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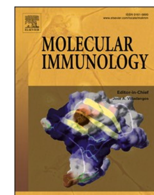

Genomic characterization and transcription analysis of European sea bass (*Dicentrarchus labrax*) *rtp3* genes

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A R T I C L E I N F O

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A B S T R A C T

Fish RTP3, belonging to the receptor-transporting protein family, display several functions, including a putative antiviral role as virus-responsive gene. In this work, we have identified and characterized two different European sea bass *rtp3* genes. In addition, an *in vivo* transcription analysis in response to LPS, poly I:C and betanodavirus infection (RGNNV genotype) has been performed. The sequence analysis showed that European sea bass displays two *rtp3* genes, X1 and X2, composed of two exons and a single intron (1007-bp and 888-bp long, respectively), located within the ORF sequence. The full-length cDNA is 1969 bp for *rtp3* X1, and 1491 bp for *rtp3* X2. Several ATTTA motifs have been found in the intron sequence of both genes, whereas *rtp3* X1 also contains this motif in both untranslated regions. The transcription analyses revealed significant level of *rtp3* X2 mRNA in brain and head kidney after LPS and poly I:C inoculation; however, the induction elicited by RGNNV infection was much higher, suggesting an essential role for this protein in controlling NNV infections.

1. Introduction

Receptor-transporting proteins (RTPs) are involved in numerous functions, including transport of G proteins-coupled receptors to the cell surface and inhibition of viral replication (Boys et al., 2021). In mammals, this family is composed of 4 proteins, whereas fish show between 1 and 3 receptor-transporting proteins, including RTP3, which was first identified as transmembrane protein 7 (TMEM7) (Kiss et al., 2002). Mammalian RTP3 shares homology with other RTPs; however, it seems to be involved in different processes (Saito et al., 2004). In particular, *rtp3* has been described as an interferon-α (IFN-α)-responsive gene (Zhou et al., 2007), and as a novel candidate gene for femoral neck bone geometry (Zhao et al., 2010). In addition, several authors have detected changes in *rtp3* transcription related with different types of cancer, such as cell renal carcinoma and human hepatocellular carcinoma (Zhou et al., 2007; Wrzesinski et al., 2015), and *rtp3* has been included, along with five more genes, in a potential diagnostic model for predicting metabolic steatohepatitis (Liao et al., 2021).

Regarding fish *rtp3*, recent studies have reported strong induction in response to heat stress (Lyu et al., 2018) or viral infections, which has led to its description as a virus-responsive gene (VRG). Specifically, transcription of Atlantic salmon (*Salmo salar*) *rtp3* is strongly induced following the infection with infectious salmon anaemia virus (ISAV), infectious pancreatic necrosis virus (IPNV), piscine myocarditis virus (PMCV), salmonid alphavirus (SAV) or piscine orthoreovirus (PRV) (Krasnov et al., 2011; Johansen et al., 2015). In addition, a remarkable induction of *rtp3* has also been reported after nervous necrosis virus (NNV) infection, regardless of the fish species analysed (Krasnov et al., 2013; Liu et al., 2016, 2017; Labella et al., 2018; Moreno et al., 2020; Gemez-Mata et al., 2021; Pereiro et al., 2023), which suggests that *rtp3* may have a relevant role within the host defence against this virus. In fact, *rtp3* has been described as a resistance gene against NNV in Asian sea bass (*Lates calcarifer*) (Liu et al., 2016). However, little information is available about fish *rtp3* gene structure and the role of RTP3 proteins during viral infections.

NNV (*Nodaviridae* family, *Betanodavirus* genus) is the causative agent of the viral nervous necrosis, the main viral disease hampering European sea bass (*Dicentrarchus labrax*) culture. It is an icosahedral, naked virus,

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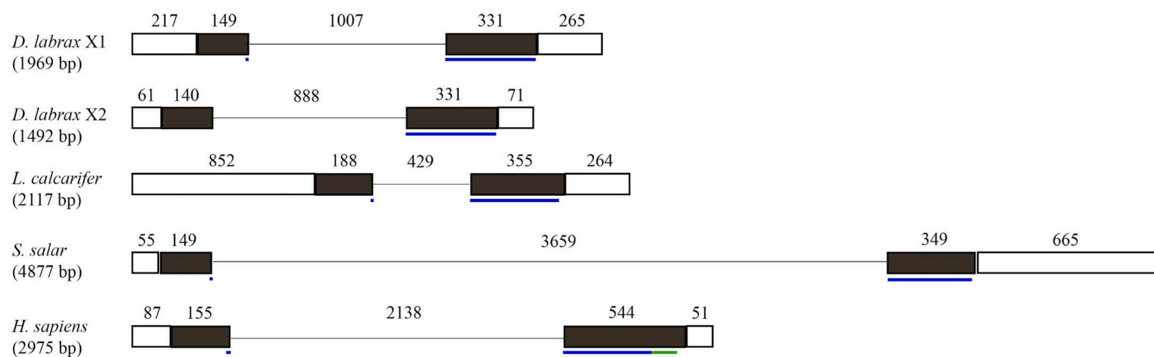

Fig. 1. Comparison between European sea bass *rtp3* genomic structure and other known *rtp3* genes. Exons are represented by boxes and introns by lines. Coding regions are in black, untranslated regions in white, zinc-binding domains are in blue, and the transmembrane domain in green. Size of each element is indicated by a number (bp). GenBank accession no. of fish species included in this study: *Lates calcarifer* (KY070548.1), *Salmo salar* (NM_001141116.2), *Homo sapiens* (AC098613.2).

with a genome composed of two single-stranded, positive-sense RNA segments: RNA1 (coding the viral polymerase), and RNA2 (coding the capsid protein). Fish betanodaviruses have been classified into four species, although red-spotted nervous necrosis virus (RGNNV) is the only one causing high mortalities in European sea bass (Vendramin et al., 2014; Souto et al., 2015; Carballo et al., 2016).

The aim of this study has been to analyse the full genomic structure of European sea bass *rtp3* and the transcription profile after injection with *Escherichia coli* lipopolysaccharides (LPSs), polyinosinic:polycytidylic acid (poly I:C), or RGNNV. The present work contributes to further characterize the European sea bass response against RGNNV, being the first step in elucidating the role of sea bass *rtp3* in the course of infections.

2. Material and methods

2.1. Sequencing of European sea bass rtp3

Genomic DNA was obtained from sea bass caudal fin by saline precipitation (Martinez et al., 1988), and cDNA was obtained from sea bass (n = 5) intraperitoneally injected with poly I:C (25 mg/kg, Sigma). At 24 h post-injection (p.i.), animals were euthanized by anaesthetic overdose (MS-222, Sigma), and brains were stored in TRI Reagent solution (Sigma) at 4 °C for the subsequent RNA extraction, according to manufacturer instructions. Resulting RNA was treated with DNase (DNase I, Roche), and cDNA was finally synthesised using the Transcriptor First Strand cDNA Synthesis Kit (Roche), following manufacturer's recommendations.

Two European sea bass *rtp3* genes (named as *rtp3* X1 and *rtp3* X2) were identified within the European sea bass genome database (www.seabass.mpipz.de) by alignment with a 124-bp fragment amplified by PCR using rtp3-qPCR-F/rtp3-qPCR-R primers (Moreno et al., 2020). Both sequences were used as template to design pairs of primers (shown in Table S1) to amplify full-length *rtp3* X1 and *rtp3* X2 in two 50-μL PCR mixtures containing: 500 ng of DNA or cDNA, 15 μM Dlrtp3X1–5′UTR-F/ Dlrtp3X1–3′UTR-R or Dlrtp3X2–5′UTR-F/ Dlrtp3X2–3′UTR-R pairs of primers (for *rtp3* X1 and *rtp3* X2, respectively, Table S1), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1x Green GoTaq® Reaction Buffer, and 1.75 U GoTaq® DNA polymerase (Promega). Thermal profile was: 95 °C for 3 min, 35 cycles at 95 °C for 15 s, 55–58 °C for 30 s and 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. Amplified products were purified from 2% agarose gels stained with RedSafe (INtRON Biotechnology) with the GeneJet gel extraction kit (Thermo scientific), following manufacturer guidelines, and sequenced with the ABI 3730 (STABVIDA) system.

2.2. Sequence analysis and phylogenetic study

Untranslated regions (UTRs) were identified using the Promoter 2.0 software (BIMAS), and introns were identified by the alignment of the resulting cDNA and DNA sequences.

Amino acid sequences were obtained with the EditSeq software (DNASTAR Lasergene 7). For comparison purposes, RTP3 sequences from other species were retrieved from the GeneBank database, aligned using Clustal Omega, and used to build a phylogenetic tree according to the Neighbour-Joining and Poisson correction methods, with a bootstrap of 100 replicates, using the Seaview5 software.

2.3. Virus and cell culture

The RGNNV isolate SpDl_IAusc965.09, obtained from European sea bass, was propagated on the E11 cell line as described by Moreno et al. (2019). Viral titration was undertaken on E11 cells, according to the 50% tissue culture infective dose ($TCID_{50}$) method (Reed and Müench, 1938).

2.4. Fish challenge

Juvenile sea bass specimens (4 g, average weight) were distributed in 25-L tanks at 25 °C in order to stablish 4 groups (n = 25): (i) L-15-injected group; (ii) intraperitoneally poly I:C-injected group (25 mg/kg, Sigma) (Moreno et al., 2016); (iii) intramuscularly LPS (Sigma)-injected group (3 mg/kg) (Bich Hang et al., 2013); and (iv) intramuscularly RGNNV-infected group (2.5×10^5 $TCID_{50}$/g).

Five animals per experimental group were euthanized by anaesthetic (MS-222) overdose at different times (ranging from 6 to 168 h p.i.). Brains and head kidneys were collected and individually stored in TRI reagent at − 80 °C. In the L-15-injected group, liver, spleen, heart, gut, muscle, skin and gill were also collected at 3 days p.i.

Fish were handled according to the European Union guidelines for the handling of laboratory animals (Directive 2010/63/UE) and the Spanish directive (RD53/2013). Trials were accomplished in accordance to the Bioethics Committee of the Junta de Andalucia (no. 04/05/2021/072).

2.5. Quantification of rtp3 mRNA and viral genome

Total RNA extraction and cDNA synthesis were performed following the above described procedures. The transcription of *rtp3* X1 and *rtp3* X2 was quantified by qPCR, using Dlrtp3X1-qPCRF2/R3 or Dlrtp3X2-qPCRF2/R3 primers (Table S1), the Lightcycler 96 thermocycler (Roche), and the Fast Start Essential DNA Green Master Mix. Amplification conditions and thermal profiles were those described by Moreno et al. (2019), using cDNA generated from 50 ng RNA. Fold change (FC) values were calculated according to Pfaffl (2004), using ribosomal 18 S as housekeeping gene (Scapigliati et al., 2010) (Table S1).

Virus replication was analysed in brain by absolute quantification of

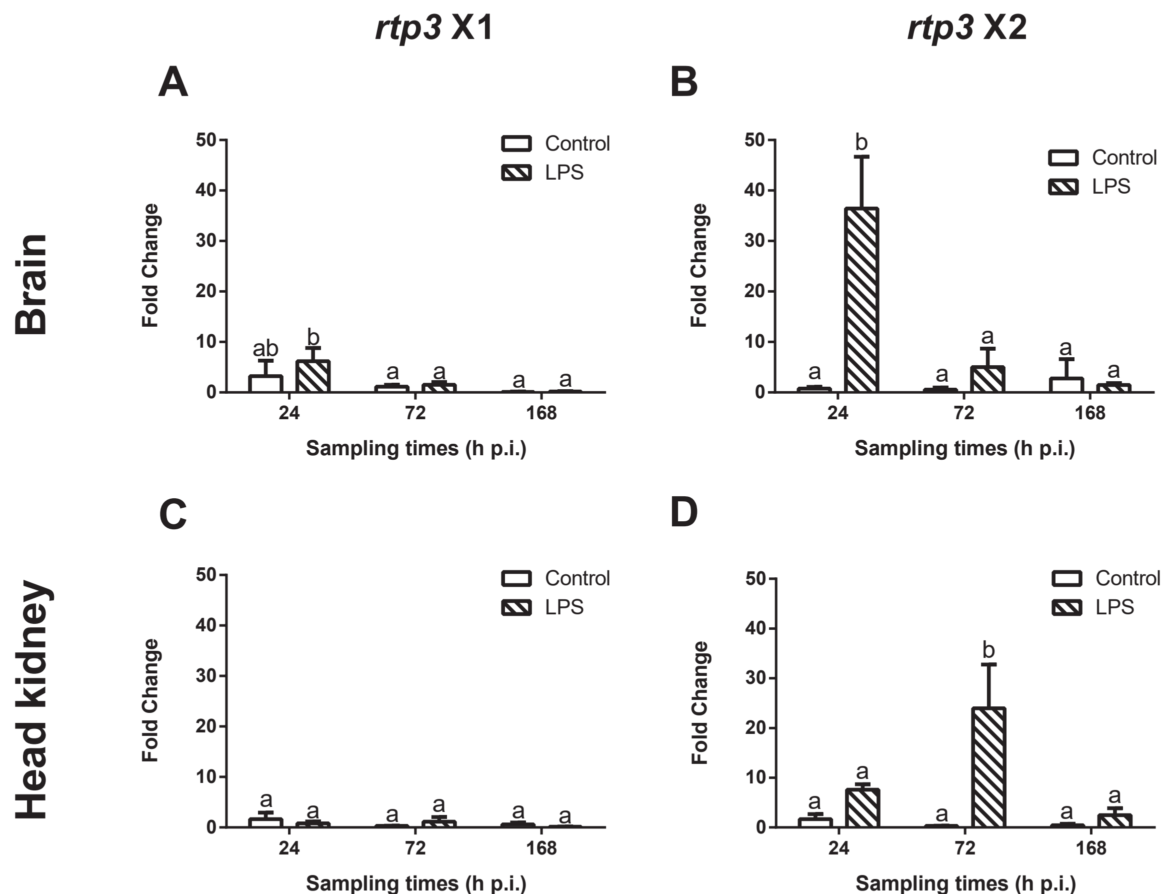

Fig. 2. *rtp3* transcription in brain and head kidney from European sea bass inoculated with LPSs. (A) *rtp3* X1 transcription in brain; (B) *rtp3* X2 transcription in brain; (C) *rtp3* X1 transcription in head kidney; (D) *rtp3* X2 transcription in head kidney. Fold change data are mean ± SD (n = 5). Different letters denote significant differences ($p < 0.05$).

the RNA2 segment (RG_965_RNA2F4/R1 primers, Table S1), using the amplification conditions described elsewhere. Standard curves were constructed according to Moreno et al. (2016).

2.6. Statistical analysis

Results were statistically analysed with the GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, USA), performing two-way ANOVA tests after verifying normality distribution of data by the Shapiro-Wilk test. Tukeýs multiple comparison analysis was carried out as post-hoc test. Values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Genomic structure and sequence analyses

Sequencing results showed that *rtp3* X1 and *rtp3* X2 are similar to other fish *rtp3* genes, such as Asian sea bass or Atlantic salmon *rtp3* (Fig. 1). European sea bass *rtp3* X1 is 1969-bp long (217-bp 5′UTR, 480-bp ORF, and 265-bp 3′UTR), and it is composed of two exons and a single 1007-bp intron located within the ORF. The full length of *rtp3* X2 is 1491 bp (61-bp 5′UTR, 471-bp ORF, and 71-bp 3′UTR), and it also contains two exons and one intron (888-bp long), which is within the ORF sequence (Fig. 1). In addition, *rtp3* X1 and *rtp3* X2 sequences show low identity (63.5%), which confirms that European sea bass displays two *rtp3* genes, as it has been found in other fish species, such as Senegalese sole (*Solea senegalensis*) (Labella et al., 2018), or turbot (*Scophthalmus maximus*) and rainbow trout (*Oncorhynchus mykiss*), by analysing DNA sequences available in databases (www.ncbi.nlm.nih.gov/genbank/). However, there are no studies on the transcriptional differences between both genes in any of these species.

European sea bass *rtp3* X1 and X2 display 2 ATTTA motifs in the intron sequence, and *rtp3* X1 also contains one ATTTA motif in the 5′UTR and two motifs in the 3′UTR region (Fig. S1). These motifs are related with mRNA maturation and stability (Malter, 1989; Zou et al., 1999; Moreno et al., 2016; Gay and Babajko, 2000); therefore, *rtp3* X1 mRNA may have a shorter half-live than *rtp3* X2 mRNA. In addition, a recent study on Asian sea bass *rtp3* reported a microsatellite region in the 3′UTR region, whose variability was correlated with fish resistance to NNV infection (Liu et al., 2017). This region is not present in the *rtp3* genes described in the present work, which may indicate different genetic evolution.

Based on the deduced protein sequences, we have stablished that *rtp3* X1 and *rtp3* X2 encode two 18-kDa proteins, composed of 159 (RTP3 X1) and 156 (RTP3 X2) amino acids. Both proteins contain a zinc-binding domain, which has been detected in all proteins included in the receptor-transporting family, but lack the transmembrane domain (Fig. S2), which is present in all mammalian RTP3. This protein structure is common in all fish RTP3 analysed. The functional implication of the transmembrane domain is unknown, although genomic diversification studies have revealed that this domain is not essential for the receptor trafficking or the antiviral functions of RTP proteins in mammals (Boys et al., 2021). In humans, RTP3 has a cytoplasmic location, related with the endoplasmic reticulum, as it has also been described for Asian sea bass RTP3 (Wrzesinski et al., 2015; Liu et al., 2017).

The identity between European sea bass RTP3 X1 and X2 was 67.9% (Fig. S2), and both proteins clustered together in the phylogenetic tree, along with the RTP3 protein from striped bass (*Morone saxatilis*), perch

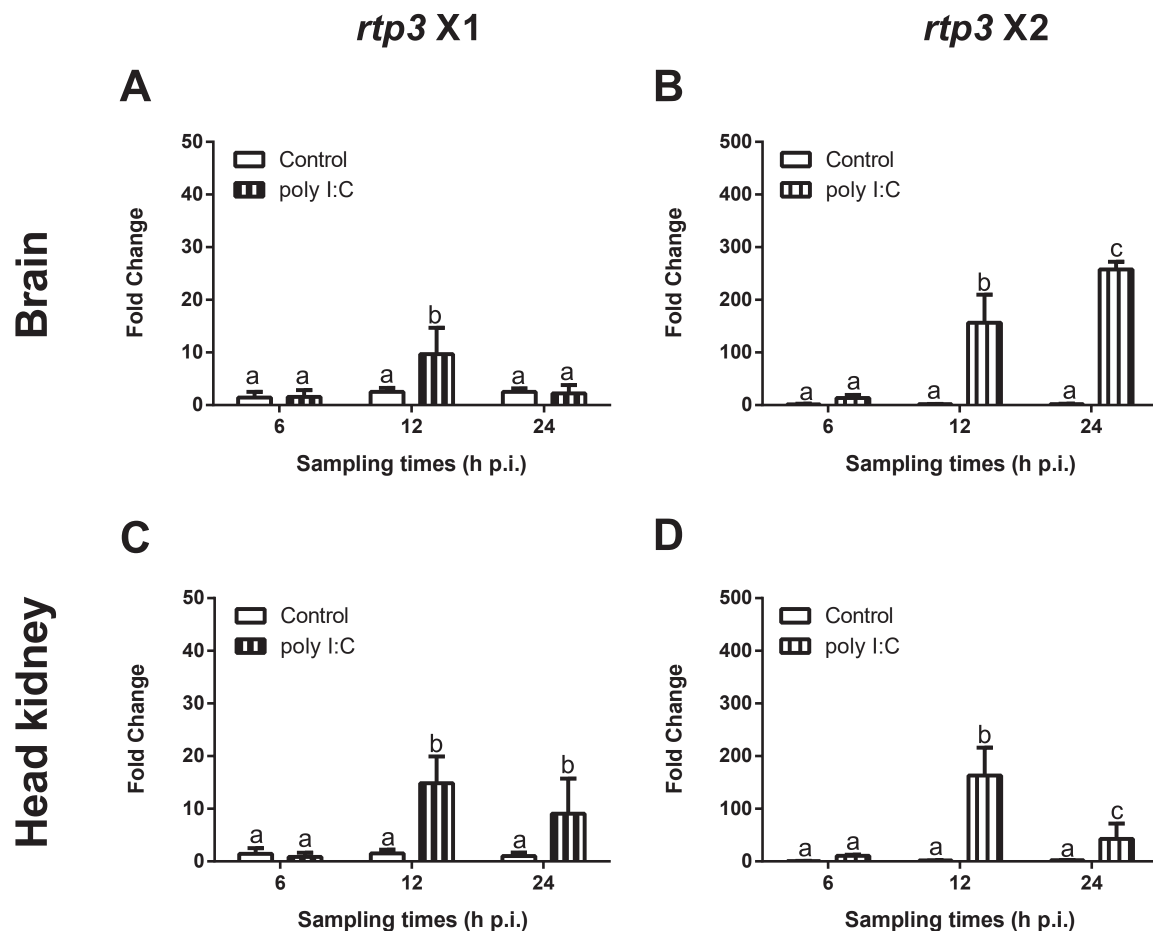

Fig. 3. *rtp3* transcription in poly I:C-inoculated European sea bass. (A) *rtp3* X1 transcription in brain; (B) *rtp3* X2 transcription in brain; (C) *rtp3* X1 transcription in head kidney; (D) *rtp3* X2 transcription in head kidney. Fold change data are mean ± SD (n = 5). Different letters denote significant differences ($p < 0.05$).

(*Perca fluviatilis*) and Southern rock bream (*Oplegnatus fasciatus*), also belonging to Perciformes order (Fig. S3). Within this cluster, sea bass RTP3 X1 and X2 identities were maximum with striped bass RTP3 X1 and X2, 93.7% and 92.9%, respectively (Fig. S2). Identities with other fish RTP3 varied between 32.9% and 93.7%. The protein with the lowest identity with both European sea bass RTP3 proteins was turbot RTP3 (32.9% and 34.2% with sea bass RTP3 X1 and X2, respectively).

3.2. Transcription analyses

The transcription of *rtp3* X1 and *rtp3* X2 was negligible in all organs sampled from control fish (Fig. S4), ranging from 1.3×10^{-3} to 1×10^{-5} relative values, which suggests that both are inducible genes. This downward trend in basal transcription is similar to previous results in Asian sea bass (Liu et al., 2017). On the contrary, a substantial level of transcription was recorded in specimens inoculated with immunostimulant compounds, such as LPSs, and poly I:C, mimicking bacterial or viral infections, or RGNNV infection. Particularly, *rtp3* X1 was not induced in brain or head kidney following LPS injection (Fig. 2A, C), whereas LPSs triggered a significant *rtp3* X2 transcription ($p < 0.0001$) at 1 day p.i. in brain (36.45 mean FC value), and at 3 days p.i. in head kidney (24 mean FC value). Previous studies have described the relationship between viral infections and *rtp3* transcription. Thus, Zhou et al. (2007) reported human *rtp3* upregulation by IFN-α, and several fish *rtp3* genes have been described as VRGs (Liu et al., 2017; Moreno et al., 2020; Valero et al., 2021). However, this is the first study that stablishes a link between LPSs and *rtp3* transcription. LPSs bind toll-like receptor 4 (TLR4), and enhance the expression of inflammatory genes, such as *il-1β*, *il-6*, and *tnf-α*, as well as *ifn-I* (Thompson and Locarnini, 2007; Cochet and Peri, 2017; Piaszyk-Borychowska et al., 2019; Bao et al., 2022). The induction of *rtp3* X2 after LPS injection suggests a putative antibacterial role for this protein, which remains to be investigated.

The induction of *rtp3* elicited by an analogue of viral RNA (poly I:C) or by RGNNV was also analysed. Poly I:C induced an early and strong transcription of both *rtp3* genes in brain and head kidney (Fig. 3). Maximum transcription of *rtp3* X1 was at 12 h p.i. in both organs (Fig. 3 A and C), being the value recorded at this time in brain (9.7 mean FC value) lower than that recorded in head kidney (14.8). Regarding *rtp3* X2 (Fig. 3B and D), poly I:C triggered a much higher induction compared with FC values recorded for *rtp3* X1. In both organs, *rtp3* X2 was upregulated at 12 and 24 h p.i., recording maximum figures at 24 h p.i. in brain (257 mean FC value), and at 12 h p.i. in head kidney (163).

The strong *rtp3* inducibility after viral infections is well documented in several fish species, such as Senegalese sole, Atlantic cod (*Gadus morhua*), Atlantic salmon, Asian sea bass and European sea bass (Krasnov et al., 2011, 2013; Liu et al., 2016, 2017; Moreno et al., 2020; Gemez-Mata et al., 2021; Valero et al., 2021). However, *rtp3* implication in the fish innate immune system as an interferon-stimulated gene (ISG) is not clear. In this regard, the *in vivo* poly I:C analysis carried out in this work supports this idea, since poly I:C is a direct stimulator of the IFN I system. Moreover, these results are in concordance with those described by Krasnov et al. (2011, 2013) and Muller et al. (2015) for *rtp3* induction after poly I:C inoculation in Atlantic salmon, Atlantic cod, and Sockeye salmon (*Oncorhynchus nerka*), which indicates that fish *rtp3* genes could be considered as ISG.

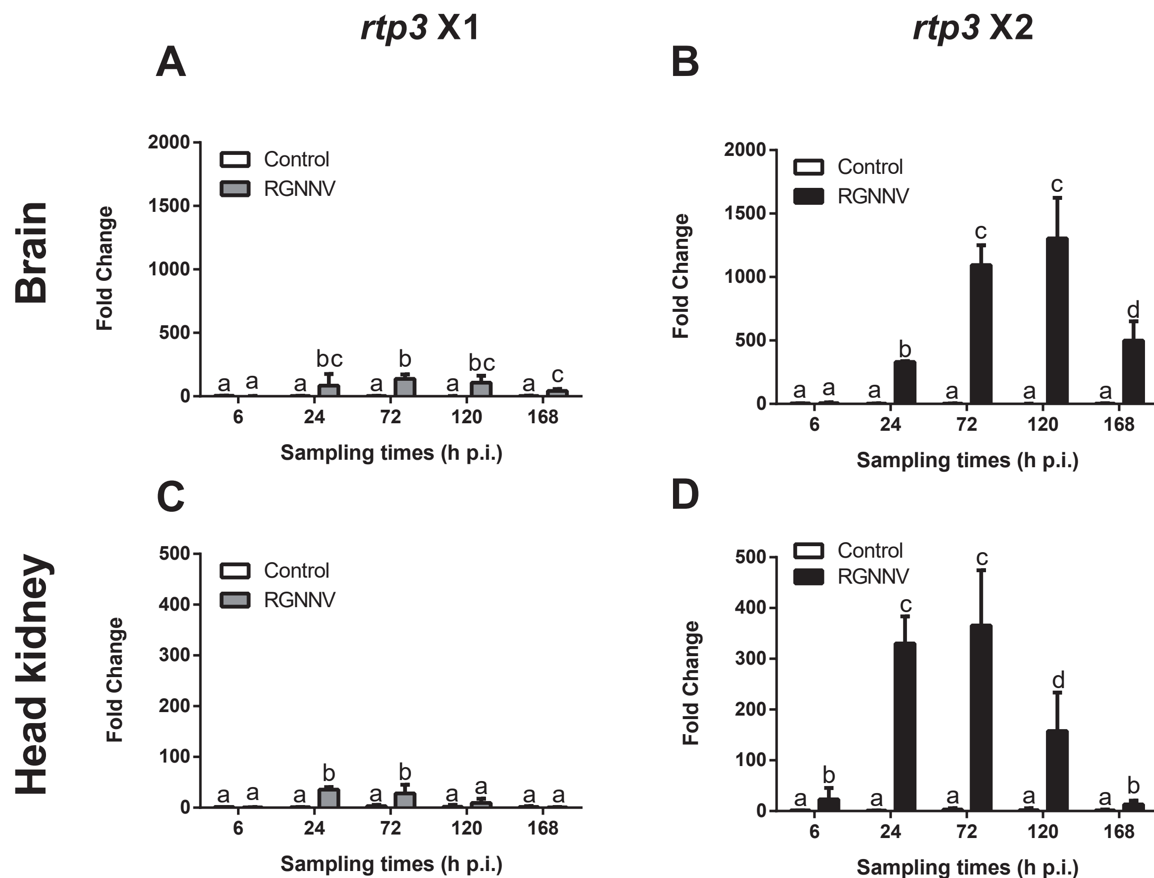

Fig. 4. *rtp3* transcription in RGNNV-inoculated European sea bass. (A) *rtp3* X1 transcription in brain; (B) *rtp3* X2 transcription in brain; (C) *rtp3* X1 transcription in head kidney; (D) *rtp3* X2 transcription in head kidney. Fold change data are mean ± SD (n = 5). Different letters denote significant differences ($p < 0.05$).

Finally, the highest transcription of both *rtp3* genes was triggered by RGNNV infection (Fig. 4). The upregulation of *rtp3* X2 was extremely high in brain (Fig. 4B), the target organ for RGNNV replication, which is in concordance with previous studies in European sea bass (Moreno et al., 2020). However, this is the first work analysing the transcription of both *rtp3* genes separately. Results depicted in Fig. 4A reveal that *rtp3* X1 induction in brain starts at 24 h p.i. (83.6 FC value), and remains constant up to 120 h p.i. A similar induction pattern was recorded in head kidney (Fig. 4C), although reporting values much lower than those obtained in brain (35 and 28 mean FC values at 24 and 72 h p.i., respectively). Regarding *rtp3* X2 (Fig. 4B and D), induction kinetics was also similar for both organs, although starting earlier in head kidney (at 6 h p.i., 23.2 mean FC value), whereas the transcription onset in brain was at 24 h p.i. (331 FC value). Moreover, gene induction reached maximum values earlier in head kidney (at 72 h p.i., 365 mean FC value) than in brain (at 120 h p.i., 1304 FC value). Surprisingly, unlike it happens with other ISGs, *rtp3* X2 upregulation in response to RGNNV is higher than after poly I:C injection, which suggests an essential role of this protein in controlling NNV infections. This idea is supported by a recent study, which described levels of *rtp3* transcription in RGNNV-infected sea bream (*Sparus aurata*), resistant to RGNNV infection, much higher than those obtained in RGNNV-challenged European sea bass (Pereiro et al., 2023), highly susceptible to this infection.

The high *rtp3* upregulation in brain may be elicited by virus multiplication in this organ, which has been demonstrated in this work by qPCR (Fig. S5). A significant increase in the RNA2 copy number was recorded from 1 day (7.7 log RNA2 copies/μg RNA) to 3 days p.i. (10.4 log RNA2 copies/μg RNA). This result is coinciding with the interval of maximum *rtp3* X2 transcription in this tissue. However, although the number of viral genome copies remains high at 7 days p.i., a reduction of *rtp3* X2 transcription was detected (Fig. 4B), which may indicate that a long-lasting high induction of this gene could be deleterious, as it has been described for other components of the IFN-I system (Poisa-Beiro et al., 2008; Labella et al., 2018).

This work has shown stimulation of European sea bass *rtp3* genes by immunomodulating substances and by viral infection, which suggests a putative role of these genes in the immune response of this fish species. It is especially remarkable the upregulation of *rtp3* X2, which may indicate that RTP3 X2 plays a more relevant role against infections, especially against RGNNV, than RTP3 X1.

In summary, this work has described two *rtp3* genes within European sea bass genome, and has analysed their transcription profiles. Our results point out the importance of sea bass *rtp3* X1 and X2 as putative resistance genes, especially *rtp3* X2, not only against RGNNV infection, but also against bacterial infections. The presence of two *rtp3* genes within sea bass genome opens the door to future studies about the involvement of each RTP3 protein in restricting RGNNV replication in sea bass.

CRediT authorship contribution statement

PM: investigation, methodology, formal analyses. **JG-M:** investigation, methodology, formal analyses. **DA-T:** methodology. **EG-R:** conceptualization, reviewing and editing. **JB:** conceptualization, reviewing and editing. **MCA:** conceptualization, writing-original draft, supervision, funding acquisition.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2023.10.009.

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