins-Smith, Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides, Virology 323 (2004) 268–275, http://dx.doi.org/10.1016/j.virol.2004.02.029.
[63] B.J. Sun, H.X. Xie, Y. Song, P. Nie, Gene structure of an antimicrobial peptide from mandarin fish, *Siniperca chuatsi* (Basilewsky), suggests that moronecidins and pleurocidins belong in one family: the piscidins, J. Fish. Dis. 30 (2007) 335–343, http://dx.doi.org/10.1111/j.1365-2761.2007.00789.x.
[64] C.-Y. Pan, T.-Y. Tsai, B.-C. Su, C.-F. Hui, J.-Y. Chen, Study of the antimicrobial activity of Tilapia piscidin 3 (TP3) and TP4 and their effects on immune functions in hybrid Tilapia (*Oreochromis spp.*), PLoS One 12 (2017), http://dx.doi.org/10.1371/journal.pone.0169678 e0169678.