Patricia Moreno García

Tesis Doctoral

2019

UNIVERSIDAD DE MÁLAGA

FACULTAD DE CIENCIAS
DEPARTAMENTO DE MICROBIOLOGÍA

Betanodavirus virulence to European sea bass (*Dicentrarchus labrax*): a new insight into virus-host interaction

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Betanodavirus virulence to European sea bass (*Dicentrarchus labrax*): a new insight into virus-host interaction

Memoria presentada por **Dña. Patricia Moreno García** para optar al grado de Doctor con Mención de Doctorado Internacional.

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Noviembre 2018

UNIVERSIDAD DE MÁLAGA

Departamento de Microbiología
Prof. Dr. J.J. Borrego

26 de Noviembre de 2018

Juan José Borrego García, Catedrático de Microbiología y como tutor de la Tesis Doctoral de Dª Patricia Moreno García titulada: "Betanodavirus virulence to European sea bass (*Dicentrarchus labarx*): A new insight into virus-host interaction",

Informa: Que la citada Tesis Doctoral está concluida, por lo que autorizo a que pueda ser defendida.

Y firmo el presente, a petición de la Comisión de Doctorado, en fecha "ut supra"

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Departamento de Microbiología

Facultad de Ciencias

Dña. MARÍA DEL CARMEN ALONSO SÁNCHEZ, Profesora Titular del Departamento de Microbiología de la Universidad de Málaga

INFORMA:

Que, Dña. **PATRICIA MORENO GARCÍA** ha realizado bajo su dirección el trabajo experimental conducente a la elaboración de la presente Memoria de Tesis Doctoral. Los resultados de dicho trabajo experimental han sido parcialmente publicados en las contribuciones que avalan la tesis, las cuales no han sido utilizadas para avalar tesis anteriores.

Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, expide el presente informe,

Málaga, a 26 de noviembre del 2018

Fdo.: M. Carmen Alonso Sánchez

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INFORME DE EVALUACIÓN DEL DIRECTOR

Isabel Bandín Matos, Profesora Titular del Departamento de Microbiología y Parasitología de la Universidad de Santiago de Compostela,

Como co-directora del trabajo de Tesis Doctoral titulado "Betanodavirus virulence to European sea bass (*Dicentrarchus labrax*): a new insight into virus-host interaction" presentado por Dña **Patricia Moreno García**, matriculada en el Programa de Doctorado de la Universidad de Málaga "Biología Celular y Molecular"

Autoriza la presentación de la tesis indicada considerando que reúne todos los requisitos exigidos por la normativa vigente

Asimismo, informa que todas las publicaciones que avalan esta tesis no han sido presentadas en trabajos de tesis anteriores

Y para que así conste a los efectos oportunos, firmo la presente en Santiago de Compostela a 23 de Noviembre de 2018.

Fdo. Isabel Bandín Matos

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Los estudios realizados en la presente Tesis Doctoral han sido financiados por los siguientes proyectos:

- Patogenicidad de betanodavirus en lenguado cultivado (*Solea senegalensis*) y su relación con la respuesta inmune del hospedador.
  Ref. AGL2014-54532-C2-1-R

- Susceptibilidad de la lubina (*Dicentrarchus labrax*) a betanodavirus: interacción virus-hospedador (MINECO/AEI/FEDER, UE).
  Ref. AGL2017-84644-R

La Doctoranda **Patricia Moreno García** ha disfrutado de una beca de Formación de Profesorado Universitario (FPU), otorgada por el Ministerio de Educación, Cultura y Deporte, durante el periodo 2013-2017, con el número identificador FPU12/00265.

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Parte de los resultados obtenidos en la presente Tesis Doctoral han sido publicados y comunicados en las siguientes revistas y congresos:

Publicaciones:

- Moreno, P., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. (2016). Genetic characterization and transcription analyses of the European sea bass (*Dicentrarchus labrax*) *isg15* gene. *Fish and Shellfish Immunology*, *55*, 642-646.

- Moreno, P., Lopez-Jimena, B., Randelli, E., Scapigliati, G., Buonocore, F., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. (2018). Immuno-related gene transcription and antibody response in nodavirus (RGNNV and SJNNV)-infected European sea bass (*Dicentrarchus labrax* L.). *Fish and Shellfish Immunology*, *78*, 270-278.

- Moreno, P., Alvarez-Torres, D., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. (2018). Differential antiviral activity of European sea bass interferon-stimulated 15 protein (ISG15) against RGNNV and SJNNV betanodaviruses. *Fish and Shellfish Immunology*, *83*, 148-157.

Congresos internacionales:

- Moreno, P., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. Antiviral activity of European sea bass ISG15 against betanodavirus infections. *10th International Symposium on Viruses of Lower Vertebrates*, p. 45, 4-7 Junio 2017, Budapest, Hungría.

Congresos nacionales:

- Moreno, P., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. Papel del inmunogen *isg15* de lubina (*Dicentrarchus labrax*) en las infecciones por betanodavirus. *XI Congreso de Microbiología del Medio Acuático*, Sociedad Española de Microbiología, p. 62, 20-22 Julio 2016, Oviedo, España.
    - Premio a la mejor comunicación oral en la "Sesión Patología I"

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Parte de los conocimientos científicos empleados en la presente Tesis Doctoral han sido adquiridos por la doctoranda **Patricia Moreno García** a través de la realización de varias estancias breves en centros de calidad:

- Estancia Nacional en el Departamento de Microbiología y Parasitología del Instituto de Acuicultura de la Universidad de Santiago de Compostela. 2014.

- Estancia Internacional en el *Dipartimento per la innovazione nei sistemi biologici, agroalimentari e forestali (DIBAF), Universidad degli studi della Tuscia*, Viterbo (Italia). 2016.

Además, la doctoranda posee el certificado de Competencia de bienestar animal en animales utilizados para experimentación y otros fines científicos, categoría B, y un curso de Buenas Prácticas de Laboratorio (BPL), ambos emitidos por la Junta de Andalucía, lo que la capacita para realizar los experimentos con peces descritos en la presente Tesis Doctoral.

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This PhD Thesis has been supported by the following projects:

- Patogenicidad de betanodavirus en lenguado cultivado (*Solea senegalensis*) y su relación con la respuesta inmune del hospedador.
  Ref. AGL2014-54532-C2-1-R

- Susceptibilidad de la lubina (*Dicentrarchus labrax*) a betanodavirus: interacción virus-hospedador (MINECO/AEI/FEDER, UE).
  Ref. AGL2017-84644-R

The PhD student **Patricia Moreno García** has been supported by a FPU (*Formación de Profesorado Universitario*) fellowship from *Ministerio de Educación, Cultura y Deporte*, Spanish Government, with the identification number FPU12/00265.

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The results derived from the present PhD Thesis have been partially published in the following scientific publications and conferences:

Scientific publications:

- Moreno, P., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. (2016). Genetic characterization and transcription analyses of the European sea bass (*Dicentrarchus labrax*) *isg15* gene. *Fish and Shellfish Immunology, 55*, 642-646.

- Moreno, P., Lopez-Jimena, B., Randelli, E., Scapigliati, G., Buonocore, F., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. (2018). Immuno-related gene transcription and antibody response in nodavirus (RGNNV and SJNNV)-infected European sea bass (*Dicentrarchus labrax* L.). *Fish and Shellfish Immunology*, *78*, 270-278.

- Moreno, P., Alvarez-Torres, D., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. (2018). Differential antiviral activity of European sea bass interferon-stimulated 15 protein (ISG15) against RGNNV and SJNNV betanodaviruses. *Fish and Shellfish Immunology*, *83*, 148-157.

International conferences:

- Moreno, P., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. Antiviral activity of European sea bass ISG15 against betanodavirus infections. *10th International Symposium on Viruses of Lower Vertebrates*, p. 45, 4-7 June 2017, Budapest, Hungary.

National conferences:

- Moreno, P., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. Papel del inmunogen *isg15* de lubina (*Dicentrarchus labrax*) en las infecciones por betanodavirus. *XI Congreso de Microbiología del Medio Acuático*, Sociedad Española de Microbiología, p. 62, 20-22 July 2016, Oviedo, Spain.
    - Scientific conference award for the best oral presentation in "*Sesión Patología I*"

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During the present PhD Thesis, **Patricia Moreno García** has carried out the following national and international stays as part of her scientific training:

- National stay in the Microbiology and Parasitology Department, Aquaculture Institute, University of Santiago de Compostela (Spain). 2014.

- International stay in the *Dipartimento per la innovazione nei sistemi biologici, agroalimentari e forestali (DIBAF), Universidad degli studi della Tuscia*, Viterbo (Italy). 2016.

Furthermore, the PhD student holds the License to perform animal experiments, according to the Spanish Act of Animal Experimentation, Law 80/2011, Category B, and the Good Laboratory Practice (GLP) course, both approved by the Andalusian Government, allowing her to carry out the fish challenges performed in the present PhD Thesis.

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El presente manuscrito corresponde a la versión definitiva de la Tesis Doctoral titulada *Betanodavirus virulence to European sea bass (Dicentrarchus labrax): a new insight into virus-host interaction*, presentada por la doctoranda **Patricia Moreno García**. En esta versión se han tenido en cuenta las recomendaciones de los revisores externos, realizando únicamente los cambios sugeridos por los mismos, sin llevar a cabo otras modificaciones respecto a la versión anterior.

A los que me han servido de guía, dado sus mentes, manos y tiempo, brindado sus sonrisas y servido de apoyo. A mis padres, a mi familia de dos patas (y a la de cuatro), a mi marido y a mi otro corazón.

Gracias

PAGE

CHAPTER 2B: Evaluation of the antiviral activity of European sea bass ISG15 protein against betanodavirus infections using an *in vitro* approach

SUMMARY

European sea bass (*Dicentrarchus labrax*) culture has become a very important activity in Europe; however, the intensive production of this fish species has resulted in the appearance of infectious diseases in the aquaculture facilities, with viral diseases being especially important.

The major viral disease affecting European sea bass nowadays is viral nervous necrosis, caused by nervous necrosis virus (NNV, *Nodaviridae* family, *Betanodavirus* genus). It is a small (20-34 nm), non-enveloped virus, with an icosahedral capsid. Genetically, it is a single-stranded, positive-sense RNA virus, with a genome composed of two segments, RNA1, which encodes the 100-kDa RNA-dependent RNA polymerase; and RNA2, encoding the 42-kDa capsid protein (CP). In addition, a sub-genomic segment, RNA3, is synthesized during RNA replication from the RNA1 3'-end. This segment comprises two open reading frames (ORFs) that encode the two non-structural proteins B1 and B2.

On the basis of the variability in the T4 region, within the RNA2 segment, four betanodavirus species have been recognized by the International Committee on Taxonomy of Viruses (ICTV): red-spotted grouper- (RGNNV), striped jack- (SJNNV), barfin flounder- (BFNNV) and tiger puffer- (TPNNV) nervous necrosis virus, showing different host ranges and optimal replication temperatures. RGNNV and SJNNV are the only viral species detected in sea bass to date. RGNNV is highly virulent to this fish species (Carballo et al., 2016; Souto et al., 2015b; Vendramin et al., 2014), whereas SJNNV causes very low or no mortality (Souto et al., 2015b; Vendramin et al., 2014); however, SJNNV reference strain (SJ93Nag) replicates in sea bass brain, although it seems to fail in developing disease lesions (Carballo et al., 2016; Souto et al., 2015b).

This difference between betanodavirus species regarding the capacity of developing disease in sea bass makes interesting the study of the main factors involved in the interaction NNV-sea bass, including those determining the NNV virulence and the antiviral sea bass defence mechanisms.

Regarding pathogen factors, it is important to highlight the role of the C-terminus region of the CP (Ito et al., 2008; Iwamoto et al., 2004). This region contains the protruding domain (P-domain), which contains hyper-variable regions located in the protrusion surface, being proposed to be involved in cell-receptor binding and host-cell specificity (Chen et al., 2015). The identity of the viral amino acids involved in cellular

binding is not fully characterized; however, two amino acids located in the P-domain of the CP (at positions 247 and 270) have been proposed as possible determinants of betanodavirus virulence and host specificity (Olveira et al., 2009; Souto et al., 2015a).

Regarding the host defence mechanisms, fish display a complete immune system, composed of innate and adaptive responses.

The innate response is the first barrier, playing an important role against viral infections, especially the type I interferon (IFN I) system and the inflammatory response.

IFN I is a cytokine expressed and secreted in response to viral infections. Once in the extracellular medium, it is detected by neighbouring cells, inducing the expression of interferon-stimulated genes (ISGs), such as mx dynamin-like GTPase (*mx*), interferon-stimulated gene 15 (*isg15*), and interferon-stimulated gene 12 (*isg12*), coding for proteins with proven antiviral activity (Alvarez-Torres et al., 2016; Avunje and Jung, 2017; Jung and Jung, 2017).

Specifically, *isg15* encodes for a 15-kDa protein (ISG15) composed of two ubiquitin-like domains (UBLs), connected by a short linker sequence, and a conserved RLRGG motif in the C-terminal end. In fish, *isg15* has been demonstrated to be one of the earliest and most highly-expressed ISGs following viral infections (Alvarez-Torres et al., 2017; 2018; Furnes et al., 2009; Lin et al., 2015; Moreno et al., 2016; 2018; Zhang et al., 2007). In addition, previous studies have demonstrated the antiviral activity of several fish ISG15 proteins (Huang et al., 2013; Langevin et al., 2013a), as well as their immuno-modulatory activity (Langevin et al., 2013a), and extracellular location (Liu et al., 2010; Wang et al., 2012).

The inflammatory response is also an essential process in the innate response against viral infections, highlighting the role of the tumour necrosis factor alpha (TNF-α) and other cytokines, such as interleukin 1β (IL-1β), which promote the activation of several cytokines, such as interleukin 8 (IL-8) and IL-6 (Al-Banna et al., 2018). To control this process, the anti-inflammatory cytokines (IL-10, IL-11 or TFG-β) play a homeostatic role.

The adaptive response is delayed, although it is essential for long-lasting immunity (Secombes and Wang, 2012). This immune pathway is mainly mediated by

two groups of lymphocytes, B- and T-cells.

B-cells mediate antibody response, and three isotypes have been identified in fish: IgM, IgD and IgT (Secombes and Wang, 2012), although most of the studies have been set up by the quantification of systemic IgM (Li et al., 2015; Peñaranda et al., 2011; Xing et al., 2017).

T-cells organize cell-mediated response. Antigens presented by major histocompatibility complex (MHC) molecules are recognized by T-cells through T-cell receptors (TR). In fish, a complete set of genes coding for molecules related to T-cell activities has been reported (Boschi et al., 2011; Buonocore et al., 2012; Nuñez-Ortiz et al., 2014; Picchietti et al., 2011).

In summary, this PhD Thesis is a comprehensive study on the interaction between European sea bass and NNV, looking into the main factors involved in the pathogen-host interaction.

CHAPTER 1: ANALYSIS OF MOLECULAR DETERMINANTS IN BETANODAVIRUS VIRULENCE TO EUROPEAN SEA BASS

In this chapter, the importance of the CP amino acid positions 247 and/or 270 as betanodavirus virulence determinants to European sea bass has been evaluated.

To fulfil this aim, the genome of the SpDl_IAusc965.09 isolate (RGNNV), named as *wild type* Dl965 (*wt*Dl965), highly virulent to sea bass, has been sequenced following a two-step strategy: (i) sequencing of RNA1 and RNA2 ORFs, using the *primer walking* approach; (ii) sequencing of 5'- and 3'-ends by the Rapid Amplification of cDNA Ends (RACE) methodology.

The results obtained revealed that *wt*Dl965 RNA1 is a 3,104-bp segment, comprising a 78-bp 5'-untranslated region (UTR), a 2,949-bp coding region and a 77-bp 3'-UTR sequence, whereas the 1,432-bp RNA2 segment displays a 26-bp 5'-UTR, 1,017-bp ORF, and a 389-bp 3'-UTR region.

According to the comparative analysis carried out, *wt*Dl965 RNA1 5'- and 3'-UTR sequences are similar to those displayed by other RGNNV isolates; however, a higher variability was recorded in the RNA2 3'-UTR sequence, with changes affecting

nucleotides at positions 1,134, 1,159, 1,174, 1,195, 1,261, 1,270, 1,299, 1,302, 1,330 and 1,422. These variations could modify the stem loop structure (3'SL) formed in the RNA2 3'-end, which may modulate viral replication efficiency. In fact, a recent study has reported that punctual mutations in this region cause a significant decrease in the virulence of a reassortant isolate to Senegalese sole (*Solea senegalensis*) (Souto et al., 2018).

Once the genome of *wt*Dl965 isolate has been sequenced, infective viral particles have been recovered from viral cDNA using a reverse genetics approach based on the T7 RNA polymerase system (Biacchesi, 2011; Takizawa et al., 2008). First step was the amplification of the full-length RNA1 and RNA2 cDNAs, including in their sequences several motifs required for cloning and sub-cloning in the expression vector pBSδRiboT7t (Biacchesi et al., 2000). The constructions obtained, pBSRNA1 and pBSRNA2, were used to transfect BSRT7/5 cells (Buchholz et al., 1999), leading to the recovery of infective viral particles, genetically identical to the *wt*Dl965 isolate. These viruses were labelled as *reverse* Dl965 (*r*Dl965), and they were propagated on E-11 cells, observing cytopathic effects (CPEs) after the first cell inoculation (P0), and obtaining a titre of $7.1x10^7$ $TCID_{50}$/ml (P2 on E-11 cells).

The construction pBSRNA2 was used as template to mutate amino acids 247 and 270, introducing mutations affecting nucleotide 759 (thymine to guanine), which switches serine in position 247 to alanine, and nucleotide 830 (guanine to adenine), changing the amino acid in position 270 (serine to asparagine). Plasmids pBSRNA1, in combination with Mut247pBSRNA2, Mut270pBSRNA2 or Mut247+270pBSRNA2, were used for BSRT7/5 cell transfection.

The infective viral particles obtained were propagated on E-11 cells, causing CPEs after one blind passage (P1). The titres of these viral suspensions were $1.3x10^8$, $7.1x10^7$ and $2.3x10^7$ $TCID_{50}$/ml for Mut247Dl965, Mut270Dl965 and Mut247+270Dl965, respectively.

The *in vitro* replication of *wt*Dl965, *r*Dl965, Mut247Dl965, Mut270Dl965 and Mut247+270Dl965 was comparatively analysed. E-11 monolayers were inoculated with each virus at 0.1 multiplicity of infection (MOI), collecting the supernatants at 1, 2, 3, 5 and 7 days post-inoculation (d p.i.) to be titrated by the $TCID_{50}$ method (Reed and Muench, 1938). *wt*Dl965 and *r*Dl965 viruses replicate similarly on E-11 cells, whereas

Mut247+270Dl965 showed significant differences, compared to *wt*Dl965, at 2 and 5 d p.i. (with mean titres of $1.3x10^3$ and $3.9x10^4$ $TCID_{50}$/ml, respectively, compared with $1.3x10^4$ and $1.9x10^5$ $TCID_{50}$/ml, respectively, obtained for *wt*Dl965). On the other hand, Mut247Dl965 and Mut270Dl965 replication differs from *wt*Dl965 at 7 d p.i., with mean titres significantly higher ($1.9x10^5$ and $7.1x10^5$ $TCID_{50}$/ml, respectively) than that recorded for *wt*Dl965 ($3.9x10^4$ $TCID_{50}$/ml). Therefore, these modifications could affect viral entry, by altering host receptor recognition.

Furthermore, the virulence of *r*Dl965 and mutant viruses was comparatively evaluated by different experimental challenges.

In a first challenge, *wt*Dl965 and *r*Dl965 virulence was comparatively analysed, inoculating sea bass (6 g, average weight) by 1-h immersion in seawater containing a final viral concentration of 10^5 $TCID_{50}$/ml. A control group, maintained in Leibovitz L-15 medium, was also included. Each group was set up in duplicate (n = 30 each) to monitoring signs of disease and mortality (tanks A), or to sample sea bass brains for virological analyses (tanks B). Samples were taken at 12 h p.i., as well as at 1, 3, 5 and 7 d p.i. (3 specimens per time).

Typical signs of disease and mortality were recorded in both infected groups, obtaining similar cumulative mortality curves, with final values of 63.3% and 73.3% for *wt*Dl965- and *r*Dl965-inoculated groups, respectively. The statistical comparison (Kaplan-Meyer survival curve and log-rank Mantel Cox analysis) showed no significant differences between both groups (p = 0.369). No mortalities were recorded in the negative control group at any time.

Viral replication in brain was evaluated by an absolute PCR protocol, using serial dilutions of a vector containing the *wt*Dl965 RNA2 sequence as standard curves. The viral RNA was detected in sea bass infected with both viruses at all sampling times analysed, recording no significant differences between the number of RNA2 copies in *wt*Dl965- and *r*Dl965-inoculated fish at any time ($p > 0.05$).

These results indicated that the virulence of the virus generated by reverse genetic (*r*Dl965) is similar to the *wild type* virus (*wt*Dl965) virulence, showing similar replication both *in vitro* (on E-11 cells) and *in vivo* (in brain of experimentally infected European sea bass).

In a second challenge, possible virulence changes resulting from amino acid modifications were analysed, infecting sea bass (8 g, average weight) with *r*Dl965, Mut247Dl965, Mut270Dl965, or Mut247+270Dl965. Each experimental group was composed of three tanks (n = 25 each). The inoculation was by intramuscular injection ($2x10^6$ $TCID_{50}$/fish), and a control group, injected with L-15, was also set up. Fish from two of the triplicate tanks were daily monitored to record mortality and disease signs, and specimens from the remaining tanks were sampled at 12 h p.i., as well as at 1, 3 and 5 d p.i. Brains were collected and individually processed for virological analysis.

The course of the disease in those groups inoculated with the mutated viruses was different, compared with animals inoculated with the non-mutated *r*Dl965, with clinical signs less severe and delayed in time. The cumulative mortality at the end of the experiment was 22.5%, 20% and 20% for Mut247Dl965-, Mut270Dl965-, and Mut247+270Dl965-infected groups, respectively. Furthermore, significant differences between the survival distribution estimated for Mut247Dl965-, Mut270Dl965- and Mut247+270Dl965-inoculated groups, in comparison to the *r*Dl965-infected group, were observed ($p < 0.0001$), although the survival distribution after inoculation with the three mutated viruses assayed was the same.

These results indicate that amino acids 247 and 270 are relevant virulence determinants, supporting the results previously obtained by Souto et al. (2015a) in Senegalese sole, in which the change of both amino acids in a reassortant isolate (RGNNV/SJNNV) resulted in a 40% decrease of the mortality rate. The mortality caused by the mutated viruses in this study was comparable to those reported in sea bass inoculated with SJNNV isolates, which oscillates from 10% (Vendramin et al., 2014) to no mortalities (Carballo et al., 2016; Souto et al., 2015b). In contrast, mortality rate obtained for *r*Dl965-infected sea bass agrees with previous studies, reporting mortality rates ranging between 36% and 75% after challenging with different RGNNV isolates (Carballo et al., 2016; Nuñez-Ortiz et al., 2016; Pascoli et al., 2016; Souto et al., 2015b; Vendramin et al., 2014).

To complete this study, viral RNA2 segment was quantified in dead, surviving and sampled fish by absolute PCR, using serial dilutions of a vector containing the *wt*Dl965 RNA2 sequence as standard curves. In dead animals, the number of RNA2 copies in Mut247Dl965- and Mut270Dl965-inoculated groups was significantly lower

($3.2x10^8$ and $1.4x10^8$ RNA2 copies/µg RNA, respectively) compared to *r*Dl965-infected group ($4.1x10^9$). This lower viral load could be related to the low mortality caused by these viruses.

In surviving animals, the number of RNA2 copies was lower than that reported in dead animals for all experimental groups, which may suggest the establishment of an asymptomatic infection, and the role of sea bass as asymptomatic carrier.

Regarding sampled animals, at 72 and 120 h p.i. fish inoculated with mutant viruses showed a number of RNA2 copies significantly lower than fish from the *r*Dl965-infected group, reaching *r*Dl965, Mut247Dl965 and Mut270Dl965 their maximal RNA2 copies mean values at 120 h p.i. ($4.4x10^9$, $4.8x10^8$ and $2.3x10^8$ RNA2 copies/µg RNA, respectively). In contrast, no significant increase of Mut247+270Dl965 RNA2 copy number was recorded up to 5 d p.i. ($p > 0.05$); however, surviving animals from this experimental group displayed similar genome levels to those recorded in *r*Dl965-inoculated surviving sea bass. This *in vivo* kinetics of replication is similar to that previously observed *in vitro*, reporting a lagged replication on E-11 cells compared to the *wild type* virus replication. These variations could be related with alterations in cell receptor binding.

Possible modifications in the predictive secondary structure of the *wt*Dl965 CP caused by the amino acid changes generated in this study have been analysed using the Geneious 4.8.4 software (Biomatters Ltd., New Zealand). The results showed that only the amino acid change from serine to asparagine at position 270 modified the structure, introducing a turn and displacing a beta strand. In addition, a protein modelling based on structure homology was performed using the Swiss Model server (ExPASy web server), showing that the mutations considered did no modify the capsid tertiary structure. However, the amino acids studied have been localized within the P-domain (Chen et al., 2015), and their alteration could modify the protuberance surface in structure and/or physicochemical properties, and therefore, could alter cell-receptor recognition and virulence.

CHAPTER 2: UNDERSTANDING THE ROLE OF THE EUROPEAN SEA BASS ISG15 PROTEIN AGAINST BETANODAVIRUS INFECTIONS

In this chapter, the role of the European sea bass ISG15 protein against betanodavirus infections has been studied, carrying out the characterization of the sea bass *isg15* gene (chapter 2A), and evaluating the anti-betanodavirus activity of the sea bass ISG15 protein using an *in vitro* system (chapter 2B).

CHAPTER 2A: GENETIC CHARACTERIZATION AND TRANSCRIPTIONAL ANALYSIS OF EUROPEAN SEA BASS *isg15* GENE

The European sea bass *isg15* gene and ISG15 protein sequences have been characterized in this part of the present PhD Thesis.

First of all, the full-length sequence of sea bass *isg15* gene has been determined. Sea bass *isg15* cDNA was obtained from head kidneys of juvenile specimens (n = 3) (6 g, average weight) intraperitoneally injected with polyinosinic:polycytidylic acid (poly I:C, 1 mg/fish), and sampled at 8 h p. i. Genomic DNA (gDNA) was isolated from caudal fin by saline precipitation (Martinez et al., 1998).

Primers to amplify the sea bass *isg15* gene were designed based on the non-annotated sea bass genome available in GenBank (accession no. HG916840), in which the *isg15* gene was located by alignment with the sea bass *isg15*-expressed sequence tag (EST) published by Seppola et al. (2007). Combinations of these primers were used to amplify the *isg15* ORF, as well as the 5'- and 3'-UTR regions, using as template both, cDNA and gDNA. Sequences obtained were edited, aligned and translated into amino acid sequences using the EditSeq and MegaAlign software (DNASTAR Lasergene 7). Intron screening was performed by alignment of the resulting cDNA and gDNA sequences. The deduced amino acid sequence was aligned with other ISG15 sequences using the Clustal Omega (EMBL-EBI) software. The results obtained were used for phylogenetic analyses by implementation of the neighbour joining-clustering method using the MEGA7 software.

Sea bass *isg15* displays the typical structure for fish *isg15* genes. It is a 1,143-bp long gene, composed of a single 276-bp intron, which is located in the 102-bp long 5'-UTR region, a 474-bp long ORF, and a 291-bp 3'-UTR. This 3'-UTR sequence contains one AATAAA polyadenylation signal, one cytoplasmic polyadenylation element (CPE, U-rich sequence), and four mRNA instability motifs (ATTTA), which have also been detected within the intron sequence (containing three ATTTA motifs).

The mRNA instability motifs within the intron sequence are infrequent, and they seem to be related with heterogeneous nuclear RNA (hnRNA or pre-RNA) splicing regulation, as well as mRNA expression (Zou et al., 1999). However, location of these ARE motifs in 3'-UTR sequences is quite common and are related with regulation of mRNA, such as mRNA degradation (Herjan et al., 2013) or stability (Akashi et al., 1994). In this study, the number of ATTTA sequences found in the 3'-UTR sequence (four) suggests that *isg15* mRNA may have a short half-life. In addition, the sea bass *isg15* 3'-UTR sequence contains one CPE element, which is a regulatory motif required for cytoplasmic elongation of the nuclear poly A tail, able to repress o exert translation depending on the cellular type (Ross, 1995). The presence of these regulatory motifs, some of them in high number, suggests a possible strong post-transcriptional regulation of European sea bass *isg15* gene.

The deduced amino acid sequence (157 amino acids) shows two typical UBL domains, and one RLRGG conjugation motif at the 3'-end, with no additional amino acids downstream, which reveals that a post-translational process is not required.

In order to complete the sea bass *isg15* gene characterization, a comparative analysis of transcription in head kidney and brain following poly I:C or virus inoculation has been conducted.

Sea bass specimens (6 g, average weight) were set up in three groups, consisting of poly I:C-, RGNNV (SpDl_IAusc965.09 isolate)- and L-15-inoculated fish (n = 40, each). Poly I:C was inoculated by intraperitoneal injection (1 mg/fish), whereas RGNNV (10^5 $TCID_{50}$/fish) and L-15 were intramuscularly injected. Head kidneys and brains from three animals per group were sampled at 4, 6, 8, 12, 24, 48 and 72 h p.i. Sea bass *isg15* mRNA was quantified by relative real-time PCR (using ribosomal 18S RNA as endogenous gene), and the RGNNV RNA2 genomic segment was quantified in brain

by absolute PCR (using serial dilutions of a vector containing the RNA2 segment of the SpDl_IAusc965.09 isolate as standard curves).

According to our results, poly I:C triggers *isg15* transcription earlier and at higher level than RGNNV in both organs. Poly I:C is a direct stimulator of the IFN I system, whereas the induction triggered by virus requires viral replication. In both organs, the maximal transcription in response to the viral infection was at 72 h p.i., with higher values in brain than in head kidney. Furthermore, viral multiplication in the target organ was recorded, with a significant increase in the RNA2 copy number from 6 ($9.1x10^6$ RNA2 copies/g tissue) to 72 h p.i. ($3.6x10^9$), being the maximal transcription in response to the viral infection in brain coincident with the maximum number of RNA2 copies (at 72 h p.i.).

The important up-regulation of *isg15* transcription in the viral replication organ after RGNNV infection suggests a crucial role of the protein encoded by this gene against betanodavirus infections, which has been studied in the second part of this chapter.

CHAPTER 2B: EVALUATION OF THE ANTIVIRAL ACTIVITY OF EUROPEAN SEA BASS ISG15 PROTEIN AGAINST BETANODAVIRUS INFECTIONS USING AN *in vitro* APPROACH

In this chapter, an *in vitro* system, consisting of E-11 cells permanently transfected with the sea bass *isg15* ORF, has been established to evaluate the sea bass ISG15 (Dl_ISG15) anti-betanodavirus activity.

To develop this system, the first step has been the complete *isg15* amplification, using as template cDNA from head kidney of poly I:C-stimulated sea bass (chapter 2A). The *isg15* sequence was cloned in the pGemT vector (Dl_ISG15_pGemT construction), and sub-cloned in the pcDNA™4/HisMax B vector (Dl_ISG15_pcDNA). Finally, E-11 cells were transfected with the Dl_ISG15_pcDNA plasmid according to Iwamoto et al. (2001), obtaining the Dl_ISG15_E11 cell line.

The characterization of this cell line has been performed by analysing cellular growth kinetics, quantifying sea bass *isg15* transcription, and detecting and determining sub-cellular ISG15 location.

The growth rate of Dl_ISG15-transfected and non-transfected E-11 cells was similar, which indicates that the expression of the exogenous gene does not affect important cellular functions. Furthermore, sea bass *isg15* transcription was stable, not being affected by the number of cellular sub-cultures or the presence of viruses. In addition, the intracellular Dl_ISG15 protein has been detected from cellular extracts by western blotting, showing a single 20-25-kDa band. A single band with the same molecular weight has been exclusively detected in the medium of Dl_ISG15_E11 cells. This extracellular ISG15 protein seems to be secreted, as it is suggested by the absence of beta-actin (cellular control protein) in the extracellular fraction, which may rule out cell disruption as cause for ISG15 presence in the medium.

In fish, ISG15 has been detected extracellularly from cultures of red drum (*Sciaenops ocellatus*) and tongue sole (*Cynoglossus semilaevis*) IFN I-activated head kidney lymphocytes (Liu et al., 2010; Wang et al., 2012). Moreover, other cell types have also been described as ISG15-releasing cells (Swaim et al., 2017). In fact, Langevin et al. (2013a) reported zebrafish (*Danio rerio*) ISG15 protein in the medium of transiently transfected epithelioma papulosum cyprini (EPC) cells.

Finally, the immunofluorescence study revealed the presence of the European sea bass ISG15 protein in the cytoplasm of ISG15-producing cells.

These results show that the *in vitro* system developed is adequate to determine the antiviral activity of the intracellular and extracellular Dl_ISG15 protein against betanodavirus species RGNNV (SpDl_IAusc965.09 isolate) and SJNNV (SJ93Nag).

The intracellular activity has been evaluated analysing viral replication on Dl_ISG15_E11 and E-11 cells by viral quantification and cellular survival assays. For viral quantification, each virus was inoculated at 0.1 MOI on monolayers of Dl_ISG15_E11 and E-11 cells. Supernatants and cells from three wells were separately collected at 0, 24, 48 and 72 h p.i. The titre of infective viral particles in supernatants was determined by the $TCID_{50}$ method, whereas cells were processed for viral genome quantification by absolute PCR, using serial dilutions of vectors containing the RGNNV or SJNNV RNA2 sequences as standard curves. In the cellular survival assay, viruses were inoculated at 0.1 MOI on Dl_ISG15_E11 and E-11 monolayers. Cells from three wells were collected and counted at 0, 48 and 72 h p.i.

The number of copies of RGNNV RNA2 was similar in both cell types at all sampling times. However, the number of SJNNV RNA2 copies was lower in Dl_ISG15-producing cells at 48 h and 72 h p.i., recording mean values of $3.2x10^{10}$ and $2.4x10^{9}$ RNA2 copies/µg RNA at 48 h p.i. in E-11 and Dl_ISG15_E11 cells, respectively. The virus titration yielded similar results, being observed SJNNV titre in supernatants of Dl_ISG15_E11 cells ($2.2x10^{3}$ $TCID_{50}$/ml) significantly lower than the titre obtained in E-11 cell supernatants ($1.2x10^{4}$ $TCID_{50}$/ml) at 48 h p.i. (p = 0.01). These results were confirmed by the survival assay, revealing similar survival percentages for RGNNV-inoculated E-11 and Dl_ISG15_E11 cells. However, the percentage of cells surviving SJNNV infection was significantly higher for cells expressing the sea bass ISG15 protein than for E-11 cells at 48 and 72 h p.i. (p = 0.01 and p = 0.005, respectively). Thus, the survival rate of Dl_ISG15_E11 cells was 100% at 48 h p.i. and 95% at 72 h p.i., whereas E-11 survival rates were 83% at 48 h p.i., and 85% at 72 h p.i.

According to these results, the course of the RGNNV infection was not altered in ISG15-producing cells, whereas sea bass ISG15 compromises SJNNV replication. This differential antiviral activity of the sea bass ISG15 protein against both viral species suggests that this protein may play different roles in the course of RGNNV and SJNNV infections.

In addition, the possible modulation of the transcription of *mx*, toll-like receptor 3 (*tlr3*) and ubiquitin ligase E3 (*e3*) has also been evaluated. E-11 and Dl_ISG15_E11 cells were inoculated with RGNNV or SJNNV at 0.01 MOI and gene transcription were quantified by relative real-time PCR at 3, 6 and 24 h p.i. Beta-actin was the endogenous reference gene used.

The transcription of *tlr3* and *e3* was similar in SJNNV-infected Dl_ISG15_E11 and E-11 cells at all times, whereas *tlr3* and *e3* transcription was significantly different in both types of cells inoculated with RGNNV. Thus, *tlr3* transcription was significantly induced in E-11 cells at all sampling times, with the maximum mean transcription value at 6 h p.i. (12.6), whereas this transcription was knocked out in RGNNV-infected Dl_ISG15_E11 cells, which may suggest that the presence of sea bass ISG15 protein could be preventing the IFN I system activation and, therefore, could be protecting the virus from the host innate immune system, as previously suggested for hepatitis C virus

(Broering et al., 2010; Chua et al., 2009). The transcription of the *e3* gene was induced in Dl_ISG15_E11 cells at all sampling times analysed, with the maximal transcription recorded at 24 h p.i. (4.6 mean fold change value), being significantly higher than the induction recorded in E-11 cells at the same sampling time, suggesting the presence of a high number of proteins functionally altered in Dl_ISG15_E11 cells infected with this isolate.

Previous studies have evaluated and reported differential antiviral effect of several fish ISG15 proteins (Huang et al., 2013; Langevin et al., 2013a), although a differential role against different species of the same virus had not been described to date. The two viral species considered in this study show different virulence to sea bass, and our results seem to suggest that differences in virulence to a specific host can be related with different mechanisms of interaction between the virus and the host immune system. Understanding the mechanisms underlining this interaction is crucial to develop prophylactic measures against viral infections.

Finally, the cytokine-like activity of the extracellular Dl_ISG15 protein against RGNNV and SJNNV infections has been evaluated by co-culture of E-11 and Dl_ISG15_E11 cells, as well as by E-11 incubation with ISG15-containing medium.

The protocol used (Parreño et al., 2016) allows the co-culture of two cell lines (donor and target cells) in droplets within the same well. Cells were inoculated with RGNNV or SJNNV (10^4 $TCID_{50}$/ml), and incubated until fully CPE appearance. The possible protective effect of sea bass ISG15-producing cells was measured by crystal violet staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction quantification. In addition, the role of the secreted Dl_ISG15 protein in the protection of E-11 cells against RGNNV has been further investigated by culturing E-11 cells with medium from the sea bass ISG15-producing cell line. Cells were incubated until fully CPE development, and the protection was visualized by MTT reduction.

Results showed putative protective effect of the Dl_ISG15 protein to co-cultured E-11 cells inoculated with either, RGNNV or SJNNV. The intensity of the crystal violet staining was maximum for non-inoculated E-11 cells, minimum for inoculated E-11 cells where ISG15-producing cells were not seeded, and medium for inoculated E-11 cells sharing the medium with ISG15-producing cells. These results were confirmed by

the MTT assay. Thus, the survival rate of RGNNV-inoculated cells increased significantly, from 25% to 46%, when these cells shared medium with ISG15-producing cells, being obtained a similar result after SJNNV inoculation, from 27% to 51%.

In addition, the incubation with medium containing ISG15 resulted in a significant increase of cellular survival (p = 0.0005), which was 33% in control cells and increased up to 99% in presence of sea bass ISG15 protein.

This PhD Thesis constitutes the first work analysing the cytokine-like activity of a fish ISG15 protein on non-immune cells. Although it has not been completely ruled out the possible activation of IFN I system in Dl_ISG15-producing cells as partially responsible for the protection recorded, it is clear that the presence of sea bass ISG15 in the medium confers cellular protection, increasing the survival rate of target cells.

CHAPTER 3: TRANSCRIPTIONAL PROFILE AND SEROLOGICAL STUDIES OF THE EUROPEAN SEA BASS IMMUNE RESPONSE AGAINST BETANODAVIRUS INFECTIONS

This chapter is a comprehensive analysis of the European sea bass host immune response against viral isolates showing different virulence to this fish species, either different viral species, or different attenuated mutated RGNNV viruses obtained by reverse genetics in chapter 1. We have analysed the transcription of genes coding for innate and adaptive molecules, with important roles in the fish antiviral response, in the course of two different *in vivo* analyses (challenges 1 and 2).

In challenge 1, the immunogene transcription after inoculation with RGNNV (ERV378/102-5/04) or SJNNV (SJ93Nag) isolates has been comparatively evaluated. Sea bass specimens (5g, average weight) were set up in three groups (n = 50 each) and were intramuscularly injected with RGNNV or SJNNV ($5x10^5$ $TCID_{50}$/fish). In addition, a control group injected with L-15 medium was also considered. Five specimens per group were sampled at 3, 12, 24 and 72 h p.i., and brains and head kidneys were collected for virological and immunological analyses.

The transcription of *mxA*, *isg12*, *isg15*, *ifn-I*, *mhcII-β*, *il-8*, *il-10*, *tgf- β*, *tnf-α* and *tr-γ* has been quantified by relative real-time PCR protocols, using ribosomal 18S RNA as endogenous gene. In addition, the copy number of the RNA2 segment was quantified

in brain by absolute PCR using serial dilutions of vectors containing the full sequence of the RGNNV or SJNNV RNA2 segments to generate standard curves.

The results revealed that betanodaviruses are strong inducers of the sea bass IFN I system-related genes (*isg15*, *isg12* and *mxA*) in both organs.

In head kidney, RGNNV is a strong inductor of the transcription of *isg15*, *isg12* and *mxA* from 12 h p.i. onwards. However, SJNNV triggers the induction of these genes earlier, at 3 h p.i., suggesting that this viral species induces a more rapid systemic response, as it has been previously reported by McBeath et al. (2014) for different strains of infectious salmon anaemia virus (ISAV). Regarding transcription values in brain, the induction of IFN I system is weaker in this organ than in head kidney at earlier stages of infection. For RGNNV- and SJNNV-inoculated fish, maximal levels of transcription of *isg12* and *isg15* were recorded at 72 h p.i. (with significant mean fold change values of 1,921.7 and 7,674.7, respectively, in the RGNNV-infected group, and 565.4 and 1,151.4, respectively, in sea bass inoculated with SJNNV), and significant differences between viral species were recorded for *isg15*, being RGNNV a stronger inductor of this gene compared to SJNNV.

Regarding the pro-inflammatory response, in head kidney, an increase of *il-8* transcripts over the time was recorded for both viral isolates, whereas *tnf-α* transcription was up-regulated from 24 h p.i. In brain, induction of pro-inflammatory genes was stronger than in head kidney, with maximal mean fold change values at 72 h p.i. (184.1 and 29.3 for *il-8*, and 1,628.5 and 73.4 for *tnf-α*, in RGNNV and SJNNV groups, respectively). Anti-inflammatory genes were induced at the same level in both organs, with up-regulation of *il-10* and *tgf-β* mainly at 24 and 72 h p.i.

The importance of the inflammatory process in the course of betanodavirus infections has been previously suggested (Poisa-Beiro et al., 2008), and it seems to be supported by the results obtained in this study. Thus, the massive inflammatory process may be responsible for the eventual damage in nervous tissues, as it has been proposed in a recent study analysing the transcriptomic response (RNAseq) of Senegalese sole after infection with high and low virulent reassortant betanodaviruses (Labella et al., 2018). However, the role in the prevention of lesions associated to a long-lasting anti-inflammatory response after infection with the less virulent isolate, previously

suggested by Poisa-Beiro et al. (2008), has not being determined, being required the analysis of samples collected at longer times p.i.

Regarding the transcription of adaptive response genes (*mhcII-β* and *tr-γ*), our results suggest the importance of the sea bass acquired response against betanodavirus not only at a systemic level, but also at a local level.

Viral genome quantification showed an increase in the number of RNA2 copies of both viral isolates throughout the time, indicating viral replication. SJNNV-inoculated fish displayed a significantly higher number of RNA2 copies, compared with animals in the RGNNV group, at 3 h and 12 h p.i., obtaining the maximal genome level at 72 h p.i. ($7.1x10^{10}$ RNA2 copies/µg RNA). However, in the RGNNV-infected group the increase in the number of RNA2 copies from 3 h ($5.5x10^{4}$) to 72 h p.i. ($4.7x10^{9}$) was higher than in the SJNNV group, obtaining also the maximal mean RNA2 copy number at 72 h p.i. Betanodavirus replication in fish species not showing disease symptoms, as it happens for SJNNV in this study, has been previously described (Castric et al., 2001; Grove et al., 2003; Korsnes et al., 2009). Our results suggest a higher replication rate for RGNNV, but a faster spread of SJNNV from the injection site to the brain (based on the RNA2 copy number recorded at 3 h p.i.). Previous studies have shown that viral isolates less virulent have advantage at the entry stage in bath challenges, inducing an early protective immune response (McBeath et al., 2014); similarly, low virulent isolates may also have advantage in spreading from the inoculation site to the target organ.

In challenge 2, the possible differential response triggered by *r*Dl965 (obtained from the SpDl_IAusc965.09 isolate), and by Mut247Dl965, Mut270Dl965 or Mut247+270Dl965 (obtained by mutation of *r*Dl965) has been analysed. Juvenile European sea bass (8 g, average weight), distributed in five groups (n = 60 each), were intramuscularly injected with *r*Dl965, Mut247Dl965, Mut270Dl965, or Mut247+270Dl965 ($2x10^{6}$ $TCID_{50}$/fish), or with L-15 medium (control group). Five animals per group were sampled at 12 h p.i., as well as at 1 and 3 days p.i., and their brains were individually collected for immunological analysis. In addition, 15 fish per group were anesthetized for blood extraction at 30 days p.i. Three samples, composed of blood from 5 specimens, were used to conduct antibody analyses.

The transcription of *mxA*, *isg15* and *tnf-α* was quantified by relative real-time PCR, using ribosomal 18S RNA as endogenous gene. Antibodies in sera have been

evaluated following the indirect Enzyme-Linked ImmunoSorbent-Assay (ELISA) procedure (Lopez-Jimena et al., 2012a).

Regarding the transcription of *mxA* and *isg15*, marker of the IFN I system, *r*Dl965 was the strongest inductor of both ISGs at the first sampling time (12 h p.i.), recording the maximal mean transcription value at 72 h p.i., with mean fold change values of 336 (*mxA*) and 470 (*isg15*). Similarly, maximal *mxA* and *isg15* transcription in Mut247Dl965- and Mut270Dl965-inoculated fish was also at 72 h p.i., although recording induction values significantly lower than those obtained for *r*Dl965-inoculated sea bass ($p < 0.0001$).

The low virulent mutated viruses caused a reduced induction of these genes, which has been especially evidenced at 72 h p.i. Moreover, as it has been observed in challenge 1 and chapter 2A (Moreno et al., 2016), the induction of these genes in brain is coherent with viral replication in this organ, which was described in chapter 1. In fact, no significant induction of *mxA* and *isg15* has been reported in Mut247+270Dl965-inoculated sea bass, in which no viral replication was recorded (chapter 1).

The results regarding the transcription of the pro-inflammatory gene *tnf-α* are also in concordance with those described in challenge 1. The transcription was maximal at 72 h p.i. for *r*Dl965-inoculated fish (mean fold change values of 483.5), the most virulent virus. Previous studies have also reported maximum values at 72 h p.i. (Poisa-Beiro et al., 2008), or even at longer times p.i. (15 d p.i.) (Valero et al., 2015a). In contrast, the level of *tnf-α* transcription in sea bass inoculated with the low virulent viruses was more stable and lower at all sampling times, statistically comparable with the transcription in the control group ($p > 0.05$). Therefore, this study may be a new evidence supporting the importance of the inflammatory reaction in the pathological process of the disease.

Finally, anti-betanodavirus antibodies in sera have also been analysed. Moreover, to further analyse changes in betanodavirus serological properties caused by the mutations considered, a cross-reaction assay has also been performed using the ELISA methodology.

A significant production of antibodies in *r*Dl965-, Mut270Dl965- and Mut247+270Dl965-inoculated fish has been recorded. Moreover, ELISA analyses have

evidenced changes in the viral serological properties as a consequence of the mutations included in the RGNNV CP sequence. Thus, the seroconversion after Mut270Dl965 or Mut247+270Dl965 inoculation was significantly higher than in sea bass inoculated with the non-mutated *r*Dl965 virus ($p < 0.0001$), whereas Mut247Dl965 inoculation did not result in a level of antibody measurable by the ELISA procedure.

Furthermore, antibodies in sera from *r*Dl965- and Mut247+270Dl965-inoculated sea bass recognized RGNNV and SJNNV antigens, which has been previously reported using other RGNNV isolates (Lopez-Jimena, 2012b; Mori et al., 2003; Pascoli et al., 2017), whereas antibodies in sera from Mut270Dl965-inoculated fish did not react with SJNNV antigens (SJ93Nag reference strain), recognizing only Mut270Dl965, used as positive control, and *r*Dl965 as coating viruses. Therefore, the mutation in amino acid 270 seems to modify the serological properties of the *r*Dl965 virus.

RESUMEN

El cultivo de la lubina (*Dicentrarchus labrax*) se ha convertido en una importante actividad económica a nivel europeo; sin embargo, su producción intensiva ha llevado asociada la aparición de enfermedades infecciosas en las instalaciones acuícolas, siendo especialmente importantes aquellas de etiología viral.

La principal enfermedad vírica que afecta al cultivo de la lubina es la necrosis nerviosa viral, causada por el virus de la necrosis nerviosa (NNV, familia *Nodaviridae*, género *Betanodavirus*). Es un virus pequeño (20-34 nm), desnudo y con cápside icosaédrica. Su material genético está compuesto por dos segmentos de ARN monocatenario de polaridad positiva: ARN1, que codifica la ARN polimerasa-ARN dependiente (100 kDa) y ARN2, que codifica la proteína de la cápside (CP, 42 kDa). Además, durante la replicación viral se sintetiza un segmento sub-genómico (ARN3) a partir del ARN1. Dicho segmento codifica las proteínas no estructurales B1 y B2.

En base a la variabilidad de la región T4, dentro del segmento ARN2, el Comité Internacional de Taxonomía de Virus (ICTV) reconoce cuatro especies de betanodavirus: *red-spotted grouper*- (RGNNV), *striped jack*- (SJNNV), *barfin flounder*- (BFNNV) and *tiger puffer*- (TPNNV) *nervous necrosis virus*, que presentan diferente rango de hospedación y temperatura óptima de replicación. En lubina solo se han detectado virus de las especies víricas RGNNV y SJNNV. La primera es altamente virulenta para esta especie de teleósteo (Carballo et al., 2016; Souto et al., 2015b; Vendramin et al., 2014), mientras que SJNNV causa una mortalidad muy baja, o incluso no causa mortalidad (Souto et al., 2015b; Vendramin et al., 2014). Sin embargo, la cepa de referencia de SJNNV (SJ93Nag) replica en el cerebro de lubina, aunque sin desarrollar las lesiones típicas causantes de la enfermedad (Carballo et al., 2016; Souto et al., 2015b).

Estas diferencias en la capacidad de ambas especies víricas de desarrollar la enfermedad en lubina hacen interesante el estudio de los factores que intervienen en la interacción NNV-lubina, incluyendo aquellos que afectan a la virulencia del NNV y los mecanismos de defensa que se activan en la lubina en el curso de la infección.

Entre los factores que determinan la virulencia del patógeno destaca la región C-terminal de la CP (Ito et al., 2008; Iwamoto et al., 2004). Recientemente se ha publicado la estructura cristalina de esta proteína (Chen et al., 2015), describiéndose el dominio-P (*protruding domain*) en la región C-terminal. Dicho dominio presenta regiones híper-

variables localizadas en la superficie del virión que podrían estar implicadas en la unión del virus a la célula y en la especificidad de hospedador (Chen et al., 2015). Aunque aún no se han caracterizado los aminoácidos virales que participan en dicha unión, varios estudios proponen los aminoácidos en posiciones 247 y 270 como determinantes de virulencia y especificidad de hospedador en betanodavirus (Olveira et al., 2009; Souto et al., 2015a).

Respecto a los mecanismos de defensa del hospedador, los peces disponen de un sistema de defensa completo, compuesto por el sistema inmune innato y el adaptativo.

El sistema inmune innato constituye la primera línea de defensa frente a infecciones víricas, destacando el sistema del interferón tipo I (IFN I) y la respuesta inflamatoria.

El IFN I es secretado por las células infectadas y detectado por las células vecinas, en las cuales activa una cascada de señales que desemboca en la expresión de los denominados genes estimulados por interferón (ISG). Entre los ISG descritos en peces destacan el gen que codifica la proteína tipo-dinamina mx (*mx*), y los genes estimulados por interferón 15 (*isg15*) y 12 (*isg12*), los cuales codifican proteínas con actividad antiviral (Alvarez-Torres et al., 2016; Avunje y Jung, 2017; Jung y Jung, 2017).

En este trabajo se ha caracterizado el gen *isg15* y evaluado la actividad anti-betanodavirus de la proteína codificada por dicho gen, la proteína ISG15 (15-kDa). Dicha proteína está compuesta por dos dominios tipo ubiquitina (UBL) conectados por una región de unión, y un motivo RLRGG en el extremo C-terminal. En peces, el gen *isg15* se induce con gran intensidad y en fase muy temprana tras una infección viral (Alvarez-Torres et al., 2017; 2018; Furnes et al., 2009; Lin et al., 2015; Moreno et al., 2016; 2018; Zhang et al., 2007). Además, estudios previos han demostrado actividad antiviral de diversas proteínas ISG15 de peces (Huang et al., 2013; Langevin et al., 2013a), así como su actividad inmuno-moduladora (Langevin et al., 2013a) y su localización extracelular (Liu et al., 2010; Wang et al., 2012).

La respuesta inflamatoria es un proceso esencial en la respuesta innata frente a infecciones víricas. En esta respuesta destaca el papel del factor de necrosis tumoral alpha (TNF-α) y otras citoquinas, como la interleucina 1β (IL-1β), las cuales promueven

la activación de numerosas citoquinas que intervienen en el proceso, como la interleucina 8 (IL-8) y la IL-6 (Al-Banna et al., 2018). Para controlar esta reacción intervienen las citoquinas anti-inflamatorias, como las IL-10, IL-11 o el TFG-β.

Finalmente, el sistema inmune adaptativo en peces resulta esencial en la protección a largo plazo (Secombes y Wang, 2012), estando constituido fundamentalmente por dos grupos de linfocitos: las células B y las células T.

Las células B intervienen en la respuesta mediada por anticuerpos, habiéndose descrito tres isotipos de anticuerpos en teleósteos: IgM, IgD e IgT (Secombes y Wang, 2012), aunque la mayoría de los trabajos se han centrado en la determinación de los niveles de IgM en suero (Li et al., 2015; Peñaranda et al., 2011; Xing et al., 2017).

Las células T son los mediadores de la respuesta adaptativa celular. A través de los complejos principales de histocompatibilidad (MHC), los antígenos son presentados por las células presentadoras de antígenos y reconocidos por los receptores de los linfocitos T (TR), desencadenándose la respuesta celular. En peces se han descrito numerosos genes que codifican moléculas que intervienen en esta ruta (Boschi et al., 2011; Buonocore et al., 2012; Nuñez-Ortiz et al., 2014; Picchietti et al., 2011).

En resumen, la presente Tesis Doctoral es un estudio de la interacción entre la lubina y el virus de la necrosis nerviosa, profundizando en los factores que intervienen en dicha interacción, tanto por parte del patógeno como del hospedador.

CAPÍTULO 1: ANÁLISIS DE DETERMINANTES DE VIRULENCIA DE BETANODAVIRUS EN LUBINA

En este capítulo se ha evaluado la importancia de los aminoácidos 247 y/o 270 de la secuencia de la CP como determinantes de la virulencia de betanodavirus en lubina.

Para ello, se han generado virus con mutaciones en los aminoácidos anteriormente mencionados mediante genética inversa, utilizando como molde un aislado virulento en lubina (SpDl_IAusc965.09, RGNNV), cambiándolos por aquellos aminoácidos presentes en la secuencia de la CP de aislados SJNNV, poco virulentos.

El primer paso ha sido la secuenciación de los segmentos genómicos del aislado,

identificado en este capítulo como *wild type* Dl965 (*wt*Dl965). Con este fin se han secuenciado los marcos abiertos de lectura (ORF), usando la estrategia molecular *primer walking*, así como los extremos 5' y 3', usando la técnica de amplificación rápida de los extremos de ADN complementario (ADNc) o RACE.

El aislado *wt*Dl965 presenta un segmento ARN1 de 3.104 pb de longitud, compuesto por una región 5'-UTR de 78 pb, un ORF de 2.949 pb y una región 3'-UTR de 77 pb. El segmento ARN2, más pequeño, presenta una longitud de 1.432 pb dispuesto en una región 5'-UTR de 26 pb, un ORF de 1.017 pb y una región 3'-UTR de 389 pb.

El análisis comparativo con otras secuencias de virus RGNNV indica que la regiones 5'- y 3'-UTR del segmento ARN1 están altamente conservadas; en cambio, la región 3'-UTR del segmento ARN2 presenta mayor variabilidad nucleotídica, con cambios en las posiciones 1.134, 1.159, 1.174, 1.195, 1.261, 1.270, 1.299, 1.302, 1.330 y 1.422. Esta variabilidad puede suponer la modificación de la estructura *stem loop*, necesaria para la replicación viral, lo que puede llevar a cambios en la eficiencia de replicación y en la virulencia. De hecho, un estudio reciente ha demostrado que mutaciones puntuales en esta región conllevan una disminución de la virulencia de un aislado recombinante de betanodavirus en lenguado senegalés (*Solea senegalensis*) (Souto et al., 2018).

Una vez secuenciado el genoma del aislado *wt*Dl965, el siguiente paso ha sido la obtención de partículas víricas infectivas a partir del ADNc, usando la tecnología de genética inversa basada en el sistema de la polimerasa T7 (Biacchesi, 2011; Takizawa et al., 2008). La metodología ha consistido en la amplificación de los segmentos completos, incluyendo en las secuencias de ADNc varios motivos nucleotídicos necesarios para la posterior clonación y sub-clonación en un vector de expresión. El vector de expresión en eucariotas utilizado ha sido pBSδRiboT7t (Biacchesi et al., 2000), obteniéndose las construcciones pBSARN1 y pBSARN2, que se utilizaron para la transfección de células BSRT7/5 (Buchholz et al., 1999), recuperándose partículas víricas genéticamente idénticas al aislado *wt*Dl965, identificadas como *r*Dl965. Dichas partículas víricas fueron propagandas en la línea celular E-11, observándose efectos citopáticos (ECP) tras la primera inoculación (P0). El título viral obtenido fue de $7{,}1x10^7$ $TCDI_{50}$/ml (P2 en E-11).

El siguiente paso ha sido la realización de mutaciones puntuales en las posiciones 247 y/o 270 en la secuencia de la CP, usando como base la construcción que contenía el segmento ARN2 (pBSARN2). La mutación en el aminoácido 247 se realizó cambiando una timina por una guanina en la posición nucleotídica 759, generando el cambio de una serina hacia una alanina. En el caso de la posición 270, la modificación de una guanina por una adenina en la posición nucleotídica 830 modificó el aminoácido serina por una asparragina. La construcción pBSARN1, en combinación con Mut247pBSARN2, Mut270pBSARN2 y/o Mut247+270pBSARN2, se usaron para transfectar células BSRT7/5, recuperándose partículas víricas infectivas que fueron propagadas en células E-11, observándose ECP tras un pase ciego (P1), alcanzando títulos víricos de $1{,}3x10^8$, $7{,}1x10^7$ y $2{,}3x10^7$ $TCDI_{50}$/ml para Mut247Dl965, Mut270Dl965 y Mut247+270Dl965, respectivamente.

Una vez obtenidos los virus, se analizó comparativamente su cinética de replicación *in vitro*. Para ello, monocapas de células E-11 se inocularon a una multiplicidad de infección (MOI) de 0,1, recogiéndose los sobrenadantes a los 1, 2, 3, 5 y 7 días post-inoculación (d p.i.). Dichos sobrenadantes se titularon siguiendo el método $TCDI_{50}$ (Reed y Muench, 1938). Los resultados mostraron que *wt*Dl965 y *r*Dl965 replican de manera similar en las células E-11. En cambio, el virus recombinante Mut247+270Dl965 mostró un título vírico significativamente inferior ($p < 0.0001$), comparado con *wt*Dl965, a los 2 y 5 d p.i., mientras que el título de los virus Mut247Dl965 y Mut270Dl965 fue significativamente superior a los 7 d p.i. ($1{,}9x10^5$ y $7{,}1x10^5$ $TCDI_{50}$/ml, respectivamente, frente a $3{,}9x10^4$ $TCDI_{50}$/ml obtenido para *wt*Dl965). Por tanto, las modificaciones consideradas pueden estar alterando el reconocimiento del receptor de la célula hospedadora.

La posible alteración en la virulencia causada por las modificaciones realizadas se ha analizado *in vivo*, a través de diferentes infecciones experimentales.

En una primera infección se evaluó la virulencia de *wt*Dl965 y *r*Dl965, inoculando juveniles de lubina (6 g, peso medio) mediante inmersión durante 1-h en agua de mar que contenía una dosis vírica final de 10^5 $TCDI_{50}$/ml. Un tercer grupo tratado con medio Leibovitz L-15 (control) también fue considerado. De cada grupo experimental se establecieron dos tanques (n = 30 cada tanque), uno de los cuales se utilizó para la vigilancia diaria de signos de enfermedad y mortalidad (tanques A),

mientras que en el segundo tanque se realizaron muestreos aleatorios de ejemplares (tanques B). Los muestreos se realizaron a las 12 h p.i., así como a los 1, 3, 5 y 7 d p.i. (3 individuos por tiempo), extrayéndose los cerebros, que fueron conservados a -80 ^{o}C para el posterior análisis de carga viral.

Se observaron signos típicos de la enfermedad y mortalidad en ambos grupos infectados, no en el grupo control, registrándose una mortalidad acumulada final de 63,3 y 73,3% para *wt*Dl965 y *r*Dl965, respectivamente. El análisis estadístico (Kaplan-Meyer seguido del test de rangos logarítmicos Mantel Cox) no mostró diferencias significativas entre las curvas de supervivencia de lubinas inoculadas con cada uno de los virus (p = 0,369).

La replicación viral en el órgano diana (cerebro) fue analizada mediante PCR absoluta, usando como curva estándar diluciones seriadas de un vector que contiene el segmento ARN2 del aislado *wt*Dl965. El ARN viral se detectó exclusivamente en el cerebro de los ejemplares de los grupos infectados, no observándose diferencias significativas en el número de copias de ARN2 entre ambos grupos experimentales (p > 0,05).

Estos resultados indican que el virus generado por genética inversa (*r*Dl965) presenta una virulencia similar al virus natural (*wt*Dl965), propagándose de forma similar tanto *in vitro* como *in vivo*.

En una segunda infección se evaluaron los cambios en la virulencia causados por las modificaciones aminoacídicas llevadas a cabo. Para ello, juveniles de lubina (8 g, peso medio) distribuidos en distintos grupos experimentales se inocularon mediante inyección intramuscular ($2x10^6$ $TCDI_{50}$/pez) con el virus *r*Dl965 o los virus mutados Mut247Dl965-, Mut270Dl965-, o Mut247+270Dl965, por triplicado (n = 25, cada grupo). Un grupo inyectado con L-15 fue dispuesto como control negativo. Los ejemplares de dos de los tanques triplicados se observaron diariamente en busca de signos de enfermedad o recuento de mortalidad, mientras que los individuos del tanque restante se muestrearon a las 12 h p.i. y a los 1, 3 y 5 d p.i. Los cerebros de estos individuos se procesaron para su análisis virológico.

El curso de la enfermedad se vio alterado en aquellos grupos inoculados con los virus mutados, observándose signos clínicos más débiles y retrasados en el tiempo, así

como una mortalidad acumulada al final de la infección del 22,5, 20 y 20% para los grupos inoculados con Mut247Dl965, Mut270Dl965, y Mut247+270Dl965, respectivamente. Además, el análisis estadístico indicó diferencias significativas ($p < 0,0001$) en la supervivencia de los grupos experimentales inoculados con los virus mutados con respecto al grupo infectado con *r*Dl965, aunque no mostró diferencias en la supervivencia de los ejemplares inoculados con los virus mutados entre sí.

Los resultados indican que los aminoácidos 247 y 270 son importantes determinantes de virulencia, apoyando los resultados obtenidos por Souto et al. (2015a) en lenguado senegalés, en los cuales la modificación de ambos aminoácidos en un aislado recombinante (RGNNV/SJNNV) causó una reducción de la mortalidad de un 40%. En el presente trabajo, la mortalidad se ha visto alterada por las mutaciones consideradas, registrándose mortalidades muy similares a las registradas por otros autores en lubinas inoculadas con aislados SJNNV (Carballo et al., 2016; Souto et al., 2015b; Vendramin et al., 2014). Por otro lado, la mortalidad en el grupo inoculado con *r*Dl965 está en concordancia con las mortalidades registradas tras la inoculación con aislados RGNNV (Carballo et al., 2016; Nuñez-Ortiz et al., 2016; Pascoli et al., 2016; Souto et al., 2015b; Vendramin et al., 2014).

Para completar el estudio se cuantificó el segmento ARN2 en cerebro de individuos muertos, supervivientes y muestreados. Se utilizó un protocolo de PCR absoluta, usando como curva estándar diluciones seriadas de un vector que contiene el segmento ARN2 del virus *wt*Dl965. En los individuos muertos en los grupos inoculados con Mut247Dl965 y Mut270Dl965 el número de copias de ARN2 fue significativamente inferior ($3,2x10^8$ y $1,4x10^8$ copias ARN2/µg ARN, respectivamente) al registrado en el grupo infectado con *r*Dl965 ($4,1x10^9$). Esta menor carga viral podría estar relacionada con la baja mortalidad generada por estos virus.

En los peces supervivientes, el número de copias de ARN viral fue inferior al registrado en los peces muertos, lo que sugiere que el virus podría establecer infección asintomática.

En los peces muestreados a las 72 y 120 h p.i. los individuos inoculados con los virus mutados mostraban un número de copias de ARN2 en el cerebro significativamente menor al observado en los peces inoculados con *r*Dl965, registrándose el máximo número de copias a las 120 h p.i. (con valores medios de

$4{,}4x10^9$, $4{,}8x10^8$ y $2{,}3x10^8$ copias ARN2/µg ARN, en los grupos inoculados con *r*Dl965, Mut247Dl965 y Mut270Dl965, respectivamente). Por el contrario, en los individuos inoculados con Mut247+270Dl965 no se observó incremento en el número de copias de ARN2 hasta los 5 d p.i. Sin embargo, los animales supervivientes de este grupo presentan un número de copias de ARN2 en el cerebro similar al cuantificado en los animales supervivientes inyectados con *r*Dl965, lo que sugiere una posible replicación tardía de este virus. De forma similar, este virus también mostró una replicación *in vitro* retrasada en el tiempo en comparación con el virus no mutado, lo que indica de nuevo que las mutaciones pueden estar alterando la unión con el receptor celular.

Para confirmar esta hipótesis, se ha realizado un estudio de las modificaciones que se pudiesen generar en la estructura secundaria de la CP de *wt*Dl965 a causa de los cambios introducidos, utilizando, para ello, el programa informático Geneious 4.8.4 (Biomatters Ltd., New Zealand). Los resultados muestran que solo el cambio en la posición 270 modifica la estructura de la CP, introduciendo un giro que provoca un desplazamiento en la lámina beta. Además, la estructura terciaria de la proteína también fue analizada realizando un modelaje basado en homología de estructuras con el servidor Swiss Model (ExPASy servidor web), no destacándose ningún cambio relevante debido a las mutaciones introducidas.

CAPÍTULO 2: ESTUDIO FUNCIONAL DE LA PROTEÍNA ISG15 DE LUBINA FRENTE A LAS INFECCIONES POR BETANODAVIRUS

En este capítulo se ha estudiado la función de la proteína ISG15 de lubina frente a infecciones por betanodavirus, llevando a cabo la caracterización del gen *isg15* (capítulo 2A), y evaluando la actividad antiviral de la proteína ISG15 de lubina usando un sistema *in vitro* (capítulo 2B).

CAPÍTULO 2A: CARACTERIZACIÓN GÉNICA Y ANÁLISIS TRANSCRIPCIONAL DEL GEN *isg15* DE LUBINA

En este capítulo se han caracterizado las secuencias del gen *isg15* y de la proteína ISG15 de lubina.

En primer lugar se amplificó y secuenció el gen *isg15* usando ADNc obtenido de

riñón cefálico de juveniles de lubina (n = 3) (6 g, peso medio) inoculados mediante inyección intraperitoneal con ácido poliinosínico-policitidílico (poli I:C, 1 mg/pez) y muestreados a las 8 h p.i., y ADN genómico (ADNg) obtenido de aleta caudal mediante precipitación salina (Martinez et al., 1998).

Los cebadores utilizados se diseñaron en base a una secuencia genómica de lubina no anotada disponible en GenBank (HG916840), en la cual el gen *isg15* se localizó mediante su alineamiento con un marcador de secuencia expresada (EST) de *isg15* de lubina publicado por Seppola et al. (2007). Distintas combinaciones de los cebadores permitieron la amplificación y secuenciación tanto del ORF como de las regiones 5'- y 3'-UTR (*untranslated region*). Las secuencias obtenidas fueron editadas, alineadas y traducidas a secuencias aminoacídicas usando los programas EditSeq y MegaAlign (DNASTAR Lasergene 7). La secuencia del intrón se localizó mediante el alineamiento del ADNc y el ADNg. La secuencia aminoacídica fue utilizada en un alineamiento múltiple usando el programa Clustal Omega (EMBL-EBI), y analizada filogenéticamente mediante el método *neighbour joining* utilizando el programa MEGA7.

El gen *isg15* de lubina presenta la estructura típica descrita para otros genes *isg15* de peces. Es un gen de 1.143 pb de longitud, compuesto por una región 5'-UTR de 102 pb, en la que se encuentra un intrón de 276 pb, un ORF de 474 pb y una región 3'-UTR de 291 pb. En la región 3'-UTR se localizan una señal de poliadenilación AATAAA, un motivo de poliadenilación citoplasmática (CPE, secuencia rica en uracilo), y cuatro motivos de inestabilidad de ARNm (ATTTA), también presentes en la secuencia del intrón (concretamente tres).

Los motivos de inestabilidad de ARNm en el intrón parecen estar relacionados tanto con la maduración mediante corte y empalme del ARN heterólogo nuclear (hnARN o pre-ARN), como con la regulación de la expresión del ARNm (Zou et al., 1999), aunque la presencia de estos motivos en el intrón no es frecuente. En cambio, su aparición en la región 3'-UTR suele ser más común, estando relacionados con la regulación de la degradación y la estabilidad del ARNm (Akashi et al., 1994; Herjan et al., 2013). En nuestro caso, el número de motivos localizados en la región 3'-UTR (cuatro) sugiere que el ARNm del gen *isg15* puede tener una vida media corta. Adicionalmente, en esta misma región se ha localizado un elemento de poliadenilación

citoplasmática, necesario para la elongación de la cola poli A en el citoplasma capaz de inducir o reprimir la traducción dependiendo del tipo celular (Ross, 1995). La presencia de motivos reguladores, algunos de ellos en amplio número, sugiere que el gen *isg15* de lubina presenta una fuerte regulación post-transcripcional.

Respecto a la secuencia aminoacídica, ISG15 es una proteína de 157 aminoácidos compuesta por dos dominios UBL, y un motivo de conjugación RLRGG en la región C-terminal que no presenta aminoácidos adicionales, por lo que la proteína ISG15 de lubina no necesita un proceso de maduración post-traduccional.

Por último, se ha llevado a cabo un análisis transcripcional comparativo, tanto en riñón cefálico como en cerebro, tras la inoculación de ejemplares de lubina con poli I:C o con un aislado de la especie RGNNV (SpDl_IAusc965.09).

Se establecieron tres grupos experimentales de ejemplares de lubina (6 g, peso medio) inoculados con poli I:C (inyección intraperitoneal, 1 mg/pez), con el virus RGNNV (inyección intramuscular, 10^5 $TCDI_{50}$/pez), o con L-15 (inyección intramuscular). El riñón cefálico y el cerebro de tres individuos se muestrearon a las 4, 6, 8, 12, 24, 48 y 72 h p.i., cuantificándose la transcripción del gen *isg15* mediante PCR a tiempo real relativa (usando el gen ARN ribosomal 18S como endógeno). Además, también se evaluó el número de copias de ARN2 en el cerebro mediante PCR absoluta (usando como curva estándar diluciones seriadas de un vector que contiene el segmento ARN2 del aislado SpDl_IAusc965.09).

Se registró una transcripción del gen *isg15* más potente y a tiempos más tempranos en ambos órganos en los individuos inoculados con poli I:C. El poli I:C es un estimulador directo del sistema IFN I, mientras la inducción activada por el virus requiere la replicación previa del mismo. En ambos órganos, el valor máximo de transcripción en respuesta al virus se produjo a las 72 h p.i., observándose mayores valores en el cerebro que en el riñón cefálico. Adicionalmente, se observó multiplicación viral en el órgano diana (cerebro), registrándose un incremento significativo en el número de copias de ARN2 entre las 6 ($9{,}1x10^6$ copias ARN2/g tejido) y las 72 h p.i. ($3{,}6x10^9$), tiempo de muestreo en el que el número de copias fue máximo.

Esta importante inducción de la transcripción del gen *isg15* de lubina frente a la infección por RGNNV en el órgano de replicación viral sugiere que la proteína codificada por este gen puede ejercer una función importante frente a las infecciones por betanodavirus, lo cual ha sido analizado en la segunda parte del presente capítulo.

CAPÍTULO 2B: EVALUACIÓN *in vitro* DE LA ACTIVIDAD ANTIVIRAL DE LA PROTEÍNA ISG15 DE LUBINA FRENTE A BETANODAVIRUS

Se ha desarrollado un sistema *in vitro,* consistente en células E-11 transfectadas de manera estable con el ORF del gen *isg15* de lubina, con el objetivo de evaluar la actividad anti-betanodavirus de la proteína ISG15 de lubina (Dl_ISG15). Para establecer este sistema, en primer lugar se amplificó el gen *isg15* de lubina, usando como molde ADNc obtenido según se explica en el capítulo 2A. El fragmento obtenido se clonó en el vector pGemT y se sub-clonó en el vector pcDNA™4/HisMax B, obteniéndose la construcción Dl_ISG15_pcDNA. Dicha construcción se utilizó para la transfección de células E-11, siguiendo la metodología descrita por Iwamoto et al. (2001), obteniéndose la línea celular Dl_ISG15_E11.

Dicha línea ha sido caracterizada mediante el análisis de su curva de crecimiento, del carácter estable de la transcripción del gen *isg15* de lubina, y la inmuno-detección y localización de la proteína ISG15.

La curva de crecimiento de las células Dl_ISG15_E11 y E-11 fue similar, indicando que la expresión del gen *isg15* de lubina no afecta a funciones celulares importantes. Además, la transcripción de dicho gen fue estable, independiente del número de sub-cultivos celulares o de la presencia de virus. La proteína recombinante se detectó en el interior celular (localización citoplasmática), y en el medio extracelular de las células transfectadas (estimándose un tamaño aproximado de 20-25 kDa). La localización extracelular de la proteína parece ser consecuencia de un proceso de secreción, ya que no se detectó beta-actina (control de proteínas celulares) en la fracción extracelular, descartando la rotura celular como causa de la presencia de la proteína extracelularmente.

Diversas proteínas ISG15 de teleósteos se han detectado en la fracción extracelular de cultivos de linfocitos de riñón cefálico activados, es el caso de la corvina

roja (*Sciaenops ocellatus*) y el lenguado (*Cynoglossus semilaevis*) (Liu et al., 2010; Wang et al., 2012). Además, se han descrito tipos celulares no implicados en respuesta inmune como secretores de ISG15 (Swaim et al., 2017), es el caso de la línea celular EPC (*epithelioma papulosum cyprini cells*) transfectada de manera transitoria con el gen i*sg15* de pez cebra (*Danio rerio*) (Langevin et al., 2013a).

Este sistema ha permitido estudiar la actividad antiviral intracelular y extracelular de Dl_ISG15 frente a RGNNV (aislado SpDl_IAusc965.09) y SJNNV (cepa de referencia SJ93Nag).

La actividad intracelular se ha evaluado mediante el análisis de la replicación viral y la supervivencia celular. Para el análisis de la replicación viral se inocularon células Dl_ISG15_E11 y E-11 con cada virus a una MOI de 0,1, muestreándose, por separado, sobrenadante y células a las 0, 24, 48 y 72 h p.i. El título de partículas infectivas se determinó a partir de la fracción extracelular (método $TCDI_{50}$), mientras que el número de copias de ARN2 se estimó a partir del ARN celular (PCR absoluta). Para el ensayo de supervivencia las células se inocularon con los distintos virus a una MOI 0,1, realizándose el recuento celular a las 0, 48 y 72 h p.i.

El número de copias de RGNNV fue similar en ambos tipos celulares a todos los tiempos analizados. Sin embargo, el número de copias de ARN2 de SJNNV en las células Dl_ISG15_E11 fue significativamente menor al registrado en las células E-11 a las 48 y 72 h p.i., con valores medios de $3{,}2x10^{10}$ y $2{,}4x10^{9}$ copias ARN2/µg ARN en E-11 y Dl_ISG15_E11, respectivamente, a las 48 h p.i. Los resultados obtenidos mediante el análisis de partículas víricas infectivas fueron similares, estimándose un título significativamente menor en el sobrenadante de las células Dl_ISG15_E11 a las 48 h p.i. ($2{,}2x10^{3}$ $TCDI_{50}$/ml) respecto al registrado en el sobrenadante de las células E-11 ($1{,}2x10^{4}$ $TCDI_{50}$/ml). El ensayo de supervivencia celular corroboró estos resultados, estimándose una mayor supervivencia de las células transfectadas inoculadas con el aislado SJNNV a las 48 h p.i. (100%, comparado con el 83% en células E-11) y a las 72 h p.i. (95 y 85% para células transfectadas y no transfectadas, respectivamente).

De acuerdo con estos resultados, la infección por el aislado RGNNV no se ve alterada por la presencia de la proteína Dl_ISG15. En cambio, esta proteína afecta a la propagación del aislado SJNNV, promoviendo la supervivencia celular.

Esta actividad antiviral diferencial frente a dos especies distintas de un mismo virus sugiere que Dl_ISG15 presenta diferentes funciones celulares dependiendo de que la infección sea generada por una especie viral u otra.

De manera complementaria, se evaluó la modulación de la transcripción de varios genes endógenos (*mx*, *tlr3* y *e3*) en las líneas celulares Dl_ISG15_E11 y E-11 inoculadas con ambos virus. Cada línea celular se inoculó a una MOI de 0,01, analizándose la transcripción de los genes endógenos a las 3, 6 y 24 h p.i. mediante PCR a tiempo real relativa, utilizando beta-actina como gen de referencia.

El patrón de transcripción de *tlr3* y *e3* en el transcurso de la infección por SJNNV fue similar en ambos tipos celulares, mientras que en las células inoculadas con el aislado RGNNV se detectaron diferencias. En concreto, en las células E-11 se registró inducción del gen *tlr3* en todos los tiempos analizados, obteniéndose el valor máximo a las 6 h p.i. (12,6), frente a las células Dl_ISG15_E11, en las que no se observó inducción, lo que sugiere que la proteína ISG15 podría estar previniendo la detección del virus y la posterior activación del sistema IFN I, tal y como se ha sugerido en el caso del virus de la hepatitis C (Broering et al., 2010; Chua et al., 2009). Con respecto al gen *e3*, se observó transcripción en las células Dl_ISG15_E11 a todos los tiempos analizados, registrándose el valor máximo a las 24 h p.i. (4,6 valor medio de *fold change*) y siendo este valor significativamente superior (p = 0,001) al registrado en las células E-11, sugiriendo la presencia de un elevado número de proteínas celulares funcionalmente alteradas en las células Dl_ISG15_E11 infectadas con RGNNV.

Otros estudios han descrito actividad antiviral diferencial de varias proteínas ISG15 de peces frente a distintos virus (Huang et al., 2013; Langevin et al., 2013a), aunque una función diferencial frente a diferentes especies dentro del mismo virus no había sido descrito hasta el momento. Las dos especies consideradas en este trabajo presentan diferente virulencia en lubina, y los resultados parecen indicar que estas diferencias podrían estar relacionadas con diferentes mecanismos de interacción entre el virus y el sistema inmune del hospedador. El conocimiento de los mecanismos que intervienen en esta interacción es vital en el desarrollo de medidas profilácticas frente a las infecciones virales.

Para completar el estudio, se ha analizado la actividad tipo citoquina de la proteína ISG15 de lubina extracelular mediante co-cultivo e incubación de células infectadas con medio procedente de células productoras de Dl_ISG15.

Mediante el protocolo de co-cultivo en gotas descrito por Parreño et al. (2016) se ha realizado el cultivo de los dos tipos celulares (Dl_ISG15_E11 como donador y E-11 como diana) en un mismo espacio de crecimiento. Las células en co-cultivo fueron inoculadas con cada virus (10^4 $TCDI_{50}$/ml) e incubadas hasta la aparición de ECP a término final. El efecto protector de las células productoras de ISG15 se determinó mediante tinción con cristal violeta y reducción de bromuro de 3-(4,5- dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT).

De manera complementaria, la protección sobre las células E-11 inoculadas con el aislado RGNNV también se ha investigado mediante el cultivo de dichas células con medio proveniente de células productoras de ISG15. Las células fueron incubadas hasta el desarrollo de ECP y el efecto de protección se cuantificó mediante reducción de MTT.

Los resultados muestran protección de las células productoras de ISG15 de lubina sobre las células E-11 en co-cultivo. Dicha protección se produce tanto frente a RGNNV como a SJNNV. Así, la intensidad de la tinción con cristal violeta de las células E-11 fue máxima en las células no inoculadas (control negativo), mínima en las células inoculadas en ausencia de células productoras de ISG15, y media en las células que compartían medio con células productoras. El ensayo de reducción de MTT confirmó estos resultados, observándose aumento de la supervivencia celular en las células co-cultivadas con células productoras tanto frente a RGNNV (25% a 46%) como a SJNNV (27% a 51%).

La incubación con medio procedente de células productoras de ISG15 confirmó esta protección, observándose aumento de la supervivencia de las células E-11, pasando de un 33% en las células control a un 99% en las células con medio que contenía ISG15 de lubina.

El presente Trabajo Doctoral constituye el primer estudio en el que se aborda el análisis de la actividad tipo citoquina de una ISG15 de teleósteo en células no inmunes,

aunque no ha podido descartarse completamente la intervención del sistema IFN I de las células productoras de ISG15 en la protección observada.

CAPÍTULO 3: ANÁLISIS TRANSCRIPCIONAL Y ESTUDIO SEROLÓGICO DE LA RESPUESTA INMUNE DE LUBINA FRENTE A INFECCIONES POR BETANODAVIRUS

Este capítulo constituye un estudio amplio de la respuesta inmune de la lubina frente a aislados con distinta virulencia, tanto distintas especies virales como diversos virus RGNNV atenuados obtenidos por genética inversa (capítulo 1). Dicho estudio se ha llevado a cabo analizando la transcripción de genes que codifican moléculas del sistema inmune innato y adaptativo, y que presentan funciones en la lucha frente a la infección viral. El análisis de transcripción se ha llevado a cabo mediante dos infecciones experimentales (infección 1 e infección 2).

En la infección 1 se ha analizado la transcripción de genes inmunes en respuesta a la infección con RGNNV (aislado ERV378/102-5/04) o SJNNV (aislado SJ93Nag). Juveniles de lubina (5g, peso medio) fueron distribuidos en tres grupos (n = 50) e inoculados mediante inyección intramuscular con RGNNV, SJNNV ($5x10^5$ $TCDI_{50}$/pez) o L-15, como grupo control. Se muestrearon cinco ejemplares de cada grupo a las 3, 12, 24 y 72 h p.i., analizándose el riñón cefálico y el cerebro de cada individuo para su estudio inmunológico y virológico.

Se cuantificó la transcripción de *mxA*, *isg12*, *isg15*, *ifn-I*, *mhcII-β*, *il-8*, *il-10*, *tgf-β*, *tnf-α* y *tr-γ* mediante PCR a tiempo real relativa, usando el gen de ARN ribosomal 18S como endógeno. Además, el número de copias de ARN2 se cuantificó en cerebro mediante PCR absoluta, usando como curva estándar diluciones de vectores que contienen el segmento ARN2 de RGNNV o de SJNNV.

Los valores de transcripción de los genes relacionados con el sistema IFN I (*isg15*, *isg12* y *mxA*) ponen de manifiesto que los betanodavirus son fuertes inductores de este sistema de defensa en lubina en ambos órganos analizados. En el riñon cefálico, RGNNV es un potente inductor de la transcripción de *isg15*, *isg12* y *mxA* desde las 12 h p.i. en adelante. Sin embargo, SJNNV promueve la transcripcion de estos genes en tiempos más tempranos, a las 3 h p.i., sugiriendo que esta especie viral induce una respuesta inmune sistémica más inmediata, tal y como ha sido descrito para diferentes

cepas poco virulentas del virus de anemia infecciosa del salmón (ISAV) (McBeath et al., 2014). En cerebro, la inducción del sistema IFN I es más débil que en el riñón cefálico en los estados iniciales de la infección, observándose los valores máximos de transcripción de *isg12* e *isg15* a las 72 h p.i. (con valores medios de *fold change* de 1.921,7 y 7.674,7, respectivamente, en el grupo inoculado con RGNNV, y 565,4 y 1.151,4, respectivamente, en el caso de inoculación con SJNNV), registrándose, además, diferencias significativas en el caso de *isg15*, siendo RGNNV un mayor inductor de este gen en comparación con SJNNV.

Respecto a la respuesta inflamatoria, en el riñón cefálico se observa un aumento de la transcripción del gen *il-8* a todos los tiempos analizados en los animales inoculados con ambas especies virales, mientras la transcripción del gen *tnf-α* solo se indujo a las 24 h p.i. En cerebro, la inducción de estos genes pro-inflamatorios fue mayor que en el riñón cefálico, registrándose los valores máximos de transcripción a las 72 h p.i. (184,1 y 29,3 para *il-8*, y 1.628,5 y 73,4 para *tnf-α*, en RGNNV y SJNNV, respectivamente). La inducción de la transcripción de los genes anti-inflamatorios fue similar en ambos órganos, produciéndose una transcripción de *il-10* y *tgf-β* principalmente a las 24 y 72 h p.i.

Estudios previos han sugerido la importancia de la respuesta inflamatoria en el desarrollo de la enfermedad generada por betanodavirus (Poisa-Beiro et al., 2008), y los resultados presentados parecen apoyar dicha hipótesis. Así, una respuesta inflamatoria no controlada parece ser parcialmente responsable de los daños producidos en los tejidos nerviosos, teoría propuesta en un estudio reciente que analiza la respuesta transcriptómica (ARNseq) del lenguado senegalés frente a infecciones por betanodavirus con diverso grado de virulencia (Labella et al., 2018). Por el contrario, el papel de las citoquinas anti-inflamatorias en la prevención de la aparición de lesiones histopatológicas en el curso de infecciones por aislados de SJNNV, propuesto por Poisa-Beiro et al. (2008), no ha podido ser determinado, siendo necesaria la realización de estudios que impliquen el análisis de tiempos mayores.

Respecto a los valores de transcripción de genes relacionados con la respuesta adaptativa (*mhcII-β* y *tr-γ*), los resultados sugieren la intervención de dicha respuesta en la lubina frente a las infecciones por betanodavirus no solo a nivel sistémico, sino también a nivel local.

La cuantificación de genoma viral muestra un incremento del número de copias de ARN2 de RGNNV y SJNNV a lo largo del tiempo, observándose un mayor número de copias en el caso de SJNNV en tiempos tempranos (3 y 12 h p.i.), y alcanzando el máximo número a las 72 h p.i. ($7{,}1x10^{10}$ copias ARN2/µg ARN). En el caso de RGNNV, se produce un fuerte incremento del número de copias de ARN2 desde las 3 h ($5{,}5x10^{4}$) a las 72 h p.i. ($4{,}7x10^{9}$). Estos resultados sugieren una mayor tasa de replicación de RGNNV, pero una dispersión más rápida desde el sitio de inoculación en el caso de SJNNV. Estudios previos han descrito que aislados víricos menos virulentos presentan una ventaja en la entrada al hospedador mediante infección por baño, induciendo una respuesta inmune temprana en el hospedador (McBeath et al., 2014); por lo que, de manera similar, el aislado menos virulento (SJNNV) podría presentar una ventaja en la dispersión desde el lugar de inoculación hacia el órgano diana.

En la infección 2 se ha estudiado la respuesta inmune tras la inoculación con virus no mutados *r*Dl965 (RGNNV) y con virus mutados obtenidos en el capítulo 1, con menor virulencia en lubina (Mut247Dl965, Mut270Dl965 o Mut247+270Dl965). Juveniles de lubina (8 g, peso medio) fueron distribuidos en cinco grupos e inoculados mediante inyección intramuscular con cada uno de los virus ($2x10^{6}$ $TCDI_{50}$/pez), o con L-15 como control negativo. Se muestrearon cinco individuos por grupo a las 12 h p.i. y a los 1 y 3 d p.i., extrayéndose los cerebros, que se procesaron individualmente para el análisis de transcripción de *mxA*, *isg15* y *tnf-*α mediante PCR a tiempo real relativa, usando el gen ARN ribosomal 18S como endógeno. Además, se extrajo la sangre de 15 ejemplares por grupo a los 30 d p.i. (3 muestras, compuestas por sangre de 5 individuos). Los anticuerpos presentes en el suero se analizaron mediante la técnica ELISA (*Indirect Enzyme-Linked ImmunoSorbent-Assay*) (Lopez-Jimena et al., 2012a).

El análisis de la transcripción de los genes del sistema IFN I (*mxA* e *isg15*) muestra que el virus no mutado, y más virulento, *r*Dl965, fue el inductor más potente de ambas ISG a las 12 h p.i., registrándose los valores máximos de transcripción a las 72 h p.i. De manera similar, los valores máximos de transcripción de estos genes en respuesta a los virus Mut247Dl965 y Mut270Dl965 también se registraron a las 72 h p.i. (con valores medios de *fold change* de 336 para *mxA* y 470 para *isg15*), aunque significativamente menores que los obtenidos en el curso de la infección con *r*Dl965 (p <0,0001). Por tanto, los virus mutados provocan una menor inducción del sistema IFN I. Además, en concordancia con los resultados obtenidos en la infección 1 y en el capítulo

2A (Moreno et al., 2016), la inducción de estos genes en cerebro es coherente con la replicación viral en este órgano (descrito en el capítulo 1). De hecho, la inoculación con el virus Mut247+270Dl965 (del cual no se observa replicación) no induce significativamente los genes del sistema IFN I *mxA* e *isg15*.

Respecto a la transcripción de genes inflamatorios, los resultados son similares a los obtenidos en la infección 1, observándose máxima transcripción del gen *tnf-α* a las 72 h p.i. en las lubinas inoculadas con el virus más virulento, *r*Dl965 (valor medio de 483,5). Valores máximos de transcripción a las 72 h p.i. han sido previamente descritos (Poisa-Beiro et al., 2008), o incluso a mayores tiempos p.i. (15 d p.i.) (Valero et al., 2015a). Por el contrario, los niveles de transcripción del gen *tnf-α* en los animales inoculados con los virus menos virulentos son similares a los del grupo control. En resumen, este análisis supone una nueva evidencia que apoya la hipótesis de la importancia de la respuesta inflamatoria en el desarrollo del proceso patológico causado por los aislados de betanodavirus virulentos.

Finalmente, se han analizado los anticuerpos anti-betanodavirus presentes en el suero de los individuos infectados a los 30 d p.i. Además, se ha realizado un ensayo de reactividad cruzada, empleando la técnica ELISA, para evaluar los posibles cambios serológicos provocados por las mutaciones introducidas.

En los individuos inoculados con *r*Dl965, Mut270Dl965 o Mut247+270Dl965 se registra una producción significativa ($p < 0,0001$) de anticuerpos. Además, se han observado cambios serológicos provocados por las mutaciones, ya que la inoculación con Mut270Dl965 o Mut247+270Dl965 generó una mayor seroconversión ($p < 0,0001$) respecto a la registrada en las lubinas inoculadas con el virus no mutado *r*Dl965.

Además, los anticuerpos presentes en el suero de las lubinas inoculadas con *r*Dl965 y Mut247+270Dl965 reconocieron antígenos tanto de RGNNV (aislado *r*Dl965) como de SJNNV (aislado SJ93Nag). Este doble reconocimiento ha sido previamente descrito usando otros aislados de RGNNV (Lopez-Jimena, 2012b; Mori et al., 2003; Pascoli et al., 2017). En cambio, los anticuerpos presentes en el suero de ejemplares inoculados con Mut270Dl965 no reconocen el antígeno de SJNNV, reconociendo únicamente los virus *r*Dl965 y Mut270Dl965 (control positivo). Estos resultados indican que la mutación en el aminoácido 270 en la secuencia de la CP de RGNNV parece modificar las propiedades serológicas del virus *r*Dl965.

GENERAL INTRODUCTION

1. OVERVIEW

European sea bass, *Dicentrarchus labrax*, L. 1758, (Perciformes order, *Moronidae* family) is widely distributed in the Mediterranean area, being present in the Mediterranean sea, Black sea and East-Atlantic coasts from Great Britain to Senegal (Moretti et al., 1999; Pickett and Pawson, 1994). It is a eurythermal and euryhaline species, showing high tolerance to salinity and temperature fluctuations (Bento et al., 2016; Masroor et al., 2018; Moretti et al., 1999; Pickett and Pawson, 1994; Tine et al., 2014), being present in marine and brackish environments, such as estuarine areas, and in regions with temperature from 2 to 32 °C. Thus, it can inhabit the open sea, coastal waters, lagoons, estuaries and rivers, depending on its life cycle stage (Dufour et al., 2009; Pickett et al., 2004).

The culture of European sea bass in Europe has become a very important activity in the last decades, since 96.2% of the consumed sea bass comes from the aquaculture industry. In 2017, the total cultured sea bass production in European countries was 192,557 tons, reaching 200,000 tons in 2018. In Spain, this fish species is becoming quite relevant. In fact, its production in tons and its market value exceed those of other relevant fish species cultured in the Mediterranean area, such as sea bream (*Sparus aurata*). Thus, Spanish sea bass production in 2017 was 21,267 tons, being expected a production of 23,100 tons in 2018 (APROMAR, 2018).

This intensive production has resulted in the appearance of infectious diseases in the aquaculture facilities, with viral diseases being specially important, since they are more difficult to control than bacterial diseases, due to several factors, such as the high fish susceptibility at early age of development, the great mortality rates recorded, the lack of therapeutic treatments, and the limited knowledge about natural fish resistance mechanisms against viral infection (Kibenge et al., 2012). In addition, viruses, and particularly RNA viruses, show high rates of mutation (Domingo, 2010), and can induce dual (Alonso et al., 1999; Kibenge et al., 2000; Kuo et al., 2012; Lopez-Jimena et al., 2010; Rodriguez et al., 1995) and asymptomatic infections (Altuntas and Ogut, 2010; Toffan et al., 2017; Valverde et al., 2017), favouring the appearance of asymptomatic carriers, and promoting the spread of the disease in aquaculture facilities.

Thus, European sea bass has been reported to be susceptible to several viral diseases, such as viral erythrocytic infection (VEI) (Pinto et al., 1991; 1992), and viral

haemorrhagic septicaemia (VHS) (Castric and De Kinkelin, 1984), although the major disease threatening the culture of this fish species nowadays is viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER) (OIE, 2017).

Due to the great relevance of this disease to sea bass, several aspects of the viral nervous necrosis disease and its causative agent, the nervous necrosis virus (NNV), will be addressed in this PhD Thesis.

2. VIRAL NERVOUS NECROSIS

This disease was first reported in Australian barramundi (*Lates calcarifer*), with affected animals showing brain lesions (Glazebrook and Campbell, 1987), and in European sea bass larvae and juveniles reared in the Caribbean area, showing encephalitis associated with viral particles in nervous cells. Afterwards, several authors visualized picorna-like viral particles associated to diseased barramundi (Glazebrook et al., 1990) and Japanese parrotfish (*Oplegnathus fasciatus*) (Yoshikoshi and Inoue, 1990), determining this virus as the causative agent of this encephalopathy, and naming the disease as nervous necrosis. This virus was subsequently detected in other fish species, such as turbot (*Scophthalmus maximus*), European sea bass or red-spotted grouper (*Epinephelus akaara*) (Bloch et al., 1991; Breuil et al., 1991; Mori et al., 1991; Renault et al., 1991). Finally, Munday et al. (1992) reported very similar symptoms in larvae and juveniles of barramundi, naming the disease as viral-induced vacuolating encephalopathy and retinopathy, or, as it is usually called, viral encephalopathy and retinopathy (VER) (OIE, 2017).

The viral characterization and classification within *Nodaviridae* family was finally performed by Mori et al. (1992), purifying the virus from striped jack (*Pseudocaranx dentex*) larvae. This virus was named as striped jack nervous necrosis virus (SJNNV).

2.1. Clinical signs and tissue distribution

The clinical signs of this disease are mainly a consequence of the lesions in central nervous system and retina derived from the viral infection. Thus, these lesions are responsible for alterations in the swimming behaviour, visual anomalies and loss of appetite (Figure 1) (Maltese and Bovo, 2007). Although these are the most frequent external symptoms, other signs have been reported, such as changes in the body

pigmentation and hyperinflation of the swim bladder (Le Breton et al., 1997; Breuil et al., 1991; Maltese and Bovo, 2007; Munday et al., 2002).

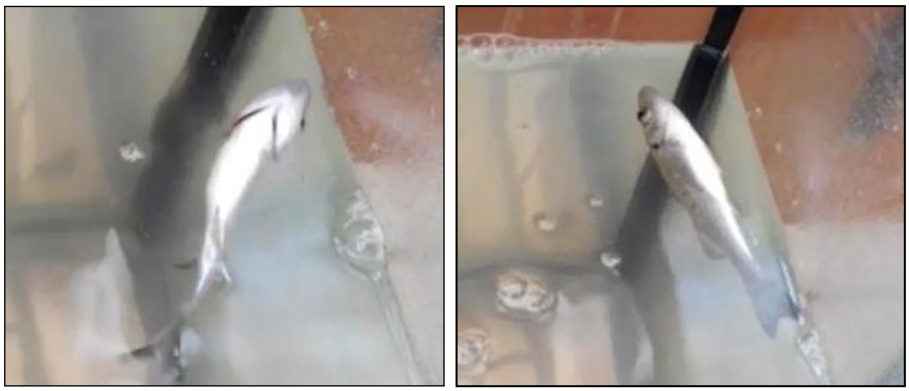


Figure 1. External clinical signs of NNV-infected sea bass. Affected fish show abnormal swimming behaviour resulting from lesions in the central nervous system.

Internally, important histopathological lesions, consisting of vacuolation and necrosis of cells in brain, spinal cord and retina, have been reported (Figure 2) (Doan et al., 2017; Lopez-Jimena et al., 2012a; Maltese and Bovo, 2007).

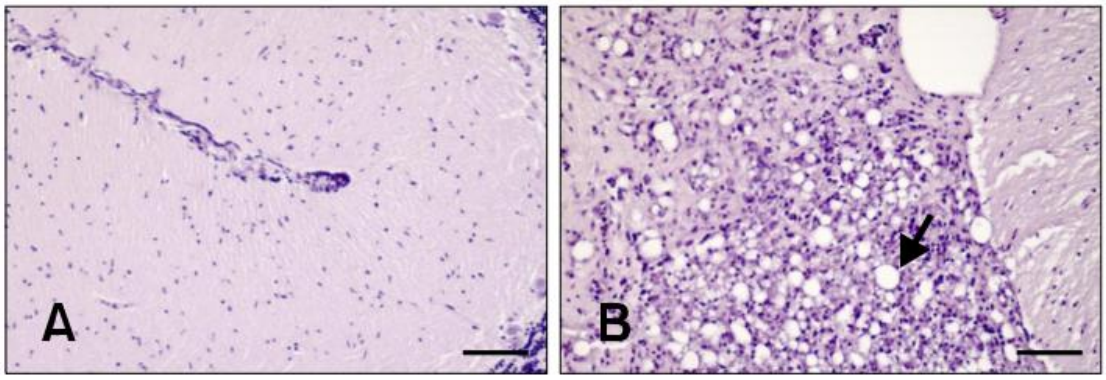


Figure 2. Haematoxylin-eosin-stained sections of brain (cerebellum) from non-infected- (A) and NNV-infected sea bass (B). Infected fish show the typical vacuoles (arrow). Scale bars = 25 µm (A), and 50 µm (B) (Lopez-Jimena et al., 2012a).

Vacuolation in brain and retina has been associated with intracytoplasmic inclusions in European sea bass, barramundi, Japanese parrotfish, and brown-spotted grouper (*Epinephelus malabaricus*) (Breuil et al., 1991; Munday et al., 2002), being the number and size of these vacuoles higher in larval and juvenile stages than in adults (Maltese and Bovo, 2007). Transmission electron microscopy studies revealed inclusion bodies within the cytoplasm of nervous cells associated in paracrystalline arrays (Breuil

et al., 1991; Renault et al., 1991), and accumulations of viral particles in vacuole membranes and degenerated organelles (Munday et al., 1992).

These signs in nervous tissues show that NNV is a neurotropic agent. The results obtained by Korsnes et al. (2009) suggest an initial viremia phase, followed by a neurotropic process. In this regard, Su et al. (2015) detected the virus in brain, kidney, spleen, gills, heart, and muscle in juvenile pompano (*Trachinotus ovatus*) at a very early stage of the infection. In addition, other authors have also detected NNV in non-nervous tissues, including liver, kidney, intestinal tissue, heart, spleen, gonadal fluid, fins and gills, of several fish species (Grove et al., 2003; Korsnes et al., 2009; Lopez-Jimena et al., 2011; Valero et al., 2015a); however, the low viral titre recorded in these non-nervous tissues suggests that no significant viral replication occurs in those organs (Lopez-Jimena et al., 2011). Viral replication in testis of European sea bass and sea bream has been recently demonstrated (Valero et al., 2015a).

All these external and internal signs of disease are frequently associated with episodes of mass mortality, especially in larval and juvenile stages (Binesh et al., 2013; Bovo et al., 1999; Hellberg et al., 2010; Munday and Nakai, 1997; Ransangan and Manin, 2010); however, serious mortality episodes have also been reported in adult specimens of several fish species, such as sevenband grouper (*Epinephelus septemfasciatus*) (Fukuda et al., 1996), striped jack (Nguyen et al., 1997), European sea bass (Bovo et al., 1999), spotted coral grouper (*Plectropomus maculates*) (Nopadon et al., 2009) and Atlantic cod (*Gadus morhua*) (Hellberg et al., 2010).

2.2. The causative agent: nervous necrosis virus

The nervous necrosis virus (*Nodaviridae* family, *Betanodavirus* genus) is a small (20-34 nm) non-enveloped virus, with an icosahedral capsid (T = 3) composed of 180 copies of a 42-kDa capsid protein (CP) (Lu and Lin, 2003; Nishizawa et al., 1994). The CP crystal structure has been characterized by Chen et al. (2015), describing four regions: (i) N-terminal arm (N-Arm), between amino acids 34 and 51; (ii) shell domain (S-domain), from amino acids 52 to 213; (iii) linker region (amino acids 214-220); and (iv) protusion domain (P-domain), at the C-terminal end (amino acids 221-338), which is located on the virion surface (Figure 3).

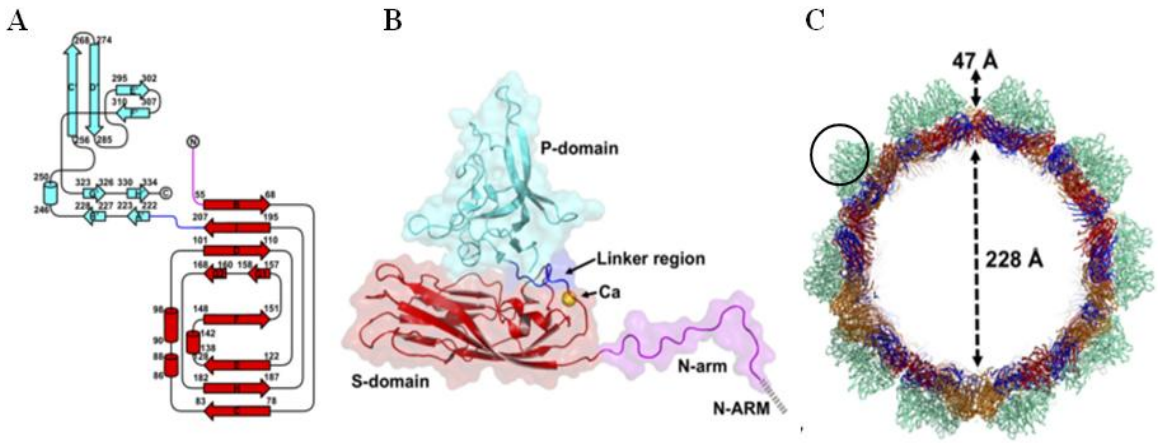


Figure 3. Capsid protein structure. (A) Topology diagram with helices and strands represented by cylinders and arrows, respectively. (B) Ribbon presentation of the protein, showing the four CP regions. (C) Viral particle central cavity representation, with protusions on the capsid surface highlighted with a circle (Chen et al., 2015).

Genetically, it is a single-stranded, positive-sense RNA virus, with a bipartite genome composed of two segments, RNA1 and RNA2 (Mori et al., 1992), which are capped in the 5'-end and lack the poly-A structure at the 3'-end (Mori et al., 1992; Nagai and Nishizawa, 1999; Tan et al., 2001). The largest segment, RNA1 (ca. 3.1 kb), encodes the non-structural 100-kDa RNA-dependent RNA polymerase (RdRp), also called protein A (Nagai and Nishizawa, 1999), whereas the RNA2 segment (1.4 kb) encodes the CP, also named protein α (Nishizawa et al., 1994; Tan et al., 2001) (Figure 4).

In addition, a sub-genomic segment, RNA3 (0.4 kb), is synthesized during RNA replication from the RNA1 3'-end. This segment comprises two open reading frames (ORFs) that encode two non-structural proteins: B1 (111 amino acids), and B2 (75 amino acids) (Su et al., 2018) (Figure 4). B1 protein is expressed during early replication, and targets the nucleus, being proposed as a protecting protein against necrotic cell death (Chen et al., 2009; Su et al., 2018). Regarding B2, this protein has been detected in recently infected fish, disappearing when the infection becomes chronical (Mezeth et al., 2009; Sommerset and Nerland, 2004). It is involved in regulating the host small interfering RNA (siRNA) silencing response at early stages of replication (Fenner et al., 2006a; 2006b; Iwamoto et al., 2005; Su et al., 2009), in the up-regulation of the apoptotic host pathway at the middle stage of replication (Chiu et al., 2017; Su et al., 2009), and in the induction of H_2O_2-mediated cell death through alteration of mitochondrial homeostasis, promoting mitochondrial fragmentation and

oxidative stress-mediated cell death (Su et al., 2014).

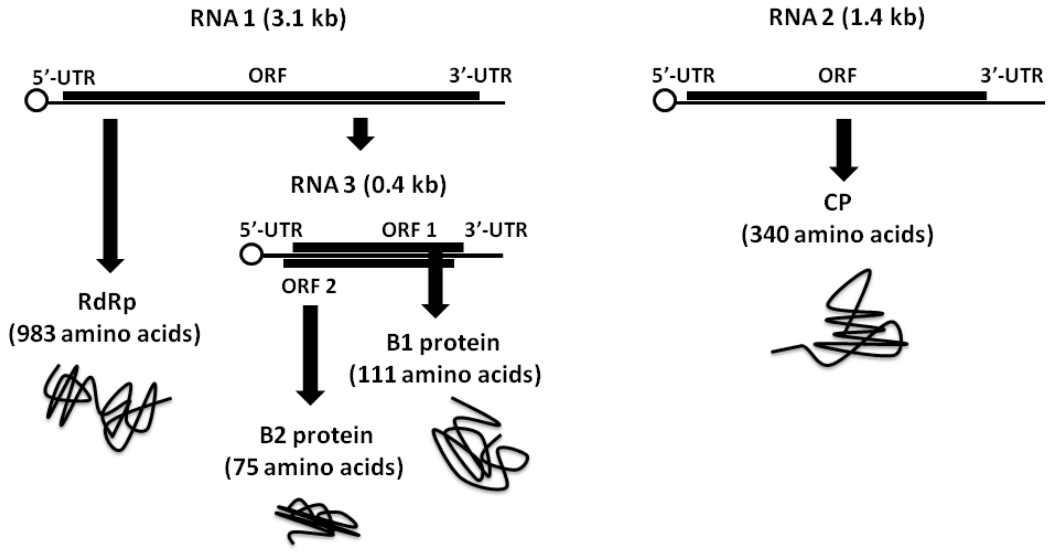


Figure 4. Scheme of betanodavirus genome organization.

2.2.1. Taxonomy and phylogeny

On the basis of the RNA2 sequence, Nishizawa et al. (1997) localized a variable region within the CP gene, labelled as T4 region, which was used for the first phylogenetic classification. Thus, betanodaviruses were clustered into four groups or genotypes: red-spotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), tiger puffer (*Takifugu rubripes*) nervous necrosis virus (TPNNV) and barfin flounder (*Verasper moseri*) nervous necrosis virus (BFNNV). Afterwards, Thiéry et al. (2004), also based on the T4 region, clustered betanodaviruses into four clades or groups: group I (previously designated as RGNNV), subdivided in Ia and Ib, group II (BFNNV), also subdivided in subtypes IIa, IIb and IIc, group III (TPNNV), and group IV (SJNNV).

In addition to this classical organization, new viral genotypes have been proposed. Thus, Johansen et al. (2004a) suggested a fifth genotype, turbot nodavirus (TNV), on the basis of RNA2 phylogenetic analyses of betanodaviruses isolated from turbot, whereas Nylund et al. (2008) proposed a new species-specific clade within the BFNNV group. Moreover, Korean shellfish nervous necrosis virus (KSNNV) has been recently reported as a new type of betanodavirus affecting shellfish (Kim et al., 2018).

Although most of studies are based on the RNA2 segment sequence, phylogenetic analyses based on the RNA1 sequence have also been performed, supporting the classical organization in four genotypes (Toffolo et al., 2007). Furthermore, this study also reported the appearance of reassortant betanodaviruses, harbouring SJNNV-type RNA1 and RGNNV-type RNA2 segments (SJNNV/RGNNV reassortants). The existence of reassortant isolates has been widely confirmed, having been also reported RGNNV/SJNNV isolates (Olveira et al., 2009; Panzarin et al., 2012; Toffan et al., 2017).

These established genotypes partially correlate with three different serotypes, identified by virus neutralization assays using polyclonal antibodies (Mori et al., 2003; Panzarin et al., 2016); however, the serological classification differs between authors. Thus, Mori et al. (2003) organized the serotypes as follows: serotype A consists of SJNNV strains, serotype B corresponds to TPNNV viruses, and serotype C comprises RGNNV and BFNNV genotypes, whereas Panzarin et al. (2016) established that serotype A comprises SJNNV and RGNNV/SJNNV viruses, whereas BFNNV, together with TPNNV, are grouped in the serotype B, and serotype C consists of RGNNV and SJNNV/RGNNV strains. This last classification is based on the analysis of a higher number of isolates from the Mediterranean area.

To date, four different betanodavirus species have been officially recognized by the International Committee on Taxonomy of Viruses (ICTV): red-spotted grouper- (RGNNV), striped jack- (SJNNV), barfin flounder- (BFNNV) and tiger puffer- (TPNNV) nervous necrosis virus (King et al., 2011; Pascoli et al., 2017), being used both terms, species and genotypes.

2.2.2. Host range

The nervous necrosis virus has been detected in at least 70 fish species, and the viral nervous necrosis disease has been reported worldwide, with the exception of South America (Doan et al., 2017), affecting mainly groupers (*Epinephelus* genus), European sea bass and flatfish species. Some of the fish species susceptible to this virus are of great importance for the aquaculture industry, such as Atlantic cod (Hellberg et al., 2010), Senegalese sole (*Solea senegalensis*) (Cutrin et al., 2007; Souto et al., 2015b), sea bream (Toffan et al., 2017) or European sea bass (Souto et al., 2015b; Thiery et al., 1999; Vendramin et al., 2014), although this virus can also be detected in wild marine

and freshwater fish species (Bandin et al., 2014; Bigarre et al., 2009; Furusawa et al., 2007; Keawcharoen et al., 2015; Korsnes et al., 2017; Ma et al., 2015; Moreno et al., 2014; Vendramin et al., 2012), and even in invertebrate species (Fichi et al., 2015; Volpe et al., 2017; 2018; Kim et al., 2018).

In addition, RGNNV/SJNNV and SJNNV/RGNNV reassortant isolates have been reported to infect several fish species, such as Senegalese sole, sea bream and European sea bass (Doan et al., 2017; Olveira et al., 2009; Panzarin et al., 2012; Souto et al., 2015b; Toffan et al., 2017).

Different viral species show different host range and optimal replication temperatures. Thus, RGNNV affects a wide variety of warm-water fish species (optimal replication temperature of 25–30 °C), such as barramundi, European sea bass and groupers (Doan et al., 2017), whereas BFNNV has been detected in cold-water marine fish species, showing an optimal temperature between 15–20 °C. TPNNV infects a single species (tiger puffer) (20 °C), and, although SJNNV was initially reported to affect only a few species in Japan (20–25 °C) (Munday et al., 2002; Nishizawa et al., 1994), this betanodavirus species has been detected in some fish species in Southern Europe, such as Senegalese sole, sea bream, white sea bream (*Diplodus sargus*), meagre (*Argyrosomus regius*) or European sea bass (Cutrin et al., 2007; Lopez-Jimena et al., 2010; Moreno et al., 2014; Olveira et al., 2009).

Regarding host specificity, it has been suggested to be determined by the protruding positions at the C-terminus region of the CP (Ito et al., 2008; Iwamoto et al., 2004), which has been supported by the CP crystal structure recently published (Chen et al., 2015).

The betanodavirus host range has been studied by experimental infections with different viral isolates and diverse host species. Thus, a viral isolate obtained from Atlantic halibut (*Hippoglossus hippoglossus*) was used to inoculate Atlantic cod, showing viral multiplication but absence of mortality. This isolate was also used in cohabitation challenges with Atlantic salmon (*Salmo salar*), turbot or Atlantic cod (Korsnes et al. 2009; 2012). Moreover, studies using recombinant viruses mutated in the CP sequence showed different results depending on the host species inoculated (Souto et al., 2015a; 2016), revealing how differences in the CP sequence could affect differently depending on the host species.

In addition, the RNA dependent-RNA polymerase has also been reported to have an important role in the viral adaptation to a new host. Viral replication has been described as a temperature-sensitive process (Hata et al., 2010; Panzarin et al., 2012; 2014; Toffan et al., 2016). Therefore, the water temperature and the mutation rate of the viral polymerase constitute other important factors to determine the betanodavirus host specificity and host range (Panzarin et al., 2012).

2.2.3. Transmission

Betanodaviruses show both, vertical and horizontal transmission (Doan et al., 2017). Vertical transmission has been described in several fish species, detecting the virus in broodstock gonads or in early larval stages (Breuil et al., 2002; Kai et al., 2010; Mao et al., 2015; Valero et al., 2015a; 2015b), whereas horizontal transmission has been detected in aquaculture facilities and experimental challenges (Athanassopoulou et al., 2004; Jaramillo et al., 2017a; Korsnes et al., 2012; Souto et al., 2015c; Totland et al., 1999). Furthermore, in this regard, species used as food, such as brine shrimp, rotifer or mollusc, have been reported as betanodavirus carriers (Costa and Thompson, 2016; Volpe et al., 2018), and several fish species, such as sea bream and Atlantic halibut, have also been described as asymptomatic carriers, constituting an important source of viral particles to the medium (Castric et al., 2001; Johansen et al., 2004b; Toffan et al., 2017).

2.2.4. Diagnosis and detection

In addition to the observation of clinical signs and electron microscopy studies, over years, the main reference method to detect betanodaviruses has been viral isolation on cell cultures. The cell lines frequently used for viral replication are grouper (*Epinephelus coioides*) GF-1 cells (Chi et al., 1999) and striped snakehead (*Channa striata*) SSN-1 and E-11 cells (E-11 is a clone of SSN-1) (Iwamoto et al., 2000), in which betanodavirus replication results in characteristic cytopathic effects (CPEs), mainly vacuolation, which ends up in granular cells that eventually lose cellular adhesion (Iwamoto et al., 1999; 2000). However, cell culture requires time, experience, and some viral isolates do not develop clear CPEs (Doan et al., 2017). For these reasons, other methods, such as molecular or immunological techniques, are more frequently used nowadays.

Regarding molecular techniques, RT-PCR and/or nested-PCR have been widely

used (Dalla Valle et al., 2000; Lopez-Jimena et al., 2010; Moreno et al., 2014; Nishizawa et al., 1994; Rajan et al., 2016; Thiéry et al., 2004); however, the development of new methods less time-consuming and with less risk of cross-contamination, such as real-time PCR, has made possible an easier, quicker, and more reliable viral diagnosis (Doan et al., 2017). A standardized SYBR Green-based real-time PCR procedure, detecting the four viral species, and a TaqMan-based real-time PCR protocol have been validated for betanodavirus detection (Dalla Valle et al., 2005; Hick and Whittington, 2010). To date, the TaqMan-based method developed by the world organisation for animal health (OIE) Reference Laboratory, and validated according to OIE standards, seems to be the most suitable method for betanodavirus detection (OIE, 2017; Panzarin et al., 2010). Even so, new detection procedures have been developed, such as a TaqMan-based real-time PCR protocol detecting the RNA1 segment (Baud et al., 2015) or several loop-mediated isothermal amplification (LAMP) procedures (Gao et al., 2018; Hwang et al., 2016; Suebsing et al., 2012).

Regarding immunological techniques, the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2017) still recognizes the immunostaining assay as the best procedure for diagnostic purposes, using it after cell culture or even with histological preparations. In addition, enzyme-linked immunosorbent assay (ELISA) is routinely used by fish farms to screen breeders, since it is a non-lethal procedure, taking account the cut-off point as critical threshold to make the distinction between virus-free status and viral infection (Jaramillo et al., 2016; 2017b; Nuñez-Ortiz et al., 2016a).

2.3. Control and prophylactic measures

There are no simple and effective procedures to treat this viral disease once established; for this reason, main efforts focus on the development of tools to prevent entry, spread and persistence of the virus, establishing strict hygiene measures and eradication of infected populations as main prophylactic measures (Doan et al., 2017).

To prevent horizontal and vertical transmission, ozone and ultraviolet light are used to treat fertilized eggs and the water in aquaculture facilities while rearing larval and juvenile specimens (Shetty et al., 2012). However, betanodaviruses are considerably resistant to ultraviolet light, requiring a dose much higher than that suggested by the OIE for its inactivation (Frerichs et al., 2000; Valero et al., 2018). In addition, vertical

transmission is also controlled by detecting viral RNA in breeders using molecular techniques, and/or by detecting anti-betanodavirus antibodies in serum using serological tests (ELISA), in order to eliminate positive individuals (Breuil et al., 2002; Lopez-Jimena et al., 2010; Watanabe et al., 2000).

Vaccination has been considered as an effective procedure for controlling VNN. A variable number of vaccines, consisting of inactivated betanodaviruses, virus-like particles (VLPs), DNA vaccines containing the RNA2 segment, recombinant capsid proteins and synthetic peptides obtained from the CP, have been tested (Kim et al., 2015; Lai et al., 2014; Lin et al., 2016; Nishizawa et al., 2012; Nuñez-Ortiz et al., 2016b; Oh et al., 2013; Pascoli et al., 2017; Valero et al., 2016). The main inconvenient of these types of vaccines is that they are usually applied by injection, not being suitable for large-scale vaccination, causing too much stress, and limiting their use to grow-out size fish. However, several alternative studies based on bath immunization (Kai and Chi, 2008) or vaccine encapsulation into brine artemia (Lin et al., 2007), commercial diet (Chien et al., 2018), or yeasts (Cho et al., 2017), have been carried out. Furthermore, a recent study has even used a plant-based method, in which the CP has been expressed in tobacco chloroplasts and used as food (Cho et al., 2018).

The diversity within betanodaviruses, the high cost of vaccine application and the poor protection generated have been other disadvantages, causing that only two inactivated RGNNV vaccines had been commercialized to date (Brudeseth et al., 2013).

Finally, other recent studies have focused on fish natural resistance against viral infections, applying genetic selection (Liu et al., 2016; Palaiokostas et al., 2018; Wang et al., 2017), or immuno-stimulants to generate viral protection. Thus, the administration of compounds able to stimulate the antiviral immune system, such as interferons, polyinosinic:polycytidylic acid (poly I:C), or viruses non-pathogenic to that fish species, such as aquabirnaviruses, has been proposed by several authors (Kuan et al., 2012; Oh et al., 2012; Yamashita et al., 2009). In this regard, the better understanding of the immune response mechanisms involved in the course of betanodavirus infections can be crucial to develop strategies to control the impact of this disease.

3. ANTIVIRAL FISH IMMUNE SYSTEM

Every organism possesses different biological structures and processes as defence mechanisms against pathogens, which constitute the immune system. Fish display a complete immune system, composed of innate and adaptive responses. The innate immune response is crucial in the resistance against infectious disease, being the forefront of the immune defence, and having an early, unspecific and powerful reaction. On the contrary, the adaptive response is delayed, although it is essential for long-lasting immunity (Secombes and Wang, 2012).

Both immune responses are mediated by a variety of cells and secreted mediators, such as lymphocytes, phagocytes (macrophages, neutrophils and eosinophils), auxiliary cells (basophils, mast cells), and cytokines. These cells are organized into tissues and organs in order to perform their functions most effectively, named as lymphoid organs, which are classified as primary (or central, where immune cells are produced) and secondary (or peripheral, where cells display their functions). In teleost fish, the lymphoid organs are thymus, kidney, spleen, liver and gut-associated lymphoid tissue. Head kidney has been described as the primary organ for antibody production (Secombes and Wang, 2012).

3.1. Innate immune system

The innate immune system comprises physical barriers, as well as cellular and humoral components, including lytic enzymes, the complement pathway, antibacterial peptides, chemokines and cytokines (Magnadóttir, 2006). One of the most important cytokines involved in the defence against viral infections in fish are interferons (IFNs).

IFNs are secreted proteins, belonging to the soluble glycoprotein class (Lin and Young, 2014), that induce a cellular antiviral state. In teleost fish, two families of IFNs have been distinguished on the basis of the gene sequence, protein structure and functional properties: type I IFN (IFN I), that includes group I and group II IFN I according to the number of cysteine residues: two for group I and four for group II (Langevin et al., 2013b; Poynter et al., 2015; Zou and Secombes, 2016), and type II IFN (IFN II), including IFN-γ. IFN I is induced by viral infection in most cells, whereas IFN II is produced by natural killer cells (NK) and lymphocytes T (T-cells) in response to interleukin-12 (IL-12), IL-18, mitogens or antigens, being involved in both, innate and adaptive immunity (Robertsen, 2006).

In mammals, IFN I shows multiple modulatory effects, including the increase of the activation of NK cells, the induction of the major histocompatibility complex (MHC) class I expression, the increase of T-cell survival, and the stimulation of dendritic cell maturation; however, the induction of a cellular antiviral state is one of the most important roles played by this cytokine (Robertsen, 2006). Regarding fish, IFN I system is also crucial against viral infections and, for this reason, it will be deeply explained below.

3.1.1. Type I IFN system

The IFN I response against viral infections is based on the detection of virus associated molecular patterns (VAMPs) by pattern recognition receptors (PRRs). Several molecules can be VAMPs, such as single-stranded (ss)RNA, which contains guanosine- and uridine-rich sequences or 5' triphosphates, double-stranded (ds)RNA (produced during viral replication) and DNA (Bauer et al., 2008; Poynter et al., 2015); cytosine- and guanine-rich unmethylated DNA sequences (CpG-DNA) (Ohto et al., 2015); imidazoquinolines (Shukla et al., 2012; Shi et al., 2012) or synthetic analogues of dsRNA, such as poly I:C (Fasciano and Li, 2006; Hafner et al., 2013).

Several PRRs have been described in fish. They can be located in the cellular membrane, in the endosomal membrane or in the cytoplasm (Poynter et al., 2015). On the cell surface, toll-like receptor 22 (TLR22) detects long dsRNA molecules, whereas class A scavenger receptor (SR-As) binds and transports dsRNA and DNA into endosomes. Regarding endosomal receptors, TLR3 recognizes dsRNA (including poly I:C), and TLR7 and TLR8 detect ssRNA and imidazoquinolines (Shi et al., 2012). Furthermore, TLR9 and TLR21, also located in the endosome membrane, recognize DNA and CpG-DNA (Ohto et al., 2015). Cytosolic receptors, referred as nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) (Zhang et al., 2018) and retinoic acid-inducible gene-I-like receptors (RLRs), bind to viral dsRNA, whereas DNA is recognized by the cytosolic DNA sensor (CDS) DDX41 (Figure 5).

Once PRRs bind to their specific ligands, the activation of their associated adaptor proteins occurs, leading to the activation of specific signalling pathways responsible for the eventual phosphorylation of the interferon regulatory factors 3 and/or 7 (IRF3, IRF7) and the nuclear transcription factor NF-kappa β (NF-kβ). Once

activated, these transcription factors translocate to the nucleus, bind to specific regulatory domains sited in the promoter region of IFN I-coding genes, and induce the transcription of this cytokine (Poynter et al., 2015) (Figure 5).

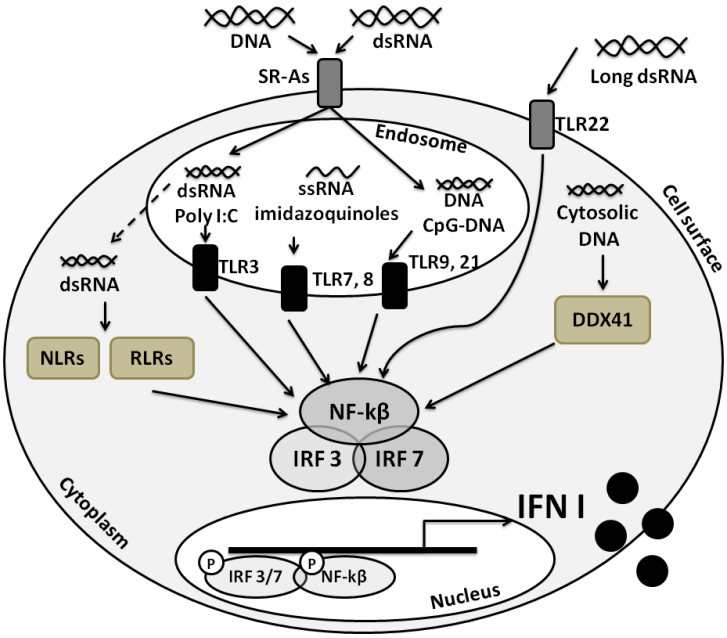


Figure 5. Signalling pathways of fish PRRs to activate the IFN I system (adapted from Poynter et al., 2015).

In fish, as in higher vertebrates, IFN is secreted in virus-infected cells, being recognized by neighbouring cells through specific IFN I receptors. These receptors are present in all nucleated cells, and, in fish, they are composed of two subunits of the cytokine receptor family B (CRFB). Different combinations of CRFB subunits have been reported in zebrafish (*Danio rerio*), Atlantic salmon and Dabry's sturgeon (*Acipenser dabryanus*) (Langevin et al., 2013b; Luo et al., 2018; Sun et al., 2014). The specific recognition activates the Janus tyrosine kinases JAK1 and TYK2, which, in turn, activate the signal transducers and activators of transcription STAT1 and STAT2, constituting the JAK-STAT pathway. Activated STATs bind to IRF9 to form the STAT1/STAT2/IRF9 complex, which is translocated to the nucleus, where recognizes and binds to interferon-stimulated response element (ISRE) sequences in the promoter region of interferon-stimulated genes (ISGs) (Coccia and Battistini, 2015; Poynter et al., 2015; Robertsen, 2006; Verrier et al., 2011) (Figure 6). This binding triggers the transcription of ISGs, establishing an antiviral state.

In fish, several ISGs have been identified, such as mx dynamin-like GTPase

(*mx*), dsRNA-activated protein kinase (*pkr*), virus inhibitory protein (viperin, *vig-1*), interferon-stimulated gene 15 (*isg15*), interferon-stimulated gene 12 (*isg12*), and tripartite motif family (TRIM) protein (Der and Lau, 1995; Fitzgerald, 2011; Gjermandsen et al., 2000; Langevin et al., 2017; Lindqvist et al., 2018; Lu and Liao, 2011; Morales and Lenschow, 2013; Okumura et al., 2013; van der Aa et al., 2012; Verhelst et al., 2013; Verrier et al., 2011). The antiviral activity of Mx proteins has been widely reported in fish (Alvarez-Torres et al., 2013; Chen et al., 2008; Novel et al., 2013; Thanasaksiri et al., 2014; Wu et al., 2016); however, recent studies on *isg15* transcription and ISG15 antiviral activity (Alvarez-Torres et al., 2017; 2018; Langevin et al., 2013b) have suggested that this ISG can also have an important antiviral role. For this reason, this ISG will be further explained and deeply studied in chapter 2 of the present PhD Thesis.

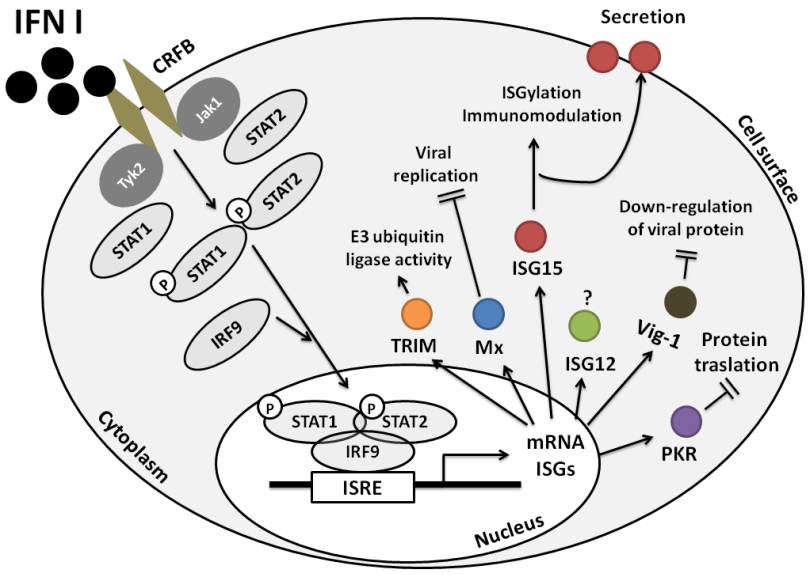


Figure 6. IFN I system activation by the JAK/STAT pathway. Induction of ISG transcription and ISGs identified in fish with their functions (adapted from Robertsen, 2006; Verrier et al., 2011).

3.1.2. Inflammatory response

Another essential process within the innate immune system is the inflammatory response. It is a complex physiological process to counter injuries, which may occur from trauma or cellular stress derived from bacterial or viral infections. Immediately after detecting danger, populations of granulocytes and leucocytes, including macrophages and lymphocytes, appear at the pathogen site, being involved in the clearance of the pathogen. Once the inflammation is resolved, cellular and tissue debris

are cleared up by neutrophils and macrophages, and healing processes finally restore homeostasis (Rebl and Goldammer, 2018).

As it has been described previously, the detection of VAMPs by PRRs in infected cells leads to the activation of NF-κB and IRFs, which initiate IFN I transcription (Figure 5) as well as the transcription of pro-inflammatory cytokines, including the tumour necrosis factor alpha (TNF-α), a pleiotropic pro-inflammatory cytokine produced by numerous immune cells during acute inflammation (Ordas et al., 2007), interleukin 1β (IL-1β), IL-12 and other immune mediators (Shukla et al., 2012; Poynter et al., 2015; Rebl and Goldammer, 2018). These pro-inflammatory cytokines are conserved among vertebrates, including fish, being mainly involved in the chemotactic attraction of leucocytes, vascular dilation, the enhanced production of prostaglandins and reactive oxygen species (Rebl and Goldammer, 2018), or in the induction of other cytokine transcription.

These molecules bind to their cell receptors and activate signalling pathways promoting the transcription of more than 50 genes that are known to be activated upon inflammation (Baeuerle, 1998). In fish, among these genes, IL-8 and IL-6 recruit and activate innate immune cells, whereas IFN-γ follows the recruitment and activation of T-cells and promotes the production of nitric oxide (NO) by monocytes/macrophages (Yang et al., 2013).

To antagonize these effects and to control the "cytokine storm", which may derive in pathologically high concentrations of cytokines in the systemic circulation, the anti-inflammatory cytokines (IL-10, IL-11 or TFG-β) play a homeostatic role and immuno-regulate the process. Among their functions, these cytokines suppress the synthesis or activation of inflammatory cytokines, surface receptors and co-stimulatory molecules (Rebl and Goldammer, 2018).

3.2. Adaptive immune system

In addition to drive inflammatory signals, cytokines regulate antigen presenting cells (APCs) and their migration, to activate lymphocytes and initiate the adaptive immune response (Wang and Secombes, 2013). Thus, this response is mediated by two groups of lymphocytes, B- and T-cells. Lymphocytes B mediate antibody response, secreting antibodies, which specifically bind to viral antigens, block viral entry to host cells and mark viruses for their destruction. Lymphocytes T organize cell-mediated

response, reacting against an antigen previously presented by APCs via MHC proteins (Secombes and Wang, 2012). This system allows a highly specific response, very effective against infections and with immunological memory.

3.2.1. Antibody response

The antibody response is mediated by lymphocytes B, which recognize antigens through their immunoglobulin (Ig) receptors, activating a first signal in the cell. If a second recognition takes place, this second signal triggers B-cell proliferation and the intense production and secretion of antibodies against the pathogen.

Antibodies are heterodimeric glycoproteins belonging to the Ig superfamily. Their basic structure consists of two heavy (H) and two light (L) chains, each containing one N-terminal variable domain (VH or VL) and one C-terminal constant domain (CH or CL). Variable domains are involved in antigen recognition, whereas constant domains mediate effector functions of the antibody molecule (Chistiakov et al., 2007; Mashoof and Criscitiello, 2016). The VL and VH domains contain three hypervariable regions, called complementarity-determining regions (CDRs), which are paired and create the specific antigen-binding site. Combinations of CDRs in VL and CDRs in VH diversify this site, creating millions of combinations, which enables to recognize a wide range of antigens (Mashoof and Criscitiello, 2016; Secombes and Wang, 2012).

In fish, three different Ig heavy chain isotypes (IgM, IgD and IgT/Z) and some Ig light chains, such as three in zebrafish (Mashoof and Criscitiello, 2016; Scapigliati et al., 2018), have been identified.

The predominant isotype in blood/serum is IgM (Scapigliati et al., 2018), appearing as tetrameric structures (molecular weight, MW, 450 kDa). It can be expressed on B-cells surface or be secreted. This antibody contributes to both, innate and adaptive fish immunity, since it is involved in complement activation, agglutination, phagocytosis processes, and cellular cytotoxicity (Mashoof and Criscitiello, 2016; Ye et al., 2013).

Both, IgM and IgT can be expressed in fish from very early developmental stages, and IgT expression increases more rapidly compared with IgM, suggesting that IgT plays an important role in the protection of fish larvae. In most adult fish, IgM is the dominant isotype, although, in some situations, IgT is the predominant isotype at

mucosal sites (Zhang et al., 2011). IgT/Z was first identified in rainbow trout (*Oncorhynchus mykiss*) (IgT) and zebrafish (IgZ), being subsequently described in most model teleost species. It appears as monomers in serum, and as tetramers in gut mucus (Zhang et al., 2011).

Regarding IgD, it has been studied at molecular level, being expressed in a monomeric form with a putative MW of 150 kDa. IgD-secreting cells can be found in the anterior and posterior kidney, spleen and gills (Mashoof and Criscitiello, 2016), but little is known on its physiological role in fish (Scapigliati et al., 2018).

3.2.2. Cell-mediated response

Activated lymphocytes T are the main effectors of the cell-mediated adaptive response. Fish and mammalian T-cells share some characteristics: (i) display T-cell receptors (TCRs) to recognize antigens presented by MHC molecules; (ii) display the CD3 complex, through which the antigen-TCR interaction promotes cellular activation (Picchietti et al., 2011); and (iii) express the TCR co-receptor CD8 or CD4, which stabilize the TCR/MHC interaction and enhance the activation of the CD3 pathway. The type of co-receptor expressed determines the specificity to MHC class I- or MHC class II-presented antigens, dividing T-cell in two sub-populations: T-cytotoxic cells (Tc), and T-helper (Th) cells (Secombes and Wang, 2012). Thus, lymphocytes T CD8 recognize peptides associated with MHC class I molecules, activating this population as Tc cells, which are able to destroy infected cells. In contrast, CD4 marks Th cells and recognizes antigens associated with MHC class II. This recognition induces the production of cytokines that promote the activation of other immune cells, enhancing the immune response.

MHC class I complex appears in most of the nucleated cells, whereas APCs, including macrophages, monocytes, B-cells and even activated T-cells, display MHC class II.

Finally, regarding TCRs, T-cells recognize antigens through a CD3/TCR complex expressed on the cell surface. There are two variants of TCRs: (i) αβ TR, which is displayed by the majority of mature T-cells in secondary lymphoid organs; and (ii) γδ TR, which is present in T-cells in epithelial layers of mucosal tissues, such as skin and intestinal epithelium, constituting a first line of defence as effector cells (Buonocore et al., 2012).

AIMS

Nervous necrosis virus (NNV) is the main viral pathogen affecting European sea bass (*Dicentrarchus labrax*). Viral isolates belonging to the red-spotted nervous necrosis virus species (RGNNV) show high virulence to this fish species, causing high mortality in aquaculture facilities, whereas isolates within the striped jack nervous necrosis virus (SJNNV) viral species, although replicate in sea bass brain, are low virulent. Viral disease appearance is the result of the pathogen virulence, the host immunity mechanisms, and the interaction between them. On the basis of these premises, the **main aim** of the current PhD Thesis has been to study the main pathogen and host factors involved in the interaction between European sea bass and NNV. In order to fulfil this aim, the following **specific aims** have been addressed:

1. Study on the implication of amino acids 247 and/or 270, located in the C-terminal region of the capsid protein, in betanodavirus virulence to European sea bass.

2. Evaluation of the role of European sea bass ISG15 protein against betanodavirus infections.

3. Study on the modulation of immunogene transcription and antibody response following betanodavirus infections in European sea bass.

CHAPTER 1

Analysis of molecular determinants in betanodavirus virulence to European sea bass

1. INTRODUCTION

Pathogenicity has been described as the ability of microorganisms to cause disease, whereas virulence refers to variations in degree of pathogenicity (Read, 1994). Virulence depends on several factors involving host, pathogen and host-pathogen interaction.

Regarding host, several variables, such as age, rearing conditions, feeding and immunological state, may influence the severity of a viral disease. On this matter, it is important to highlight the role of the innate immune system as the first barrier against virus infections in fish (Robertsen, 2006). Regarding the pathogen, factors involved in host-cell recognition and entry, as well as viral genome replication efficiency, along with the presence of immune system antagonistic mechanisms, should be considered as important viral virulence factors.

In betanodavirus, the C-terminus region of the capsid protein (CP) has been described as an important determinant of virulence and host specificity. Specifically, the protruding positions in this region (amino acids 238–340 in striped jack, *Pseudocaranx dentex*, nervous necrosis virus, SJNNV, and in red-spotted grouper, *Epinephelus akaara*, nervous necrosis virus, RGNNV), have been considered good candidates as host specificity determinants (Ito et al., 2008; Iwamoto et al., 2004). In addition, a recent study on the crystal structure of the betanodavirus CP indicates that those positions described by Iwamoto et al. (2004) are located on the protruding domain (P-domain). Moreover, this domain shows hyper-variable regions, which coincide structurally with the protrusion surface, suggesting their role in cell-receptor binding and, therefore, in host-cell specificity (Chen et al., 2015).

In the context of host-pathogen interaction, the identity of the specific receptor in nervous cells of brain and retina, as well as the identity of the viral amino acids involved in cellular binding, are not fully characterized; however, betanodavirus entry mechanisms have been described for several cell lines. Thus, dragon grouper (*Epinephelus lanceolatus*) nervous necrosis virus (DGNNV) entry in striped snakehead (*Channa striata*) fry cells (SSN-1) is by micro- and macro-pinocytosis (Liu et al., 2005), whereas entry in Asian sea bass (*Lates calcarifer*) cells (SB cells) is through the clathrin-mediated endocytosis pathway (CME) (Huang et al., 2017). In addition, the sialic acid and the cellular heat shock cognate protein 70 (HSC70) have been reported to

be essential for betanodavirus binding to SSN-1 and grouper (*Epinephelus coioides*) fin cells (GF-1), respectively (Chang and Chi 2015; Liu et al., 2005).

Two amino acids located in the P-domain of the CP have been suggested as possible determinants of betanodavirus virulence and host specificity. Olveira et al. (2009), based on studies on RGNNV/SJNNV-type reassortant strains, proposed the importance as host determinants of amino acids 247 and 270 in the CP sequence, which was corroborated by Souto et al. (2015a) studying the reassortant strain SpSs-IAusc160.03, pathogenic to Senegalese sole (*Solea senegalensis*). This strain harbours RGNNV-type RNA1 and SJNNV-type RNA2 segments, although the RNA2 segment contains mutations affecting amino acids 247 and 270, which are those present in RGNNV-type CP sequences. These authors modified, by reverse genetics, these amino acids to those in the CP sequence of the SJNNV reference strain, resulting in a 40% decrease of mortality rate, in comparison with mortality caused by the natural reassortant.

Betanodavirus replication is a temperature-sensitive process (Hata et al., 2010; Panzarin et al., 2014) controlled by the RNA dependent-RNA polymerase. Thus, Toffan et al. (2016) showed that RGNNV isolates are highly virulent to European sea bass (*Dicentrarchus labrax*) only at high temperature (25-30 ^{o}C), whereas SJNNV is detected in sea bass brain at medium temperature, and reassortant isolates (RGNNV/SJNNV and SJNNV/RGNNV) are less temperature-dependent than both parental viral species, although being influenced by the genetic type of the polymerase gene. Therefore, water temperature is an environmental factor playing an important role in betanodavirus virulence.

Finally, the role of the non-structural B2 protein as antagonistic factor of host RNA interference has been demonstrated (Fenner et al., 2006a; 2006b), as well as its implication in cellular apoptosis, triggering cellular mitochondria-mediated necrotic death, and inducing mitochondrial oxidative stress by hydrogen peroxide production (Su et al., 2009; 2014).

Regarding European sea bass, all stages of this fish species, specially larvae and juveniles, are highly susceptible to RGNNV infection (Buonocore et al., 2017). Susceptibility to RGNNV isolates is well documented, causing mortalities ranging from 36% to 75% (Bovo et al., 1999; Carballo et al., 2016; Chérif et al., 2009; Le Breton et

al., 1997; Panzarin et al., 2012; Vendramin et al., 2014), whereas sea bass susceptibility to SJNNV and reassortant isolates has only been recorded in a few studies (Vendramin et al., 2014; Souto et al., 2015b). These authors reported that, although SJNNV isolates can infect and develop productive infections in sea bass, they cause very low mortality, or even no mortality, depending on the viral isolate. However, the RGNNV/SJNNV-type reassortant strain SpSs-IAusc160.03, despite displaying a SJNNV-type CP, caused 33% mortality in experimentally infected sea bass (Souto et al., 2015b), which has been associated with a switch in SJNNV host range caused by the RGNNV-type amino acids 247 and 270 of the reassortant CP sequence. Moreover, the CP sequence analysis of several RGNNV/SJNNV-type reassortant isolates naturally pathogenic to sea bream (*Sparus aurata*) showed the presence of RGNNV-type amino acids at positions 247 and 270 (serine in both positions) (Toffan et al., 2017). Altogether, these previous reports suggest the possible implication of these amino acids not only as virulence determinants, but also as host determinants.

The reverse genetics technology is an excellent tool for the study of virulence determinants, since it allows creating recombinant infective viral particles, which can be easily modified by site-directed mutagenesis, being a good tool for virulence and host-specificity studies. This technique has been successfully used with the main viruses affecting a variety of fish species cultured worldwide, such as infectious salmon anaemia virus (ISAV) (Toro-Ascuy et al., 2015), infectious pancreatic necrosis virus (IPNV) (Yao and Vakharia, 1998), infectious hematopoietic necrosis virus (IHNV) (Biacchesi et al., 2000; Rouxel et al., 2016) and viral haemorrhagic septicaemia virus (VHSV) (Kwak et al., 2017). The reverse genetics procedure has been applied to betanodavirus studies for the first time by Iwamoto et al. (2001), who recovered infective particles by transfecting RNA transcripts into E-11 cells. Recently, a more stable methodology, based on the T7 RNA polymerase system constitutively expressed by BSRT7/5 cells, has been established (Biacchesi, 2011; Souto et al., 2015a; Takizawa et al., 2008).

The aim of the present study has been the evaluation of amino acids 247 and 270 in the CP sequence as betanodavirus virulence determinants to European sea bass. To fulfil this aim, we have conducted punctual mutations of the above mentioned amino acids using as back-bone the genome of a RGNNV isolate highly virulent to sea bass, in order to apply a "loss of virulence" approach.

2. MATERIALS AND METHODS

2.1. VIRAL ISOLATE AND CELL CULTURE

A RGNNV isolate obtained from diseased European sea bass in the Aquaculture Institute of Santiago de Compostela (Spain) (SpDl_IAusc965.09) has been used in this study. From now on, this isolate will be named as *wild type* Dl965, or *wt*Dl965, to establish a clear differentiation with the isolate generated by reverse genetics (hereafter *r*Dl965).

Viruses were propagated on the E-11 cell line (Iwamoto et al., 2000). E-11 cells were grown in 25-cm^2 flasks (Nunc) using Leibovitz L-15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 10 mg/ml streptomycin (Sigma) (growth medium) at 25 ^oC until confluence. Cells were then inoculated and maintained in L-15 medium supplemented with 2% FBS, 100 units/ml penicillin and 10 mg/ml streptomycin (maintenance medium) at the same temperature until fully cytopathic effect (CPE) development. At that moment, supernatants and cells were collected, subjected to 3 cycles of freeze/thawing, and cellular debris was removed by centrifugation at 1,500 x *g* for 5 min. The resulting viral suspensions were titrated on 96-well plates (Nunc) following the 50% tissue culture infective dose method ($TCID_{50}$, Reed and Müench, 1938). These viral stocks were stored at -80 ^oC until used.

BSRT7/5 cells (Buchholz et al., 1999), kindly provided by Dr. K.K. Conzelmann (Ludwig-Maximilians-Universität Munich, Germany), were used to generate infective viral particles by reverse genetics. This cell line, derived from the Baby Head Kidney 21 cell line (BHK-21, Sanders and Burford, 1964), expresses constitutively the phage T7 RNA polymerase under the control of the cytomegalovirus promoter (Buchholz et al., 1999). These cells were cultured using Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS, 200 mM glutamine (Sigma), 100 units/ml penicillin, and 10 mg/ml streptomycin. Cells were incubated at 37 ^oC in a 5% CO_2 atmosphere, adding 1 mg/ml geneticin (G418, Sigma) every other sub-culture.

2.2. SEQUENCING OF RNA1 AND RNA2 GENOMIC SEGMENTS

A two-step strategy has been followed to obtain the complete *wt*Dl965 sequence: (i) sequencing of RNA1 and RNA2 open reading frames (ORFs), using the *primer walking* approach; (ii) sequencing of 5'- and 3'-ends by the Rapid Amplification of cDNA Ends (RACE) methodology.

2.2.1. Open reading frame sequencing

2.2.1.1. RNA extraction and cDNA synthesis

Total RNA was extracted from viral suspensions using the E.Z.N.A. Total RNA Kit I (Omega bio-tek) following manufacturer guidelines. The resulting RNA was quantified at 260 nm using the NanoDrop-1000 system (ND-1000, ThermoFisher), being stored at -80 ^{o}C until cDNA synthesis.

cDNA was obtained with the Transcriptor First Strand cDNA Synthesis Kit (Roche) in a reaction carried out in two steps. In a first step, RNA (1 µg) and 60 µM random hexamer primers were mixed in a 13-µl reaction, followed by incubation at 65 ^{o}C for 10 min. In a second step, the remaining components were added up to 20 µl. These components were: 1x Transcriptor Reverse Transcriptase Reaction Buffer, Protector RNase Inhibitor (20 U), 1 mM Deoxynucleotide Mix, and Transcriptor Reverse Transcriptase (10 U). This mixture was incubated at 25 ^{o}C for 10 min, followed by 50 ^{o}C for 60 min and 85 ^{o}C for 5 min. cDNA was stored at -20 ^{o}C until used.

2.2.1.2. ORF sequencing by the *primer walking* approach

Amplifications of the coding regions within *wt*Dl965 RNA1 and RNA2 segments were performed as described by Olveira et al. (2009). Primers used were designed according to the SJ93Nag strain sequences, with the GenBank accession numbers AB056571 and AB056572. These primers amplify 527-655-bp long overlapping regions in the sequence of both viral segments (Table 1, Figure 1).

Amplifications were carried out in 50-µl mixtures composed of 1x Pfx Amplification Buffer, 0.3 mM dNTPs, 1 mM $MgSO_4$, 0.3 µM specific primers (Table 1), Platinum™ Pfx DNA Polymerase (1 U, Invitrogen) and cDNA (200 ng). The thermal profile was: denaturation at 94 ^{o}C for 5 min, followed by 35 amplification cycles of 94 ^{o}C for 15 s and 58 ^{o}C for 30 s. As final extension, mixtures were incubated

at 68 ^{o}C for 5 min. Resulting products were visualized on 2% agarose gels stained with ethidium bromide (Sigma), and DNA bands were purified for the subsequent sequencing.

Purification was conducted with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) following manufacturer instructions. Purified DNA was quantified at 260 nm using the NanoDrop-1000 system, and maintained at -20 ^{o}C until sequencing, which was performed with the ABI 3730 (STABVIDA) system. Each DNA fragment was sequenced using the specific primers shown in Table 1 in separate reactions, and each reaction was conducted in triplicate. Sequences were analysed and aligned using the EditSeq and MegAlign software (DNASTAR Lasergene 7).

Table 1. Primers used for *wt*Dl965 cDNA amplification (Olveira et al., 2009).

Name	Segment	Sequence (5'-3')	Amplicon size (bp)
NNVs1_B1F	RNA1	CGCAAGGTTACCGTTTAGC	613
NNVs1_B1R		CATGGTATCCTGGTTGTAGTTCC	
NNVs1_B2F		CAAGTACTGTGTCCGGAGAGGTTA	568
NNVs1_B2R		GAGCAAGATCGCCAGGTTTATG	
NNVs1_B3F		AACATCCGCACTGCATACGAACTG	615
NNVs1_B3R		ATGCTGGAGAACACTGGCTTTGAA	
NNVs1_B4F		AACGCCCCAAATTACCCCAGGAAC	552
NNVs1_B4R		GCCCGGCACCAATGACCAATAAAA	
NNVs1_B5F		CGACATTGGCTGGGCTAAGACACA	653
NNVs1_B5R		GCCGAAGCGTAGGACAGCATAAAG	
NNVs2_RG1F	RNA2	TCCATCACCGCTTTGCAATCAC	527
NNVs2_RG1R		ACACAGGAGTATCAGCCGACCAG	
NNVs2_RG2F		CTTCCTGCCTGATCCAACTGACAACG	568
NNVs2_RG2R		CCAGATGCCCCAGCGAAACCA	
NNVs2_RG3F		AGCCTTGGAACTGGAGATGT	655
NNVs2_RG3R		ACGCGGAGCTAACGGTAAC	

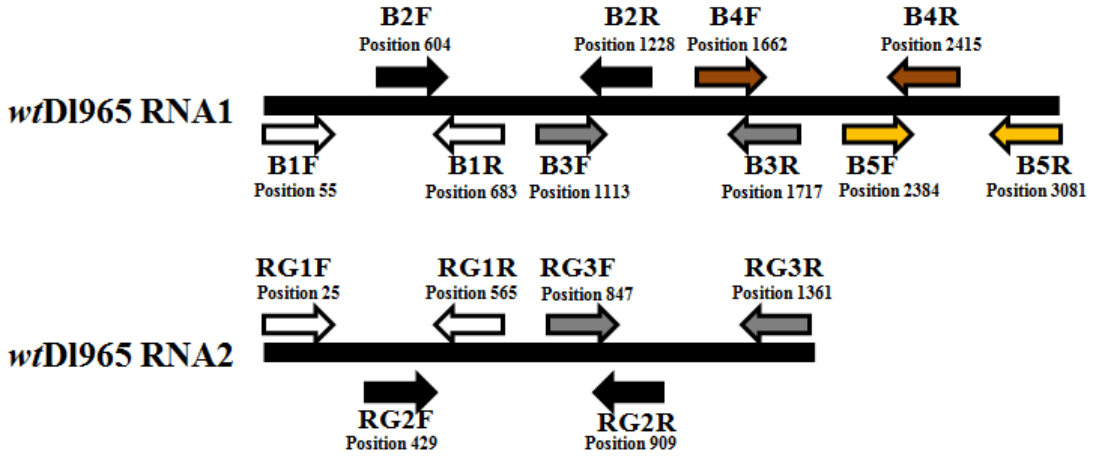


Figure 1. Position of primers used for RNA1 and RNA2 ORF amplification using the *primer walking* approach.

2.2.2. Sequencing of viral segment ends

RACE protocol requires high amount of good-quality RNA template; for this reason, viral suspensions were semi-purified prior to the sequencing strategy.

2.2.2.1. Semi-purification of *wt*Dl965 viral particles

E-11 cells grown in 75-cm^2 flasks were inoculated with *wt*Dl965 (section 2.1.), and incubated until CPE appearance. The resulting viral suspensions were collected, pooled and centrifuged at 3,000 x *g* for 15 min, discarding the cellular debris. Virus particles were pelleted by ultracentrifugation at 25,000 rpm at 10 ^{o}C for 1 h. Then, 500 µl of 1x saline-sodium citrate buffer (SSC, Sigma) plus 500 µl of sucrose solution (30%, in 1x SSC) were carefully added to the virus-containing pellet. After a second 1-h ultracentrifugation (35,000 rpm at 10 ^{o}C), virus particles were diluted in 1x SSC (150-200 µl), and stored at -80 ^{o}C until used. All ultracentrifugations were conducted with the Beckman Coulter Optima L-90K Ultracentrifuge (SW55Ti and SW32Ti rotors, Beckman Coulter).

2.2.2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from semi-purified viral particles using the TRI reagent solution (Sigma). After adding 1 ml of this reagent, virus suspensions were incubated at room temperature for 5 min. Afterwards, a volume of 200 µl of 1-Bromo-3-chloropopane (Sigma) was added and mixed until acquiring milky appearance.

Samples were then incubated at room temperature for 15 min, and subsequently centrifuged at 12,000 x *g* at 4 ^{o}C for 15 min. The aqueous phase was transferred to a new vial, and 500 µl of isopropanol were added. After mixing and incubating at room temperature for 10 min, RNA was pelleted by centrifugation at 12,000 x *g* at 4 ^{o}C for 15 min. The resulting RNA was washed with 75% ethanol, dried and resuspended in diethyl pyrocarbonate (DEPC, Sigma)-treated water (50 µl) by incubation at 55 ^{o}C for 10 min. RNA was quantified at 260 nm using the NanoDrop-1000 system, and stored at -80 ^{o}C until used.

According to RLM-Race Kit (Ambion) guidelines, first step to obtain the 5'-end sequence (5'-RACE assay) was RNA treatment (1 µg) with Calf Intestine Alkaline Phosphatase (CIP) at 37 ^{o}C for 1 h, which removes free 5'-phosphates and avoids incomplete RNA, fragmented mRNA and contaminating genomic DNA. Afterwards, RNA was purified following RACE guidelines, precipitated with isopropanol, washed with 75% ethanol, dried and resuspended in nuclease-free water. The subsequent RNA treatment with Tobacco Acid Pyrophosphatase (TAP), at 37 ^{o}C for 1 h, removes the cap structure, exposing a 5'-monophosphate residue, which is required for 5'-RACE adapter ligation.

For 5'-RACE adapter ligation, 1x RNA Ligase Buffer, 5'-RACE adapter (0.3 µg), and T4 RNA ligase (5 U) were added to the CIP/TAP RNA mixture up to 10 µl. The reaction was incubated at 37 ^{o}C for 1 h prior to reverse transcription. cDNA was obtained in a 20-µl mixture consisting of 2 µl of ligated RNA, 2.5 mM dNTP mix, 50 mM random decamer primers, 1x RT Buffer, RNase inhibitor (10 U), and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV). The reaction was incubated at 42 ^{o}C for 1 h and subsequently stored at -20 ^{o}C until used.

For the 3'-RACE assay, first step was to add the poly-A tail at the 3'-end of both segments, using the Poly (A) Tailing Kit (Applied Biosystems). In order to do this, 1x E-PAP Buffer, 25 mM $MnCl_2$, 10 mM ATP Solution and poly-A polymerase E-PAP (8 U) were mixed with RNA (2 µg) in a 100-µl reaction. This mixture was incubated at 37 ^{o}C for 1 h. RNA was then purified with the MEGAclear Kit (Ambion) following commercial instructions. Poly-A tail binding is required for the RNA reverse transcription following the guidelines described for the 5'-RACE assay, although using the 3'-RACE adaptor as reverse primer.

2.2.2.3. Amplification of 5'- and 3'-ends by RACE methodology

Amplification of 5'- and 3'-ends of both viral segments was performed following the RLM-Race Kit guidelines. PCRs were conducted in 50-µl mixtures composed of cDNA (20 ng), 1x Accuprime PCR Buffer I, AccuPrime™ *Taq* DNA polymerase (1 U) and specific primers (10 pmol) (Table 2).

For 5'-end amplification, forward primers (for PCR and nested-PCR, if it was necessary) were those supplied by the commercial system, whereas reverse primers (R1-5' Outer, R1-5' Inner, R2-5' Outer and R2-5' Inner, Table 2) were designed in this study on the basis of the sequences obtained in section 2.2.1. For 3'-end amplification, forward primers (R1-3' Outer, R1-3' Inner, R2-3' Outer and R2-3' Inner, Table 2) were designed, whereas reverse primers were those supplied by the commercial system. The thermal profile for PCR and nested-PCR was: denaturation at 94 ^{o}C for 3 min, followed by 35 amplification cycles at 94 ^{o}C for 30 s, 60 ^{o}C for 30 s and 68 ^{o}C for 1 min. A final elongation at 68 ^{o}C for 7 min was carried out. Amplified products were visualized on 2% agarose gels stained with RedSafe™ Nucleic Acid Staining Solution (European Bioteck), and they were subsequently purified using the Nucleospin Gel PCR and Clean-up column system (Macherey-Nagel), following commercial indications.

2.2.2.4. Cloning and sequencing of 5'- and 3'-ends

The RLM-RACE amplified products, corresponding to 5'- and 3'-ends in both *wt*Dl965 genomic segments, were cloned using the pGEM®-T Easy Vector System (Promega). Ligation reactions were set up in 10 µl (final volume) mixing 1x Rapid Ligation Buffer T4 DNA Ligase (Promega), pGEM®-T Easy Vector (50 ng), DNA template (3:1, insert:vector) and T4 DNA Ligase (Promega, 3 U). These mixtures were incubated at 4 ^{o}C overnight. Ligation products were used to transform *Escherichia coli* DH5α cells using a standard electroporation procedure.

Transformed bacterial clones were grown in Luria-Bertani (LB) broth (Sigma) with ampicillin (100 µg/ml, Sigma) at 37 ^{o}C overnight for plasmid extraction, which was performed using the High Pure Plasmid Isolation Kit (Roche), according to manufacturer instructions. Purified plasmids were quantified at 260 nm using the NanoDrop-1000 system, and insert presence was checked by PCR using the vector primers M13 Forward (5'-GTTTTCCCAGTCACGAC-3') and M13 Reverse (5'-

CAGGAAACAGCTATGAC-3'), following the GoTaq® DNA Polymerase (Promega) protocol. Thus, plasmids (500 ng) were amplified in 50-µl mixtures consisting of 1x Green GoTaq® Reaction Buffer, 0.2 mM PCR Nucleotid Mix, 1.5 mM $MgCl_2$, 15 pmol M13 primers and GoTaq® DNA Polymerase (1.75 U). The amplification profile was as follows: denaturation at 95 ^oC for 5 min, followed by 35 cycles at 95 ^oC for 1 min, 50 ^oC for 30 s, 72 ^oC for 1 min, and a final elongation at 72 ^oC for 10 min. Amplified products were visualized on 2% agarose gels stained with ethidium bromide. Those plasmids positive for the insert presence were sequenced in triplicate with the ABI 3730 (STABVIDA) system. These sequences were analysed with the EditSeq and MegAlign software (DNASTAR Lasergene 7).

Table 2. Primers designed to amplify 5'- and 3'-ends of both viral segments.

Name	Sequence (5'-3')	Product length (bp) in combination with primers supplied by the RLM-RACE Kit
R1-5' Outer	ACGGTAGTCTTGGCGAAAATCTGC	454
R1-5' Inner	ACGGGGTGATGGTGAAATGAC	384
R1-3' Outer	AGCATCTTGCACTCCTGGAT	541
R1-3' Inner	CCCACGTCTGACAACATCAA	499
R2-5' Outer	ACTGTTCTGCTTTCCCACCA	544
R2-5' Inner	TGTCAGTTGGATCAGGCAGGAAGC	464
R2-3' Outer	GATGAGCAGCCTCGTCAAAT	504
R2-3' Inner	ATCCGGTTCCCTAGTGCGTATCGT	428

2.3. REVERSE GENETICS STRATEGY FOR *r*Dl965 RECOVERY

Infective virus particles have been recovered from viral cDNA using a reverse genetics approach based on the T7 RNA polymerase system (Takizawa et al., 2008; Biacchesi, 2011), which is summarized in Figure 4. First step was to amplify the full-length RNA1 and RNA2 cDNAs.

Total RNA was extracted from semi-purified viral particles (section 2.2.2.1.) following the methodology described in section 2.2.2.2., and cDNA synthesis was carried out using the SuperScript™ III Reverse Transcriptase (Invitrogen) protocol as follows. In a first reaction, RNA (1 µg) was mixed with random primers (100 ng) and 10 mM dNTP, and incubated at 65 ^oC for 5 min in a 10-µl mixture. In a second step, 1x

First-strand Buffer, 0.1 M DTT, RnaseOUT (40 U), and SuperScript™ III RT (200 U) were added to the previous mixture up to 20-µl (final volume). This reaction was incubated at 65 ^{o}C for 5 min, 50 ^{o}C for 30 min and 75 ^{o}C for 15 min. cDNA was stored at -20 ^{o}C until used.

2.3.1. Full-length amplification and cloning of both *wt*Dl965 genomic segments

External primers designed to amplify the complete cDNA sequences were T7_5'RNA1_965 and 3'RNA1_965, for RNA1 amplification, and T7_5'RNA2_965 and 3'RNA2_965, to amplify the RNA2 segment (Table 3). These primers have been designed based on the *wt*Dl965 sequence previously obtained (section 2.2.), and they contain several sequences required for cloning and reverse genetics. Thus, forward primers (T7_5'RNA1_965 and T7_5'RNA2_965) include *BamHI* and *SacII* restriction sites, the T7 polymerase promoter sequence, and two guanine residues. These residues up-regulate the transcription of T7 RNA polymerase (Roy and Lieber, 2009; Takizawa et al., 2008). Reverse primers (3'RNA1_965 and 3' RNA2_965) display a blunt-end *SfoI* restriction site (Table 3). Unfortunately, all the attempts to amplify the complete RNA1 sequence using the pair of primers T7_5'RNA1_965 and 3'RNA1_965 failed. For this reason, the 3,104-bp long RNA1 sequence was divided in two smaller sequences (labelled as RNA1_1 and RNA1_2), which were amplified and cloned separately as described below.

Table 3. External primers designed to amplify the full-length RNA1 and RNA2 segments. Several motifs are represented in colour: *BamHI* cut site in grey, *SacII* cut site in green, the T7 promoter sequence in blue, two guanine residues in red and *SfoI* blunt-ended cut site in orange.

Name	Sequence (5'-3')	Product length (bp)	Segment
T7_5'RNA1_965	GGATCCCCGCGGTAATACGACTCACTATA GGTAACATCCCTTTCTTGCTCTGTT	3,104	RNA1
3'RNA1_965	GGCGCCGAAGCGTAGGACAGCATAAAGC		
T7_5'RNA2_965	GGATCCCCGCGGTAATACGACTCACTATA GGTAATCCATCACCGCTTTGCAATC	1,432	RNA2
3'RNA2_965	GGCGCCGAGTTGAAAAGCGATCAGCGG		

The amplification of the RNA2 segment was performed with T7_5'RNA2_965 and 3' RNA2_965 primers and the high fidelity DNA polymerase Pfx Platinum, following the protocol described in section 2.2.1.2. The amplification profile was: denaturation at 95 ^{o}C for 5 min, followed by 35 cycles of amplification at 95 ^{o}C for 1 min, 60 ^{o}C for 30 s, 72 ^{o}C for 1.5 min, and a final elongation at 72 ^{o}C for 10 min. Amplified products were visualized on 2% agarose gels stained with ethidium bromide, purified using the Illustra GFX PCR DNA and Gel Band Purification Kit, quantified using the NanoDrop-1000 system, and finally cloned in the CloneJET Vector (Fermentas). For ligation, a 20-µl mixture, composed of 1x Reaction Buffer, 0.15 pmol RNA2 products, 0.5 pmol pJET vector and T4 DNA ligase (5 U), was incubated at room temperature for 30 min. These products were used to transform, by electroporation, *E. coli* DH5α cells. Insert presence was corroborated by PCR using the GoTaq® DNA Polymerase (section 2.2.2.4.), and confirmed by sequencing using the primers pJET1.2 Fw (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2 Rw (5'-AAGAACATCGATTTTCCATGGCAG-3'). The amplification profile was: 95 ^{o}C for 3 min, followed by 35 cycles at 94 ^{o}C for 30 s, 60 ^{o}C for 30 s and 72 ^{o}C for 1.5 min. The resulting plasmid was named as pJET_RNA2.

As it has been previously mentioned, due to the amplification problems of the RNA1 segment, internal primers were designed in order to amplify separately two smaller sequences, labelled as RNA1_1 and RNA1_2 (Figure 2). RNA1_1 was amplified using the external T7_5'RNA1_965 forward primer (previously designed) and the internal BamHI_1315Rw primer (Table 4), whereas the internal forward primer BamHI_1315Fw (Table 4) in combination with the external reverse primer 3'RNA1_965 were used to amplify the RNA1_2 sequence. Both internal primers introduced a *BamHI* cut site in both sequences (RNA1_1 and RNA1_2), which is required for their subsequent ligation.

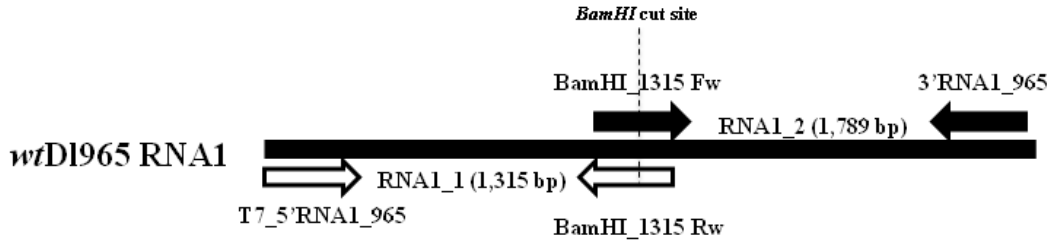


Figure 2. Strategy followed for the full-length RNA1 amplification, showing primers used for RNA1_1 and RNA1_2 amplification.

Table 4. Primers designed to amplify RNA1_1 and RNA1_2. Several motifs are represented in colour: *BamHI* cut site in grey, *SacII* cut site in green, the T7 promoter sequence in blue, two guanine residues in red and *SfoI* blunt-ended cut site is in orange.

Name	Sequence (5'-3')	Product length (bp)	Target
T7_5'RNA1_965	GGATCCCCGCGGTAATACGACTCACTATAGG TAACATCCCTTTCTTGCTCTGTT	1,315	RNA1_1
BamHI_1315 Rw	CTGCTCGGCTGGATCCGTGTCGTAGGCATTA		
BamHI_1315 Fw	TAATGCCTACGACACGGATCCAGCCGAGCAG	1,789	RNA1_2
3'RNA1_965	GGCGCCGAAGCGTAGGACAGCATAAAGC		

The amplifications of RNA1_1 and RNA1_2 were carried out with the AccuPrime™ *Taq* DNA Polymerase, high fidelity, as described in section 2.2.2.3. Amplified products were visualized on 2% agarose gels stained with RedSafe™ Nucleic Acid Staining Solution, purified with the Nucleospin Gel PCR and Clean-up system, following commercial indications, and finally quantified using the NanoDrop-1000 system. These products were cloned in the pGEM®-T Easy Vector (section 2.2.2.4.), yielding the pGemT RNA1_1 and pGemT RNA1_2 constructions.

Finally, RNA1_1 and RNA1_2 sequences were joined in a single cloning vector using the following procedure: RNA1_1 was set free from pGemT_RNA1_1 vector, and pGemT_RNA1_2 vector was linearized. Both reactions were carried out by *BamHI* digestion at 37 ^{o}C for 3 h. Afterwards, linearized pGemT_RNA1_2 vector and RNA1_1 insert were ligated and used to transform *E. coli* DH5α cells, according to the standard pGEM®-T Easy Vector clonation procedure, as described in section 2.2.2.4. Selected clones were confirmed by sequencing. The resulting pGemT vector, containing the complete sequence of the RNA1 viral segment, was labelled as pGemT_BamHI_RNA1.

Bacterial clones harbouring the RNA1 or RNA2 segment (pGemT_BamHI_RNA1 or pJET_RNA2 constructions, respectively), were subjected to a maxi plasmid extraction using the NucleoBond®PC 500 system (Macherey-Nagel), according to manufacturer guidelines. These plasmids were kept at -20 ^{o}C until used.

Next step in the reverse genetics protocol is cloning both viral segments in an expression vector. The construction of the RNA1 expression vector requires the previous elimination of the *BamHI* restriction site, which was introduced in the RNA1

sequence as a tool for RNA1_1 and RNA1_2 ligation (section 2.3.1.). In addition, RNA1 sequence contains a *SfoI* cut site, which also needs to be eliminated, since *SfoI* enzyme will be used for cloning in the expression vector. Both restriction sites were eliminated by introducing a silent mutation, using the directed mutagenesis procedure described below. Thus, the GGATCC *BamHI* restriction site was replaced by TGATCC, and the GGCGCC *SfoI* cut site was replaced by GACGCC.

2.3.2. Point mutation in *BamHI* and *SfoI* restriction sites (RNA1 segment)

For site-directed mutagenesis, the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) was used in combination with the mutagenesis primers MutBamHI_1315 (5'-TAATGCCTACGACACTGATCCAGCCGAGCAG-3') and MutSfoI_1186 (5'-CGCCGGTCAGGACGCCCGTGCAA-3'), which eliminate the *BamHI* and *SfoI* restriction sites, respectively, within the RNA1 sequence. Briefly, mutant-strand synthesis reaction was prepared in 25-µl mixtures adding 1x QuickChange Multi reaction buffer, dNTP mix, mutagenesis primers (100 ng), QuickChange Multi enzyme blend (*Pfu turbo* DNA polymerase, 2.5 U) and DNA (50 ng). The amplification profile was 95 ^{o}C for 1 min, followed by 30 cycles at 95 ^{o}C for 1 min, 55 ^{o}C for 1 min and 65 ^{o}C for 12 min. Resulting products were treated with *DpnI* (10 U, at 37 ^{o}C for 1 h), which specifically degrades methylated and hemimethylated template DNA (non-mutated). This treated DNA was used to transform *E. coli* DH5α cells by electroporation, selecting those clones displaying the correct punctual mutations, which was confirmed by sequencing. The resulting vector (pGemT_RNA1) was purified with the NucleoBond®PC 500 kit, according to manufacturer instructions. The elimination of *BamHI* and *SfoI* restriction sites was confirmed by digesting with these enzymes at 37 ^{o}C for 1 h, followed by 1% agarose gel electrophoresis.

2.3.3. Construction of RNA1 and RNA2 expression plasmids

The eukaryotic expression plasmid used was pBSδRiboT7t (Biacchesi et al., 2000), which was kindly supplied by Dr. Bremont (Unité de Virologie et Immunologie Moléculaires, INRA, Jouy en Josas, France). This plasmid contains the hepatitis δ virus (HDV) self-cleaving ribozyme, and T7 RNA polymerase terminator sequences, which allow to cut the RNA segment during transcription just at the 3'-end of each genomic segment, without adding any extra nucleotide (Figure 3).

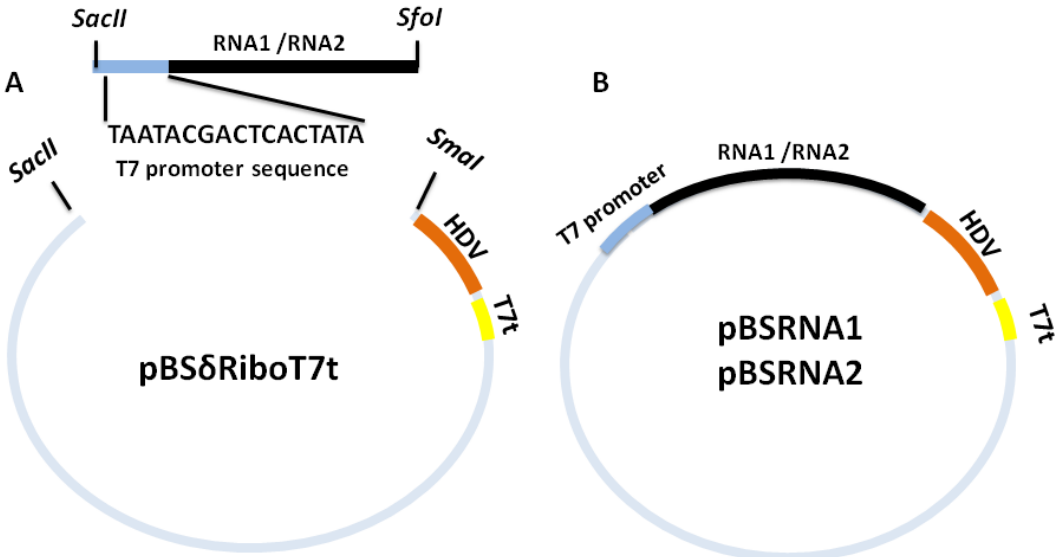


Figure 3. (A) Structure of the pBSδRiboT7t vector and RNA1/RNA2 cDNA. (B) Structure of the pBSRNA1/pBSRNA2 constructions. The T7 promoter sequence is represented in blue, HDV ribozyme is in orange, and the T7 terminator sequence is in yellow.

For pBSδRiboT7t linearization, digestions with *SmaI* (30 ^{o}C for 90 min) and *SacII* (37 ^{o}C for 90 min) enzymes were consecutively performed. To prevent vector recirculation, phosphate groups were removed with the FastAP Thermosensitive Alkaline Phosphatase (Fermentas) reaction, following this protocol: linear plasmids (2 µg) were mixed with 1x AP Reaction Buffer and FastAP Thermosensitive Alkaline Phosphatase (1 U), in a final volume of 50 µl. The reaction was incubated at 37 ^{o}C for 45 min, at 80 ^{o}C for 20 min and stored at 4 ^{o}C or -20 ^{o}C until used.

As a first step for cloning into the pBSδRiboT7t plasmid, RNA1 and RNA2 sequences were set free from pGemT_RNA1 and pJET_RNA2 vectors, respectively, by simultaneous digestion with *SacII* and *SfoI* at 37 ^{o}C for 2-4 h. Free inserts were purified from 1% agarose gels using the QIAquick® Gel Extraction Kit (Qiagen) according to manufacturer instructions. The resulting sequences were ligated to the linearized pBSδRiboT7t vector in a 20-µl mixture composed of 1x Ligation Buffer, T4 DNA ligase (1 U) and DNA template (3:1, insert:vector). Incubation was at 15 ^{o}C overnight.

Afterwards, *E. coli* DH5α cells were transformed, and bacterial clones were checked for the RNA1 or RNA2 insert presence, using the NNVs1_B3F/NNVs1_B3R, or NNVs2_RG2F/NNVs2_RG2R pairs of primers (Table 1), respectively. Amplified products were visualized on 2% agarose gels stained with ethidium bromide and sequenced. Plasmid (labelled as pBSRNA1 and pBSRNA2) were purified with the

Genopure Plasmid Maxi Kit (Roche) according to manufacturer guidelines, and stored at -20 ^{o}C until used.

2.3.4. Transfection of BSRT7/5 cells

Cell transfection was carried out using the Lipofectamine® 2000 Transfection Reagent (Invitrogen) in two separate reactions. In reaction A, 125 µl of serum-free OptiMEM medium (Gibco) and 6 µl of Lipofectamine® 2000 Transfection Reagent were mixed. In reaction B, each construction (pBSRNA1 and pBSRNA2, 1 µg) was added to 125 µl of serum-free OptiMEM medium. Both reactions were separately incubated at room temperature for 5 min to be finally mixed. The resulting mixture was incubated at room temperature for 30 min and finally added in droplets over confluent monolayers of BSRT7/5 cells grown on 6-well plates (Nunc). Then, the cells were incubated at 37 ^{o}C overnight in a 5% CO_2 atmosphere. After this incubation, the lipofectamine mixture was removed and cells were maintained at 28 ^{o}C in DMEM supplemented with 2% FBS, 200 mM glutamine, 100 units/ml penicillin, 10 mg/ml streptomycin and 1% HEPES (Gibco). After 7 days, cells and supernatants were harvested together, subjected to 3 cycles of freeze/thawing, and centrifuged at 1,500 x *g* at 4 ^{o}C for 5 min. Virus-containing supernatants were stored at -80 ^{o}C until used.

2.3.5. Recovery of infective viral particles

Viral suspensions obtained after BSRT7/5 transfection were diluted in L-15 medium (1/10, 1/100 and 1/1000) to be inoculated on E-11 cells grown on 24-well plates (Nunc). These inoculated cells were cultured in L-15 maintenance medium at 25 ^{o}C for 7 days. Several blind passages on new E-11 cultures were performed once a week until the development of CPEs. Viral particles obtained (*r*Dl965) were propagated and titrated on E-11 cells, as it has been described in section 2.1. Viruses were stored at -80 ^{o}C until used.

2.4. GENERATION OF RECOMBINANT MUTATED VIRUSES

The construction pBSRNA2 was used as template to mutate amino acids 247 and 270 within the viral CP sequence. Specifically, Mut247Dl965 primer (5'-GTCCATCCTCCTAGGAGCCACACCACTGGAC-3') introduces a mutation affecting nucleotide 759 (thymine to guanine), which switches serine in position 247 to alanine. The change generated by Mut270Dl965 primer (5'-

TCCGCTGTCTATTGACTACAACCTTGGAACTGGAG-3') occurs in the nucleotide 830 of the RNA2 sequence (guanine to adenine), changing the amino acid in position 270 (serine to asparagine). These modifications were carried out using the QuikChange Multi Site-Directed Mutagenesis Kit (section 2.3.2.). Mutations were confirmed by PCR, using T7_5'RNA2_965/3'RNA2_965 primers (Table 3), and the subsequent sequencing of the amplified products.

Three different *E. coli* DH5α clones, named as Mut247pBSRNA2, Mut270pBSRNA2 and Mut247+270pBSRNA2, were obtained following this procedure. Plasmid extraction was performed using the Genopure Plasmid Maxi Kit, according to manufacturer guidelines, and kept at -20 ^{o}C until transfection.

The plasmid pBSRNA1, in combination with Mut247pBSRNA2, Mut270pBSRNA2 or Mut247+270pBSRNA2, were used for BSRT7/5 cell transfection following the procedure described in sections 2.3.4. and 2.3.5. The mutations in viral particles recovered were demonstrated by sequencing. Viral RNA was treated with DNase I Recombinant from bovine pancreas (Roche) to remove plasmids remaining after transfection. Total RNA (350 ng) was mixed with 1x Incubation Buffer and DNase I Recombinant (10 U) in a 25-µl reaction. This mixture was incubated at 37 ^{o}C for 30 min, followed by incubation at 75 ^{o}C for 10 min to stop the reaction. Treated RNA was reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit and amplified, using NNVs2_RG2F/NNVs2_RG2R primers (Table 1). Amplified products were subsequently sequenced.

Mutated viral particles were propagated and titrated on E-11 cells, as it has been described in section 2.1. Viruses were stored at -80 ^{o}C until used.

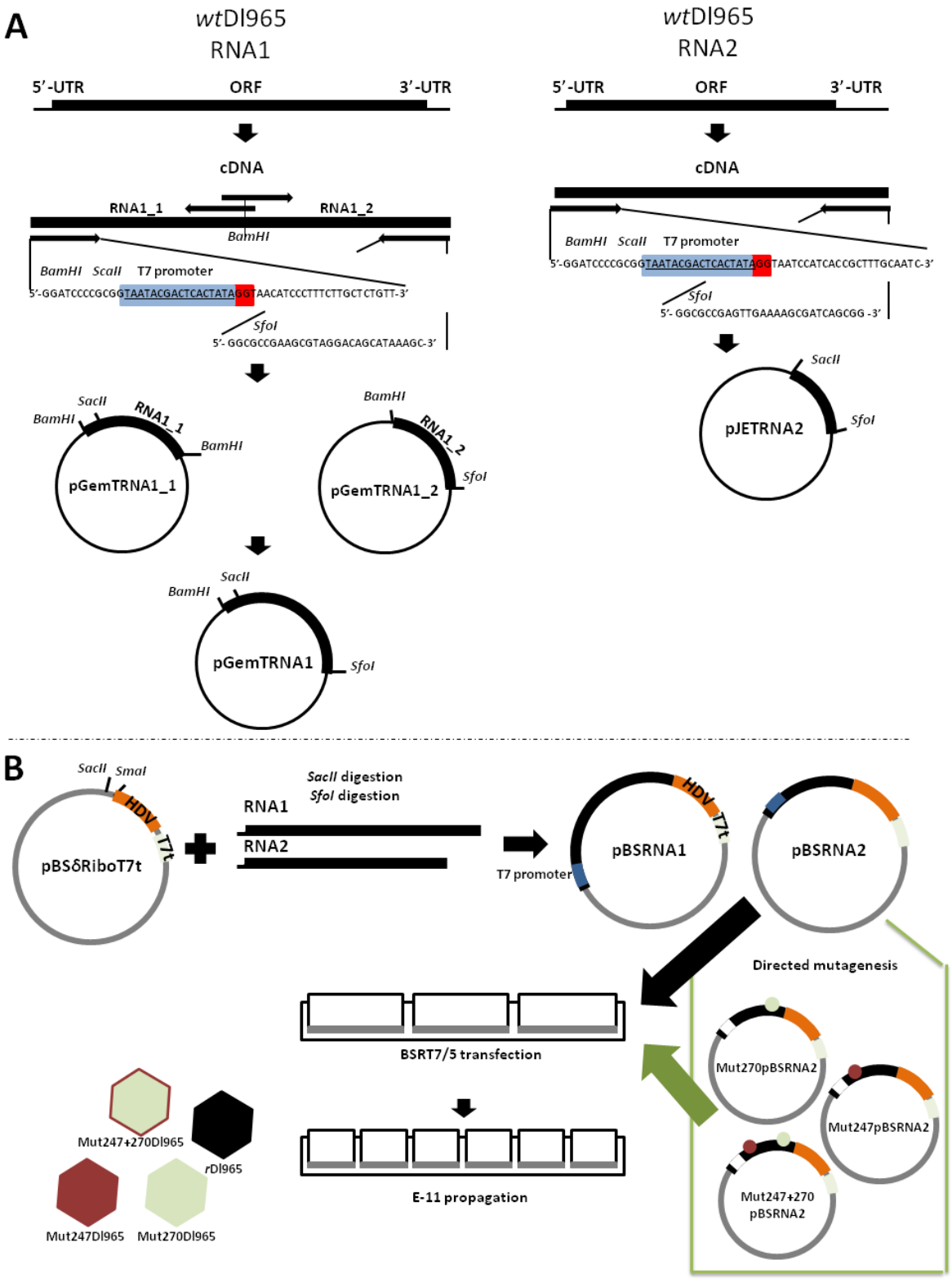


Figure 4. Procedure followed to obtain infective viral particles using the reverse genetics methodology. (A) Viral segments were amplified adding restriction sites at 5'- and 3'-ends, as well as the T7 RNA polymerase promoter (blue) and two guanine residues (red) at the 5'-end. (B) These constructions were cloned in the pGemT or pJET vectors and sub-cloned in the pBSδRiboT7t expression vector, which contains ribozyme HDV (orange) and T7 terminator sequence (white). pBSRNA1 in combination with pBSRNA2 (or modifications of this vector generated by directed mutagenesis) were used for BSRT7/5 cell transfection. Viral particles generated were propagated on E-11 cells.

2.5. *In vitro* VIRAL REPLICATION

The replication on E-11 cells of *wt*Dl965, *r*Dl965 and the mutant viruses Mut247Dl965, Mut270Dl965 and Mut247+270Dl965 was comparatively analysed. Each virus was inoculated at 0.1 multiplicity of infection (MOI) on E-11 cells grown on 24-well plates. Inoculated cells were maintained in L-15 medium supplemented with 100 units/ml penicillin and 10 mg/ml streptomycin (inoculation medium) at 25 ^{o}C for 1 h for virus adsorption. After this incubation, the viral suspension was removed, and L-15 maintenance medium was added. Supernatants from three wells were collected at 1, 2, 3, 5 and 7 days post-inoculation (d p.i.) to be titrated on E-11 cells by the $TCID_{50}$ method. All titrations were conducted in triplicate.

2.6. *In vivo* VIRAL VIRULENCE ANALYSIS

The virulence of *r*Dl965 and mutant viruses was comparatively evaluated by a series of challenges carried out with juvenile European sea bass specimens maintained in 100-l seawater tanks at 23-25 ^{o}C. Animals were always handled according to the European Union guidelines for the handling of laboratory animals (Directive 2010/63/UE). The lowest stress-generating conditions of light (8-h light and 16-h darkness photoperiod), oxygen (6 ± 0.5 ppm) and feeding were applied throughout all the experimental challenges. On arrival, animals were acclimated for 15 days and, during this period, checked for the presence of betanodavirus in brain according to Lopez-Jimena et al. (2010). Sampled and surviving animals were killed by MS-222 (Sigma) anaesthetic overdose.

2.6.1. *wt*Dl965 *versus r*Dl965

2.6.1.1. Experimental challenge and sample processing

Juvenile sea bass specimens (6 g, average weight) were distributed into 6 tanks in order to establish 3 experimental groups in duplicate (tanks A and B) (n = 30 each): (i) *wt*Dl965-infected group, (ii) *r*Dl965-infected group, and (iii) L-15-treated group, as negative control. Infections were performed by 1-h immersion in seawater containing a final viral concentration of 10^5 $TCID_{50}$/ml. Afterwards, animals were maintained during 30 days under the above described conditions.

Fish from tanks A were daily monitored to record clinical signs and mortality. Dead animals were collected and stored at -80 ^{o}C until processing. Fish from tanks B were sampled at 12 h post-inoculation (h p.i.), as well as at 1, 3, 5 and 7 d p.i. (3 specimens per time point). Their brains were aseptically collected, immediately frozen in liquid nitrogen, and maintained at -80 ^{o}C until the virological analysis. Sampled fish from tanks B, as well as survivors from tanks A, were euthanized as previously described.

Brains were individually homogenized (10%, w/v) in TRI reagent solution (Sigma) by wet grinding, using the Mixer Mill MM400 (Retsch). Total RNA was extracted as described in section 2.2.2.2. and stored at -80 ^{o}C. After treatment with DNase I Recombinant from bovine pancreas (section 2.4.), cDNA was synthesized using 1 µg of RNA and the Transcriptor First Strand cDNA Synthesis Kit (section 2.2.1.1.). cDNA was quantified with the NanoDrop-1000 system, and maintained at -20 ^{o}C until viral replication analyses.

2.6.1.2. Absolute quantification of the RNA2 viral segment

Viral RNA in brain was quantified using an absolute real-time PCR protocol, which amplifies a 126-bp fragment within the RNA2 segment. The specific primers RG_965_RNA2 F4 (5'-ACCGTCCGCTGTCTATTGACTA-3') and RG_965_RNA2 R1 (5'-CAGATGCCCCAGCGAAACC-3') were designed and used in this study, and serial dilutions of the pJET vector containing the complete *wt*Dl965 RNA2 sequence were used to generate reference standard curves. All the amplifications were conducted with the LightCycler 96 Thermocycler (Roche), using the Fast Start Essential DNA Green Master Mix (Roche), and cDNA generated from 50 ng of RNA. PCR mixtures were composed of 1x Fast Start Essential DNA Green Master and 10 pmol specific primers, in 20 µl (final volume). Amplification conditions were 95 ^{o}C for 10 min, followed by 45 cycles at 95 ^{o}C for 10 s, 60 ^{o}C for 10 s and 72 ^{o}C for 10 s. To obtain melting curves, the following profile was conducted: 95 ^{o}C for 10 s, 65 ^{o}C for 60 s and 97 ^{o}C for 1 s.

2.6.2. *r*Dl965 *versus* mutant viruses

2.6.2.1. Experimental challenge and sample processing

In order to analyse possible virulence changes resulting from amino acid modifications, fish (8 g, average weight) were infected with *r*Dl965-, Mut247Dl965-, Mut270Dl965-, or Mut247+270Dl965. In addition, a control group, injected with L-15, was set up. Each experimental group consisted of 3 tanks (n = 25 each). Infection was performed by intramuscular injection of previously anesthetized sea bass ($2x10^6$ $TCID_{50}$/fish). Animals were maintained at 23-25 ^oC during 30 days.

Fish from two of the triplicate tanks were daily monitored to record mortality and signs of disease, and dead animals were collected and stored at -80 ^oC. Specimens from the remaining tanks were sampled at 12 h p.i., as well as at 1, 3 and 5 d p.i. Five animals were killed per sampling time and group, and their brains were collected and frozen in liquid nitrogen for the subsequent virological analysis. Brains were processed as described in section 2.6.1.1.

2.6.2.2. Viral genome quantification

The viral RNA2 segment was quantified in sampled, dead and surviving fish following the procedure described in section 2.6.1.2. Brains from sampled fish were individually processed, whereas samples from dead and surviving specimens were processed as pools of 2-3 brains.

2.7. CAPSID PROTEIN STRUCTURE

Possible modifications in the predictive secondary structure of the *wt*Dl965 CP caused by the amino acid changes generated in this study (serine to alanine in position 247, and serine to asparagine in position 270) have been analysed using the Geneious 4.8.4 software (Biomatters Ltd., New Zealand), using Garnier Osguthorpe Robson algorithm (GOR method). In addition, a protein modelling based on structure homology was performed using the Swiss Model server (ExPASy web server). The crystal structure of the orange-spotted grouper (*Epinephelus coioides*) nervous necrosis virus (OSGNNV, RGNNV species) CP (Chen et al., 2015) was used as template for the search model. The resulting protein structures were aligned and compared using the PDB structure alignment v. 4.0 implemented in Geneious (Geneious v. 9.0.5,

Biomatters, http://www.geneious.com/). The UCSF Chimera 1.11.2 software (RBVI, University of California) was used to visualize the 3D structures.

2.8. STATISTICAL ANALYSES

Statistical analyses were performed with the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA). Values of $p < 0.05$ were considered significant. Mortality rates were analysed by the survival curves, using the Kaplan-Meyer test. To determine significant differences in survival distributions between experimental groups, a log-rank Mantel Cox test was carried out. Viral replication results in section 2.6.1.2. were analysed by the t-student test, whereas viral replication from section 2.6.2.2. were analysed by the one-way ANOVA test, using Bonferroni´s multiple comparison test as post test. Normality distribution was verified by Shapiro-Wilk test.

3. RESULTS

3.1. SEQUENCE OF *wt*Dl965 GENOMIC SEGMENTS

*wt*Dl965 RNA1 is a 3,104-bp segment, comprising a 78-bp 5'-untranslated region (UTR), a 2,949-bp coding region and a 77-bp 3'-UTR sequence. The 1,432-bp RNA2 segment displays a 26-bp 5'-UTR, and a 389-bp 3'-UTR region. The RNA2 ORF is 1,017-bp long. The alignment of these sequences with other RGNNV sequences available in GenBank (isolated from *Ephinephelus septemfasciatus*, *E. tauvina* and *Lateolabrax japonicas*, accession numbers RNA1/RNA2: KM095958/KM095959, AF326776/AF318942 and KP455643/KP455642, respectively) corroborated that both segments of the *wt*Dl965 isolate belong to the RGNNV species.

The comparative analysis of *wt*Dl965 RNA1 showed that the 5'- and 3'-UTR sequences are similar to those displayed by other RGNNV isolates. The only exceptions are nucleotides at positions 8 and 10 in the 5'-UTR region, and the presence of an additional nucleotide (as it happens in the SGWak97 sequence) at position 3,055 within the 3'-UTR region (Figure 5). The analysis of the deduced RNA polymerase sequence showed that it is fairly conserved, with the RNA replication GDD motif at positions 686-688 (Figure 5).

The 5'-UTR sequence of *wt*Dl965 RNA2 segment is also highly conserved, presenting a single change affecting nucleotide 24, which is thymidine instead of adenine (Figure 5). The highest variability was recorded in the 3'-UTR sequence, with changes affecting nucleotides located at positions 1,134; 1,159; 1,174; 1,195; 1,261; 1,270; 1,299; 1,302; 1,330 and 1,422 (Figure 5). The deduced *wt*Dl965 CP sequence differs from other RGNNV sequences at positions 35, 234, 289 and 290 (Figure 5).

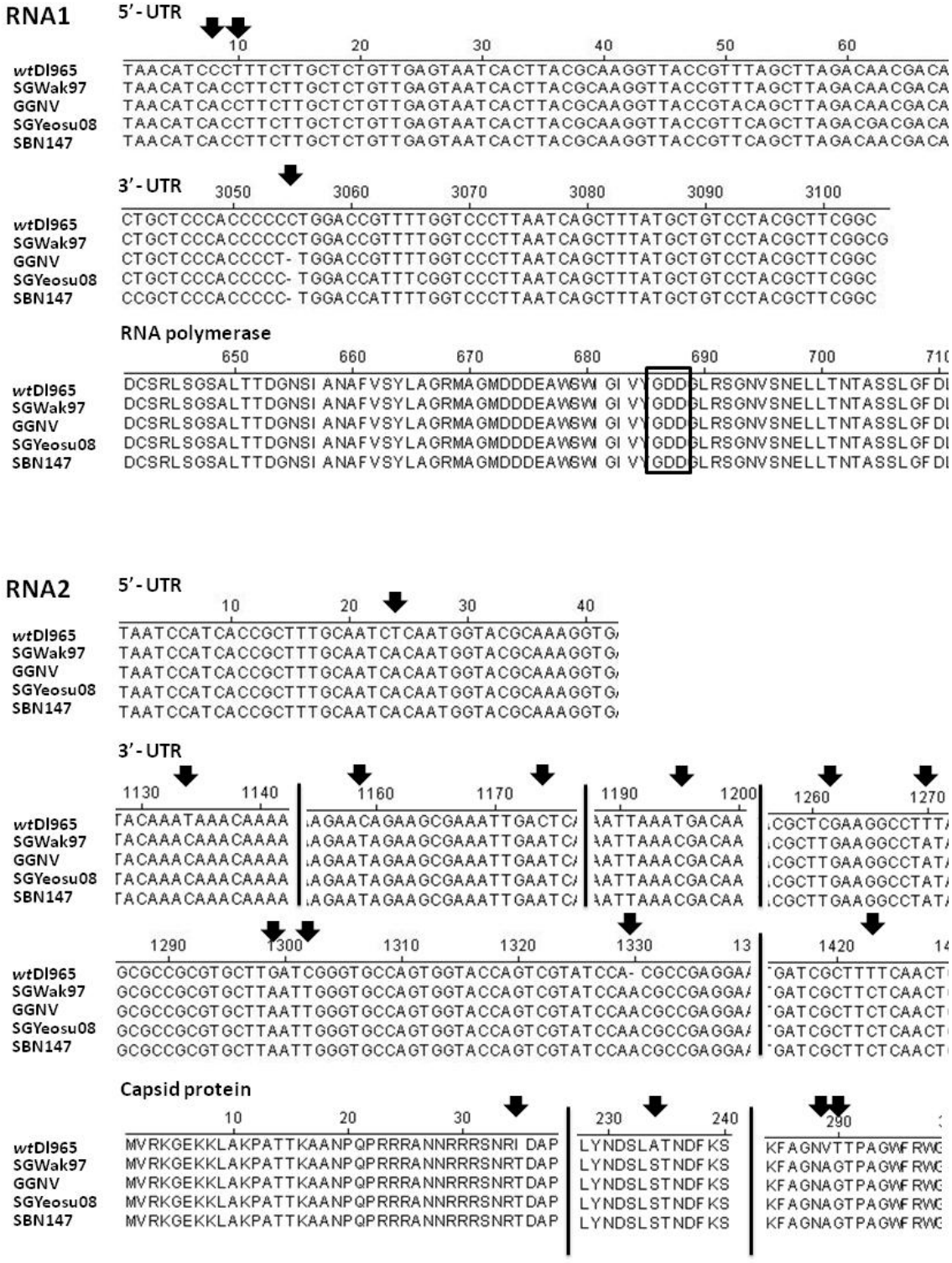


Figure 5. Alignment of *wt*Dl965 RNA1 and RNA2 segments with sequences from other RGNNV isolates: SGWak97 (AY324869/AY324870), GGNV (AF319555/AF318942), SGYeosu08 (KM095958/KM095959) and SBN147 (KP455643/KP455642) (RNA1/RNA2 accession no.). Variations in the *wt*Dl965 sequence are indicated with arrows. The box contains the RNA replication GDD motif. Alignments were conducted with the MegAlign software (DNAstar Lasergene 7).

3.2. *r*Dl965 RECOVERY BY REVERSE GENETICS

The reverse genetics methodology has allowed the recovery of infective *r*Dl965 virus particles from viral cDNA, showing identical sequence to *wt*Dl965, which has been corroborated by sequencing. The infective viral particles obtained after BSRT7/5 transfection were propagated on the E-11 cell line, observing CPEs after the first cell inoculation (P0), with either 1/10 or 1/100 viral dilutions. The titre of the viral stock used for the subsequent virulence analyses (P2 on E-11 cells) was $7.1x10^7$ $TCID_{50}$/ml.

3.3. POINT MUTATIONS IN THE CODING REGION OF THE RNA2 GENOMIC SEGMENT

As a result of the mutations introduced in the RNA2 sequence, amino acids at positions 247 (serine) and 270 (serine) within the *wt*Dl965 CP gene (RGNNV isolate) were replaced by those amino acids at the same positions in SJNNV isolates (alanine and asparagine, respectively). Therefore, the capsid protein of Mut247Dl965, Mut270Dl965 and Mut247+270Dl965 partially resembles the capsid protein of SJNNV isolates, which do not cause disease outbreaks in European sea bass. Mutations were confirmed by PCR, obtaining a single band with the expected size, followed by sequencing (Figure 6).

Infective viral particles obtained after BSRT7/5 transfection were subsequently propagated on the E-11 cell line. All mutant viruses caused CPEs on this cell line, although one blind passage (P1) was required. The titres of these viral suspensions, propagated on E-11 cells (P2), were $1.3x10^8$, $7.1x10^7$ and $2.3x10^7$ $TCID_{50}$/ml for Mut247Dl965, Mut270Dl965 and Mut247+270Dl965, respectively. These viral stocks were used for the subsequent *in vivo* virulence analyses.

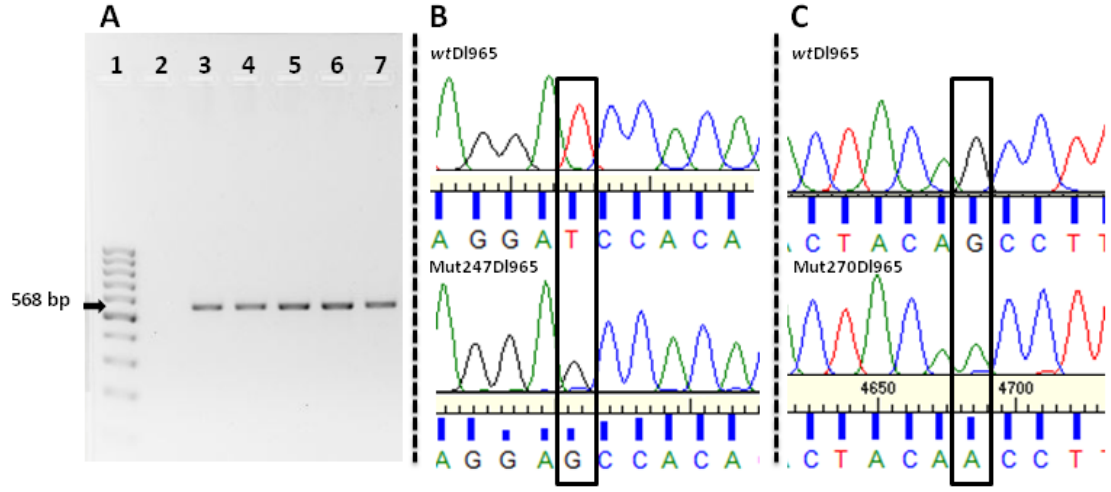


Figure 6. Confirmation of recombinant viruses after successive passages on E-11 cells. (A**)** Amplification of the RNA2 segment using s2NNVRG2F/s2NNVRG2R primers (Table 1). Lane 1. DNA ladder: Generuler 100 bp; lane 2, negative control; lane 3, *r*Dl965; lane 4, Mut247Dl965; lane 5, Mut270Dl965; lane 6, Mut247+270Dl96; lane 7, positive control. (B**)** Nucleotide modification at position 247. (C) Nucleotide modification at position 270.

3.4. REPLICATION KINETICS OF RECOMBINANT VIRUSES

Replication of the different recombinant viruses was analysed on E-11 cells in comparison to *wt*Dl965 replication. According to the results shown in Figure 7, all viruses replicate on E-11 cells, with a significant increase of the viral titres from 24 h p.i. onwards. Maximal mean titres for *wt*Dl965, *r*Dl965 and Mut247Dl965 were recorded at 5 d p.i. ($1.9x10^5$, $5.5x10^5$ and $3.8x10^5$ $TCID_{50}$/ml, respectively), whereas Mut270Dl965 and Mut247+270Dl965 maximal titres were at 7 d p.i., with mean values of $7.1x10^5$ and $1x10^5$ $TCID_{50}$/ml, respectively.

Replication curve analysis indicated that *wt*Dl965 and *r*Dl965 viruses replicate similarly on E-11 cells at all sampling times, except at 2 d p.i. (Figure 7). At this sampling time, *r*Dl965 showed a significant decrease in the viral titre compared to *wt*Dl965 (p = 0.0001), obtaining mean viral titres of $1.8x10^3$ and $1.3x10^4$ $TCID_{50}$/ml, respectively. Regarding recombinant viruses, Mut247+270Dl965 was the only mutated virus with significant differences, compared to *wt*Dl965. Specifically, titres recorded for this virus at 2 and 5 d p.i. were $1.3x10^3$ and $3.9x10^4$ $TCID_{50}$/ml, respectively, which were significantly lower (p < 0.0001) than those obtained for *wt*Dl965 at the same sampling times ($1.3x10^4$ and $1.9x10^5$ $TCID_{50}$/ml, respectively). However, Mut247Dl965 and Mut270Dl965 replication differs from *wt*Dl965 only at the last sampling time. Thus, at 7 d p.i. the mean titres observed for Mut247Dl965 and Mut270Dl965 were

$1.9x10^5$ and $7.1x10^5$ $TCID_{50}$/ml, respectively, which were significantly higher (p = 0.015 and 0.0013, respectively) that those recorded for *wt*Dl965, $3.9x10^4$ $TCID_{50}$/ml.

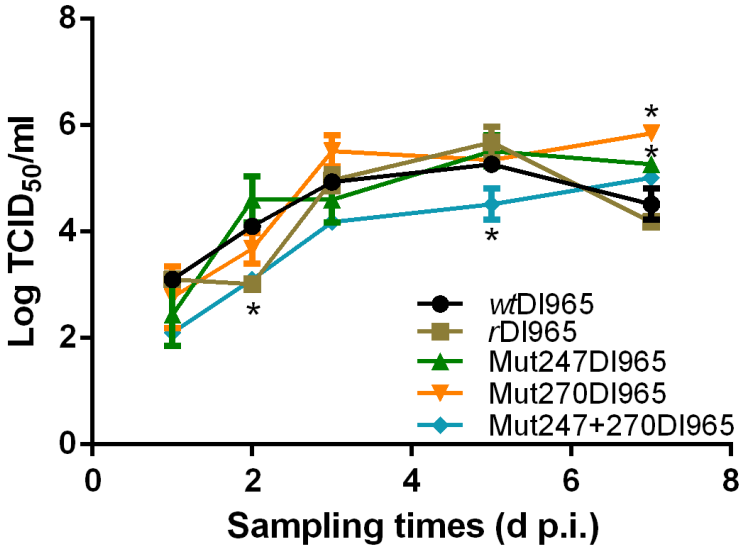


Figure 7. Viral replication on the E-11 cell line inoculated at 0.1 MOI. The one-way ANOVA with Bonferroni´s multiple comparison test were the statistical analyses carried out. Values of $p < 0.05$ were considered significant. * represents significant differences compared to *wt*Dl965 within each sampling time. Results are mean ± standard deviation (SD) (n = 3).

3.5. *In vivo* VIRULENCE STUDY

3.5.1. Comparative analysis of *wt*Dl965 and *r*Dl965 virulence

Mortality and viral replication in brain of experimentally inoculated juvenile European sea bass (bath challenge) have been evaluated. Typical signs of disease and mortality were recorded in both, *wt*Dl965 and *r*Dl965 groups. Specifically, first disease signs, consisting of dark skin and lack of appetite, were recorded at 3 d p.i., whereas typical neurological signs, such as abnormal swimming, appeared later on, at 5 d p.i.

The cumulative mortality curves recorded for *wt*Dl965- and *r*Dl965-inoculated groups were very similar (Figure 8A). In *wt*Dl965-inoculated group, mortality onset was at 5 d p.i., with an acute episode of mortality at 6 d p.i., increasing the cumulative mortality from 6% to 50% in just one day. After that significant event, mortality stabilized, reaching a final value of 63.3% (Figure 8A). In *r*Dl965-inoculated group, first mortalities were at 6 d p.i., with the maximal daily mortality at day 8 p.i. After this episode, mortality stabilized and animals recovered. Mortality rate at the end of the

challenge was 73.3%. No mortalities were recorded in the negative control group (L-15-exposed fish) at any time (Figure 8A).

The statistical comparison showed no significant differences (p = 0.369) between cumulative survival rates for *wt*Dl965- and *r*Dl965-infected groups (Figure 8B).

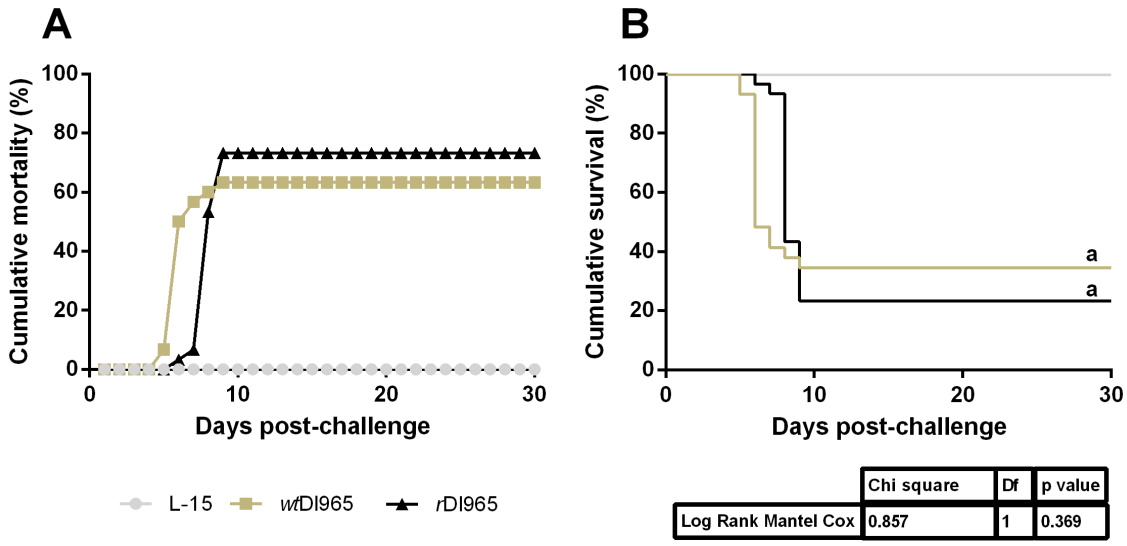


Figure 8. Comparative analysis of *wt*Dl965 and *r*Dl965 virulence. (A) Cumulative mortality. (B) Kaplan-Meyer survival curves. Log Rank Mantel Cox Test results are represented below the Kaplan-Meyer survival curve. Values of $p < 0.05$ were considered significant. Same letters indicate no significant differences.

To complete this study, the number of RGNNV RNA2 copies was quantified, by RT absolute real-time PCR, in sea bass brains sampled at different times p.i. Results depicted in Figure 9 showed that the viral RNA was detected in sea bass infected with both viruses at all sampling times analysed, whereas no viral genome was recorded in the negative control group. In addition, significant differences between the number of RNA2 copies in *wt*Dl965- and *r*Dl965-inoculated fish were not recorded at any time ($p > 0.05$), indicating that the pathogenesis of both viruses, in terms of replication and migration to brain, is similar.

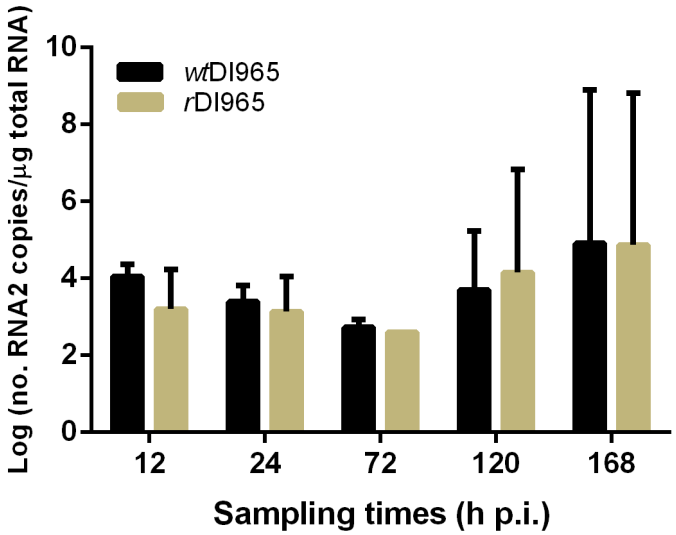


Figure 9. Viral genome quantification in European sea bass brain. Viral genome was quantified by RT absolute real-time PCR. The t-student test was used to compare the number of RNA2 copies in fish from different experimental groups within each sampling time. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 3).

3.5.2. Involvement of CP amino acids 247 and 270 in betanodavirus virulence

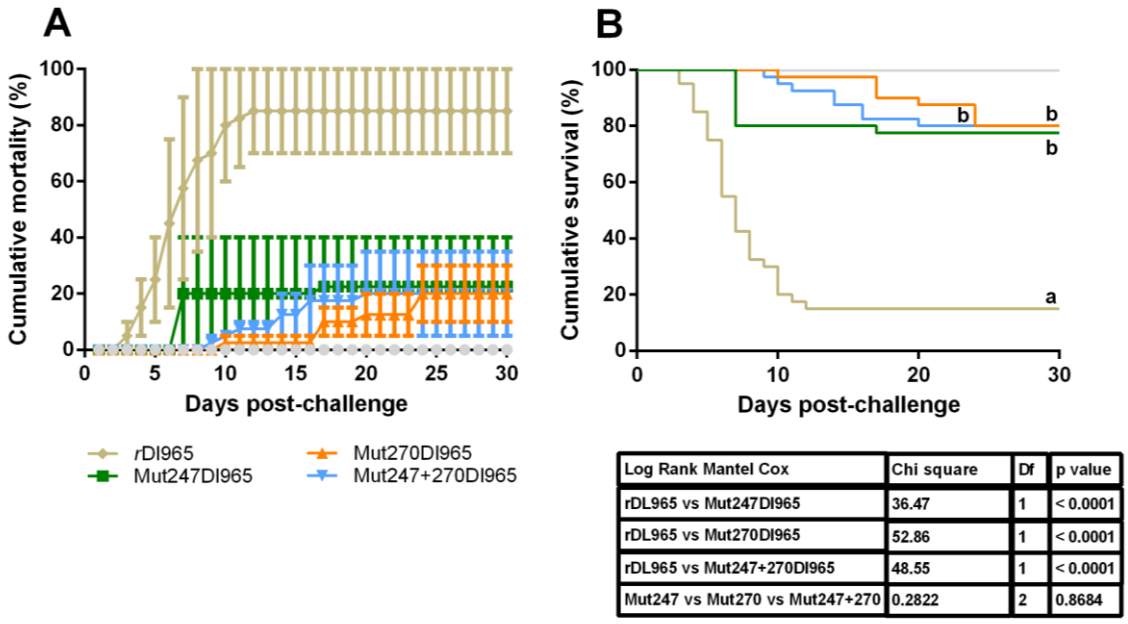
The aim of this challenge has been to evaluate the effect on betanodavirus virulence to European sea bass of mutations in the amino acids 247 and/or 270, which were changed to those amino acids at the same positions in SJNNV isolates. In this challenge, *r*Dl965 has been used as positive control, since it shows the same virulence as *wt*Dl965 (as it has been evidenced in section 3.5.1.), and it has been generated by reverse genetics, which is the methodology used to obtain the mutated viruses.

The course of disease in *r*Dl965-infected group was similar to that described in section 3.5.1. In this group, typical signs of disease (dark pigmentation, lack of appetite and abnormal swimming) were observed from 3 to 12 d p.i., with a peak of daily mortality at day 7 p.i. After this period, mortality stabilized and animals recovered. Cumulative mortality at the end of the experiment (30 d p.i.) was 85% (Figure 10A).

The course of the disease in those groups inoculated with the mutated viruses was different (Figure 10). In Mut247Dl965-inoculated group, a peak of daily mortality was also recorded at day 7 p.i. From that time on, the mortality stabilized. The cumulative mortality at the end of the experiment was 22.5% (Figure 10A). In Mut270Dl965-infected group, clinical signs appeared later and were less severe. The mortality onset was at 10 d p.i., and the mortality at the end of the experiment was 20%.

In Mut247+270Dl965-inoculated group, disease signs appearance and mortality onset were also delayed (compared to the *r*Dl965-infected group). The mortality onset was at 9 d p.i., and cumulative mortality at the end of the challenge was 20% (Figure 10A).

The log-rank Mantel Cox analysis showed significant differences ($p < 0.0001$) between the survival distribution estimated for Mut247Dl965-, Mut270Dl965- and Mut247+270Dl965-inoculated groups in comparison to *r*Dl965-infected group (Figure 10B). However, the survival distribution after inoculation with the three mutated viruses assayed was the same (Figure 10B).



Log Rank Mantel Cox	Chi square	Df	p value
rDL965 vs Mut247Dl965	36.47	1	< 0.0001
rDL965 vs Mut270Dl965	52.86	1	< 0.0001
rDL965 vs Mut247+270Dl965	48.55	1	< 0.0001
Mut247 vs Mut270 vs Mut247+270	0.2822	2	0.8684

Figure 10. European sea bass mortality (A) and Kaplan-Meyer survival curves (B) after viral inoculation. The log Rank Mantel Cox Test results are in a box below the Kaplan-Meyer curves. Different letters indicate significant differences between experimental groups. Values of $p < 0.05$ were considered significant.

Brains from dead or surviving fish were pooled and processed for RNA2 quantification. Viruses were detected in brain of both, dead and surviving fish, obtained from all the experimental groups. In dead animals, the number of RNA2 copies in Mut247Dl965- and Mut270Dl965-inoculated groups was significantly lower ($3.2x10^8$ and $1.4x10^8$ RNA2 copies/µg RNA, respectively) compared to the *r*Dl965-infected group, showing $4.1x10^9$ RNA2 copies/µg RNA (Figure 11).

Regarding surviving animals, the mean number of RNA2 copies was significantly lower ($p < 0.0001$) in the Mut270Dl965-inoculated group ($1x10^7$ RNA2 copies/µg RNA) than in the *r*Dl965-infected group ($3.6x10^7$). On the contrary, brains

from Mut247+270Dl965-inoculated surviving sea bass presented a mean number of RNA2 copies ($1.5x10^8$ RNA2 copies/µg RNA) significantly higher than samples from the *r*Dl965-infected group ($p < 0.0001$) (Figure 11).

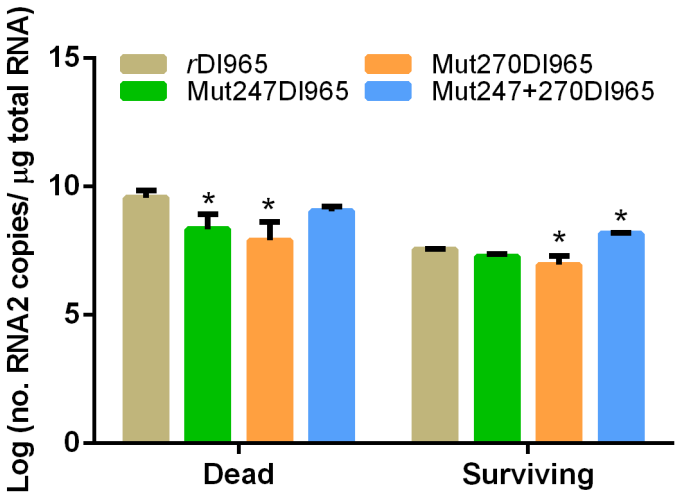


Figure 11. Viral RNA2 quantification in brain from dead and surviving fish. The analysis was performed by RT absolute real-time PCR. The one-way ANOVA was the statistical analysis conducted. * indicates significant differences, compared to the *r*Dl965 group, within each group of animals (dead or surviving). Values of $p < 0.05$ were considered significant. Results are mean ± SD of two pools of three brains.

In order to complete the virulence analysis, replication of all these recombinant viruses has been analysed in brain from fish sampled at different time points. Viral genome was not recorded in fish from the negative control group, whereas all recombinant viruses were detected at all sampling times considered (Figure 12).

At early stage of infection, viral genome load was statistically similar in samples from all the experimental groups, except for the Mut247Dl965-inoculated group at 12 h p.i. However, at 72 and 120 h p.i. fish inoculated with mutant viruses showed a number of RNA2 copies significantly lower than fish from the *r*Dl965-infected group ($p < 0.0001$). In addition, the number of *r*Dl965, Mut247Dl965 and Mut270Dl965 RNA2 copies increased throughout the time, with maximal mean values at 120 h p.i. ($4.4x10^9$, $4.8x10^8$ and $2.3x10^8$ RNA2 copies/µg RNA, respectively). In contrast, no significant increase of the Mut247+270Dl965 RNA2 copy number was recorded up to 5 d p.i. (Figure 12).

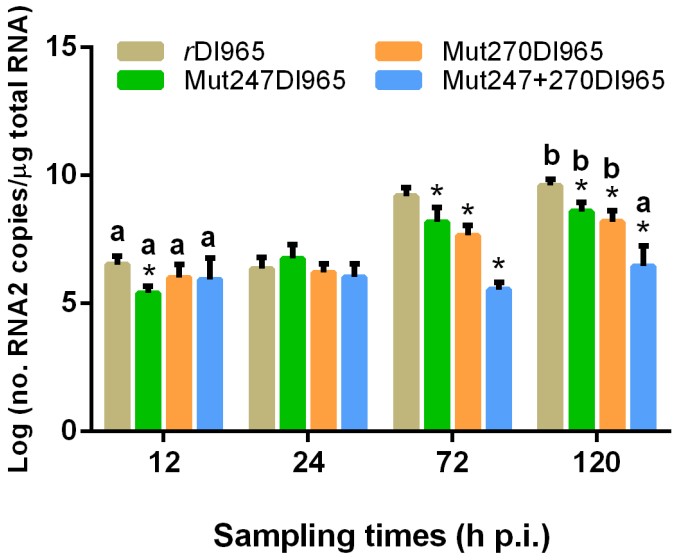


Figure 12. RNA2 quantification in brain from sampled sea bass. The one-way ANOVA and t-student tests were the statistical analyses performed. * indicates significant differences between each mutated virus and *r*Dl965 within each sampling time. Different letters indicate significant differences between 12 h and 5 d p.i. within each experimental group. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 5).

3.6. CAPSID PROTEIN STRUCTURE

In order to analyse possible modifications in the predicted secondary structure of the *wt*Dl965 CP as a consequence of the mutations considered, a protein structure study has been carried out. The results obtained (Figure 13) showed that the mutation of the amino acid 247 (serine to alanine) did not generate a substantial change in the secondary structure of the capsid protein. On the contrary, amino acid change from serine to asparagine at position 270 modified the structure, introducing a turn and displacing a beta strand (Figure 13). Moreover, the presence of both mutations simultaneously does not generate additional changes in the structure, only the modification introduced by the mutation at position 270.

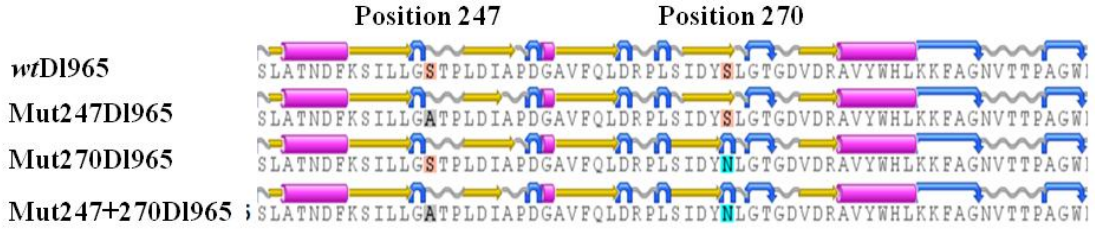


Figure 13. Capsid protein secondary structure of *wt*Dl965 and mutant viruses. The analysis was conducted with the Geneious 4.8.4. software, using Garnier Osguthorpe Robson algorithm (GOR method). Structures are indicated in colour: beta strand in yellow, alpha helix in pink, turn in blue and coil in grey.

In addition, a protein modelling based on structure homology was performed using the Swiss Model server. The resulting protein model was visualized with the UCSF Chimera software. The results obtained showed that the mutations considered did no generate a significant modification of the capsid structure (Figure 14).

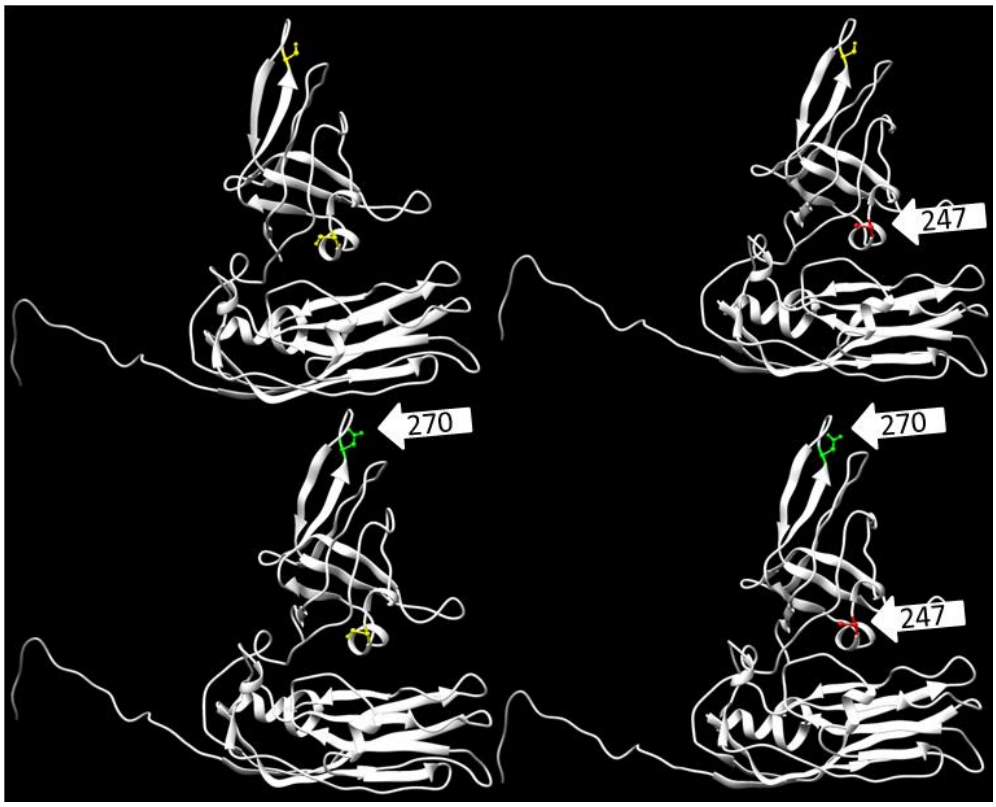


Figure 14. Protein modelling. Amino acid changes are in colour and indicated by arrows: *wild type* amino acids are in yellow (two serines), alanine in amino acid 247 is in red, and asparagine in amino acid 270 in green.

4. DISCUSSION

The aim of the present study has been to evaluate the role of the CP amino acids 247 and 270 as betanodavirus virulence determinants to European sea bass. First of all, the full-length RNA1 and RNA2 sequences of the *wt*Dl965 isolate (RGNNV species) have been obtained. Afterwards, recombinant viruses displaying mutations in the above mentioned positions were accomplished by the reverse genetics technique. These amino acids were modified to those present in SJNNV isolates, which are low virulent to European sea bass (Carballo et al., 2016; Souto et al., 2015b; Toffan et al., 2016; Vendramin et al., 2014). The virulence of these viruses has been evaluated by determining cumulative mortality and viral replication in brain, following a "loss of virulence" approach.

The alignment of the *wt*Dl965 sequences with other RGNNV sequences showed that the RNA1 segment is quite conserved within RGNNV isolates. On the contrary, the *wt*Dl965 RNA2 segment includes a 3'-UTR region with several nucleotide modifications. The importance of 5'- and 3'-ends in RNA2 replication efficiency has been previously reported for the related *Alphanodavirus* genus (Albariño et al., 2003; Eckerle et al., 2003). These variations, compared to other RGNNV isolates, could modify the stem loop structure (3'SL) formed in the RNA2 3'-end, which has been reported to be an essential cis-acting signal for Nodamura virus (NoV, *Alphanodavirus* genus) replication (Rosskopf et al., 2010). This element is structurally conserved among betanodaviruses, including SJNNV isolates and greasy grouper (*Epinephelus tauvina*) nervous necrosis virus (GGNNV), and its modifications may modulate viral replication efficiency. In fact, a recent study has reported that punctual mutations in this region cause a significant decrease in the virulence of a reassortant isolate to Senegalese sole (Souto et al., 2018).

The reverse genetics technique allows to manipulate viral RNA genomes by modifying the cDNA sequences. This technique has been successfully used to recover and study both, negative- and positive-stranded RNA fish viruses (Biacchesi, 2011; Kwak et al., 2017; Souto et al., 2015a; Takizawa et al., 2008). In the present study, this methodology has been applied to generate a genetically accurate RGNNV virus (*r*Dl965), which showed similar virulence to the *wild type* virus (*wt*Dl965) in bath-challenged juvenile European sea bass. Both viruses caused statistically similar

cumulative mortality ($p > 0.05$) (63.3% and 73.3% for *wt*Dl965 and *r*Dl965, respectively) (Figure 8), and replicated similarly both *in vitro* (on E-11 cells) and *in vivo* (in brain of experimentally infected European sea bass). Several authors have previously reported similar *in vitro* replication of recombinant and *wild type* viruses (Shuai et al., 2017; Souto et al., 2015a; Wu et al., 2016), indicating the absence of modifications affecting cellular entry of the virus.

Several recombinant viruses harbouring amino acids changed at positions 247 (serine to alanine) and/or 270 (serine to asparagine) have been obtained in this work (Mut247Dl965, Mut270Dl965 and Mut247+270Dl965). The effect of these modifications has been first analysed by evaluating viral replication on E-11 cells. The comparison between *wt*Dl965 and mutant virus replication showed lower titres for the virus with the double mutation at 2 and 5 d p.i., and higher titres for viruses harbouring the single 247 or 270 mutation at 7 d p.i. (Figure 7). Therefore, these modifications could affect viral entry, by altering host receptor recognition, which would affect viral propagation.

The ability of recombinant viruses to infect and develop disease in European sea bass has been evaluated, and the results obtained indicated that amino acids 247 and 270 are relevant as virulence determinants, supporting the results previously obtained by Souto et al. (2015a) in Senegalese sole. These authors analysed the role of both amino acids in the virulence to Senegalese sole of a reassortant isolate (RGNNV/SJNNV), which displays an SJNNV-type CP with several modifications compared to the SJNNV reference strain (SJ93Nag), including the amino acids 247 and 270. In this study, Souto et al. (2015a) reported that the change of both amino acids resulted in a 40% decrease in the mortality rate of infected sole.

The mortality rate of *r*Dl965-infected sea bass was 85% (Figure 10A), which agrees with previous studies, reporting mortality rates ranging between 36% and 75% after challenging with different RGNNV isolates (Carballo et al., 2016; Nuñez-Ortiz et al., 2016b; Pascoli et al., 2016; Souto et al., 2015b; Vendramin et al., 2014), depending on the infection route, temperature, salinity, viral isolate or viral dose. Mortality caused by mutated viruses was 60% lower (22.5%, 20% and 20%, for Mut247Dl965-, Mut270Dl965- and Mut247+270Dl965-inoculated fish, respectively) (Figure 10A). In addition, the course of the disease was altered, and clinical signs were weaker in

intensity and duration. Mortalities caused by mutant viruses were comparable to those reported in sea bass inoculated with SJNNV isolates, which oscillate from 10% (Vendramin et al., 2014) to no mortalities (Carballo et al., 2016; Souto et al., 2015b).

Noteworthy, an additive effect on betanodavirus virulence was not observed after inoculation with the double-mutated virus, since mortality caused by this virus (Mut247+270Dl965) is statistically comparable (p = 0.868) to mortality caused by Mut247Dl965 and Mut270Dl965 (Figure 10B). A similar result has been previously reported by Souto et al. (2015a) in Senegalese sole inoculated with single- and double-mutated viruses; however, the same double-mutant caused higher mortality than single-mutated viruses in turbot (*Scophthalmus maximus*) (Souto et al., 2016). These results suggest the importance of unknown host factors (probably different cellular receptor), and support the importance of these amino acids also as host determinants.

Another aspect analysed in this study has been the quantification of viral genome in brain from dead, surviving and sampled sea bass. Regarding dead animals, the RNA2 copy number in Mut247Dl965- and Mut270Dl965-inoculated sea bass was lower than in *r*Dl965-infected fish (Figure 11). Previous studies have described the RNA2 copy number in brain from dead fish at different phases of the disease, showing a decrease from the initial phase of mortality onwards (Souto et al., 2015b). Unfortunately, we could not conduct this kind of approach, since most of the mortalities occurred in one or two acute episodes; however, the lower viral load in Mut247Dl965- and Mut270Dl965-inoculated dead fish could be related to the low mortality caused by these viruses.

Viral genome was detected in all surviving fish analysed, although the number of RNA2 copies was lower than that reported in dead animals, as it has been previously reported by Souto et al. (2015b) after betanodavirus experimental infection in European sea bass and Senegalese sole. This result may suggest the establishment of an asymptomatic infection, and the role of sea bass as asymptomatic carrier. However, additional experiments would be necessary to test fish at long term and to check if animals would eventually eliminate the virus or would establish a persistent infection.

Comparative analyses of the RNA2 copy number in sampled animals revealed that amino acid modifications affect viral replication in sea bass brain (Figure 12). The number of RNA2 copies in Mut247Dl965-, Mut270Dl965- and Mut247+270Dl965-

infected animals was lower than in *r*Dl965-inoculated sea bass at 72 h and 5 d p.i., which is coherent with the lower mortality caused by these viruses. Furthermore, virus harbouring double mutation did not replicate throughout the sampling times considered (5 d p.i.); however, surviving animals from this experimental group displayed similar genome levels to those recorded in *r*Dl965-inoculated surviving sea bass. All together, these results suggest that, although Mut247+270Dl965 replicates at a lower level in sea bass brain at the early stage of infection, it may replicate at a higher level later on, reaching virus levels in brain similar to those reported in fish inoculated with the non-mutated virus. In addition, this *in vivo* kinetics of replication is similar to that previously observed *in vitro*, reporting a lagged replication on E-11 cells compared to the *wild type* virus replication.

These variations could be related with alterations in cell receptor binding. In fact, a single change (leucine at position 226) in the influenza virus hemagglutinin receptor binding domain has been reported to change the cell-receptor recognition (Zhang et al., 2013). Although the structural analysis carried out in this study did not reveal modifications in the CP tertiary structure after 247 and/or 270 mutations, the amino acids studied have been localized within the P-domain (Chen et al., 2015), and their alteration could modify the protuberance surface in structure and/or physicochemical properties, and therefore, could alter cell-receptor recognition.

In conclusion, this study has demonstrated the importance of amino acids 247 and 270, sited in the P-domain within the C-terminus region of the CP sequence, as virulence determinants in betanodavirus. Modifications of these amino acids to those present in a SJNNV-type CP caused a significant decrease in viral virulence to sea bass; however, a complete attenuated virus has not been obtained.

CHAPTER 2

Understanding the role of the European sea bass ISG15 protein against betanodavirus infections

1. INTRODUCTION

Type I interferon (IFN I) is a key cytokine secreted by virus-infected cells and detected by neighbouring cells, inducing the transcription of numerous interferon-stimulated genes (ISGs) (Seif et al., 2017; Wilson and Brooks, 2013), including the interferon-stimulated gene 15 (*isg15*) (Der and Lau, 1995; Fitzgerald, 2011; Lin et al., 2006; Lindqvist et al., 2018; Okumura et al., 2013; Sadler and Williams, 2008; Verhelst et al., 2013).

The *isg15* gene encodes a 15-kDa protein (ISG15) fairly conserved in vertebrates. ISG15 is composed of two ubiquitin-like domains (UBLs) connected by a short linker sequence (Figure 1), and shows a conserved RLRGG motif in the C-terminal end.

ISG15 was the first UBL protein described, and displays N- and C-terminal domains with 32% and 37% similarity, respectively, with ubiquitin in vertebrates (Swaim et al., 2017). In higher vertebrates, as it happens for ubiquitin, ISG15 is synthetized as an inactive 17-kDa precursor, exposing the C-terminal RLRGG motif after a post-translational maturation process (Bogunovic et al., 2013; Campbell and Lenschow, 2013; Narasimhan et al., 2005).

Mature mammalian ISG15 proteins are effector proteins with a proven antiviral role against a wide variety of viruses, including influenza A and B viruses, Sindbis virus, HIV-1, herpes simplex-1, murine herpesvirus, West Nile virus, Chikungunya virus and dengue virus (Dai et al., 2011; Morales and Lenschow, 2013; Villarroya-Beltri et al., 2017; Werneke et al., 2011; Zhao et al., 2014). ISG15 has been found inside the producing cells free or conjugated to cellular or viral proteins, through a process called ISGylation, which requires the RLRGG motif.

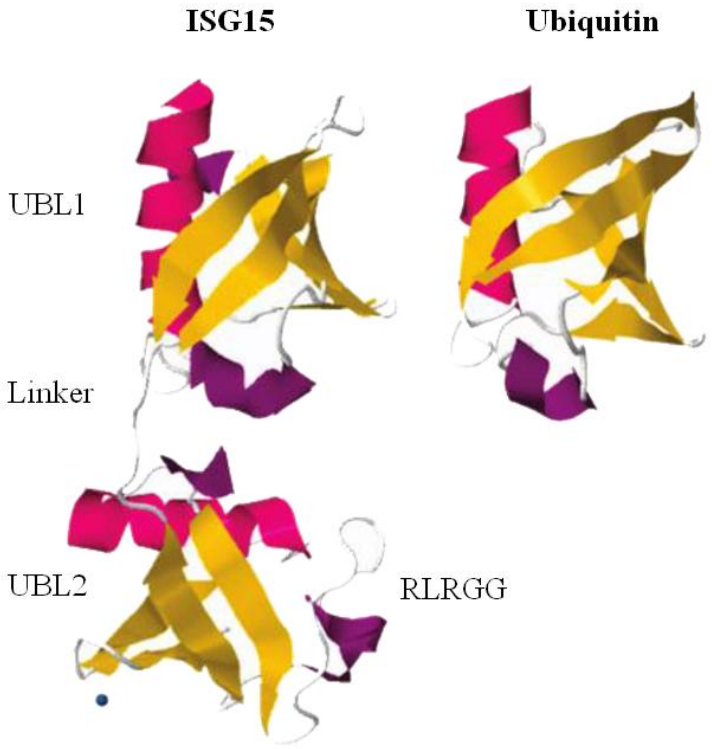


Figure 1. Tertiary structure of ISG15 and ubiquitin proteins (Bogunovic et al., 2013).

ISGylation occurs through a pathway similar to that of ubiquitination (Morales and Lenschow, 2013), resulting in the modification of some characteristics of the conjugated proteins, such as location, stability and activity (Zhang and Zhang, 2011). The ISGylation process is described in Figure 2. The first step of this process is the generation of a thioester bond between the two C-terminal glycine residues (-GG) of ISG15 proteins and a cysteine residue within the ubiquitin-activating enzyme E1-like protein (Ube1L or UBA7 proteins). Subsequently, ISG15 is transferred from E1 to a cysteine residue of the E2 enzyme (also known as ubiquitin-carrier protein H8, UbcH8 or UBE2L6) through a thioester bond, and, together with E2 enzyme, the E3 ligase (HERC5, EFP or TRIM25, and HERC6) transfers ISG15 protein to a lysine residue in the target protein. This is a reversible process, since ISG15 can be deconjugated via ISG15-specific proteases (USP18 or UBP43) (Bade et al., 2012; Harty et al., 2009; Morales and Lenschow, 2013; Takeuchi et al., 2005; Villarroya-Beltri et al., 2017; Zhao et al., 2014). The transcription of all enzymes involved in this process is regulated by IFN I.

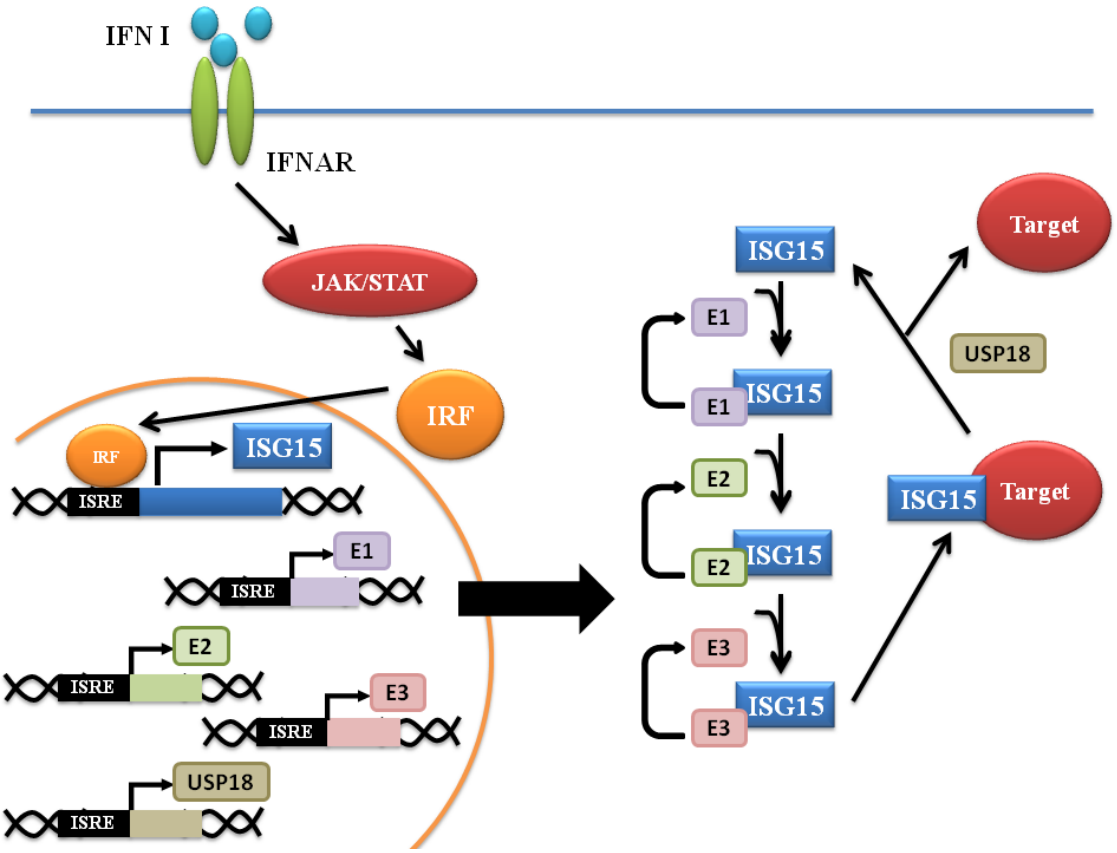


Figure 2. Scheme representing the ISG15 ISGylation pathway. The transcription of genes coding ISG15-conjugating and deconjugating enzymes is controlled by IFN I-regulated enzymes.

Hundreds of proteins, involved in all stages of cellular biology, have been described as ISGylation-target (Giannakopoulos et al., 2005; Villarroya-Beltri et al., 2017; Zhao et al., 2005). Regarding viral infections, ISG15 has been described to limit virus release by conjugating cellular proteins, such as Nedd4, TSG101 and CHMP5, in Ebola virus, influenza virus and HIV-1 virus, respectively (Okumura et al., 2008; Pincetic et al., 2010; Sanyal et al., 2014) or directly conjugating viral proteins, such as influenza NS1 and NP proteins (Zhao et al., 2010; 2016). In addition to this role as conjugating protein, ISG15 can also act as free un-conjugated protein, both intracellularly or as secreted protein, acting, therefore, as cytokine.

Intracellularly, although its pathway is unclear, free ISG15 has been described as an antiviral protein, probably acting by modulating the transcription of several immunogenes and, therefore, by regulating the production of pro-inflammatory cytokines and chemokines (Domingues et al., 2015; Werneke et al., 2011).

Although the ISG15 secretion mechanism is still unknown, this protein has been

detected extracellularly in IFN I-treated human leukocyte and monocyte cultures (D' Cunha et al., 1996; Knight and Cordova, 1991). Thus, secreted ISG15 promotes IFN-γ production by immune system cells, mainly natural killer (NK) cells and lymphocytes T (Bogunovic et al., 2012; 2013; D' Cunha et al., 1996; Xiang et al., 2017), generating an immune activation against pathogens. The possible ISG15 cell surface receptor in human NK cells (LFA-1, CD11a/CD18; aLb2 integrin) has been recently reported (Swaim et al., 2017). The activity of secreted human ISG15 seems to require IL-12, which drives cytokine expression, whereas ISG15 would be responsible for cytokine secretion (Swaim et al., 2017).

In fish, the *isg15* gene has been demonstrated to be one of the earliest and most highly-expressed ISGs following viral infections (Alvarez-Torres et al., 2017; 2018; Furnes et al., 2009; Lin et al., 2015; Moreno et al., 2016; 2018; Zhang et al., 2007). Several studies have characterized the genomic structure of *isg15* genes for some fish species, such as Atlantic cod (*Gadus morhua*), zebrafish (*Danio rerio*), turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*) and Japanese flounder (*Paralichthys olivaceus*) (Alvarez-Torres et al., 2017; Lin et al., 2015; Seppola et al., 2007; Yasuike et al., 2011). The genomic structure described for all these *isg15* genes is similar, being composed of one 471-480-bp long open reading frame (ORF), flanked by the 5'- and 3'-untranslated regions (UTRs). The length of these UTR sequences varies between species, although all sequences described display an intron located in the 5'-UTR region. In contrast, mammalian *isg15* genes display the intron sequence within the coding region, just after the start codon.

In addition, several studies on the antiviral activity of ISG15 proteins from several fish species, such as grouper (*Epinephelus coioides*) and zebrafish, have revealed antiviral mechanisms against several fish viruses, such as grouper nervous necrosis virus (GGNV) (Huang et al., 2013), infectious hematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), and epizootic haematopoietic necrosis virus (EHNV) (Langevin et al., 2013a). Furthermore, the intracellular immuno-modulatory activity of fish ISG15 has also been reported (Langevin et al., 2013a), and fish ISG15 proteins have been detected extracellularly, in cultures of IFN I-stimulated lymphocytes of red drum (*Sciaenops ocellatus*) and tongue sole (*Cynoglossus semilaevis*) (Liu et al., 2010; Wang et al., 2012).

The main aim of the present study has been to study the role of the European sea bass (*Dicentrarchus labrax*) ISG15 protein against betanodavirus infections. The first step to fulfil this objective has been the characterization of the sea bass *isg15* gene, which has been carried out by analysing the gene structure and transcription (chapter 2A). The *isg15* ORF has been subsequently cloned into a eukaryotic expression vector in order to develop an *in vitro* system suitable for evaluating the anti-betanodavirus activity of the sea bass ISG15 protein, which has been addressed in chapter 2B.

CHAPTER 2A

Genetic characterization and transcriptional analysis of European sea bass *isg15* gene

Moreno, P., Garcia-Rosado, E., Borrego, J. J., Alonso, M. C. (2016). Genetic characterization and transcription analyses of the European sea bass (*Dicentrarchus labrax*) *isg15* gene. *Fish and Shellfish Immunology*, *55*, 642–646.

The European sea bass (*Dicentrarchus labrax*) *isg15* gene and ISG15 protein sequences have been characterized in this study. First of all, the full-length sequence of European sea bass *isg15* gene has been obtained, and the regulatory motifs found have been described. Afterwards, based on the sequence obtained, the genomic structure and the protein phylogenetic relationships have been evaluated. In addition, an *in vivo* transcriptional analysis in response to red-spotted (*Epinephelus akaara*) nervous necrosis virus (RGNNV) and polyinosinic:polycytidylic acid (poly I:C) has been performed.

2. MATERIALS AND METHODS

2.1. STRUCTURAL CHARACTERIZATION OF EUROPEAN SEA BASS *isg15* GENE

2.1.1. cDNA synthesis and genomic DNA extraction

Sea bass *isg15* cDNA was obtained from juvenile specimens (n = 3) (6 g, average weight) intraperitoneally injected with poly I:C (CalBiochem, 1 mg/fish), a strong inductor of the interferon I (IFN I) system. A negative control group, consisting of fish intramuscularly injected with Leibovitz L-15 medium (Gibco), was also set up. At 8 h post-infection (p.i.), fish were killed by anaesthetic overdose (MS-222, Sigma), and head kidneys were collected, individually frozen in liquid nitrogen, and maintained at -80 ^{o}C until used. Animals were handled according to the European Union guidelines for the handling of laboratory animals (Directive 2010/63/UE), and they were maintained in 50-l tanks under the conditions described in chapter 1, section 2.6.

Head kidneys were individually homogenized (10%, w/v) in L-15 medium supplemented with 2% foetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 10 mg/ml streptomycin (Sigma) by wet grinding, using the Mixer Mill MM400 (Retsch). Total RNA was extracted with the TRI reagent solution (Sigma) (procedure described in chapter 1, section 2.2.2.2.), quantified at 260 nm using the NanoDrop-1000 system (ND-1000, ThermoFisher) and stored at -80 ^{o}C until used. After RNA treatment with DNase I Recombinant from bovine pancreas (Roche) (chapter 1, section 2.4.), cDNA was synthesized using 1 μg of RNA and the Transcriptor First Strand cDNA Synthesis Kit (Roche), following guidelines described in chapter 1, section 2.2.1.1. The resulting cDNA was maintained at -20 ^{o}C until used.

Genomic DNA (gDNA) was isolated from caudal fin by saline precipitation (Martinez et al., 1998). This tissue (10 mg) was lysed in a mixture composed of buffer I (0.1 M Tris-HCl pH 8.0 and 0.1 M ethylenediaminetetraacetic acid, EDTA, pH 8.0) and buffer II (0.1 M Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0 and 1% sodium dodecyl sulphate, SDS) (1:1). Subsequently, 6 M NaCl (precipitation solution, 32 µl) and proteinase K (30 µl, Roche) were added to the mixture. After incubation at 55 ^{o}C overnight, RNAse A (Roche) was included (10 mg/ml, final concentration), and the reaction was incubated at 37 ^{o}C for 1 h. Afterwards, precipitation solution (160 µl) was added, incubated at 4 ^{o}C for 10 min and centrifuged at 13,000 rpm at 4 ^{o}C for 10 min. DNA was precipitated with isopropanol by centrifugation at 13,000 rpm at 4 ^{o}C for 3 min, washed with 70% ethanol, dried and diluted in diethyl pyrocarbonate (DEPC, Sigma)-treated water (30 µl). Resulting gDNA was quantified at 260 nm using the NanoDrop-1000 system, and stored at 4 ^{o}C until used. Centrifugations were carried out using the 2-16PK centrifuge (12148-H rotor, Sigma).

2.1.2. Primer design

Primers to amplify the sea bass *isg15* gene were designed based on the non-annotated sea bass genome available in GenBank (accession no. HG916840), in which the *isg15* gene was located by alignment (BLAST, NCBI, software) with the sea bass *isg15*-expressed sequence tag (EST) published by Seppola et al. (2007) (accession no. CV186275). The *in silico* genomic sequence obtained was analysed with the PROSCAN v 7.1 (BIMAS) software to predict the putative start site and polyadenylation signal. This sequence was subsequently used to design the specific primers shown in Table 1. Combinations of these primers were used to amplify the *isg15* open reading frame (ORF), as well as the 5'- and 3'-untranslated regions (UTRs), using as template both, sea bass cDNA and gDNA, following the strategy depicted in Figure 1.

Table 1. Primers used for European sea bass *isg15* gene amplification and sequencing.

Name	Sequence (5'-3')	Annealing temperature (°C)
Dl ISG15 NotI-F	GATCACAAGGGCGGCCGCATGATGGATATAACC	65
Dl ISG15 XhoI-R	TATGAGCCTCGAGGGTGCTCAGCCTCCTCTCAG	65
polyAISG15-Rw	TTTTTTTTTTTTTACTTTAAATAGTTTC	48
StarsiteISG15-F1	CACAGCTGTTCCTAACAATCCTC	57
StarsiteISG15-F2	CTGTGGGGAGCTGAAACCTCT	58
qISG15-F2	CGACTCAAAGCCTCTCTGCTACT	60
qISG15-R2	CGTTTCTGACGAACACCTGGAT	60

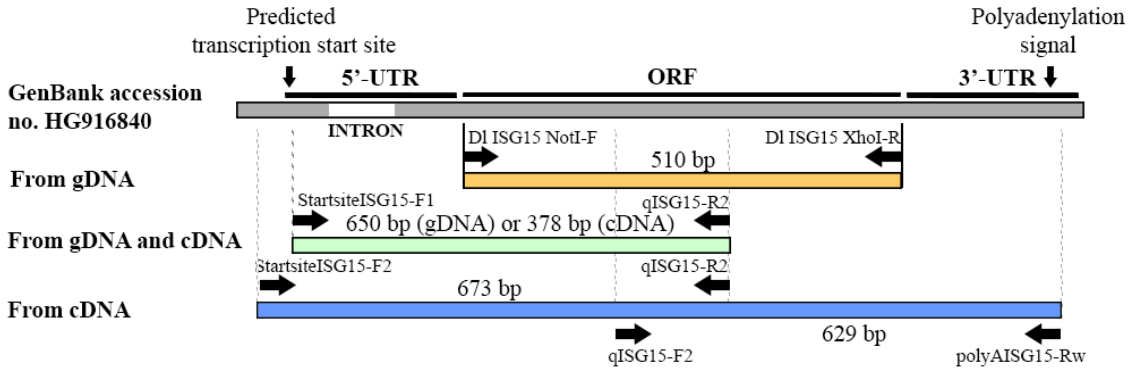


Figure 1. Strategy followed for European sea bass *isg15* gene sequencing using as template cDNA or gDNA. The scheme shows primer positions and the length (bp) of the amplified products.

2.1.3. Amplification, sequencing and analysis of sea bass *isg15* cDNA and gDNA

Amplification reactions were conducted in 50-µl mixtures containing cDNA or gDNA (400 ng), 2x Universe Buffer, 10 mM dNTPs, Universe High-Fidelity Hot Start DNA polymerase (Biotools, 1 U) and 10 µM each primer (Table 1). The amplification profile was: denaturation at 95 °C for 3 min, followed by 35 cycles at 95 °C for 15 s, 48-65 °C for 15 s (Table 1) and 72 °C for 15 s. Final elongation was at 72 °C for 5 min. Amplified products were purified from ethidium bromide (Sigma)-stained 2% agarose gels with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), following manufacturer instructions. They were quantified at 260 nm with the NanoDrop-1000 system, and maintained at -20 °C until sequencing, which was performed, in triplicate, with the ABI 3730 (STABVIDA) system.

Sequences were edited, aligned and translated into amino acid sequences using the EditSeq and MegaAlign software (DNASTAR Lasergene 7). Intron screening was performed by alignment of the resulting cDNA and gDNA sequences. The deduced amino acid sequence was aligned with other ISG15 sequences using the Clustal Omega (EMBL-EBI) software. The results obtained were used for phylogenetic analyses by implementation of the neighbour joining-clustering method using the MEGA7 software. Reference sequences from fish species belonging to Pleuronectiformes, Gadiformes, Cypriniformes, Perciformes, Salmoniformes, Siluriformes, Scorpaeniformes, and Tetraodontiformes orders were included. Sequences of ISG15 proteins from species in Rodentia and Primates orders were included as outgroups.

2.2. ANALYSIS OF *isg15* GENE TRANSCRIPTION

2.2.1. Virus and viral propagation

The RGNNV viral isolate used in this study (SpDl_IAusc965.09) has been described in chapter 1 (section 2.1.). From now on, in this chapter, this isolate will be referred as RGNNV.

Viral propagation was on E-11 cells (Iwamoto et al., 2000) following the methodology described in chapter 1, section 2.1. The resulting viral stock was titrated on E-11 cells grown on 96-well plates (Nunc), following the 50% tissue culture infective dose method ($TCID_{50,}$ Reed and Muënch, 1938), and stored at -80 ^{o}C until used.

2.2.2. Experimental challenge and sample processing

The juvenile sea bass specimens (6 g, average weight) used in this study have been ruled out as betanodavirus carriers using the protocol described by Lopez-Jimena et al. (2010). Afterwards, three groups, consisting of poly I:C-, RGNNV- and L-15-inoculated fish (n = 40, each group), were considered. Poly I:C was inoculated by intraperitoneal injection (1 mg/fish), whereas RGNNV (10^5 $TCID_{50}$/fish) and L-15 were intramuscularly injected. Animals were maintained at 23-25 ^{o}C, and head kidneys and brains were sampled at 4, 6, 8, 12, 24, 48 and 72 h p.i. Three animals per group were killed by anaesthetic overdose at each sampling time. Organs were individually frozen in liquid nitrogen and maintained at -80 ^{o}C until processing. Total RNA extraction and

cDNA synthesis were conducted as it has been described in section 2.1.1. cDNA was kept at -20 ^{o}C until transcription analyses.

2.2.3. Quantification of *isg15* transcription

Sea bass *isg15* mRNA was quantified in brain and head kidney by relative real-time PCR, using the LightCycler 480 Thermocycler (Roche) and the ribosomal 18S RNA as reference endogenous gene (Table 2). Amplification reactions were conducted in 20-µl mixtures composed of cDNA generated from 50 ng of RNA, 1x Fast Start Essential DNA Green Master Mix (Roche) and 10 pmol primers (Table 2). The amplification profile was denaturation at 95 ^{o}C for 10 min, and 45 cycles at 95 ^{o}C for 10 s, 60 ^{o}C for 10 s and 72 ^{o}C for 10 s. Melting curves were obtained at 95 ^{o}C for 10 s, 65 ^{o}C for 60 s and 97 ^{o}C for 1 s. Relative fold change values were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 2. Primers used for relative quantification of European sea bass *isg15* transcription.

Name	Sequence (5'-3')	Product length (bp)	Reference
qISG15-F2	CGACTCAAAGCCTCTCTGCTACT	100	Moreno et al. (2016)
qISG15-R2	CGTTTCTGACGAACACCTGGAT		
18S rRNA Fw	CCAACGAGCTGCTGACC	208	Scapigliati et al. (2010)
18S rRNA Rw	CCGTTACCCGTGGTCC		

2.2.4. Viral genome quantification

The RGNNV RNA2 genomic segment was quantified in brain by absolute real-time PCR using RG_965_RNA2 F4 (5'-ACCGTCCGCTGTCTATTGACTA-3') and RG_965_RNA2 R1 (5'-CAGATGCCCCAGCGAAACC-3') primers, which amplify a 126-bp fragment within the viral capsid protein gene. Amplifications were performed using the LightCycler 480 Thermocycler and the Fast Start Essential DNA Green Master Mix. Serial dilutions of the pJET easy vector containing the complete RNA2 segment of the RGNNV isolate SpDl_IAusc965.09 (chapter 1, section 2.3.1.) were used to generate standard curves. The amplification mixtures and cycling conditions have been described in section 2.2.3.

2.3. STATISTICAL ANALYSES

Data have been statistically analysed with the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA). Normality distribution was verified by Shapiro-Wilk test. The one-way ANOVA was the statistical test used, and the Tukey test for multiple comparisons was used as post test. Values of $p < 0.05$ were considered significant.

3. RESULTS

3.1. ANALYSIS OF EUROPEAN SEA BASS *isg15* GENE SEQUENCE

The present study has added *isg15* to the list of antiviral genes characterized in European sea bass. Sea bass *isg15* is a 1,143-bp long gene, displaying a single 276-bp intron, which is located in the 102-bp long 5'-UTR region (Figure 2, positions 37-365). The complete ORF sequence, estimated on the basis of the putative location of the start and stop codons, is 474-bp long (Figure 2, positions 366-840). The 291-bp 3'-UTR sequence (positions 841-1132) contains one AATAAA polyadenylation signal, one cytoplasmic polyadenylation element (CPE, U-rich sequence), and four mRNA instability motifs (ATTTA), which have also been detected within the intron sequence (containing three ATTTA motifs). Sea bass *isg15* displays the typical structure for fish *isg15* genes (Figure 3), in which the intron is always located within the 5'-UTR sequence, whereas mammalian *isg15* genes display the intron inside the coding region.

The deduced amino acid sequence depicted in Figure 2 (157 amino acids) shows two typical ubiquitin-like domains (UBLs), and a C-terminal RLRGG conjugation motif at the 3'-end, with no additional amino acids downstream this motif.

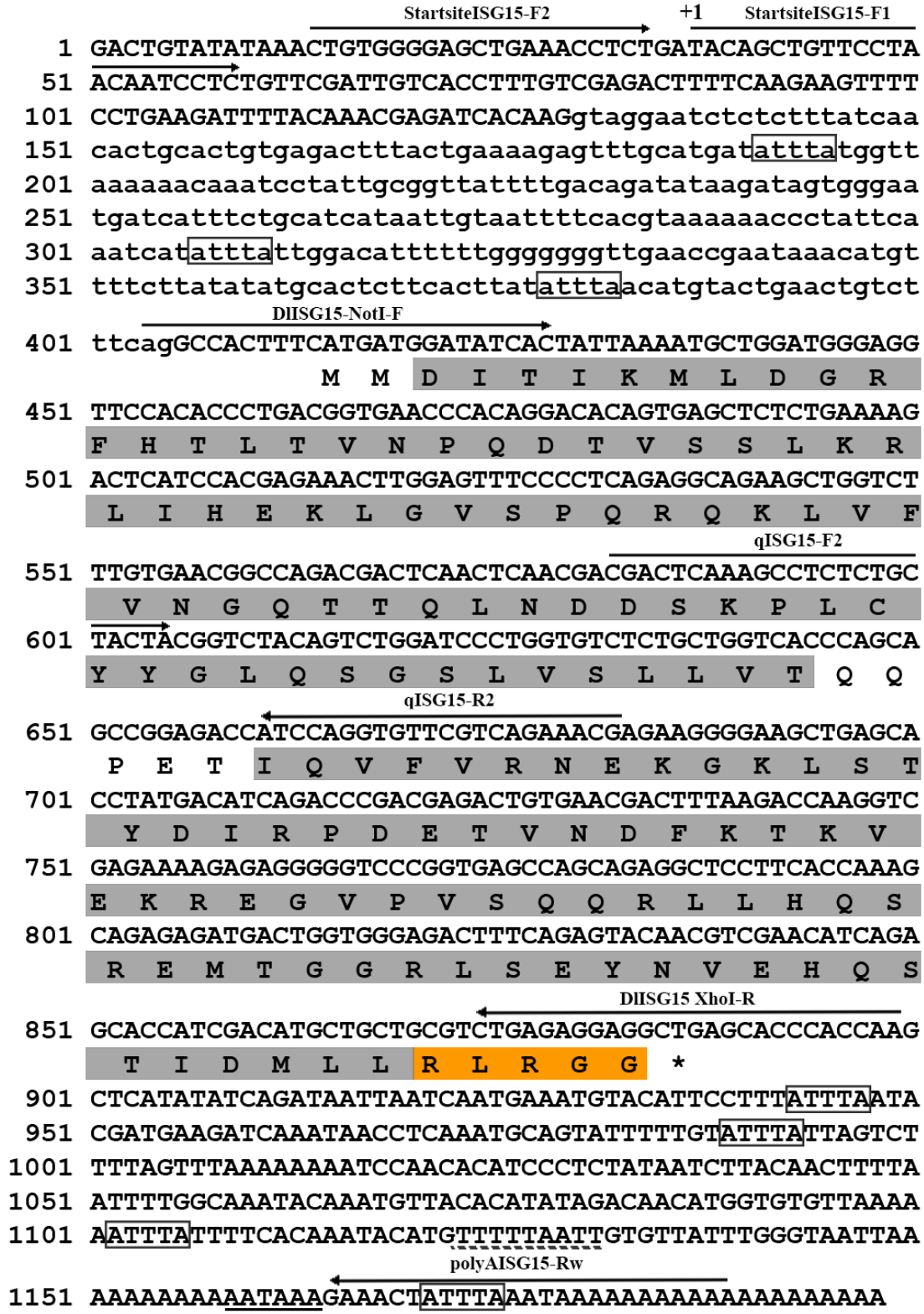


Figure 2. Nucleotide and putative amino acid sequences of European sea bass *isg15*. The intron is shown in lower case letters. The ORF appears with the putative amino acid sequence below (both UBL motifs are indicated in grey, and the conjugation motif is in orange). Asterisk indicates the stop codon. The instability motifs (ATTTA) are boxed. The CPE sequence is underlined with a dotted line, and the polyadenylation signal with a solid line. The positions of the primers are indicated with arrows.

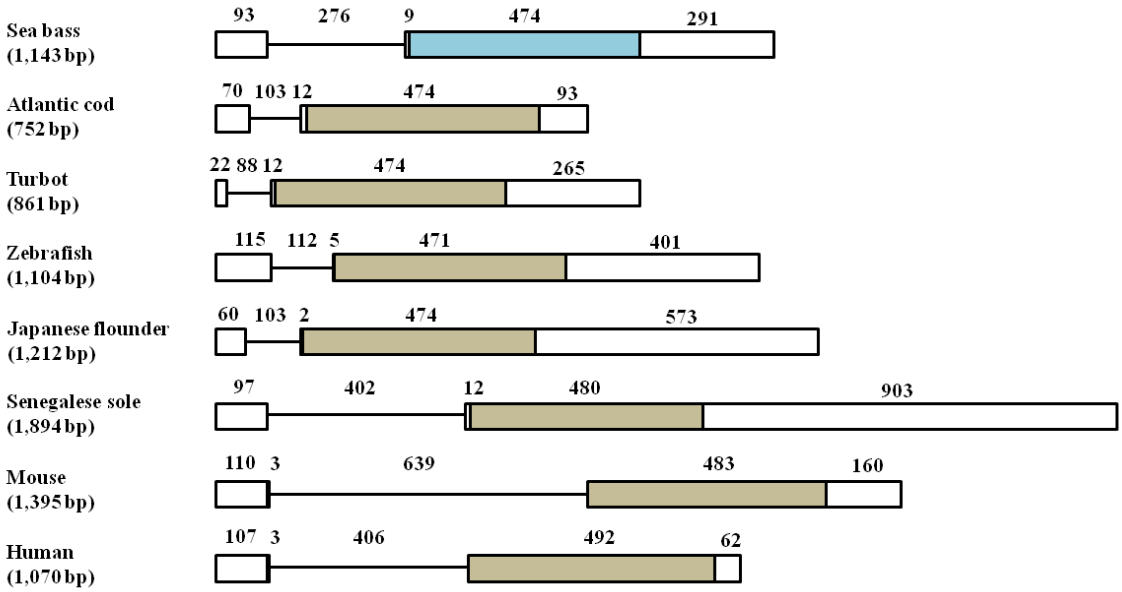


Figure 3. Comparison between the European sea bass *isg15* genomic structure and other *isg15* genes. Lines indicate introns, and boxes are exons. Coding regions are in blue for sea bass *isg15*, and in grey for other *isg15* genes. Untranslated regions are in white. Numbers above exons and introns represent the size (bp) of each element. Accession no. or references: European sea bass (*Dicentrarchus labrax*) (this study, Moreno et al., 2016), Atlantic cod (*Gadus morhua*) (Seppola et al., 2007), turbot (*Scophthalmus maximus*) (Lin et al., 2015), zebrafish (*Danio rerio*) (Seppola et al., 2007), Japanese flounder (*Paralichthys olivaceus*) (Yasuike et al., 2011), Senegalese sole (*Solea senegalensis*) (Alvarez-Torres et al., 2017), mouse (*Mus musculus*) (ENSMUSG00000035692) and human (*Homo sapiens*) (NG_033033).

The alignment of the putative sea bass ISG15 amino acid sequence with other ISG15 proteins (Figure 4) shows that UBL domain 2 is more conserved than UBL domain 1, displaying 5 amino acids shared by all species analysed (tyrosine, valine, leucine, leucine and tyrosine, at positions 496, 504, 524, 537 and 540, respectively), and several amino acids shared by most of the species. In addition, several amino acids involved in the immunomodulation function of secreted ISG15 proteins have been recently identified at positions 97, 100, 102, 103 and 104, which are highly conserved in mammals (Swaim et al., 2017). The alignment performed in this study has reported for the first time differences in amino acids at positions 102 and 103 between mammalian and fish ISG15 sequences (Figure 4). Specifically, the amino acid 102 in fish ISG15 sequences is variable (in sea bass is aspartic acid, in mammals is always threonine), whereas the amino acid 103 in fish is always glutamic acid (glutamine in mammals).

The similarity with other homologous fish ISG15 proteins ranged from 46.1 to 75.5% (Table 3), with the highest sequence similarity (75.5%) with sea bream (*Sparus*

aurata) ISG15, followed by red drum (*Sciaenops ocellatus*) and striped beakfish (*Oplegnathus fasciatus*) ISG15, with 73.5% and 73% similarity, respectively. The lowest similarities were with Atlantic cod (*Gadus morhua*) (46.1%), goldfish (*Carassius auratus*) (46.1%) and zebrafish (*Danio rerio*) (46.7%) ISG15 proteins (Table 3).

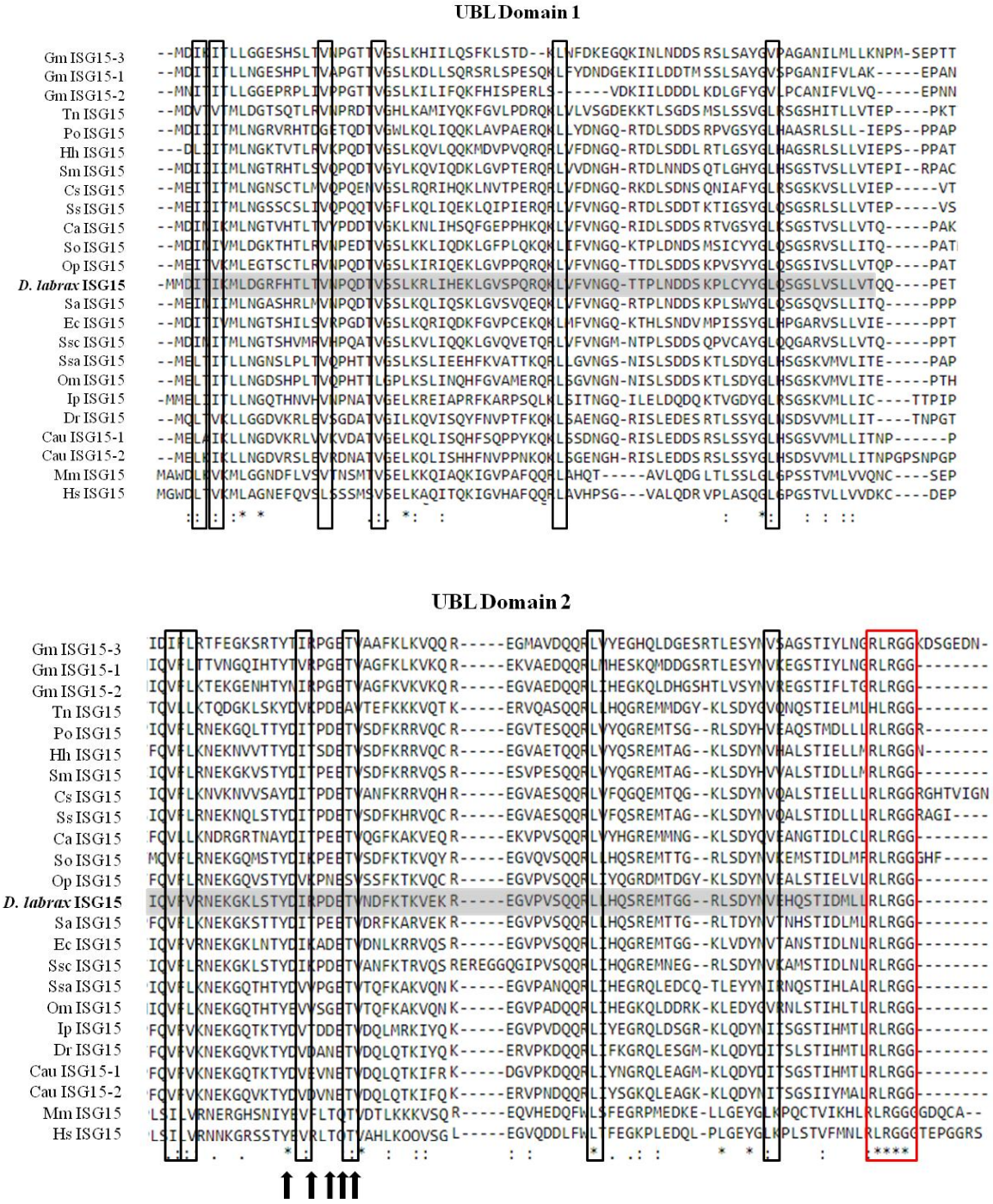


Figure 4. Alignment of the European sea bass ISG15 amino acid sequence with other ISG15 proteins. Ubiquitin like domains (UBL) are highlighted in grey, the RLRGG motif is within a red box, and the six invariant aliphatic residues in each UBL domain are within black boxes. Residues involved in immunomodulation are indicated with arrows. Symbols below alignment represent positions that have a single fully conserved residue (*), conservation between groups of strongly similar properties with a score > 0.5 in the Gonnet PAM 250 matrix (:), and conservation between groups of weakly similar properties with a score =< 0.5 in the Gonnet PAM 250 matrix (.). Abbreviations: Gm (*Gadus morhua*), Tn (*Teaodron nigroviridis*), Po (*Paralichthys olivaceus*), Hh (*Hippoglossus hippoglossus*), Sm (*Scophthalmus maximus*), Cs (*Cynoglossus semilaevis*), Ss (*Solea senegalensis*), Ca (*Channa argus*), So (*Sciaenops ocellatus*), Op (*Oplegnathus fasciatus*), Sa (*Sparus aurata*), Ec (*Epinephelus coioides*), Ssc (*Sebastes schlegelli*), Ssa (*Salmo salar*), Om (*Oncorhynchus mykiss*), Ip (*Ictalurus punctatus*), Dr (*Danio rerio*), Cau (*Carassius auratus*), Mm (*Mus musculus*) and Hs (*Homo sapiens*).

Table 3. Amino acid similarity (%) between European sea bass ISG15 and ISG15 proteins from other species.

Protein	Species	Accession no. or reference	Similarity (%)
ISG15	*Sparus aurata*	HS987162	75.5
	Sciaenops ocellatus	BAJ16365	73.5
	Oplegnathus fasciatus	ADJ57326	73.0
	Sebastes schlegelli	BAG672218	69.7
	Epinephelus coioides	AGC26172	65.1
	Solea senegalensis	Alvarez-Torres et al. (2017)	65.1
	Scophthalmus maximus	AHW76805	64.7
	Paralichthys olivaceus	BAI48419	61.9
	Channa argus	ABK63480	61.9
	Cynoglossus semilaevis	AFR33115	59.3
	Hippoglossus hippoglossus	AER92772	58.7
	Tetraodon nigroviridis	CAG08614	56.4
	Salmo salar	AY926456	52.2
	Oncorhynchus mykiss	AAO14689	50.3
	Ictalurus punctatus	CB940275	48.4
ISG15-2	*Gadus morhua*	FJ883792	47.9
ISG15-1	*Carasiuss auratus*	AY303810	47.4
ISG15-3	*Gadus morhua*	ABD60150	46.7
	Danio rerio	NP_001191098	46.7
ISG15-2	*Carasiuss auratus*	AY303811	46.1
ISG15-1	*Gadus morhua*	FJ883793	46.1
	Homo sapiens	AAH09507	35.4
	Mus musculus	NP_056598	35.2

According to the phylogenetic tree (Figure 5), European sea bass ISG15 has the closest phylogenetic relationship with ISG15 proteins from other fish species within the same order (Perciformes), such as *S. aurata*, *S. ocellatus* and *O. fasciatus*, followed by Scorpaeniformes, Pleuronectiformes and Tetraodontiformes orders. Species in mammalian orders, Primates and Rodentia, are grouped in a different cluster.

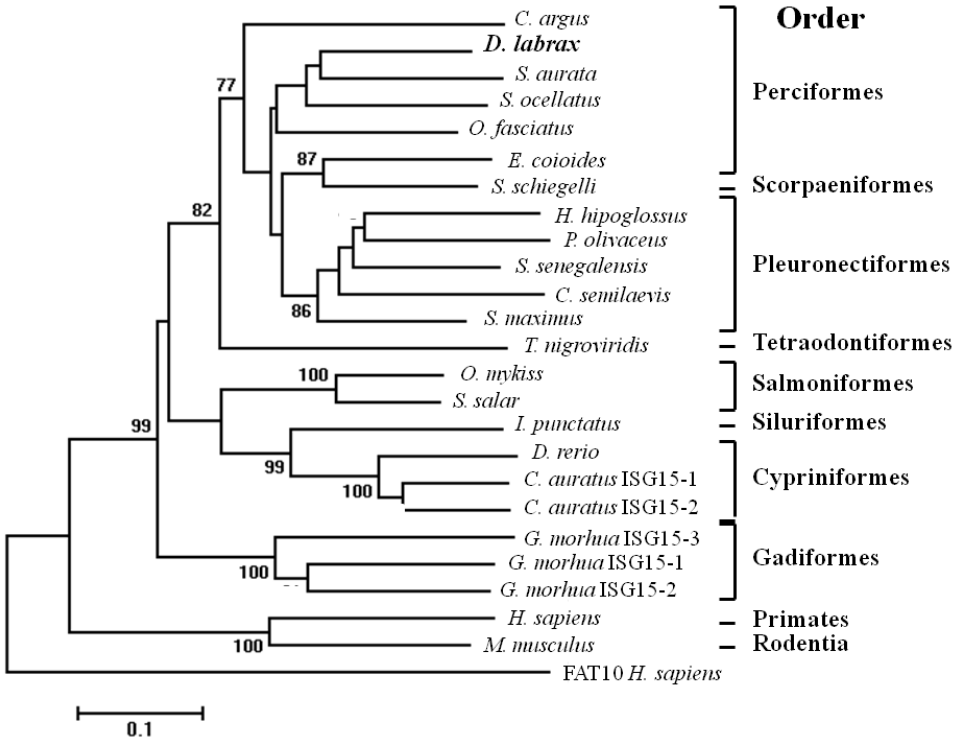


Figure 5. ISG15 phylogenetic tree constructed with the neighbour-joining method using the MEGA7 software. Numbers on nodes indicate the bootstrap confidence value based on 1000 replications. 0.1 indicates the genetic distance.

3.2. ANALYSIS OF *isg15* GENE TRANSCRIPTION

The transcription of the sea bass *isg15* gene after poly I:C or RGNNV inoculation has been comparatively analysed in brain and head kidney. The results obtained, displayed in Figures 6A and 6B, showed that *isg15* transcription is induced earlier and at higher level after poly I:C injection than after viral inoculation in both organs analysed.

In head kidney (Figure 6A), poly I:C stimulated *isg15* transcription from 4 to 24 h p.i. (47 and 4.6 mean fold change values, respectively), with maximum transcription recorded at 8 h p.i. (70 mean fold change value). On the contrary, RGNNV-induced transcription was only recorded at 48 and 72 h p.i., showing the maximum mean fold change value (11.7) at 72 h p.i.

Regarding brain (Figure 6B), kinetics of poly I:C-induced *isg15* transcription is similar to that described in head kidney, with the maximum mean fold change value (62) at 8 h p.i. However, the induction promoted by the virus started earlier than in head kidney, with significant ($p < 0.01$) transcription at 12 and 24 h p.i. (12 and 8 mean fold change values, respectively), and the maximum value (59) at 72 h p.i.

In addition, viral genome has been quantified in brain by absolute real-time PCR (Figure 6C), demonstrating viral multiplication in the target organ, with a significant increase in the RNA2 copy number (p = 0.02) from 6 ($9.1x10^6$ RNA2 copies/g tissue) to 72 h p.i. ($3.6x10^9$ RNA2 copies/g tissue).

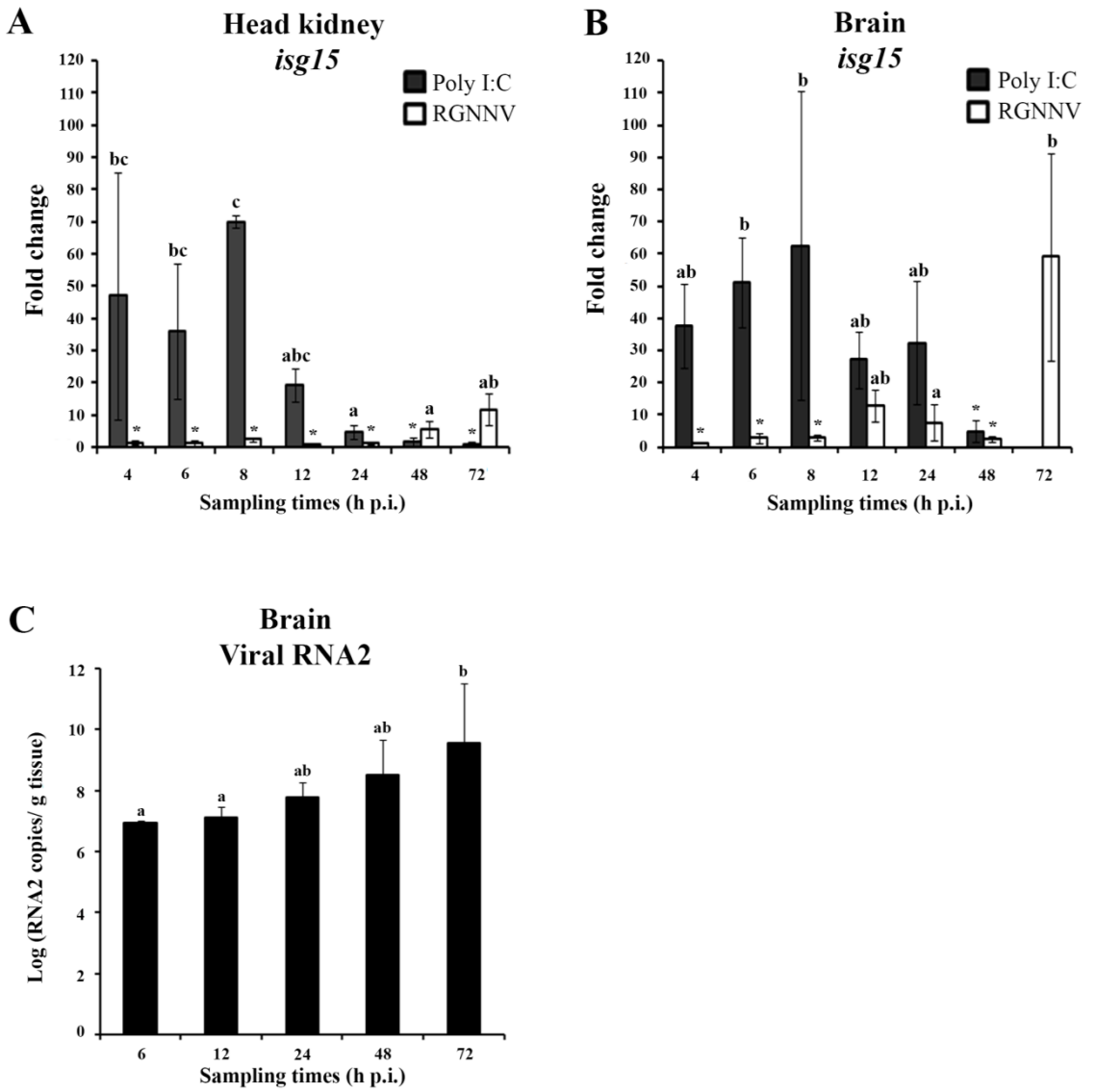


Figure 6. (A) *isg15* gene transcription in head kidney from poly I:C- or RGNNV-inoculated sea bass. (B) *isg15* gene transcription in brain from fish inoculated with poly I:C or RGNNV. (C) Absolute quantification of the RNA2 viral segment in brain. Results are mean ± standard deviation (SD) (n = 3). * indicates samples with fold change values below 1.5 (non-induced samples). Different letters denote significant differences. Values of $p < 0.05$ were considered significant.

4. DISCUSSION

The characterization of European sea bass *isg15* gene and ISG15 protein sequences has been accomplished in this study, allowing the description of several regulatory motifs, the analysis of the genomic structure, and the evaluation of the protein phylogenetic relationships. In addition, an *in vivo* transcriptional analysis in response to a RGNNV isolate and poly I:C has also been performed.

European sea bass *isg15* is a 1,143-bp long gene composed of a 102-bp long 5'-UTR region, in which a single 276-bp intron is located, followed by a 474-bp long ORF sequence, and a 291-bp long 3'-UTR sequence (Figure 2).

The intron location in the 5'-UTR sequence, which has also been described for other fish *isg15* genes, has been related with the mRNA maturation process, being involved in translational silencing and, therefore, in the regulation of the ISG15 protein level (Matsumoto et al., 1998; Seppola et al., 2007).

In addition, three mRNA instability motifs (ATTTA, AU-rich elements or AREs) have been observed within the intron sequence (Figure 2), which seems to be related with heterogeneous nuclear RNA (hnRNA or pre-RNA) splicing regulation, as well as mRNA expression (Zou et al., 1999). Instability motifs in introns are not frequent, and they have only been described in a few genes, such as the Atlantic cod *isg15* gene (Seppola et al., 2007), which contains a single ATTTA motif, and rainbow trout (*Oncorhynchus mykiss*) *interlaeukin-1β* gene (Zou et al., 1999); however, location of these ARE motifs in 3'-UTR sequences is quite common, and they have been described in genes coding for short-life cytokines (Akashi et al., 1994), growth factors, tumour suppressors, as well as in proto-oncogenes and genes involved in regulation of cell cycle (Barrett et al., 2012). The 3'-UTR sequence of European sea bass *isg15* contains four ARE motifs. The number of these motifs in the 3'-UTR region in fish *isg15* genes varies from one in zebrafish to six in Japanese flounder (*Paralichthys olivaceus*), whereas no motifs have been found in *isg15* from mammals (human, mouse and cow). The function of ARE sequences is mediated by specific RNA-binding proteins, which modulate mRNA degradation (Herjan et al., 2013), altering mRNA stability (Akashi et al., 1994), promoting the decay of mRNA, modulating translation efficiency or even increasing protein production in a tissue- or cell type-specific manner (Barrett et al., 2012). In this study, the number of ATTTA sequences found in the 3'-

UTR sequence of sea bass *isg15* gene (four) suggests that *isg15* mRNA may have a short half-life, being modulated its degradation by specific RNA-binding proteins.

In addition, the sea bass *isg15* 3'-UTR sequence contains one cytoplasmic polyadenylation element (CPE, U-rich sequences, Figure 2), which is required for cytoplasmic elongation of the nuclear poly A tail, able to repress or exert translation depending on the cellular type (Ross, 1995). This mRNA regulatory element is also present in *isg15* genes from other fish species, such as sea bream, zebrafish, Japanese flounder and turbot (*Scophthalmus maximus*), but it has not been found in mammals (human, mouse and cow), suggesting that *isg15* post-transcriptional regulation may be stronger in fish (and specifically in sea bass) than in higher vertebrates.

Regarding the deduced amino acid sequence, two typical UBL domains, connected by a linker region, and the RLRGG conjugating motif at the C-terminal end have been described (Figure 4). The UBL domains are highly conserved in fish and mammalian ISG15 proteins, specially UBL domain 2, highlighting the presence of six aliphatic residues, which are crucial for the hydrophobic core of the ubiquitin-like protein structure (Chen et al., 2015; Lin et al., 2015). Regarding these aliphatic amino acids, European sea bass ISG15 conserves these essential residues, although, as it happens in other fish ISG15 proteins (Alvarez-Torres et al., 2017), it lacks the cysteine residue at position 78, within the linker region, which is highly conserved in mammals, being essential for the stabilization of the structure of mammalian ISG15 proteins (Chen et al., 2015). Recently, five amino acids that are essential for the immunomodulatory action of secreted human ISG15 have been reported (Swaim et al., 2017). European sea bass ISG15 conserves several of these amino acids; however, the sequence analysis has shown two amino acids, at positions 102 and 103, which are different in mammals and fish (Figure 4), suggesting that there could be differences in the cytokine-like activity of these proteins, regarding either ISG15-receptor interaction, or the signalling cascade for the immunomodulatory ISG15 action.

The RLRGG motif of ISG15 proteins is essential for their antiviral activity through ISGylation (Harty et al., 2009; Morales and Lenschow, 2013; Zhao et al., 2014). The presence of additional amino acids downstream this conjugation motif has been described in mammals (except ruminants) and some fish species, such as Atlantic cod, red drum, tongue sole (*Cynoglossus semilaevis*), Atlantic halibut (*Hippoglossus*

hippoglossus), Japanese flounder and Senegalese sole (*Solea senegalensis*) (Alvarez-Torres et al., 2017). In these species, ISG15 is primary synthesized as a precursor, which is post-translationally processed, exposing the RLRGG motif (Harty et al., 2009); however, sea bass ISG15 lacks additional amino acids, which would allow the exposition of the conjugation motif without post-translational processing.

According to the similarity analysis and the phylogenetic tree (Table 3, Figure 5), European sea bass ISG15 displays the closest phylogenetic relationship with ISG15 proteins from other fish species within the same order (Perciformes), such as *S. aurata*, *S. ocellatus* and *O. fasciatus*, followed by Scorpaeniformes, Pleuronectiformes and Tetraodontiformes orders, which exhibit similarity percentages higher than 50% with the ISG15 protein of European sea bass. The similarity between European sea bass ISG15 and ISG15 proteins in Salmoniformes, Siluriformes, Cypriniformes and Gadiformes orders varied between 46% and 52%, whereas ISG15 proteins from species in mammalian orders, Primates and Rodentia, were grouped in a different cluster. These results are in concordance with those reported by other authors (Alvarez-Torres et al., 2017; 2018; Lin et al., 2015). In fact, Alvarez-Torres et al. (2018) proposed an interspecies diversification of this protein, indicating differences at a functional level, although additional studies would be necessary to understand functional differences between fish ISG15 proteins and species-specificity against viral infections.

In order to complete the sea bass *isg15* gene characterization, a comparative analysis of transcription in head kidney and brain following poly I:C or virus inoculation has been conducted. Poly I:C triggered *isg15* transcription earlier and at higher level than RGNNV in both organs. In head kidney, the maximum transcription value was recorded at 8 h p.i. (Figure 6A), as it has been evidenced for other fish species, such as Senegalese sole (Alvarez-Torres et al., 2016) or Atlantic cod (Seppola et al., 2007). Poly I:C is a direct stimulator of the IFN I system, whereas the induction triggered by virus requires viral replication. In both organs, the maximum transcription in response to the viral infection was at 72 h p.i., with higher values in brain than in head kidney. Moreover, the high mean fold change value recorded in brain at 72 h p.i. (Figure 6B) may constitutes a second peak in the transcription kinetics, suggesting the possible role of the sea bass ISG15 protein up-regulating the IFN I system. Thus, sea bass ISG15 could be involved in a positive amplification loop of the antiviral innate immunity, as it has been reported in zebrafish (Langevin et al., 2013a).

In addition, the maximum transcription in response to the viral infection in brain, the main target organ for viral replication, coincided with the maximum number of viral RNA2 copies (Figure 6C). This coherence between *isg15* transcription and viral replication has also been recorded in the experimental infections carried out in chapter 3, using a different RGNNV isolate, and in Senegalese sole infected with a reassortant betanodavirus (SpSs-IAusc160.03 isolate) (Alvarez-Torres et al., 2017).

Most of the previous studies on ISGs in brain from RGNNV-infected fish have focused on *mx* gene, which is also highly induced after this viral infection (Chaves-Pozo et al., 2012; Valero et al., 2015b). Although *mx* transcription has been traditionally considered as a good marker of IFN I activity, the results derived from recent works (Alvarez-Torres et al., 2017; 2018; Huang et al., 2013), along with the results derived from this study, suggest that *isg15* could also be a useful marker of this activity. Finally, the important up-regulation of *isg15* transcription observed in the viral replication organ suggests a crucial role of this protein against betanodavirus infections, which has been previously suggested for grouper (*Epinephelus coioides*) (Huang et al., 2013) and Senegalese sole (Alvarez-Torres et al., 2017), and will be deeply analysed in the second part of this chapter using an *in vitro* approach.

In summary, this study describes the structure of the European sea bass *isg15* gene, which is similar to other fish *isg15* genes. Furthermore, the putative amino acid sequence is also similar, showing the closest phylogenetic relationship with ISG15 proteins from other fish species within the Perciformes order. Finally, a differential transcription in brain and head kidney in response to poly I:C and RGNNV has been recorded, with poly I:C being a short-term inducer in both organs. The up-regulation of *isg15* transcription after RGNNV infection was especially high in brain, suggesting the possible anti-betanodavirus role of the European sea bass ISG15 protein, which will be studied in the second part of this chapter.

CHAPTER 2B

Evaluation of the antiviral activity of European sea bass ISG15 protein against betanodavirus infections using an *in vitro* approach

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The sequencing of the European sea bass *isg15* gene, and the *in vivo* transcription analysis in response to RGNNV infection (described in chapter 2A) constitute the basis for the present chapter, in which an *in vitro* system stably expressing the sea bass ISG15 protein has been established to evaluate the sea bass ISG15 anti-betanodavirus activity.

2. MATERIALS AND METHODS

2.1. VIRUSES AND VIRAL PROPAGATION

Viral isolates SpDl_IAusc965.09 (red-spotted grouper, *Epinephelus akaara*, nervous necrosis virus, RGNNV) and SJ93Nag (striped jack, *Pseudocaranx dentex*, nervous necrosis virus, SJNNV) were propagated and titrated on the E-11 cell line (Iwamoto et al., 2000) at 25 ^{o}C or 20 ^{o}C, respectively, as described in chapter 1, section 2.1. From now on, in this chapter, these isolates will be named as RGNNV and SJNNV. Cells were cultured as described in the above mentioned section.

2.2. ESTABLISHMENT OF A CELL LINE CONSTITUTIVELY EXPRESSING SEA BASS ISG15 PROTEIN (Dl_ISG15)

An *in vitro* system (Dl_ISG15_E11), consisting of E-11 cells permanently transfected with a construction containing the sea bass *isg15* open reading frame (ORF) under the control of the cytomegalovirus immediate-early promoter (CMV), has been developed in this study.

2.2.1. Amplification and cloning of the full-length *isg15* ORF

Primers for *isg15* amplification were DlISG15 NotI-F (5'-GATCACAAGGGCGGCCGCATGATGGATATAACC-3') and DlISG15 XhoI-R (5'-TATGAGCCTCGAGGGTGCTCAGCCTCCTCTCAG-3') (Moreno et al., 2016). These primers harbour *NotI* (DlISG15 NotI-F primer) and *XhoI* (DlISG15 XhoI-R primer) restriction sites, which are required for sub-cloning in the expression vector.

Amplification was carried out with the Universe High-Fidelity Hot Start DNA polymerase (Biotools), following the protocol described in chapter 2A (section 2.1.3.), using as template cDNA (400 ng) obtained from head kidney of polyinosinic:polycytidylic acid (poly I:C, CalBiochem)-stimulated sea bass (chapter 2A,

section 2.1.1.). Annealing temperature was 65 ^{o}C. Amplified products were purified from ethidium bromide (Sigma)-stained 2% agarose gels using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), following manufacturer instructions. Purified DNA was quantified with the NanoDrop-1000 system (ND-1000, ThermoFisher), sequenced, and maintained at -20 ^{o}C until cloning in the pGemT easy vector (Promega).

Before cloning, the poly-A tail was added at the 3'-end of the *isg15* sequence in a 10-µl mixture composed of 1x Colorless GoTaq® Reaction Buffer, 0.2 mM PCR Nucleotide Mix, GoTaq® DNA Polymerase (Promega, 1 U) and DNA (50 ng). This reaction was incubated at 72 ^{o}C for 8 min, chilled on ice, and purified with the Illustra GFX PCR DNA and Gel Band Purification Kit, to be finally cloned into the pGemT vector, as described in chapter 1, section 2.2.2.4. The insert presence was confirmed by amplification with the vector M13 primers (Forward, 5'-GTTTTCCCAGTCACGAC-3'; Reverse, 5'-CAGGAAACAGCTATGAC-3'). Selected clones were labelled as Dl_ISG15_pGemT.

2.2.2. Construction of the Dl_ISG15 expression plasmid

The plasmid used for over-expressing the sea bass ISG15 recombinant protein in E-11 cells was pcDNA™4/HisMax B (Invitrogen), containing the CMV promoter and a N-terminal fusion peptide encoding the Xpress™ epitope, as well as a polyhistidine metal-binding tag. Furthermore, this plasmid also harbours the bovine growth hormone polyadenylation sequence (BGH pA), with the sequence motif AAUAAA, which promotes both, polyadenylation and transcription termination, as well as the Zeocin™ resistance gene (Figure 1).

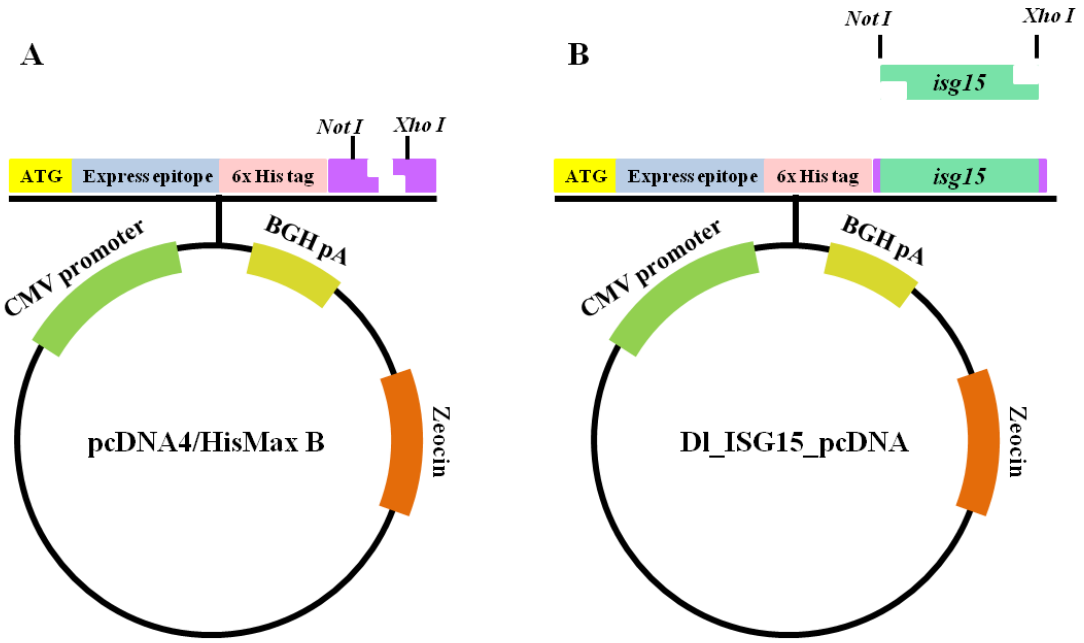

Figure 1. (A) Structure of the pcDNA4/HisMax B vector. (B) Structure of the European sea bass *isg15* cDNA and Dl_ISG15_pcDNA construction. Different sequences are indicated in colour: CMV promoter is represented in green, transcription initiation codon (ATG) is in yellow, N-terminal express epitope is in blue, 6x His tag is in pink, BHG pA sequence is in pale yellow, zeocin-resistance gene is in orange, and *isg15* gene is in turquoise.

For pcDNA4/HisMax B linearization, *NotI* and *XhoI* digestions were simultaneously performed at 37 ^{o}C for 2-4 h. Phosphate groups were removed with the FastAP Thermosensitive Alkaline Phosphatase (Fermentas) reaction (chapter 1, section 2.3.3.).

The *isg15* sequence was set free from the Dl_ISG15_pGemT construction by simultaneous *NotI* and *XhoI* digestions. The linearized vector and the *isg15* ORF were purified from ethidium bromide-stained 1% agarose gels using the Illustra GFX PCR DNA and Gel Band Purification Kit, and they were ligated at 4 ^{o}C overnight. Ligation mixture (40 µl, final volume) consisted of 1x Ligation Buffer, T4 DNA ligase (2 U, Invitrogen) and DNA (3:1, insert:vector). *Escherichia coli* DH5α cells were transformed by electroporation, and bacterial clones were grown and checked for the *isg15* insert presence as described in chapter 1, section 2.2.2.4., using DlISG15 NotI-F and DlISG15 XhoI-R primers (section 2.2.1.), and the GoTaq® DNA Polymerase. Amplified products (510-bp long) were visualized on 2% agarose gels stained with ethidium bromide and sequenced. The plasmid (Dl_ISG15_pcDNA) was finally purified with the Genopure

Plasmid Maxi Kit (Roche), according to manufacturer instructions, and stored at -20 ^{o}C until cell transfection.

Sequencings were always conducted with the ABI 3730 (STABVIDA) system. Sequence analyses and alignments were carried out using the EditSeq and MegAlign software (DNASTAR Lasergene 7).

2.2.3. Transfection of E-11 cells

Cells were transfected using the Lipofectamine® 2000 Transfection Reagent (Invitrogen), following the methodology described by Iwamoto et al. (2001). Briefly, the lipofectamine reagent (15 µl) and the Dl_ISG15_pcDNA construction (15 µg) were added to two separate reactions containing 125 µl of serum-free OptiMEM medium (Gibco). After 5-min incubation at room temperature, both reactions were mixed, and the resulting mixture was incubated for 30 min on ice to be finally added in droplets, along with Leibovitz L-15 medium (Gibco) (1 ml), over previously washed (with L-15 medium) monolayers of E-11 cells grown on 6-well plates (Nunc). These cells were incubated at 25 ^{o}C for 60-120 min. After this incubation, the lipofectamine mixture was removed, and cells were maintained at 25 ^{o}C for 24 h in L-15 medium supplemented with 2% foetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 10 mg/ml streptomycin (Sigma) (maintenance medium). Transfected cells grown in 25-cm^2 flasks (Nunc) were selected with zeocine (1,250 µg/ml, Invitrogen), which was added every other sub-culture.

The presence of sea bass *isg15* DNA and mRNA in transfected cells was demonstrated using DlISG15 NotI-F and DlISG15 XhoI-R primers (section 2.2.1.), and the GoTaq® DNA Polymerase (chapter 1, section 2.2.2.4.). Cellular DNA/RNA was extracted using the Illustra tripleprep kit (GE Healthcare, Life Science) following manufacturer guidelines. Plasmids remaining after transfection were removed by RNA treatment with DNase I Recombinant from bovine pancreas (Roche) following the procedure carried out in chapter 1, section 2.4. Reverse-transcription was conducted with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using 1 µg of RNA as template (chapter 1, section 2.2.1.1.). E-11 cells transfected with the empty expression plasmid (mocked transfected), as well as non-transfected cells, were used as negative controls. However, mocked-transfected cells did not progress after several steps of

zeocine treatment; for this reason, non-transfected cells were the only negative control considered in the subsequent experiments.

2.3. CHARACTERIZATION OF THE Dl_ISG15_E11 CELL LINE

Analyses of cellular growth kinetics, ISG15 detection by immunoblotting, sub-cellular ISG15 location, and *isg15* gene transcription have been performed.

2.3.1. Cellular growth kinetics

Multiplication of Dl_ISG15_E-11 and non-transfected E-11 cells has been comparatively analysed. Both cell lines were seeded ($1.4x10^5$ cells/ml) on 24-well plates (Nunc), being maintained in L-15 medium supplemented with 10% FBS, 100 units/ml penicillin and 10 mg/ml streptomycin (growth medium) at 25 ^oC. Cells from three wells were washed with 1x phosphate buffer saline (PBS, Sigma) and collected by 1x trypsin addition (Sigma) at 2, 3, 4, 5, 7 and 8 days to be counted, in triplicate, using the Z1 coulter particle counter (Beckman Coulter). This assay has been performed in duplicate.

2.3.2. Immunoblotting detection of sea bass ISG15 protein

Both cell lines, E-11 and Dl_ISG15_E11, were grown in 75-cm^2 flasks (Nunc) at 25 ^oC until confluence. Supernatants and cells were then separately collected and processed to detect, independently, extracellular and intracellular recombinant Dl_ISG15 protein.

Extracellular Dl_ISG15 proteins were purified and concentrated with an immuno-precipitation kit (Roche), using protein A/G agarose beads, the 6xHis-protein tag, and the HisG Epitope Tag Monoclonal Antibody (Invitrogen, Thermo). As a first step, Dl_ISG15-containing medium was precleared with protein A/G agarose, to reduce background caused by non-specific binding. Thus, a volume of 50 µl of protein A/G agarose was added, and this mixture was incubated at 4 ^oC overnight, followed by centrifugation at 12,000 x *g* at room temperature for 20 s. Recombinant protein in this precleared medium was bound to the HisG Epitope Tag Monoclonal Antibody (2.5 µg) by incubation at 4 ^oC for 1 h. Protein A/G agarose (50 µl) was subsequently added, and this mixture was incubated at 4 ^oC overnight to complex formation, which were washed and collected by successive centrifugation (12,000 x *g* at room temperature for 20 s) and

wash cycles (with buffer 1, 2 and 3). Finally, loading buffer (50 µl) was added, denatured by heating at 100 ^{o}C for 3 min, and analysed by western blot.

For intracellular ISG15 detection, cells were washed twice with 1x PBS, sonicated at 4 ^{o}C for 10 min (Ultrasons H-D, Selecta) and centrifuged at 1,500 x *g* at 4 ^{o}C for 5 min. Cellular debris was discarded, and proteins in the medium were concentrated, using the centrifugal filter Amicon® Ultra-15 (Merck) with a molecular weight cut-off (MWCO) of 3,000, at 5,000 x *g* at room temperature for 30 min. The resulting sample was analysed by western blot.

For western blotting, proteins were separated by 12.5% SDS-PAGE electrophoresis, and subsequently transferred to an Immobilon membrane (Millipore) using a standard protocol (Mahmood and Yang, 2012). The HisG Epitope Tag Monoclonal Antibody (1/1,000 dilution in 5% skimmed-powder milk-PBS-Tween20) was used to detect the 6xHis-tagged ISG15 protein. The monoclonal Anti-β-Actin Antibody (Sigma, 1/5,000 in 5% skimmed-powder milk-PBS-T) was used to detect beta-actin, used as cellular control. The secondary antibody used was HRP-conjugated goat anti-mouse IgG (Sigma, 1/20,000 dilution in 5% skimmed-powder milk-PBS-T). The specific band was visualized using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo) and the ChemiDoc™ Imaging Systems (Bio-Rad), following manufacturer guidelines.

2.3.3. Sub-cellular location of sea bass ISG15 protein

The Dl_ISG15 protein has been located by indirect immunofluorescence, using as target the 6xHis tag fusioned to the N-terminal end of the recombinant protein.

Cells were seeded on 8-well slides (Lab-Tek™ Chamber Slide System, Nunc) and cultured at 25 ^{o}C in L-15 growth medium. Once confluent monolayers were observed, cells were washed three times with 1x PBS for 5 min, fixed with methanol/acetone (1/1, v/v) at -20 ^{o}C for 20 min, and finally washed three times (1x PBS) for 10 min. All washes were carried out at room temperature.

Fixed cells were incubated with Triton-X (0.3% in PBS, v/v, Sigma) for 10 min for cell membrane permeabilization. Afterwards, they were washed three times with 1x PBS for 10 min, and blocked with 5% skimmed-powder milk (Sigma) for 20 min. After blocking, the HisG Epitope Tag Monoclonal Antibody (1/1,000 dilution in 5%

skimmed-powder milk-PBS) was added and incubated for 1 h, followed by three washes with 1x PBS and the incubation with the goat anti-mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 (Thermo, 1/1,000 dilution in 5% skimmed-powder milk-PBS) for 1 h in darkness. After several 10-min washes with 1x PBS, slides were finally mounted with mounting medium for immunofluorescence microscopy (Sigma) and visualised with the confocal microscope SP5 II (Leica). All incubations were conducted at room temperature.

2.3.4. European sea bass *isg15* gene transcription

In order to evaluate the transcription of sea bass *isg15* in Dl_ISG15_E11 cells, *isg15* mRNA has been analysed by semi-quantitative RT PCR throughout several cellular sub-cultures. Cells grown in 25-cm^2 flasks were harvested, by scraping, at 2, 4, 6 and 8 sub-cultures, and RNA was extracted with the E.Z.N.A. Total RNA Kit I (Omega bio-tek). After RNA treatment with DNase I Recombinant from bovine pancreas, cDNA was obtained with the Transcriptor First Strand cDNA Synthesis Kit, using 1 μg of RNA as template (chapter 1, section 2.2.1.1.). Sea bass *isg15* cDNA amplification was performed with the GoTaq® DNA Polymerase (chapter 1, section 2.2.2.4.), and the specific primers DlISG15 NotI-F and DlISG15 XhoI-R (section 2.2.1.) (Moreno et al., 2016). Amplified products were visualized on 2% agarose gels stained with ethidium bromide.

In addition, the possible effect of viral infection on sea bass *isg15* transcription has been evaluated. Transcription analyses were performed by relative real-time PCR in presence or absence of RGNNV or SJNNV. Each virus was inoculated at 0.1 multiplicity of infection (MOI) on Dl_ISG15_E-11 cells grown on 24-well plates. Inoculated cells were maintained in L-15 medium added with 100 units/ml penicillin and 10 mg/ml streptomycin (inoculation medium) at 25 ^oC or 20 ^oC for 1 h for RGNNV or SJNNV adsorption, respectively. The viral suspension was then removed, and L-15 maintenance medium was added. Cells from three wells were harvested by scraping at 24 h post-infection (p.i.), and total RNA was extracted with the E.Z.N.A. Total RNA Kit I, following manufacturer instructions. After treatment with DNase I Recombinant from bovine pancreas (chapter 1, section 2.4.), RNA (1 μg) was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (chapter 1, section 2.2.1.1.).

PCR amplifications were performed with the LightCycler 96 Thermocycler, using cDNA generated from 50 ng of RNA, 1x Fast Start Essential DNA Green Master Mix (Roche) and 10 pmol primers qISG15 F2 (5'-CGACTCAAAGCCTCTCTGCTACT-3') and qISG15 R2 (5'-CGTTTCTGACGAACACCTGGAT-3'), which amplify a 100-bp fragment within the sea bass *isg15* sequence (Moreno et al., 2016), in 20-µl mixtures. Amplifications consisted of denaturation at 95 ^{o}C for 10 min, followed by 45 cycles at 95 ^{o}C for 10 s, 52 ^{o}C for 10 s and 72 ^{o}C for 10 s. Melting curves were obtained at 95 ^{o}C for 10 s, 65 ^{o}C for 60 s and 97 ^{o}C for 1 s. Beta-actin was the endogenous reference gene used (section 2.4.1.3.). Relative values were calculated using the $2^{-\Delta Ct}$ method. This assay has been performed in duplicate.

2.4. ANTI-BETANODAVIRUS ACTIVITY OF SEA BASS ISG15

The activity of the intracellular and extracellular Dl_ISG15 protein has been evaluated against RGNNV (highly virulent to European sea bass) and SJNNV (with low virulence to this fish species).

2.4.1. Analyses of ISG15 intracellular activity

Viral replication on Dl_ISG15_E11 and E-11 cells has been comparatively analysed by two different approaches: virus quantification, and cellular survival assays. In addition, the possible modulation of the transcription of mx dynamin like GTPase (*mx*), toll-like receptor 3 (*tlr3*) and ubiquitin ligase E3 (*e3*) genes in RGNNV- and SJNNV-inoculated Dl_ISG15_E11 cells has also been evaluated.

2.4.1.1. Viral quantification: experimental design and sample processing

Each virus was inoculated at 0.1 MOI on confluent monolayers of Dl_ISG15_E11 and E-11 cells grown on 24-well plates. For virus adsorption, cells were maintained for 1 h at 25 (for RGNNV) or 20 ^{o}C (for SJNNV) in L-15 inoculation medium. After this incubation, viral suspensions were removed, and L-15 maintenance medium was added. Supernatants and cells from three wells were separately collected at 0, 24, 48 and 72 h p.i. The titre of infective viral particles in supernatants was determined by the 50% tissue culture infective dose method ($TCID_{50,}$ Reed and Muënch, 1938) in triplicate, whereas cells were processed for viral genome quantification by absolute real-time PCR.

Cellular RNA, extracted with the E.Z.N.A. Total RNA Kit I, was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (chapter 1, section 2.2.1.1.). PCRs were performed with the LightCycler 96 Thermocycler and the Fast Start Essential DNA Green Master Mix. The pairs of primers RG_965_RNA2 F4/RG_965_RNA2 R1 and SJ-RNA2-F/SJ-RNA2-R (Table 1) were used to amplify the RGNNV or SJNNV RNA2 segment, respectively. Serial dilutions of the pJET vector containing the complete RGNNV RNA2 sequence (chapter 1, section 2.3.1.), and the pCR™4-TOPO® TA vector (Invitrogen) containing the complete SJNNV RNA2 sequence (Lopez-Jimena et al., 2014) were used to generate reference standard curves. All amplifications were conducted following the procedure described in chapter 1, section 2.6.1.2. These assays have been performed in duplicate.

Table 1. Primers used for absolute quantification of the RGNNV or SJNNV RNA2 viral segment.

Name	Sequence (5'-3')	Product length (bp)	Reference
RG_965_RNA2 F4	ACCGTCCGCTGTCTATTGACTA	126	Moreno et al. (2016)
RG_965_RNA2 R1	CAGATGCCCCAGCGAAACC		
SJ-RNA2-F	GACACCACCGCTCCAATTACTAC	75	Lopez-Jimena et al. (2014)
SJ-RNA2-R	ACGAAATCCAGTGTAACCGTTGT		

2.4.1.2. Cellular survival: experimental design and sample processing

Viruses were inoculated at 0.1 MOI on confluent monolayers of Dl_ISG15_E11 and E-11 cells grown on 24-well plates, as described in section 2.4.1.1. Cells from three wells were collected by trypsin treatment at 0, 48 and 72 h p.i. to be counted with the Z1 coulter particle counter (Beckman Coulter), in triplicate. Non-inoculated cells, considered as negative control, were processed in the same way.

2.4.1.3. Modulation of immunogene transcription in the course of viral infections

This study has evaluated the possible modulation of *mx, tlr3* and *e3* transcription in E-11 cells (derived from *Channa striata*) expressing the European sea bass ISG15

protein. The transcription of these genes in inoculated E-11 and Dl_ISG15_E11 cells has been comparatively analysed by relative real-time PCR.

Confluent monolayers of E-11 and Dl_ISG15_E11 cells seeded on 24-well plates were inoculated with RGNNV or SJNNV at 0.01 MOI, as described in section 2.4.1.1. After virus inoculation, cells from three wells were collected by scraping at 3, 6 and 24 h p.i. for transcription analyses. Cellular RNA was extracted with the E.Z.N.A. Total RNA Kit I, treated with DNase I Recombinant from bovine pancreas (chapter 1, section 2.4.), and reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit, using 1 μg of RNA as template (chapter 1, section 2.2.1.1.).

The specific pairs of primers TLR3 E11 F/TLR3 E11 R and E3 E11 F/E3 E11 R (Table 2) were designed in this study to amplify conserved regions within *C. striata tlr3* and *e3* genes, respectively. All amplifications were conducted with the LightCycler 96 Thermocycler and the Fast Start Essential DNA Green Master Mix, using cDNA generated from 50 ng of RNA as template (section 2.3.4.). Beta-actin was the endogenous reference gene used (Table 2). Relative fold change values were calculated using the Pfaffl method (Pfaffl, 2004).

Table 2. Primers used for relative quantification of *tlr3*, *e3* and *mx* gene transcription from the E-11 cell line.

Name	Sequence (5'-3')	Product length (bp)	Reference
TLR3 E11 F	TGCAACACTCCACTGACTTACTTTAA	115	This study
TLR3 E11 R	AGGACAGCTGTGCTAAGTATATAA		
E3 E11 F	TGCACTTGCAAGGCTGTCA	100	This study
E3 E11 R	CTCCTAGGATACTTGCATAGAAGACAAC		
Mx protein F	GGGGTCAGAAGGAGATCACA	150	Poisa-Beiro et al. (2008)
Mx protein R	ATGATGCACCAGCTCAAGTG		
E11 beta-actin F	CACTGTGCCCATCTACGAG	200	Chen et al. (2017)
E11 beta-actin R	CCATCTCCTGCTCGAAGTC		

2.4.2. Analyses of ISG15 extracellular activity

The cytokine-like activity of the extracellular Dl_ISG15 protein against RGNNV and SJNNV infections has been evaluated by co-culture of E-11 and Dl_ISG15_E11 cells, as well as by E-11 incubation with ISG15-containing medium.

2.4.2.1. Co-culture in droplets

The methodology used in this study (Parreño et al., 2016) allows the co-culture of two different cell lines (donor and target cells) in droplets within the same well in a 6-well plate. The possible protective effect of Dl_ISG15_E11 cells (sea bass ISG15-producing cells) over RGNNV- or SJNNV-inoculated E-11 cells was measured by crystal violet staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction quantification.

Droplets (150 µl) of donor (Dl_ISG15_E11) and target (E-11) cells, containing the same number of cells ($9x10^4$) were separately seeded on the same well, and they were incubated in a humid chamber at 25 ^oC overnight for cellular attachment to the plate surface. A negative control well, in which two droplets of E-11 cells were seeded, was also set up. After that time, droplets were carefully removed, and a volume of 2 ml of L-15 growth medium was added. These cells were incubated at 25 ^oC for 24 h before virus inoculation (10^4 $TCID_{50}$/ml, final concentration), which was carried out as described in section 2.4.1.1. Inoculated cells were incubated at optimal virus replication temperature until fully cytopathic effect (CPE) appearance. A negative control, consisting of non-inoculated cells, was also included. This assay has been conducted in duplicate.

Once CPEs were developed, cells in two wells were fixed and stained with 1% crystal violet in 22% formaldehyde (Sigma) at room temperature for 10 min. After several washes with 1x PBS at room temperature for 5 min, droplets were photographed and pictures were digitally analysed by the ImageJ (NIH) software.

Since crystal violet staining is a semi-quantitative technique to estimate the level of surviving E-11 target cells after viral infection, a modification of the above explained protocol, based on MTT reduction, has been developed. This experimental design was essentially the same described previously, with only one modification: cells in droplets were attached on glass coverslips (Nunc™ Thermanox™ Coverslips, Thermo), instead

of on the plate surface. These coverslips were placed into 6-well plates for virus inoculation and incubation until CPE observation, as described previously.

Once fully CPEs were observed, coverslips were moved to a 24-well plate for MTT reduction development. Cells were then washed with 1x PBS before adding L-15 growth medium. Afterwards, MTT (Sigma, 50 µg/µl) was added and cells were incubated at 25 ^{o}C for 4 h for colorimetric reaction, which was stopped by adding acidic isopropanol (0.04 N HCl in isopropanol, 150 µl). Optical density (OD) was quantified at 550 nm using the Whittaker Microplate Reader 2001 (Anthos Labtec). The resulting values were normalized by subtracting the OD values obtained for the negative controls (L-15 medium in absence of cells).

2.4.2.2. Culture of E-11 cells with sea bass ISG15-containing medium

The role of the secreted Dl_ISG15 protein in the protection of E-11 cells from viral infection has been further investigated by culturing target E-11 cells with medium from the sea bass ISG15-producing cell line.

In order to obtain this medium, sea bass ISG15-producing (Dl_ISG15_E11) and non-producing (E-11) cells were seeded in 25-cm^2 flasks and cultured at 25 ^{o}C for 72 h. After this period, the medium was collected, aliquoted and stored at -20 ^{o}C until used. All the experiments described below have been conducted with these batches of ISG15-containing and ISG15-free media.

E-11 cells grown on 96-well plates were inoculated with RGNNV (10^4 $TCID_{50}$/ml) in L-15 inoculation medium at 25 ^{o}C for 1 h. Afterwards, viruses were removed, and L-15 maintenance medium mixed with medium from sea bass ISG15-producing cells (1:1) was added. A control group, consisting of inoculated cells incubated with medium from non-producing E-11 cells (ISG15-free medium, in the same proportion), was also included. Plates were incubated at 25 ^{o}C until fully CPE development, and the protective effect was visualized from three wells by MTT addition, as described in section 2.4.2.1. This assay has been conducted in duplicate.

2.5. STATISTICAL ANALYSES

Data were statistically analysed with the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA). Normality distribution was verified by the Shapiro-Wilk test, and t-student was the statistical tests used. Values of $p < 0.05$ were considered significant.

3. RESULTS

3.1. CHARACTERIZATION OF THE Dl_ISG15_E11 CELL LINE

The presence of sea bass *isg15* DNA and mRNA in Dl_ISG15_E11 cells was first visualized by PCR and RT PCR (Figure 2). The identity of the 510-bp long amplified product was confirmed by sequencing. No bands were observed when DNA or cDNA from control non-transfected E-11 cells was analysed.

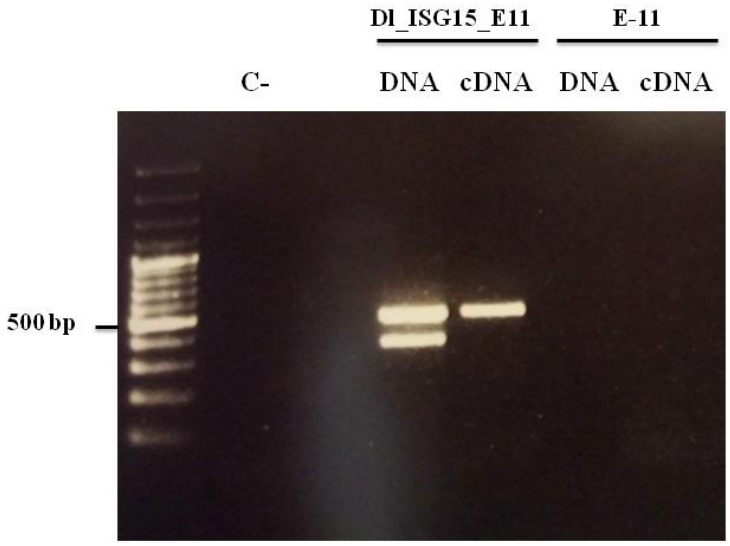

Figure 2. Amplification of sea bass *isg15* DNA or cDNA obtained from Dl_ISG15_E11 or non-transfected E-11 cells. DNA ladder: Generuler 100 bp.

This system has been further characterized, prior to perform the sea bass ISG15 antiviral studies, by analysing cellular growth kinetics, Dl_ISG15 detection, sub-cellular protein location, and sea bass *isg15* gene transcription.

3.1.1. Cellular growth kinetics

Growth curve analysis (Figure 3) showed the same growth performance for Dl_ISG15_E11 and E-11 cells ($p > 0.05$), since cell concentration was the same for both cell types at all sampling times considered. Maximum cell concentration was recorded after 7 days of incubation, with $3.9x10^5$ and $3.6x10^5$ cells/ml for E-11 and Dl_ISG15_E11 cells, respectively. Concentration of both cell types seems to reach a plateau at longer sampling times.

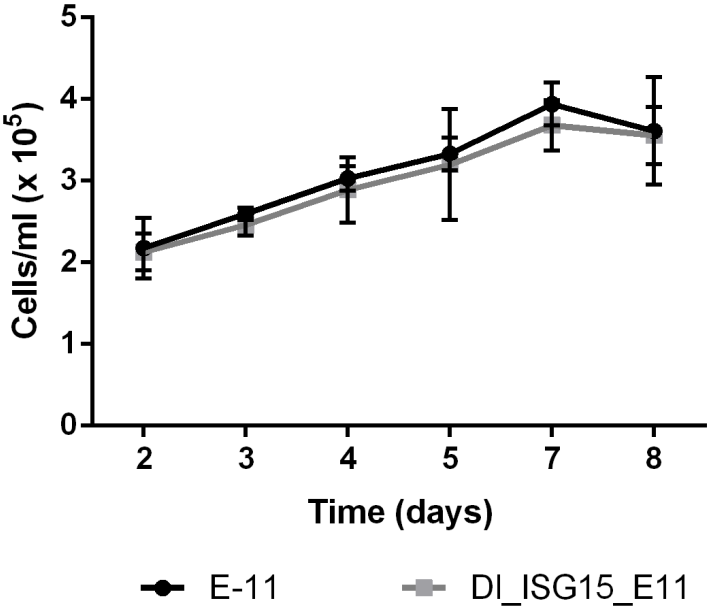

Figure 3. Growth curves of Dl_ISG15_E11 and E-11 cells. The t-student test was the statistical analysis performed. Values of $p < 0.05$ were considered significant. Results are mean ± standard deviation (SD) (n = 3).

3.1.2. Detection of Dl_ISG15 protein

The recombinant Dl_ISG15 protein has been detected by western blot both, extracellularly and intracellularly (Figure 4). The extracellular detection of Dl_ISG15 is depicted in Figure 4A, showing a single band of the expected size (20-25 kDa) after analysing medium from Dl_ISG15-producing cells (lane 4). However, ISG15 concentration by immuno-precipitation was required, since no bands were observed by processing crude Dl_ISG15-producing cell medium (lane 2). In addition, ISG15 was not observed when control E-11 medium was processed in the same way (lane 3).

In order to clarify if the extracellular Dl_ISG15 protein is secreted or is a consequence of cellular lysis, the presence of beta-actin in the extracellular fraction has been evaluated. As it is shown in Figure 4B, beta-actin was detected in concentrated extract of both cell types, Dl_ISG15_E11 (lane 7) and E-11 (lane 8), which constitutes a positive control for the beta-actin detection. Lanes 9 and 10 show absence of beta-actin in the extracellular fraction of Dl_ISG15_E11 and E-11 cells, respectively, suggesting absence of cellular proteins in the medium derived from cellular disruption.

The intracellular Dl_ISG15 detection is represented in Figure 4C, showing the presence of this protein only within Dl_ISG15_E11 cells (lane 13). E-11 cells have been analysed as negative control (lane 12).

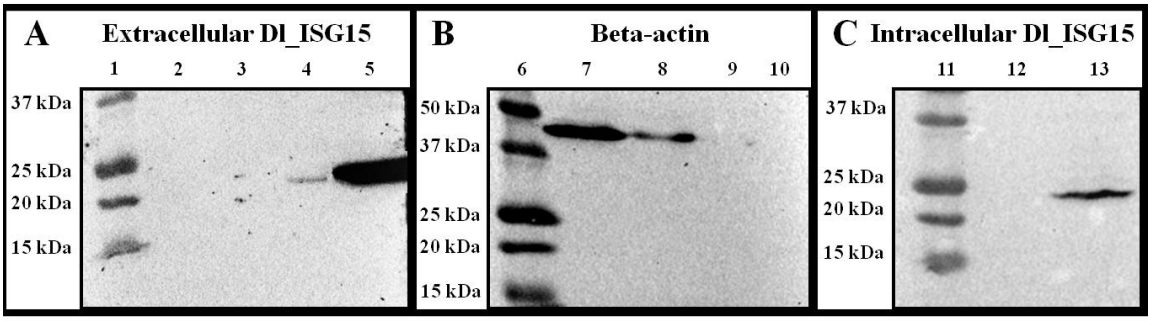

Figure 4. Detection of the recombinant Dl_ISG15 protein by western blot. Lanes 1, 6 and 11: precision plus protein standard marker (Bio-Rad). (A) Extracellular Dl_ISG15 protein. Lane 2: non-concentrated Dl_ISG15_E11 medium. Lane 3: E-11 medium. Lane 4: immuno-precipitated Dl_ISG15_E11 medium. Lane 5: positive control. (B) Beta-actin (cellular control). Lane 7: Dl_ISG15_E11 cell extracts. Lane 8: E-11 cell extracts. Lane 9: immuno-precipitated Dl_ISG15_E11 medium. Lane 10: immuno-precipitated E-11 medium. (C) Intracellular Dl_ISG15 protein. Lane 12: E-11 control cell extracts. Lane 13: Dl_ISG15_E11 cell extracts.

3.1.3. Sub-cellular location of Dl_ISG15 protein

The sub-cellular location of the recombinant Dl_ISG15 protein is visualized in Figure 5, which shows specific green immunofluorescence signal in the cytoplasm of Dl_ISG15_E11 cells (Figures 5A and 5B). No signal was detected in E-11 cells (Figure 5C). In addition, both cellular types show similar morphological characteristics, as visualized by brightfield microscopy (Figure 5).

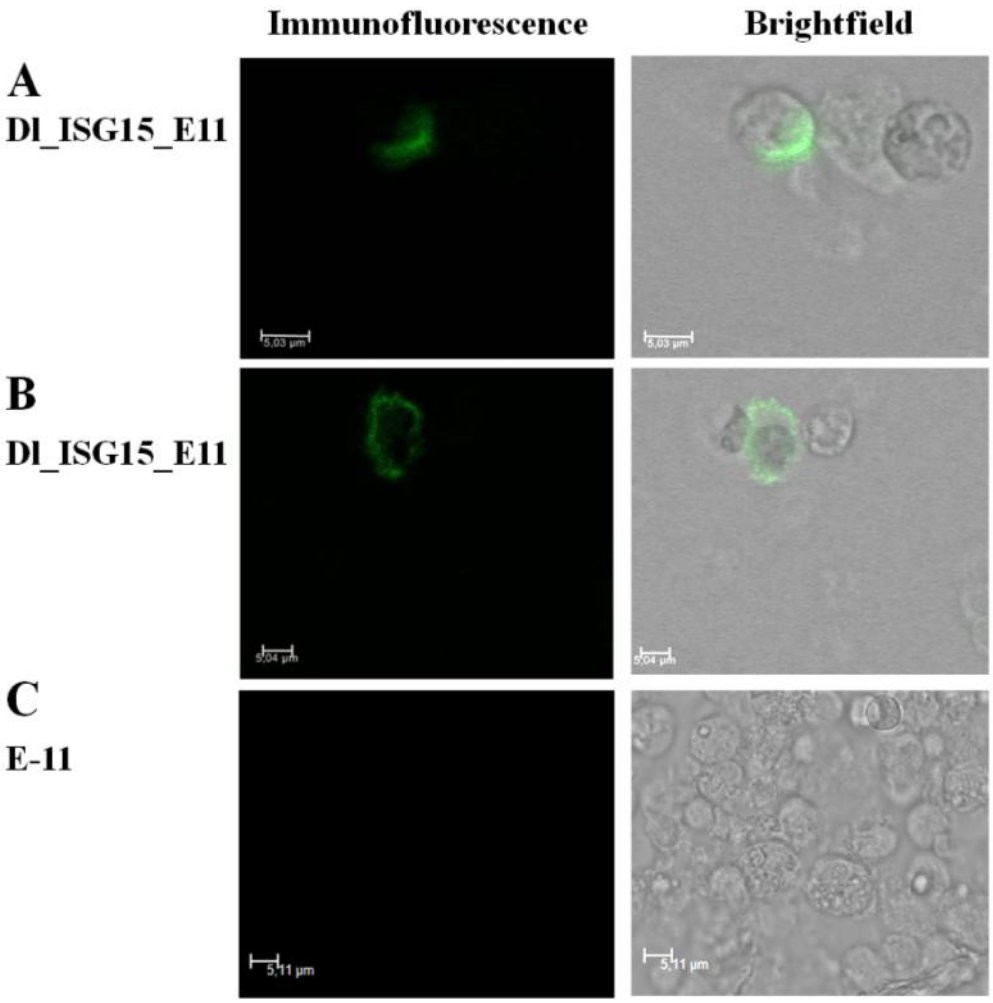

Figure 5. Expression and localization of Dl_ISG15 protein by immunofluorescence. (A, B) Dl_ISG15_E11 cells (C) Control non-transfected E-11 cells. The recombinant Dl_ISG15 protein is labelled in green.

3.1.4. Transcription of sea bass *isg15* gene

Finally, the stable transcription of sea bass *isg15* has been analysed (Figure 6). The transcription was first evaluated by RT PCR using as template cDNA obtained from different cellular sub-cultures (Figure 6A). A single 510-bp band, which was confirmed by sequencing, was obtained from all samples analysed. No bands were visualized when cDNA from control non-transfected E-11 cells was analysed.

As a second step for transcription analyses, the level of *isg15* mRNA in absence and in presence of viral infection has been analysed by relative real-time PCR. Figure 6B evidences no significant differences ($p > 0.05$) in *isg15* gene transcription due to betanodavirus infection (RGNNV or SJNNV species), with mean relative values of 0.96, 0.90 and 0.63 for non-infected, RGNNV- and SJNNV-infected cells, respectively.

Viral multiplication in these cells has been confirmed by absolute real-time PCR (data non-shown).

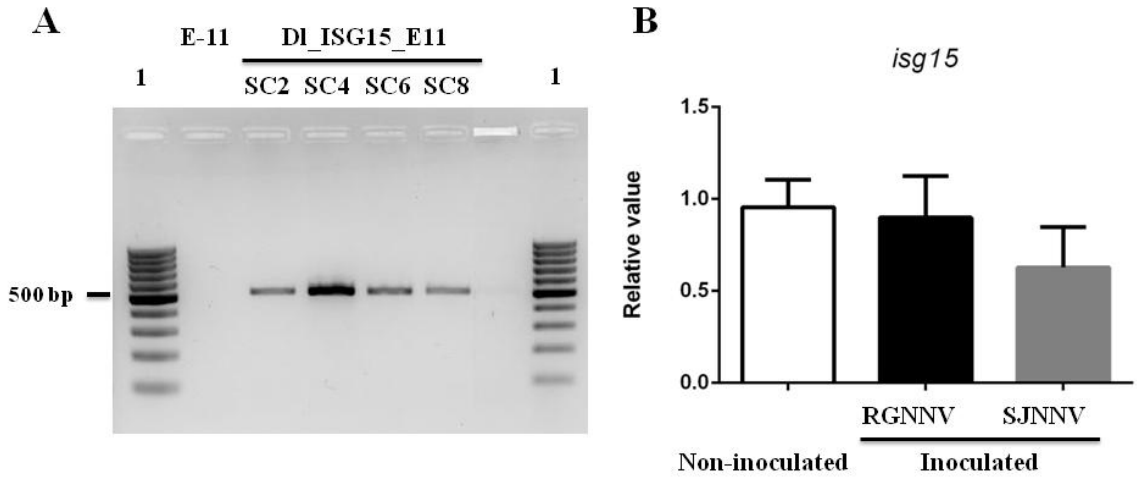

Figure 6. Sea bass *isg15* transcription. (A) Amplification of *isg15* cDNA obtained from non-transfected E-11 cells, as well as from Dl_ISG15_E11 cells at sub-culture (SC) 2, 4, 6 and 8. Lane 1. DNA ladder: Generuler 100 bp. (B) *isg15* mRNA quantification in non-inoculated, RGNNV- and SJNNV-inoculated Dl_ISG15_E11 cells. The t-student test was the statistical analysis performed. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 3).

3.2. ANTI-BETANODAVIRUS ACTIVITY OF SEA BASS ISG15 PROTEIN

The intracellular and cytokine-like activities of Dl_ISG15 have been analysed using the Dl_ISG15_E11 cell line, evaluating its antiviral role against RGNNV and SJNNV.

3.2.1. Antiviral activity of the intracellular Dl_ISG15 protein

The antiviral activity of Dl_ISG15 protein has been evaluated by comparing viral multiplication in transfected and non-transfected cells. Viral multiplication has been determined by quantification of viral genome within the cells (RT real-time PCR), and by titration of infective viral particles ($TCID_{50}$ method) in the supernatant. In addition, in a parallel assay, survival rates of inoculated cells have also been determined.

The results of viral genome quantification are shown in Figure 7. The number of copies of RGNNV RNA2 was similar in both cell types (Dl_ISG15-producing and non-producing cells) at all sampling times considered (Figure 7A, $p > 0.05$). However, the number of SJNNV RNA2 copies was lower in Dl_ISG15-producing cells at 48 h ($p = 0.004$) and 72 h ($p = 0.01$) p.i. (Figure 7B), recording mean values of $3.2x10^{10}$ and

$2.4x10^9$ RNA2 copies/µg RNA at 48 h p.i. in E-11 and Dl_ISG15_E11 cells, respectively.

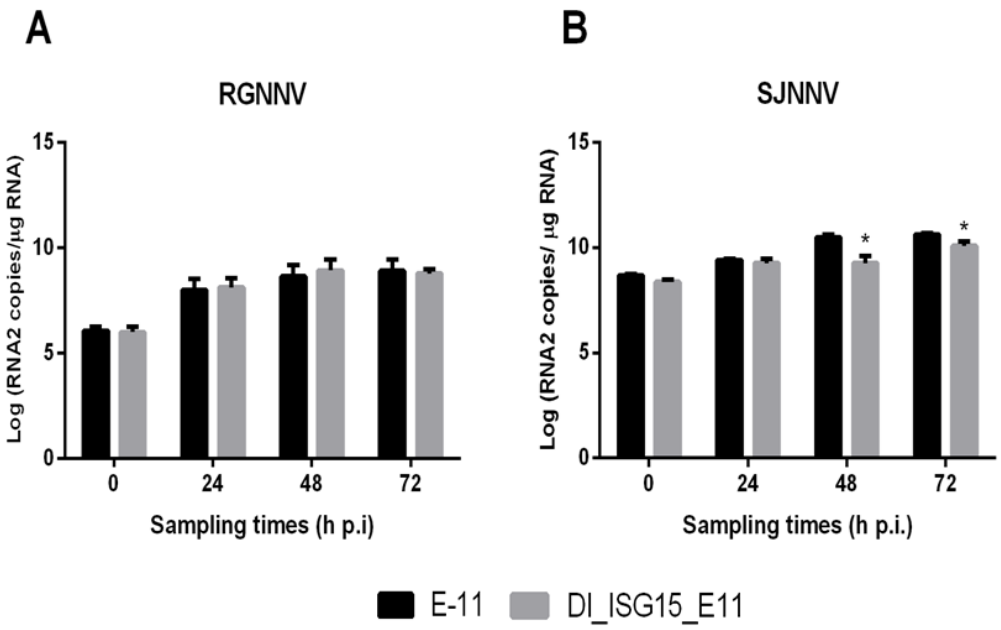

Figure 7. Number of RNA2 copies in E-11 and Dl_ISG15_E11 cells inoculated at 0.1 MOI with RGNNV (A) or SJNNV (B). The t-student test was the statistical analysis performed. * indicates significant differences between E-11 and Dl_ISG15_E11 cells within each sampling time. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 3).

The virus titration yielded similar results (Figure 8). Thus, viral titres recorded in supernatants of RGNNV-inoculated Dl_ISG15_E11 and E-11 cells (Figure 8A) were similar ($p > 0.05$) at all sampling times analysed, with maximum titres recorded at 72 h p.i. ($9.9x10^5$ and $6.4x10^5$ $TCID_{50}$/ml, for E-11 and Dl_ISG15_E11, respectively). However, significant differences ($p = 0.01$) were recorded regarding the SJNNV titre (Figure 8B). Thus, SJNNV titre in supernatants of Dl_ISG15_E11 cells ($2.2x10^3$ $TCID_{50}$/ml) was significantly lower than the titre obtained in E-11 cell supernatants ($1.2x10^4$ $TCID_{50}$/ml) at 48 h p.i. The maximum SJNNV titre was observed at 72 h p.i., with mean values of $1.9x10^5$ and $8.9x10^4$ $TCID_{50}$/ml, for E-11 and Dl_ISG15_E11 cells, respectively (Figure 8B).

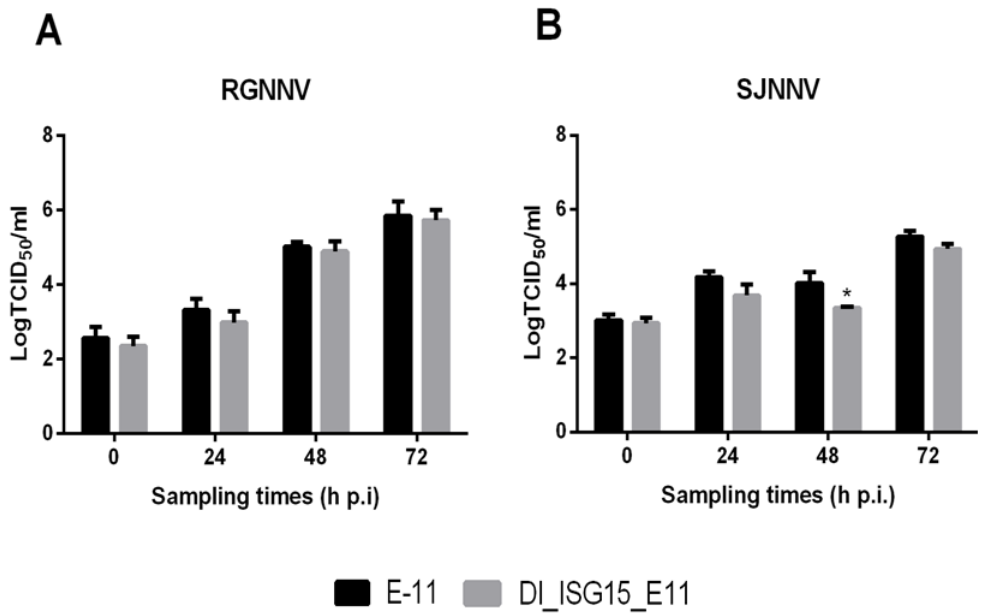

Figure 8. Infective viral particles in supernatants of E-11 and Dl_ISG15_E11 cells inoculated at 0.1 MOI with RGNNV (A) or SJNNV (B). The t-student test was the statistical analysis performed. * indicates significant differences between E-11 and Dl_ISG15_E11 cells within each sampling time. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 3).

In order to complete the evaluation of the anti-betanodavirus role of Dl_ISG15 protein, a survival assay of E-11 and Dl_ISG15_E11 cells inoculated with RGNNV or SJNNV has been performed.

Regarding RGNNV-inoculated cells, E-11 and Dl_ISG15_E11 survival percentages were statistically similar ($p > 0.05$, Figure 9A), with the minimum mean value (ca. 80%) recorded at 72 h p.i. However, the percentage of cells surviving SJNNV infection was significantly higher for cells expressing the sea bass ISG15 protein than for E-11 cells at 48 and 72 h p.i. ($p = 0.01$ and $p = 0.005$, respectively). Thus, the survival rate of Dl_ISG15_E11 cells was 100% at 48 h p.i. and 95% at 72 h p.i., whereas E-11 survival rates were 83% at 48 h p.i., and 85% at 72 h p.i. These results were corroborated by CPE observation at 72 h p.i. (Figure 10).

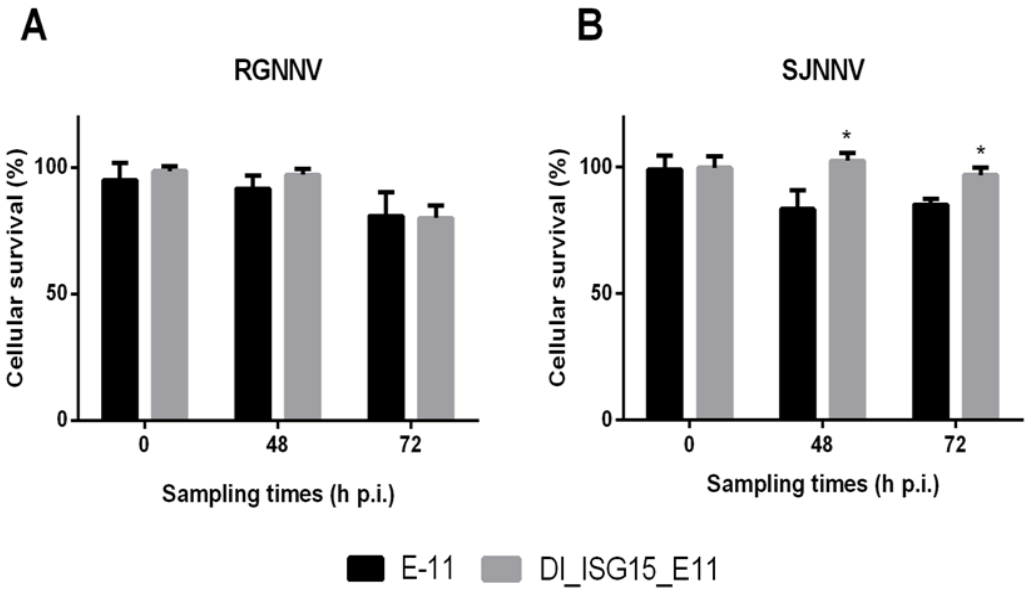

Figure 9. Survival percentage of E-11 and Dl_ISG15_E11 cells inoculated at 0.1 MOI with RGNNV (A) or SJNNV (B). The t-student test was the statistical analysis performed. * indicates significant differences between E-11 and Dl_ISG15_E11 cells within each sampling time. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 3).

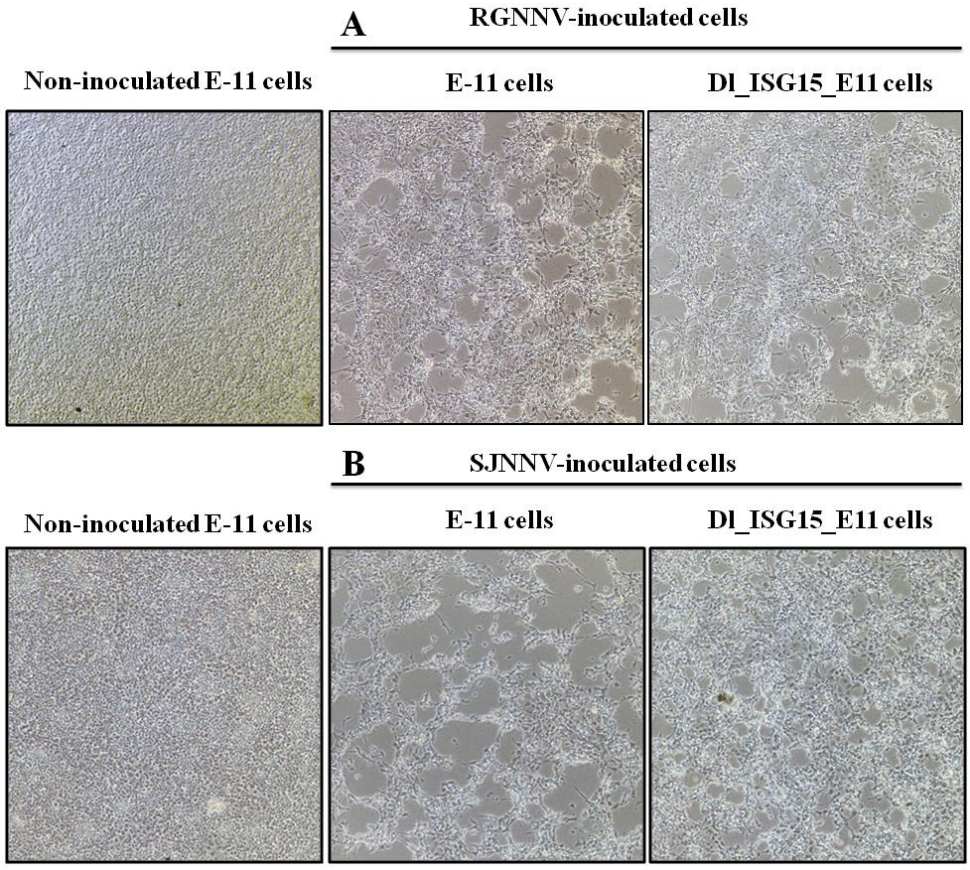

Figure 10. CPE development in RGNNV- (A) and SJNNV-inoculated cells (B) observed at 72 h p.i. (40x, Eclipse Ti microscopy, Nikon).

3.2.2. Intracellular immunomodulation

The possible modulation of the transcription of *mx*, *tlr3* and *e3* endogenous genes has been evaluated in sea bass ISG15-producing cells, both in presence and in absence of viruses, by comparison with the transcription of these genes in control E-11 cells.

In absence of viruses (Figure 11), *e3*, *tlr3* and *mx* genes were down-regulated in Dl_ISG15_E11 cells, compared with the transcription of these genes in control E-11 cells, with relative mean fold change values below 1.5 (0.17, 0.1 and 0.4, for *e3*, *tlr3* and *mx* respectively).

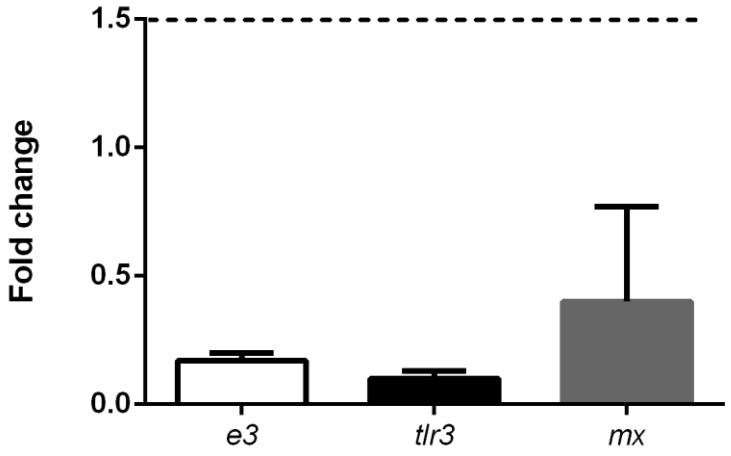

Figure 11. Relative quantification of *e3*, *tlr3* and *mx* transcription in non-inoculated Dl_ISG15_E11 cells. The cut-off value considered for induction was 1.5. The t-student test was the statistical analysis performed. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 3).

After RGNNV infection, the transcription of *mx* gene, shown in Figure 12A, was triggered only at 6 h p.i. in E-11 cells (3.19 mean fold change value). Regarding SJNNV-inoculated cells (Figure 12B), significant transcription was recorded at 3 and 6 h p.i. in E-11 cells (2.6 and 4.9 mean fold change values, respectively) and only at 6 h p.i. in Dl_ISG15_E11 cells (6.9), without significant differences between both cell lines ($p > 0.05$).

The transcription of *tlr3* gene (Figures 12C and 12D) was significantly induced in E-11 cells inoculated with RGNNV at all sampling times, with the maximum transcription mean fold change value (12.6) at 6 h p.i., whereas this transcription was knocked out in RGNNV-infected Dl_ISG15_E11 cells (Figure 12C). However, after

SJNNV infection (Figure 12D), *tlr3* transcription was only triggered at 6 h p.i. in Dl_ISG15_E11 cells, without significant differences between both cell lines ($p > 0.05$).

Finally, the transcription of the *e3* gene is induced in Dl_ISG15_E11 cells infected with RGNNV (Figure 12E) at all sampling times analysed, with the maximum transcription value recorded at 24 h p.i. (4.6 mean fold change value), being significantly higher ($p = 0.001$) than the induction value recorded in E-11 cells at the same sampling time (non-induced, mean fold change value < 1.5). In SJNNV-infected groups (Figure 12F), *e3* transcription was also observed in Dl_ISG15_E11 cells, at 3 and 24 h p.i., although without significant differences between the cell lines analysed ($p > 0.05$).

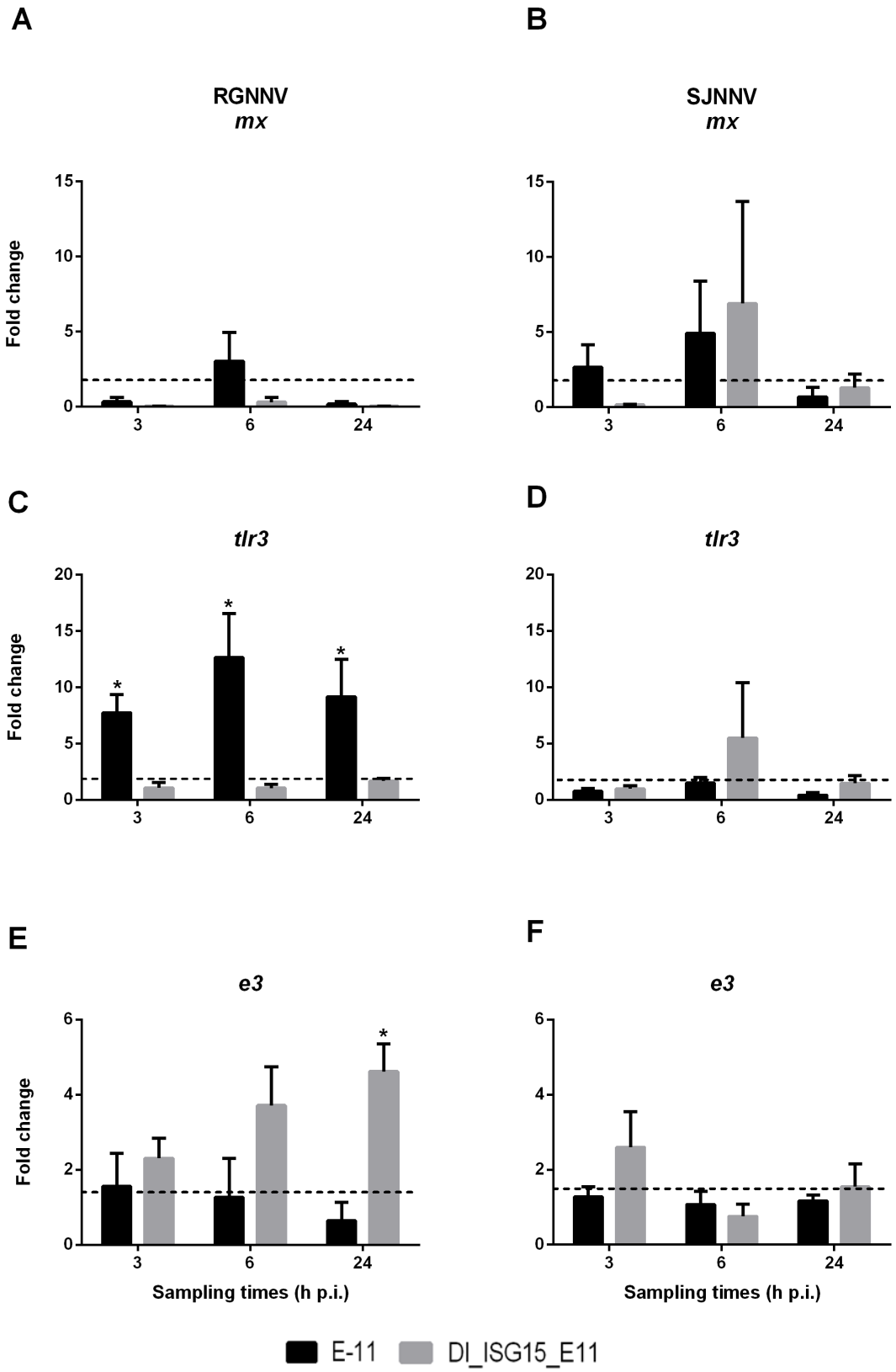

Figure 12. Relative quantification of *e3*, *tlr3* and *mx* transcription in RGNNV- (A, C, E), and SJNNV-inoculated cells (B, D, F). The t-student test was the statistical analysis performed. * indicates significant differences between E-11 and Dl_ISG15_E11 cells within each sampling time. Values of $p < 0.05$ were considered significant. Samples with mean fold change values below 1.5 were considered as non-induced compared with samples at 0 h p.i. Results are mean ± SD (n = 3).

3.2.3. Antiviral activity of the extracellular Dl_ISG15 protein

The role of European sea bass ISG15 protein as secreted cytokine, with potential protective effect against RGNNV and/or SJNNV infections, has been evaluated by co-culture of E-11 and Dl_ISG15_E11 cells in droplets, and by E-11 incubation with ISG15-containing medium obtained from Dl_ISG15-producing cells.

Results obtained by applying the droplet assay showed putative protective effect of the Dl_ISG15 protein to co-cultured E-11 cells inoculated with either, RGNNV or SJNNV (Figure 13).

Figures 13A and 13B show the results obtained estimating the percentage of surviving E-11 cells by crystal violet staining. The intensity of the staining was evaluated, being maximum for non-inoculated E-11 target cells (either E-11/E-11 or Dl_ISG15_E11/E-11 systems). After RGNNV inoculation, there were no surviving cells in control inoculated cells (E-11/E-11), where ISG15-producing cells were not seeded, whereas staining intensity was medium for E-11 target cells sharing the medium with ISG15-producing cells (Dl_ISG15_E-11/E-11). The same pattern was obtained when SJNNV-inoculated cells were analysed (Figure 13B).

The results derived from this semi-quantitative assay were confirmed by the MTT assay (Figures 13C and 13D). Thus, the survival rate of RGNNV-inoculated cells increased significantly, from 25% to 46% (p = 0.01) when these cells shared medium with ISG15-producing cells (Figure 13C). A similar result was obtained after SJNNV inoculation (Figure 13D). In this experimental group, the mean survival rate of E-11 cells co-cultured with ISG15-producing cells was 51%, whereas this value dropped to 27% (p = 0.01) when they were co-cultured with control non-producing E-11 cells.

In addition, to verify the protection exerted by the ISG15-containing medium from RGNNV infection, inoculated E-11 cells were incubated with ISG15-containing medium mixed with fresh medium (1:1). Cellular survival was determined by MTT reduction. The incubation with medium containing ISG15 resulted in a significant increase (p = 0.0005) of cellular survival, which was 33% in control cells and increased up to 99% in presence of sea bass ISG15 protein (Figure 13E).

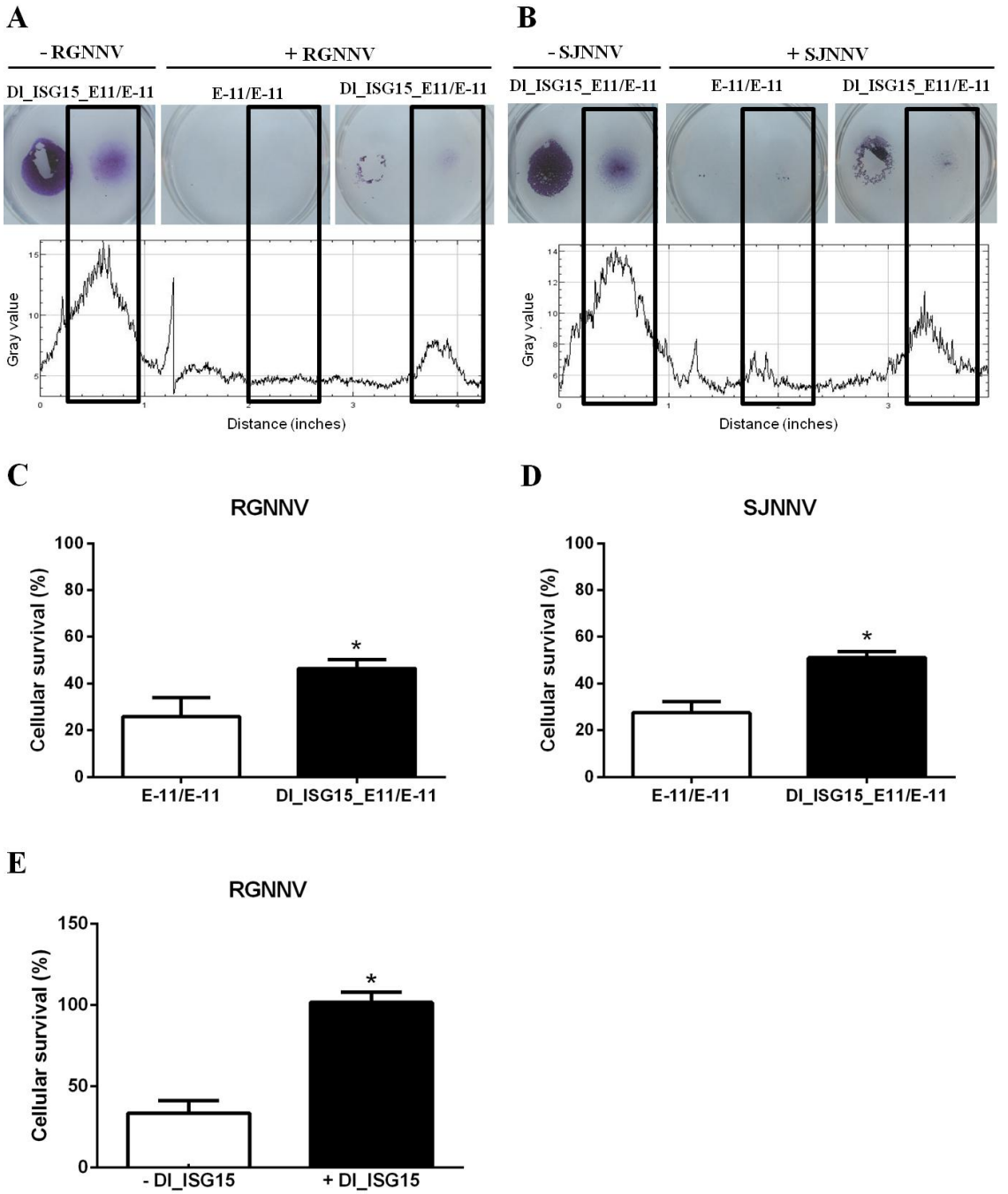

Figure 13. Extracellular Dl_ISG15 antiviral activity. (A, B) Co-culture in droplets: cellular survival after RGNNV or SJNNV inoculation visualized by crystal violet staining. (C, D) Co-culture in droplets: cellular survival after RGNNV or SJNNV infection developed by MTT reduction. (E) Incubation with ISG15-containing medium: survival percentage of RGNNV-inoculated E-11 cells incubated with Dl_ISG15-free medium (white) or Dl_ISG15-containing medium (black). The t-student test was the statistical analysis performed. * indicates significant differences. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 3).

4. DISCUSSION

The results obtained in this study, in chapters 2A and 3, demonstrated an important up-regulation of European sea bass *isg15* transcription in brain and head kidney after RGNNV and SJNNV infections (Moreno et al., 2016; 2018). These results, together with those obtained for orange-spotted grouper (*Epinephelus coioides*) (Huang et al., 2013) and Senegalese sole (*Solea senegalensis*) *isg15* transcription (Alvarez-Torres et al., 2017), suggest an important role of this protein against betanodavirus infections in different fish species.

For this reason, the present study focuses on evaluating the Dl_ISG15 activity against betanodaviruses. To fulfil this aim, an *in vitro* system, named as Dl_ISG15_E11, has been established.

4.1. CHARACTERIZATION OF THE Dl_ISG15_E11 CELL LINE

Experimental systems based on the constitutive expression of exogenous interferon-stimulated genes in stable cell lines constitute useful tools for the functional characterization of the antiviral proteins encoded by these genes. In fact, these systems have been previously applied for ISG15 studies, being the transient transfection the modality most frequently used (Furnes et al., 2009; Hishiki et al., 2014; Langevin et al., 2013a; Okumura et al., 2008; Zhao et al., 2010). The permanently transfected cell line developed in the present study is a stable tool, which has allowed to conduct different assays under steady and homogeneous conditions.

The suitability of this system to determine the Dl_ISG15 antiviral activity has been proven by conducting studies regarding cellular growth kinetics and *isg15* transcription and expression. Thus, the growth rate of Dl_ISG15-transfected and non-transfected E-11 cells is similar (Figure 3), which indicates that the expression of the exogenous gene does not affect important cellular functions. Therefore, putative differences in viral replication recorded in both cell types are not a consequence of the exogenous Dl_ISG15 protein expression, but derived from the antiviral activity of this protein itself. Furthermore, sea bass *isg15* transcription is stable, not being affected by the number of cellular sub-cultures or the presence of viruses (Figure 6).

The recombinant Dl_ISG15 protein has been detected by western blot analyses. Results obtained after processing cellular extracts showed a single 20-25-kDa band

(Figure 4), which was not present in non-transfected E-11 cells. This band corresponds to the recombinant Dl_ISG15 protein, composed of the 15-kDa ISG15 protein plus the 6xHis-tag (which has been used for western blot development) and the Xpress™ epitope, both added at the N-terminal end. Previous studies have also reported similar molecular weights (ranging from 17 to 22 kDa, depending on the fusion tag used) for recombinant ISG15 proteins over-expressed in cellular systems, such as ISG15 proteins of Atlantic cod (*Gadus morhua*, Furnes et al., 2009), zebrafish (*Danio rerio*, Langevin et al., 2013a), Atlantic salmon (*Salmo salar*, Røkenes et al., 2007) and red drum (*Sciaenops ocellatus*, Liu et al., 2010).

In addition, a single band with the same molecular weight has been exclusively detected in the medium of Dl_ISG15_E11 cells (Figure 4A), even though this protein does not have any signal peptide sequence. This extracellular ISG15 protein seems to be secreted by transfected E-11 cells, as it is suggested by the absence of beta-actin (used as cellular control protein) in the extracellular fraction, even after its concentration by immuno-precipitation (Figure 4B), which may rule out cell disruption as possible cause for ISG15 presence in the medium. ISG15 has been previously detected as protein secreted by different types of immune cells, such as IFN I-treated human leukocytes and monocytes (D' Cunha et al., 1996; Knight and Cordova, 1991). The mechanism underlying ISG15 secretion is unknown (Morales and Lenschow, 2013). In fish, ISG15 has been detected extracellularly from cultures of red drum and tongue sole (*Cynoglossus semilaevis*) IFN I-activated head kidney lymphocytes (Liu et al., 2010; Wang et al., 2012). Moreover, other cell types, such as epithelial-derived cell lines, fibroblasts and neutrophils, have also been described as ISG15-releasing cells (Swaim et al., 2017). In fact, Langevin et al. (2013a) reported zebrafish ISG15 protein in the medium of transiently transfected epithelioma papulosum cyprini (EPC) cells. All these previous investigations support our results, in which a fibroblastic cell line (E-11 cells, derived from snakehead whole fry) secretes the Dl_ISG15 protein.

The immunofluorescence study revealed the cytoplasmic location of the European sea bass ISG15 protein in ISG15-producing cells (Figure 5). ISG15, as it has been described for other ISGs, such as Mx, can show nuclear or cytoplasmic location, which may determine the antiviral specificity of these proteins (Alvarez-Torres et al., 2013; Furnes et al., 2009; Huang et al., 2013; Lin et al., 2006). Thus, orange-spotted grouper ISG15 was located in the cytoplasm of grouper spleen cells (GS) (Huang et al.,

2013), whereas Atlantic cod ISG15-1, ISG15-2 and ISG15-3 were primarily detected in the nucleus, with a weak signal in the cytoplasm, of Chinook salmon (*Oncorhynchus tschawytscha*) embryo cells (CHSE-214) (Furnes et al., 2009). Furthermore, Loeb and Haas (1994) reported association of ISG15 to cytoskeleton filaments in epithelial and mesothelial human cells.

Taken together, these results show that the *in vitro* system developed in this study is an adequate tool to determine the antiviral activity of the intracellular and extracellular Dl_ISG15 protein.

4.2. ANTIVIRAL ACTIVITY OF THE INTRACELLULAR Dl_ISG15 PROTEIN

This study has evaluated the intracellular activity of the European sea bass ISG15 protein against betanodavirus by analysing viral propagation and endogenous immunogene transcription in ISG15-expressing and non-expressing E-11 cells.

Previous studies have evaluated the antiviral effect of several fish ISG15 proteins focusing only on one aspect of the viral propagation. Thus, zebrafish ISG15 activity against infectious hematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), and epizootic haematopoietic necrosis virus (EHNV) has been evaluated by analysing viral titre and CPE appearance (Langevin et al., 2013a), whereas grouper ISG15 activity against grouper nervous necrosis virus (GNNV) has been demonstrated at a transcriptional level (Huang et al., 2013). Regarding viral propagation, the present work is a comprehensive study analysing viral genome synthesis, titration of infective viral particles and cell survival percentage.

According to our results, the course of the RGNNV infection was not altered in ISG15-producing cells. Thus, the number of RGNNV RNA2 copies was similar in ISG15-producing and non-producing cells at all time points analysed (Figure 7A), indicating that Dl_ISG15 does not affect viral genome synthesis. Although some ISG15 proteins have been reported to act at a post-transcriptional level, by blocking virion assembly or viral budding (Okumura et al., 2008; Røkenes et al., 2007), this possibility has been ruled out in the present study, since a significant reduction in viral titre due to Dl_ISG15 protein expression has not been observed (Figure 8A). Furthermore, these results were confirmed by analysing CPE development and cellular survival rates after

viral infection, which demonstrated that Dl_ISG15-producing cells were not protected from RGNNV infection (Figure 9A). Taken together, all these results confirmed the lack of intracellular anti-RGNNV activity of the Dl_ISG15 protein. On the contrary, sea bass ISG15 compromises SJNNV replication, since viral genome synthesis was reduced in ISG15-producing cells at 48 and 72 h p.i. (Figure 7B). This result has been confirmed by viral titration and surviving assays (Figures 8B and 9B).

This differential antiviral activity of the sea bass ISG15 protein against both viral species suggests that this protein may play different roles in the course of RGNNV and SJNNV infections. The lack of ISG15 antiviral activity against some fish viruses (both DNA and RNA viruses) has been previously evidenced. Thus, Huang et al. (2013) showed that grouper ISG15 affects GNNV replication, whereas Singapore grouper iridovirus (SGIV) replication was not inhibited. In addition, Langevin et al. (2013a) failed in the detection of zebrafish ISG15 activity against spring viremia of carp virus (SVCV), although this ISG15 protein inhibits the replication of a wide range of fish viruses.

Previous studies conducted with similar *in vitro* systems, although using transiently transfected cells, have shown that exogenous ISG15 proteins may regulate the transcription of several endogenous genes, which may be involved in cellular processes, such as osteoclastogenesis (Takeuchi et al., 2015), or may be related with antiviral activity (Langevin et al., 2013a). For this reason, in this study we have analysed the transcription of several immunogenes in Dl_ISG15_E11 cells, such as *e3*, involved in ISG15 conjugation, *mx*, as a marker of IFN I system, and *tlr3*, as a model of pathogen-associated molecular pattern (PAMP) receptor. The transcription analyses showed lack of induction of the above mentioned genes in non-infected Dl_ISG15_E11 cells (Figure 11), indicating that over-expression of the exogenous ISG15 protein did not trigger the transcription of the endogenous IFN I system (estimated by *mx* transcription), unlike it has been reported for zebrafish ISG15 expressed in transiently-transfected EPC cells (Langevin et al., 2013a); however, it would be interesting to extend the analysis to other IFN I system-related genes.

Regarding inoculated cells, the transcription of these genes was similar ($p > 0.05$) in SJNNV-infected Dl_ISG15_E11 and control E-11 cells at all time points analysed (Figures 12B, 12D and 12F), whereas *tlr3* and *e3* transcription was significantly different ($p = 0.001$) in both types of cells inoculated with RGNNV

(Figures 12C and 12E). This result shows that both betanodavirus species interact in a different way with the host immune system, which may be a consequence of different RGNNV and SJNNV adaptation to the host. In fact, a recent study has recorded differences in the transcription of several immunogenes after the infection with IPNV isolates belonging to different genogroups, which has also been reported in the present study, suggesting that genetic similarity in fish viruses does not necessarily mean functional similarity (Gamil et al., 2015).

These results, together with the results previously described, in which Dl_ISG15 did not block RGNNV propagation, suggest a differential implication of the Dl_ISG15 protein against SJNNV and RGNNV infections on E-11 cells, which is a cell line widely used for betanodavirus propagation. Specifically, the over-expression of sea bass ISG15 in RGNNV-infected cells caused a clear knock out of *tlr3* transcription (Figure 12C), which is a PAMP receptor responsible for the IFN I system activation. This result may suggest that the presence of sea bass ISG15 protein could be preventing the IFN I system activation and, therefore, could be protecting the virus from the host innate immune system, which could account for the lack of intracellular anti-RGNNV activity recorded in this study. Although ISG15 has generally been described as an antiviral effector, some studies have demonstrated that this protein can favour viral infections, having a positive effect on the virus multiplication. Thus, human ISG15 has been shown to have a pro-viral role in the course of hepatitis C virus infection in Rof-0c and con1 cells (Broering et al., 2010; Chua et al., 2009), and Chen et al. (2010) demonstrated that protein ISGylation promotes hepatitis C production. In fact, experiments conducted with fibroblastic cells have suggested that human ISG15 may be a negative regulator of the IFN system (Hermann and Bogunovic, 2017), as it may be happening in Dl_ISG15_E11 cells infected with RGNNV.

The transcription of the endogenous *e3* gene was also altered by the presence of the sea bass ISG15 protein in RGNNV-infected cells, with Dl_ISG15_E11 cells showing a significantly higher level of *e3* transcripts than E-11 cells at 24 h p.i. (Figure 12E). E3 are ligases that may be involved in ubiquitination and ISGylation, labelling proteins for degradation, or changing their properties in terms of location, half-life, activity, etc. (Zhang and Zhang, 2011). In higher vertebrates, a variable number of E3 ligases have been recorded in different species, showing different protein specificity, and therefore, regulating different cellular pathways, as it has been reported for human

cells (revised in Hermann and Bogunovic, 2017). The lack of this information regarding our experimental system (E-11 cells) makes difficult to deduce the functional implications of the up-regulation of this gene transcription; however, it corroborates the sea bass ISG15 immunomodulation in RGNNV-infected cells acting at different levels, and suggests the presence of a high number of proteins functionally altered in Dl_ISG15_E11 cells infected with this isolate.

4.3. ANTIVIRAL ACTIVITY OF THE EXTRACELLULAR Dl_ISG15 PROTEIN

The European sea bass ISG15 protein has been detected extracellularly (Figure 4A), and the next step has been the evaluation of the antiviral properties of this protein acting as a signal molecule. This evaluation has been performed by co-culture, showing that Dl_ISG15-producing E-11 cells protected neighbouring E-11 cells from both viruses analysed (Figure 13). Furthermore, to confirm the establishment of an anti-RGNNV state, E-11 cells were incubated with Dl_ISG15-producing cell medium, and the results were similar to those previously obtained, being recorded cellular protection from RGNNV infection (Figure 14).

The co-culture protocol followed in this study has been previously used by Parreño et al. (2016), who described an antiviral state in EPC target cells due to the IFN I system activation in donor cells (cells permanently infected with IPNV). However, the protection observed in the present study is unlikely to be due to the activation of the IFN I system in donor cells, since *mx* transcription has only been detected, to a certain extent, in SJNNV-inoculated cells, and without significant differences between E-11 and Dl_ISG15_E11 cells (Figures 12A and 12B). This hypothesis has been confirmed by incubating RGNNV-inoculated E-11 cells with medium from Dl_ISG15_E11 cells, which resulted in an increase of the cell survival rate from 33% to 99% (Figure 14). This medium was collected from non-inoculated transfected cells, which have been demonstrated not to have induced any of the genes tested, including *mx* (Figure 11), and, therefore, this medium would not contain cytokines derived from the IFN I system. However, to confirm this hypothesis, it would be necessary to extend the transcription analyses to a wider range of cytokine-coding genes.

In conclusion, we have developed an experimental system (Dl_ISG15_E11), which has been demonstrated to be a valuable tool to characterize the anti-betanodavirus

properties of the sea bass ISG15 protein. Based on this expression system, the antiviral action of the intracellular sea bass ISG15 protein against SJNNV has been established by demonstrating reduction of viral genome and infective viral particles, as well as by showing increased cellular survival rates. In addition, transcription of the endogenous *mx*, *tlr3* and *e3* genes has been analysed, revealing a modulation of *tlr3* and *e3* transcription in presence of Dl_ISG15 protein in the course of RGNNV infections. Finally, the Dl_ISG15-containing medium conferred protection from RGNNV and SJNNV infections. All these results suggest and promote the use of the intracellular and extracellular European sea bass ISG15 protein in future investigations as an antiviral, immunomodulator, and protective agent against betanodaviruses.

CHAPTER 3

Transcriptional profile and serological studies of the European sea bass immune response against betanodavirus infections

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1. INTRODUCTION

European sea bass (*Dicentrarchus labrax*) is highly susceptible to betanodavirus infection; however, only two out of the four betanodavirus species have been detected in this fish species: red-spotted grouper (*Epinephelus akaara*) nervous necrosis virus (RGNNV), and striped jack (*Pseudocaranx dentex*) nervous necrosis virus (SJNNV) (Le Breton et al., 1997; Cutrin et al., 2007).

Both viral species show different level of virulence to sea bass. Thus, RGNNV is highly virulent to this fish species, whereas SJNNV causes no mortality or very low mortality after intramuscular or bath challenges (Souto et al., 2015b; Vendramin et al., 2014). In this regard, Souto et al. (2015b) and Carballo et al. (2016) showed that the SJNNV reference strain (SJ93Nag) replicates in sea bass brain, although it seems to fail in developing the histopathological lesions characteristic of this disease, causing no mortality. In addition, the mortality analysis carried out in this PhD Thesis has shown that the modification of amino acids at position 247 and/or 270 in a RGNNV-type capsid protein (CP) sequence, to those amino acids in a SJNNV-type CP sequence, causes a significant depletion of the RGNNV virulence to sea bass (chapter 1). The different level of viral virulence may be the result of the differential interaction between the virus and the host immune system. Thus, previous studies have suggested an inverse relationship between virulence and immune response in a specific host (Cano et al., 2016; Carballo et al., 2016; McBeath et al., 2014). However, this suggestion has been based on the study of only a few genes, mainly those involved in the interferon type I system (IFN I).

The fish immune response is complete, being able to induce a protective response against viral pathogens, including the production of specific antibodies (Verrier et al., 2011); however, the first antiviral defence corresponds to the innate immune system.

Fish innate immune system consists of cellular and molecular components, including those involved in the IFN I system and inflammatory responses. IFN I is a cytokine expressed and secreted in response to viral infections, which is detected by neighbouring cells, inducing the expression of interferon-stimulated genes (ISGs), such as mx dynamin-like GTPase (*mx*), interferon-stimulated gene 15 (*isg15*), and interferon-stimulated gene 12 (*isg12*), coding for proteins with proven activity against a wide

number of fish viruses (Alvarez-Torres et al., 2016; Avunje and Jung, 2017; Jung and Jung, 2017). The transcription of some of the IFN I system genes in sea bass after RGNNV infection has been previously reported (Carballo et al., 2016; Chaves-Pozo et al., 2012; Moreno et al., 2016; Novel et al., 2013; Poisa-Beiro et al., 2008; Scapigliati et al., 2010). In contrast, there is only one study addressing the IFN I response after SJNNV inoculation in this fish species (Carballo et al., 2016), analysing only *mx* transcription.

The Mx proteins have been widely characterized, existing in nearly all vertebrates, and being active mainly against RNA viruses (Verhelst et al., 2013). These proteins belong to the dynamin family of large GTPases (Schusser et al., 2011; Verhelst et al., 2013), in which the GTPase activity and the ability to form oligomers are important properties for the antiviral activity. Thus, Mx proteins can hamper viral replication by inhibiting viral genome synthesis (Alvarez-Torres et al., 2013; Caipang et al., 2002; Fernandez-Trujillo et al., 2011; Lin et al., 2006), by hindering viral assembly, or by sequestering viral proteins to be degraded (Wu et al., 2016). Vertebrates and invertebrates show a variable number of Mx isoforms (Haller et al., 2007; Rothman et al., 1990; Shirozu et al., 2016). Regarding fish, a variable number of isoforms has been described depending on the species considered (Fernandez-Trujillo et al., 2011; Lin et al., 2006; Peng et al., 2012; Zenke and Kim, 2009). European sea bass displays two isoforms, MxA and MxB, with different response against betanodavirus, suggesting differences in their mechanism of action (Novel et al., 2013).

The *isg15* gene encodes a 15-kDa protein (ISG15) very conserved in vertebrates. In addition, the antiviral activity of several fish ISG15 proteins has been demonstrated (Huang et al., 2013; Langevin et al., 2013a), and this PhD Thesis has described the anti-betanodavirus activity of the European sea bass ISG15 protein (chapter 2B).

The *isg12* gene has been proven to be induced by IFN I in several human and mouse cell lines (Gjermandsen et al., 2000; Kelly et al., 1986). ISG12 is a small hydrophobic protein, with a molecular weight of 12 kDa, composed of 122 amino acids (Gjermandsen et al., 2000). Although its functions are not completely known, this protein is involved in activating the intrinsic apoptosis pathway (Lu and Liao, 2011), and it has an antiviral role against various viruses affecting higher vertebrates (Ji et al., 2017; Labrada et al., 2002). In fish, different ISG12 proteins have been identified (Parker and Porter, 2004), including European sea bass ISG12 (Pallavicini et al., 2010).

The initiation of the inflammatory process is mainly controlled by the tumour necrosis factor alpha (TNF-α) and other cytokines, such as interleukin 1β (IL-1β), which promote the activation of several cytokines, such as interleukin 8 (IL-8) (Al-Banna et al., 2018). TNF-α is a widely studied cytokine (Chu, 2013; Old, 1985; Pfeffer, 2003; Wajant et al., 2003), with homologues from humans to invertebrates (Secombes et al., 2016). It is involved in cell survival, apoptosis, necrosis, and plays an essential role in inflammation: inducing pro-inflammatory factors, enhancing the recruitment and activity of phagocytic cells, inducing nitric oxide production, and regulating non-specific cytotoxic cells (Secombes et al., 2016). This cytokine has been identified in a wide number of fish species, including European sea bass (Hirono et al., 2000; Nascimento et al., 2007; Ordas et al., 2007; Saeij et al., 2003; Zou et al., 2002; 2003). As it has been previously mentioned, IL-8 is a pro-inflammatory cytokine, belonging to the CXC chemokine family (Laing and Secombes, 2004), that promotes the leukocyte chemoattraction (Sepulcre et al., 2007). In fish, the transcription of *il-8* gene in response to ectoparasitic, bacterial and viral infections has been demonstrated (Corripio-Miyar et al., 2007; Covello et al., 2009; Hwang et al., 2016; Sepulcre et al., 2007; Zhonghua et al., 2008). Furthermore, the inflammatory response has been suggested to be especially important in the course of RGNNV infections, since it has been proposed to be responsible for the intense degeneration, mainly vacuolation, observed in brain, retina and spinal cord of affected animals (Chaves-Pozo et al., 2012; Poisa-Beiro et al., 2008).

The inflammatory response is tightly regulated, mainly at a transcriptional level, by anti-inflammatory cytokines. In this contest, interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) play a key role in preventing a massive inflammatory response, which may cause tissue damage. Both cytokines have been related in fish, as in mammals, with the inhibition of the nitric oxide response, the reduction of reactive oxygen species and the down-regulation of pro-inflammatory genes (Grayfer et al., 2011; Haddad et al., 2008; Piazzon et al., 2015; Yang et al., 2014). Specifically, the transcription of both anti-inflammatory cytokines have been studied in European sea bass in response to RGNNV infection (Scapigliati et al., 2010).

Although the relative importance of the adaptive immunity is lower in fish than in mammals, fish adaptive response is crucial for long-lasting immunity, and involves two major groups of lymphocytes, B-cells (humoral response) and T-cells (cellular response) (Secombes and Wang, 2012). Regarding humoral response, three isotypes of

antibodies have been identified in fish: IgM, IgD and IgT (Secombes and Wang, 2012). Most of the studies on fish adaptive immune response, including infection and vaccination challenges, have been set up by the quantification of systemic IgM (Li et al., 2015; Peñaranda et al., 2011; Xing et al., 2017). Thus, several studies have reported that RGNNV infection elicits the production of specific antibodies in sea bass (Lopez-Jimena et al., 2012a; Pascoli et al., 2017; Scapigliati et al., 2010; Skliris and Richards, 1999).

T-cells are important immune cells that require the major histocompatibility complex (MHC) molecules, class I and II, to be activated. Antigens presented by MHC molecules are recognized by T-cells through T-cell receptors (TR). All the *tr* genes have been identified in multiple fish species and are very conserved (Secombes and Wang, 2012). There are two T-cell populations according to the receptor they express: TR-αβ and TR-γδ. T-cells expressing the TR-γδ receptor are especially abundant in the epithelial layer of mucosal tissues, and display cytotoxic activity, promoting the lysis of infected macrophages, and, therefore, limiting the pathogen spreading (Buonocore et al., 2012). In fish, a complete set of genes coding for molecules related to T-cell activities has been reported, having been described MHC molecules and TR receptors in sea bass (Boschi et al., 2011; Buonocore et al., 2012; Nuñez-Ortiz et al., 2014; Picchietti et al., 2011). In addition, RGNNV has been described to modulate *trγ* transcription in intestine and head kidney of infected sea bass (Buonocore et al., 2012).

In this study, we have analysed the transcription of genes coding for some of the above described molecules, with important roles in the fish antiviral response. Thus, this chapter is a comprehensive analysis of the European sea bass host immune response against viral isolates showing different virulence to this fish species, either different viral species, or different attenuated mutated RGNNV viruses obtained by reverse genetics (chapter 1).

2. MATERIALS AND METHODS

2.1. VIRAL ISOLATES AND CELL CULTURE

The viral isolates ERV378/102-5/04 (RGNNV), provided by Dr. G. Bovo (Istituto Zooprofilattico Sperimentale delle Venezie, Italy), SpDl_IAusc965.09 (RGNNV), and SJ93Nag (SJNNV) (Iwamoto et al., 1999), have been used in this study. These isolates have been propagated and titrated on the E-11 cell line (Iwamoto et al., 2000), following the protocols described in chapter 1, section 2.1.

2.2. FISH INFECTIONS

Two different *in vivo* analyses have been carried out in order to examine the European sea bass immunogene transcription following betanodavirus infections (challenges 1 and 2).

In challenge 1, the immunogene transcription after inoculation with RGNNV (ERV378/102-5/04) or SJNNV (SJ93Nag) isolates has been comparatively evaluated. Hereafter, in this chapter, these isolates will be referred as RGNNV and SJNNV, respectively.

In challenge 2, the possible differential response triggered by *r*Dl965 (obtained from the SpDl_IAusc965.09 isolate), and by Mut247Dl965, Mut270Dl965 or Mut247+270Dl965 (obtained by mutation of *r*Dl965) has been analysed. Viruses used in this challenge are those obtained in chapter 1 by reverse genetics (*r*Dl965) and the subsequent mutations in amino acids 247 and/or 270 of the CP sequence (Mut247Dl965, Mut270Dl965 and Mut247+270Dl965). The reverse genetics technique and the generation of recombinant mutated viruses are described in chapter 1, sections 2.3. and 2.4.

European sea bass specimens were always handled according to the European Union guidelines for the handling of laboratory animals (Directive 2010/63/UE), following the handling and maintenance conditions described in chapter 1, section 2.6. Prior to the experimental infections, fish were checked for betanodavirus presence according to Lopez-Jimena et al. (2010). Sampled and surviving animals were killed by MS-222 (Sigma) anaesthetic overdose.

2.2.1. Challenge 1: transcription of immunogenes following RGNNV or SJNNV infection

Juvenile sea bass specimens (5g, average weight) were set up in three 100-l tanks (n = 50 each) and were intramuscularly injected with RGNNV or SJNNV ($5x10^5$ $TCID_{50}$/fish). In addition, a negative control group, consisting of fish intramuscularly injected with Leibovitz (L-15) medium (Gibco), was also considered. For optimal viral propagation, water temperature was maintained at 22-25 ^oC throughout the challenge. Five specimens per experimental group were sampled at 3, 12, 24 and 72 hours post inoculation (h p.i.). Brains and head kidneys were aseptically collected and individually stored in liquid nitrogen to be subsequently homogenized.

Homogenization (10%, w/v) was performed by wet grinding in QIAzol Lysis Reagent (Qiagen), using Lysis Matrix tubes designed for the Fast-Prep24 system (MP Biomedical). Total RNA was extracted with the RNeasy Lipid Tissue Mini Kit (Quiagen) following commercial guidelines, quantified using the NanoDrop-1000 system (ND-1000, ThermoFisher), and stored at -80 ^oC until used. cDNA was synthesized with the iScript cDNA synthesis Kit (BioRad) in 20-µl mixtures composed of RNA (1 µg), 1x iScript Reaction Mix and iScript Reverse Transcriptase (1 µl). Mixtures were incubated at 25 ^oC for 5 min, 46 ^oC for 30 min, and 85 ^oC for 5 min for transcriptase inactivation. Resulting cDNA was stored at -20 ^oC until immunological and virological analyses.

2.2.1.1. Quantification of immunogene transcription

The transcription of *mxA*, *isg12*, *isg15*, *ifn-I*, *mhcII-β*, *il-8*, *il-10*, *tgf- β*, *tnf-α* and *tr-γ* has been quantified by relative real-time PCR protocols using the Mx3000P thermocycler (Stratagene). Reactions were performed in 20-µl mixtures by adding cDNA generated from 10 ng of RNA, 1x Brilliant II Sybr Green qPCR Master Mix complemented with 0.002 mM Rox (Agilent Technologies), and 10 pmol specific primers (Table 1). Rox was included as a passive reference dye to compensate for non-PCR related variations in fluorescence. Ribosomal 18S RNA was amplified as reference endogenous gene (Table 1). The amplification profile was: denaturation at 95 ^oC for 10 min followed by 35 cycles of 95 ^oC for 45 s, 52 ^oC for 45 s and 72 ^oC for 45 s. The melting curve was analysed at 95 ^oC for 1 min, 52 ^oC for 30 s and a final step at 95 ^oC for 30 s.

The MxPro software was used to calculate relative fold change values using the Pfaffl method (Pfaffl, 2004).

Table 1. Primers used for relative quantification of European sea bass immunogene transcription.

Name	Sequence (5'-3')	Product length (bp)	Reference
rRNA18S Fw	CCAACGAGCTGCTGACC	208	Scapigliati et al. (2010)
rRNA18S Rw	CCGTTACCCGTGGTCC		
Dl_MxA Fw	ATTCTGAGTTCTTGCTGAAGG	113	Novel et al. (2013)
Dl_MxA Rw	CCTCTAGAACTCCACCAGG		
TGF-β Fw	GACCTGGGATGGAAGTGG	225	Scapigliati et al. (2010)
TGF-β Rw	CAGCTGCTCCACCTTGTG		
IFN-I Fw	GGCTCTACTGGATACGATGGCT	200	Scapigliati et al. (2010)
IFN-I Rw	GCGTCCAAAGCATCAGCT		
MHCII-β Fw	CAGAGACGGACAGGAAG	240	Picchieti et al. (2015)
MHCII-β Rw	CAAGATCAGACCCAGGA		
IL-8 Fw	GTGCTCCTGGCGTTC	220	Sepulcre et al. (2007)
IL-8 Rw	CTTCACCCAGGGAGC		
TR-γ Fw	CTGCTGTGTGTGGCCTCAGAC	200	Buonocore et al. (2012)
TR-γ Fw	GTGCTGGACGGAGCAGTGGTA		
ISG12 Fw	CCTGGTACAGCTGCTGT	199	Pallavicini et al. (2010)
ISG12 Rw	AGCTGCTCCTGCTGACT		
qISG15 F2	CGACTCAAAGCCTCTCTGCTACT	100	Moreno et al. (2016)
qISG15 R2	CGTTTCTGACGAACACCTGGAT		
IL-10 Fw	ACCCCGTTCGCTTGCCA	164	Scapigliati et al. (2010)
IL-10 Rw	CATCTGGTGACATCACTC		
Dl_TNFα Fw	CGACTGGCGAACAACC	220	Nascimento et al. (2007)
Dl_TNFα Rw	GCTGTCCTCCTGAGC		

2.2.1.2. Quantification of viral genome

The copy number of the RNA2 genomic segment was quantified in brain by absolute real-time PCR using the primers shown in Table 2. Serial dilutions of pCR™4-

TOPO® TA (Invitrogen) vectors containing the full sequence of the RGNNV or SJNNV RNA2 segments were used to generate standard curves. Amplification conditions were those described in chapter 1, section 2.6.1.2. Amplifications were performed with the LightCycler 96 Thermocycler (Roche), using the Fast Start Essential DNA Green Master Mix (Roche).

Table 2. Primers used for absolute quantification of RGNNV or SJNNV RNA2 viral segment.

Name	Sequence (5'-3')	Product length (bp)	Reference
RG-RNA2-F	CGTCCGCTGTCCATTGACTA	100	Lopez-Jimena et al. (2011)
RG-RNA2-R	CTGCAGGTGTGCCAGCATT		
SJ-RNA2-F	GACACCACCGCTCCAATTACTAC	75	Lopez-Jimena et al. (2014)
SJ-RNA2-R	ACGAAATCCAGTGTAACCGTTGT		

2.2.2. Challenge 2: transcription of immunogenes and antibody production following *r*Dl965 or attenuated mutated *r*Dl965 viruses

In order to analyse possible changes in the immunogene transcription resulting from modifications of amino acids 247 and/or 270 within the RGNNV CP sequence (to those amino acids present in the SJNNV CP sequence), juvenile European sea bass (8 g, average weight), distributed in five experimental groups (n = 60 each group), were intramuscularly injected with *r*Dl965, Mut247Dl965, Mut270Dl965, or Mut247+270Dl965 ($2x10^6$ $TCID_{50}$/fish), or with L-15 medium (control group). Fish were maintained at 20-25 ^oC throughout the challenge. Five animals per group were randomly sampled at 12 h p.i., as well as at 1 and 3 days p.i., and their brains were aseptically collected and immediately frozen in liquid nitrogen until being processed. In addition, 15 fish per group were anesthetized for blood extraction from the caudal vein at 30 days p.i. Three samples, composed of blood from 5 specimens, were used to conduct antibody analyses. Sera were obtained by overnight incubation at 4 ^oC for clotting. After this incubation, supernatants were centrifuged twice at 400 x *g* for 15 min at 4 ^oC, and the resulting sera were stored at -20 ^oC.

Brains were processed for transcription analysis as described in chapter 1, section 2.6.1.1.

2.2.2.1. Analysis of immunogene transcription

The transcription of *mxA, isg15* and *tnf-α* genes was quantified by relative real-time PCR. All amplifications were conducted with the LightCycler 96 Thermocycler and the Fast Start Essential DNA Green Master Mix (Roche), using as template cDNA generated from 50 ng of RNA (chapter 2B, section 2.3.4.). Ribosomal 18S RNA was the reference endogenous gene used (Table 1), and relative fold change values were calculated using the Pfaffl method (Pfaffl, 2004).

2.2.2.2. Analysis of anti-betanodavirus antibodies

Antibodies in sera have been evaluated following the indirect Enzyme-Linked ImmunoSorbent-Assay (ELISA) procedure described by Lopez-Jimena et al. (2012a). Briefly, 96-well polystyrene plates (Immulon 4HBX, Thermo) were coated with lysates (0.4 mg) from infected or non-infected (control) E-11 cells. Viral protein concentration was estimated with Bicinchoninic acid (BCA, Sigma), using serial dilutions of bovine serum albumin (BSA) as standard curve. Quantification was performed at 562 nm with the NanoDrop-1000 system. Plates were incubated at 4 ^{o}C overnight for coating, and a glutaraldehyde solution (0.05% in PBS, v/v) was subsequently added to be incubated at room temperature for 20 min. Afterwards, plates were washed 3 times with low salt wash buffer (LSW, 2.4 g/l Trizma base, 22.2 g/l NaCl, 0.1 g/l merthiolate, and 0.5 ml/l Tween20), blocked with 3% skimmed-powder milk (Sigma), and washed again with LSW. Finally, aliquots of 1/64-diluted (in PBS) sera (100 µl) were added in triplicate. Negative controls (PBS instead of fish sera) were included in each plate. After incubation at 4 ^{o}C overnight, plates were washed five times with high salt wash buffer (HSW, 2.4 g/l Trizma base, 29.2 g/l NaCl, 0.1 g/l merthiolate, and 1 ml/l Tween20). The anti-European sea bass IgM monoclonal antibody (F01, Aquatic Diagnostic, 1/33 dilution in 1% BSA-PBS) was added and incubated at room temperature for 1 h, followed by five washes with HSW and 1-h incubation with HRP-conjugated goat anti-mouse IgG (Sigma, 1/4,000 dilution in 1% BSA-LSW) at room temperature. Reactions were visualized by adding 3, 3',5,5'-tetramethylbenzidine (TMB, Sigma), incubating for 10 min at room temperature, and were stopped with the Stop Reagent for TMB Substrate (Sigma). The optical density (OD) at 450 nm was measured using the ELISA Whittaker Microplate Reader 2001 (Anthos Labtec). Resulting OD values were normalized by subtracting the OD of the negative control wells.

2.3. STATISTICAL ANALYSES

Statistical analyses have been performed with the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA). Immunogene transcription and viral quantification results from challenge 1 (section 2.2.1.) were analysed by the two-way ANOVA test, whereas immunogene transcription and ELISA results from challenge 2 (section 2.2.2.) were analysed by the one-way ANOVA test. The Bonferroni´s multiple comparison test was always used as post test. Normality distribution was verified with the Shapiro-Wilk test. Values of $p < 0.05$ were considered significant.

3. RESULTS

3.1. COMPARATIVE ANALYSIS OF INMMUNOGENE TRANSCRIPTION IN EUROPEAN SEA BASS CHALLENGED WITH RGNNV OR SJNNV

The mortality caused by the viral isolates used in this study has been previously published by Souto et al. (2015b), being observed typical signs of disease and mortality only in RGNNV-inoculated fish. In this experimental group, the cumulative mortality recorded at the end of the experiment in intramuscularly-injected fish was 47%. The analysis of immunogene transcription performed in the present study is a step forward in the study of RGNNV and SJNNV infections in sea bass. This analysis has been performed by the transcription quantification of some IFN I system-related genes (*ifn-I*, *isg15*, *isg12* and *mxA*); inflammatory response genes (*il-8*, *tnf-α*, *il-10* and *tgf-β*); and adaptive response genes (*mhcII-β* and *tr-γ*) in brain (viral replication organ) and head kidney.

3.1.1. Transcription of IFN I system-related genes

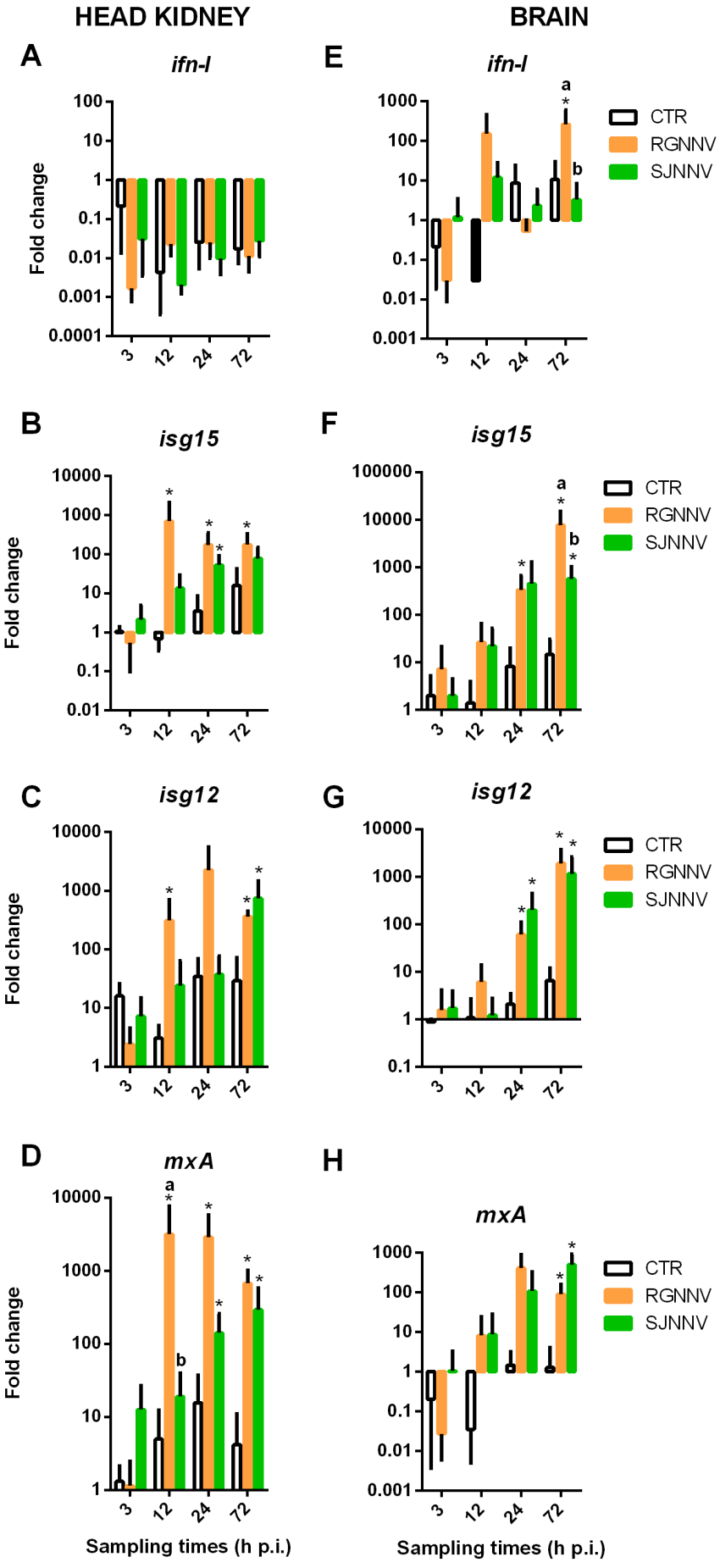
The results, shown in Figure 1, revealed a strong induction of *isg15*, *isg12* and *mxA* in both organs, being observed different kinetics of transcription.

In head kidney, RGNNV is a strong inductor of the transcription of *isg15*, *isg12* and *mxA* from 12 h p.i. onwards (Figures 1B, 1C and 1D). However, SJNNV triggers the induction of these genes earlier, at 3 h p.i., increasing their transcription throughout the time, and reaching significant mean fold change values at 24 h p.i. for *isg15* (53.1), and at 72 h p.i. for *isg12* (739.7) and *mxA* (293.4), whereas the maximal transcript values in RGNNV-inoculated sea bass were observed at 12 h (for *isg15* and *mxA*), with significant differences between viral species, or 72 h p.i. (for *isg12*) (Figures 1B, 1C and 1D). The induction of *ifn-I* transcription was not recorded at any time analysed in any experimental group (Figure 1A).

Regarding transcription values in brain (Figures 1E, 1F, 1G and 1H), the induction of the IFN I system is weaker in this organ than in head kidney at earlier stages of infection. For RGNNV- and SJNNV-inoculated fish, maximal levels of transcription of *isg12* and *isg15* genes were recorded at 72 h p.i., with significant mean fold change values of 1,921.7 and 7,674.7, respectively, in the RGNNV-infected group (Figures 1F and 1G), and 565.4 and 1,151.4, respectively, in sea bass inoculated with

SJNNV. In addition, at this time, significant differences between viral species were recorded for *isg15*, being RGNNV a stronger inductor of this gene compared to SJNNV (Figure 1F). Similarly, RGNNV also induced higher level of *ifn-I* transcripts at this sampling time (263.1 and 3.32 mean fold change values for RGNNV- and SJNNV-infected fish, respectively) (Figure 1E). Regarding *mxA* (Figure 1H), significant levels of transcription after RGNNV and SJNNV infection were recorded at 72 h p.i., with mean fold change values of 89.7 and 493.2, respectively.

Figure 1. Analyses of IFN I-system related genes. Relative quantification of *ifn-I*, *isg15*, *isg12* and *mxA* transcription in head kidney (A, B, C and D) and brain (E, F, G and H) from juvenile European sea bass inoculated with RGNNV (orange) or SJNNV (green). Data were statistically analysed with the two-way ANOVA test. * indicates significant differences between L-15-injected (negative control) and virus-inoculated fish within each sampling time. Different letters denote significant differences between fish inoculated with RGNNV or SJNNV at the same sampling time. Values of $p < 0.05$ were considered significant. Results are mean ± standard deviation (SD) (n = 5).



3.1.2. Transcription of inflammatory response genes

The transcription of genes involved in the inflammatory response is depicted in Figures 2 and 3. Regarding the pro-inflammatory response, in head kidney (Figures 2A and 2B), an increase of *il-8* transcripts over the time was recorded for both viral isolates (Figure 2A), whereas *tnf-α* transcription was up-regulated from 24 h p.i. (Figure 2B). In brain (Figures 2C and 2D), induction of pro-inflammatory genes was stronger than in head kidney, with maximal mean fold change values, recorded at 72 h p.i., of 184.1 and 29.3 for *il-8*, and 1,628.5 and 73.4 for *tnf-α*, in RGNNV and SJNNV groups, respectively; however, significant differences between viral species were not observed (Figures 2C and 2D).

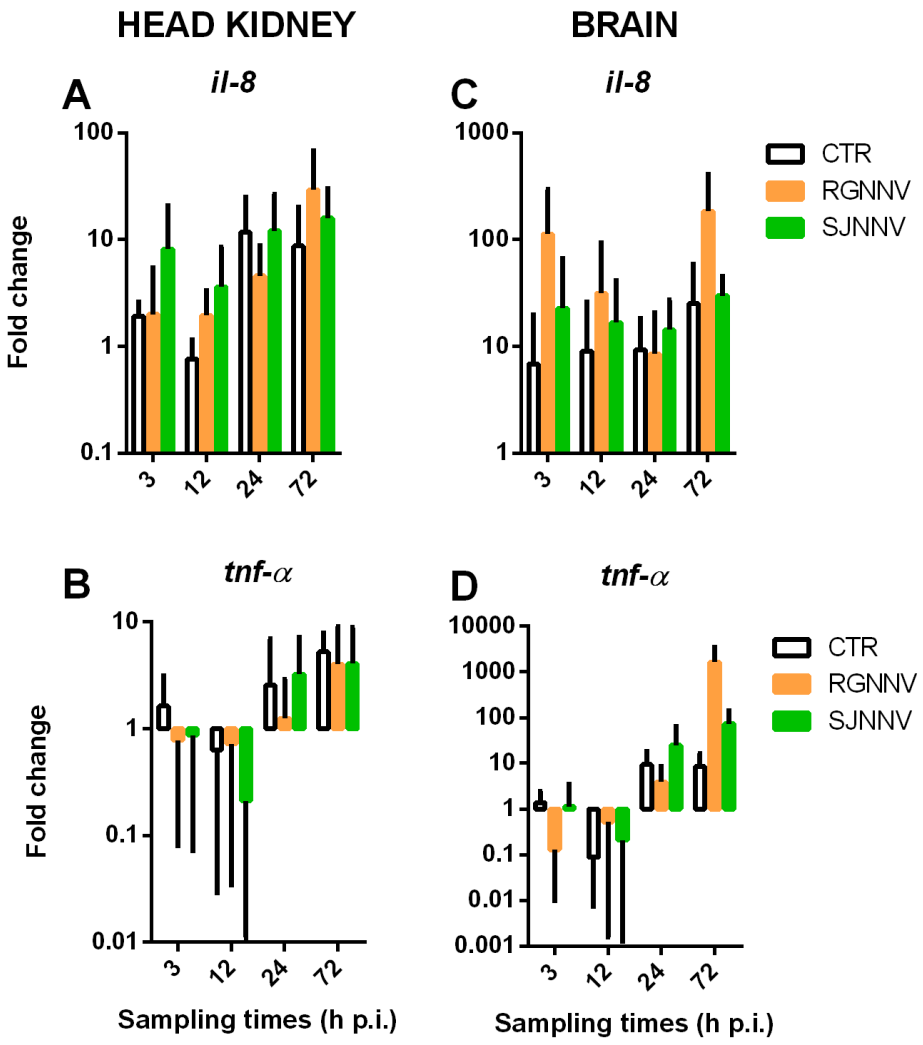


Figure 2. Analyses of pro-inflammatory genes. Relative quantification of *il-8* and *tnf-α* transcription in head kidney (A and B) and brain (C and D) from juvenile European sea bass inoculated with RGNNV (orange) or SJNNV (green). Data were statistically analysed with the two-way ANOVA test. Results are mean ± SD (n = 5).

Unlike what happens with the transcription of pro-inflammatory genes, in general, anti-inflammatory genes were induced at the same level in both organs, with up-regulation of *il-10* and *tgf-β* mainly at 24 and 72 h p.i. (Figure 3). In head kidney, maximal mean fold change values were recorded at 24 h p.i. in RGNNV-inoculated fish (24.5 and 6.6 for *il-10* and *tgf-β*, respectively), and at 72 h p.i. in the SJNNV group (6.6 and 13.7 fold change values for *il-10* and *tgf-β*, respectively) (Figures 3A and 3B). In brain, maximal transcription for *il-10* and *tgf-β* was recorded at 72 h p.i., for both viral isolates, with mean fold change values of 19.5 and 86, respectively, in the RGNNV group, and 12.5 and 14.6, respectively, in the SJNNV group (Figures 3C and 3D), although significant differences between viral species were not observed.

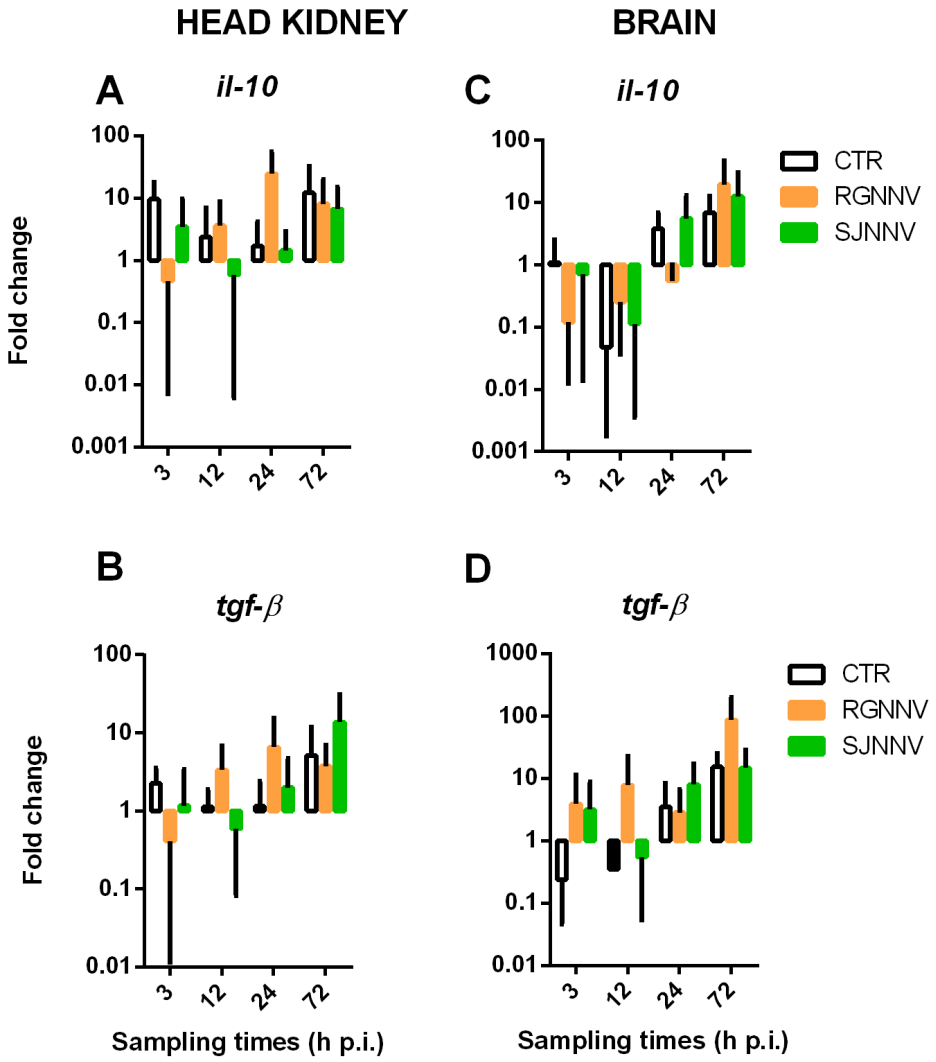


Figure 3. Analyses of anti-inflammatory genes. Relative quantification of *il-10* and *tgf-β* transcription in head kidney (A and B) and brain (C and D) from juvenile European sea bass inoculated with RGNNV (orange) or SJNNV (green). Data were statistically analysed with the two-way ANOVA test. Results are mean ± SD (n = 5).

3.1.3. Transcription of adaptive response genes

In head kidney, both adaptive genes considered, *mhcII-β* and *tr-γ*, were mostly down-regulated at the first sampling times (Figures 4A and 4B). The up-regulation of these genes was recorded later, at 24 or 72 h p.i. (depending on the viral isolate), with maximal mean fold change values of 13.1 at 72 h p.i., for *mhcII-β* in the SJNNV-infected group, and 4.3 at 72 h p.i., for *tr-γ* in the RGNNV-infected group (Figures 4A and 4B).

In brain (Figures 4C and 4D), the kinetics of *mhcII-β* transcription was similar to that previously described in head kidney, with maximal fold change values at 72 h p.i. for both, RGNNV (14 fold change value) and SJNNV (87.5) groups. However, the temporal pattern of *tr-γ* transcription was different, with a strong up-regulation early after infection (from 3 h p.i.).

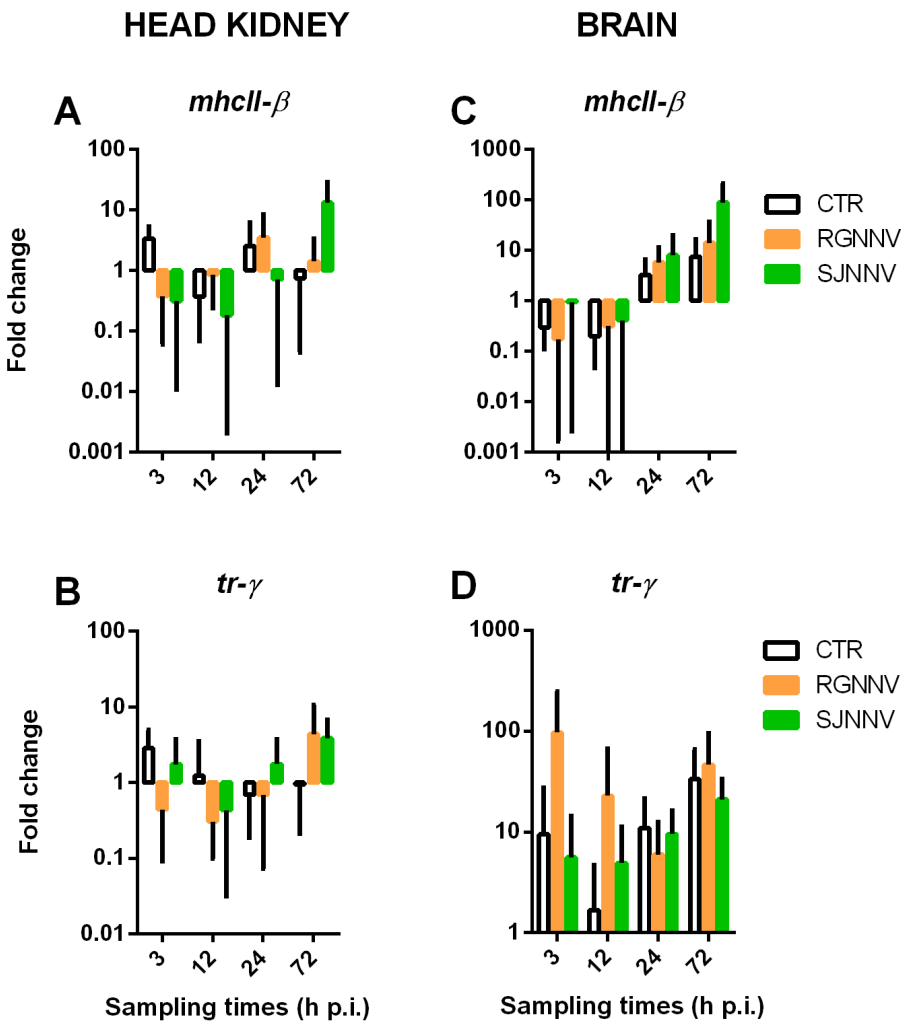


Figure 4. Analyses of adaptive response genes. Relative quantification of *mhcII-β* and *tr-γ* transcription in head kidney (A and B) and brain (C and D) from juvenile European sea bass inoculated with RGNNV (orange) or SJNNV (green). Data were statistically analysed with the two-way ANOVA test. Results are mean ± SD (n = 5).

3.1.4. Viral genome quantification

Results depicted in Figure 5 showed a significant increase ($p < 0.05$) in the number of RGNNV and SJNNV RNA2 copies throughout the time. Moreover, SJNNV-inoculated fish displayed a significantly higher number of RNA2 copies, compared with animals in the RGNNV group, at 3 h ($p = 0.006$) and 12 h p.i. ($p = 0.008$), obtaining the maximal genome level at 72 h p.i. (7.1×10^{10} RNA2 copies/µg RNA). However, in the RGNNV-infected group the increase in the number of RNA2 copies from 3 h (5.5×10^{4}) to 72 h p.i. (4.7×10^{9}) was higher than in the SJNNV group, obtaining also the maximal mean RNA2 copy number at 72 h p.i., 4.7×10^{9} copies/µg RNA.

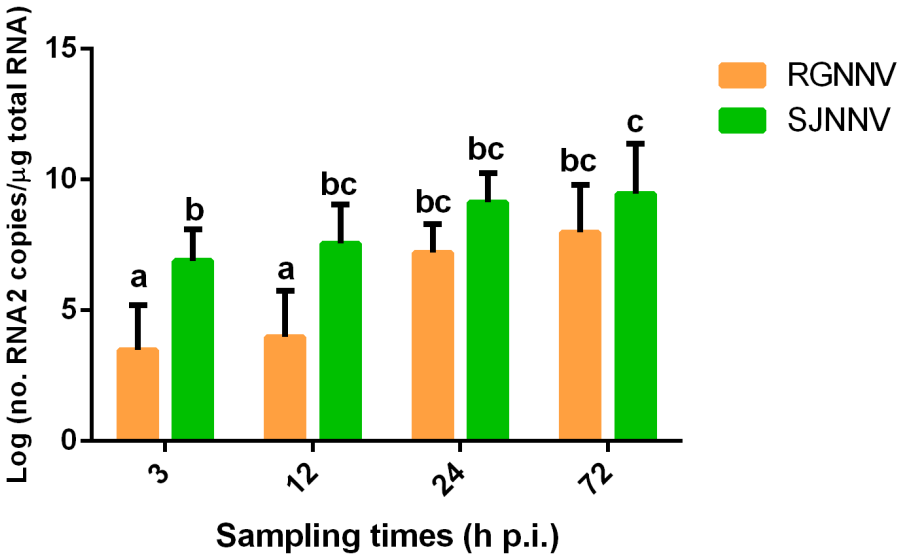


Figure 5. Absolute quantification of the viral RNA2 segment in brain from juvenile European sea bass inoculated with RGNNV (orange) or SJNNV (green). Data were statistically analysed with the two-way ANOVA test. Different letters denote significant differences. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 5).

3.2. INMMUNOGENE TRANSCRIPTION AND ANTIBODY PRODUCTION IN SEA BASS CHALLENGED WITH RECOMBINANT MUTATED VIRUSES

Possible changes in the immunogene transcription caused by the mutations at positions 247 and/or 270 of the RGNNV CP sequence have been evaluated. In this study, *r*Dl965 has been used as positive control on the basis of the virulence results described in chapter 1, section 3.4.1. This virus caused 73.3% mortality after intramuscular inoculation, whereas the mortality caused by Mut247Dl965, Mut270Dl965 and Mut247+270Dl965 was 22.5, 20, and 20%, respectively (chapter 1, section 3.4.2.). The study of the immune response, in terms of immunogene transcription and antibody production, against these recombinant viruses constitutes another aspect analysed regarding the RGNNV pathogenesis in European sea bass.

3.2.1. Transcription of immunogenes

The innate immune response has been partially analysed by quantification of *mxA*, *isg15* and *tnf-α* transcription in brain. These genes have been selected on the basis of the results obtained in challenge 1 (section 3.1.).

Regarding the transcription of *mxA* and *isg15* (Figures 6A and 6B), *r*Dl965 was the strongest inductor of both ISGs at the first sampling time (12 h p.i.), with mean fold

change values of 12.5 and 25.2 for *mxA* and *isg15*, respectively. In addition, the transcription of these genes was maximal at 72 h p.i. in the *r*Dl965 group, with fold change values of 336 (*mxA*) and 470 (*isg15*). Similarly, maximal *mxA* and *isg15* transcription in Mut247Dl965- and Mut270Dl965-inoculated fish was also observed at 72 h p.i., although recording induction values significantly lower than those obtained for *r*Dl965-inoculated sea bass ($p < 0.0001$). Thus, Mut247Dl965 and Mut270Dl965 induced, respectively, 145 and 130 fold change values for *mxA*, and 189 and 178.4 for *isg15*. However, the pattern of transcription after Mut247+270Dl965 inoculation was different, obtaining steady and low transcription values, comparable to those reported in fish from the control group, at all sampling times analysed.

Concerning the transcription of the pro-inflammatory cytokine *tnf-α* (Figure 6C), it was maximal at 72 h p.i. for *r*Dl965-inoculated fish, and at 24 h p.i. for the Mut247+270Dl965-infected group, with mean fold change values of 483.5 and 206, respectively. However, the transcription of this gene in fish inoculated with Mut247Dl965 or Mut270Dl965 was more stable and lower at all sampling times.

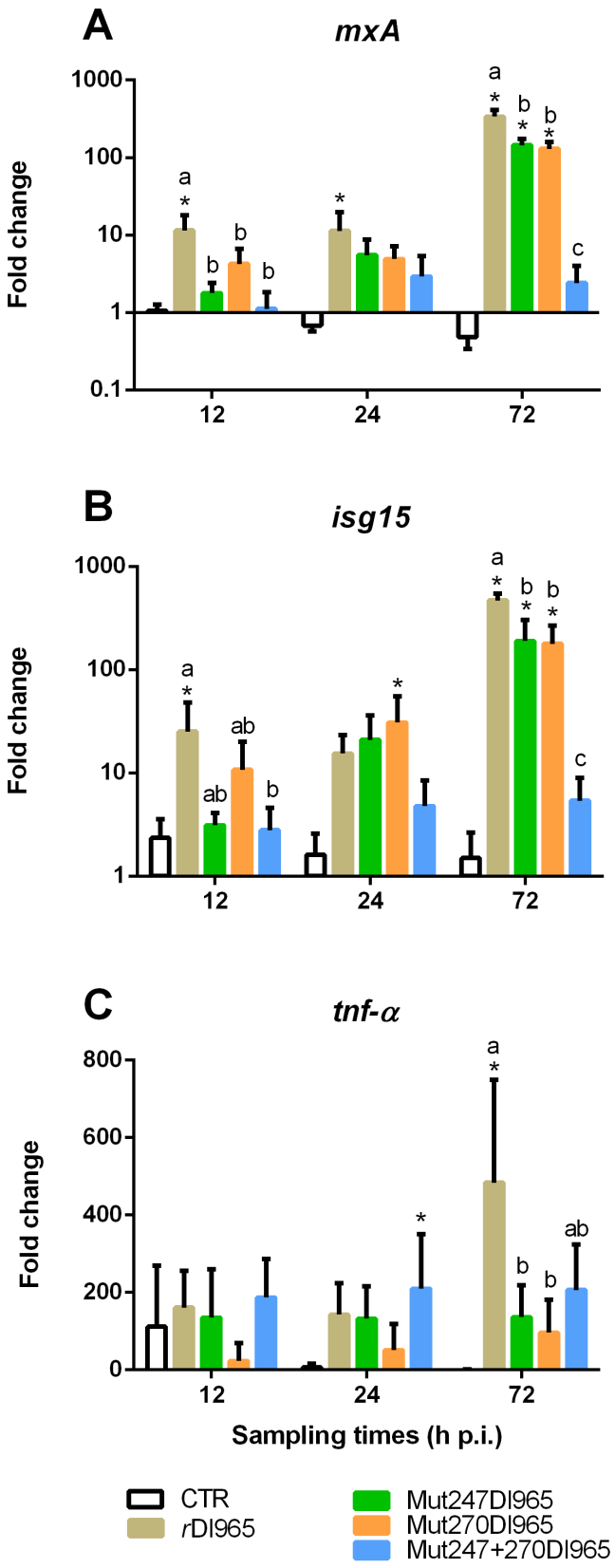


Figure 6. Relative quantification of *mxA*, *isg15* and *tnf-α* transcription after inoculation with *r*Dl965 or mutated viruses. Data were statistically analysed with the one-way ANOVA test. * indicates significant differences between L-15-injected (negative control) and virus-inoculated fish within each sampling time. Different letters denote significant differences among experimental groups at the same sampling time. Values of $p < 0.05$ were considered significant. Results are mean ± SD (n = 5).

3.2.2. Analysis of anti-betanodavirus antibodies in sera

In addition to quantify *mxA*, *isg15* and *tnf-α* transcription, anti-betanodavirus antibodies in sera have also been analysed at 30 d p.i. Moreover, to further analyse possible changes in betanodavirus serological properties caused by the mutations considered, a cross-reaction assay has also been performed. For comparative purposes, all sera were analysed at 1/64 dilution.

The results showed a significant production of antibodies in *r*Dl965-, Mut270Dl965- and Mut247+270Dl965-inoculated fish, compared to the negative control group (L-15-injected fish) ($p < 0.0001$) (Figure 7). Moreover, the antibody production was significantly higher in sea bass infected with these mutated viruses than in fish from the *r*Dl965 group (Figure 7) ($p < 0.0001$). In contrast, a measurable level of antibodies was not reported in sera from the Mut247Dl965-infected group, obtaining mean OD values statistically comparable to those reported for sera from L-15-injected sea bass.

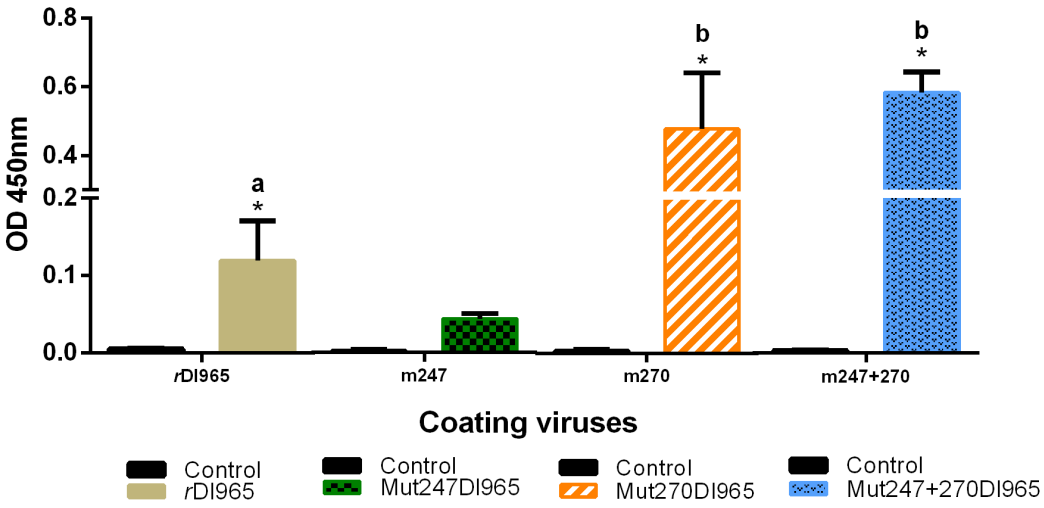


Figure 7. Level of anti-betanodavirus antibodies evaluated by indirect ELISA. Data were statistically analysed with the one-way ANOVA test. * indicates significant differences between control (sera from L-15-injected fish) and infected groups. Different letters denote significant differences among infected groups. Values of $p < 0.05$ were considered significant. Results are mean ± SD of three samples composed of sera from five fish. All sera were analysed at 1/64 dilution.

Furthermore, antibodies in sera from *r*Dl965- and Mut247+270Dl965-inoculated sea bass recognized RGNNV and SJNNV antigens (Figure 8), whereas antibodies in sera from Mut270Dl965-inoculated fish did not react with SJNNV antigens (SJ93Nag

reference strain), recognizing only Mut270Dl965, used as positive control, and *r*Dl965 as coating viruses. Maximal mean OD values were obtained for sera from Mut247+270Dl965-inoculated sea bass. These values were 0.15, 0.22 and 0.58 for *r*Dl965, SJNNV and Mut247+270Dl965 coating viruses, respectively (Figure 8).

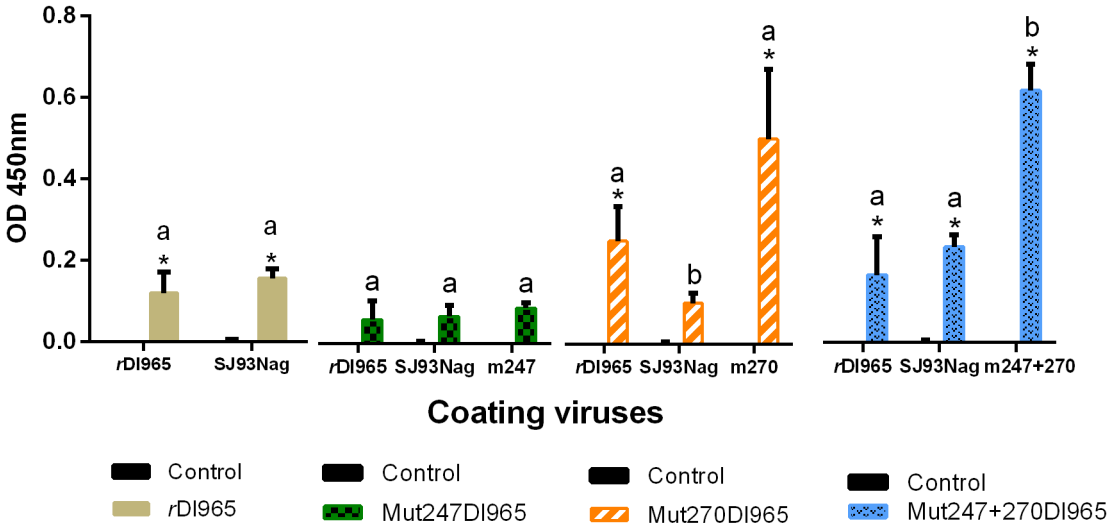


Figure 8. Cross-reaction between antibodies in sera from European sea bass inoculated with *r*Dl965, Mut247Dl965, Mut270Dl965 or Mut247+270Dl965 (1/64 dilution), and different viral antigens (lysates of E-11 cells inoculated with the different viruses considered). Data were statistically analysed with the one-way ANOVA test. * indicates significant differences between control (sera from L-15-injected fish) and infected groups. Different letters denote significant differences among different coating viruses within each experimental group. Values of $p < 0.05$ were considered significant. Results are mean ± SD of three samples composed of sera from five fish.

4. DISCUSSION

The development of infectious diseases does not depend only on pathogen virulence, but also on host defence mechanisms, and the interactions established between them. For this reason, in this chapter, the European sea bass immune response following infections with betanodaviruses has been evaluated. In a recent study, Pascoli et al. (2017) described a poor *in vivo* long-term cross-protection between RGNNV and SJNNV isolates in sea bass, which evidences different host response against both viral species and the importance of this investigation. Based on this premise, the transcriptional profiles of different immunogenes after infection with RGNNV, highly virulent to this fish species, or SJNNV, less virulent to this fish species, have been analysed. In addition, the immunogene transcription and the antibody response after infection with RGNNV viruses attenuated by mutation have also been evaluated.

The immune response after infection with RGNNV or SJNNV has been comparatively analysed by quantifying the transcription of genes involved in the IFN I system response, the inflammatory response, and the adaptive response.

The analysis of *ifn-I*, *isg12*, *isg15* and *mxA* transcription showed that betanodaviruses are strong inducers of the sea bass IFN I system in brain and head kidney, which suggests the importance of this innate defence mechanism in controlling this viral infection. Noteworthy, RGNNV seems to induce a more intense response of the IFN I system than SJNNV in both organs, although SJNNV induced an early gene transcription in head kidney. The earlier and quicker systemic response induced by the less virulent viral strains, and the strong up-regulation of IFN I-related genes at later stage of infection by highly virulent strains have been previously described by McBeath et al. (2014) in Atlantic salmon (*Salmo salar*) inoculated with different strains of infectious salmon anaemia virus (ISAV). These authors suggest that the more rapid systemic response induced by the less virulent virus might provide a sufficient level of protection to prevent this strain from reaching the damaging levels obtained for the highly virulent virus.

The *ifn-I* transcription was not recorded in head kidney at any time, whereas *isg12*, *isg15* and *mxA* were up-regulated in this organ. Although the induction in an IFN-independent way of some ISGs, such as *isg15* and *mx*, has been recorded (Domingues et al., 2015; Levraud et al., 2007; Lopez-Muñoz et al., 2010), it is unlikely

this to happen for the three ISGs analysed. The *ifn-I* gene induction in sea bass head kidney seems to occur at a very early stage of infection, being very transitory and self-limiting, and, in consequence, difficult to record, as it has been described by Scapigliati et al. (2010). On the contrary, the transcription of this gene in brain seems to last longer, being recorded at 72 h p.i., at least in RGNNV-inoculated sea bass.

The ISG most frequently analysed in sea bass is *mx*. Thus, numerous studies have evaluated *mx* transcription in this fish species after RGNNV infection, mainly in head kidney, reporting induction of *mx* mRNA at times post-infection ranging from 6 to 72 h (Chaves-Pozo et al., 2012; Novel et al., 2013; Poisa-Beiro et al., 2008; Scapigliati et al., 2010). In the present study, a strong induction triggered by RGNNV has been found at 12 h p.i. in head kidney (3,174.1 folds) (Figure 1D), with significant differences between both viral species, and at 72 h p.i. in brain (Figure 1H). These results are in contrast to those of Carballo et al. (2016), which demonstrated that sea bass head kidney *mx* gene was induced at higher level by SJNNV than by RGNNV isolates. Similarly, a high induction of *mx* transcription in brain has been recorded in sea bream (*Sparus aurata*) (resistant to the infection) in comparison with sea bass (susceptible) (Poisa-Beiro et al., 2008). These previous reports refer to *mx* gene, without distinguishing between *mxA* and *mxB*. The present work has used primers detecting specifically *mxA*, which transcribes earlier and at higher level than *mxB* in head kidney of RGNNV-infected sea bass (Novel et al., 2013). An under-estimation of *mxA* transcription by the primers used in previous studies, as well as differences in fish age or viral isolates, could explain these discrepancies. In fact, fish age is an important factor determining the appearance of viral nervous necrosis (Grove et al., 2003), which can be the result of differences in the host immune response.

Regarding *isg15*, on the whole, the transcription of this gene is higher after RGNNV inoculation than in SJNNV-inoculated sea bass, especially at the last sampling time analysed. The potent induction elicited by the RGNNV isolate in brain is coherent with the results obtained in chapter 2A, and supports the anti-betanodavirus role of the sea bass ISG15 protein. In addition, results described in chapter 2B, although obtained in an *in vitro* system that differs from sea bass, highlight different mechanisms of action of sea bass ISG15 against RGNNV and SJNNV.

A similar pattern of induction has been described for *isg12* (Figures 1C and 1G). The high induction recorded after RGNNV and SJNNV infection in this work points out

an anti-betanodavirus role of the sea bass ISG12 protein comparable to the role of more extensively analysed ISGs, such as *mx* and *isg15*. To our knowledge, the only previous study on *isg12* induction has been performed with sea bass leukocytes stimulated with poly I:C (Pallavicini et al., 2010).

The importance of the inflammatory process in the course of betanodavirus infections has been previously suggested (Poisa-Beiro et al., 2008), and it seems to be supported by the results obtained in the present study. Thus, the transcription of *il-8*, which attracts and activates neutrophils, and *tnf-α*, which regulates inflammation and apoptosis, has been higher in brain than in head kidney, with the RGNNV being a stronger inductor in brain (Figures 2C and 2D). Although inflammation is essential to control betanodavirus multiplication in brain (Huang et al., 2015; Montes et al., 2010; Poisa-Beiro et al., 2008; 2009), an uncontrolled process may cause tissue damage. In fact, one of the main differences between the immune response against betanodavirus infection in sea bass and sea bream is the long-lasting anti-inflammatory response in sea bream (Poisa-Beiro et al., 2008), which would prevent the typical histological lesions in brain. Although this could also be an important difference in sea bass immune response against SJNNV and RGNNV, no differences have been recorded in the induction of the anti-inflammatory genes *il-10* and *tgf-β* (Figure 3); therefore, analyses at longer times p.i. would be required in order to prove this hypothesis. In a previous study, the induction of *il-10* and *tgf-β* was recorded up to 10 days p.i. in head kidney from RGNNV-challenged sea bass, although at low level (Scapigliati et al., 2010); however, no data about SJNNV-triggered transcription are available.

Regarding the transcription of adaptive response-related genes, our results suggest that the importance of the sea bass acquired response against betanodavirus is not only at a systemic level, but also at a local level. Thus, the immune response to betanodavirus in brain is characterized by an early *tr-γ* transcription (Figure 4D), which is a T-cell receptor, suggesting an important role of the acquired immunity in controlling viral replication locally, in the target organ, as it has been previously reported in brain of experimentally-challenged sea bream and Atlantic halibut (*Hippoglossus hippoglossus*) (Lopez-Muñoz et al., 2012; Øvergård et al., 2012). On the contrary, in head kidney the increase of *tr-γ* transcription was not recorded at 3, 12 or 24 h p.i., which may indicate either a quick migration of T-cells to the brain as target organ, or down-regulation in the transcription of this gene. This result is surprising,

since this gene has been classically considered to be important mainly in mucosal tissues, such as skin, gills or intestine (Buonocore et al., 2012). Regarding *mhcII-β* (Figures 4A and 4C), the lack of transcription of this gene recorded at the beginning of the infection in several tissues has also been described for different experimental systems, including Atlantic salmon infected with infectious pancreatic necrosis virus (IPNV) (Ingerslev et al., 2009).

Viral genome quantification showed an increase in the number of RNA2 copies of both viral isolates throughout the time (Figure 5), indicating viral replication, even when no signs of disease or mortality were observed in the SJNNV group (Souto et al., 2015b). Betanodavirus replication in fish species not showing disease signs, as it happens for SJNNV in this study, has been previously described for Atlantic halibut, Atlantic cod (*Gadus morhua*) and sea bream (Castric et al., 2001; Grove et al., 2003; Korsnes et al., 2009). In addition, Souto et al. (2015b) reported similar RNA2 copy number in brains from RGNNV- and SJNNV-inoculated sea bass at 30 days p.i., suggesting SJNNV replication. Our results suggest a higher replication rate for RGNNV (considering the difference in the number of RNA2 copy number from 3 to 72 h p.i.), but a faster spread of SJNNV from the injection site to the brain (based on the RNA2 copy number recorded at 3 h p.i.). Previous studies have shown that viral isolates less virulent to a specific host have advantage at the entry stage in bath challenges, inducing an early protective immune response in the host (McBeath et al., 2014); similarly, low virulent isolates may also have advantage in spreading from the inoculation site to the target organ.

In order to complement this study, the transcriptional analysis of several selected immunogenes (*mxA, isg15* and *tnf-α*) in sea bass brain in response to attenuated mutated RGNNV viruses has also been included. The results reveal an immunogene response against attenuated RGNNV viruses similar to that described after SJNNV inoculation.

The transcription of *mxA* and *isg15* has been evaluated as marker of the IFN I system. The low virulent mutated viruses caused a reduced induction of these genes, which has been especially evidenced at 72 h p.i. (Figures 6A and 6B). Moreover, as it has been observed in challenge 1, and, additionally, in chapter 2A (Moreno et al., 2016), the induction of these genes in brain is coherent with the viral replication in this organ, which was described in chapter 1. In fact, no significant induction of *mxA* and *isg15* has been reported in Mut247+270Dl965-inoculated sea bass, in which no viral replication

was recorded (chapter 1). Maximal levels of transcription were always at 72 h p.i., as it has been previously reported (Moreno et al., 2016; 2018; Novel et al., 2013; Poisa-Beiro et al., 2008).

The results regarding the transcription of the pro-inflammatory gene *tnf-α* are also in concordance with those described in challenge 1. Thus, the most virulent virus (*r*Dl965) induced the highest level of *tnf-α* transcription, obtaining the maximal mean value of fold induction at 72 h p.i. (483.5) (Figure 6C). Previous studies have also reported maximal values at 72 h p.i. (Poisa-Beiro et al., 2008), or even at longer times p.i. (15 d p.i.) (Valero et al., 2015a). In contrast, the level of *tnf-α* transcription in sea bass inoculated with the low virulent viruses was statistically comparable with the transcription in the control group. Therefore, this study may be new evidence supporting the importance of the inflammatory reaction in the pathological process of the disease.

Finally, ELISA analyses have evidenced changes in the viral serological properties as a consequence of the mutations included in the RGNNV CP sequence. Thus, the seroconversion after Mut270Dl965 or Mut247+270Dl965 inoculation was significantly higher ($p < 0.0001$) than in sea bass inoculated with the non-mutated *r*Dl965 virus (Figure 7), whereas Mut247Dl965 inoculation did not result in a level of antibody measurable by the ELISA procedure followed in this study (analysis of 1/64-diluted sera). Unfortunately, the analysis of a lower serum dilution was not possible due to the limited amount of serum obtained.

The anti-*r*Dl965 antibody production recorded in the present study is lower than those reported by several authors after inoculation with different RGNNV isolates (Nuñez-Ortiz et al., 2016; Pascoli et al., 2017; Scapigliati et al., 2010). Furthermore, previous studies have compared the production of antibodies in sea bass inoculated with betanodaviruses with different level of virulence to this fish species, obtaining very different results. Thus, Pascoli et al. (2017) reported a higher antibody production in sea bass inoculated with inactivated RGNNV than after inoculation with inactivated SJNNV, whereas Moreno et al. (2018) showed that a SJNNV isolate triggered a higher level of antibodies in experimentally-infected sea bass than a RGNNV isolate. These contradictory results point out the variability in the level of antibodies recorded depending on experimental details, including the ELISA protocol, viral isolates, fish age or inoculation route.

To further evaluate the serological changes in mutated viruses, a cross-reaction assay has been conducted by indirect ELISA. Antibodies produced by *r*Dl965- and Mut247+270Dl965-infected fish recognized both, RGNNV and SJNNV antigens at similar level (Figure 8), which has been previously reported using other RGNNV isolates (Lopez-Jimena, 2012b; Mori et al., 2003; Pascoli et al., 2017), whereas the pattern of reaction of antibodies in Mut270Dl965-inoculated sea bass was different, recognizing only the RGNNV-type antigen used. Therefore, the mutation in amino acid 270 within the RGNNV CP sequence seems to modify the serological properties of the *r*Dl965 RGNNV virus.

RGNNV and SJNNV have been classified as different serotypes on the basis of neutralization assays (Panzarin et al., 2016). Thus, SJNNV belongs to serotype A, whereas RGNNV is included within serotype C. According to Panzarin et al. (2016), capsid proteins retaining RGNNV amino acids at positions 217-256 are recognized by anti-RGNNV antibodies, and proteins containing the SJNNV-amino acid sequence at positions 257-341 react with anti-SJNNV antibodies, as it has been demonstrated by immunofluorescence (IF). The comparison between *r*Dl965 and SJNNV (SJ93Nag) CP sequences showed no similarities at positions 257-341 that could stand for the cross-reaction recorded in this study; however, *r*Dl965 comparison with other RGNNV isolates revealed a single amino acid changed at position 234, in which the *r*Dl965 isolate displays an alanine (serine in other RGNNV sequences), and a double change at positions 291-292 (valine and threonine instead of alanine and glycine). Both sets of changes are located in the RGNNV and SJNNV immuno-reactive regions, respectively (data non-shown). Based on our results, a more extensive study, using several RGNNV and SJNNV isolates, seems to be necessary to fully understand the implications of these variations in antigen-antibody cross-reactivity. Moreover, the titre of neutralizing antibodies should also be established, since reactivity does not necessarily mean neutralization, and, therefore, protection. In fact, a recent study has reported high seroconversion in sea bass vaccinated with inactivated SJNNV, although with low neutralization properties against RGNNV infections, which indicates lack of *in vivo* cross-protection (Pascoli et al., 2017).

Another important aspect to consider is the technique used. Thus, Pascoli et al. (2017) recorded cross-reactivity by ELISA and IF, whereas Panzarin et al. (2016) used IF, and Lopez-Jimena (2012b) applied the ELISA technique. Although ELISA and IF

methods are commonly used in serology and report similar results (Cunha et al., 2006; Salinas et al., 1993), there could be differences in the detection of cross-reactions, which would be interesting to further analyse.

In conclusion, this study has shown that RGNNV and SJNNV stimulated a complete immunogene response, involving the induction of numerous genes related with both, innate and adaptive responses. These responses have been recorded both in brain and head kidney, with SJNNV inducing an early immune gene transcription in head kidney, whereas RGNNV induces a strong up-regulation in both organs. Furthermore, the strong induction of the pro-inflammatory genes induced by RGNNV and *r*Dl965 in brain supports the importance of inflammation as a process involved in the nervous tissue degeneration in the course of betanodavirus infections, as previously suggested. Finally, attenuated mutated viruses triggered a clearly reduced transcription of *mxA*, *isg15* and *tnf-α*, when compared with the non-mutated virus, eliciting, however, higher production of antibodies (except for Mut247Dl965).

GENERAL REMARKS

Viral diseases are the result of the pathogen virulence, the host immunity mechanisms, and the interaction between those components, which constitutes the so called host-pathogen interaction. This PhD Thesis is a comprehensive study on the interaction between European sea bass (*Dicentrarchus labrax*) and nervous necrosis virus (NNV), which is the main viral pathogen affecting this fish species, being responsible for high mortalities and economical losses in the farming industry. Therefore, the main factors involved in this interaction, including those determining the NNV virulence and the antiviral sea bass defence mechanisms have been analysed.

This study has evaluated the role of the capsid protein (CP) amino acids 247 and 270 in the virulence of a red-spotted grouper (*Epinephelus akaara*) nervous necrosis virus (RGNNV) isolate (SpDl_IAusc965.09) to sea bass. This isolate has been sequenced and recombinant viruses, displaying mutations in the above mentioned positions, have been generated by the reverse genetics technique. The effect of these changes on viral replication and virulence has been assessed after experimental infection.

In addition, the European sea bass immune response against betanodavirus infections has been deeply analysed. First of all, the sea bass *isg15* gene and ISG15 protein have been characterized for the first time. In this regard, the structure of the sea bass *isg15* gene, including the description of regulatory motifs, has been analysed, as well as the phylogenetic relationships among different ISG15 proteins, and the transcription profile following polyinosinic:polycytidylic acid (poly I:C) inoculation or RGNNV infection. Furthermore, the antiviral activity of the sea bass ISG15 protein (Dl_ISG15) has been evaluated *in vitro*. To fulfil this aim, a cellular system, which expresses constitutively Dl_ISG15, has been established and characterized. On the basis of the suitability of this system, several studies on the intracellular and extracellular Dl_ISG15 activity against RGNNV and striped jack (*Pseudocaranx dentex*) nervous necrosis virus (SJNNV) isolates have been carried out. Finally, an extensive *in vivo* study on the European sea bass immune response has been performed by analysing the transcriptional profiles of several immunogenes, involved in both, innate and adaptive responses, following infection with RGNNV, SJNNV or attenuated mutant RGNNV viruses.

Amino acids at positions 247 and/or 270 of the RGNNV CP sequence are important virulence determinants to European sea bass (chapter 1)

Several previous studies have pointed out the importance of amino acids 247 and 270 in the CP sequence as virulence and host-specificity determinants to several fish species, such as Senegalese sole (*Solea senegalensis*), turbot (*Scophthalmus maximus*) and sea bream (*Sparus aurata*) (Souto et al., 2015a; 2016; Toffan et al., 2017). The work carried out in this chapter constitutes the first approach to study the role of those amino acids in the interaction between sea bass and a RGNNV isolate (SpDl_IAusc965.09), which is highly virulent to this fish species.

The implication of amino acids 247 and 270 has been analysed by changing those amino acids to those present in a SJNNV-type capsid. This experimental approach has highlighted their relevant role as virulence determinants, and, therefore, in the host-pathogen interaction. Thus, clinical signs observed in fish challenged with mutant viruses were weaker in intensity and in duration, causing mortalities 60% lower than that caused by the non-mutated virus (22.5%, 20% and 20% in Mut247Dl965, Mut270Dl965 and Mut247+270Dl965 groups, respectively), yielding mortality rates comparable to those recorded for SJNNV-infected sea bass in literature (Vendramin et al., 2014). Although these mutations have been responsible for a significant decrease in the viral virulence to sea bass, a totally attenuated virus has not been obtained. For this reason, it would be necessary to target additional regions of the RNA2 segment involved in betanodavirus virulence. In this regard, Olveira et al. (2009) described a third amino acid changed in the CP sequence of RGNNV/SJNNV reassortant strains. This amino acid is located at position 20, in the N-terminus region, which is involved in virus assembly and RNA packaging (Lu and Lin, 2003; Marshall and Schneemann, 2001). Furthermore, in a recent study, Souto et al. (2018) reported significant virulence modifications caused by nucleotide mutations in the 3'-untranslated region of the RNA2 segment. Both strategies could be good new targets to consider.

This reduced viral virulence of the mutant viruses has not been associated with changes in the CP tertiary structure, although some changes in the *in vitro* multiplication (on E-11 cells) have been reported. These results point out the complexity of the mechanisms involved in determining betanodavirus virulence to a specific role. Therefore, the present PhD Thesis may be a starting point to further

investigate betanodavirus virulence determinants.

In addition to virulence variations, differences in viral replication in brain were also recorded. Specifically, mutant viruses show lagged replication, or even no replication, up to 120 h post-infection (p.i.), compared to the non-mutated recombinant virus, resulting in lower viral titres in dead animals, and in consequence, in lower mortalities. These differences could be related with alterations in cell-receptor binding, since the modified amino acids are located in the protuberance surface of the CP, which is involved in interacting with host cells (Chen et al., 2015).

European sea bass *isg15*, a typical *isg15* gene with particular features (chapter 2A)

The European sea bass *isg15* gene has been sequenced and described in this study (chapter 2A). It displays the general structure described for other fish *isg15* genes, although showing some features that slightly differentiate it from other *isg15* genes.

Thus, as it happens for other fish *isg15* genes, sea bass *isg15* displays an intron sequence within the 3'-unstranslated region (UTR); however, it contains three non-frequent mRNA instability motifs (ATTTA, AU-rich elements or AREs), which have been related with heterogeneous nuclear RNA (hnRNA or pre-RNA) splicing regulation, as well as with mRNA expression (Zou et al., 1999). Another singular characteristic of the European sea bass *isg15* is the presence of four ATTTA motifs in the 3'-UTR sequence, which implies that *isg15* mRNA may have a short half-life. Furthermore, one cytoplasmic polyadenylation element (CPE, U-rich sequence), which is related with cytoplasmic elongation of the poly A tail, has also been located in this region. The presence of these regulatory motifs, some of them in high number, suggests a possible strong post-transcriptional regulation of European sea bass *isg15* gene.

Finally, the sea bass ISG15 deduced amino acid sequence also presents some characteristics that make this protein different from other mammal and fish ISG15 proteins. One of them is the lack of additional amino acids beyond the RLRGG conjugation motif. Some fish species, such as Atlantic cod (*Gadus morhua*), tongue sole (*Cynoglossus semilaevis*) or Senegalese sole, display additional residues downstream the conjugation motif, suffering a post-translational process, which is not required in the case of sea bass ISG15.

In addition, the comparison of the sea bass ISG15 sequence with other ISG15

proteins from higher vertebrates has shown differences in amino acids recently described to be crucial in the role of the ISG15 protein as cytokine (Swaim et al., 2017), being tempting to suggest that these changes may have a functional meaning, which requires further studies.

The *isg15* gene may be a useful marker of sea bass type I interferon (IFN I) activity *in vivo* (chapter 2A)

Although most studies on the fish IFN I system focus on characterizing *mx* transcription (Chaves-Pozo et al., 2012; Valero et al., 2015b), the *in vivo* analysis conducted in this PhD Thesis (chapter 2A) has determined that *isg15* is also a good marker of the IFN I system, complementing the knowledge on the IFN I response against betanodavirus infection in sea bass. Thus, *isg15* transcription was triggered in response to poly I:C and RGNNV infection both in head kidney and in brain, being the induction in response to poly I:C earlier and at higher level, whereas the *isg15* transcription triggered by the virus required viral replication, with the maximum value of transcription coinciding with the maximum number of viral copies recorded, which was also corroborated in chapter 3. RGNNV triggered a strong transcription in brain, highlighting the putative importance of this interferon-stimulated gene (ISG) in controlling betabodavirus infections.

Dl_ISG15_E11 is an *in vitro* system suitable for studying European sea bass ISG15 antiviral activity (chapter 2B)

The study of the antiviral specificity of ISGs in general, and ISG15 in particular, may be crucial to understand host-pathogen interaction. In this PhD Thesis, we have studied the antiviral activity of the European sea bass ISG15 protein (Dl_ISG15) based on the development of a stable cellular system, constitutively expressing this protein. Therefore, sea bass *isg15* transcription in this system does not depend on the activation of the IFN I pathway, which may depend on the cellular type used and/or the virus considered (Alvarez-Torres et al., 2013).

This system has been characterized, demonstrating that it expresses the Dl_ISG15 protein without affecting important cellular functions, since the cellular growth curve has not been altered. In addition, the *isg15* transcription is stable, not being affected by the number of cellular sub-cultures or the presence of viruses. All

these data demonstrate that putative changes recorded in these cells regarding viral titre and/or viral RNA synthesis are due to the antiviral activity of the Dl_ISG15 protein, and not a consequence of functional changes caused by the transfection and selection processes. In addition, the sea bass ISG15 protein has been shown to have a cytoplasmic location. Although a previous study has shown that grouper ISG15 does not alter its location in grouper (*Epinephelus coioides*) spleen cells after IFN I system stimulation with poly I:C or viral infection (Huang et al., 2013), it would be interesting to perform analyses on the sea bass ISG15 possible change of location after betanodavirus infections.

Finally, sea bass ISG15 has also been detected extracellularly, allowing the consecution of several kinds of assays.

The establishment of a permanently transfected cell line is an important advantage over previous studies based on transient transfection, since it allows a more stable and homogeneous expression of the exogenous protein, which is key to obtain reliable and reproducible results.

Intracellular sea bass ISG15 protein exerts a differential antiviral role against RGNNV and SJNNV infections (chapter 2B)

The *in vitro* analyses of intracellular Dl_ISG15 antiviral activity performed in the current work (chapter 2B) indicate that this protein may play different roles against both betanodavirus species tested, exerting antiviral action only against SJNNV, and modifying the transcription of some of the endogenous genes analysed, those involved in viral recognition (*tlr3*) and protein ligation (*e3*), in RGNNV-infected cells. We have demonstrated that Dl_ISG15 affects SJNNV replication, reducing viral genome synthesis, viral titres and increasing the survival rate of infected cells, whereas does not protect RGNNV-infected E-11 cells.

Previous studies have evaluated and reported differential antiviral effect of several fish ISG15 proteins against different fish viruses (Huang et al., 2013; Langevin et al., 2013a), although a differential role against different species of the same virus had not been described to date. The two viral species considered in this study show different virulence to sea bass, and our results seem to suggest that differences in virulence to a specific host can be closely related with different mechanisms of interaction between

the virus and the host immune system. Understanding the mechanisms underlining this interaction is crucial to develop prophylactic measures against viral infections.

Dl_ISG15-containing medium confers cellular protection from RGNNV and SJNNV (chapter 2B)

One previous study has reported the presence of a fish ISG15 protein in the medium of an established fish cell line transiently transfected (Langevin et al., 2013a); however, the present PhD Thesis constitutes the first work analysing the cytokine-like activity of a fish ISG15 protein on non-immune cells.

This cytokine-like activity has been evaluated by co-culturing Dl_ISG15_E11 (donor) and E-11 (target) cells and by incubating E-11 cells with Dl_ISG15-containing medium. Although it has not been completely ruled out the possible activation of IFN I system in Dl_ISG15-producing cells as partially responsible for the protection recorded, it is clear that the presence of sea bass ISG15 in the medium confers cellular protection, increasing the survival rate of target cells. Surprisingly, this protection is exerted against both betanodavirus species tested, RGNNV and SJNNV. In a recent study, Swaim et al. (2017) have reported five amino acids that are essential for the immuno-modulatory action of the secreted human ISG15 protein. The alignment performed in chapter 2A of this PhD Thesis revealed that these amino acids are only partially conserved in fish compared to higher vertebrates, which suggests that this signalling molecule could act with different mechanisms in higher vertebrates and teleost fish.

Thus, although additional experiments are required in order to further elucidate numerous aspects of the antiviral action of the secreted sea bass ISG15 protein, the results obtained suggest that Dl_ISG15 is a good protective agent against betanodavirus infection.

European sea bass exerts a complete immunogene response, involving innate and adaptive immune systems, against RGNNV and SJNNV infections (chapter 3)

Understanding the host-pathogen interaction is the main goal of studying the immune response, which should be applied to develop strategies to increase the fish resistance to viral diseases. In this study, the induction of the European sea bass immune system in response to betanodavirus infections has been analysed by quantification of the transcription of immunogenes involved in the IFN I system, as well

as in the inflammatory and adaptive responses. The results obtained reveal that European sea bass exerts a strong and quick response, involving both innate and adaptive pathways.

The analysis has been carried out in two different situations: (i) evaluating the response against RGNNV and SJNNV isolates in head kidney and brain (challenge 1), and (ii) studying the response against RGNNV attenuated viruses (harbouring mutations in amino acids 247 and/or 270 of the CP sequence, challenge 2), which were generated by reverse genetics (chapter 1). The aim of these challenges was to compare the sea bass immune response against betanodaviruses with different virulence to sea bass. Both challenges yielded comparable results.

The analysis of the innate defence (*ifn-I*, *mxA*, *isg15* and *isg12* transcription) points out the importance of this mechanism in response to betanodavirus and its relevant role in controlling viral infections. The results obtained showed a strong induction of these IFN I system genes, being this induction more intense in response to the viruses highly virulent to sea bass in all the organs analysed (RGNNV, in challenge 1, in head kidney and brain; and non-mutated virus, in challenge 2, in brain); however, this response was quicker in head kidney of SJNNV-inoculated sea bass, suggesting that this viral species induces a more rapid systemic response, as it has been previously reported by McBeath et al. (2014) for different strains of infectious salmon anaemia virus (ISAV). In addition, the induction of these genes in brain is coherent with the viral replication recorded.

The inflammatory response has also been evaluated, analysing the induction of the pro-inflammatory genes *il-8* and *tnf-α*, and the anti-inflammatory genes *il-10* and *tgf-β*. The results derived from both challenges, in which RGNNV (challenge 1) and the non-mutated virus (challenge 2) triggered a strong transcription of these pro-inflammatory genes in brain, provide evidences about the importance of the inflammatory process in betanodavirus infection. Thus, the massive inflammatory process may be responsible for the eventual damage in nervous tissues, which would lead to the fish dead, as it has been recently proposed for betanodavirus pathogenesis in Senegalese sole on the basis on a RNAseq study after infection with high and low virulent reassortant viruses (Labella et al., 2018). However, the role of a long-lasting anti-inflammatory response in preventing the histopathological lesions in nervous tissue

after infection with the less virulent isolate, as suggested by Poisa-Beiro et al. (2008), has not been possible to determine, being necessary the analysis of samples collected at longer times p.i.

Finally, regarding adaptive response, only evaluated in challenge 1, *tr-γ* and *mhc-β* transcription values suggest that the European sea bass adaptive response against betanodaviruses constitutes another important factor in the immune response, not only at a systemic level, but also at a local level.

Mutations in amino acids 247 and/or 270 in the RGNNV CP sequence cause serological changes (chapter 3)

The serological studies, using the ELISA technique and sera obtained from sea bass infected with the mutated viruses (chapter 1), have shown that the mutations considered (specially the mutation in amino acid 270) result in changes in some viral serological properties, reporting higher seroconversion in sea bass inoculated with Mut270Dl965 and Mut247+270Dl965, compared with the seroconversion elicited by the non-mutated virus, and changing antigen recognition by Mut270Dl965.

Noteworthy, the double mutation resulted in the generation of a virus (Mut247+270Dl965) able to induce the highest seroconversion, and antibodies in sera from these animals recognized both, RGNNV and SJNNV antigens. For these reasons, this virus may be a valuable candidate for anti-betanodavirus vaccine development. Bremont (2005) also proposed the use of attenuated rhabdoviruses, generated by reverse genetics, as safe vaccines, and Panzarin et al. (2016) suggested a quimeric protein as a candidate with particular interest in the development of an anti-betanodavirus vaccine due to its cross-reaction.

However, additional studies, including the use of a wider range of viral isolates as coating viruses in the ELISA protocol, and the analysis of the antibody neutralization properties, are required.

Final remark

European sea bass is an important commercial fish species on which important efforts have been focused at an industrial and scientific level. In this regard, the knowledge about the interaction between this fish species and the main viral pathogen threatening its culture, the nervous necrosis virus, is essential.

Some of the biotechnological approaches that have been described to date in order to control viral infections in fish farms include the use of vaccines, genetic selection or immuno-stimulants (Dalmo, 2018; Oh et al., 2012; Palaiokostas et al., 2018), which makes necessary to know both, the pathogen mechanism and the host response. The current PhD Thesis has addressed both aspects of this virus-host system, describing some important virulence determinants of RGNNV betanodaviruses to sea bass, obtaining attenuated viruses; increasing the knowledge of the sea bass immune response against betanodavirus species with different virulence to this fish species, and developing a cellular system to analyse the antiviral role of an specific interferon-stimulated protein, ISG15, and its possible use as protective agent.

Progress on these issues may help to develop new control measures and tools to prevent and overcome betanodavirus infections in sea bass aquaculture facilities.

CONCLUSIONS

1. Amino acids 247 and/or 270 in the RGNNV (red-spotted grouper nervous necrosis virus) capsid protein (CP) sequence (SpDl_IAusc965.09 isolate) play a key role as betanodavirus virulence determinants to European sea bass (*Dicentrarchus labrax*). The modification of theses amino acids to those in a SJNNV (striped jack nervous necrosis virus)-type CP sequence, generates attenuated virus to this fish species; however, a totally attenuated virus has not been obtained, suggesting the involvement of additional amino acids as virulence determinants.

2. The European sea bass interferon-stimulated gene 15 (*isg15*) displays the typical organization described for fish *isg15* genes, showing several regulatory motifs that suggest a strong post-transcriptional regulation.

3. European sea bass ISG15 protein (Dl_ISG15) exerts an *in vitro* antiviral action against SJNNV (SJ93Nag isolate), whereas RGNNV (SpDl_IAusc965.09 isolate) is not affected. The recorded antiviral activity is caused, at least partially, by reducing viral RNA synthesis.

4. Dl_ISG15 protein modulates the transcription of several E-11 endogenous genes, although only in the course of the RGNNV infection (SpDl_IAusc965.09 isolate), suggesting a differential antiviral role of this protein against SJNNV and RGNNV infections in E-11 cells.

5. Permanently transfected E-11 cells secrete European sea bass ISG15 protein, and the Dl_ISG15-containing medium protects non-transfected E-11 cells from RGNNV and SJNNV infection (SpDl_IAusc965.09 and SJ93Nag isolates).

6. The European sea bass immunogene response against betanodavirus infections involves a strong induction of interferon type I (IFN I) system genes (*isg15*, *isg12* and *mxA*) in brain and head kidney, and an important transcription of inflammatory (*il-8* and *tnf-α*) and adaptive response genes (*mhcII-β* and *tr-γ*) in the viral replication organ, especially after challenging with highly virulent viruses, belonging to the RGNNV species (ERV378/102-5/04 and SpDl_IAusc965.09 isolates).

7. Attenuated viruses, mutated in amino acids 247 and/or 270 in the RGNNV CP sequence, trigger a reduced transcription of genes involved in the IFN I system response (*mxA* and *isg15*) and in the inflammatory response (*tnf-α*) in brain, inducing, however, higher production of antibodies than the non-mutated *r*Dl965 virus (except for Mut247Dl965).

CONCLUSIONES

1. Las posiciones aminoacídicas 247 y/o 270 en la secuencia de la proteína de la cápside (CP) de RGNNV (*red-spotted grouper nervous necrosis virus*, aislado SpDl_IAusc965.09) constituyen importantes determinantes de virulencia de betanodavirus en lubina (*Dicentrarchus labrax*), generándose virus atenuados en base a la modificación de estos aminoácidos hacia aquellos presentes en la secuencia de la CP de SJNNV (*striped jack nervous necrosis virus*). Sin embargo, la falta de una atenuación total sugiere la implicación de aminoácidos adicionales como determinantes de virulencia.

2. El gen estimulado por interferón 15 (*isg15*) de lubina presenta una organización típica, similar a la de otros genes *isg15* de teleósteos, mostrando varios motivos de regulación que sugieren una fuerte regulación post-transcripcional.

3. La proteína ISG15 de lubina (Dl_ISG15) presenta actividad antiviral *in vitro* frente a la infección por SJNNV (aislado SJ93Nag), mientras la infección por RGNNV (aislado SpDl_IAusc965.09) no se ve afectada. Esta actividad antiviral se debe, al menos en parte, a la inhibición de la síntesis de ARN viral.

4. La proteína Dl_ISG15 modula la transcripción de varios genes en las células E-11 en las que se expresa, aunque solo en el curso de la infección por RGNNV (aislado SpDl_IAusc965.09), lo que sugiere una función antiviral diferencial de esta proteína frente a las infecciones por SJNNV y RGNNV en las células E-11.

5. Las células E-11 permanentemente transfectadas secretan la proteína ISG15 de lubina, y el medio procedente de estas células protege a las células E-11 no transfectadas frente a la infección por RGNNV y SJNNV (aislados SpDl_IAusc965.09 y SJ93Nag).

6. La respuesta inmune de la lubina frente a las infecciones por betanodavirus implica una fuerte inducción de los genes del sistema interferón tipo I (IFN I) *isg15*, *isg12* y *mxA*) tanto en riñón cefálico como en cerebro, y una importante transcripción de genes pro-inflamatorios (*il-8* y *tnf-α*) y genes implicados en la respuesta adaptativa (*mhcII-β* y *tr-γ*) en el órgano de replicación vírica, especialmente durante la infección con los virus que presentan mayor virulencia,

pertenecientes a la especie viral RGNNV (aislados ERV378/102-5/04 y SpDl_IAusc965.09).

7. Los virus atenuados, mutados en los aminoácidos 247 y/o 270 en la secuencia de la CP de RGNNV, inducen una menor transcripción de genes implicados en el sistema IFN I (*mxA* e *isg15*) y de respuesta inflamatoria (*tnf-α*) en cerebro, promoviendo, por el contrario, un mayor nivel de anticuerpos en suero con respecto al virus no mutado *r*Dl965 (excepto en el caso de Mut247Dl965).

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Short communication

Genetic characterization and transcription analyses of the European sea bass (*Dicentrarchus labrax*) *isg15* gene

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ABSTRACT

Fish interferons are cytokines involved in its resistance to viral infections by inducing the transcription of several interferon-induced genes, such as *isg15*. The aim of the present study was the genetic characterization of the European sea bass *isg15* gene, describing the regulatory motifs found in its sequence. In addition, an in vivo analysis of transcription in response to betanodavirus (RGNNV genotype) and poly I:C has been performed. The analysis of the resulting sequences showed that sea bass isg15 gene is composed of two exons and a single 276-bp intron located at the 5′-UTR region. The full length cDNA is 1143-bp, including a 102-bp 5′-UTR region, a 474-bp ORF, and a 291-bp 3′-UTR region. Several mRNA-regulatory elements, including three unusual ATTTA instability motifs in the intron, and four ATTTA motifs along with a cytoplasmic polyadenylation element in the 3′-UTR region, have been found in this sequence. The *in vivo* analyses revealed a similar kinetics and level of transcription in fish brain and head kidney after poly I:C inoculation; however, the induction caused by RGNNV started earlier in brain, where the upregulation of *isg15* gene transcription was high. The present study contributes to further characterize the European sea bass IFN I response against RGNNV infections.

Type I interferon (IFN I) is one of the main factors involved in the fish anti-viral innate response, inducing the transcription of numerous interferon-stimulated genes (ISG). One of the earliest and most highly-expressed ISGs upon viral infections is the interferon-stimulated gene 15 (*isg15*), coding for ISG15, which are 15-kDa effector proteins with a proven antiviral role [1]. ISG15 activity occurs through the covalent conjugation (ISGylation) of the LRGG motif, sited in the C-terminal end of all ISG15 proteins, to cellular or viral proteins. ISGylation is controlled by IFN-induced enzymes [2,3], and occurs through pathways similar to those of ubiquitination, resulting in the modification of some characteristics of the conjugated proteins, such as location, stability and activity [3,4]. In addition, unconjugated ISG15 may also play an important role in host response [5], since they can be secreted, acting as cytokines [6].

One of the main threats to the culture of European sea bass (*Dicentrarchus labrax*) is the appearance of viral nervous necrosis (VNN) outbreaks. This disease is characterized by damages in nervous tissues, which are responsible for the typical external symptoms, such as altered swimming and floatability, anorexia and visual dysfunction [7]. VNN is caused by Nervous Necrosis Virus (NNV, *Betanodavirus* genus, *Nodaviridae* family), with a single-stranded, positive-sense RNA bipartite genome [8]. RNA1 and RNA2 viral segments encode the viral polymerase and the capsid protein (CP), respectively. Fish nodaviruses have been grouped into four genotypes [9], being red-spotted grouper nervous necrosis virus (RGNNV) the only one associated with mass mortality episodes in sea bass to date. Although some aspects of the immune response of this fish species against NNV infections have been characterized, no information about the role of sea bass *isg15* is available. The present study contributes to further characterize the European sea bass IFN I response against RGNNV infections, addressing the *isg15* gene structure and transcription after polyinosinic:polycytidylic acid (poly I:C) and RGNNV inoculation.

For *isg15* gene characterization, sea bass cDNA and DNA were obtained and sequenced. Primers were designed according to the sea bass genome database (GenBank accession no. HG916840) by alignment with the expressed sequence tag (EST) of sea bass *isg15* (accession no. CV186275) [10] following the diagram displayed in Fig. 1. Those primers used to sequence and characterize the untranslated regions (UTR) were designed considering the polyadenylation signal and the transcription start site predicted with

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1050-4648/

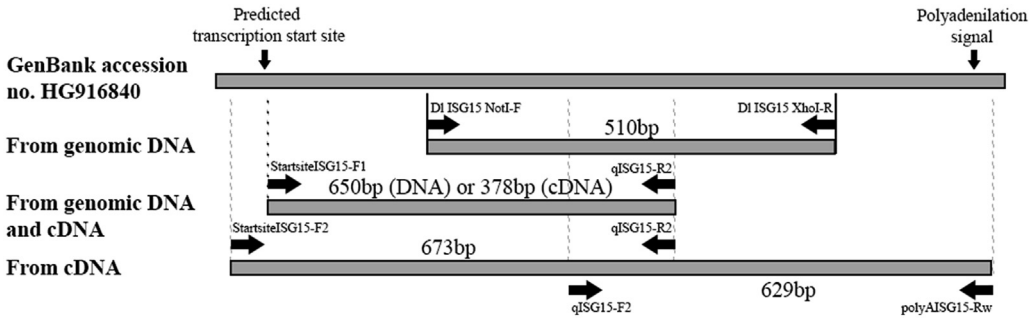

Fig. 1. Diagram showing fragments amplified and primers used for elucidating the genomic structure of the European sea bass *isg15*.

the PROSCAN v1.7 (BIMAS) software.

To obtain the *isg15* cDNA, juvenile European sea bass were intraperitoneally injected with poly I:C (25 mg/kg, CalBiochem). Animals were killed by anaesthetic overdose (MS-222, Sigma) at 8 h post-injection, and head kidneys were immediately collected and homogenized (10%, w/v) in Leibovitz medium (L15, Gibco) with 2% foetal bovine serum (FBS, Gibco), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Lonza). Homogenates were clarified, and total RNA extraction and cDNA synthesis were conducted using TRI Reagent solution (Sigma) and the Transcriptor First Strand cDNA Synthesis Kit (Roche), respectively, following manufacturer's recommendations. After the DNase I treatment (Roche), cDNA was amplified in 50-μl PCR mixtures containing 400 ng cDNA, 10 μM each primer (Table 1, Fig. 1, S1), 10 mM dNTPs, 2x Universe Buffer (Biotool.com), and 1 U Universe High-Fidelity Hot Start DNA polymerase (Biotool.com). Amplification conditions were: 95 °C for 3 min, 35 cycles at 95 °C for 15 s, 48–65 °C (depending on the primers used, Table 1) for 15 s and 72 °C for 15 s, and a final extension at 72 °C for 5 min.

Genomic DNA was isolated from caudal fin by saline precipitation [11], and the non-coding regions were sequenced and characterized by PCR amplification using the primers shown in Table 1 (Fig. 1, S1), and the above described conditions and mixture reactions.

Sequencing was conducted with the ABI 3730 (STABVIDA) system. Nucleotide sequence was translated into aminoacid sequence using the EditSeq (DNASTAR Lasergene 7) software. Intron screening was performed by alignment of the resulting cDNA and genomic DNA sequences.

The analysis of the resulting sequences showed the typical structure for fish *isg15* genes (Fig. 2). European sea bass *isg15* gene is composed of two exons and a single 276-bp intron, flanking by the canonical splicing GT/AG motif (S1). The intron region is located within the 5′-UTR, as it happens in other fish *isg15* genes (Fig. 2).

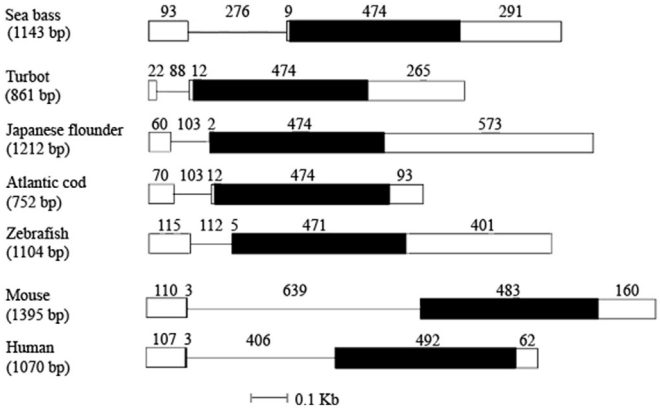

Fig. 2. Comparison between the European sea bass *isg15* genomic structure and other known *isg15* genes. Lines represent introns, and boxes are exons. Coding regions are in black, and the untranslated regions in white. Numbers above exons and introns indicate the size (bp) of each element.

This location suggests that the intron may be involved in repressing the silencing, and, therefore, in regulating ISG15 protein level [10]. Surprisingly, the sea bass *isg15* intron contains three instability motifs (ATTTA) (S1), which has only been described in a few genes, such as Atlantic cod (*Gadus morhua*) *isg15* (with a single ATTTA motif in the intron) or rainbow trout (*Onchorynchus mykiss*) interlaeukin-1β gen [12]. The ATTTA motifs in introns seem to have a role in regulating the splicing of heterogeneous nuclear RNA or the mRNA expression [12].

Based on the putative start and stop codons, the estimated complete ORF is 474-bp long. The 5′-UTR starts 102 bp upstream the start codon (Fig. 2, S1), whereas the 3′-UTR region is 291-bp long, containing one AATAAA polyadenylation signal and four instability motifs (S1). These motifs are frequently found in 3′-UTR

Table 1
Primers used in this study.

Name	Sequence	Annealing temperature (°C)	Usage
DlISG15 *Not*I-F	5′-GATCACAAGGGCGGCCGCATGATGGATATAACC-3′	65	*isg15* ORF
DlISG15 *Xho*I-R	5′-TATGAGCCTCGAGGGTGCTCAGCCTCCTCTCAG-3′	65	*isg15* ORF
PolyAISG15-Rw	5′-TTTTTTTTTTTTTACTTTAAATAGTTTC-3′	48	*isg15* cDNA sequencing
StartsiteISG15-F1	5′-CACAGCTGTTCCTAACAATCCTC-3′	57	*isg15* cDNA, DNA sequencing
StartsiteISG15-F2	5′-CTGTGGGGAGCTGAAACCTCT-3′	58	*isg15* cDNA, DNA sequencing
qISG15-F2	5′-CGACTCAAAGCCTCTCTGCTACT-3′	60	*isg15* qPCR, cDNA sequencing
qISG15-R2	5′-CGTTTCTGACGAACACCTGGAT-3′	60	*isg15* qPCR, cDNA, DNA sequencing
RG_965_RNA2-F4	5′-ACCGTCCGCTGTCTATTGACTA-3′	60	RGNNV qPCR
RG_965_RNA2-R1	5′-CAGATGCCCCAGCGAAACC-3′	60	RGNNV qPCR
18S rRNA–Fw[a]	5′-CCAACGAGCTGCTGACC-3′	60	Housekeeping gene qPCR
18S rRNA-Rw[a]	5′-CCGTTACCCGTGGTCC-3′	60	Housekeeping gene qPCR

[a] [24].

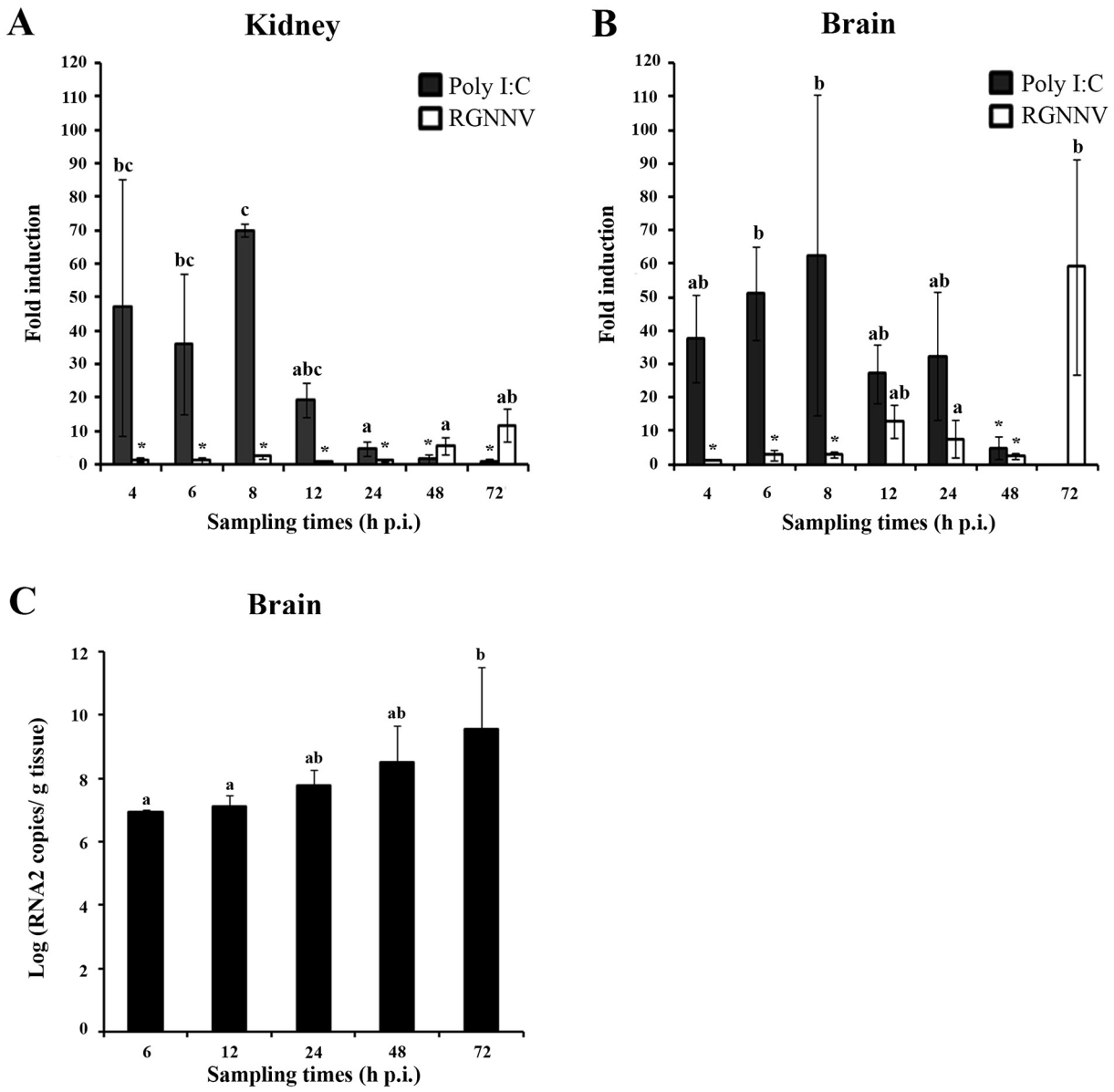

Fig. 3. (A) *isg15* gene transcription in head kidney of fish inoculated with poly I:C or RGNNV; (B) *isg15* gene transcription in brain of fish inoculated with poly I:C or RGNNV; (C) Absolute quantification of the NNV RNA2 segment in brain. Values are mean ± SD obtained from three samples. Different letters denote significant differences ($p < 0.05$). Asterisks indicate non-induced samples.

regions of interleukins and other signalling proteins-coding genes [13], being involved in mRNA stability and translation efficiency. Specifically, the number of ATTTA motifs in fish *isg15* genes varies from one in zebrafish (*Danio rerio*, NP_001191098) to six in Japanese flounder (*Paralichthys olivaceus*, BA148419), whereas no instability motifs have been found in 3′-UTR regions of *isg15* mRNAs from mammals, such as *Homo sapiens* (AAH09507), *Mus musculus* (NP_056598), *Bos taurus* (BC102318), *Tupaia belangeri* (JN866608), or *Ovis ammon* (KC481400). The high number of ATTTA motifs in the 3′-UTR region of sea bass *isg15* (four) suggests that these transcripts may have a short half-life, as has been previously reported for the human insulin-like growth factor mRNA [14], which is more efficiently cleaved by the E-like endonuclease if it contains more than one ATTTA motif. In addition, the sea bass *isg15* 3′-UTR region contains one cytoplasmic polyadenylation element (CPE, S1), which are U-rich sequences required for the cytoplasmic elongation of the nuclear poly-A tail, able to repress or exert translation depending on the cellular type [15]. This mRNA regulatory element is also present in *isg15* genes from some fish species, such as zebrafish, Japanese flounder, or turbot (*Scophthalmus maximus*, AHW76805), but has not been found in the above mentioned mammals. Taken together, these results suggest that ISG15 expression may be subjected to a stronger post-transcriptional regulation in fish than in higher vertebrates.

The deduced aminoacid sequence showed two typical ubiquitin like (UBL) domains and the LRGG motif at the 3′-end (S1). Additional aminoacids downstream the LRGG motif have been described in mammals (except ruminants) and some fish species, such as Atlantic cod, red drum (*Sciaenops ocellatus*), tongue sole (*Cynoglossus semilaevis*), Atlantic halibut (*Hippoglosssus hippoglossus*), three spined stickleback (*Gasterosteus aculeatus*) and Japanese flounder [10]. However, those additional aminoacids have not been found in the sea bass ISG15 sequence, indicating that this protein does not suffer post-translational processing to expose the

conjugating motif. The alignment with other fish ISG15 proteins yielded identities ranging from 46.1 to 73.5% (S2), showing the highest identity with ISG15 proteins from other species within the Perciformes order, such as *Sciaenops ocellatus*, *Oplegnathus fasciatus*, and *Epinephelus coioides*, and the lowest identity with ISG15 from goldfish (*Carassius auratus*) and zebrafish.

In order to complete the sea bass *isg15* gene characterization, a comparative analysis of transcription in head kidney and brain following poly I:C and virus inoculation was conducted. Fish used in this study were handled following the European Union guidelines for the handling of laboratory animals (directive 2010/63/UE). Temperature, lighting and noise were strictly controlled in order to minimize stress. Prior to the challenges, 9 fish were randomly collected, and their brains and eyes were analyzed according to Lopez-Jimena et al. [16] to discard a possible NNV asymptomatic carrier state (data not shown).

NNV-free European sea bass specimens (6 g, average weight) were split into two groups ($n = 40$ per group) to be intramuscularly challenged with the D1965.05 isolate (RGNNV genotype, 10^5 $TCID_{50}$/fish), or intraperitoneally injected with poly I:C (25 mg/kg). In addition, a negative control group, intramuscularly injected with L15, was also considered. The RGNNV isolate was propagated on E-11 cells [17] at 25 °C in L15 medium plus 2% FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin until displaying extensive cytopathic effects. The resulting virus suspension was titrated by the 50% tissue culture infective dose method [18].

Three animals per group were killed by anaesthetic overdose at 4, 6, 8, 12, 24, 48, and 72 h post-infection (p.i.). Head kidneys and brains were aseptically collected and individually stored in liquid nitrogen. Organ homogenization, RNA isolation and cDNA synthesis were conducted as described previously.

Isg15 gene transcription was quantified by real-time PCR using qISG15F2/R2 primers and the 18S RNA-coding gene as housekeeping (Table 1). Amplification was undertaken according to Alvarez-Torres et al. [19] using the LightCycler 480 termocycler (Roche). Relative fold induction was calculated by the $2^{-\Delta\Delta Ct}$ method [20]. These values were log-transformed, and data normality distribution was verified by Shapiro-Wilk test. Finally, the results were analyzed by the one-way analysis of variance (ANOVA) and the Tukey post hoc test for multiple comparisons. Values of $p < 0.05$ were considered significant. Samples with relative values statistically similar to those recorded in mock-injected animals ($p < 0.05$) were considered as non-induced.

Virus replication in brain was analyzed by absolute quantification of the RNA2 segment. Serial dilutions of the pJET easy vector (Thermo scientific) containing the complete RNA2 segment of the Dl965.05 isolate were used as standard curve. Amplification was performed according to Alvarez-Torres et al. [19] using RG_965_RNA2-F4/R1 primers (Table 1), the LightCycler 480 termocycler, and the Fast Start Essential DNA Green Master (Roche).

The results displayed in Fig. 3A and B showed that poly I:C triggers an earlier and higher *isg15* gene transcription than RGNNV in both organs analyzed, which has also been reported in other fish species [10,19]. This is not a surprising result, since poly I:C is a direct stimulator of the IFN I system, whereas the induction triggered by viruses requires viral replication. In head kidney, poly I:C stimulated *isg15* gene transcription from 4 h (47 mean fold induction) to 24 h p.i. (4.6) (Fig. 3A), whereas NNV-induced transcription was only recorded at 48 and 72 h p.i., with mean values of 5.6 and 11.7, respectively (Fig. 3A). In brain, the kinetics and level of transcription following poly I:C inoculation was similar to that recorded in head kidney; however, the induction caused by the virus started earlier (at 12 h p.i., 12.7 mean fold induction) (Fig. 3B). In both organs, the maximum transcription in response to virus infection was at 72 h p.i., being the values recorded at this time in brain (59.1 mean fold induction) higher than those recorded in kidney (11.7). The high mean fold induction value in brain at 72 h p.i. constitutes a second peak in the transcription kinetics, which could be consequence of a positive loop in the induction of the IFN system caused by ISG15 proteins, which has been previously demonstrated for zebrafish ISG15 [21].

Viral multiplication in brain has been demonstrated by the significant increase in the RNA2 copy number from 6 h (6.7 log copies/g tissue) to 72 h p.i. (9.6) (Fig. 3C). The maximum number of RNA2 copies was at 72 h p.i., coinciding with the maximum induction of *isg15* gene in this tissue, suggesting a possible relation between *isg15* and CP genes expression.

This study describes the genomic structure of sea bass *isg15* gene, showing a differential transcription in brain and head kidney in response to poly I:C and RGNNV, with poly I:C being a short-term inducer in both organs. The upregulation of *isg15* gene transcription was especially high in brain (59.1 mean fold induction at 72 h p.i.), which is the main target organ for viral replication, suggesting an important anti-NNV role of this protein in sea bass, as has been previously established for grouper ISG15 [22]. In fact, the evaluation of the anti-NNV activity of sea bass ISG15 is an important issue to be addressed for the characterization of the sea bass-NNV interaction.

Previous studies on ISG transcription in brain from NNV-infected fish have been focused on the *mx* gene, which is also highly induced after viral infection from 72 h p.i. onwards in turbot and European sea bass [23,24]. Although *mx* gene transcription has traditionally been considered as a good marker of IFN I activity, the results derived from this work indicate that *isg15* gene can also be a useful marker of this activity.

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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.2016.06.043.

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Full length article

Differential antiviral activity of European sea bass interferon-stimulated 15 protein (ISG15) against RGNNV and SJNNV betanodaviruses

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ABSTRACT

ISG15 is an antiviral protein acting intracellularly, by conjugation to viral or cellular proteins, or extracellularly, as cytokine. In this work, an *in vitro* system, consisting of E-11 cells over-expressing European sea bass ISG15 (Dl_ISG15_E11 cells), has been developed to evaluate the European sea bass ISG15 protein activity against RGNNV and SJNNV isolates. Regarding RGNNV, RNA2 copy number and viral titres were similar in E-11 and Dl_ISG15_E11 cells, and the cellular survival analyses demonstrated that Dl_ISG15_E11 cells were not protected from this virus. In contrast, ISG15 compromises SJNNV replication, since a reduction of the SJNNV genome synthesis has been recorded. The ISG15 anti-SJNNV activity was confirmed by viral titration and survival assays. In addition, a role of the intracellular ISG15 in modulating the transcription of endogenous genes has being recorded, with *tlr3* gene being knocked out and *e3* gene being up-regulated in RGNNV-inoculated Dl_ISG15_E11 cells. Sea bass ISG15 has also been detected extracellularly, and its activity has been evaluated by co-culture. The survival rate of RGNNV-inoculated E-11 cells increased from 25% to 46% when they were co-cultured with ISG15-producing cells. Similarly, the survival rate of SJNNV-inoculated E-11 cells increased from 27% to 51% in co-culture with ISG15-producing cells. To our knowledge, this is the first description of a differential antiviral activity of an ISG15 protein against two betanodavirus species, and the first evaluation of the cytokine-like activity of a fish ISG15 protein on non-immune cells.

1. Introduction

Type I interferon (IFN I) is a cytokine involved in the antiviral innate immune system of vertebrates [1–3]. After viral infection, cells secrete IFN I, which is detected by neighbouring cells, inducing the transcription of numerous interferon-stimulated genes (ISGs), including the interferon-stimulated gene 15 (*isg15*). The *isg15*-encoding protein (ISG15) is a 15-kDa protein composed of two ubiquitin-like domains (UBL), connected by a short linker sequence, and a conserved C-terminal RLRGG motif, which is required for conjugation to viral or cellular proteins, in a process called ISGylation.

ISGylation occurs through a pathway similar to that of ubiquitination [4], resulting in the modification of some characteristics of the conjugated proteins, such as location, stability and activity [5]. In mammals, hundreds of proteins, involved in all stages of cellular biology, have been described as ISGylation-target proteins [6–8]. Regarding viral infections, ISG15 has been described to limit virus release by conjugating cellular proteins involved in ligase activity and secretory pathways, such as Nedd4, TSG101 and CHMP5, in Ebola virus, influenza virus and HIV-1 virus infections, respectively [9–11], or conjugating viral proteins, such as influenza NS1 and NP proteins [12,13].

ISG15 proteins have also been found extracellularly, acting as cytokine [14,15]. Thus, this protein has been detected in IFN I-treated human leukocyte and monocyte cultures [14,16], promoting IFN-γ production by immune system cells, mainly natural killers (NK) and lymphocytes T [14,17–19].

Viral nervous necrosis is a widely spread neuropathological disease that affects European sea bass (*Dicentrarchus labrax*), specially at larval and juvenile stages [20–24]. Main signals of this disease are lesions in nervous tissues, causing alterations in swimming and floatability, anorexia and visual anomalies. The etiological agent is the nervous necrosis virus (NNV, *Betanodavirus* genus), a single-stranded, positive-sense RNA virus, with a bipartite genome composed of RNA1 and RNA2 segments, coding the viral polymerase and the capsid protein, respectively [25]. Based on the nucleotide variability in the RNA2 segment, betanodaviruses have been clustered into four species (former genotypes) [26,27]; however, only red-spotted grouper- (RGNNV) and

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striped jack- (SJNNV) nervous necrosis virus have been detected in sea bass, which is susceptible to both viral species, although causing very different mortality rates, as it has been demonstrated by experimental infections [28,29].

In fish, *isg15* is one of the earliest and most highly-expressed ISGs following viral infections [30–34]. Recently, Moreno et al. [35] have described the structure of European sea bass *isg15*, which is similar to that described for other fish *isg15* genes, being composed of one 510-bp long open reading frame (ORF), with an intron located in the 5′-untranslated region (UTR). The *in vivo* transcription of this gene after RGNNV and SJNNV inoculation has also been evaluated, demonstrating that both viral species are strong *isg15* inductors in sea bass brain and head kidney [35,36].

In addition, studies on the antiviral activity of ISG15 proteins from several fish species, such as grouper (*Epinephelus coioides*) and zebrafish (*Danio rerio*), have revealed activity against several fish viruses, such as grouper nervous necrosis virus (GGNV) [37], infectious haematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), and epizootic haematopoietic necrosis virus (EHNV) [38].

The aim of the present study has been to study the role of the European sea bass ISG15 protein against betanodavirus infections. As a first step, the *isg15* ORF has been cloned into an expression vector in order to develop an *in vitro* system suitable for evaluating the antiviral activity of this protein.

2. Materials and methods

2.1. Viruses and viral propagation

Viral isolates SpDl_IAusc965.09 (RGNNV) [35] and SJ93Nag (SJNNV) [39] were propagated and titrated on the E-11 cell line [40].

E-11 cells were grown in Leibovitz L-15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco), 100 unit/ml penicillin and 10 mg/ml streptomycin (Sigma) (growth medium) at 25 °C until confluence. Once inoculated, these cells were maintained in L-15 supplemented with 2% FBS, 100 units/ml penicillin and 10 mg/ml streptomycin (maintenance medium) at 25 °C (RGNNV) or 20 °C (SJNNV), until cytopathic effect (CPE) development. Viral suspensions were titrated on E-11 cells following the 50% tissue culture infective dose method ($TCID_{50}$) [41].

2.2. Establishment of a cell line constitutively expressing the sea bass ISG15 protein (Dl_ISG15)

Sea bass *isg15* cDNA was obtained from juvenile specimens (n = 3) (6 g, average weight) intraperitoneally injected with polyinosinic:polycytidylic acid (poly I:C, CalBiochem, 1 mg/fish). At 8 h post-infection (p.i.), fish were killed by anaesthetic overdose (MS-222, Sigma), and head kidneys were frozen in liquid nitrogen until used. Head kidneys were individually homogenized, and total RNA was extracted with the TRI reagent solution (Sigma). After RNA treatment with DNase I Recombinant from bovine pancreas (Roche), cDNA was synthesized using 1 μg of RNA and the Transcriptor First Strand cDNA Synthesis Kit (Roche).

Sea bass *isg15* was amplified using the pair of primers DlISG15 NotI-F (5′-GATCACAAGGGCGGCCGCATGATGGATATAACC-3′) and DlISG15 XhoI-R (5′-TATGAGCCTCGAGGGTGCTCAGCCTCCTCTCAG-3′), and the Universe High-Fidelity Hot Start DNA polymerase (Biotools), as described by Moreno et al. [35]. Amplified products were cloned in the pGemT easy vector (Promega) and subsequently sub-cloned in the expression vector pcDNA™4/HisMax B (Invitrogen), containing the cytomegalovirus (CMV) promoter, a polyhistidine metal-binding tag, and the Zeocin™ resistance gene. Purified plasmids (labelled as Dl_ISG15_pcDNA) were stored at −20 °C until cell transfection.

Monolayers of E-11 cells grown on 6-well plates were transfected using the Lipofectamine® 2000 Transfection Reagent (Invitrogen), according to Iwamoto et al. [42]. Transfected cells were sub-cultured in 25-cm^2 flasks and selected with zeocine (1250 μg/ml, Invitrogen) to obtain the Dl_ISG15_E11 cell line.

2.3. Characterization of the Dl_ISG15_E11 cell line

Analyses of cellular growth kinetics, ISG15 immuno-detection, and *isg15* gene transcription have been performed.

For the cellular growth analysis, multiplication of Dl_ISG15_E-11 and E-11 cells has been comparatively evaluated. Both cell lines were seeded (1.4×10^5 cells/ml) on 24-well plates and maintained in growth medium at 25 °C. Cells from three wells were collected at 2, 3, 4, 5, 7 and 8 days to be counted, in triplicate, using the Z1 coulter (Beckman Coulter). This assay was performed in duplicate.

The Dl_ISG15 protein has been detected by western blotting. Supernatants and cells from both cell lines, grown in 75-cm^2 flasks, were separately collected and processed to detect extracellular and intracellular recombinant Dl_ISG15 protein.

Extracellular Dl_ISG15 was purified and concentrated with an immuno-precipitation kit (Roche), using A/G agarose beads, and the HisG Epitope Tag Monoclonal Antibody (Invitrogen, Thermo), according to commercial guidelines.

For intracellular Dl_ISG15 detection, cells were washed with PBS, sonicated at 4 °C for 10 min, and centrifuged at 1500 × *g* at 4 °C for 5 min. Proteins in the resulting supernatant were concentrated using the centrifugal filter Amicon® Ultra-15 (Merck), with a molecular weight cut-off of 3000, at 5000 × *g* for 30 min. The resulting sample was analysed by western blot.

For western blotting, proteins separated by 12.5% SDS-PAGE electrophoresis were transferred to an Immobilon membrane (Millipore) [43]. The HisG Epitope Tag Monoclonal Antibody (1/1000 in 5% skimmed-powder milk-PBS-Tween20) was used to detect the 6xHis-tagged recombinant ISG15 protein. The monoclonal Anti-β-Actin Antibody (Sigma, 1/5000 in 5% skimmed-powder milk-PBS-T) was used as cellular control. The secondary antibody used was HRP-conjugated goat anti-mouse IgG (Sigma, 1/20000 in 5% skimmed-powder milk-PBS-T). The specific band was visualized using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo) and the ChemiDoc™ Imaging Systems (Bio-Rad).

The effect of viral infection on sea bass *isg15* transcription has been evaluated by relative RT real-time PCR in presence or in absence of RGNNV or SJNNV. Each virus was inoculated at 0.1 multiplicity of infection (MOI) on Dl_ISG15_E-11 cells grown on 24-well plates. Cells from three wells were harvested at 24 h p.i., and total RNA was extracted with the E.Z.N.A. total RNA Kit I. After treatment with DNase I Recombinant from bovine pancreas, RNA (1 μg) was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit. Amplifications of *isg15* were performed following the methodology described by Moreno et al. [35], using the specific primers shown in Table 1, and *beta-actin* as endogenous reference gene. Relative values were calculated by the $2^{-\Delta Ct}$ method. This assay was performed in duplicate.

2.4. Anti-betanodavirus activity of intracellular sea bass ISG15 protein

Viral replication on Dl_ISG15_E11 and E-11 cells has been comparatively analysed by virus quantification and cellular survival assays.

For virus quantification analyses, Dl_ISG15_E11 and E-11 cells grown on 24-well plates were inoculated with RGNNV or SJNNV at 0.1 MOI. At 0, 24, 48 and 72 h p.i., supernatants and cells from three wells were separately collected for viral titration, or viral genome quantification, respectively. This assay was performed in duplicate.

Viral genome was quantified by absolute real-time PCR. Cellular RNA and cDNA were obtained as previously described. All the amplifications were conducted with the LightCycler 96 Thermocycler in 20-μl

Table 1
Primers used in this study.

Target gene	Sequence (5′-3′)	Amplicon size (bp)	Reference
RGNNV RNA2	ACCGTCCGCTGTCTATTGACTA CAGATGCCCCAGCGAAACC	126	[35]
SJNNV RNA2	GACACCACCGCTCCAATTACTAC ACGAAATCCAGTGTAACCGTTGT	75	[44]
Sea bass isg15	CGACTCAAAGCCTCTCTGCTACT CGTTTCTGACGAACACCTGGAT	100	[35]
E-11 *tlr3*	TGCAACACTCCACTGACTTACTTTAA AGGACAGCTGTGCTAAGTATATAA	115	This study
E-11 *e3*	TGCACTTGCAAGGCTGTCA CTCCTAGGATACTTGCATAGAAGACAAC	100	This study
E-11 *mx*	GGGGTCAGAAGGAGATCACA ATGATGCACCAGCTCAAGTG	150	[58]
E-11 *beta-actin*	CACTGTGCCCATCTACGAG CCATCTCCTGCTCGAAGTC	200	[59]

mixtures containing cDNA generated from 50 ng of RNA, 1 × Fast Start Essential DNA Green Master Mix (Roche) and 10 pmol specific primers (Table 1). Amplification conditions were 95 °C for 10 min, and 45 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Melting curves were obtained at 95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s. Serial dilutions of the pJET vector (Thermo) containing the RGNNV RNA2 sequence, and the pCR™4-TOPO® TA vector (Invitrogen) containing the SJNNV RNA2 sequence [44] were used to generate reference standard curves.

In order to determine the cellular survival rate after virus infection, Dl_ISG15_E11 and E-11 cells grown on 24-well plates were inoculated with RGNNV or SJNNV at 0.1 MOI. Cells from three wells were collected at 0, 48 and 72 h p.i. to be counted in triplicate (Z1 coulter, Beckman Coulter). Non-inoculated cells, negative control, were processed in the same way.

2.5. Immunomodulation activity of intracellular sea bass ISG15 protein

The possible modulation of the transcription of mx dynamin like GTPase (*mx*), toll-like receptor 3 (*tlr3*) and ubiquitin ligase E3 (*e3*) genes in Dl_ISG15_E11 cells has also been evaluated. Monolayers of E-11 and Dl_ISG15_E11 cells seeded on 24-well plates were inoculated with RGNNV or SJNNV at 0.01 MOI. Cells from three wells were collected at 0, 3, 6 and 24 h p.i., and RNA and cDNA were obtained as previously described.

All amplifications were conducted with the LightCycler 96 Thermocycler and the Fast Start Essential DNA Green Master Mix, using cDNA generated from 50 ng of RNA and the primers shown in Table 1. Amplifications consisted of 95 °C for 10 min followed by 45 cycles at 95 °C for 10 s, 52 °C for 10 s and 72 °C for 10 s. Melting curves were obtained at 95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s. *Beta-actin* was the endogenous reference gene used (Table 1). Relative fold change values were calculated by the Pfaffl method [45].

2.6. Anti-betanodavirus activity of extracellular sea bass ISG15 protein

These analyses have been conducted by co-culture of E-11 and Dl_ISG15_E11 cells, as well as by E-11 incubation with ISG15-containing medium.

The co-culture protocol used [46] allows the culture of two different cell lines (donor and target cells) in droplets within the same well on a 6-well plate. The possible protection of Dl_ISG15_E11 cells over inoculated E-11 cells was measured by crystal violet staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction quantification.

Droplets (150 µl) of donor (Dl_ISG15_E11) and target (E-11) cells, containing 9 × 10^4 cells, were separately seeded in the same well, and they were incubated in a humid chamber at 25 °C overnight for cell attachment. A negative control, in which two droplets of E-11 cells were seeded, was also set up. After that time, droplets were removed, and a volume of 2 ml of L-15 growth medium was added. These cells were incubated at 25 °C for 24 h before virus inoculation (10^4 $TCID_{50}$/ml). Inoculated cells were incubated at optimal virus replication temperature until fully CPE appearance. A negative control, non-inoculated cells, was also included.

Once CPEs were observed, cells in two wells were fixed and stained with 1% crystal violet in 22% formaldehyde (Sigma) at room temperature for 10 min. After several washes with PBS at room temperature for 5 min, droplets were photographed and pictures were analysed with the ImageJ (NIH) software.

Since crystal violet staining is a semi-quantitative technique, a modification of the above explained protocol, based on MTT reduction, has been developed. In this modified protocol, cells in droplets were attached on glass coverslips (Nunc™ Thermanox™ Coverslips, Thermo), which were placed into 6-well plates for virus inoculation and the subsequent incubation, as described previously. Once fully CPEs were observed, coverslips were moved to a 24-well plate for MTT reduction development [47]. Optical density (OD) was quantified at 550 nm using the Whittaker Microplate Reader 2001 (Anthos Labtec).

The role of the secreted Dl_ISG15 protein in protecting E-11 cells from betanodavirus has been further investigated by culturing these cells with medium from Dl_ISG15_E11 cells. This medium was obtained from sea bass ISG15-producing and non-producing (control) cells cultured at 25 °C for 72 h. All the experiments have been conducted with these batches of ISG15-containing and ISG15-free media.

E-11 cells grown on 96-well plates were inoculated with RGNNV (10^4 $TCID_{50}$/ml). After 1-h incubation at 25 °C, the viral suspension was removed and replaced by L-15 maintenance medium mixed with medium from ISG15-producing cells (1/1). A control group, consisting of inoculated cells incubated with medium from non-producing E-11 cells (in the same proportion), was also included. Plates were incubated until CPE appearance, and the protective effect was then visualized by MTT addition in triplicate.

2.7. Statistical analyses

Data were analysed with the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA). Normality distribution was verified by the Shapiro-Wilk test, and the t-student was the statistical tests used. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Characterization of the Dl_ISG15_E11 cell line

Growth curve analysis (Fig. 1) showed no significant differences ($P > 0.05$) between the growth performance of Dl_ISG15_E11 and control E-11 cells, since cell concentration was the same for both cell types at all sampling times. Maximum cellular concentration was recorded after 7 days of incubation, with 3.9 × 10^5 and 3.6 × 10^5 cells/ml for E-11 and Dl_ISG15_E11, respectively.

In addition, the recombinant Dl_ISG15 protein has been detected by western blot extracellularly and intracellularly (Fig. 2). The extracellular detection of Dl_ISG15 is depicted in Fig. 2A, showing a band of the expected size after analysing medium from Dl_ISG15-producing cells immuno-precipitated with an anti-His monoclonal antibody (lane 4). However, no bands were observed by processing crude Dl_ISG15-producing cell medium (lane 2). Moreover, ISG15 was not observed when control E-11 medium was processed in the same way (lane 3).

In order to clarify if extracellular Dl_ISG15 protein is secreted or is a consequence of cellular lysis, the presence of beta-actin in the extracellular medium was evaluated (Fig. 2B). Beta-actin was detected in concentrated extracts of Dl_ISG15_E11 (lane 7) and E-11 (lane 8) cells,

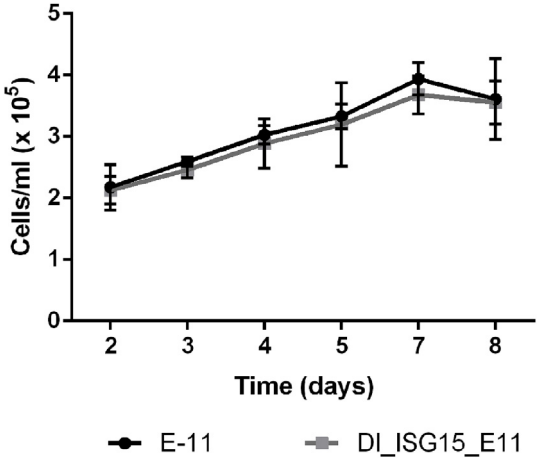

Fig. 1. Growth curves of Dl_ISG15_E11 and E-11 cells. The t-student test was used to compare cell concentration within each sampling time. Values of $P < 0.05$ were considered significant. Results are mean ± standard deviation (SD) (n = 3).

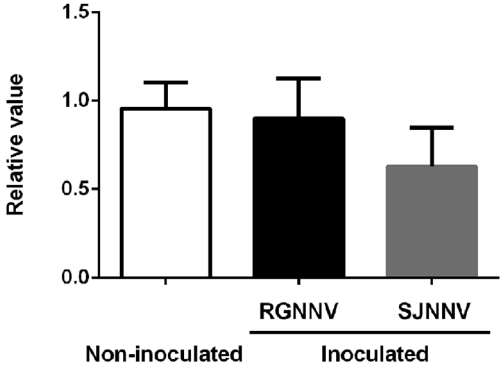

Fig. 3. Sea bass *isg15* mRNA quantification in non-inoculated, RGNNV- and SJNNV-inoculated Dl_ISG15_E11 cells analysed at 24 h p.i. Data were statistically analysed with the t-student test. Values of $P < 0.05$ were considered significant. Results are mean ± SD (n = 3).

which constitutes a positive control for the beta-actin detection. Lanes 9 and 10 show absence of beta-actin in the extracellular fraction of Dl_ISG15_E11 and E-11 cells, respectively, suggesting absence of cellular proteins in the medium. Intracellular Dl_ISG15 detection is represented in Fig. 2C, showing the presence of this protein only within Dl_ISG15_E11 cells (lane 13). E-11 cells have been analysed as negative control (lane 12).

Finally, the level of *isg15* mRNA in absence and in presence of viral infection has been analysed. Fig. 3 evidences no significant differences ($P > 0.05$) in *isg15* transcription due to viral infection, with mean relative values of 0.96, 0.90 and 0.63 for non-infected, RGNNV- and SJNNV-infected cells, respectively. Viral multiplication in these cells has been confirmed by absolute PCR (data non-shown).

3.2. Anti-betanodavirus activity of the intracellular Dl_ISG15 protein

Viral multiplication in Dl_ISG15_E11 and E-11 cells has been determined by quantification of viral genome within the cells, and by titration of infective viral particles in the supernatant. In addition, in a parallel assay, survival rates of inoculated cells have also been determined.

The results of quantitative analysis of viral genome are shown in Fig. 4A. The copy number of RGNNV RNA2 was similar in both cell types at all sampling times ($P > 0.05$). However, the number of SJNNV RNA2 copies was lower in Dl_ISG15-producing cells at 48 h ($P = 0.004$) and 72 h ($P = 0.01$) p.i., recording mean values of 3.2×10^{10} and 2.4×10^{9} RNA2 copies/μg RNA at 48 h p.i. in E-11 and Dl_ISG15_E11 cells, respectively.

The virus titration yielded similar results (Fig. 4B). Thus, viral titres recorded in supernatants of RGNNV-inoculated Dl_ISG15_E11 and E-11 cells were similar ($P > 0.05$) at all sampling times analysed, with maximum titres at 72 h p.i. (9.9×10^5 and 6.4×10^5 $TCID_{50}$/ml, for E-11 and Dl_ISG15_E11 cells, respectively). However, SJNNV titre in supernatants of Dl_ISG15_E11 cells (2.2×10^3 $TCID_{50}$/ml) was significantly lower than the titre obtained in E-11 supernatants (1.2×10^4 $TCID_{50}$/ml) at 48 h p.i. ($P = 0.01$). The maximum SJNNV titre was observed at 72 h p.i., with mean values of 1.9×10^5 and 8.9×10^4 $TCID_{50}$/ml, for E-11 and Dl_ISG15_E11 cells, respectively (Fig. 4B).

In order to complete this analysis, a survival assay of inoculated E-11 and Dl_ISG15_E11 cells has been performed (Fig. 4C). Regarding RGNNV-inoculated cells, results showed similar survival percentages for both cell lines ($P > 0.05$) at all sampling times, with the minimum mean value (ca. 80%) at 72 h p.i. However, the percentage of cells surviving SJNNV infection was significantly higher for cells expressing the sea bass ISG15 protein at 48 and 72 h p.i. ($P = 0.01$ and $P = 0.005$, respectively). Thus, the survival rate of Dl_ISG15_E11 cells was 100% at 48 h p.i. and 95% at 72 h p.i., whereas the survival percentage recorded for E-11 cells was 83% at 48 h p.i., and 85% at 72 h p.i.

3.3. Modulation of endogenous immunogene transcription

The transcription of *mx, tlr3* and *e3* has been evaluated in sea bass ISG15-producing cells both in presence and in absence of viruses.

In non-inoculated Dl_ISG15_E11 cells (Fig. 5A), *e3*, *tlr3* and *mx* genes were down-regulated (in relation to the transcription of these genes in E-11 cells), showing relative mean fold change values below 1 (0.17, 0.1 and 0.4 for *e3*, *tlr3* and *mx* respectively). After RGNNV inoculation, *mx* transcription (Fig. 5B) was triggered only at 6 h p.i. in E-11 cells (3.19 mean fold change value). Regarding SJNNV-inoculated cells, significant transcription was recorded at 3 and 6 h p.i. in E-11 cells (2.6

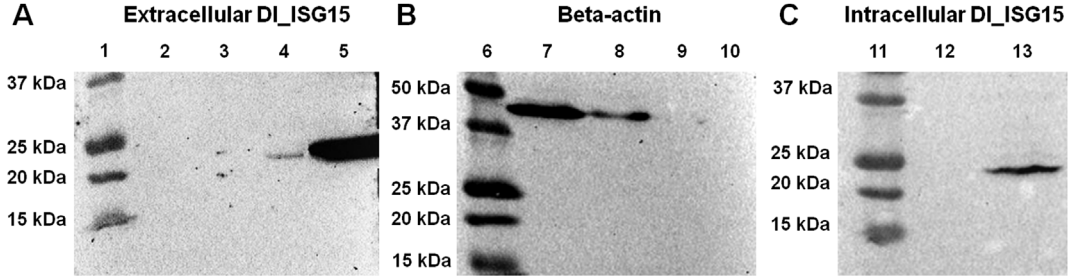

Fig. 2. Detection of the recombinant Dl_ISG15 protein by western blot. Lanes 1, 6 and 11: precision plus protein standard marker (Bio-Rad). (A) Extracellular Dl_ISG15. Lane 2: non-concentrated Dl_ISG15_E11 medium. Lane 3: immuno-precipitated E-11 medium. Lane 4: immuno-precipitated Dl_ISG15_E11 medium. Lane 5: positive control. (B) Intracellular and extracellular beta-actin. Lane 7: Dl_ISG15_E11 cell extracts. Lane 8: E-11 cell extracts. Lane 9: immuno-precipitated Dl_ISG15_E11 medium. Lane 10: immuno-precipitated E-11 medium. (C) Intracellular Dl_ISG15. Lane 12: E-11 cell extracts. Lane 13: Dl_ISG15_E11 cell extracts.

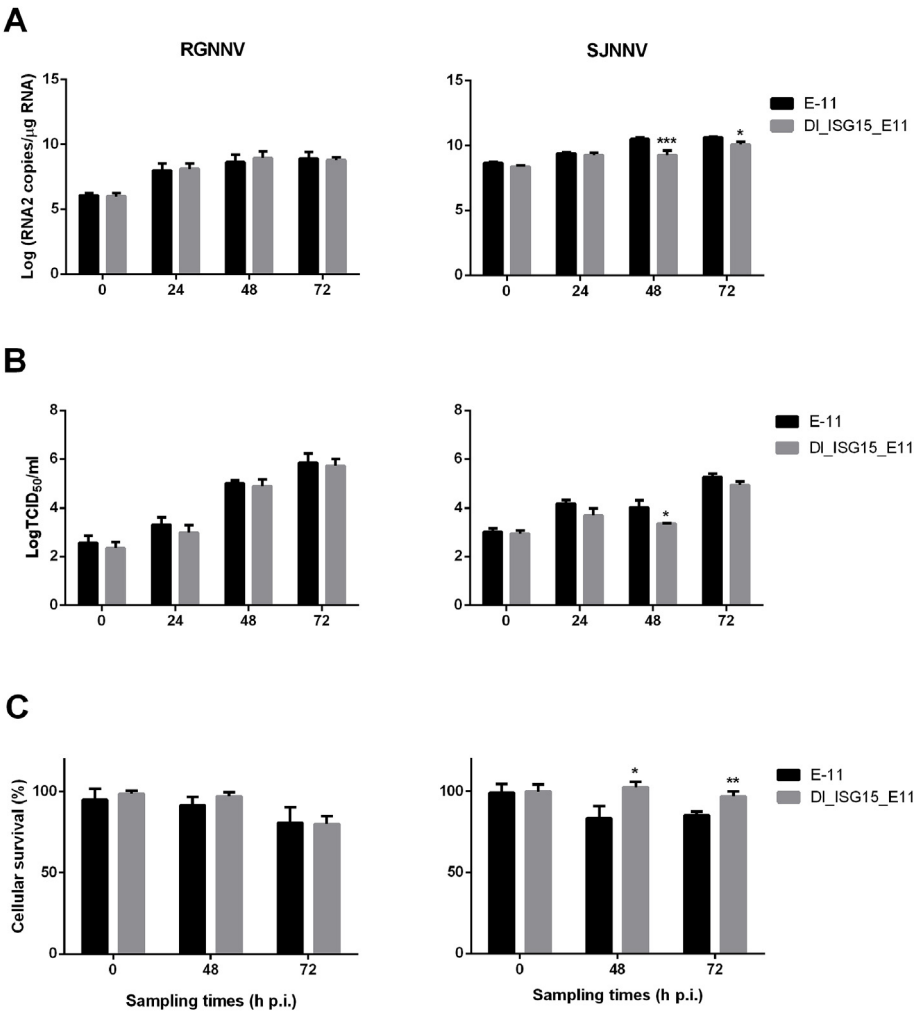

Fig. 4. Intracellular Dl_ISG15 antiviral activity. (A) Viral RNA2 copies in E-11 and Dl_ISG15_E11 cells inoculated with RGNNV or SJNNV. (B) Infective viral particles in supernatants of E-11 and Dl_ISG15_E11 cells inoculated with RGNNV or SJNNV. (C) Survival percentage of E-11 and Dl_ISG15_E11 cells inoculated with RGNNV or SJNNV. Data were statistically analysed with the t-student test. Asterisks indicate significant differences between cell types within each sampling time: $*P = 0.01$; $**P = 0.005$; $***P = 0.004$. Results are mean ± SD (n = 3).

and 4.9 mean fold change values, respectively) and only at 6 h p.i. in Dl_ISG15_E11 cells (6.9 mean fold change value), not being observed significant differences between both cell lines ($P > 0.05$).

The transcription of *tlr3* (Fig. 5C) was significantly induced in E-11 cells inoculated with RGNNV at all sampling times, with the maximum transcription fold change value (12.6) at 6 h p.i. ($P = 0.005$), whereas this transcription was knocked out in RGNNV-infected Dl_ISG15_E11 cells. However, in SJNNV-infected cells, *tlr3* transcription was induced at 6 h p.i. in Dl_ISG15_E11 cells, without significant differences between both cell lines ($P > 0.05$).

Finally, *e3* transcription is induced in Dl_ISG15_E11 cells infected with RGNNV at all sampling times (Fig. 5D), with the maximum transcription value at 24 h p.i. (4.6), being significantly higher ($P = 0.001$) than the induction value recorded in E-11 cells at the same sampling time (non-induced, mean fold change < 1.5). In SJNNV-infected groups, *e3* transcription was also observed in Dl_ISG15_E11 cells, at 3 and 24 h p.i., although without significant differences between the cell lines analysed ($P > 0.05$).

3.4. Anti-betanodavirus activity of the extracellular Dl_ISG15 protein

The role of the sea bass ISG15 protein as secreted cytokine has been evaluated by co-culture of E-11 and Dl_ISG15_E11 cells, and by E-11 incubation with ISG15-containing medium obtained from Dl_ISG15-producing cells.

Results obtained by co-culture (Fig. 6A and B) showed putative protective effect of the Dl_ISG15 protein to co-cultured E-11 cells inoculated with either, RGNNV or SJNNV. Fig. 6A shows the results obtained estimating the level of surviving E-11 cells by crystal violet staining. Staining intensity of the target E-11 cells was maximal in non-inoculated cells. After RGNNV infection, there were no surviving cells for control inoculated cells (E-11/E-11), where ISG15-producing cells were not seeded, whereas the staining intensity was intermediate for E-11 cells sharing the medium with ISG15-producing cells (Dl_ISG15_E-11/E-11). The same pattern was obtained when SJNNV-inoculated cells were analysed. These results were confirmed by the MTT assay (Fig. 6B). Thus, the survival rate of RGNNV-inoculated cells increased significantly, from 25% to 46% ($P = 0.01$), when these cells shared medium with ISG15-producing cells. A similar result was obtained after SJNNV inoculation. In this experimental group, survival rate of E-11 cells co-cultured with ISG15-producing cells was 51%, whereas this value dropped to 27% ($P = 0.01$) when they were co-cultured with non-producing E-11 cells.

In addition, RGNNV-inoculated E-11 cells were incubated with ISG15-containing medium mixed with fresh medium (1/1). Cellular survival was determined by MTT reduction, showing a significant increase ($P = 0.0005$) of cellular survival, which was 33% for control cells (incubated with ISG15-free medium) and increased up to 99% in

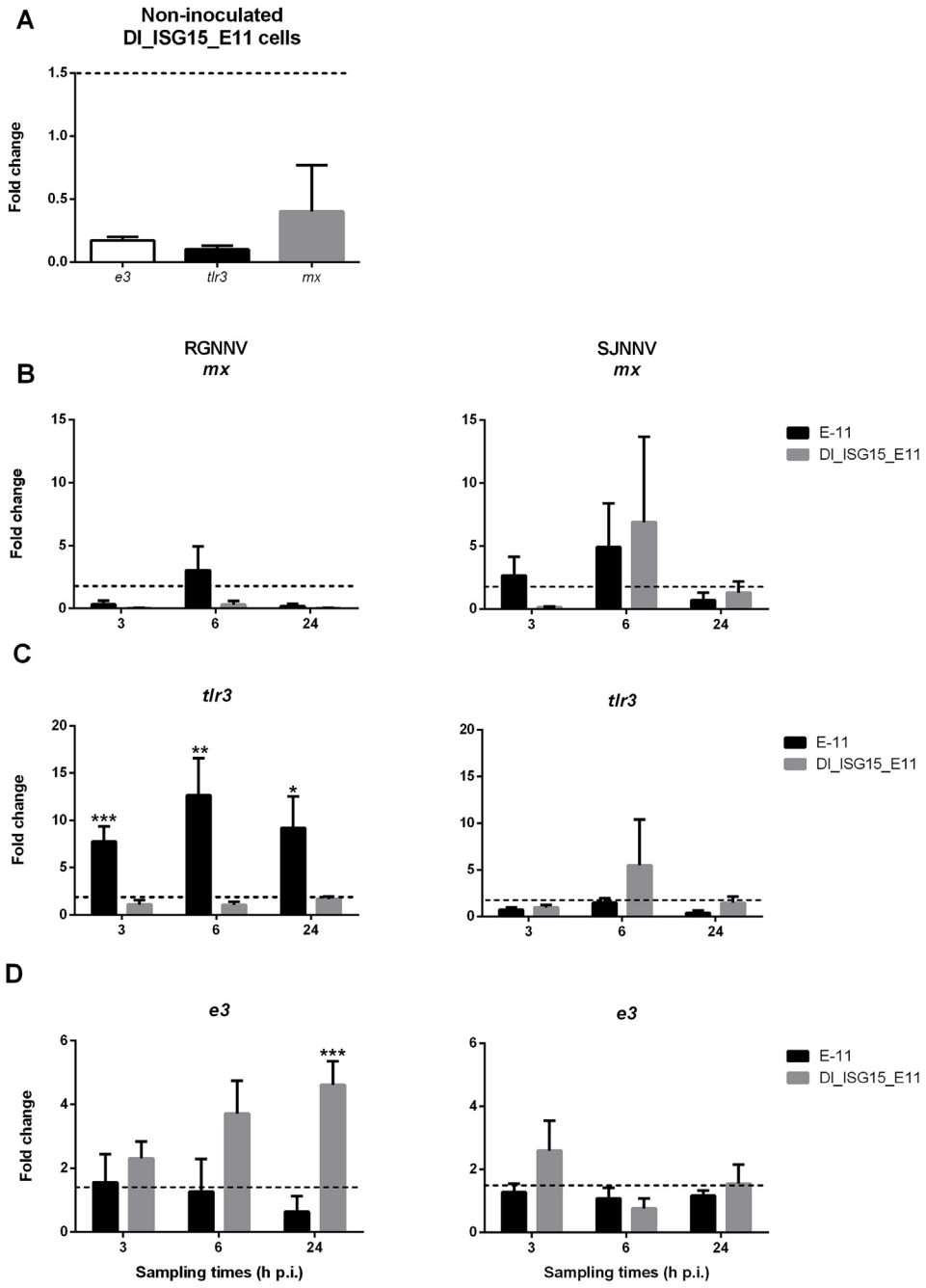

Fig. 5. Transcription analyses of endogenous genes. (A) *e3*, *tlr3* and *mx* transcription in non-inoculated Dl_ISG15_E11 cells. (B, C, D) *mx*, *tlr3* and *e3* transcription in RGNNV- and SJNNV-inoculated Dl_ISG15_E11 cells. The cut-off value considered for induction was 1.5. Data were statistically analysed with the t-student test. Asterisks indicate significant differences between cell types within each sampling time: $*P = 0.01$; $**P = 0.005$; $***P = 0.001$. Results are mean ± SD (n = 3).

presence of sea bass ISG15 protein (Fig. 6C).

4. Discussion

Recent *in vivo* studies have demonstrated an important up-regulation of European sea bass *isg15* transcription after RGNNV or SJNNV infection [35,36], which may suggest an important role of this protein against betanodavirus infections in this fish species. For this reason, the present study is focused on evaluating the Dl_ISG15 activity against betanodaviruses. To fulfil this aim, the Dl_ISG15_E11 cell line has been developed.

Experimental systems based on the constitutive expression of exogenous ISG15 proteins have been previously applied, being the transient transfection the modality most frequently used [9,12,32,38,48]. The development of a permanently transfected cell line performed in the present study provides a stable tool, which has allowed to conduct different assays under steady and homogeneous conditions.

The suitability of this cell line to determine the Dl_ISG15 anti-betanodavirus activity has been evaluated by conducting studies on cellular growth kinetics and *isg15* gene transcription and expression. The growth performance of Dl_ISG15-transfected and non-transfected E-11 cells is similar (Fig. 1), which indicates that the expression of the exogenous gene does not affect important cellular functions and, therefore, putative differences in viral replication recorded in both cell types are not consequence of the exogenous Dl_ISG15 protein

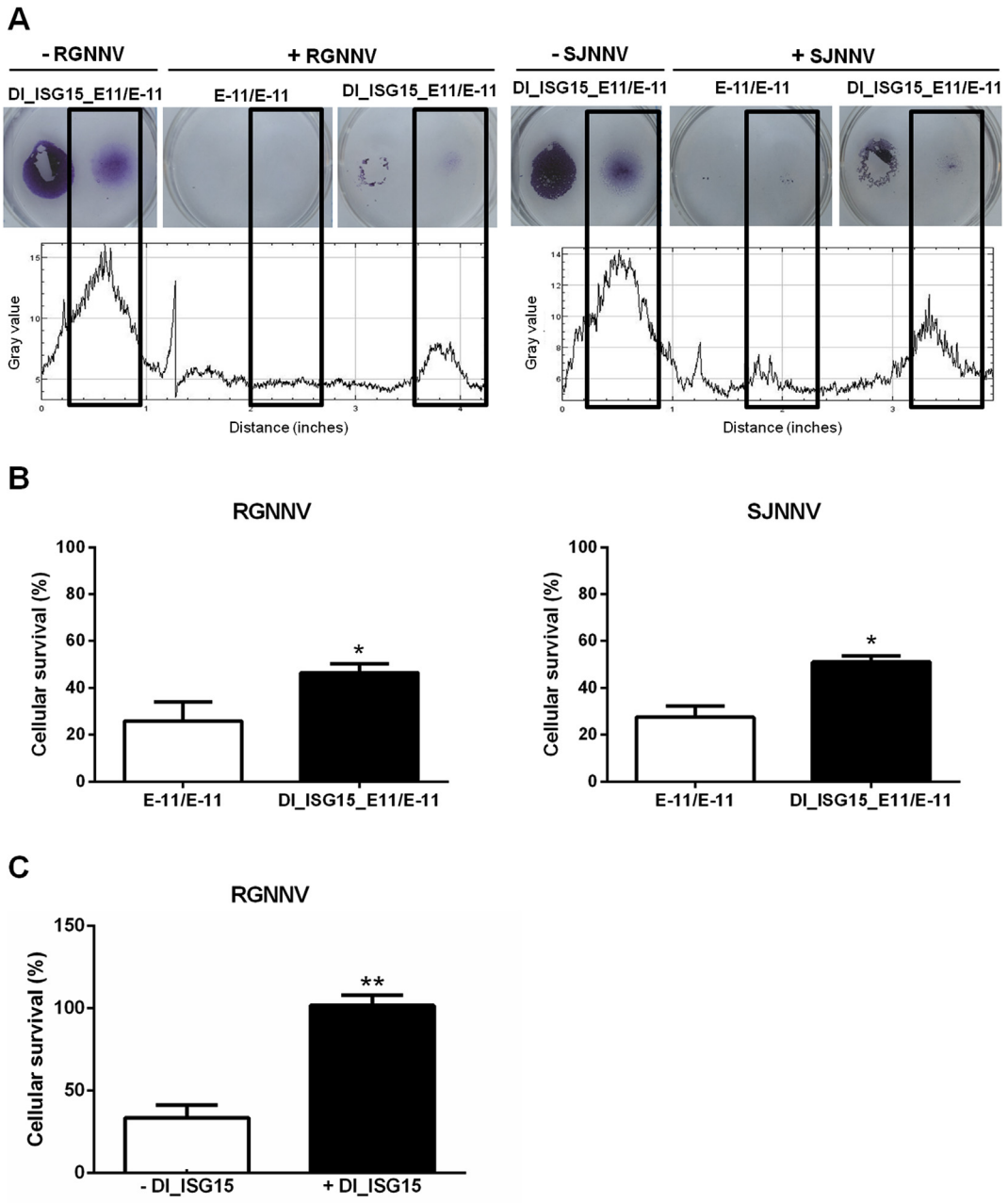

Fig. 6. Extracellular Dl_ISG15 antiviral activity. (A) Co-culture in droplets: cellular survival after RGNNV or SJNNV inoculation visualized by crystal violet staining. (B) Co-culture in droplets: cellular survival after RGNNV or SJNNV infection developed by MTT reduction. (C) Incubation with ISG15-containing medium: survival percentage of RGNNV-inoculated E-11 cells incubated with Dl_ISG15-containing medium (black) or Dl_ISG15-free medium (white). The t-student test was the statistical analysis performed. Asterisks indicate significant differences: $*P = 0.01$; $**P = 0.0005$. Results are mean ± SD (n = 3).

expression, but derived from the antiviral activity of this protein itself. In addition, *isg15* transcription is stable, not being affected by the presence of viruses.

The recombinant Dl_ISG15 protein has been detected by western blot after processing Dl_ISG15_E11 cellular extracts (Fig. 2C), showing the presence of a single 20–25 kDa band, corresponding to the 15-kDa ISG15 protein plus the protein tags. Previous studies have also reported similar molecular weights for recombinant ISG15 proteins expressed in cellular systems [38,49,50]. Moreover, this protein has been located in the cytoplasm of the producing cells by immunofluorescence (data non-shown). In addition, a single band with the same molecular weight has also been detected in the medium of these cells (Fig. 2A), even though this protein does not have any signal peptide sequence. This extracellular ISG15 protein seems to be secreted, as it is suggested by the absence of beta-actin in the extracellular fraction, even after its concentration by immuno-precipitation (Fig. 2B), which may rule out cell disruption as possible cause for ISG15 presence in the medium, as previously suggested by Wang et al. [51]. ISG15 has been previously detected as protein secreted by immune cells, such as IFN I-treated human leukocytes and monocytes [14,16], although the mechanism underlying ISG15 secretion is unknown [4]. In fish, ISG15 has been detected extracellularly in cultures of red drum (*Sciaenops ocellatus*) and tongue sole (*Cynoglossus semilaevis*) IFN I-activated head kidney lymphocytes [49,51]. Furthermore, other cell types, such as epithelial-derived cell lines, fibroblasts and neutrophils, have also been described as ISG15-releasing cells in vertebrates [15]. In fact, Langevin et al. [38] reported extracellular zebrafish ISG15 in transfected epithelioma papulosum cyprini (EPC) cell cultures. All these previous investigations

support our results, in which a fibroblastic cell line (E-11 cells, derived from whole fry snakehead fish, *Channa striata*) secretes Dl_ISG15. Taken together, these results show that Dl_ISG15_E11 cell line is an adequate tool to determine the antiviral activity of the intracellular and extracellular Dl_ISG15 protein.

The intracellular activity of Dl_ISG15 has been evaluated by comparison of viral replication in ISG15-producing and non-producing cells (Fig. 4). The number of RGNNV RNA2 copies was similar in both cellular types at all times analysed, indicating that Dl_ISG15 does not affect viral genome synthesis. Although some ISG15 proteins have been reported to act at a post-transcriptional level, by blocking virion assembly or viral budding [9,50], this possibility has been ruled out in the present study, since no significant reduction in viral titre due to the Dl_ISG15 protein expression has been observed (Fig. 4B). Furthermore, these results were confirmed by analysing survival rates, which demonstrated that Dl_ISG15-producing cells were not protected from RGNNV infection (Fig. 4C). All these results confirmed the lack of intracellular anti-RGNNV activity of the Dl_ISG15 protein.

On the contrary, sea bass ISG15 compromises SJNNV replication, since a reduction of the SJNNV genome synthesis was recorded (Fig. 4A). This result has been confirmed by viral titration and surviving assays (Fig. 4B and C), concluding that intracellular sea bass ISG15 exerts an antiviral role against SJNNV infection.

This differential antiviral activity of Dl_ISG15 against both viral species suggests that this protein may play different roles in the course of RGNNV and SJNNV infections. The lack of ISG15 activity against some fish viruses (both DNA and RNA viruses) has been previously evidenced. In addition, Langevin et al. [38] failed in the detection of zebrafish ISG15 activity against spring viremia of carp virus (SVCV), a rhabdovirus, although this ISG15 protein inhibits the replication of a wide range of fish viruses (both RNA and DNA), even other member of the *Rhabdoviridae* family.

Previous studies conducted with similar *in vitro* systems, although using transiently transfected cells, have shown that exogenous ISG15 proteins may regulate the transcription of endogenous genes, which may be involved in cellular processes, such as osteoclastogenesis [52], or antiviral activity [38]. For this reason, this study has addressed the transcriptional analyses of several genes in Dl_ISG15_E11 cells, such as *mx, tlr3*, and *e3*. These analyses showed that the over-expression of the exogenous ISG15 protein did not trigger the transcription of the endogenous IFN I system (estimated by *mx* transcription), unlike it has been reported for zebrafish ISG15 expressed in transiently-transfected EPC cells [38]; however, it would be interesting to extend the analysis to other IFN I system-related genes. On the other hand, the transcription of these IFN I-related genes following SJNNV inoculation was similar ($P > 0.05$) in Dl_ISG15_E11 and E-11 cells at all times analysed. On the contrary, *tlr3* and *e3* transcription was significantly different ($P < 0.05$) in both types of cells inoculated with RGNNV (Fig. 5). Thus, a clear knock out of *tlr3* transcription has been detected in ISG15-producing cells. This gene encodes for a receptor responsible for the IFN I system activation, and its low transcription may suggest that the presence of sea bass ISG15 could be preventing the IFN I system activation and, therefore, could be protecting the virus from the innate immune system, which could partially account for the lack of intracellular anti-RGNNV activity recorded in this study. Although ISG15 has generally been considered as an antiviral effector, some studies have described that this protein can favour viral infections. Thus, human ISG15 has been shown to have a pro-viral role in the course of hepatitis C virus infection [53,54], and Chen et al. [55] demonstrated that protein ISGylation promotes hepatitis C production. In fact, experiments conducted with fibroblastic cells have suggested that human ISG15 may be a negative regulator of the IFN system [56], as it may be happening in E-11 cells over-expressing the sea bass ISG15 protein infected with RGNNV.

The transcription of the endogenous *e3* gene was also altered by the presence of the sea bass ISG15 protein in RGNNV-infected cells, with Dl_ISG15_E11 cells showing a significantly higher level of *e3* transcripts than E-11 cells at 24 h p.i. (Fig. 5D). E3 are ligases that may be involved in ubiquitination and ISGylation. In higher vertebrates, a variable number of E3 ligases has been recorded in different host-species, showing different protein specificity, and therefore, regulating different pathways, as it has been reported for human cells [56]. The lack of this information regarding our experimental system (E-11 cells) makes difficult to deduce the functional implications of the up-regulation of this gene transcription; however, it corroborates sea bass ISG15 immunomodulation in RGNNV-infected cells acting at different levels, and suggests the presence of a high number of cellular proteins functionally altered in Dl_ISG15_E11 cells infected with RGNNV. Therefore, Dl_ISG15 immunomodulation is different in RGNNV- and SJNNV-infected cells, which shows that both viral species interact in a different way with the host immune system. This result may be consequence of different RGNNV and SJNNV adaptation to host. In fact, a recent study has recorded differences in the transcription of several immunogenes after the infection with IPNV isolates belonging to different genogroups, which has also been reported in the present study, suggesting that genetic similarity in fish viruses does not necessarily mean functional similarity [57].

In this study, sea bass ISG15 has been detected extracellularly, and its antiviral properties as signal molecule have been evaluated. To our knowledge, there is only one previous study reporting ISG15 in medium of a fish cell line. Thus, Langevin et al. [38] detected zebrafish ISG15 in the medium of transiently transfected EPC cells over-expressing this protein. However, no antiviral activity of this secreted ISG15 was recorded, which was suggested to be due to a concentration problem. In this regard, it is important to highlight the advantage of using a permanently transfected cell line, which assures that all cells are expressing the exogenous protein and, therefore, the concentration of this protein in the medium is increased.

The co-culture assays have evidenced that ISG15-producing cells exert protection over E-11 cells infected with either RGNNV or SJNNV, which is unlikely to be due to the activation of the IFN I system in donor cells, since *mx* transcription has only been detected in SJNNV-inoculated cells, and without significant differences between E-11 and Dl_ISG15_E11 cells. This hypothesis has been confirmed by incubating RGNNV-inoculated E-11 cells with medium from Dl_ISG15_E11 cells, which resulted in a significant increase of surviving cells, from 33% to 99% (Fig. 6C). This medium was collected from non-inoculated transfected cells, which have been demonstrated not to have induced any of the genes tested, including *mx*, and, therefore, their medium would not contain cytokines derived from the IFN I system. However, to confirm this hypothesis, it would be necessary to extend the transcription study to cover a wider range of cytokine-coding genes.

In conclusion, in this study an *in vitro* experimental system (Dl_ISG15_E11), which has been demonstrated to be a valuable tool to characterize the anti-betanodavirus properties of the sea bass ISG15 protein, has been developed. The antiviral action of the intracellular sea bass ISG15 protein against SJNNV has been stablished by demonstrating reduction of viral genome and infective viral particles, as well as by showing increased cellular surviving. In addition, the transcription of several endogenous genes has been analysed, revealing modulation of *tlr3* and *e3* transcription in Dl_ISG15-producing cells in the course of RGNNV infection. Finally, the Dl_ISG15-containing medium conferred protection from RGNNV and SJNNV infections. These results suggest and promote the use of the intracellular and extracellular sea bass ISG15 protein in future investigations as an antiviral, immunomodulator and protective agent against betanodaviruses [58,59].

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Immuno-related gene transcription and antibody response in nodavirus (RGNNV and SJNNV)-infected European sea bass (*Dicentrarchus labrax* L.)

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ABSTRACT

The immune response of European sea bass to RGNNV and SJNNV infections has been evaluated by quantifying the transcription of some genes involved in the IFN I system, as well as in the inflammatory and adaptive immune mechanisms. The transcription of IFN-I, ISG-12, ISG-15 and MxA genes has been analyzed in brain and head kidney, showing that RGNNV genotype induces a more intense response of the IFN I system than SJNNV in both organs. In addition, the results obtained indicate the importance of the inflammatory response in nodavirus pathogenesis, with the transcription of IL-8 and TNF-α significantly higher in brain than in head kidney, being RGNNV the strongest inductor. An important difference between the immune response induced by both genotypes refers to the IgM titre in sera, which was higher in SJNNV-inoculated fish. The acquired response is also important locally, since TR-γ transcription is higher in brain than in head kidney (especially in the RGNNV-inoculated group). To our knowledge, this is the first study addressing the sea bass anti-SJNNV immune response.

1. Introduction

Viral nervous necrosis is a neurological fish disease caused by Nervous Necrosis Virus (NNV), *Betanodavirus* genus, a naked icosahedral virus with two single-stranded, positive-sense RNA segments (RNA1 and RNA2). RNA1 encodes the RNA-dependent RNA polymerase, and RNA2 encodes the capsid protein. Based on the RNA2 segment, betanodaviruses have been clustered in four genotypes [1], now officially considered as species. The genotypes Red-spotted Grouper Nervous Necrosis Virus (RGNNV) and Striped Jack Nervous Necrosis Virus (SJNNV) have been detected in several fish species, including European sea bass (*Dicentrarchus labrax* L.) [2–4]. In addition, the coexistence of both genotypes within the same individual, which may lead to the reassortment of both viral segments, seems to be a frequent event [5]. In fact, a recent study conducted with isolates from the Mediterranean has shown that a high percentage of the betanodavirus isolates in this geographical area are reassortants displaying RGNNV-type RNA1 and SJNNV-type RNA2 segments [6]. Reassortment may be an important mechanism involved in modifying viral virulence to a certain fish species and/or host range.

RGNNV is the only genotype highly virulent to sea bass, whereas SJNNV causes no mortality or very low mortality after intramuscular or bath challenges [7,8]. In fact, Souto et al. [8] and Carballo et al. [9] showed that SJNNV replicates in sea bass brain, although it seems to fail in developing the histopathological lesions characteristic of this disease. This different level of virulence to sea bass may be the result of a differential interaction between the virus and the host immune system. Thus, previous studies have suggested an inverse relationship between viral virulence and immune response in a specific host [9–12]. However, this suggestion has been based on the study of a few genes, mainly those involved in the type I interferon system (IFN I). For this reason, a more comprehensive analysis of the host immune response is required in order to get more insight into the immune genes involved in controlling the viral disease onset.

Fish innate immune system consists of cell and molecular components, including those involved in the IFN I and inflammatory responses. Interferons are cytokines acting through the induction of interferon-stimulated genes (ISGs), such as Mx, ISG-15 and ISG-12, which encode for proteins with proven antiviral activity in several fish species. This system has been shown to be crucial in limiting replication of a wide number of fish viruses [13–16], and the transcription of some of these genes in sea bass after RGNNV infection has been reported

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[9,17–21]. In contrast, to our knowledge, there is only one study addressing the IFN I response after SJNNV inoculation in this fish species [9], analysing only the Mx transcription. The inflammatory response has been suggested to be especially important in the course of RGNNV infections, since it has been proposed to be responsible for the intense degeneration, mainly vacuolation, observed in brain, retina and spinal cord of affected animals [17,18]. In addition, the importance of the inflammatory response in limiting NNV infection has been demonstrated in corticosteroid-treated turbot (*Scophthalmus maximus*), which resulted in an earlier mortality onset [22]. The inflammatory response is generated by pro-inflammatory cytokines, including Interleukin 8 (IL-8) and Tumour Necrosis Factor alpha (TNF-α), which are tightly regulated, mainly at a transcriptional level, by anti-inflammatory cytokines. In this contest, Interleukin 10 (IL-10) and Transforming Growth Factor beta (TGF-β) play a key role in preventing a massive inflammatory response, which may cause tissue damage.

Although the relative importance of the adaptive immunity is lower in fish than in mammals, sea bass-RGNNV infection elicits the production of high levels of antibodies in sera [18,23–25], and the local antibody production by peripheral non-lymphoid tissue has been demonstrated in NNV-infected halibut (*Hippoglossus hippoglossus*) [24]. In addition, T-cells have also been demonstrated to play an important role, being the major histocompatibility complex (MHC) molecules, class I and II, required for their activation. Specifically, MHC II displays antigens to T-helper cells, which leads to the induction of an immune response specific to that antigen [27,28]. Class II molecules consist of MHC-encoded α and β chains [28]. Antigen recognition by T-cells is through antigen-specific T-cell receptors (TR); there are two T-cell populations according to the receptor they express: TR-αβ and TR-γδ. TR-γδ is an antigen pattern recognition receptor, T-cells expressing this receptor are especially abundant in mucosal tissues, and display cytotoxic activity [29]. These cells have been functionally considered as a bridge between innate and acquired immunity [30].

To go deeper inside the mechanisms of antiviral immune response in fish, the present work describes a local (brain) and systemic (head kidney) response induced in European sea bass by high virulent (RGNNV) and a low virulent (SJNNV) nodavirus isolates. The results obtained are important for a better understanding of some features of antiviral responses, and can be of interest to find strategies to combat this pathology.

2. Materials and methods

2.1. Viruses and cell culture

The isolates ERV378/102-5/04 (RGNNV genotype) [25] and SJ93Nag (SJNNV reference strain) [8] have been used in this study.

Both viral isolates were propagated on the E−11 cell line [31]. These cells were grown in Leibovitz L15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco), penicillin (Sigma, 100 units/mL) and streptomycin (Sigma, 10 mg/mL) at 25 °C until confluence before virus inoculation. Once inoculated, cells were maintained in L15 medium supplemented with FBS (2%), penicillin (100 units/mL) and streptomycin (10 mg/mL) at the same temperature, and the resulting viral suspensions were titrated using 96-well plates (Nunc) following the 50% tissue culture infective dose method ($TCID_{50}$) [32].

2.2. Experimental challenge

European sea bass specimens were ruled out as NNV carriers following the methodology described by Lopez-Jimena et al. [5]. Animals were maintained in 100-L aquaria with aeration, and the temperature was between 22 and 25 °C throughout the challenge. Fish were handled following the European Union guidelines for the handling of laboratory animals (Directive 2010/63/UE).

Fish (5 g, average weight) were split into two different groups (n = 50, each group) in duplicate (tanks A and B) to be intramuscularly injected with the previously described NNV isolates, at a dose of 10^5 $TCID_{50}$/g of fish. Fish from tanks A were used for the mortality curve analysis, and fish from tanks B for sampling to conduct immunological studies. A negative control group, consisting of L-15-injected fish, was included. Five specimens were randomly sampled per experimental group at 3, 12, 24 and 72 h post-inoculation (p.i.). Animals were killed by anesthetic overdose (MS-222, Sigma), and their brains and head kidneys were aseptically collected and individually stored in liquid nitrogen until immunological and virological analyses. In addition, blood samples were extracted from the caudal vein of 15 fish per experimental group at 30 days p.i. Blood from 5 fish was pooled, and sera were obtained after overnight incubation at 4 °C for clotting. Supernatants were then centrifuged twice at 400 x *g* for 15 min, and the resulting sera were stored at −20 °C until used. A total of 3 samples, each one composed of sera from 5 animals, were analyzed in each experimental group.

2.3. Sample processing

Brains and head kidneys were individually homogenized (10% w/v) in QIAzol Lysis Reagent (Qiagen) using the Lysis Matrix tubes designed for the Fast-Prep24 (MP Biomedical) system. Total RNA was extracted with the RNeasy Lipid Tissue Mini Kit (Qiagen) following commercial guidelines. The resulting RNA was analyzed by agarose gel electrophoresis, quantified with the NanoDrop-1000 system (ND-1000, ThermoFisher), and stored at −80 °C until used. The cDNA synthesis was performed with the iScript cDNA synthesis Kit (BioRad), mixing 1 μg of RNA, 4 μL of 5x iScript Reaction Mix, and 1 μL of iScript Reverse Transcriptase in a final volume of 20 μL. Mixtures were incubated at 25 °C for 5 min, followed by 30 min at 46 °C and 5 min at 85 °C for transcriptase inactivation. The resulting cDNA was stored at −20 °C.

2.4. Viral genome quantification

RGNNV and SJNNV RNA2 genomic segments have been quantified by two specific absolute real-time PCR protocols [33,34]. Serial dilutions of pCR™4-TOPO® TA vector (Invitrogen) containing the complete RGNNV- or SJNNV-RNA2 segment were used as reference standard curves. All the reactions were conducted using the LightCycler 96 Termocycler (Roche) and the Fast Start Essential DNA Green Master Mix (Roche) using SYBR Green technology. PCRs were carried out in 20-μL mixtures containing cDNA generated from 50 ng of RNA, 10 μL of Fast Start Essential DNA Green Master 2x, 1 μL of each primer (10 pmol) and 6 μL of water. Termocycling conditions were: initial denaturation at 95 °C for 10 min, followed by 45 amplification cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. To obtain melting curves, the following profile was conducted: 95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s. Statistically significant differences were analyzed by the two-way ANOVA test and Bonferroni's test as a post hoc, using the GraphPad Prism 5.0 software. Values with $p < 0.05$ were considered significant.

2.5. Immunogene transcription analyses

The transcription of MxA, ISG-12, ISG-15, IFN-I MHCII-β, IL-8, IL-10, TGF-β, TNF-α and TR-γ was quantified in brain and head kidney by relative real-time PCR protocols using the Mx3000P thermocycler (Stratagene), in combination with Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies), following manufacturer's guidelines. Rox was included as a passive reference dye to compensate for non-PCR related variations in fluorescence. Mixtures were prepared with cDNA generated from 10 ng of RNA, 12.5 μL of 2x Brilliant II SYBR Green qPCR Master Mix complemented with 0.375 μL of Rox (0.002 mM), and 1 μL of each primer (10 pmol) in a final volume of 20 μL. The amplification profile was as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. In addition, melting

Table 1
Primers used in this study.

Target gene	Sequence (5´-3´)	Amplicon size (bp)	Reference
rRNA18S	CCAACGAGCTGCTGACC CCGTTACCCGTGGTCC	208	[18]
MxA	ATTCTGAGTTCTTGCTGAAGG CCTCTAGAACTCCACCAGG	113	[20]
TGF-β	GACCTGGGATGGAAGTGG CAGCTGCTCCACCTTGTG	225	[18]
IFN-I	GGCTCTACTGGATACGATGGCT GCGTCCAAAGCATCAGCT	200	[18]
MHCII-β	CAGAGACGGACAGGAAG CAAGATCAGACCCAGGA	240	[57]
IL-8	GTGCTCCTGGCGTTC CTTCACCCAGGGAGC	220	[58]
TR-γ	CTGCTGTGTGTGGCCTCAGAC GTGCTGGACGGAGCAGTGGTA	200	[56]
ISG-12	CCTGGTACAGCTGCTGT AGCTGCTCCTGCTGACT	199	[49]
ISG-15	CGACTCAAAGCCTCTCTGCTACT CGTTTCTGACGAACACCTGGAT	100	[21]
IL-10	ACCCCGTTCGCTTGCCA CATCTGGTGACATCACTC	164	[18]
TNF-α	CGACTGGCGAACAACC GCTGTCCTCCTGAGC	220	[59]
CP_RGNNV	CGTCCGCTGTCCATTGACTA CTGCAGGTGTGCCAGCATT	100	[33]
CP_SJNNV	GACACCACCGCTCCAATTACTAC ACGAAATCCAGTGTAACCGTTGT	75	[34]

curves were obtained following this profile: 95 °C for 1 min, 52 °C for 30 s, and a final step at 95 °C for 30 s. Reactions were performed with five biological samples in triplicate.

The annealing temperature of all the primers used was 52 °C, and the amplification product size ranged between 75 and 240 bp (Table 1). Relative quantification was performed comparing with the transcription level of the target gene in control sample (L15-injected fish) at 3 h p.i. Ribosomal 18S RNA was the reference endogenous gene used [18], and relative fold change values (Pfaffl method) were calculated with the MxPro software. Results were expressed as mean relative fold change values ± standard deviation (SD). Statistically significant differences were analyzed by the two-way ANOVA test and Bonferroni's test as a post hoc, using the GraphPad Prism 5.0 software. Values with $p < 0.05$ were considered significant.

2.6. Anti-NNV antibody quantification

Quantification of anti-NNV antibodies was conducted by indirect ELISA, as reported by Lopez-Jimena et al. [25]. Briefly, 96-well polystyrene plates (Immulon 4HBX, Thermo) were coated with 0.4 mg of lysates from RGNNV- or SJNNV-infected E−11 cells. Wells coated with lysates from non-infected (control) E−11 cells were also included. Positive and negative controls were included in each plate. After washing with low salt wash buffer (LSW) and blocking with 3% skimmed-powder milk (Sigma), two-fold dilutions of sera (100 µL) were added. Each dilution was analyzed in triplicate. After incubation at 4 °C overnight, plates were washed five times with high salt wash buffer (HSW). The anti-European sea bass IgM monoclonal antibody (F01, Aquatic Diagnostic) (1/33 dilution) was finally added and incubated for 1 h, followed by 1-h incubation at room temperature with HRP-conjugated goat anti-mouse IgG (1/4000 dilution). Plates were washed five times with HSW between incubations. The reactions were visualized by adding 3, 3′,5,5′-tetramethylbenzidine (TMB, Sigma). The optical density (OD) was measured at 450 nm using an ELISA Whittaker Microplate Reader 2001 (Anthos Labtec). Three times the mean OD value of the negative controls was considered as the cut-off threshold. The highest serum dilution above this value is the anti-RGNNV antibody end point titre for that sample [25].

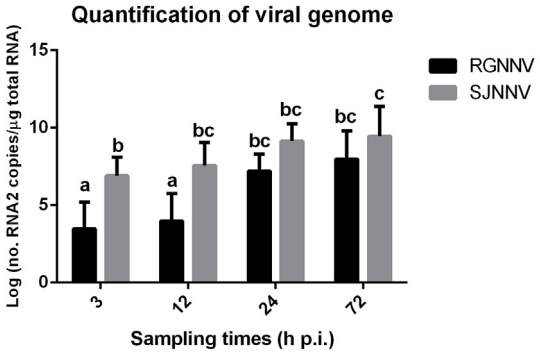

Fig. 1. Absolute quantification of NNV RNA2 segment in brain from juvenile European sea bass experimentally challenged with RGNNV or SJNNV. Data are mean ± SD obtained from five different samples. Different letters denote significant differences ($p < 0.05$).

3. Results

3.1. Clinical signs and mortality

Typical signs of the disease and mortalities were only observed in the RGNNV-inoculated group. External signs of the disease were loss of appetite, erratic swimming, hyperactivity and skin darkening. Internally, fish showed hyperinflation of the swim bladder. Mortality onset was at 5 days p.i., and cumulative mortality at the end of the experiment was 47% [8].

3.2. Viral genome quantification

Viral quantification in brain showed a significant increase ($p < 0.05$) in the number of RGNNV and SJNNV RNA2 copies through time (Fig. 1). SJNNV-inoculated fish displayed a higher number of RNA2 copies compared to animals in the RGNNV group within each sampling time; however, the increase in the number of log RNA2 copies from 3 to 72 h p.i. was higher for RGNNV (4.49) than for SJNNV (2.56). Viral genome levels at the last sampling time (72 h p.i.) were 7.77 and 9.25 log RNA2 copies/µg RNA, for RGNNV- and SJNNV-inoculated fish, respectively.

3.3. IFN I system induction

A strong induction of ISG-15, ISG-12 and MxA genes was recorded from 3 h p.i. onwards, with similar fold change values in both organs, although with different temporal patterns (Fig. 2).

In head kidney, RGNNV is a strong inductor of the transcription of several ISGs (Fig. 2 F, G, H), especially at 12 and 24 h p.i. However, SJNNV triggers the induction earlier than RGNNV, with fold change values of 2.1, 7.2 and 12.6 for ISG-15, ISG-12 and MxA, respectively, at 3 h p.i., establishing, therefore, a quicker systemic response. In addition, ISG-15, ISG-12 and MxA transcription in SJNNV group increased throughout the time, with maximum fold change values always at 72 h p.i. (77.3, 739.7, 293.4, respectively), whereas maximal transcript values in RGNNV-inoculated fish were at 12 h (for ISG-15 and MxA genes) or 24 h (for ISG-12) (Fig. 2 F, G, H). IFN-I transcription was not recorded at any analyzed time (Fig. 2 E).

In brain (Fig. 2 A, B, C, D), the induction of IFN system is slower than in head kidney, with values of ISG transcripts at 12 h p.i. lower in this tissue than in head kidney. The temporal pattern of ISG-15 and ISG-12 transcription in brain from RGNNV-inoculated fish was consistent with viral replication in this organ, showing maximum levels at 72 h p.i., with mean fold change values of 1921.7 and 7674.7 for ISG-12 and ISG-15, respectively (Fig. 2 B, C). Significant differences between genotypes were only recorded for ISG-15 gene at 72 h p.i. At this time,

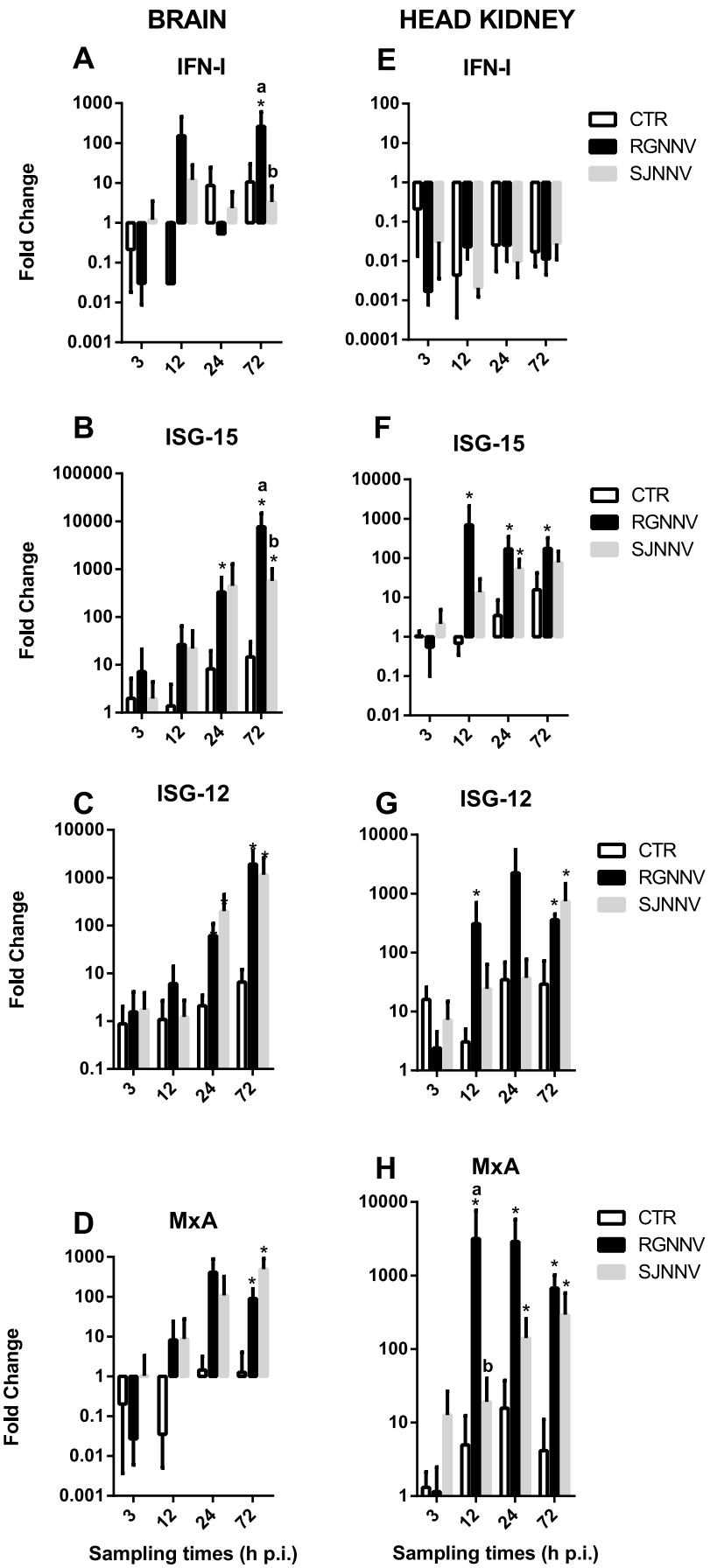

Fig. 2. Relative quantification of the indicated IFN I system-involved genes in brain (A, B, C, D) and head kidney (E, F, G, H) from juvenile European sea bass experimentally challenged with RGNNV or SJNNV. Data are mean ± SD obtained from five different samples. Different letters denote significant differences ($p < 0.05$) between genotypes within each sampling time. Asterisk refers to significant differences ($p < 0.05$) with control fish.

RGNNV was a stronger inductor of this gene compared to SJNNV (Fig. 2 B). Similarly, RGNNV also induced higher level of IFN-I transcripts at this sampling time (263.1 and 3.32 folds for RGNNV- and SJNNV-infected fish, respectively) (Fig. 2 A). Regarding MxA (Fig. 2 D), the transcription of this gene in brain from RGNNV-infected fish showed a peak at 24 h p.i. (412.3 folds), whereas maximum levels of transcription after SJNNV infection were recorded at 72 h p.i. (493.2 folds), being these values higher than those obtained in the RGNNV group at the same sampling time (Fig. 2 D).

3.4. Inflammatory genes

The analysis of genes encoding pro-inflammatory cytokines showed that transcription of IL-8 increased quickly after infection in both organs (at 3 h p.i.), brain and head kidney (Fig. 3 A, E), whereas the levels of TNF-α mRNA increased later (from 24 h p.i.) (Fig. 3 B, F), with a clear down-regulation up to 12 h p.i. in both organs.

In brain, induction of pro-inflammatory genes (Fig. 3 A, B), especially TNF-α, was stronger than in head kidney, and RGNNV induces higher levels of transcription of both pro-inflammatory genes compared to SJNNV at the last sampling time (maximum mean fold change values of 1628.5 and 73.4 for TNF-α, 184.1 and 29.3 for IL-8 in RGNNV and SJNNV groups, respectively) (Fig. 3 A, B).

In head kidney, SJNNV triggers a higher induction of both pro-inflammatory genes compared to RGNNV at 3 h p.i. for IL-8 (8.1 mean fold change; Fig. 3 E), and at 24 h p.i. for TNF-α (3.2 mean fold change; Fig. 3 F). In addition, a clear increase of IL-8 transcripts over the time was observed for both genotypes (Fig. 3 E, F).

Unlike what happens with pro-inflammatory gene transcription, in general, anti-inflammatory genes were induced at the same level in both organs, with clear up-regulations of IL-10 and TGF-β genes at 24 and 72 h p.i. (Fig. 3 C, D, G, H). In both organs, the IL-10 gene was down-regulated up to 12 h p.i.

3.5. Transcription of genes involved in adaptive response

When the acquired response was evaluated, it was observed that in head kidney both genes analyzed, MHCII-β and TR-γ, were down-regulated at the first sampling times (Fig. 4 C, D). The up-regulation of these genes was recorded later, at 24 or 72 h p.i. (depending on the viral genotype), with maximum fold change values of 13.1 at 72 h p.i., for MHCII-β gene in the SJNNV-infected group, and 4.3 at 72 h p.i., for TR-γ in the RGNNV-infected group (Fig. 4 C, D).

In brain (Fig. 4 A, B), the kinetics of MHCII-β transcription was similar to that previously described in head kidney, with maximum fold change values at 72 h p.i. for both, RGNNV (14 folds) and SJNNV (87.5 folds) groups. However, the temporal pattern of TR-γ transcription is different, with a strong up-regulation early after infection (from 3 h p.i.), especially in the RGNNV-infected group (96.2 folds) (Fig. 4 B).

3.6. Antibody response

A measurable level of specific anti-NNV antibodies was recorded only in sera from challenged animals, with important differences between genotypes (Fig. 5). The results showed an intense production of anti-SJNNV antibodies at 30 days p.i., with titres of 1/4,096, whereas the anti-RGNNV antibody titre was 1/1,024.

4. Discussion

In this study, it has been possible to determine transcriptional profiles of different immuno-genes, as well as the antibody response of European sea bass after infection with RGNNV, highly virulent to this fish species, and SJNNV, less virulent to this fish species. Recently, Pascoli et al. [24] have reported a poor *in vivo* cross-protection between these two genotypes in sea bass, which evidences the importance of investigating the immune response triggered by both genotypes.

Viral genome quantification by real-time PCR showed an increase in the number of RNA2 copies of both genotypes throughout the time, which may indicate viral replication. Nodavirus replication in fish species not showing disease symptoms, as it happens for SJNNV genotype in this study, has been previously described for Atlantic halibut, Atlantic cod and sea bream [19,35–37]. In addition, Souto et al. [8] reported similar RNA2 copy number in brains from RGNNV- and SJNNV-inoculated surviving sea bass, suggesting similar replication levels for both genotypes. Our results suggest a higher replication rate for RGNNV (considering the difference in the number of RNA2 copy number from 3 to 7 h p.i.), which agrees with previous studies showing that viral isolates highly virulent to a specific host display faster kinetics of replication than low virulent isolates [10,38,39].

In addition, a higher number of RNA2 copies was recorded at 3 h p.i. in the SJNNV group, which may indicate a faster spread from the injection site to the brain; however, this aspect needs to be further investigated. Although the number of RNA2 copies was always higher in the SJNNV group, this fact did not give raise to a more intense immune response, meaning that a lower number of copies of the RGNNV genotype can induce a similar o even more intense immune response.

The transcription analysis of IFN-I, ISG-12, ISG-15 and MxA showed that NNV is a strong inducer of sea bass IFN I system in brain and head kidney, which confirms the importance of this innate defence mechanism in controlling this viral infection. Noteworthy, RGNNV seems to induce a more intense response of the IFN I system compared to SJNNV. The IFN-I transcription was not recorded in head kidney at any time, whereas ISG-15, ISG-12 and MxA were clearly up-regulated in this organ. Other authors [18] also failed in the detection of IFN-I transcription in sea bass kidney. Although the induction in an IFN-independent way of some ISGs, such as ISG-15 and Mx, has been recorded [40–42] it is unlikely this to happen for the three ISGs analyzed. Therefore, the IFN-I induction in head kidney seems to be very transitory, and, in consequence, difficult to record. On the contrary, the transcription of this gene in brain seems to last longer, as it has also been reported by Scapigliati et al. [18].

Mx gene is the most frequently analyzed ISG in sea bass, having been studied as a marker of the IFN I system. Thus, numerous studies have evaluated Mx transcription in this fish species after RGNNV infection, mainly in head kidney, with first detection of Mx mRNA at times post-infection ranging from 6 to 72 h [17–20]. In this study, a strong induction triggered by RGNNV was found at 12 h p.i. in head kidney (3174.1 folds) (Fig. 2 H), with significant differences between both genotypes at 12 h p.i. This result is in contrast to Carballo et al. [9], who demonstrated that sea bass head kidney Mx gene was induced at higher level by SJNNV than RGNNV. Similarly, a highest induction of Mx transcription has been recorded in sea bream (resistant to the infection) in comparison with sea bass (susceptible) [17]. Based on these observations, Mx has been suggested to be essential in controlling NNV infections. In fact, the direct anti-NNV activity of this protein has been demonstrated in cells over-expressing the seven-banded grouper Mx gene [43]. These previous reports refer to Mx gene, without distinguishing between MxA and MxB. The present work has used primers detecting specifically MxA, which transcribes earlier and at higher level than MxB in kidney of RGNNV-infected sea bass [20]. An underestimation of MxA transcription by the primers used in previous studies, as well as differences in fish age or isolates, could explain these discrepancies. In fact, fish age is an important factor determining the appearance of betanodavirus disease, as it has been previously suggested [44], which can be the result of differences in the host immune response. In addition, the high transcription of other ISGs analyzed in this study following RGNNV infection is consistent with the MxA transcription recorded. Furthermore, the analysis of the MxA transcription in brain (Fig. 2 D) also corroborates the ability of the highly virulent RGNNV isolate to induce this gene transcription. The decrease of MxA transcription in head kidney over the time in RGNNV-inoculated

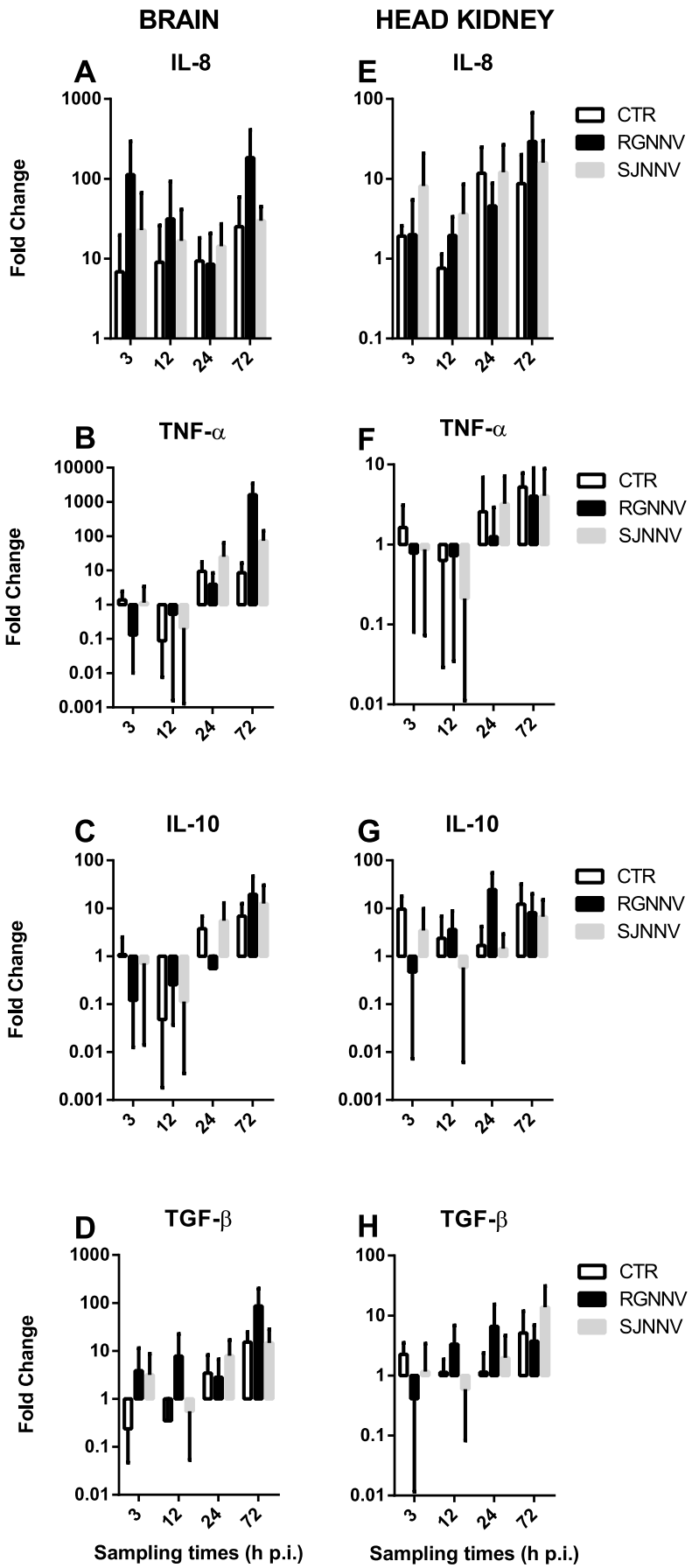

Fig. 3. Relative quantification of the indicated inflammatory response-involved genes in brain (A, B, C, D) and head kidney (E, F, G, H) from juvenile European sea bass experimentally challenged with RGNNV or SJNNV. Data are mean ± SD obtained from five different samples.

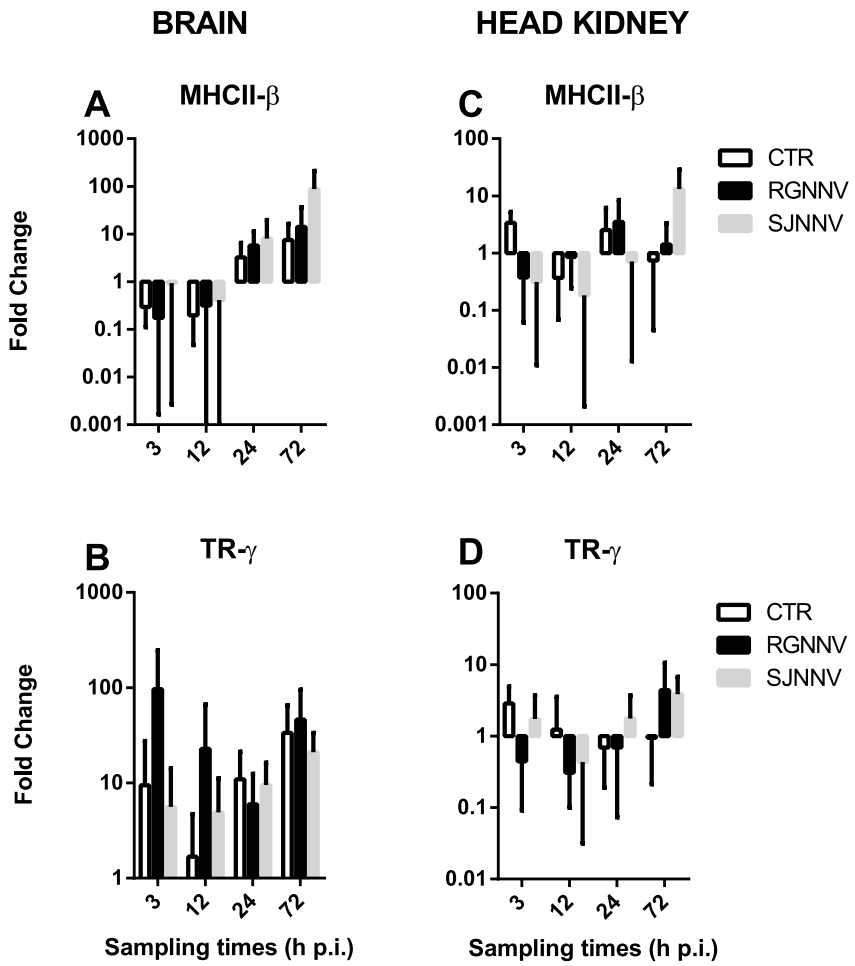

Fig. 4. Relative quantification of the indicated response-involved genes in brain (A, B) and head kidney (C, D) from juvenile European sea bass experimentally challenged with RGNNV or SJNNV. Data are mean ± SD obtained from five different samples.

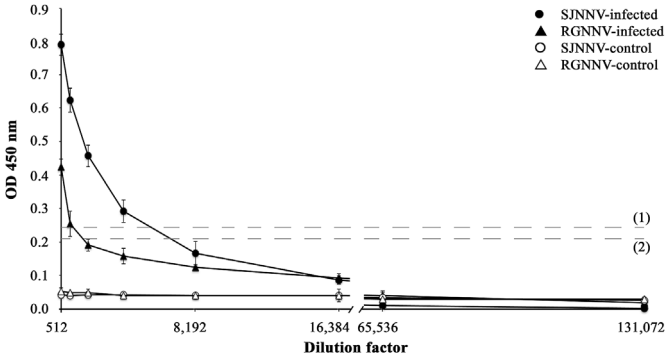

Fig. 5. Titration of anti-RGNNV (▲) and anti-SJNNV (●) antibodies in sera from experimentally challenged European sea bass at 30 days p.i. White symbols represent antibodies in control groups. (1) and (2) are the thresholds for samples from the SJNNV- and RGNNV-infected fish, respectively. Data represent mean OD values ± SD obtained by the ELISA analysis of 3 samples composed of sera from 5 different fish.

animals has also been recorded by Scapigliati et al. [18].

ISG-15 is an important ISG, which can display direct antiviral activity by conjugating with cellular or viral proteins, or can have cytokine-like activity [45]. A recent study has demonstrated high levels of ISG-15 transcription in brain and head kidney of experimentally RGNNV-infected sea bass [21]. This result is consistent with the induction profile obtained for both organs in the present study (Fig. 2 B, F). In addition, a clear relation between viral replication and ISG-15 transcription in the viral target organ has been evidenced. On the whole, RGNNV is an ISG-15 inducer stronger than SJNNV, especially at the last sampling time analyzed. The potent induction elicited locally by the RGNNV isolate can also be involved in an increased inflammatory reaction in brain, as it has been reported for some mammalian ISG-15 proteins. A similar pattern and intensity of induction has been described for ISG-12 gene (Fig. 2 C, G). ISG-12 is a small protein involved in activating the intrinsic apoptosis pathway [46], and its antiviral activity has been demonstrated for various viruses affecting high vertebrates, including neurotropic viruses [47,48]. The high induction recorded after RGNNV and SJNNV infection in this work points out an anti-NNV role of the sea bass ISG-12 protein, comparable to those of more extensively analyzed ISGs, such as Mx and ISG-15. To our knowledge, the only previous study on ISG-12 induction has been performed with sea bass leukocytes stimulated with poly I:C [49].

The importance of the inflammatory process in the course of NNV infections has been previously suggested, and it seems to be supported by the results obtained in the present study. Thus, the transcription of IL-8, which attracts and activates neutrophils, and TNF-α, which regulates inflammation and apoptosis, has been significantly higher in brain than in head kidney, with the RGNNV being a stronger inductor (Fig. 3 A, E). Although inflammation is essential to control NNV multiplication in brain [17,22,50,51], an uncontrolled process may cause tissue damage. In fact, one of the main differences between the immune response against nodavirus infection in sea bass and sea bream is the long-lasting anti-inflammatory response in sea bream [17], which would prevent the typical histological lesions in brain. Although this may also be an important difference in sea bass immune response

against SJNNV and RGNNV, no differences have been recorded in the induction of the anti-inflammatory genes IL-10 and TGF-β, therefore analyses at longer times p.i. would be required in order to probe this hypothesis. In a previous study, induction of IL-10 and TGF-β was recorded up to 10 days p.i. in head kidney from RGNNV-challenged sea bass, although at low level [18]; however, no data about SJNNV-triggered transcription are available.

The most significant difference between the immune response induced by both genotypes refers to the antibody titre in sera, with the SJNNV eliciting the production of a higher titre of antibodies than RGNNV at 30 d p.i. Both genotypes belong to different serotypes, with the C-terminal end of the capsid protein containing the immune reactive portion [52]. Previous studies have shown the presence of antibodies in sera from RGNNV-infected or immunized fish [18,23–25,53], and antibody detection has been widely used to detect previous exposures to the virus. In this study, the titre of anti-nodavirus antibodies was higher in SJNNV-inoculated fish and, therefore, it is tempting to suggest that this higher antibody response could be partly responsible for the lack of disease in sea bass after inoculation with this genotype. However, in a recent study Pascoli et al. [26] reported a higher antibody production in sea bass inoculated with inactivated RGNNV than after inoculation with inactivated SJNNV. This discrepancy may be consequence of differences regarding the viral isolate used. In fact, the level of antibodies produced in response to RGNNV infections varies widely depending on the challenge considered [18,25,26]. In addition, in the previous study [26] sea bass specimens were injected with the same dose of inactivated RGNNV and SJNNV isolates, whereas in the present study the replication of both genotypes has been recorded, with SJNNV reaching a higher number of RNA2 copies at all sampling times, which may also be an important difference to be considered. Therefore, a more extensive study, using a wide range of RGNNV and SJNNV isolates, would be necessary.

The importance of the acquired response is not only at a systemic level, but also at a local level, since TR-γ transcription is significantly higher in brain than in head kidney of infected fish (especially in the RGNNV group) (Fig. 4 B, D). In addition, the time course of the induction of this gene in both tissues is different. The immune response to NNV in brain is characterized by an early TR-γ response (Fig. 4 B), which is a T-cell-related receptor, suggesting an important role of the acquired immunity in controlling viral replication in the target organ, as it has been previously reported in brain of experimentally-challenged sea bass and Atlantic halibut [54,55]. On the contrary, in head kidney no increase of TR-γ transcription at 3, 12 and 24 h p.i. was recorded, which may indicate either a quick migration of T-cells to the brain as target organ or down-regulation of this gene transcription. This result is surprising, since this gene has been classically considered to be important mainly in mucosal tissues, such as gills or intestine, and kidney and brain are the organs showing the lowest basal levels of gene expression [55]. Regarding MHCII-β (Fig. 4 A, C), the lack of transcription of this gene recorded at the beginning of the infection in both tissues has also been described for different experimental systems, including rainbow trout infected with Infectious Pancreatic Necrosis Virus (IPNV) [28]. Our results support the putative role of the local adaptive immune response suggested by an important up-regulation of the gene coding the IgM light chain following RGNNV challenge in sea bass [18].

This study has shown that RGNNV and SJNNV stimulated a complete immune response, involving the induction of numerous genes related with both, innate and adaptive responses. These responses have been recorded both at a local (brain) and systemic (head kidney) level, with SJNNV inducing an early immune gene transcription in kidney. Thus, the quicker systemic response induced by SJNNV, together with a higher production of antibodies, may be partly responsible for the resistance of sea bass to this genotype.

The present work complements and extends previous studies, and poses questions to be considered in future investigations, such as, for instance, that both, RGNNV and SJNNV, stimulated an immune response that includes the induction of genes involved in innate and adaptative responses, as well as a humoral antibody response.

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