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# Betanodavirus Quantification and IFN I-Antagonism Detection Using Luciferase Reporter Systems Based on Fish *mx* Promoters

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**Received:** 31 March 2025 | **Revised:** 30 May 2025 | **Accepted:** 4 June 2025

**Funding:** This work was supported by the UMA20-FEDERJA-020 project (Regional Government). Daniel Álvarez-Torres has been granted funding by the project PTA2020-018984-I. Funding for open access publishing: Universidad de Málaga/CBUA.

**Keywords:** betanodavirus | IFN I-antagonism | luciferase reporter system | *mx* promoter

## ABSTRACT

*Mx* genes display strong and quick induction in response to viral infections, which varies according to the viral virulence; furthermore, *mx* transcription is blocked by several viruses as part of their immune evasion strategies. Therefore, the level and time course of *mx* induction reflect virus–host interplay. This idea prompted the development of in vitro experimental systems consisting of cells stably expressing luciferase under the control of fish *mx* promoters. In this study, two RTG-2 cell lines, expressing luciferase under the control of Senegalese sole (*Solea senegalensis*) *mx* (*ssmx*) promoter, and under the control of gilthead sea bream (*Sparus aurata*) *mx2* (*saumx2*) promoter, have been applied to study betanodavirus–host interaction. Both systems were inoculated with nervous necrosis virus, NNV, isolates belonging to different genotypes. The combination of both systems has been proved to be useful to detect all of them, although each isolate triggered a characteristic profile in each system. In addition, a protocol to estimate the titre of the RGNNV isolate in sea bass (*Dicentrarchus labrax*) brain has been established, and a clear dose-dependent antagonistic effect of this NNV isolate was recorded. Thus, both cell lines are useful tools to study betanodavirus–host interaction and, therefore, contribute to developing measures to fight viral infections in aquaculture.

## 1 | Introduction

The impact of fish diseases in aquaculture extends beyond individual farms, with potential consequences for wild fish populations, ecosystems and food security. Fish infectious diseases encompass a diverse array of pathogens, including viruses belonging to several families in which different genotypes or species with particular host specificities or virulence in different fish species have been identified (Lafferty et al. 2015). Understanding the dynamics of virus–fish interactions is vital

for elucidating disease mechanisms and for designing strategies to control viral infections to safeguard fish health and, therefore, aquaculture productivity and sustainability.

Traditional methods in Virology, such as cell culture-based techniques, are time-consuming procedures that have limitations in terms of sensitivity or specificity, and, in addition, they also have knowledge and expertise requirements. Therefore, the development of in vitro experimental systems that facilitate the study of virus–host interplay, or that can serve as tools to

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measure viraemia, determine the virulence of different viral isolates, or significantly reduce in vivo experimental infections is needed (Collet et al. 2018).

Innate antiviral immune response in fish is regulated by type I interferon (IFN I), which induces the transcription of interferon-stimulated genes (ISGs), including *mx* genes, which are main effectors of the IFN I response due to their proven direct antiviral activity (Poynter and DeWitte-Orr 2016). In addition, *mx* transcription is blocked by several viruses as part of their strategies of immune evasion (García-Sastre 2017), which is also related with isolate virulence. Therefore, the level of *mx* induction in the course of a viral infection has been related with the susceptibility/resistance of different fish species to specific viral isolates (Collet et al. 2007; Jørgensen et al. 2007; Sun et al. 2011). In this scenario, in vitro reporter systems based on luciferase expression under the control of fish *mx* promoters (*pmx*-luciferase systems) have been developed and used to compare the IFN I response triggered by different viral isolates (Alvarez-Torres et al. 2014; Cano et al. 2016), to measure viremia in fish serum (Collet et al. 2013) or to study and compare the antagonistic activity of several viral isolates against the host IFN I response (Collet et al. 2004, 2007; Gémez-Mata et al. 2020; González-Mariscal et al. 2016; Lester et al. 2012).

Two *pmx*-luciferase systems, consisting of RTG-2 cells stably expressing luciferase under the control of Senegalese sole (*Solea senegalensis*) *mx* (*ssmx*) promoter: RTG *pssmx*-luc cells (Gémez-Mata et al. 2020); and under the control of gilthead seabream (*Sparus aurata*) *mx2* (*saumx2*) promoter: RTG *psaumx2*-luc (González-Mariscal et al. 2016) had been developed in our research group. This study aims to validate and compare these two experimental systems as tools to study the interaction between fish immune system and nervous necrosis virus (NNV, *Nodaviridae* family), that has a bisegmented genome consisting of two single-stranded, positive-sense RNA molecules (RNA1 and RNA2), and it is the aetiological agent of the viral encephalopathy and retinopathy (VER), which represents one of the main viral diseases in marine aquaculture (Yang et al. 2022), affecting mainly Senegalese sole, European sea bass (*Dicentrarchus labrax*) and gilthead seabream in the Mediterranean area (Bandin and Souto 2020).

## 2 | Material and Methods

### 2.1 | Cell Culture

The cell lines RTG-2, derived from gonad tissue of rainbow trout (*Oncorhynchus mykiss*) (Wolf and Quimby 1962); RTG *pssmx*-luc (Gémez-Mata et al. 2020); RTG *psaumx2*-luc (González-Mariscal et al. 2016); and CHSE-214, derived from chinook salmon embryonic cells (Fryer et al. 1965), were cultured on 25-cm<sup>2</sup> flasks (Nunc, ThermoFisher) at 20°C with Leibovitz L-15 medium (L-15, Gibco) supplemented with 10% foetal bovine serum (FBS, Lonza), 4 mM L-glutamine (Gibco) and 100 units/mL penicillin, 100 µg/mL streptomycin (Sigma) (growth medium). E-11 cells (Iwamoto et al. 2000) were grown in 25-cm<sup>2</sup> flasks at 25°C with L-15 medium supplemented with 5% FBS and 100 units/mL penicillin, 100 µg/mL streptomycin.

### 2.2 | Viruses and Virus Propagation

Three betanodavirus isolates were used in this study: NNV SpDI\_IAusc965.09 (RGNNV genotype, obtained from sea bass, Moreno et al. 2019); NNV SJ93Nag (SJNNV genotype, Iwamoto et al. 2000); and NNV SpSs-IAusc160.03 (reassortant isolate RGNNV/SJNNV; RG/SJ; with a genome consisting of RGNNV-type RNA1 and SJNNV-type RNA2 segments, Oliveira et al. 2009).

All NNV isolates were propagated on E11 cells, at 25°C (RGNNV and RG/SJ) or 20°C (SJNNV). Inoculated E-11 cells were incubated in maintenance medium without L-glutamine. Supernatants from cultures displaying extensive cytopathic effects (CPEs) were collected and centrifuged at 5000×g for 10 min at 4°C to remove cellular debris. Viral suspensions were titrated on E11 cells by the 50% tissue culture infective dose (TCID<sub>50</sub>) method (Reed and Muench 1938) and stored at −80°C until used.

### 2.3 | Viral Detection in Cell Supernatants Using RTG-Pmx-Luciferase Systems

RTG *pssmx*-luc and RTG *psaumx2*-luc cells were seeded (100,000 cells per well) on 24-well cell culture plates (Nunc) and incubated for 24 h at 20°C in growth medium. Afterwards, cells were washed with phosphate-buffered saline (PBS) and inoculated with the viral isolates at  $5 \times 10^2$  and  $5 \times 10^3$  TCID<sub>50</sub>/mL in maintenance medium without FBS (inoculation medium). After 1 h adsorption, viral suspensions were removed and replaced by maintenance medium. Incubations were conducted at the optimum replication temperature for each virus, described in Section 2.2. Luciferase activity in cells was measured at 18, 24 and 36 h post-inoculation (p.i.). At those times, culture medium was removed, and cell monolayers were washed with PBS and subsequently lysed with Passive Lysis Buffer (100 µL, Luciferase assay system, Promega). Cellular lysates were stored at −80°C until used. Three replicates per condition in three independent experiments were carried out, and control non-infected cells were also included in each assay.

### 2.4 | Quantification of Luciferase Activity

For luciferase quantification, each cellular lysate (20 µL) was mixed with 100 µL of Luciferase Assay Reagent II (Promega) in a white opaque 96-well plate (Nunc). Afterwards, light emission (Relative Light Units, RLU) was determined with continuous measurements every 2 s during 10 s, using a GloMax 96 Microplate Luminometer (Promega). Inducibility was calculated as the ratio between each RLU value and that of non-infected control cells. Data were presented as mean ± standard deviation (SD). Differences between samples were tested using a two-tailed unpaired Student's t-test;  $p < 0.05$  was considered statistically significant.

### 2.5 | Quantification of RGNNV in Brain Samples

The following brain samples from a previous experimental infection of juvenile sea bass with the RGNNV strain (Moreno

et al. 2023) were used to set up the RTG *pmx-luc* systems to quantify RGNNV in tissue samples: three samples from non-infected control fish, five from infected fish that survived to the infection, and three from infected fish that died in the course of the infection. Brains were independently homogenised in inoculation medium (10% w/v). Afterwards, homogenates were treated with penicillin–streptomycin (100 mL/mL) for 24 h at 4°C, and subsequently centrifuged at 5000×g for 5 min at 4°C. Supernatants were stored at –80°C until used.

The three homogenates from non-infected control fish were mixed in a pool and diluted 1:100 in inoculation medium, to avoid cytotoxic effects on cells. Then, the RGNNV strain was added to the diluted homogenate at  $10^1$ ,  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$  and  $10^6$  TCID<sub>50</sub>/mL (final concentration). RTG *pssmx-luc* and RTG *psaumx2-luc* cells were seeded on 24-well culture plates as described in Section 2.3, incubated for 24 h, and finally inoculated with the above-described spiked samples (250 µL). After 1-h adsorption, maintenance medium (750 µL) was added, and cells were incubated at 25°C for 24 h. At that time, luciferase activity was measured, and inducibility values were calculated as described above. Three independent replicates were performed for each assay, and non-inoculated cells were also included. Differences between samples were tested using a two-tailed unpaired Student's t-test;  $p < 0.05$  was considered statistically significant. A regression analysis between inducibility (*y*-axis) and TCID<sub>50</sub>/mL (*x*-axis) was conducted using the *GraphPad Prism 9* software (GraphPad Software Inc. La Jolla, USA).

Brain homogenates (1:100 dilution) from infected fish (survivors, S and dead, D) were independently added to the RTG *pmx-luc* systems as described above. Luciferase activity was measured and inducibility values were calculated at 24 h p.i. Inducibility values were used to estimate the viral titre (TCID<sub>50</sub>/mL) of brain homogenates using the regression equations obtained for each RTG *pmx-luc* system. In parallel, brain homogenates from infected fish were also titrated on E11 cells by the standard TCID<sub>50</sub>/mL method, described in Section 2.2, and differences between values obtained by both methodologies were tested using the two-way ANOVA test using the *GraphPad Prism 6* software. Differences of  $p < 0.05$  were considered significant.

## 2.6 | Unravelling RGNNV Antagonistic Effect Using RTG-Pmx-Luc Systems

As a first step, the activation pattern of *mx* promoters in response to polyinosinic-polycytidylic acid (poly I:C) was determined. In order to do that, RTG *pssmx-luc* and RTG *psaumx2-luc* cells were seeded on 24-well cell culture plates (100,000 cells per well) and incubated in growth medium for 24 h at 20°C. Afterwards, cells were treated with poly I:C at 10 µg/mL (final concentration). Luciferase activity was measured at 6, 18, 24 and 36 h post-treatment. Three replicates per sampling time in three independent experiments were analysed.

To study the antagonistic activity of the RGNNV strain against the IFN-I system, both RTG *pmx-luc* cell lines were seeded on 24-well cell culture plates (100,000 cells per well) as described above. After 24 h at 20°C, cells were washed with PBS and inoculated

with the RGNNV strain at  $5 \times 10^2$  and  $5 \times 10^3$  TCID<sub>50</sub>/mL in maintenance medium. At 24 h p.i., poly I:C (10 µg/mL, final concentration) was added, and luciferase activity was measured 18 h after poly I:C treatment (time of maximum induction by poly I:C, Figure S1). Uninfected cells stimulated with poly I:C were used as a positive control of *mx* promoter stimulation. Infected cells non-stimulated with poly I:C, and non-stimulated and non-infected cells were used as negative controls. Data, presented as mean RLU ± standard deviation (SD), were statistically analysed with the two-way ANOVA test using the *GraphPad Prism 6* software. Differences of  $p < 0.05$  were considered significant.

## 2.7 | Quantification of Endogenous Ifn I and mx Transcription After RGNNV Inoculation

RTG-2 cells were grown on 24-well plates for 24 h as described above and inoculated with the RGNNV strain ( $5 \times 10^4$  TCID<sub>50</sub>/mL). At 24 h p.i., poly I:C (10 µg/mL, final concentration) was added to the infected cells. Uninfected cells stimulated with poly I:C were used as positive controls. Infected cells non-stimulated with poly I:C, and non-stimulated and non-infected cells were used as negative controls. RNA was extracted at 2 and 4 h (*ifn*) or at 18 h (*mx*) after poly I:C treatment using the EZNA total RNA kit I (Omega). After RNA treatment with DNase (DNase I recombinant RNase-free, Sigma), cDNA was synthesised with the Transcriptor First Strand cDNA Synthesis Kit (Roche), using 1000 ng of RNA and random primers. Transcription quantification of *ifnI*, *mx1* and *mx3* was performed according to (Gómez-Mata et al. 2020). Fold change values were calculated according to Pfaffl (2004), using *ef1-a* as the endogenous reference gene, and uninfected cells as the calibrator. Primers used are listed in Table 1. Data were statistically analysed with the two-way ANOVA test using the *GraphPad Prism 6* software. Differences of  $p < 0.05$  were considered significant.

## 3 | Results

### 3.1 | Detection of Betanodaviruses in Cell Supernatants

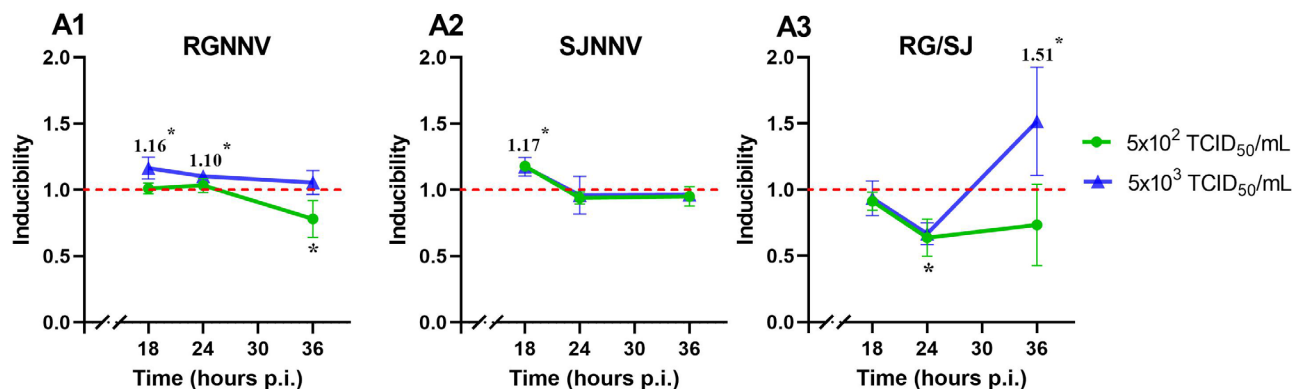
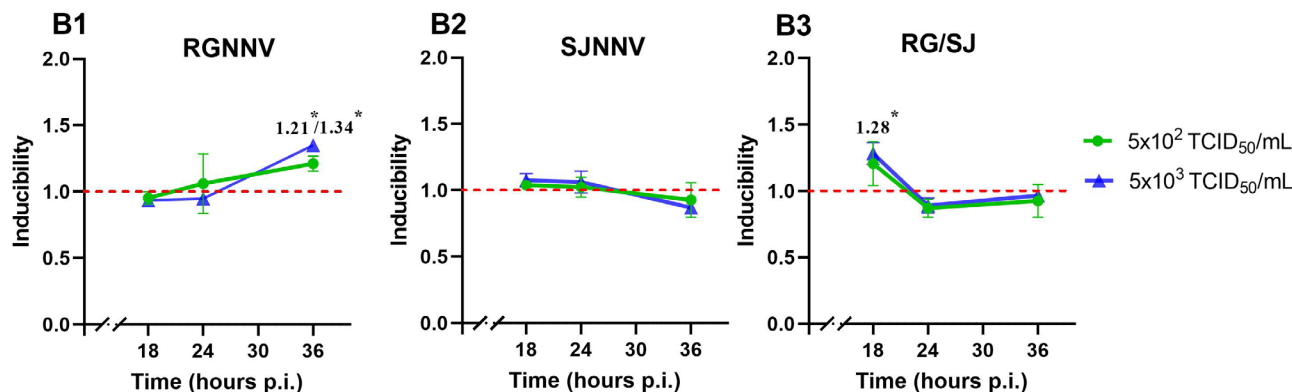
All betanodaviruses induced the *ssmx* promoter (Figure 1A). The RGNNV and SJNNV strains triggered an early induction, whereas induction was recorded only at 36 h p.i. in RG/SJ-inoculated cells. Regarding sensitivity, SJNNV was the only viral isolate inducing a significant increase of luciferase activity at the lowest viral dose tested ( $5 \times 10^2$  TCID<sub>50</sub>/mL).

In RTG *psaumx2-luc* cells, a significant induction was only observed after inoculation with the isolates RGNNV (at 36 h p.i.) and RG/SJ (at 18 h p.i.) (Figure 1B). The RGNNV strain was the only one inducing a significant increase of luciferase activity at the lowest dose tested ( $5 \times 10^2$  TCID<sub>50</sub>/mL).

Therefore, only the RGNNV and RG/SJ strains triggered significant inducibility values in both cell lines, being RGNNV able to induce the *saumx2* promoter at the lowest dose tested. For this reason, the RGNNV strain was selected to validate both RTG *pmx-luc* systems in further assays.

**TABLE 1** | Primers used in this study (Ballesteros et al. 2015).

Gene	Abbreviation	Primer sequence (5'-3')
<i>ifn1</i>	IFN-1-F	AAAAC TGT T T GAT G GGA AT AT G AAA
	IFN-1-R	CGTTTCAGTCTCCTCTCAGGTT
<i>mx1</i>	Mx-1-F	GGTTGTGCCATGCAACGTT
	Mx-1-R	GGCTTGTCAGGATGCCTAAT
<i>mx3</i>	Mx-3-F	AGCTCAAACGCCTGATGAAG
	Mx-3-R	TGAATATGTCTGTTATCCTCCAAA
<i>elf1α</i>	RTeF1α-F	GATCCAGAAGGAGGTCACCA
	RTeF1α-R	TTACGTTTCGACCTTCCATCC

**A. RTG-*pssmx-luc*****B. RTG-*psaumx2-luc***

**FIGURE 1** | Induction of luciferase in RTG *pssmx-luc* (A) and RTG *psaumx2-luc* (B) cells after viral infections at  $5 \times 10^2$  (green) and  $5 \times 10^3$  (blue) TCID<sub>50</sub>/mL with RGNNV (A1, B1); SJNNV (A2, B2); and RG/SJ (A3, B3) strains. Mean inducibility values  $\pm$  standard deviation (SD) obtained from three different samples are shown. Asterisks indicate significant differences in comparison with non-inoculated control cells at each time point,  $p < 0.05$ . Numbers are fold induction values. The dashed red line indicates the baseline, corresponding to the inducibility value in control non-inoculated cells.

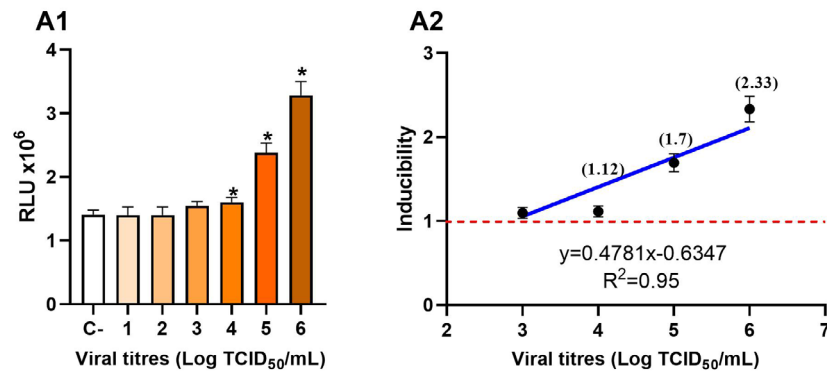
### 3.2 | Detection of RGNNV in Tissue Samples Using the RTG-Pmx-Luc Systems

The analysis of sea bass brain homogenates (added with the RGNNV strain from  $10^4$  to  $10^6$  TCID<sub>50</sub>/mL) showed a clear dose-dependent induction in both cell lines (Figure 2A1,B1). The sensitivity limit for RGNNV detection in sea bass brain using both cell systems is between  $10^3$  and  $10^4$  TCID<sub>50</sub>/mL at 24 h p.i.

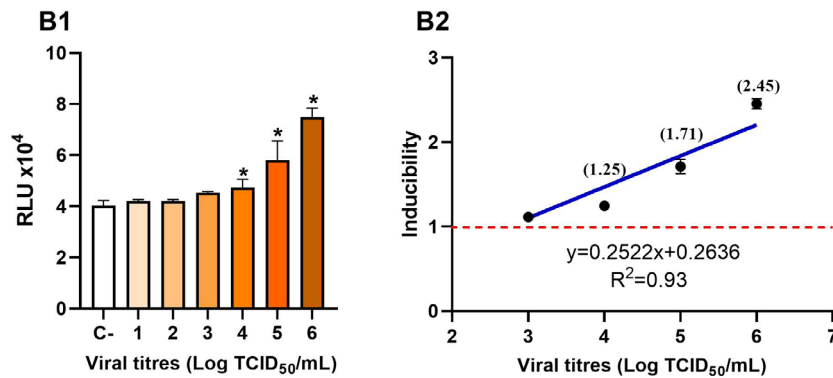
Although the sensitivity of both cellular systems is similar, the *psaumx2* promoter activation seems to be stronger (Figure 2B1), reaching  $7.49 \times 10^4$  RLU values.

To establish the RTG *pmx-luc* experimental systems as tools to estimate RGNNV load in brain samples, a linear regression between values of inducibility (y-axis) and TCID<sub>50</sub>/mL (x-axis) was performed using samples added with the RGNNV strain from

## A RTG *pssmx*-luc



## B RTG *psaumx2*-luc



**FIGURE 2** | Detection of RGNNV in sea bass brain homogenates using RTG *pssmx*-luc (A) and RTG *psaumx2*-luc cells (B). Different doses of the RGNNV strain (from  $10^1$  to  $10^6$  TCID<sub>50</sub>/mL) were added to samples from non-infected sea bass. Luciferase activity was measured at 24 h p.i. In A1 and B1, luciferase activity is shown. Asterisks indicate significant differences with control non-inoculated cells,  $p < 0.05$ . In A2 and B2, the lineal regression analysis of inducibility values obtained in samples and the viral doses inoculated is shown. Bars indicate mean  $\pm$  standard deviation (SD) obtained from three different measures. The dashed red line indicates the baseline.

$10^3$  to  $10^6$  TCID<sub>50</sub>/mL.  $R^2$  values were 0.95 and 0.93, for RTG *pssmx*-luc and RTG *psaumx2*-luc cells, respectively, indicating that both cell lines could be used to estimate load in sea bass brain samples (Figure 2A2,B2).

To confirm this, inducibility in cells inoculated with brain homogenates from experimentally challenged survivors (asymptomatic fish) and dead sea bass specimens was calculated using both RTG *pssmx*-luc systems (Table S1). Afterwards, TCID<sub>50</sub>/mL values predicted according to the regression equations were compared with TCID<sub>50</sub>/mL values determined following the standard TCID<sub>50</sub>/mL method, based on CPE appearance on E11 cells (Figure 3, Table S1). No differences between both predicted values were observed. Similarly, no differences between predicted and measured TCID<sub>50</sub>/mL values were recorded, with the exception of the D3 sample.

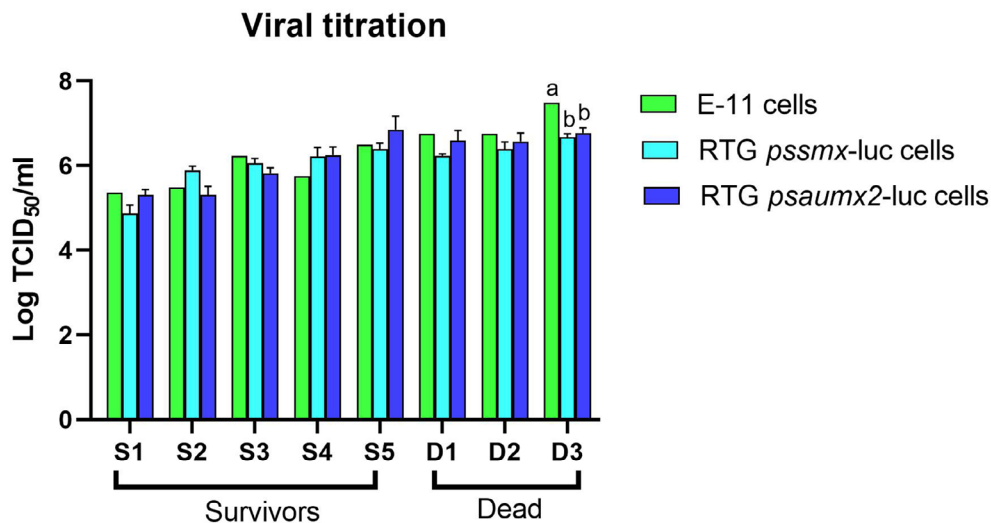
### 3.3 | RTG Pmx-Luc Systems Reveal RGNNV Antagonistic Activity on the IFN-I System

To evaluate the RGNNV antagonistic activity on the IFN-I system, the possible interference of RGNNV infection with the well-known induction triggered by poly I:C was investigated in both systems. When cells were inoculated with the RGNNV strain 24 h

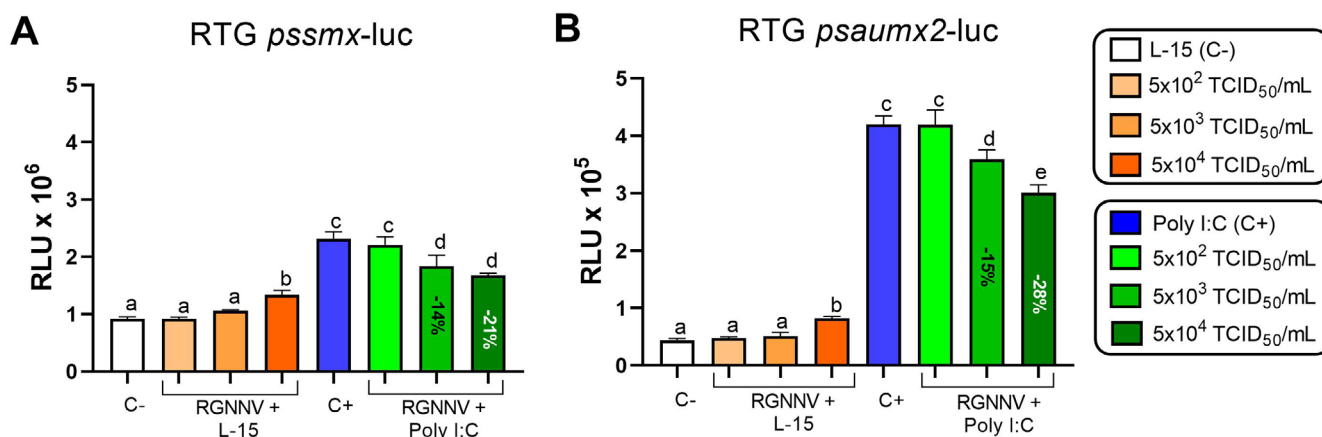
before poly I:C treatment, a significant decrease in the induction elicited by poly I:C was recorded (Figure 4A,B), suggesting the RGNNV antagonistic effect on *ssmx* and *saumx2* promoters. This effect was only detected in cells infected with  $5 \times 10^3$  and  $5 \times 10^4$  TCID<sub>50</sub>/mL, being higher at the highest viral dose tested, indicating a dose-dependent effect. In RGNNV-infected cells not treated with poly I:C, only the highest viral dose caused a significant increase in luciferase activity (Figure 4A,B).

### 3.4 | Transcription of Endogenous *ifn I* and *mx* Genes

In order to determine if the RGNNV antagonistic activity observed also affects the endogenous IFN I-system response, *ifn I*, *mx1* and *mx3* transcription was quantified in RTG-2 cells inoculated with the virus and subsequently treated with poly I:C. Regarding *ifn I*, the infection with the RGNNV strain ( $5 \times 10^4$  TCID<sub>50</sub>/mL) did not affect the transcription elicited by poly I:C (Figure 5A). In contrast, the poly I:C-induced transcription of *mx1* and *mx3* was lower in RGNNV-infected cells (19.2 and 39.71% reduction, respectively) ( $p < 0.05$ , Figure 5B). Therefore, the RGNNV strain interfered with the transcription of both endogenous *mx* genes, affecting the ISG signalling pathway but not *ifn I* transcription.



**FIGURE 3** | RGNNV titres in sea bass samples estimated using the regression equations obtained with the RTG *pmx*-luc systems (light and dark blue) and calculated on E-11 cells using the standard TCID<sub>50</sub> method (green). Different letters indicate significant differences between columns of the same sample  $p < 0.05$ . Raw data are shown in Table S1.



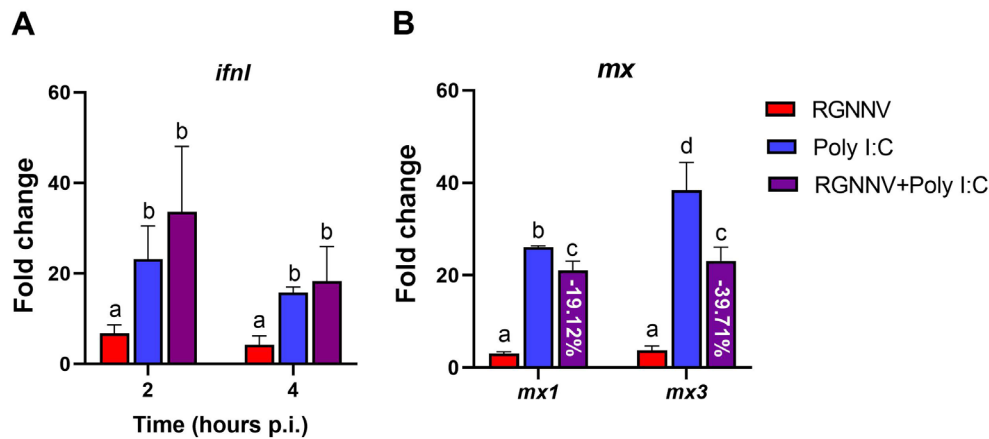
**FIGURE 4** | Antagonistic activity of the RGNNV strain on RTG *pssmx*-luc (A) and RTG *psaumx2*-luc (B). Luciferase activity in cells inoculated with RGNNV (orange) at different titres ( $5 \times 10^2$ – $5 \times 10^4$ ) was measured at 42 h p.i. In cells inoculated first with the RGNNV strain and subsequently (24 h p.i.) treated with poly I:C (10  $\mu$ g/mL) (green), luciferase activity was measured 18 h after poly I:C treatment (42 h after RGNNV infection). Non-treated cells were used as a negative control (white), and non-infected cells treated with poly I:C were used as positive control (blue). Results are represented as relative light units (RLU). Bars indicate mean  $\pm$  standard deviation (SD) obtained from three different samples. Different letters indicate significant differences,  $p < 0.05$ . Numbers indicate the percentage of inducibility decrease with respect to the positive control.

## 4 | Discussion

Firstly, different betanodavirus isolates were added to both experimental systems to establish the minimum viral dose detectable by the luciferase-reporter systems and to compare the time course response of the two promoters to each isolate. RGNNV and RG/SJ NNV strains triggered luciferase activity only when they were inoculated at  $5 \times 10^3$  TCID<sub>50</sub>/mL, thus this could be considered the detection limit of the systems for these isolates, which is in concordance with previous studies (Collet et al. 2004; Jørgensen et al. 2007). Although standard titration has a higher sensitivity ( $10^{1.5}$ – $10^{1.8}$  TCID<sub>50</sub>/mL) (Padrós et al. 2022), the method is time-consuming and highly dependent on the development of CPEs in permissive cell lines, as well as on the subjective recognition and interpretation of those CPEs, whereas the proposed experimental systems are

able to detect virus which do not develop CPEs on RTG-2 cells, such as nodavirus.

Some differences in the magnitude and kinetics of luciferase activity induction were recorded between the two *pmx*-luc systems. For instance, only *pssmx* was activated by the SJNNV strain, whereas RGNNV and RG/SJ strains activated both promoters, although with a different profile: the RGNNV strain triggered an early response on *pssmx* and a late response on *psaumx*, whereas the RG/SJ strain triggered a late response on *pssmx* and an early induction on *psaumx*. These differences might probably be related to the structural characteristics of each promoter. In fact, whereas *pssmx* presents three potential IFN-stimulated response elements (ISREs) and a single gamma interferon activation site (GAS) (Alvarez-Torres et al. 2013), *psaumx* has a single ISRE motif, four GAS elements and a putative interleukin-6 binding



**FIGURE 5** | Relative quantification of endogenous *ifnI* (A) and *mx1* and *mx3* (B) transcription in RTG-2 cells inoculated with the RGNNV strain ( $5 \times 10^4$  TCID<sub>50</sub>/mL) and treated with poly I:C (10  $\mu$ g/mL) 24 h later. Quantification was performed at 2 and 4 h after poly I:C treatment for *ifnI* and at 18 h for *mx1* and *mx3*. Bars indicate mean  $\pm$  standard deviation (SD) obtained from three different samples. Different letters indicate significant differences,  $p < 0.05$ . Numbers indicate the reduction in fold change values compared to the positive control (poly I:C-treated cells).

site (González-Mariscal et al. 2014). These structural differences support the idea that the fine-tuning regulation of *mx* expression occurs at the *mx* promoter level. Actually, this fine regulation was also observed when the response of the three seabream *mx* promoters to IPNV and VHSV was compared (González-Mariscal et al. 2016). Regarding the induction triggered by different isolates on each promoter, the differences observed in the induction patterns after infection with the three NNV isolates as well as with the IPNV isolate and the two VHSV isolates tested on our reporter systems (Figure S2) agree with previous reports (Alvarez-Torres et al. 2014; Cano et al. 2016; Gémez-Mata et al. 2020; González-Mariscal et al. 2016). Even the decrease in luciferase activity observed in cells inoculated with the RGNNV and RG/SJ strains has also been recorded when RTG-P1 cells (RTG-2 cells expressing luciferase under the control of rainbow trout *mx1* promoter) were infected with IPNV or salmon pancreas disease virus, SPDV (Jørgensen et al. 2007), pointing to a response highly dependent on the interplay between each isolate and each promoter, which, in turn, might also be influenced by cell-specific factors (Alvarez-Torres et al. 2014). In spite of this, the combination of both systems has been proven to be useful to detect the three betanodavirus isolates evaluated in this study, even if these viruses are not normally propagated in RTG-2 cells. Furthermore, although both reporter systems are polyclonal cell lines, their sensitivity was similar to that recorded for other systems based on monoclonal cell lines, such as RTG-P1 (Collet et al. 2004) or CHSE-Mx10 (Jørgensen et al. 2007). Thus, it is tempting to think that the systems could probably also be induced by unknown isolates responsible for field outbreaks or by isolates that do not generate CPE, making this CPE-independent viral detection method particularly beneficial (Collet et al. 2018).

Once the RGNNV strain was chosen to continue with the *pmx*-luc systems validation, a protocol to detect this virus in brain, the target tissue of betanodaviruses, was established. A 1:1000 dilution was required to avoid cytotoxicity, allowing RGNNV detection with a sensitivity limit between  $10^3$  and  $10^4$  TCID<sub>50</sub>/mL using both systems. Furthermore, the systems proved to be useful to estimate viral titre. Actually, *pmx*-luc systems provided results after less than 48 h p.i., a very short period of time

compared with 10–14 days required for CPE generation in E-11 cells. Detection of viral pathogens prior to CPE development was established for the first time in RTG-P1 cells, where Salmonid Alphavirus (SAV) isolates were quantified in trout serum (Collet et al. 2013). Then, SPDV viraemia in serum was also estimated using these cells (Collet et al. 2018). Thus, our results add RGNNV in brain to the group of samples where viral titre can be estimated using luciferase quantification, and encourage the establishment of similar protocols using other tissues/isolates combinations.

Finally, a clear dose-dependent antagonistic effect of the RGNNV strain was recorded in both *pmx*-luc systems. Therefore, both, *ssmx* and *saumx* promoters can be antagonised by RGNNV infection, although this effect does not completely abolish the activation of the *mx* promoters, at least at high viral dose ( $10^4$  TCID<sub>50</sub>/mL, Figure 5). Poly I:C induction of luciferase was also suppressed by IPNV in RTG-P1 cells and in *psaumx2* cells (Collet et al. 2007; González-Mariscal et al. 2016; Jørgensen et al. 2007). Regarding VHSV, its antagonistic effect was detected in RTG-P1 cells (Cano et al. 2016), as well as in *pssmx*-luc cells, where the differential antagonistic activity recorded for different isolates was related with their virulence in a specific host species (Gémez-Mata et al. 2020).

The antagonistic activity of RGNNV had been previously suggested (Álvarez-Torres et al. 2017; Carballo et al. 2016; Mai et al. 2018). Regarding the mechanism responsible for this activity, it has been recently reported that the RGNNV non-structural proteins B1 and B2 interfere with the host immune response acting as transcription inhibition factors targeting RNA polymerase II (Qin et al. 2021, 2023). Thus, our two *pmx*-luc systems can become useful tools to investigate more in depth this process. Actually, in contrast with the differential modulation of *mx* induction occurring at promoter level described for VHSV antagonism (Gémez-Mata et al. 2020), RGNNV effect seems to occur very similarly in the two systems, and a clear interference with endogenous *mx1* and *mx3* was also evidenced, thus suggesting that the effect is not dependent on the specific *mx* promoter. However, endogenous *ifnI* transcription was not affected by RGNNV infection, and similar

Ct values (ranging from 19.76 to 20.67) for the housekeeping gene *ef1-a* were recorded. These results suggest a specific interference triggered by the RGNNV strain that occurs at the ISG signalling pathway, that is, after IFN I synthesis. Further investigations must be conducted to get more understanding about RGNNV antagonistic activity, a key point in virus-host interplay.

In summary, this study validates two *pmx*-luc systems as tools to detect betanodavirus isolates belonging to different genotypes; to estimate RGNNV viral titre from sea bass brain samples; and to study virus evasion strategies. Both systems can therefore help to identify the molecular mechanisms underlying these processes and can contribute to the design of strategies to fight viral diseases in fish aquaculture.

#### Author Contributions

**Esther García-Rosado, M. Carmen Alonso and Julia Béjar:** conceptualization. **Daniel Álvarez-Torres and Patricia Moreno:** investigation. **Daniel Álvarez-Torres and Julia Béjar:** writing, original draft preparation. **Patricia Moreno, Esther García-Rosado and M. Carmen Alonso:** writing, review and editing. **Esther García-Rosado, M. Carmen Alonso and Julia Béjar:** supervision. **Esther García-Rosado, M. Carmen Alonso and Julia Béjar:** funding acquisition. All authors have read and agreed to the published version of the manuscript.

#### Conflicts of Interest

The authors declare no conflicts of interest.

#### Data Availability Statement

The data that support the findings of this study are available within the article and its Figures S1 and S2, Table S1.

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### Supporting Information

Additional supporting information can be found online in the Supporting Information section.