Elsevier Editorial System(tm) for Fish and Shellfish Immunology

Manuscript Draft

Manuscript Number: FSIM-D-18-00106R1

Title: Immuno-related gene transcription and antibody response in nodavirus (RGNNV and SJNNV)-infected European sea bass (Dicentrarchus labrax L.)

Article Type: Full Length Article

Keywords: RGNNV, SJNNV, sea bass, Dicentrarchus labrax, IFN I system, anti-viral immune system, antibody response

Corresponding Author: Professor M. Carmen Alonso,

Corresponding Author's Institution: University of Malaga

First Author: Patricia Moreno

Order of Authors: Patricia Moreno; Benjamin Lopez-Jimena; Elisa Randelli; Giuseppe Scapigliati; Francesco Buonocore; Esther Garcia-Rosado; Juan J Borrego; M. Carmen Alonso, Ph.D.

Abstract: 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-□□ 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-□□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.

cover letter

Dear editor,

The manuscript entitle “immuno-related gene transcription and antibody response in nodavirus (RGNNV and SJNNV)-infected European sea bass (*Dicentrarchus labrax* L.)” (MS no. FSIM-D-18-00106) has been revised according to the referee suggestions. The revised MS shows the changes, which are highlighted in yellow. All the suggestions have been assumed.

Sincerely yours,

M. Carmen Alonso
Department of Microbiology
Faculty of Sciences
University of Malaga (Spain)

Answers to the specific comments (in red):

Reviewer #1:

COMMENT-1) Authors said that they performed a challenge with betanodavirus, ma no data of cumulative mortality are reported. In my opinion, this is a very important information that is needed

This work is part of an integrated study focused on the comparison between RGNNV and SJNNV infection in juvenile seabass. This comprehensive study has yielded a great amount of data in collaboration with two different research groups. For this reason, they are going to be published in two different articles. The first one, comprising the results about viral pathogenesis (viral quantification in dead and surviving fish and mortality rate), has already been published (Souto et al. 2015), whereas the present manuscript addresses the host immune response to those infections. Therefore, the present study complements the previous one, and both of them yield a more complete knowledge on the differences between RGNNV and SJNNV pathogenesis in seabass.
Nevertheless, following referee suggestion, a section describing the clinical signs and mortality, along with the reference in which these data have been published, have been added in 214-219 paragraph. In addition, the material and method section (lines 127-131), has also be modified to include the fish tank to record mortality.

COMMENT-2) in M&M, authors said that they used 50 fish per group, then they explain the sampling points and number of animals sampled, but if you sum the numbers there are less fish than those used, so where are the others? Since there is no data on mortality, I cannot assume that they are dead. Moreover, how authors could predict that they will have at least 15 fish per group at the end of the challenge?

Samples were: 5 fish per time (4 sampling times), that makes a total of 20 specimens, plus 15 specimens to obtain serum at 30 days p.i. Total: 35 specimens. Although there was some mortalities (cumulative mortality at 30 days was 46%), we have enough number of fish to conduct our analyses. In addition, sera for antibody analyses were obtained from surviving fish from both replicate tanks (see material and methods lines 127-131).

COMMENT-3) Authors said that they used fish of 5 g average weight, then they collected blood from 15 fish per group and pooled them into 3 groups of 5 sample each. Why they pooled the blood, since the quantity you can obtain from such fish size will be enough to perform the analyses? Doing this, they obtained just 3 samples per group, that is very few to be strong statistically supported.

Although, on form, blood extracted from 5-g fish should be enough to obtain serum for ELISA analyses, we did not succeed (especially considering the volume reduction after clotting and centrifugation). For this reason, we had to pool samples from different animals.

In addition, on the basis of previous works on nodavirus immunization and ELISA assays, considering the low amount of sera from single fish (ca. 20 ul) and the dilution to be employed, for a great part of individuals the serum is not enough to make replicates.

Although increasing the number of biological samples analyzed would have given us more information about significant differences between samples, as referee suggests, we believe that the differences between RGNNV- and SJNNV-inoculated fish are big enough to be considered reliable. In addition of analyzing three samples, each of them has been analyzed in triplicate.

COMMENT-4) Please explain which kind of statistical analyses you performed to obtain the sample size of 50 animals per group (test, power, etc.)

We always intend to use the minimum amount of animals able to give us the maximum information taking account both, ethical issues, and the capacity of our installation (in order to avoid stress by overcrowding).

COMMENT-5) lines 148-150: authors said that they analyzed viral REPLICATION in brain using a real-time PCR targeting the RNA2. In betanodavirus, replication is leaded by RNA1 segment, since it is the one coding for polymerases. So, why authors decided to use a PCR that target the RNA2 instead of one targeting the RNA1, if their aim was to QUANTIFY the viral replication?

When we designed the experiments we planned to quantify the RNA2 segment assuming that an increase in the number of copies of this viral segment would be indicating viral replication (as it has been previously considered in other articles), even when this replication would be driven by the RNA polymerase encoded by RNA1. Nevertheless, since this idea seems to be a

bit confusing, the term “viral replication” has been replaced by “viral quantification” or “increase in RNA2 copy number” throughout the MS, according to the referee suggestion.

COMMENT-6) As above, in Results, paragraph 3.1, authors said that they quantified viral replication and they reported RNA2 quantity. In this case, they quantified the viral PRESENCE, and not the REPLICATION.

The suggested change has been performed.

COMMENT-7) As already said, the first paragraph of the Results should be the challenge results, so data and analyses on mortality are necessary.

A new section describing challenge results has been added in “Results” (paragraph 214-219). The cumulative mortality curve cannot be added to this MS, since, as I have previously explained, it has been already published. The reference of this previous publication appears in this new section.

COMMENT-8) in Fig.1 and Fig.2 there are no data of Controls group. I think that they are necessary

Data of control group have been added.

COMMENT-9) Discussion: at lines 297-300 authors said that SJNNV is less immunogenic than RGNNV. At lines 371, they said that SJNNV is more antigenic than RGNNV. This is very interesting, also if you consider that the previous study by Pascoli et al. you cited in the manuscript reported a lower seroconversion in fish vaccinated with inactivated SJNNV respect to those vaccinated with inactivated RGNNV. How can authors explain these findings? I think they need a deeper explanation in discussion.

A paragraph to discuss the results of seroconversion after SJNNV and RGNNV inoculation has been added in the MS (lines 402-413).

Reviewer #2:

COMMENT-1. Be consequent about the way RNA2 is written (with or without space in between).

The manuscript has been carefully revised and RNA2 has always been written without space in between.

COMMENT-2. Address the situation about the reassortment of RGNNV/SJNNV and its implication on pathogenicity and immunity in the introduction and discussion.

Information about RGNNV/SJNNV reassortants has been included in lines 49-56.

COMMENT-3. Mat and methods: Difficult to understand the method used for collecting serum where blood is incubated at 37C for 10 minutes before overnight incubation at 4C and centrifugation twice. Why incubate at such high temperature? Does it affect the quality of downstream analysis?

The protocol we follow to obtain serum was developed to be applied for mammalian blood, which explains the previous incubation at 37 C for improving clotting. We modify this protocol by eliminating this step (as it was described by Lopez-Jimena et al. 2012). This incubation was included in the MS by mistake, it has been now deleted.

COMMENT-4. Lines 188-189: it is meant to be - RGNNV-, SJNNV-infected or non-infected (right?) please correct.

Referee is right, this mistake has been corrected.

COMMENT-5. Line 191: please change double-diluted to two-fold dilution 6. Line 200: Move "considered" from end of sentence such that it is: negative controls was considered as the cut-off threshold 7. Results: Viral replication- The figure 1 should be 1 figure including both SJNNV and RGNNV in one graph. Moreover, statistics between the virus amounts for both viruses at each time point should be carried out for better comparison, and include the new statistics comparison in results section.

All these suggestions have been assumed. In addition the description of these results has also been changed, according with the new graph (lines 221-228).

The new statistical test performed has been described in lines 166-168. Figure caption has also been changed (line 655).

COMMENT-6. Section 3.3: In general, mention the down regulation of both TNFa and IL-10 in both brain and head kidney up to 12 hrs before upregulation. Lines 250-254 - the results do not match the figure and the figure section cited is also wrong. Also, since the ratio from higher for SJNNV at 24 hrs to higher for RGNNV at 72 hrs should be mentioned.

The description of transcription of inflammatory genes (section 3.4) has been modified in order to make it less confusing.

The down-regulation of TNF-alpha and IL-10 genes up to 12 h p.i. has been mentioned in line 262.

COMMENT-7. Line 282: SJNNV, non-pathogenic.... is it completely true? may be rewrite to less pathogenic will be better. The results in this study show that there is replication for both viruses, and the differences are not really huge.

SJNNV is now referred as to be “less virulent” to sea bass, meaning that this genotype cause less mortality than RGNNV

COMMENT-8. line 319: The study which the results here are compared to had used similar dose in fish almost 3 times in size. Can the dose/weight of fish have any influence? This should be discussed.

Fish age is an important variable to take into account regarding sea bass susceptibility to RGNNV. This idea has been added in lines 349-352.

COMMENT-9. Line 356: Remove the word "fact" at the end of the sentence. previous results "suggests" role of inflammatory...and thus it does not become a fact.

This phrase has been changed

COMMENT-10. Line 412: correct the spelling for antibody. Done.

Figure 5: Please add the which symbols represent RGNNV. SJNNV and negative control in the figure for better readability

Figure 5 has been changed.

Immuno-related gene transcription and antibody response in nodavirus (RGNNV and SJNNV)-infected European sea bass (*Dicentrarchus labrax* L.)

Patricia Moreno[1], Benjamin Lopez-Jimena[2], Elisa Randelli[3], Giuseppe Scapigliati[3], Francesco Buonocore[3], Esther Garcia-Rosado[1], Juan J Borrego[1], M Carmen Alonso[1*]

[1]*Universidad de Málaga, Departamento de Microbiología, Facultad de Ciencias, 29071 Málaga, Spain*

[2]*Mast Group Ltd., Mast House, Derby Road, Bootle, Merseyside, L20 1EA, England, United Kingdom*

[3]*Department for Innovation in Biological, Agrofood and Forest Systems, University of Tuscia, Viterbo, Italy*

Running title: European sea bass immune system modulation after NNV infections

[*]Correspondence:
M. Carmen Alonso, Department of Microbiology, Faculty of Sciences, University of Malaga, Campus Teatinos, 29071-Malaga, Spain.
Phone: 34-952-137588
Fax: 34-952-136645
E-mail: mdalonso@uma.es

Keywords: RGNNV, SJNNV, sea bass, *Dicentrarchus labrax*, IFN I system, anti-viral immune system, antibody response

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 recognized 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 [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 ^{o}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 ^{o}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 ^{o}C for clotting. Supernatants were then centrifuged twice at 400 x *g* for 15 min, and the resulting sera were stored at -20 ^{o}C until used. A total of 3 samples, each one composed of sera from 5 animals, were analyzed in each experimental group.

141

142 *2.3. Sample processing*

143 Brains and head kidneys were individually homogenized (10% w/v) in QIAzol Lysis
144 Reagent (Qiagen) using the Lysis Matrix tubes designed for the Fast-Prep24 (MP
145 Biomedical) system. Total RNA was extracted with the RNeasy Lipid Tissue Mini Kit
146 (Qiagen) following commercial guidelines. The resulting RNA was analyzed by agarose
147 gel electrophoresis, quantified with the NanoDrop-1000 system (ND-1000,
148 ThermoFisher), and stored at -80 ^{o}C until used. The cDNA synthesis was performed
149 with the iScript cDNA synthesis Kit (BioRad), mixing 1 µg of RNA, 4 µL of 5x iScript
150 Reaction Mix, and 1 µL of iScript Reverse Transcriptase in a final volume of 20 µL.
151 Mixtures were incubated at 25 ^{o}C for 5 min, followed by 30 min at 46 ^{o}C and 5 min at
152 85 ^{o}C for transcriptase inactivation. The resulting cDNA was stored at -20 ^{o}C.

153

154 *2.4. Viral genome quantification*

155 RGNNV and SJNNV RNA2 genomic segments have been quantified by two specific
156 absolute real-time PCR protocols [33, 34]. Serial dilutions of pCR™4-TOPO® TA
157 vector (Invitrogen) containing the complete RGNNV- or SJNNV-RNA2 segment were
158 used as reference standard curves. All the reactions were conducted using the
159 LightCycler 96 Termocycler (Roche) and the Fast Start Essential DNA Green Master
160 Mix (Roche) using SYBR Green technology. PCRs were carried out in 20-µL mixtures
161 containing cDNA generated from 50 ng of RNA, 10 µL of Fast Start Essential DNA
162 Green Master 2x, 1 µL of each primer (10 pmol) and 6 µL of water. Termocycling
163 conditions were: initial denaturation at 95 ^{o}C for 10 min, followed by 45 amplification
164 cycles of 95 ^{o}C for 10 s, 60 ^{o}C for 10 s and 72 ^{o}C for 10 s. To obtain melting curves, the
165 following profile was conducted: 95 ^{o}C for 10 s, 65 ^{o}C for 60 s and 97 ^{o}C for 1 s.

166 Statistically significant differences were analyzed by the two-way ANOVA test and
167 Bonferroni´s test as a post hoc, using the GraphPad Prism 5.0 software. Values with $p <$
168 0.05 were considered significant.
169
170 *2.5. Immunogene transcription analyses*
171 The transcription of MxA, ISG-12, ISG-15, IFN-I MHCII-β, IL-8, IL-10, TGF-β,
172 TNF-α and TR-γ was quantified in brain and head kidney by relative real-time PCR
173 protocols using the Mx3000P thermocycler (Stratagene), in combination with Brilliant
174 II SYBR Green qPCR Master Mix (Agilent Technologies), following manufacturer´s
175 guidelines. Rox was included as a passive reference dye to compensate for non-PCR
176 related variations in fluorescence. Mixtures were prepared with cDNA generated from
177 10 ng of RNA, 12.5 µL of 2x Brilliant II SYBR Green qPCR Master Mix
178 complemented with 0.375 µL of Rox (0.002 mM), and 1 µL of each primer (10 pmol) in
179 a final volume of 20 µL. The amplification profile was as follows: 95 ^{o}C for 10 min,
180 followed by 35 cycles of 95 ^{o}C for 45 s, 52 ^{o}C for 45 s and 72 ^{o}C for 45 s. In addition,
181 melting curves were obtained following this profile: 95 ^{o}C for 1 min, 52 ^{o}C for 30 s, and
182 a final step at 95 ^{o}C for 30 s. Reactions were performed with five biological samples in
183 triplicate.
184 The annealing temperature of all the primers used was 52 ^{o}C, and the amplification
185 product size ranged between 75 and 240 bp (Table 1). Relative quantification was
186 performed comparing with the transcription level of the target gene in control sample
187 (L15-injected fish) at 3 h p.i. Ribosomal 18S RNA was the reference endogenous gene
188 used [18], and relative fold change values (Pfaffl method) were calculated with the
189 MxPro software. Results were expressed as mean relative fold change values ± standard
190 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 ^{o}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 (1/4,000 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].

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, 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 sea bass, 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 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 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.

414 The importance of the acquired response is not only at a systemic level, but also at a
415 local level, since TR-γ transcription is significantly higher in brain than in head kidney
416 of infected fish (especially in the RGNNV group) (Fig. 4 B, D). In addition, the time
417 course of the induction of this gene in both tissues is different. The immune response to
418 NNV in brain is characterized by an early TR-γ response (Fig. 4 B), which is a T-cell-
419 related receptor, suggesting an important role of the acquired immunity in controlling
420 viral replication in the target organ, as it has been previously reported in brain of
421 experimentally-challenged sea bass and Atlantic halibut [54, 55]. On the contrary, in
422 head kidney no increase of TR-γ transcription at 3, 12 and 24 h p.i. was recorded, which
423 may indicate either a quick migration of T-cells to the brain as target organ or down-
424 regulation of this gene transcription. This result is surprising, since this gene has been
425 classically considered to be important mainly in mucosal tissues, such as gills or
426 intestine, and kidney and brain are the organs showing the lowest basal levels of gene
427 expression [55]. Regarding MHCII-β (Fig. 4 A, C), the lack of transcription of this gene
428 recorded at the beginning of the infection in both tissues has also been described for
429 different experimental systems, including rainbow trout infected with Infectious
430 Pancreatic Necrosis Virus (IPNV) [28]. Our results support the putative role of the local
431 adaptive immune response suggested by an important up-regulation of the gene coding
432 the IgM light chain following RGNNV challenge in sea bass [18].
433 This study has shown that RGNNV and SJNNV stimulated a complete immune
434 response, involving the induction of numerous genes related with both, innate and
435 adaptive responses. These responses have been recorded both at a local (brain) and
436 systemic (head kidney) level, with SJNNV inducing an early immune gene transcription
437 in kidney. Thus, the quicker systemic response induced by SJNNV, together with a

438 higher production of antibodies, may be partly responsible for the resistance of sea bass
439 to this genotype.
440 The present work complements and extends previous studies, and poses questions to
441 be considered in future investigations, such as, for instance, that both, RGNNV and
442 SJNNV, stimulated an immune response that includes the induction of innate- and
443 adaptive response-related genes as well as a humoral antibody response.
444
445 **Acknowledgments**
446 This study has been partly supported by a research contract signed with Novartis
447 Animal Health Inc. (Canada), the project AGL2014-54532-C2-1-R from Ministerio de
448 Economía y Competitividad (Spanish Government), and the project AGL2017-84644-R
449 (MINECO/AEI/FEDER, UE). P. Moreno was supported by a fellowship from
450 Ministerio de Educación, Cultura y Deporte (FPU12/00265, Spanish Government).

References

[1] T. Nishizawa, M. Furuhashi, T. Nagai, T. Nakai, K. Muroga, Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene, Appl. Environ. Microbiol. 63 (1997) 1633-1636.

[2] G.N. Frerichs, H.D. Rodger, Z. Peric, Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*, J. Gen. Virol. 77 (1996) 2067-2071.

[3] A. Le Breton, L. Grisez, J. Sweetman, F. Ollevier, Viral nervous necrosis (VNN) associated with mass mortalities in cage-reared seabass, *Dicentrarchus labrax* (L.), J. Fish Dis. 20 (1997) 145-151.

[4] J.M. Cutrin, C.P. Dopazo, R. Thiery, P. Leao, J.G. Olveira, J.L. Barja, I. Bandin, Emergence of pathogenic betanodaviruses belonging to the SJNNV genogroup in farmed fish species from the Iberian Peninsula, J. Fish Dis. 30 (2007) 225-232.

[5] B. Lopez-Jimena, N. Cherif, E. Garcia-Rosado, C. Infante, I. Cano, D. Castro, S. Hammami, J.J. Borrego, M.C. Alonso, A combined RT-PCR and dot-blot hybridization method reveals the coexistence of SJNNV and RGNNV betanodavirus genotypes in wild meagre (*Argyrosomus regius*), J. Appl. Microbiol. 109 (2010) 1361-1369.

[6] V. Panzarin, A. Fusaro, I. Monne, E. Cappelloza, G. Bovo, I. Capua, E.C. Holmes, G. Cattoli, Molecular epidemiology and evolutionaty dynamics of betanodavirus in Southern Europe, Gennet. Evol. 12 (2012) 63-70.

[7] N. Vendramin, A. Toffan, M. Mancin, E. Cappellozza, V. Panzarin, G. Bovo, G. Cattoli, I. Capua, C. Terregino, Comparative pathogenicity study of ten different

475 betanodavirus strains in experimentally infected European sea bass, *Dicentrarchus*
476 *labrax* (L.), J. Fish Dis. 37 (2014) 371-383.
477 [8] S. Souto, B. Lopez-Jimena, M.C. Alonso, E. Garcia-Rosado, I. Bandin,
478 Experimental susceptibility of European sea bass and Senegalese sole to different
479 betanodavirus isolates, Vet. Microbiol. 177 (2015) 53-61.
480 [9] C. Carballo, E. Garcia-Rosado, J.J. Borrego, M.C. Alonso, SJNNV down-regulates
481 RGNNV replication in European sea bass by the induction of the type I interferon
482 system, Vet. Res. 47 (2016) 6.
483 [10] A.J.A. McBeath, Y.M. Ho, M. Aamelfot, M. Hall, D.H. Christiansen, T.
484 Markussen, K. Falk, I. Matejusova, Low virulent infectious salmon anaemia virus
485 (ISAV) replicates and initiates the immune response earlier than a highly virulent
486 virus in Atlantic salmon gills, Vet. Res. 45 (2014) 83.
487 [11] K. Julin, L.-H. Johansen, A.-I. Sommer, J.B. Jorgensen, Persistent infections with
488 Infectious Pancreatic Necrosis Virus (IPNV) of different virulence in Atlantic
489 salmon *Salmo salar* L., J. Fish Dis. 38 (2015) 1005-1019.
490 [12] I. Cano, B. Collet, C. Pereira, R. Paley, R. Van Aerle, D. Stone, N.G.H. Taylor, *In*
491 *vivo* virulence of Viral Haemorrhagic Septicaemia Virus (VHSV) in rainbow trout
492 *Oncorhynchus mykiss* correlates inversely with *in vitro* Mx gene expression, Vet.
493 Microbiol. 187 (2016) 31-40.
494 [13] D. Alvarez-Torres, A.M. Podadera, J. Bejar, I. Bandin, M.C. Alonso, E. Garcia-
495 Rosado, Role of the IFN I system against the VHSV infection in juvenile
496 Senegalese sole (*Solea senegalensis*), Vet. Res. 47 (2016) 3.
497 [14] J. Liu, X. Huang, Y. Yu, J. Zhang, S. Ni, Y. Hu, Y. Huang, Q. Qin, Fish DDX3X
498 exerts antiviral function against grouper nervous necrosis virus infection, Fish
499 Shellfish Immunol. 71 (2017) 95-104.

[15] M.H. Jung, S.J. Jung, Innate immune responses against rock bream iridovirus (RBIV) infection in rock bream (*Oplegnathus fasciatus*) following poly (I:C) administration, Fish Shellfish Immunol. 71 (2017) 171-176.

[16] S. Avunje, S. Jung, Poly (I:C) and imiquimod induced immune responses and their effects on the survival of olive flounder (*Paralichthys olivaceus*) from viral haemorrhagic septicaemia, Fish Shellfish Immunol. 71 (2017) 338-345.

[17] L. Poisa-Beiro, S. Dios, A. Montes, R. Aranguren, A. Figueras, B. Novoa, Nodavirus increases the expression of Mx and inflammatory cytokines in fish brain, Mol. Immunol. 45 (2008) 218-225.

[18] G. Scapigliati, F. Buonocore, E. Randelli, D. Casani, S. Meloni, G. Zarletti, M. Tiberi, D. Pietretti, I. Boschi et al., Cellular and molecular immune responses of the sea bass (*Dicentrarchus labrax*) experimentally infected with betanodavirus, Fish Shellfish Immunol. 28 (2010) 303-311.

[19] E. Chaves-Pozo, F.A. Guardiola, J. Meseguer, M.A. Esteban, A. Cuesta, Nodavirus infection induces a great innate cell-mediated cytotoxic activity in resistant, gilthead seabream, and susceptible, European sea bass, teleost fish, Fish Shellfish Immunol. 33 (2012) 1159-1166.

[20] P. Novel, M.A. Fernandez-Trujillo, J.B. Gallardo-Galvez, I. Cano, M. Manchado, F. Buonocore, E. Randelli, G. Scapigliati, M.C. Alvarez, J. Bejar, Two Mx genes identified in European sea bass (*Dicentrarchus labrax*) respond differently to VNNV infection, Vet. Immunol. Immunopathol. 153 (2013) 240-248.

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

[22] A. Montes, A. Figueras, B. Novoa, Nodavirus encephalopathy in turbot (*Scophthalmus maximus*): inflammation, nitric oxide production and effect of anti-inflammatory compounds, Fish Shellfish Immunol. 28 (2010) 281-288.

[23] G.P. Skliris, R.H. Richards, Induction of nodavirus disease in seabass, *Dicentrarchus labrax*, using different infection models, Virus Res. 63 (1999) 85-93.

[24] S. Grove, R. Johansen, L.J. Reitan, C.M. Press, B.H. Dannevig, Quantitative investigation of antigen and immune response in nervous and lymphoid tissues of Atlantic halibut (*Hippoglossus hippoglossus*) challenged with nodavirus, Fish Shellfish Immunol. 21 (2006) 525-539.

[25] B. Lopez-Jimena, E. Garcia-Rosado, K.D. Thompson, A. Adams, C. Infante, J.J. Borrego, M.C. Alonso, Distribution of Red Spotted Grouper Nervous Necrosis Virus (RGNNV) antigens in nervous and non-nervous organs of European seabass (*Dicentrarchus labrax*) during the course of an experimental challenge, J. Vet. Sci. 13 (2012) 355-362.

[26] F. Pascoli, A. Guazzo, A. Buratin, M. Toson, F. Buonocore, G. Scapigliati, A. Toffan, Lack of *in vivo* cross-protection of two different betanodavirus species RGNNV and SJNNV in European sea bass *Dicentrachus labrax*, Fish Shellfish Immunol. *In press*, doi: 10.1016/j.fsi.2017.10.033.

[27] M.D. Mannie, T cell-mediated antigen presentation: a potential mechanism of infectious tolerance, Immunol. Res. 23 (2001) 1-21.

[28] D.A. Chistiakov, B. Hellemans, F.A.M. Volckaert, Review on the immunology of European sea bass *Dicentrarchus labrax,* Vet. Immunol. Immunopathol. 117 (2007) 1-16.

548 [29] H. Koizumi, C.C. Liu, L.M. Zheng, S.V. Joag, N.K. Byne et al., Expression of
549 perforin and serine esterases bay human γ/δ T cells, J. Ex. Med. 173 (1991) 499-
550 502.
551 [30] W. Holtmeier, D. Kabelitz, gammadelta T cells link innate and adaptative immune
552 responses, Chem. Immunol. Allergy, 86 (2005) 151-183.
553 [31] T. Iwamoto, T. Nakai, K. Mori, M. Arimoto, I. Furusawa, Cloning of the fish cell
554 line SSN-1 for piscine nodaviruses, Dis. Aquat. Org. 43 (2000) 81-89.
555 [32] L.J. Reed, H. Muench, A simple method of estimating 50 per cent end-points,
556 Am. J. Hyg. 27 (1938) 493-497.
557 [33] B. Lopez-Jimena, M.C. Alonso, K.D. Thompson, A. Adams, C. Infante, J.J.
558 Borrego, E. Garcia-Rosado, Tissue distribution of Red Spotted Grouper Nervous
559 Necrosis Virus (RGNNV) genome in experimentally infected juvenile European
560 seabass (*Dicentrarchus labrax*), Vet. Microbiol. 154 (2011) 86-95.
561 [34] B. Lopez-Jimena, E. Garcia-Rosado, C. Infante, D. Castro, J.J. Castro, M.C.
562 Alonso, Effect of the coexistence on the replication of Striped Jack Nervous
563 Necrosis Virus (SJNNV) and Red-spotted Grouper Nervous Necrosis Virus
564 (RGNNV) using an *in vitro* approach, J. Appl. Ichthyol. 30 (2014) 912-922.
565 [35] J. Castric, R. Thiery, J. Jeffroy, P. De Kinkelin, J.C. Raymond, Sea bream *Sparus*
566 *aurata*, an asymptomatic contagious fish host for nodavirus, Dis. Aquat. Organ.
567 47 (2001) 33-38.
568 [36] S. Grove, R. Johansen, B.H. Dannevig, L.J. Reitan, T. Ranheim, Experimental
569 infection of Atlantic halibut *Hippoglossus hippoglossus* with nodavirus: tissue
570 distribution and immune response, Dis. Aquat. Organ. 53 (2003) 211-221.
571 [37] K. Korsnes, E. Karlsbakk, M. Devold, A.H. Nerland, A. Nylund, Tissue tropism
572 of Nervous Necrosis Virus (NNV) in Atlantic cod, *Gadus morhua* L., after

intraperitoneal challenge with a virus isolate from diseased Atlantic halibut, *Hippoglossus hippoglossus* (L.), J. Fish Dis. 32 (2009) 655-665.

[38] M.M. Penaranda, M.K. Purcell, G. Kurath, Differential virulence mechanisms of infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) include host entry and virus replication kinetics, J. Gen. Virol. 90 (2009) 2172-2182.

[39] M.K. Purcell, K.A. Garver, C. Conway, D.G. Elliott, G. Kurath, Infectious hematopoietic necrosis virus genogroup-specific virulence mechanisms in sockeye salmon *Oncorhynchus nerka* (Walbaum), from Redfish Lake, Isaho, J. Fish Dis. 32 (2009) 619-631.

[40] J.P. Levraud, P. Boudinot, I. Colin, A. Benmansour, N. Peyrieras, P. Herbomel, G. Lutfalla, Identification of the zebrafish IFN receptor: implications for the origin of the vertebrate IFN system, J. Immunol. 178 (2007) 4385-4394.

[41] A. Lopez-Muñoz, F.J. Roca, M.P. Sepulcre, J. Meseguer, V. Mulero, Zebrafish larvae are unable to mount a protective antiviral response against waterborn infection by spring viremia of carp virus, Dev. Comp. Immunol. 34 (2010) 546-552.

[42] P. Domingues, C.G. Bamford, C. Boutell, J. McLauchlan, Inhibition of hepatitis C virus RNA replication by ISG15 does not require its conjugation to protein substrates by the HERC5 E3 ligase, J. Gen. Virol. 96 (2015) 3236-3242.

[43] Y.M. Chen, Y.L. Su, P.S. Shie, S.L. Huang, H.L. Yang, T.Y. Che, Grouper Mx confers resistance to nodavirus and interacts with coat protein, Dev. Comp. Immunol. 32 (2008) 825-836.

[44] S. Grove, R. Johansen, B.H. Dannevig, L.J. Reitan, T. Ranheim, Experimental infection of Atlantic halibut *Hippoglossus hippoglossus* with nodavirus: tissue distribution and immune response, Dis. Aquat. Organ. 53 (2003) 211-221.

[45] D. Bogunovic, S. Boisson-Dupuis, J.-L. Casanova, ISG15: leading a double life as a secreted molecule, Exp. Mol. Med. 45 (2013) e18.

[46] M.-Y. Lu, F. Liao, Interferon-stimulated gene *ISG12b2* is localized to the inner mitochondrial membrane and mediates virus-induced cell death, Cell Death Differ. 18 (2011) 925-936.

[47] L. Labrada, X.H. Liang, W. Zheng, C. Johnston, B. Levine, Age-dependent resistance to lethal Alphavirus Encephalitis in mice: analysis of gene expression in the central nervous system and identification of a novel interferon-inducible protective gene, mouse *ISG12*, J. Virol. 76 (2002) 11688-11703.

[48] L. Ji, X. Zhou, W. Liang, J. Liu, B. Liu, Porcine interferon stimulated gene 12a restricts Porcine Reproductive and Respiratory Syndrome Virus replication in MARC-14S cells, Int. J. Mol. Sci. 18 (2017) 1613.

[49] A. Pallavicini, E. Randeli, M. Modonut, D. Casani, G. Scapigliati, F. Buonocore, Searching for immunomodulatory sequences in sea bass (*Dicentrarchus labrax* L.): transcripts analysis from thymus, Fish Shellfish Immunol. 29 (2010) 571-578.

[50] L. Poisa-Beiro, S. Dios, H. Ahmed, G.R. Vasta, A. Martinez-Lopez, A. Estepa, J. Alonso-Gutierrez, A. Figueras, B. Novoa, Nodavirus infection of sea bass (*Dicentrarchus labrax*) induces up-regulation of galectin-1 expression with potential anti-inflammatory activity, J. Immunol. 15 (2009) 6600-6611.

[51] Y. Huang, X. Huang, Y. Yang, W. Wang, Y. Yu, Q. Qin, Involvement of fish signal transducer and activator of transcription 3 (STAT3) in nodavirus infection induced cell death, Fish Shellfish Immunol. 43 (2015) 241-248.

[52] V. Panzarin, A. Toffan, M. Abbadi, A. Buratin, M. Mancin, S. Braaen et al., Molecular basis for antigenic diversity of genus *Betanodavirus*, Plos One, 11 (2016) e0158814.

[53] G. Breuil, B. Romestand, A rapid ELISA method for detecting specific antibody level against nodavirus in the serum of the sea bass, *Dicentrachus labrax* (L.): application to the screening of spawners in a sea bass hatchery, J. Fish Dis. 22 (1999) 45-52.

[54] A. Lopez-Muñoz, M.P. Sepulcre, D. Garcia-Moreno, I. Fuentes, J. Bejar, M. Manchado, M.C. Alvarez, J. Meseguer, V. Mulero, Viral nervous virus persistently replicates in the central nervous system of asymptomatic gilthead seabream and promotes a transient inflammatory response followed by the infiltration of IgM^+ B lymphocytes, Dev. Comp. Immunol. 37 (2012) 429-437.

[55] A.C. Overgard, A.H. Fiksdal, S. Patel, Atlantic halibut experimentally infected with nodavirus shows increased levels of T-cell marker and IFN gamma transcripts, Dev. Comp. Immunol. 37 (2012) 139-150.

[56] F. Buonocore, R. Castro, E. Randelli, M.P. Lefranc, A. Six, H. Kuhl, R. Reinhardt et al., Diversity, molecular characterization and expression of T cell receptor γ in a teleost fish, the sea bass (*Dicentrarchus labrax*, L.), Plos one, 7 (2012) 1-11.

[57] S. Picchietti, L. Abelli, L. Guerra, E. Randelli, F. Proietti-Serafini, M.C. Belardinelli, F. Buonocore, C. Bernini, A.M. Fausto, G. Scapigliati, MHCII-β chain gene expression studies define the regional organization of the thymus in the developing bony fish *Dicentrarchus labrax* (L.), Fish Shellfish Immunol. 42 (2015) 483-493.

[58] M.P. Sepulcre, E. Sarropoulou, G. Kotoulas, J. Meseguer, V. Mulero, *Vibrio anguillarum* evades the immune response of the bony fish sea bass

(*Dicentrarchus labrax* L.) through the inhibition of leukocyte respiratory burst and down-regulation of apoptotic caspases, Mol. Immunol. 44 (2007) 3751-3757.

[59] D.S. Nascimento, P.J. Pereira, M.I. Reis, A. do Vale, J. Zou, M.T. Silva, C.J. Secombes, N.M. dos Santos, Molecular cloning and expression analysis of sea bass (*Dicentrarchus labrax* L.) tumor necrosis factor-alpha (TNF-alpha), Fish Shellfish Immunol. 23 (2007) 701-710.

653 **Figure captions**

654

655 **Fig 1.** Absolute quantification of NNV RNA2 segment in brain from juvenile European
656 sea bass experimentally challenged with RGNNV or SJNNV. Data are mean ± SD
657 obtained from five different samples. Different letters denote significant differences (p
658 < 0.05).

659

660 **Fig 2.** Relative quantification of the indicated IFN I system-involved genes in brain (A,
661 B, C, D) and head kidney (E, F, G, H) from juvenile European sea bass experimentally
662 challenged with RGNNV or SJNNV. Data are mean ± SD obtained from five different
663 samples. Different letters denote significant differences (p < 0.05) between genotypes
664 within each sampling time. Asterisk refers to significant differences (p < 0.05) with
665 control fish.

666

667 **Fig 3.** Relative quantification of the indicated inflammatory response-involved genes in
668 brain (A, B, C, D) and head kidney (E, F, G, H) from juvenile European sea bass
669 experimentally challenged with RGNNV or SJNNV. Data are mean ± SD obtained from
670 five different samples.

671

672 **Fig 4.** Relative quantification of the indicated response-involved genes in brain (A, B)
673 and head kidney (C, D) from juvenile European sea bass experimentally challenged
674 with RGNNV or SJNNV. Data are mean ±SD obtained from five different samples.

675

676 **Fig 5.** Titration of anti-RGNNV (▲) and anti-SJNNV (●) antibodies in sera from
677 experimentally challenged European sea bass at 30 days p.i. White symbols represent

678 antibodies in control groups. (1) and (2) are the thresholds for samples from the
679 SJNNV- and RGNNV-infected fish, respectively. Data represent mean OD values ± SD
680 obtained by the ELISA analysis of 3 samples composed of sera from 5 different fish.
681

Table 1. Primers used in this study

Target gene	Sequence (5´-3´)	Amplicon size (bp)	Reference
rRNA18S	CCAACGAGCTGCTGACC CCGTTACCCGTGGTCC	208	[16]
MxA	ATTCTGAGTTCTTGCTGAAGG CCTCTAGAACTCCACCAGG	113	[18]
TGF-β	GACCTGGGATGGAAGTGG CAGCTGCTCCACCTTGTG	225	[16]
IFN-I	GGCTCTACTGGATACGATGGCT GCGTCCAAAGCATCAGCT	200	[16]
MHCII-β	CAGAGACGGACAGGAAG CAAGATCAGACCCAGGA	240	[55]
IL-8	GTGCTCCTGGCGTTC CTTCACCCAGGGAGC	220	[56]
TR-γ	CTGCTGTGTGTGGCCTCAGAC GTGCTGGACGGAGCAGTGGTA	200	[54]
ISG-12	CCTGGTACAGCTGCTGT AGCTGCTCCTGCTGACT	199	[47]
ISG-15	CGACTCAAAGCCTCTCTGCTACT CGTTTCTGACGAACACCTGGAT	100	[19]
IL-10	ACCCCGTTCGCTTGCCA CATCTGGTGACATCACTC	164	[16]
TNF-α	CGACTGGCGAACAACC GCTGTCCTCCTGAGC	220	[57]
CP_RGNNV	CGTCCGCTGTCCATTGACTA CTGCAGGTGTGCCAGCATT	100	[32]
CP_SJNNV	GACACCACCGCTCCAATTACTAC ACGAAATCCAGTGTAACCGTTGT	75	[33]

Figure 1

Click here to download high resolution image

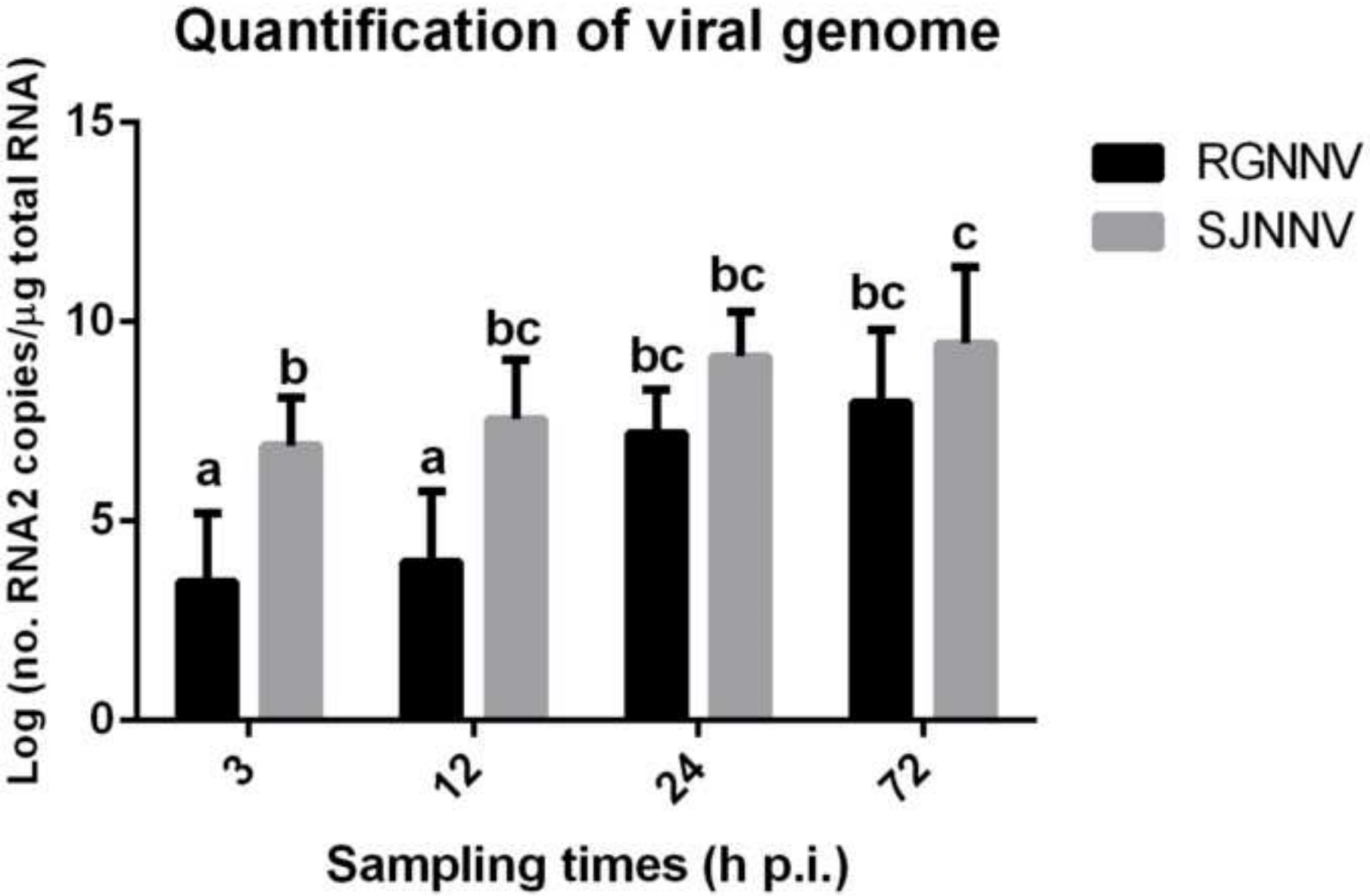


Figure 2

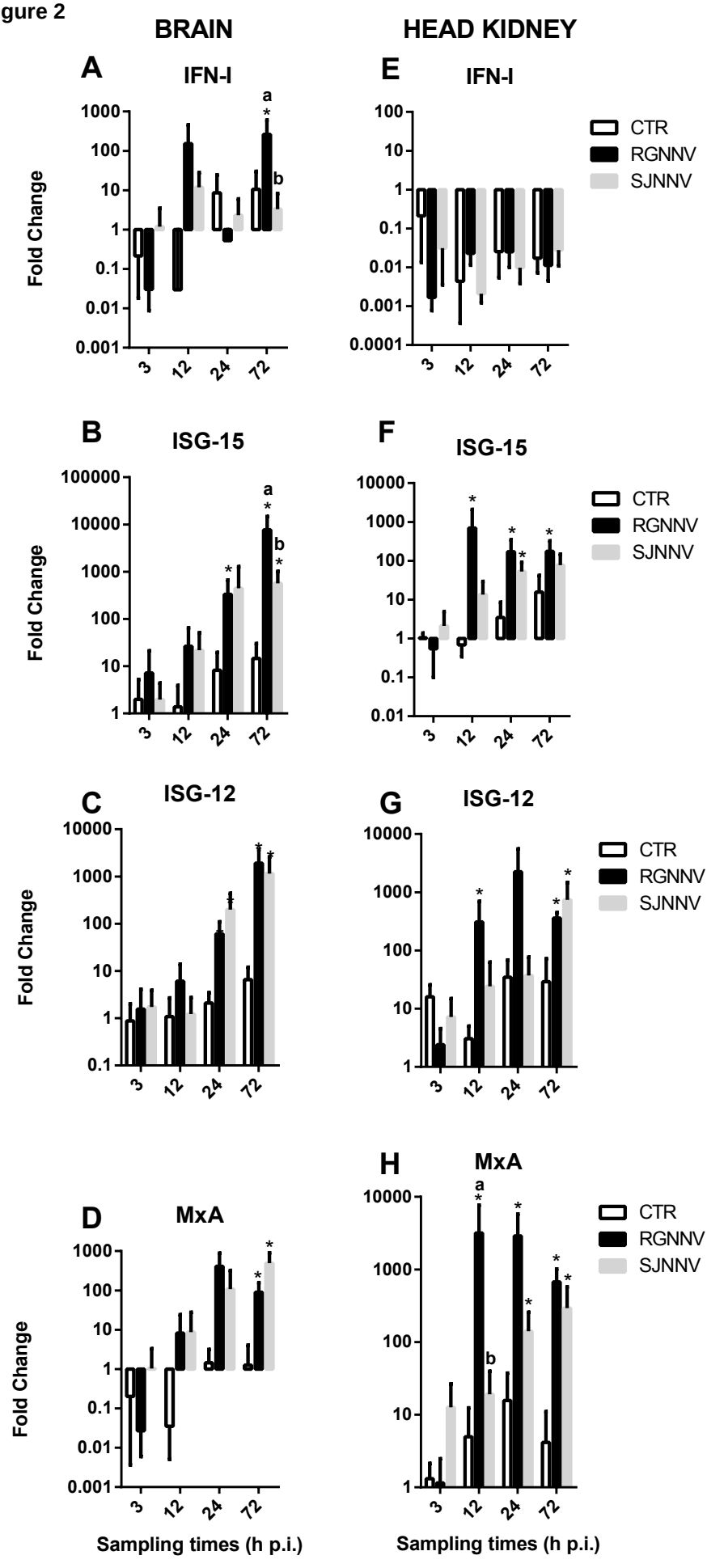

BRAIN
HEAD KIDNEY
A
IFN-I
E
IFN-I
B
ISG-15
F
ISG-15
C
ISG-12
G
ISG-12
D
MxA
H
MxA
CTR
RGNNV
SJNNV
Fold Change
Sampling times (h p.i.)
Sampling times (h p.i.)

Figure 3

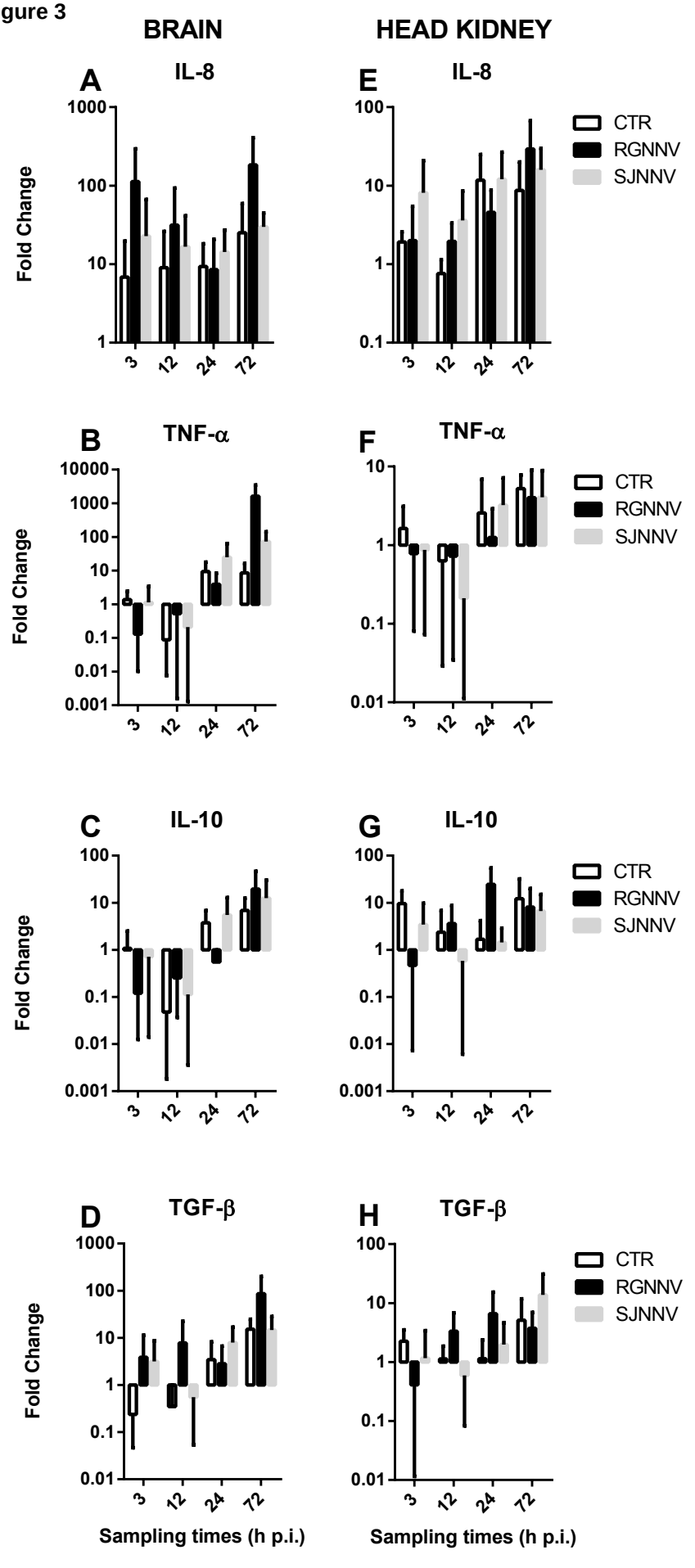

BRAIN
HEAD KIDNEY
A
IL-8
E
IL-8
B
TNF-α
F
TNF-α
C
IL-10
G
IL-10
D
TGF-β
H
TGF-β
CTR
RGNNV
SJNNV
Fold Change
Sampling times (h p.i.)
Sampling times (h p.i.)

Figure 4

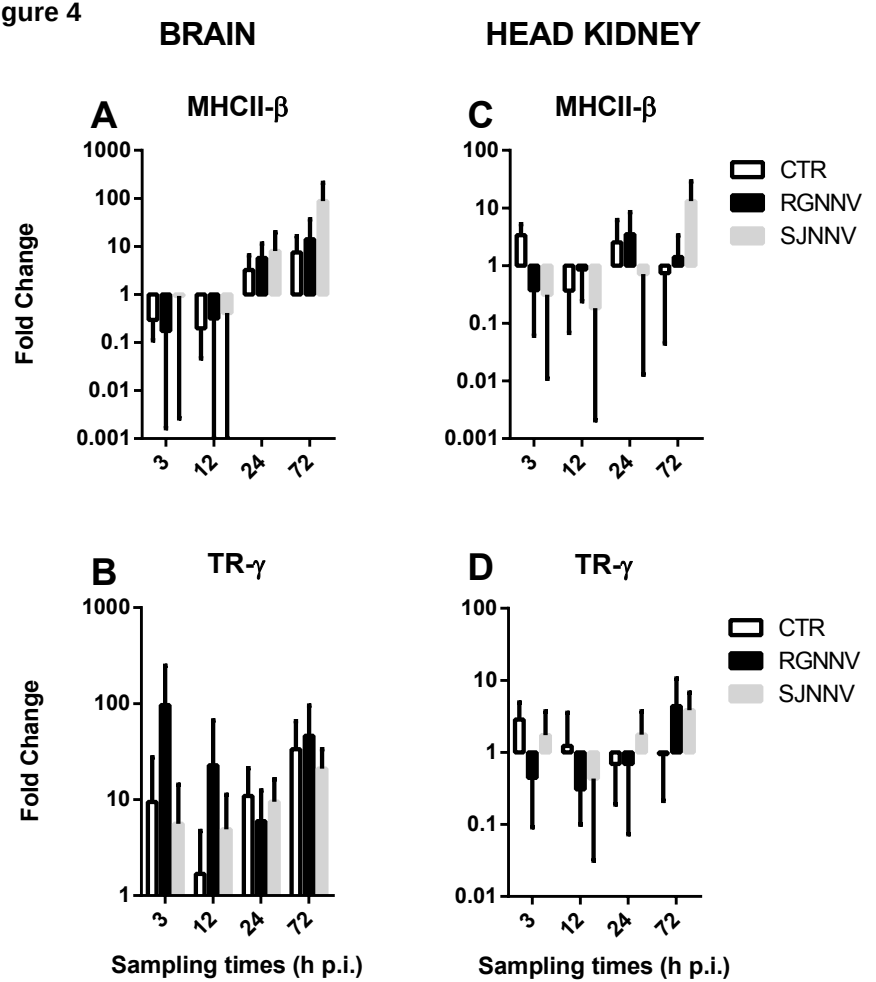
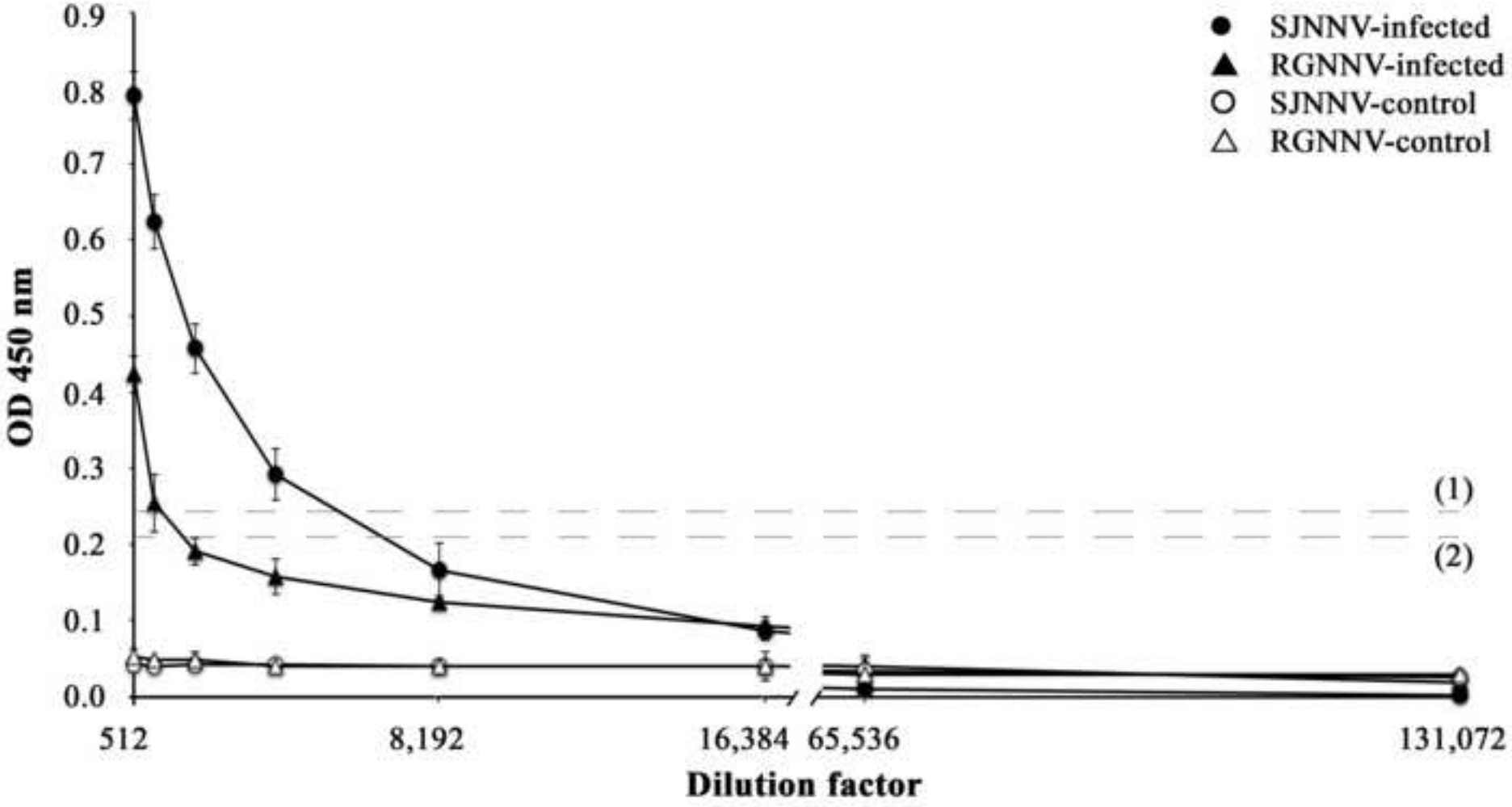

BRAIN
HEAD KIDNEY
A
MHCII-β
C
MHCII-β
Fold Change
1000
100
10
1
0.1
0.01
0.001
3
12
24
72
CTR
RGNNV
SJNNV
B
TR-γ
D
TR-γ
Fold Change
Sampling times (h p.i.)
Sampling times (h p.i.)

Figure 5

Click here to download high resolution image



HIGHLIGHTS

IFN I system response against RGNNV is more intense, especially in kidney

Transcription of inflammatory genes is higher in brain, with RGNNV being a stronger inductor

Antibody response against SJNNV is more intense

The early TR-γ response in brain suggests a key role of acquired immunity in controlling viral replication