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Identification of an interferon-stimulated gene, *isg15*, involved in host immune defense against viral infections in gilthead seabream (*Sparus aurata* L.)

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ABSTRACT

Interferons (IFNs) play a key role in the innate immunity of vertebrates against viral infections by inducing hundreds of IFN-stimulated genes (ISGs), such as *isg15*. Isg15 is an ubiquitin-like protein, which can conjugate cellular and viral proteins in a process called ISGylation, although it can also act as a cytokine-like protein. Gilthead seabream (*Sparus aurata* L.) is an important asymptomatic carrier of viral haemorrhagic septicaemia virus (VHSV) and nodavirus, representing a threat to other co-cultivated susceptible species. In order to better understand virus-host interactions in this fish species, this study addresses the identification and molecular characterization of seabream *isg15* (*sb-isg15*). In addition, the modulation of transcript levels of *sb-isg15* was analysed in SAF-1 cells and seabream acidophilic granulocytes (AGs) stimulated *in vitro* with different pathogen-associated molecular patterns (PAMPs) or inoculated with VHSV and striped jack nervous necrosis virus (SJNNV).

The full-length cDNA of *sb-isg15* gene, encoding a predicted protein of 155 amino acids, was identified and seen to share the same characteristics as other fish and mammalian *isg15* genes. Here we report the clear induction of *sb-isg15* transcript levels in SAF-1 cells and AGs stimulated with toll-like receptor (TLR) ligands, such as polyinosinic:polycytidylic acid (poly I:C) or genomic DNA from *Vibrio anguillarum* (*Va*DNA), respectively. Furthermore, VHSV and SJNNV inoculation induced a significant degree of *sb-isg15* transcription in SAF-1 cells and AGs. However, the relative levels of viral RNA transcription showed that SJNNV replication seems to be more efficient than VHSV in both *in vitro* systems. Interestingly, *sb-isg15* transcript induction elicited by *Va*DNA was reduced in VHSV- and SJNNV-inoculated AGs, suggesting an interference prompted by the viruses against the type I IFN system. Taken together, these findings support the use of seabream AGs as a valuable experimental system to study virus-host interactions, in which *sb-isg15* seems to play an important role.

1. Introduction

Interferons (IFNs) are key cytokines of the antiviral innate immune system that induce the expression of hundreds of genes through the JAK-STAT signalling pathway in vertebrates [1]. Some of the most studied interferon-stimulated genes (ISGs) are *mx*, IFN-stimulated gene 15 (*isg15*), double-stranded RNA-activated protein kinase (*pkr*), and virus inhibitory protein (*viperin*). Isg15 is an ubiquitin-like protein, which is quickly and highly upregulated by IFN stimulation or viral infections [2]. It contains two tandem ubiquitin-like domains (UBL) and a conserved C-terminal LRGG sequence that is required for covalent conjugation of Isg15 proteins with host and viral proteins in a process

Abbreviations: AGs, acidophilic granulocytes; ANOVA, analysis of variance; CPE, cytoplasmic polyadenylation element; ENA, European Nucleotide Archive; EPC, epithelioma papulosum cyprini; FBS, foetal bovine serum; FCS, foetal Calf Serum; GNNV, grouper nervous necrosis virus; HK, head kidney; IFNs, interferons; IHNV, infectious haematopoietic necrosis virus; ISGs, IFN-stimulated genes; *isg15*, interferon stimulated gene 15; L15, Leibowitz-15 medium; LPS, lipopolysaccharide; MACS, magnetic-activated cell sorting; MOI, multiplicity of infection; NJ, neighbour-joining; NNV, nervous necrosis virus; PAMPs, pathogen associated molecular patterns; PI, propidium iodide; *pkr*, double-stranded RNA-activated protein kinase; Poly I:C, polyinosinic:polycytidylic acid; ROS, reactive oxygen species; *rps18*, ribosomal protein S18; *sb-isg15*, seabream isg15; SJNNV, striped jack nervous necrosis virus; TCID50, 50% tissue culture infective dose; TLR, toll-like receptors; UBL, ubiquitin-like domains; UTR, untraslated region; *Va*DNA, genomic DNA from *Vibrio anguillarum*; VHSV, viral haemorrhagic septicaemia virus

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called ISGylation [3]. ISGylation involves a series of enzymatic reactions similar to the ubiquitin conjugation pathway and affects enzymatic degradation, subcellular localization and the half-life of target proteins [2,4]. ISGylation seems to preferentially target newly translated proteins, so that, during infections, ISGylation of viral proteins and ISGs plays an important role in both antiviral activity and the proper regulation of the host antiviral response [5]. Several studies have shown that the broad antiviral activity of mammalian Isg15 against numerous RNA and DNA viruses is ISGylation dependent [2].

As well as existing in as conjugated form, Isg15 is present in a non-conjugated form, both intracellularly and in the extracellular space. This form also plays an important role in the host response to infections, acting as an immunomodulatory protein that negatively regulates the expression of proinflammatory cytokines and chemokines, and mediates in the regulation of IFNα/β signalling, resulting in the prevention of an auto-inflammation course [6,7]. Furthermore, free Isg15 can act as a cytokine-like protein, inducing IFNγ expression in T-cells, stimulating natural killer cell proliferation, or inducing dendritic cell maturation and neutrophil recruitment [8].

Isg15 orthologues have been described in several fish species (reviewed in Ref. [9]). Fish *isg15* genes are induced upon stimulation by viral infection, treatment with double-stranded RNA (poly I:C), or by other pathogen-associated molecular patterns (PAMPs) [10]. Piscine Isg15 proteins share structural characteristics with mammalian Isg15, such as the two UBL domains and the presence of the LRGG motif, which also seems to be crucial for its antiviral activity [11,12]. Fish Isg15 proteins show direct antiviral activity against several fish viruses, such as haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), grouper nervous necrosis virus (GNNV) [12,13] or megalocytivirus [11].

Gilthead seabream (*Sparus aurata* L.) is one of the most extensively cultured species in Mediterranean aquaculture. It shows high resistance to viral infections and lymphocystis disease is the only natural viral infection reported to affect the species [14]. However, seabream could be an asymptomatic carrier and/or reservoir of VHSV [15], and nervous necrosis virus (NNV) [16], which are pathogenic to other co-cultivated fish species [17–21]. Thus, to face the threat that the presence of asymptomatic carriers would represent in fish farms, it is crucial to understand virus-host interactions in this type of organism. Hence, the aim of the current study was to characterize the *isg15* gene of gilthead seabream (*sb-isg15*) and to analyse the interference between the IFN system and viral infection by using *sb-isg15* transcription as marker of the IFN I response. For this purpose, VHSV and striped jack nervous necrosis virus (SJNNV) were inoculated into two *in vitro* systems, the established seabream cell line SAF-1, and purified seabream acidophilic granulocytes (AGs), which display similar functions to mammalian neutrophils.

2. Materials and methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*S. aurata*, Actinoperygii, Sparidae) were bred and kept at the Oceanographic Centre of Murcia (Spain) in a 14 m^3 running seawater tank (dissolved oxygen 6 ppm, flow rate 20% tank volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Burgos, Spain). Fish were fasted for 24 h before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

2.2. Amino acid sequence analysis

The nucleotide sequence of seabream *isg15* (GenBank accession number HS987162) was identified from a public EST database by alignment with the ORF of different fish *isg15* sequences. Nucleic acid sequence homology analysis was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the blast tool at Ensembl (Ensembl Genome Browser, http://www.ensembl.org). The complete Isg15 aminoacid sequences of different fish species were chosen to maximize the representativeness of the different fish orders (Table S1). The deduced amino acid sequence obtained with EditSeq (DNASTAR Lasergene 7) software was analysed with the Expert Protein Analysis System (EXPASY) (http://www.expasy.org/) and motifs were identified using the NSITE database.

Multiple amino acid sequence alignments were constructed using MegAlign software v 3.3.8. For the phylogenetic analysis, aminoacidic sequences were aligned by the neighbour-joining (NJ) algorithm within the MEGA version 7.0 software. Human FAT10, which also contains two UBL domains, was used as outgroup. Bootstrap values were calculated with 2000 replications to estimate the robustness of internal branches.

2.3. Virus propagation

A VHSV genotype III isolate (SpSm-IAusc2897, a marine isolate obtained from turbot, *Scophthalmus maximus*) and an NNV isolate SJNNV genotype (SJ93Nag, reference SJNNV isolate), both pathogenic to Senegalese sole (*Solea senegalensis*) [23,24], were used in this study. The VHSV isolate was propagated on the epithelioma papulosum cyprini (EPC) cell line (ATCC CRL-2872). Inoculated EPC cells were maintained in Leibowitz-15 (L15) medium with 2% foetal bovine serum (FBS), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Lonza) at 20 °C, and were monitored until cytopathic effect emergence. The SJNNV isolate was propagated on E11 cell (ECACC 01110916) monolayers at 25 °C in L15 with 2% FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin, until it displayed extensive cytopathic effects. Supernatants of VHSV- and SJNNV-inoculated cells were collected, centrifuged at 5000 × *g* for 10 min at 4 °C, and the resulting viral suspensions were titrated on EPC (VHSV) or E11 (SJNNV) cells grown on 96-well plates (NuncThermo Scientific). Titres were expressed as the viral dilution infecting 50% of the cell cultures ($TCID_{50}$) following the methodology described by Reed and Muench [25]. Viral suspensions were stored at −80 °C until use.

2.4. Isolation of phagocytes

Head kidneys (HK) were collected and AGs were purified by magnetic-activated cell sorting (MACS) as described [26]. Briefly, head kidney cell suspensions were incubated in a 1:10 dilution of a monoclonal antibody specific to gilthead seabream AGs (G7) [27], washed twice with PBS containing 2 mM EDTA (Sigma-Aldrich) and 5% Foetal Calf Serum (FCS) (Invitrogen) and then incubated with 100–200 µl per 10^8 cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, $G7^+$ (AGs) cell fractions were collected by MACS following the manufacturer's instructions, and their purity was analysed by flow cytometry.

2.5. Cell culture and treatments

SAF-1 cells, established from gilthead seabream fibroblasts [28] (ECACC, UK), were grown at 25 °C in L15 medium supplemented with 2% L-glutamine, 10% FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin on 12-well plates until semiconfluence. SAF-1 cells were stimulated with polyinosinic:polycytidylic acid (poly I:C) (25 µg/ml, InvivoGen) for 12 and 24 h and RNA was isolated as described below. For virus infections, VHSV or SJNNV were diluted in FBS-free L15 medium and used for cell inoculation at 0.1 multiplicity of infection (MOI). After virus adsorption at 20 °C (VHSV) or 25 °C (SJNNV) for 1 h, the inoculum was removed and immediately replaced by L15 medium with 2% FBS.

Then, cells were incubated at 20 °C (VHSV) or 25 °C (SJNNV), and total RNA was isolated at 24 h and 48 h post-inoculation (p.i.) as described below. Each assay was performed in triplicate.

Gilthead seabream purified AGs (2×10^6 cells/well) were seeded on 24-well plates (1 ml, final volume) and incubated at 23 °C in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl] supplemented with 5% FBS, and 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Biochrom). AGs were stimulated with 50 µg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (*Va*DNA) [29] and/or infected with VHSV or SJNNV at 0.1 MOI. Treatments applied to AGs were: (i) sRPMI + sRPMI (control group), (ii) *Va*DNA + sRPMI group, (iii-iv) sRPMI + VHSV or SJNNV groups, (v-vi) *Va*DNA + VHSV or SJNNV groups. RNA was isolated at 12, 24 and 48 h after treatments as described below.

The concentration of PAMPs (*Va*DNA and poly I:C) and sampling times tested have been found to be optimal for the *in vitro* activation of seabream phagocytes [30]. Each assay was performed in triplicate and repeated with AGs from three animals.

2.6. Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and the upper phases containing RNA were purified with RNAquous Micro Kit (Ambion) according to the manufacturer's protocols. Final RNA concentration was measured at 260 nm with the nanodrop system (ND-1000), and RNA quality was checked by electrophoresis. RNA was stored at −80 °C until use. Total RNA was treated with Amplification grade DNase I (1 unit/µg RNA, Life technologies). Then, SuperScrip III RNase H$^-$ Reverse Transcriptase (Life technologies) was used to synthesize first strand cDNA with random hexamer primers from 1 µg of total RNA at 50 °C for 50 min followed by 15 min at 70 °C. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reactions were performed in 10-µl mixtures containing 5 µl of 2 × SYBR Green PCR Core Reagents (Applied Biosystems), 0.15 µl of each primer (0.15 µM, final concentration), and 2.5 µl of cDNA. The amplification profile was: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C. RNA was quantified using ribosomal protein S18 (*rps18*) transcript content as housekeeping gene using the comparative Ct method ($2^{-\Delta\Delta Ct}$ for fold change and $2^{-\Delta Ct}$ for relative value). The primers used (Table 1) had an amplification efficiency of 2 and their specificity was confirmed by melting curve analysis and sequencing each amplified product (Genetic Analyser ABI PRISM 3130, Applied Biosystems) (data non-shown). In all cases, each PCR was performed with triplicate samples.

Table 1
List of primers used in this study.

Name	Sequence (5′-3′)	Accession no.	Target gene
sa-isg15-F[a]	GTGAGCTCCCTGAAGCAACT	HS987162	*isg15*
sa-isg15-R	GACCGTTTACAAACACCAGC		
rps18-F[b]	AGGGTGTTGGCAGACGTTAC	AY587263	*Rps 18 housekeeping gene*
rps18-R	CTTCTGCCTGTTGAGGAACC		
VHSV-F1[c]	AAGGCCCTCTATGCGTTCATC	AJ233396	VHSV *nucleoprotein*
VHSV-R1	GGTGAACAACCCAATCATGGT		
SJ-RNA2-F[d]	GACACCACCGCTCCAATTACTAC	D30814	SJNNV *capsid protein*
SJ-RNA2-R	ACGAAATCCAGTGTAACCGTTGT		

[a] This study.
[b] [31].
[c] [32].
[d] [33].

2.7. Cell viability

To quantify seabream AG viability, 5×10^5 cells/well were seeded on 96-well plates and incubated in sRPMI with identical treatments as described in section 2.5. On days 1–5, aliquots of cell suspensions (50 µl) were diluted in 200 µl PBS containing 40 µg/ml propidium iodide (PI). The number of red fluorescent cells (dead cells) from triplicate samples of three different fish was analysed by flow cytometry (FACSCalibur, Becton-Dickinson).

2.8. Statistical analysis

Data were analysed by ANOVA and Tukey's multiple range tests to determine differences among groups. Differences were considered statistically significant when $p < .05$.

3. Results

3.1. Identification and characterization of gilthead seabream isg15

Searches within the publicly available EST database of the European Nucleotide Archive (ENA) allowed us to identify a gene encoding for gilthead seabream Isg15 (*sb-isg15*, GenBank accession number HS987162). It contains a 5′-untraslated region (UTR) of 77 bp, an open reading frame of 468 bp coding for a 155-aminoacid protein, and a 3′-UTR of 398 bp (Fig. 1). In the 3′-UTR, three motifs ATTTA responsible for the instability of mammalian [34] and fish [35] cytokine mRNAs and a putative cytoplasmic polyadenylation element (CPE) were identified (Fig. 1). However, taken into account that the poly A sequence

```
1       GGTTTCCAGAGGTTCGAGTCTTTTTCTGAAGTTTTCAACA
41      GGAGTTTTTTCTGAAGTGATCACAGACAACTTTCATCATG
    1                                        M
81      GAGATAAACATCATTATGCTGAATGGGGCGTCCCATCGCC
    2    E  I  N  I  I  M  L  N  G  A  S  H  R
121     TGATGGTGAACCCACAGGACACCGTGAGCTCCCTGAAGCA
    15  L  M  V  N  P  Q  D  T  V  S  S  L  K  Q
161     ACTCATCCAGAGTAAACTGGGAGTCTCTGTTCAGGAGCAG
    29    L  I  Q  S  K  L  G  V  S  V  Q  E  Q
201     AAGCTGGTGTTTGTAAACGGTCAGAGGACTCCTCTCAACG
    42   K  L  V  F  V  N  G  Q  R  T  P  L  N
241     ACGACTCAAAGCCTCTCAGCTGGTACGGTCTACAGTCCGG
    55  D  D  S  K  P  L  S  W  Y  G  L  Q  S  G
281     CTCCCAGGTGTCTCTGCTGATCACCCAGCCGCCACCCTTC
    69    S  Q  V  S  L  L  I  T  Q  P  P  P  F
321     CAGGTCTTCCTCAAAAACGAGAAGGGCAAATCAACCACCT
    82   Q  V  F  L  K  N  E  K  G  K  S  T  T
361     ACGATATCACACCTGAGGAGACTGTGGACCGCTTCAAGGC
    95  Y  D  I  T  P  E  E  T  V  D  R  F  K  A
401     CAGGGTGGAGAAAAGAGAGGGGGTCCCAGTGAGCCAGCAG
    109   R  V  E  K  R  E  G  V  P  V  S  Q  Q
441     AGGCTCCTCCACCAGAGCAGAGAGATGACCACTGGAAGAC
    122  R  L  L  H  Q  S  R  E  M  T  T  G  R
481     TGACAGACTACAACGTCACCAATCACAGCACCATCGACCT
    135 L  T  D  Y  N  V  T  N  H  S  T  I  D  L
521     GATGCTTCGCCTGAGGGGAGGCTGAGGACTCTTAGTTAAT
    149   M  L  R  L  R  G  G  *
561     CACCTATAGCCTAGTTTATTTTAATTTAATAGAAATGAAA
601     ATCTTAAATTCTGTTTATTTATATAAAAAAAAATCCAAATC
641     AGCTTTATTGGTCAAGTATTGACAAATTGGACTAATTAAC
681     TGATCATCAACATCATTCCTAGATATGCAATTGTGTCATA
721     GCTTAAAATATGATTTTGTTTTGACCATACAAATGTCACA
761     CATGCATACATTTTTTATTTTTCAAAATTGCATGTTTAAAT
801     TANGTTAAATAGTCCAAACATTCCTCGATGTGTACAGAAA
841     TGACGACCTCCAATGCTGCGTTTTGTATTTACTTTTATTT
881     TTGACTTGTAACTGTGTCATATCTTAAACAGTTGTAATGG
921     CTTAATATGTTTTATTTTGTTTG
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Fig. 1. Nucleotide and deduced amino acid (bold) sequences of sb-*isg15* cDNA. Nucleotides and amino acids are numbered along the left margin. The start (ATG) and stop (TGA) codons are underlined and the ubiquitin conjugation motif (LRGG) is highlighted in grey. The instability motifs (ATTTA) are in bold, and the cytoplasmatic polyadenylation element (CPE) is in bold and underlined.

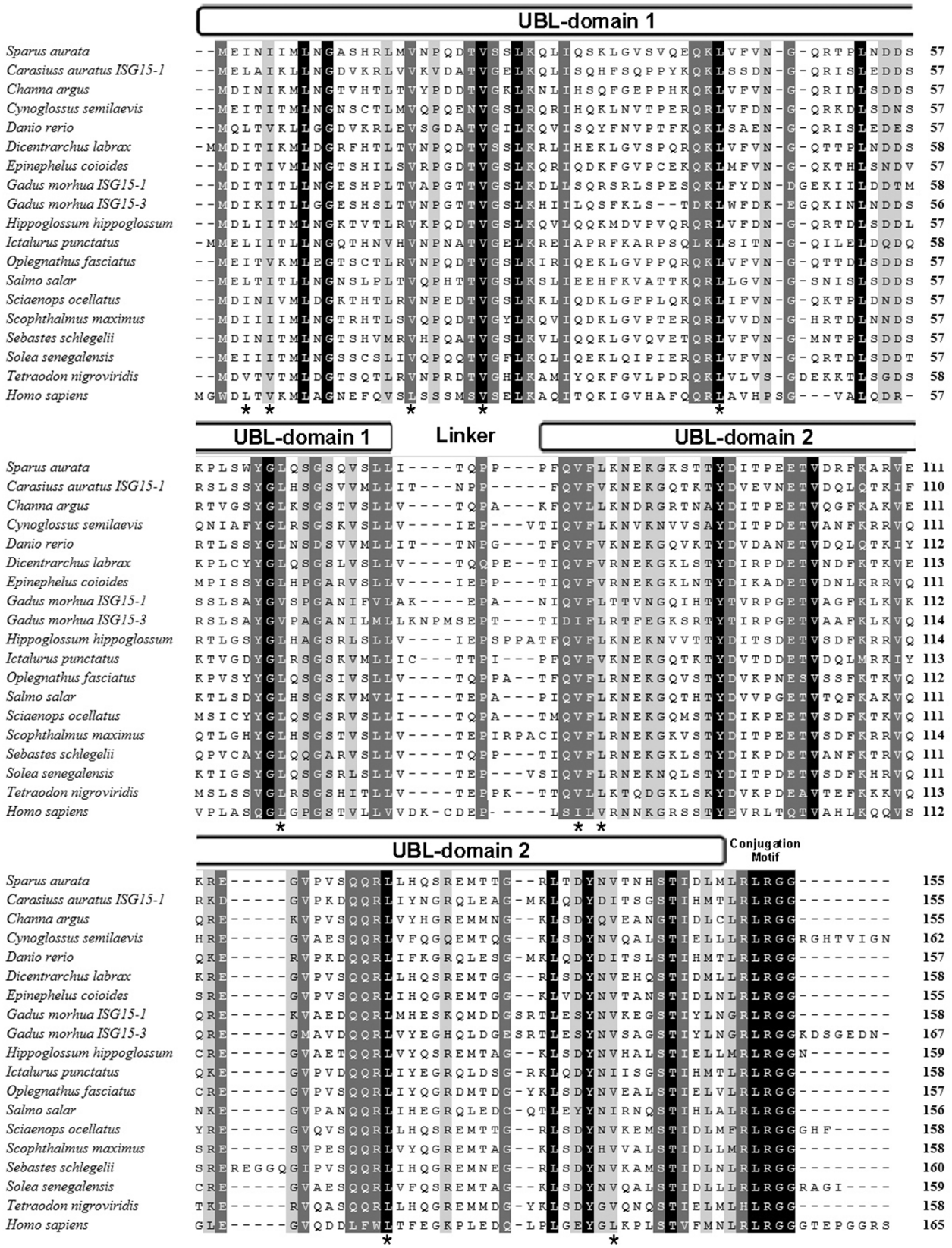

Fig. 2. Alignment of the deduced amino acid sequence of sb-Isg15 with other Isg15 homologue sequences. The predicted ubiquitin-like (UBL) domains are indicated by white boxes above the sequences. Conserved amino acid regions are shaded in black, dark grey (regions preserved in ≥85% of the species), or light grey (regions preserved in ≥75% of the species) and the six invariant aliphatic residues in each UBL domain are indicated with asterisks. Sequences accession numbers are shown in Supplementary Data, Table S1.

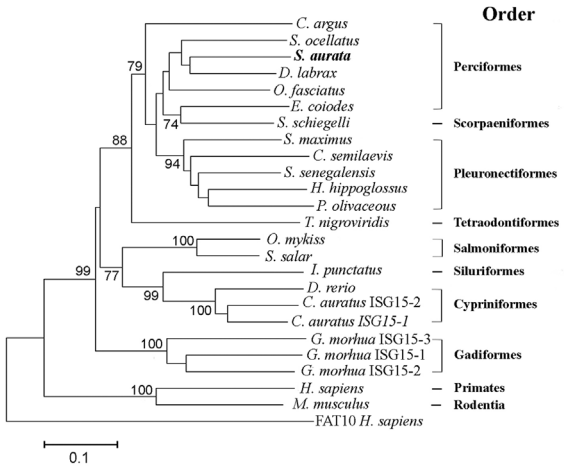

Fig. 3. Isg15 phylogenetic tree constructed by the neighbour-joining method using MEGA7 software. Numbers on nodes indicate the bootstrap confidence value based on 2000 replications. 0.1 indicates the genetic distance.

was not found in this study, hence 3′-UTR is incomplete, the existence of additional motifs could not be discarded. *In silico* analysis revealed the presence of two conserved tandem UBL domains, and a C-terminal LRGG conjugation motif in sb-Isg15 (Fig. 2). The deduced protein exhibits the highest sequence identity (75.6–63.0%) with other fish Isg15 proteins belonging to the Perciformes order, such as *Dicentrarchus labrax*, *Sciaenops ocellatus,* or *Oplegnathus fasciatus,* (Table S1). The NJ-phylogenetic tree showed that mammalian and teleost Isg15 sequences were separated into two clusters (Fig. 3) and further subdivision of the teleost sequences showed that sb-Isg15 has the closest phylogenetic relationship with other fish Isg15 belonging to the Perciformes order, followed by Scorpaeniformes, Pleuronectiformes, and Tetraodontiformes, all included in the Actinopterygii class (Fig. 3).

3.2. Modulation of sb-isg15 and viral proteins transcription in SAF-1 cells

Stimulation of SAF-1 cells with poly I:C, which mimics the dsRNA produced during viral infection, for 12 and 24 h resulted in increased mRNA levels of *sb-isg15* (Fig. 4A), being the highest degree of induction ocurring at 12 h post-stimulation. Furthermore, infection of SAF-1 cells with VHSV or with SJNNV induced *sb-isg15* transcription but with a different kinetic (Fig. 4B), the highest induction being at 48 h p.i. in VHSV-infected cells, and at 24 h p.i. in SJNNV-infected cells.

Analysis of the mRNA of viral genes in SAF-1-infected cells (*nucleoprotein* of VHSV and the *capsid protein* of SJNNV) revealed a maximum level of both viral RNAs at 48 h p.i. (Fig. 4C). Interestingly, infection of SAF cells with SJNNV resulted in higher transcription levels of viral protein compared with the VHSV ones (Fig. 4C).

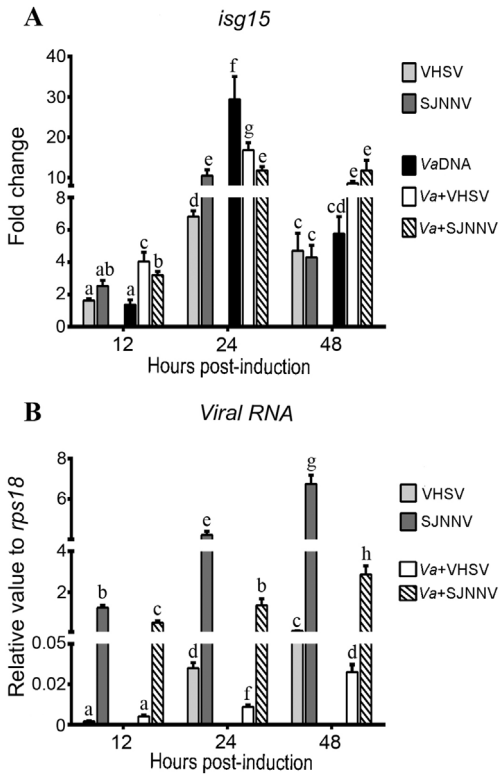

Fig. 5. Gilthead seabream *isg15* induction and viral RNA in acidophilic granulocytes (AGs). **(A)** Induction of sb-*isg15* in AGs inoculated with VHSV (light grey), SJNNV (dark grey), *Va*DNA (50 μg/ml, black) and *Va*DNA co-inoculated with VHSV (white) or SJNNV (white and black lines) at 0.1 MOI. Data were normalized with *rps18* transcription level and expressed as mean ± SD (n = 3) of fold change in inoculated cells relative to control untreated cells. **(B)** Viral RNA of VHSV *nucleoprotein* (light grey) or SJNNV *capsid protein* (dark grey) genes in inoculated AGs. Data were normalized with *rps18* transcription level and expressed as relative value to *rps18* mean ± SD (n = 3). Different letters denote statistically significant differences among the groups and within each group over the time according to a Tukey test ($p < .05$).

3.3. Modulation of sb-isg15 and viral RNA transcription in seabream AGs

Isg15 plays an important role in the regulation of macrophage responses [36], but little is known to date concerning the role of Isg15 in neutrophil functions. Therefore, we studied the modulation of *sb-isg15* transcription by viral infection in a purified fraction of professional phagocytes, AGs, which are functionally equivalent to mammalian neutrophils [27,30,37–39]. The results showed that VHSV and SJNNV inoculation increased the transcript level of *isg15* in seabream AGs from 12 h p.i. onwards (Fig. 5A). The maximum transcription levels were recorded 24 h p.i., SJNNV-infected cells showing a higher induction rate (10.51) than VHSV-infected cells (6.82) (Fig. 5A).

In a previous report [30], we demonstrated that poly I:C failed to

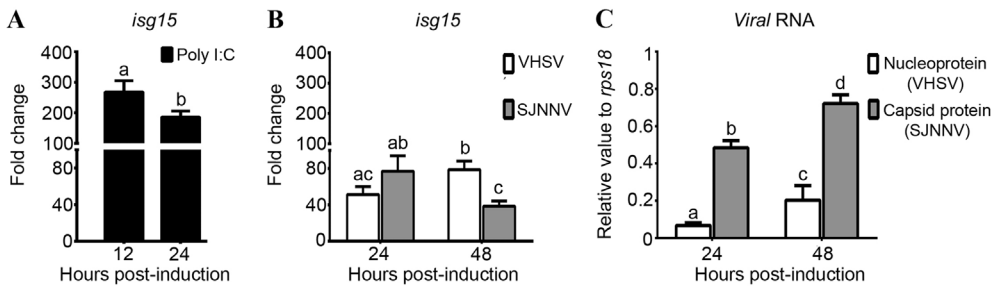

Fig. 4. Gilthead seabream *isg15* induction and viral replication in SAF-1 cells. The mRNA levels of the gene coding for Isg15 (A y B) and viral proteins (C) were determined by real-time RT-PCR. **(A)** sb-*isg15* induction after poly I:C (25 μg/ml) treatment. **(B)** sb-*isg15* induction after VHSV (white) or SJNNV (grey) inoculation (0.1 MOI). **(C)** Viral RNA induction of VHSV *nucleoprotein* (light grey) or SJNNV *capsid protein* (dark grey) genes in SAF-1-inoculated cells. Data were normalized with *rps18* transcription level and expressed as mean ± SD (n = 3) of RNA fold change levels relative to control untreated cells for sb-*isg15* and as relative value to *rps18* mean ± SD (n = 3) for viral RNA. Different letters denote significant differences ($p < .05$) among groups and within each group over the time, using one-way analysis of variance (ANOVA) and Tukey's multiple range test.

directly activate AGs. In contrast, *Va*DNA had a powerful impact on the main AGs functions [30]. So we sought to analyse the effect of stimulation with *Va*DNA alone or in combination with VHSV and SJNNV inoculation on the regulation of *sb-isg15* transcription levels in AGs. The results showed that *Va*DNA induced the transcription of *sb-isg15* in seabream AGs with a similar pattern to that observed in VHSV and SJNNV, with maximum induction at 24 h post-stimulation and a significant decrease of *sb-isg15* transcriptional induction 48 h after the treatment (Fig. 5A).

Interestingly, the stimulation of AGs with *Va*DNA accompanied by viral infection had a synergistic effect on the induction of *sb-isg15* transcription 12 and 48 h post-treatments (Fig. 5A). Furthermore, it is of interest that the kinetic of the transcription of *sb-isg15* induced by *Va*DNA or viral infection was different from that recorded in cells treated with *Va*DNA combined with VHSV or SJNNV (Fig. 5A). The stimulation of AGs with *Va*DNA and viral infection simultaneously resulted in a more sustained induction of *sb-isg15* transcript than that observed with independent treatments (Fig. 5A).

As regards viral replication in seabream AGs, VHSV and SJNNV genome levels significantly increased from 12 h to 48 h p.i. (Fig. 5B). In addition, IFN I induction elicited by *Va*DNA seemed to interfere with viral replication at 24 and 48 h p.i. in VHSV-infected cells, and at 12, 24, and 48 h p.i. in SJNNV-inoculated AGs (Fig. 5B), as indicated by the lower amount of viral genome recorded in cells stimulated with *Va*DNA and viral infection compared with cells not treated with *Va*DNA. The greatest differences in viral replication between *Va*DNA-stimulated and non-stimulated cells were recorded at 48 h p.i.: 67.24% lower VHSV-nucleoprotein RNA and 57.53% lower level of SJNNV capsid protein RNA in cells treated with *Va*DNA (Fig. 5B).

To further analyse the putative cytopathic effect induced by VHSV and SJNNV in AGs we measured the cellular mortality produced by viral infection in AGs treated or not with *Va*DNA (Fig. 6). Upon viral infection, no differences in the percentage of cellular viability at one or two days post-infection were observed, apart from the differences in viral RNA content at these time points. However, at 5 days p.i. a lower percentage of cell viability was observed in VHSV- or SJNNV-infected AGs compared with non-infected cells. Furthermore, in keeping with previous studies [38] *Va*DNA treatment increased the lifespan of AGs compared with non-treated ones (Fig. 6). Of interest was the fact that viral infection did not decrease cell viability in *Va*DNA-stimulated AGs at any time tested (Fig. 6).

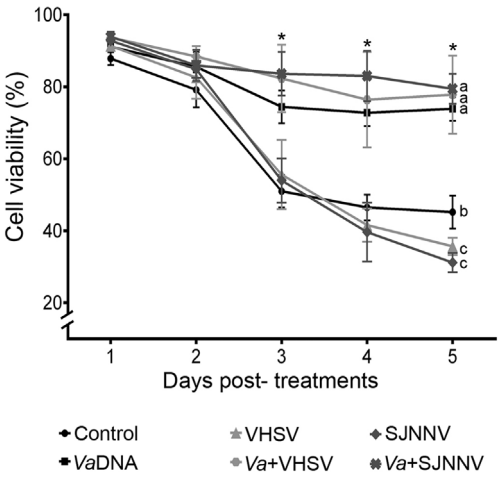

Fig. 6. Survival rate of purified seabream AGs treated with *Va*DNA (50 μg/ml), and/or inoculated with VHSV or SJNNV (0.1 MOI). Data are expressed as mean ± SD (n = 3) of the percentage of PI negative AGs determined by flow cytometry at the indicated time points. Asterisks indicate statistically significant differences ($p < .05$) between non-treated and *Va*DNA-treated groups. Different letters denote statistically significant differences ($p < .05$) among all groups according to a Tukey test of means comparison.

4. Discussion

The full-length cDNA of an *isg15* gene from *S. aurata* (*sb-isg15*) was identified. Like all known Isg15, sb-Isg15 contains two tandem UBL domains and the LRGG motif. In addition, three instability motifs were identified in the 3′-UTR of *sb-isg15*, as has also been detected in several fish *isg15* sequences, varying from one in zebrafish (*Danio rerio*) to six in Japanese flounder (*Paralichthys olivaceus*). These sequences have a direct effect on mRNA stability and translation efficiency [34,35,40]. Besides, a CPE, which is an U-rich sequence able to repress or exert translation depending on the cellular type [41], was also found in the *sb-isg15* 3′-UTR region. This motif is also present in several fish *isg15*, such as zebrafish, Japanese flounder, turbot, or seabass [42]. The presence of both types of regulatory elements in fish, but not in mammalian *isg15,* suggests that the expression of fish *isg15* genes is subjected to a tight post-transcriptional regulation.

When the deduced amino acid sequence of sb-Isg15 was compared with other fish and mammalian Isg15 sequences, a high degree of conservation of the UBL domains, especially of the six aliphatic residues that are crucial for the ubiquitin-like protein structure [43], was observed. The LRGG motif, which is essential for ISGylation and, therefore, for the direct antiviral activity of Isg15 proteins [3], is present in sb-Isg15 but without the additional amino acids that appear in other fish and mammalian Isg15 proteins [44]. In human and mouse, Isg15 is synthesized as a precursor protein which is post-translational processed by a cellular converting enzyme that cleaves additional C-terminal amino acids, exposing the LRGG residues for conjugation [44]. Interestingly, the lack of these additional amino acids on sb-Isg15 could allow the exposure of the conjugating motif and hence suggest that a post-translational processed is not required. However, additional studies are needed to determine whether sb-Isg15 is synthesized as an active protein.

Phylogenetic analysis of sb-Isg15 confirmed that it represents the orthologue of fish and mammalian Isg15. Sb-Isg15 exhibited the closest relationship with Isg15 from other species within the Perciformes order, followed by those from Gadiformes, Cypriniformes, Siluriformes or Salmoniformes, which showed identity percentages around 50%. When compared with mammalian Isg15, the identity percentages of sb-Isg15 were around 30%. Across mammals, Isg15 conservation is also poor [45]. These data suggest an interspecies diversification of this protein that would be interesting to analyse at functional level. Key functional differences have been found between murine and human Isg15, murine Isg15 showing clear antiviral activity and human Isg15 playing a more powerful role as a negative regulator of IFN signalling [7,46–48]. Further studies are necessary to disclose putative functional differences between fish Isg15 proteins, and to what extent Isg15-species specificity contributes to the different responses of fish to viral infections.

Two *in vitro* systems were used in this study to characterize the role of *sb-isg15* in the host immune defense against viral infections in gilthead seabream: the established cell line SAF-1 and AGs. In SAF-1 cells, *sb-isg15* transcription was up-regulated by the IFN inducer poly I:C, and by VHSV and SJNNV infections, which supports a putative role for sb-Isg15 in the antiviral defense system in that species. Poly I:C triggered an earlier and stronger up-regulation than that of both viruses, probably due to the direct activation of the immune system by poly I:C. In contrast, viruses have to replicate before they can generate a noticeable response. Similar results have been obtained for other fish *isg15,* both *in vivo* and *in vitro* [10,11,13,42–44,49,50]; however, grouper (*Epinephelus coioides*) *isg15* showed stronger induction after GNNV infection than after treatment with poly I:C [13].

In mammals, several viruses have been shown to induce the expression of ISGs in neutrophils [51], which are the most abundant circulating granulocytes and play an important role in the innate host response [52]. Similar physiological responses have been documented for seabream AGs and mammalian neutrophils, so seabream AGs could be considered the functional equivalents of mammalian neutrophils

[26,27,30,31,37–39]. In addition to cytotoxic cells, phagocytes (macrophages and granulocytes) play important roles in viral clearance. Therefore, seabream AGs represent a valuable experimental system to study virus-host interactions. In a previous report, we demonstrated that poly I:C fail to directly activate AGs [30]. In contrast, *Va*DNA has a powerful impact on the main biological functions of AGs like phagocytosis, respiratory burst and cytokine production [30]. Here, we have shown that the inoculation of seabream AGs with VHSV and SJNNV results in increased mRNA levels of *sb-isg15*. Interestingly, stimulation of those cells with *Va*DNA triggered the up-regulation of *sb-isg15* transcription levels more than those of VHSV or SJNNV inoculation. These findings are consistent with previous reports demonstrating that, besides viral infections, the transcription of mammalian *isg15* is also induced by other factors like lipopolysaccharide (LPS), bacterial infection, *p53*, and certain genotoxic stress factors (reviewed in Ref. [53]). In fish, mycobacterial infection induced *isg15* transcription in immune associated organs of goldfish (*Carassius auratus*) [54], formalin killed *V. anguillarum* induced *isg15* transcription in cod (*Gadus morhua*) head kidney [10], and IFN-γ induced Isg15 in Atlantic salmon (*Salmo salar*) cells [55].

Analysis of the viral genome content demonstrated that VHSV and SJNNV replicated in the two *in vitro* systems used in this study and, as expected, the viral charge increased with time. Interestingly, as in SAF-1 cells, inoculation of AGs with VHSV or with SJNNV triggered *sb-isg15* transcription up-regulation at a similar level. Interestingly, viral genome replication was lower in simultaneously *Va*DNA-stimulated and viral-infected AGs compared with viral infection alone. Furthermore, simultaneous stimulation of AGs with *Va*DNA and viral infection triggered lower induction of *sb-isg15* transcript levels than that *Va*DNA alone, but it was more sustained than that with independent treatments, suggesting that both viral infections interfered with *sb-isg15* induction, probably to prevent antiviral activity. These results suggest that *Va*DNA stimulated *sb-isg15* transcription and, probably the complete IFN response, in AGs during the early stages of the infections, thus reducing but not preventing viral replication. Then, as viruses usually antagonize the type I IFN system through non-structural proteins (reviewed in Ref. [56]), when VHSV and SJNNV protein expression started, viral proteins might have interfered with the transcription of *sb-isg15*. Interference of VHSV and SJNNV against the IFN system has previously been reported *in vivo* in Senegalese sole (*Solea senegalensis*) and Japanese flounder [50,57] and *in vitro* in RTG and EPC cells [58,59]. Thus, our results add gilthead seabream to the group of fish species where VHSV and SJNNV show antagonistic activity against the IFN response.

It is interesting that VHSV and SJNNV genome replication was higher in AGs than in SAF-1 cells; however, both viral infections triggered weak *sb-isg15* induction in AGs compared with that observed in SAF-1 cells. This phenomenon could be explained by differences in basal *sb-isg15* expression levels among SAF-1 cells and AGs. In this regard, although not statistically significant, a tendency towards lower constitutive mRNA levels of *sb-isg15* was observed in AGs compared with SAF-1 (data not shown). So, the two systems used in this study reveal the complexity of the antiviral response and the variable behavior of the two cell types to viral infection regarding their IFN response activation level, which could be due to the specific role of AGs in the immune response. To the best of our knowledge, this is the first evidence of viral replication in fish AGs. Furthermore, viral replication exerted a cytophatic effect on seabream AGs by increasing cellular mortality. Apoptosis after infection with many types of viruses is generally considered as a self-defense mechanism [36,60,61]. Besides the fact that SJNNV genome replication was higher than that of VHSV in AGs, interestingly, there were no differences in cell mortality among treatments. In previous studies we demonstrated that apoptosis is the default state of gilthead seabream AGs and that microbial products, like *Va*DNA, extend their functional lifespan in culture [38]. The results herein showed that the stimulation of AGs with *Va*DNA prevents cell death even in the presence of viral infection. The prevention of AGs mortality by TLR-agonists is mediated by a mechanism that is dependent on p38 MAPK [38]. So, in view of these results, it is tempting to speculate that the activation of downstream p38 MAPK signalling pathways in the host by VHSV and SJNNV is required for viral infection to proceed. In mammals, several viruses have been found to productively infect neutrophils, and it has been suggested that these viruses might use neutrophils as transport vehicles for dissemination in the body [62]. In addition, Zhang et al. [7] recently showed that influenza A virus can be replicated in and released by human neutrophils, and may play a significant role in the infection course. Further studies are necessary to determine whether VHSV and SJNNV viral particles are released from seabream AGs and the putative consequences that this might have regarding pathogenesis of these infections.

In summary, this study describes the molecular structure of gilthead seabream *isg15* and its deduced protein, which shows similar characteristics to that observed in other fish species. Two cellular *in vitro* models (the SAF-1 cell line and AGs), two virus infection models (VHSV and SJNNV) and two TLR-ligands (poly I:C and *Va*DNA) have been used to gain insight into the role of Isg15 in the host immune defense against viral infections in this species. Using these models, we report here the up-regulation of *sb-isg15* transcription in both cell types at different levels but with similar kinetics as well as the correlation of *sb-isg15* transcript level and viral genome replication. Furthermore both viruses exerted a similar cytopathic effect on AGs, recorded as cell death, which was abolished by concomitant virus infection and TLR activation. The results highlight the complexity of the antiviral response and the variable behavior of different cell types to viral infection and confirm the use of gilthead seabream AGs as a valuable experimental system for illustrating virus-host interactions in this fish species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.12.027.

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