Experimental susceptibility of European sea bass and Senegalese sole to different betanodavirus isolates

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Abstract

The susceptibility of juvenile European sea bass and Senegalese sole to three VNNV isolates (a reassortant RGNNV/SJNNV, as well as the parental RGNNV and SJNNV genotypes) has been evaluated by challenges using two inoculation ways (bath and intramuscular injection). The results demonstrate that these two fish species are susceptible to all the VNNV isolates tested. In European sea bass, RGNNV caused the highest cumulative mortality, reaching maximum values of viral RNA and titres. Although the SJNNV isolate did not provoke mortality or clinical signs of disease in this fish species, viral production in survivor fish was determined; on the other hand the reassortant isolate did cause mortality and clinical signs of disease, although less evident than those recorded after RGNNV infection. These results suggest that the changes suffered by the SJNNV RNA2 segment of the reassortant isolate, compared to the parental SJNNV, may have involved host-specificity and/or virulence determinants for European sea bass. Regarding Senegalese sole, although the three isolates caused 100% mortality, the reassortant strain provoked the most acute symptoms, and more quickly, especially in the bath challenge. This was also the isolate showing less difference between the number of RNA copies and viral titre, reaching the highest titres of infective viral particles in nervous tissue of infected animals. The RGNNV isolate produced the lowest values of infective viral particles. All these results suggest that the RGNNV and the reassortant isolates are the most suited for infecting European sea bass and Senegalese sole, respectively.

Keywords: Betanodavirus, genotypes, natural reassortant, experimental infection, European sea bass, Senegalese sole.

1. Introduction

Viral nervous necrosis (VNN), or viral encephalopathy and retinopathy (VER), is a serious emerging disease affecting a wide range of marine farmed and wild fish species (Munday et al., 2002), freshwater fish (Maltese and Bovo, 2007; Bigarré et al., 2009; Vendramin et al., 2012), and invertebrates (Gomez et al., 2006) worldwide.

The etiological agent is the Viral Nervous Necrosis Virus (VNNV, *Betanodavirus* genus, *Nodaviridae* family), which is responsible for high mortalities, particularly in larvae and juveniles, with deleterious economic consequences in the aquaculture industry. Affected fish display lesions in retina, brain and spinal cord. The clinical signs associated to this pathology include abnormal swimming, loss of appetite, changes in pigmentation and hyperinflation of the swim bladder (Maltese and Bovo, 2007). Betanodaviruses are small non-enveloped icosahedral viruses with a genome composed of two single-stranded positive sense RNA molecules (RNA1 and RNA2) (Mori et al., 1992; Frerichs et al., 1996). The RNA1 (3.1 Kb) encodes the RNA-dependent RNA polymerase, and the RNA2 (1.4 Kb) encodes the capsid protein (CP, 42 kDa) (Mori et al., 1992; Comps et al., 1994). It has also been reported a subgenomic transcript originated from the RNA1 segment (RNA3, 0.4 Kb), which encodes the non-structural B1 (11 kDa) and B2 (8.4 kDa) proteins (Tan et al., 2001).

Traditionally, betanodaviruses have been clustered into four genotypes: Barfin Flounder Nervous Necrosis Virus (BFNNV), Redspotted Grouper Nervous Necrosis Virus (RGNNV), Striped Jack Nervous Necrosis Virus (SJNNV) and Tiger Puffer Nervous Necrosis Virus (TPNNV) (Nishizawa et al., 1997). This classification is based on the phylogenetic analysis of a variable region within the RNA2 segment. Further studies have evidenced the importance of analysing both RNA segments for VNNV characterization, since the reassortment between genomic segments seems to be a

74 frequent event. As a matter of fact, reassortant isolates combining genomic segments
75 from the SJNNV and RGNNV genotypes have been obtained from farmed European
76 sea bass (*Dicenthrachus labrax*) and Senegalese sole (*Solea senegalensis*) (Toffolo et
77 al., 2007; Olveira et al., 2009; Panzarin et al., 2012).
78 Although the susceptibility of European sea bass to RGNNV is well-known (Skliris et
79 al., 2001; Toffolo et al., 2007; Panzarin et al., 2012), the knowledge on the
80 susceptibility of this fish species to other genotypes is very poor to date. In fact, to our
81 knowledge, there is only one study analysing the susceptibility of European sea bass to
82 different VNNV genotypes by experimental infection (Vendramin et al., 2014). The
83 studies about the Senegalese sole susceptibility to VNNV are even more scarce, since
84 only a few RGNNV/SJNNV reassortant isolates (composed of RGNNV RNA1 and
85 SJNNV RNA2 segments) have been associated with mortalities in this species so far
86 (Olveira et al., 2009).
87 In the present study, the susceptibility of European sea bass and Senegalese sole to
88 RGNNV, SJNNV, and RGNNV/SJNNV isolates was evaluated. In addition, viral RNA
89 production and viral infectivity were determined in different experimental infections.
90
91 **2. Materials and Methods**
92
93 *2.1. Virus and cell culture*
94
95 Several VNNV isolates were used in this study: (i) ERV378/102-5/04 (RGNNV
96 genotype), kindly provided by Dr G. Bovo (Istituto Zooprofilattico Sperimentale delle
97 Venezie, Italy), and previously used in European sea bass experimental infections
98 (Lopez-Jimena et al., 2011; 2012); (ii) SpSs-IAusc160.03, a RGNNV/SJNNV

reassortant isolate obtained from diseased Senegalese sole (Olveira et al., 2009), and (iii) SJ93Nag, a reference SJNNV strain.

All viral isolates were propagated on E-11 cells (Iwamoto et al., 2000) grown in Leibovitz L-15 (Gibco) medium supplemented with penicillin (Gibco, 100 units/ml), streptomycin (Gibco, 100 mg/ml), and 2% foetal bovine serum (FBS, Lonza). Inoculated cells were incubated at 25 ºC. Viral titration was performed in 96-well plates (Nunc), and expressed as the viral dilution infecting 50% of the cell cultures ($TCID_{50}$), following the methodology described by Reed and Müench (1938).

2.2. Experimental challenges

European sea bass and Senegalese sole were obtained from commercial fish farms and acclimatized for at least 7 days after arrival to the quarantine facilities sited at the IFAPA Centre El Toruño (Cádiz, Spain) (sea bass), and at the University of Santiago de Compostela (Spain) (sole). Fish were maintained at a maximum density of 100 fish in 100 l aquaria with aeration and were fed ad libitum with a commercial diet.

Prior to experimental infections, they were tested for the presence of VNNV, Infectious Pancreatic Necrosis Virus (IPNV), Viral Haemorrhagic Septicaemia Virus (VHSV) and Infectious Haematopoietic Necrosis Virus (IHNV) genome. VNNV detection was performed using a combination of RT-PCR and dot-blot hybridization, according to Lopez-Jimena et al. (2010a), whereas IPNV, VHSV and IHNV were analysed by RT-PCR and nested-PCR following the procedures described by Lopez-Jimena et al. (2010b), Lopez-Vazquez et al. (2006) and Dopazo et al. (2002), respectively. Amplified products were run on 2% agarose gels stained with ethidium bromide, using the 100-bp DNA ladder (Lonza).

124 *2.2.1. Bath challenge*

125 Both, European sea bass and Senegalese sole (2 g, average weight), were split into four
126 groups (100 fish per group): (i) ERV_{bath}/bass-sole, challenged with the RGNNV isolate
127 (ii) $Ss160.03_{bath}$/bass-sole, infected with the reassortant isolate, (iii) $SJ93Nag_{bath}$/bass-
128 sole, challenged with the SJNNV isolate and (iv) control group (negative control).
129 Animals were exposed to each virus (10^5 $TCID_{50}$/ml) in 3 l for 3 h (Senegalese sole) or
130 1 h (European sea bass) and strong aeration was supplied to the water during the
131 challenge. Negative controls were exposed to L-15 containing no viruses. Temperature
132 was maintained between 22 and 25 ºC throughout all the experiment. Mortality was
133 daily recorded, and dead fish were stored at -80 ºC until virological analyses.
134
135 *2.2.2. Intramuscular challenge*

136 Fish (5 g, average weight) were intramuscularly (i.m.) injected with 0.1 ml of viral
137 inoculum (5×10^5 or 10^5 $TCID_{50}$/fish, for sea bass and sole challenges, respectively) or
138 L-15 medium (control group), and were distributed in four groups (100 and 60
139 individuals per group in sea bass and sole trials, respectively) named as follows: (i)
140 $ERV_{i.m.}$/bass-sole, challenged with RGNNV, (ii) $Ss160.03_{i.m.}$/bass-sole, infected with
141 the reassortant, (iii) $SJ93Nag_{i.m.}$/bass-sole, challenged with SJNNV, and (iv) control
142 group, infected with 0.1 ml of L-15 medium. Temperature was maintained between 22
143 and 25 ºC throughout all the experiment. Mortality was daily recorded, and dead fish
144 were stored at -80 ºC until virological analyses.
145
146 *2.3. Processing of samples*
147

148 Samples from dead fish, brain from 5-g fish, and brain and eyes from 2-g fish, were
149 aseptically collected and pooled in samples comprising tissues from 5 fish.
150 Homogenization of Senegalese sole samples was performed as previously described
151 (Olveira et al., 2008), whereas the European sea bass samples (1:10 dilution, w:v, in L-
152 15 medium supplemented with 1% penicillin-streptomycin (Gibco), and 2% FBS) were
153 homogenized using the FastPrep® system (MP Biomedicals) and Lysing Matrix D tubes
154 (2 pulses for 40 s at 6 m/s). Homogenates were treated with penicillin-streptomycin
155 (100 µl/ml) for 24 h at 4 ºC, and then centrifuged at 7500 ×*g* for 15 min at 4 ºC.
156 Supernatants were stored at -80 ºC until used.
157 Survivor fish (SV) were killed at 30 days post-inoculation (p.i.) by overdose of MS-222
158 (Sigma). Three samples (composed of 5 pooled brains or brain and eyes, depending on
159 the fish size) from survivor fish from each group were analysed.
160
161 *2.4. Quantitative real-time PCR*
162
163 Total RNA extraction was performed using the RNeasy® Mini Kit (Qiagen) following
164 the manufacturer's instructions. The cDNA synthesis was carried out with the
165 SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Both, RNA
166 and cDNA, were quantified with the ND-1000 (NanoDrop) system at 260 nm, and
167 stored at -80 ºC (RNA) or -20 ºC (cDNA) until further use.
168 RGNNV and SJNNV RNA2 segments were quantified by two independent SYBR
169 Green I-based quantitative real-time PCR (qPCR) protocols following the conditions
170 previously described by Lopez-Jimena et al. (2011) and (2014), respectively.

Three pools (containing five fish) per virus isolate group and route of infection were analyzed by triplicate at three different time points of the mortality curve (T1, T2 and T3). One pool of surviving fish was also analyzed.

2.5. Infective viral particle quantification

Viral titration was performed following the $TCID_{50}$ method. Briefly, ten-fold serial dilutions of tissue homogenates were inoculated onto E-11 cell monolayers seeded on 96-well plates (Nunc), and incubated for 1 h at 25 °C. After this adsorption period, viral suspensions were removed, and L-15 medium supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), and 2% FBS was added. Cells were incubated for 10 days at 25 °C and viral titres were expressed as $TCID_{50}$/g.

2.6. Statistical analyses

Correlation between the RNA2 copy number and the viral titre was evaluated using the Spearman Correlation Test. Statistical analysis was performed using the Statgraphics Centurion XVI software, the significance of the tests was defined as $P<0.05$.

3. Results

3.1. Clinical signs and cumulative mortality

European sea bass and Senegalese sole were confirmed to be free from VHSV, IPNV, IHNV and VNNV prior to infection.

196 *3.1.1. European sea bass*

197

198 Typical signs of the disease were observed in sea bass challenged (both, by bath and
199 i.m. injection) with ERV378/102-5/04 (RGNNV genotype) and SpSs-IAusc160.03
200 (reassortant) isolates. Such symptoms, consisting of loss of appetite, erratic swimming,
201 hyperactivity, skin darkening and hyperinflation of the swim bladder, were always more
202 evident in RGNNV-challenged than in reassortant-challenged animals. No signs of
203 disease were observed in the SJNNV and control groups.

204 In the bath challenges, the RGNNV isolate provoked 20% cumulative mortality at 30 d
205 p.i. (the end of the experiment) (Figure 1A). Mortalities were recorded between 4 and
206 18 d p.i. The reassortant isolate caused only 5% cumulative mortality, showing less
207 severe symptoms and a shorter mortality period (4-14 d p.i.) (Figure 1A). No mortalities
208 were recorded in the SJNNV and control groups.

209 By i.m. injection, cumulative mortality in the RGNNV-challenged group was 47%
210 (Figure 1C). First symptoms (abnormal behaviour, such as spiral swimming) were
211 observed at 3 d p.i., and the onset of mortality was at 5 d p.i., with the clinical signs
212 disappearing at 15 d p.i. In the group challenged with the reassortant isolate, cumulative
213 mortality was 33%. In this group, mortalities were first recorded at 5 d p.i., and clinical
214 signs were observed from 3 to 11 d p.i. (Figure 1C). Clinical signs in this group also
215 included loss of appetite. No mortalities were recorded in the SJNNV and control
216 groups.

217 The different phases of the mortality curves considered for further analyses have been
218 named as: (i) T1, initial mortality phase (at 4-6 and 5-6 d p.i. for bath and injection,
219 respectively), (ii) T2, acute mortality phase (at 9-10 and 7-8 d p.i. for bath and injection,

220 respectively), and (iii) T3 asymptotic phase (at 13-14 and 10-11 d p.i. for bath and
221 injection, respectively) (Figures 1A and 1C).
222
223 *3.1.2. Senegalese sole*
224
225 Typical signs of the disease were observed in fish infected with the three isolates,
226 regardless of the inoculation way (bath or i.m. injection). Such signs, consisting of loss
227 of appetite, hyperactivity and erratic swimming, were always more severe in the
228 reassortant-challenged group. No symptoms were observed in the control group.
229 In the bath challenges, the three isolates provoked 100% cumulative mortality, which
230 was recorded earlier in reassortant-infected fish (at 18 d p.i.), followed by animals
231 infected with SJNNV (25 d p.i.) and, finally, the RGNNV-infected group (31 d p.i.)
232 (Figure 1B). Signs of the disease were first observed at 3 d p.i., and mortalities were
233 first recorded at 5 d p.i. in the reassortant- and RGNNV-challenged groups, and at 7 d
234 p.i. in the SJNNV-challenged group. After 11 d p.i., cumulative mortality was clearly
235 lower in the RGNNV and SJNNV groups, especially in the RGNNV group, than in the
236 reassortant group (Figure 1B).
237 The cumulative mortality after i.m. injection was also 100% for all the experimental
238 groups considered, although the mortality rate was lower than that recorded after bath
239 challenge, and, therefore, 100% mortality was reported later (from 36 to 41 d p.i.), with
240 the typical signs of the disease appearing from 8 d p.i. onwards. The onset of mortality
241 was at 10 d p.i. in the SJNNV-challenged group and at 13 d p.i. for the reassortant and
242 RGNNV groups. The analysis of the mortality curve showed that the RGNNV isolate
243 caused 50% cumulative mortality at day 17, much earlier than the SJNNV and the
244 reassortant isolates (at 31 and 32 d p.i., respectively). However, after the initial high

245 mortality rate recorded in the RGNNV-challenged group, the number of deaths was
246 stabilized and, consequently, 100% mortality in this group was reached later than in the
247 SJNNV- and reassortant-inoculated animals (Figure 1D).
248 Similarly to European sea bass, the phases of the mortality curves have been named as:
249 (i) T1, the initial mortality phase (4-7 and 10-15 d p.i. for bath and injection,
250 respectively); T2, half-time stabilized phase (10-14 and 20-25 d p.i. for bath and
251 injection, respectively), and (iii) T3, late mortality phase (15-21 and 35-40 d p.i for bath
252 and injection, respectively) (Figures 1B and 1D).
253
254 *3.2. Viral genome quantification*
255
256 Nervous tissue was aseptically collected from fish dead at different phases of the
257 mortality curves (T1, T2 and T3). In addition, samples from survivor European sea bass
258 (30 d p.i.) were also analysed. In the Senegalese sole trials, no survivor animals from
259 infected groups were available to be analysed at the end of the experiment.
260 In the bath trials (Figure 2A), samples from animals challenged with ERV (RGNNV
261 type) displayed the highest RNA2 copy number/g in both species, sea bass (3.8×10^{10}
262 RNA2 copies/g at T2) and sole (4.6×10^{10} RNA2 copies/g at T2). Although neither
263 symptoms nor mortality were observed in European sea bass challenged with the
264 SJ93Nag isolate (SJNNV), viral genome replication was determined in survivor fish, at
265 30 d p.i. (2.4×10^{8} RNA2 copies/g) (Figure 2A). With regard to sole, whereas the copy
266 number in fish challenged with RGNNV and SJNNV strains varied throughout the
267 course of the experiment, those of the reassortant stains remained stable. Viral genome
268 was not detected in control animals.

Samples from European sea bass challenged by i.m. injection also showed the highest viral copy number when fish were infected with the ERV isolate (RGNNV type), with maximum values of 5.3×10^{13} copies/g at T1. In general, the highest number of RNA2 copies/g was quantified at the initial phase of the mortality curve (T1). As in the bath challenge, the lowest RNA2 copy number/g in i.m. injected sea bass was obtained in survivor fish, at 30 d p.i. (Figure 2B). Regarding to sole challenged by i.m. injection, the maximum copy number (4.61×10^9 RNA2 copies/g at T2, Figure 2B), was obtained with the RGNNV isolate (ERV). However, at T1 the highest values were obtained the with the reassortant strain Ss160.03 (6.40×10^8 RNA2 copies/g Figure 2B). Viral genome was not detected in animals from the control group.

3.3. Determination of infective viral particles

Viral titration was carried out from the three samples previously quantified in the different phases of the mortality curve (T1, T2 and T3).

In bath-challenged groups, the highest viral titres were obtained in samples from European sea bass, with maximum values at T2 for ERV_{bath}/bass (5.6×10^7 $TCID_{50}$/g, Figure 2C). In both, ERV_{bath}/bass and $Ss160.03_{bath}$/bass groups, titres increased from T1 to T2. The viral titres in survivor sea bass challenged with the ERV isolate were similar to those obtained in the dead fish at T1, whereas samples from survivors from the $Ss160.03_{bath}$/bass group showed viral titres as high as those recorded in fish that died at T2 and T3. In the $SJ93Nag_{bath}$/bass group, only survivors were analysed, and nervous tissues from these animals showed a high viral titre (1.6×10^7 $TCID_{50}$/g), similar to viral titres in dead fish from other groups (Figure 2C).

293 In bath-challenged sole, the pattern of the viral titres in dead fish throughout time was
294 different for the three isolates (Figure 2C). The highest titres were obtained from
295 animals infected with the Ss160.03 isolate (from 1.3×10^5 $TCID_{50}$/g, at T1 and T3, to
296 6.9×10^5 $TCID_{50}$/g at T2) followed by fish in the SJ93Nag group (from 1.7×10^4
297 $TCID_{50}$/g at T2 to 7.4×10^4 $TCID_{50}$/g at T3). Regarding ERV group, the maximum titre
298 was recorded at T1 (3.9×10^4 $TCID_{50}$/g), decreasing slightly afterwards (1.3×10^4
299 $TCID_{50}$/g at T2 and T3).
300 In the i.m.-challenged groups (Figure 2D), as happened in the bath experiments, the
301 highest viral titres were from the $ERV_{i.m.}$/bass group (from 1.6×10^9 to 1.6×10^{10}
302 $TCID_{50}$/g), with the maximum titre at T2. In the remaining groups, the viral titre
303 decreases throughout time (Figure 2D). Animals infected with the reassortant strain
304 ($Ss160.03_{i.m}$/bass and $Ss160.03_{i.m}$/sole) also showed high titres, reaching 1.6×10^8
305 $TCID_{50}$/g at T1 (10-15 d p.i.) in sea bass and 1.2×10^9 $TCID_{50}$/g at T1 in sole. In the
306 i.m.-challenged sea bass groups, as in the bath challenge, viral titre was also determined
307 from nervous tissue from survivors, showing that in $ERV_{i.m}$/bass and $Ss\text{-}160.03_{i.m}$/bass
308 groups viral titre was lower than that obtained from dead fish. However, in survivors
309 from the SJ93Nag $_{i.m}$/bass group (with no deaths recorded), the viral titre was high (1.6
310 $\times 10^7$ $TCID_{50}$/g) (Figure 2D).
311 The comparison between the log10-transformed RNA2 copy number and the log10-
312 transformed viral titre is drawn in Figure 3. The viral genome copy number was, in
313 general, higher than the viral titres. Specifically, in European sea bass (bath and i.m.
314 injection challenges) the RNA2 copy number was, on average, 2.7 log higher than the
315 infective virus titre. Although the average copy number in i.m.-challenged sole was 2.5
316 log higher than viral titres (similar to sea bass), in fish from the $ERV_{i.m./}$sole group the
317 differences ranged from 3.3 log at T1 to 4.9 at T3 (Figure 3D). These differences

318 increased in the ERV_{bath}/sole groups up to 5 log (Figure 3B). The Spearman test showed
319 significant correlation between RNA2 copy number and viral titre (P=0.0102, for
320 European sea bass and P=0.0097 for Senegalese sole).
321
322 **4. Discussion**
323
324 In this study, two of the most important fish species cultured in South Europe
325 (European sea bass and Senegalese sole) were challenged with three VNNV isolates: a
326 reassortant RGNNV/SJNNV isolate (SpSs-IAusc160.03), a SJNNV reference strain
327 (SJ93Nag) and a RGNNV isolate (ERV378/102-5/04). Two different routes of infection
328 were tested: waterborne exposure, which better mimics natural VNNV infection, and
329 intramuscular injection, which ensures delivery of a standardised viral dose and causes
330 the highest mortality rates in sea bass (Péducasse et al., 1999; Skliris and Richards,
331 1999).
332 Although European sea bass is considered one of the most susceptible fish species to
333 VNNV infection (Munday et al., 2002), an increasing number of studies have reported
334 betanodavirus isolation or detection in Senegalese sole in the last years (Thiéry et al.,
335 2004; Cutrín et al., 2007; Olveira et al., 2009; Panzarin et al., 2012). According to
336 phylogenetic studies, most of these isolates are reassortants exhibiting RGNNV-type
337 RNA1 and SJNNV-type RNA2 segments, which suggests that the reassortment may
338 have promoted the VNNV colonization of this host and may have favoured infectivity
339 to this fish species (Olveira et al., 2009).
340 According to the results obtained, Senegalese sole and European sea bass are
341 susceptible to the three VNNV isolates used in this study, given that virus replication in
342 nervous tissue was always recorded; however, SJNNV inoculation did not result in

343 mortality or clinical signs of VNN in European sea bass. The cumulative mortality in
344 challenged European sea bass was moderate to low, whereas 100% mortality was
345 recorded in all inoculated Senegalese sole groups, with some differences in the
346 mortality curve depending on the VNNV isolate. Indeed, mortality in the sole
347 challenged with the reassortant was observed clearly earlier in the bath trials (all fish
348 were dead by day 18, versus day 25 and 31 in the RGNNV- and SJNNV-challenged
349 groups, respectively). However, in the i.m. trials this difference was not so evident (36 d
350 p.i. in the reassortant-challenged group versus 38-41 in the other groups). Based on this
351 result, it can be suggested that the external barriers of sole (i.e. skin, mucus) could be an
352 important factor controlling RGNNV infection in this fish species, at least at early
353 stages of infection. Regardless of the infection route, the most severe signs of the
354 disease were recorded in sole challenged with the reassortant isolate, yielding the
355 highest viral titres, and showing fewer differences between the RNA2 copy number and
356 the viral titre. Taken together, these results indicate that the reassortant might be more
357 suited for infecting Senegalese sole than the RGNNV or the SJNNV isolates, since it
358 seems to be more effective in the assembly and production of infective viral particles in
359 this fish species. The reassortant isolate exhibits a slightly modified SJNNV CP, with
360 two amino acid substitutions in the C-terminal domain compared to the SJNNV parental
361 genotype (Olveira et al., 2009). It is known that a small number of amino acid
362 substitutions in the coat proteins can have dramatic effects on the host specificity of
363 different animal viruses (Baranowski, 2001). In addition, it has been reported that the
364 VNNV infectivity to different fish species is controlled by the CP (Iwamoto et al.,
365 2001). Reassortment has also been involved in the colonization of new species by
366 betanodavirus (Toffolo et al., 2007; Olveira et al., 2009; Panzarin et al., 2012).

Therefore, our results support that the above described capsid modifications, putatively linked to the host shift event, allow reassortants to replicate better than parental strains in Senegalese sole

Regarding European sea bass, the cumulative mortalities obtained after RGNNV challenge (47% and 20% when fish were i.m.- and bath-challenged, respectively) are similar to those previously reported, ranging from 2 to 50%, and reaching 100% in larvae (Thiery et al., 1997; Skliris and Richards, 1999; Munday et al., 2002; Bigarré et al., 2010; Lopez-Jimena et al., 2011, 2012). Furthermore, the highest values of VNNV genome copy number and viral titres have been recorded in sea bass inoculated with this isolate, which may indicate the adaptation of RGNNV to this fish species. The mortality caused by RGNNV in European sea bass seems to strongly depend on the viral isolate considered. Thus, in a recent study Vendramin et al. (2014) have reported mortalities ranging from 13 to 36% caused by different RGNNV isolates in sea bass challenged under similar experimental conditions. These authors also reported mortalities caused by different RGNNV/SJNNV reassortant isolates (obtained from sea bass and sea bream, *Sparus aurata*), ranging from 7.5 to 25%, which, in many cases, were higher than the mortality caused by RGNNV isolates. The reassortant used in the present study (SpSs-IAusc160.03), although isolated from diseased Senegalese sole, caused clinical signs of VNN and mortality in European sea bass. However, the cumulative mortality (33% and 5% in i.m.- and bath-challenged groups, respectively) and severity of the symptoms were lower compared to those caused by the parental RGNNV isolate, ERV378/102-5/04. As it was mentioned earlier, the CP (encoded by the RNA2 segment) is involved in determining the viral host range (Iwamoto et al., 2004), and, therefore, a relation between the RNA2-type of the reassortant isolate and the host range should be expected (Toffolo et al., 2007; Olveira et al., 2009). However, the

392 RGNNV/SJNNV isolate used in this study caused VNN in European sea bass, whilst
393 the parental SJNNV isolate did not, suggesting that changes during the reassortment
394 could be involved in the pathogenesis/virulence for this species.
395 The differences between the cumulative mortality caused by the same viral isolate
396 depending on the route of infection have been previously reported in other fish species
397 (Aranguren et al., 2002; Nakai et al., 2009). Furthermore, as previously indicated in
398 European sea bass, intramuscular injection is the route provoking the highest mortality
399 levels and, in gilthead sea bream, it is the only way to successfully cause the disease
400 (Aranguren et al., 2002).
401 The number of RNA2 copies and the virus titre were determined in nervous tissue from
402 fish that died at different times p.i., as well as from survivor sea bass. SJNNV-
403 inoculated sea bass showed RNA2 copy number and viral titre similar to those reported
404 in reassortant-challenged animals at the end of the experiment, although mortalities and
405 symptoms of VNN were not observed in SJNNV-inoculated fish. The absence of
406 disease and mortality, even when a productive viral infection occurs, has been reported
407 in other VNNV-infected fish species, such as Atlantic halibut (*Hippoglossus*
408 *hippoglossus*), Atlantic cod (*Gadus morhua*) and gilthead sea bream (Castric et al.,
409 2001; Grove et al., 2003; Korsnes et al., 2009). These results are in contrast to those
410 reported by Vendramin et al. (2014), who demonstrated low mortality (10%) in
411 European bass after bath inoculation with one SJNNV isolate. However, variation in the
412 SJNNV isolate used, as well as in the fish size (0.2 g, mean weight) may account for the
413 different results obtained.
414 In all the trials, a statistical correlation between viral RNA copy number and viral titres
415 was established ($P<0.005$, Spearman test), although, in general, the viral copy number
416 was higher than the viral titres, especially in Senegalese sole. Differences between

417 infective viral particles and viral genome copy number have been previously reported in
418 infections caused for several human viruses (Garcia et al., 2001; Falsey et al., 2003),
419 fish viruses (Purcell et al., 2006; Hope et al., 2010) and higher vertebrate viruses
420 (Achenbach et al., 2004). The differences observed in the European sea bass groups
421 (from 1 to 4 logs) were similar to those reported for the Bovine Respiratory Syncytial
422 Virus (BRSV, Achenbach et al., 2004), the Rift Valley Virus (RVFV, Garcia et al.,
423 2001) and IPNV (Purcell et al., 2006). In the Senegalese sole groups, the differences
424 increased up to 5 and 6 log, especially in the bath challenges, and in the RGNNV- and
425 SJNNV-challenged groups.
426 Several explanations have been given to the differences between viral genome copy
427 number and viral titre. Thus, the differences found for the avian influenza H5N1 virus
428 (Dovas et al., 2010) have been associated to the virus inactivation under aquatic
429 environmental conditions. The reported VNNV ability to survive for extended periods
430 of time under a wide range of environmental conditions (Frerichs et al., 2000) makes
431 unlikely viral inactivation during sample processing. Other possibility is the loss of viral
432 infectivity in dead tissues (Rosenbergova et al., 2009). In the present work, the
433 overnight degradation of fish tissues when the individuals were found dead in the
434 following morning cannot be ruled out. This degradation would probably be more
435 intense in bath-challenged fish, due to their smaller size. Alternatively, defective viral
436 particles production or overexpression of the RNA2, as it has been reported for the S
437 segment of RVFV (Garcia et al., 2001) and BRSV F RNA (Achenbach et al., 2004),
438 could be occurring. The putative overexpression of the RNA2 segment or the generation
439 of defective particles seems to have occurred mainly in RGNNV and SJNNV infections
440 in sole, suggesting a poor adaptation to the host.
441

6. Conclusion

In conclusion, the susceptibility of juvenile European sea bass and Senegalese sole to a reassortant isolate (RGNNV/SJNNV), as well as to two isolates of the parental genotypes, RGNNV and SJNNV, has been demonstrated. In European sea bass, the reassortant isolate caused lower mortality levels than the RGNNV isolate, whereas no mortality was recorded after SJNNV inoculation. These results suggest that the amino acid changes in the reassortant CP (compared to the parental SJNNV isolate) could have involved host specificity and/or virulence determinants to/for this fish species. In sole, the three isolates caused 100% mortality, with the reassortant isolate causing the most acute symptoms, appearing more quickly, especially in bath-challenged animals, and yielding the highest number of infective viral particles. In addition, the differences observed between RNA2 copy number and viral titre were lower for this isolate than for the RGNNV and SJNNV isolates. All these results suggest that the RGNNV and the reassortant isolates used in this study are more suited for infecting European sea bass and Senegalese sole, respectively, than the SJNNV isolate assayed.

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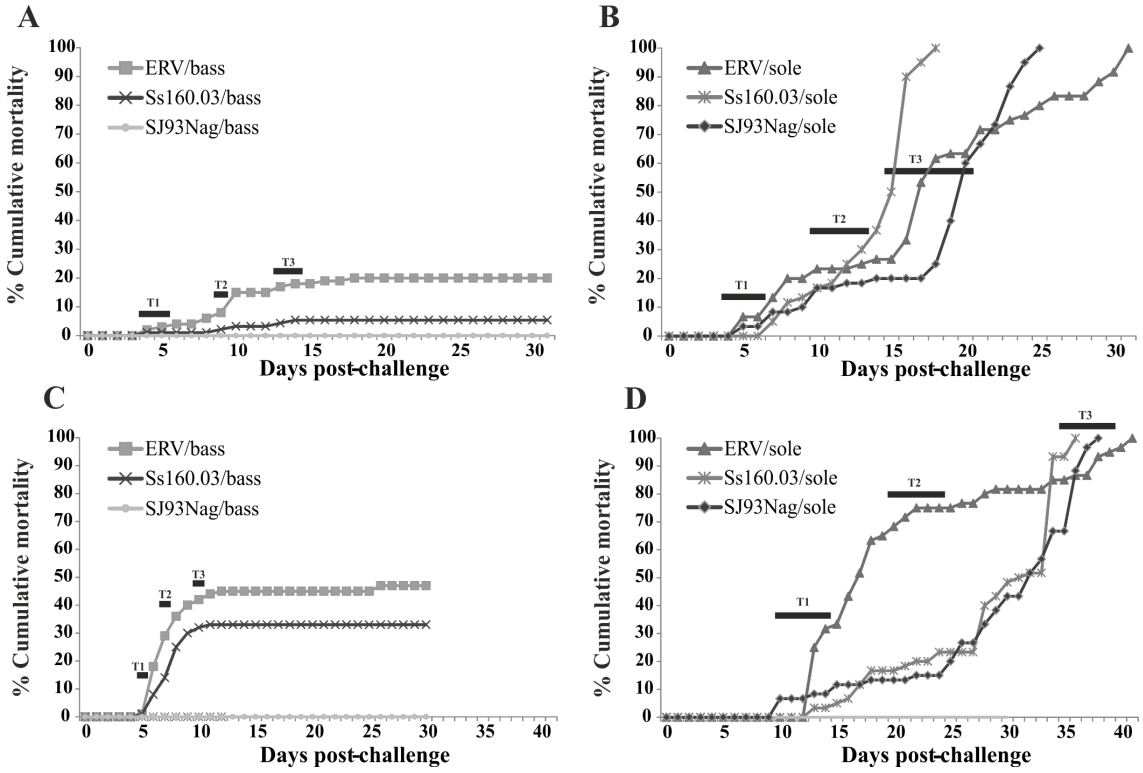
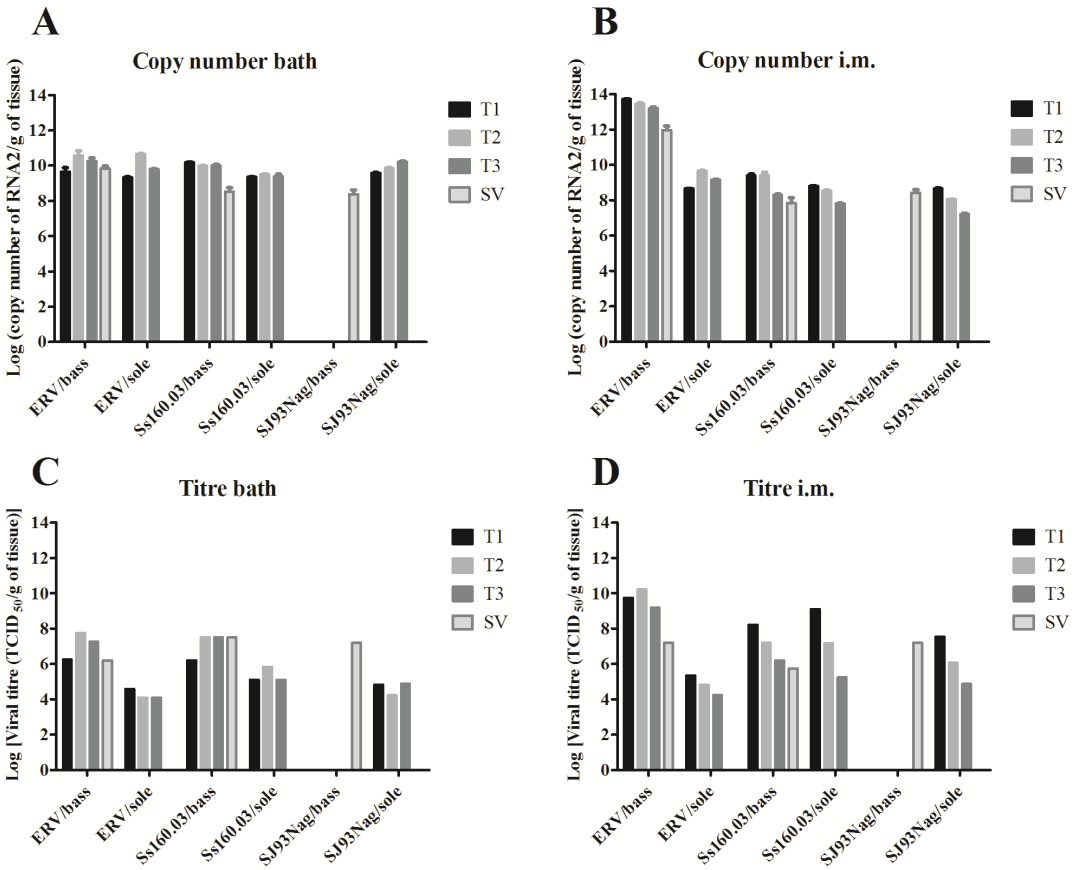
Figure 1. Cumulative mortality in juvenile European sea bass (bass) and Senegalese sole (sole) challenged by bath (A and B) or intramuscular injection (C and D). Virus isolates are: ERV378/102-5/04 (RGNNV); SpSs-IAusc160.03 (reassortant) and SJ93Nag (SJNNV). T, phases of the mortality curve; for sea bass T1, initial mortality phase; T2, acute mortality phase;T3 asymptotic phase; for sole T1, initial mortality phase; T2, half-stabilized phase; T3, late mortality phase.



628 **Figure 2.** RNA2 copy number (A and B) and viral titre (C and D) in nervous tissue
629 from dead and survivor (SV) sea bass and dead sole challenged by bath and
630 intramuscular injection. Virus isolates are: ERV378/102-5/04 (RGNNV); SpSs-
631 IAusc160.03 (reassortant) and SJ93Nag (SJNNV). Samples were considered at different
632 times p.i.: T1, T2, T3 and SV. Each bar represents the mean ± S.D. (n=3)



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635 **Figure 3.** Comparison of RNA2 copy number (grey symbols) and viral titre (black
636 symbols). The virus titre is expressed as log10 of $TCID_{50}$/g, the copy number is
637 represented as log10 of the RNA2 copies/g. Both, RNA2 copy number and $TCID_{50}$
638 values, were obtained from dead fish at different days p.i. (T1, T2 and T3), as well as
639 from survivor fish (SV). (A-B) are bath-challenged fish, (C-D) are intramuscularly-

640 challenged fish. Viral isolates are: ERV378/102-5/04 (RGNNV), SpSs-IAusc160.03

641 (reassortant) and SJ93Nag (SJNNV).

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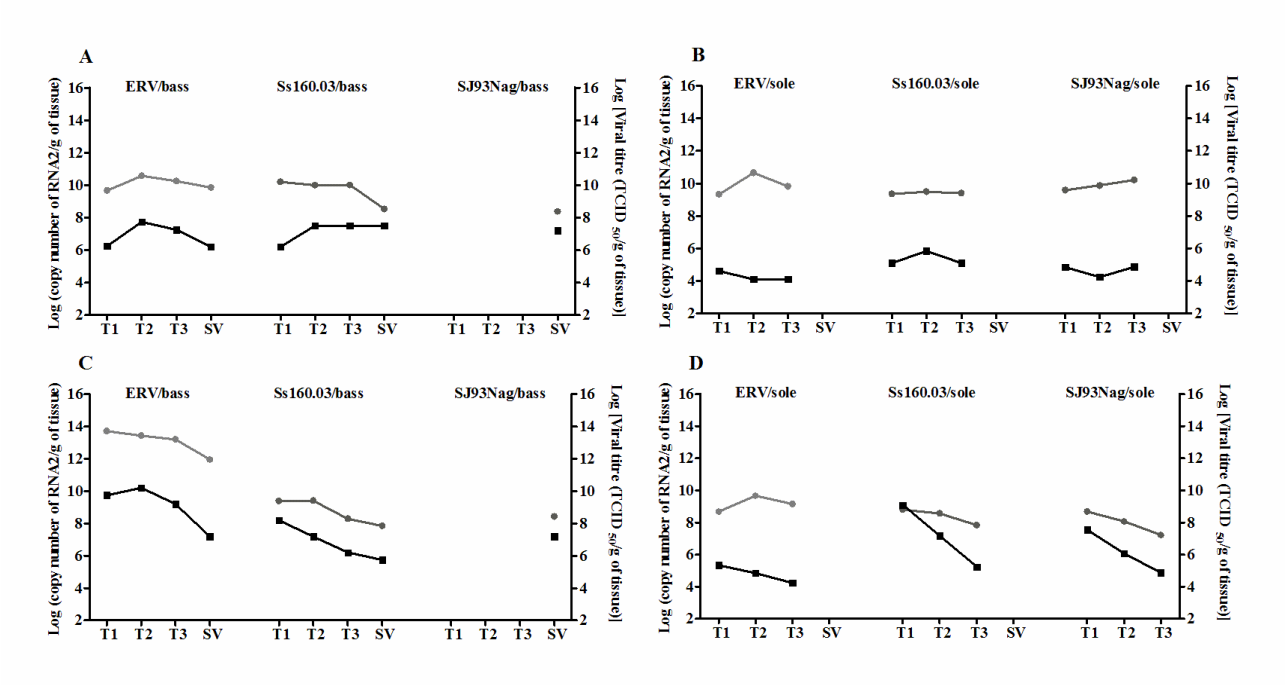

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