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Betanodavirus genotypes produce clinical signs and mortality in the shi drum (*Umbrina cirrosa*), and infective particles are isolated from the damaged brain

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ABSTRACT

Betanodavirus (NNV), the causative agent of viral retinopathy and encephalopathy (VER) disease in fish, is the greatest viral threat for aquaculture growth and diversification in the Mediterranean area, where several genotypes and reassortants have been described. The shi drum (*Umbrina cirrosa*) is an excellent candidate for marine aquaculture diversification in the Mediterranean area, as this species is easily adapted to culture conditions and shows high growth rates, low mortalities during larval development and high market value. Although outbreaks of RGNNV, the most common NNV genotype in the Mediterranean Sea, have been detected in wild and farmed shi drums, little is known about host–virus interactions, clinical signs, mortality rates and viral load in this species. In this framework, we have evaluated the mortality rates, the signs of the VER disease including behaviour, histopathological alterations in the brain and retina and the rescue of infective particles in shi drums after infection with the four NNV genotypes (RGNNV, SJNNV, BFNNV and TPNNV). We found that all of the genotypes produce mortalities and analogous time-lapses between the first signs of disease and mortalities. However, infective particles were only recovered from RGNNV-, BFNNV- and TPNNV-infected specimens. Interestingly, clinical signs and histopathological lesions in the brain and retina were different depending on the genotype used.

1. Introduction

Betanodavirus (NNV; family *Nodaviridae*, genus *Betanodavirus*) is the causative agent of viral nervous necrosis or viral retinopathy and encephalopathy (VER) disease, which mainly affects the larval and juvenile stages of fish. The virus has an acute lethal effect in larval stages, which decreases with host body size (Tanaka et al., 2004) but impacts fish growth and performance (Vendramin et al., 2014). In fact, chronic subclinical infections have been detected in adult fish, allowing viral dissemination by horizontal and vertical transmission (Bandín and Souto, 2020). Betanodaviruses are composed by the genome, two-segmented positive-sense RNA molecules called RNA1 and RNA2, into an icosahedric capsid formed by the capsid protein (CP). RNA1 encodes for the viral RNA-dependent RNA polymerase (RdRp or A) and RNA2 for the CP. A subgenomic RNA3, produced from RNA1, encodes for two proteins: B1 and B2. The B1 protein preserves the infected cells from death at early stages of the infection, while the B2 protein interferes with the RNA-silencing system of the host cell and triggers cell death at final stages of the viral cycle (Bandín and Souto, 2020).

According to the RNA2 sequence, betanodaviruses are mainly divided into four genotypes (http://www.ictv.global/report/nodaviridae): RGNNV (red-spotted grouper nervous necrosis virus), SJNNV (striped jack nervous necrosis virus), BFNNV (barfin flounder nervous necrosis virus) and TPNNV (tiger puffer nervous necrosis virus) (Nishizawa et al., 1997), although a fifth genotype has recently been accepted and two others are under consideration (Bandín and Souto, 2020). They were also divided, depending on their antigenic features, into serotypes A (SJNNV), B (TPNNV) and C (RGNNV and BFNNV) (Mori et al., 2003). However, some strains of genotypes BFNNV and TPNNV have the same antigenic features as viruses of serotype B (Panzarin et al., 2016).

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Interestingly, members of genotypes RGNNV, TPNNV and BFNNV, as well as SJ/RGNNV reassortants, also display a certain level of cross-reaction (Panzarin et al., 2016).

Betanodavirus affects a constantly increasing number of freshwater and marine fish species throughout the world, with around 177 susceptible species so far (Bandín and Souto, 2020). NNV is distributed worldwide; however, the genotypes are differently distributed based on viral thermotolerance (Bandín and Souto, 2020). Thus, RGNNV is the most widely distributed in warm waters, followed by BFNNV in cold waters, SJNNV and TPNNV. SJNNV is distributed in Japanese waters and in the Mediterranean area of the Iberian Peninsula, while TPNNV has been described in only one species in Japan (see for review Bandín and Souto (2020)). In Europe, RGNNV and BFNNV are the two genotypes traditionally detected. Also, a rapid expansion of the SJNNV genotype has been documented in Spanish waters (Cutrín et al., 2007; Thiery et al., 2004). The first NNV reassortant (SJ/RGNNV) was described in 2007 in the Mediterranean Sea (Toffolo et al., 2007); since then, some other reassortant strains of RG/SJNNV and SJ/RGNNV have been detected in several fish species producing high mortalities, even in fish species considered resistant (see for review Costa and Thompson (2016)). Moreover, the coexistence of RGNNV and SJNNV has been described in wild meagre (*Argyrosomus regius*) specimens (Lopez-Jimena et al., 2010). The host specificity of NNV is determined by some residues in the capsid protein and mutations in RNA2 might greatly alter virus–host specificity, although the specific mechanisms are still unclear (Low et al., 2017). In contrast, the thermotolerance of the betanodaviruses seems to be determined by RNA1 (Bandín and Souto, 2020).

Bearing in mind the worldwide distribution of NNV and the reassortant strains that are able to replicate and cause disease in more diverse fish species, betanodaviruses are currently a threat to the aquaculture sector. In particular, in the Mediterranean Sea, the European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) are the most important species for the aquaculture industry. NNV represents one of the major diseases for sea bass, one of its main hosts, but the apparition of outbreaks in gilthead seabream (Olveira et al., 2009; Toffan et al., 2017) unravels future problems in the aquaculture sector for these fish species. Also, the NNV isolation from many other fish species under culture development, such as the shi drum (*Umbrina cirrosa*), wreckfish (*Polyprion americanus*), sole (*Solea solea*), greater amberjack (*Seriola dumerili*) or meagre (*Argyrosomus regius*), among others (OIE, 2016), highlights the potential risk posed by this virus for the development of aquaculture and the implementation of production with new fish species.

The shi drum is a serious candidate for the diversification of Mediterranean aquaculture (Basurco and Abellán, 1999) because it does not have cannibalistic behaviour, shows a high survival rate in different rearing conditions and can be weaned on artificial diets very early during its development (Arizcun et al., 2009; Melotti et al., 1995; Papadakis et al., 2009; Zaiss et al., 2006). Although the shi drum is being cultivated so far under experimental conditions and several studies have been performed, it is necessary to go further in our knowledge of its biology to ascertain if it is worth for industrial cultivation. In that sense, studies are mainly related to reproduction (Barbaro et al., 2002, 1996; Chaves-Pozo et al., 2019; Mylonas et al., 2004), development of the digestive system and dietary requirements (Akpınar et al., 2012; Pedini et al., 2001; Zaiss et al., 2006) and growth performance (Ballarin et al., 2004; Maccatrozzo et al., 2002; Mylonas et al., 2009), while aspects related to pathology, immunity or stress have been poorly studied. In this regard, the shi drum is a susceptible species for betanodavirus (RGNNV) infection, as several natural outbreaks have been reported (Katharios et al., 2010; Pavoletti et al., 1998).

Taking into consideration that the shi drum is a promising candidate for Mediterranean aquaculture and that some studies have identified the RGNNV genome in shi drum tissues (Katharios et al., 2010), we aimed to evaluate the potential susceptibility of laboratory-reared shi drum specimens to the four common NNV genotypes, the clinical signs, the histopathological alterations in the central nervous tissues and the isolation of infective particles. This information would benefit the implementation of shi drum culture; for example, the use of selective breeding programmes for improving resistance against NNV.

2. Materials and methods

2.1. Animals

Specimens of shi drum were bred at the *Centro Oceanográfico de Murcia, Instituto Español de Oceanografía* (IEO) facilities from spawning culture broodstocks, as described elsewhere (Chaves-Pozo et al., 2019). Healthy specimens of shi drum ($n = 230$) of a mean body weight (bw) of 10.95 ± 2.66 g were randomly distributed in five tanks (46 fish/tank) with 200 L of capacity with a close re-circulated seawater flux (38‰ salinity), with a 12 h light:12 h dark photoperiod and 19 ± 1 °C of controlled temperature. The animals were fed *ad libitum* daily with a commercial pellet diet (Skretting).

Handling the specimens was always performed under the Guidelines of the European Union Council (2010/63/UE), the Bioethical Committees of the IEO (REGA code ES300261040017) and the approval of the Ministry of Water, Agriculture and Environment of the Autonomous Community Region of Murcia (Permit Number A13200602).

2.2. NNV stocks

Four genotypes of NNV (RGNNV strain It/411/96, SJNNV strain SJNag97, BFNNV strain JFIwa98 and TPNNV strain TPkag93) were propagated in the E-11 cell line as elsewhere (Frerichs et al., 1996) until the cytopathic effect (CPE) was extensive. The supernatant was harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates before use in the experiments (Reed and Müench, 1938). Propagation and titration of RGNNV was performed at 25 °C while SJNNV, BFNNV and TPNNV were at 20 °C.

2.3. NNV challenge

The main objective of this study was to evaluate under laboratory conditions whether shi drum specimens were infected by NNV genotypes and suffered the VER disease and/or mortalities, but no comparisons among genotypes were established since the infection conditions are not optimal for each of the assayed NNV isolates. Fish in each group received a single intramuscular injection of 100 μL of culture medium alone (mock-infected) or containing 10^7TCID$_{50}$/fish of RGNNV or 10^8TCID$_{50}$/fish of SJNNV, BFNNV or TPNNV. The intramuscular injection was used, since it has been proven to be the most effective route of infection (Aranguren et al., 2002). Mortality was daily recorded and the percentage of survival determined. Survivor fish were considered as those without any disease signs during the infection trial or able to overcome disease signs within three days. By contrast, susceptible fish were those that died during the trial or showed disease signs during three consecutive days, and were humanely euthanised using 40 μL of clove oil/L of seawater according to the guidelines on the care and use of fish (Batt et al., 2005).

After 8 days of infection (dpi), five fish per group, showing disease signs as abnormal swimming or incapability of movement, were sampled. After being euthanised with 40 μL/L of clove oil, fish were completely bled and the brain and one eye removed. Half of the brain was immediately frozen and stored at −80 °C until used, while the other half of the brain and one eye were processed for light microscopy as described below. All fish were weighed at the beginning of the infection trial and, when sampled, recovered from the tanks as susceptible or at the end of the trial as survivor fish.

2.4. Dot-blot

To check whether the four NNV genotypes are detected by a commercial antiserum against NNV particles (Abcam), a dot-blot technique was applied. Briefly, 2 μL of 1:10 diluted NNV stocks were applied to nitrocellulose membranes and allowed to dry. Controls consisted of phosphate buffer (PBS) or 1:10 diluted infective pancreatic necrosis virus (IPNV). Afterwards, membranes were blocked with TBT buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween20, pH 7.5) containing 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h. After washing, the antiserum to NNV was diluted 1:5000 in TBT with 0.1% BSA and incubated overnight at 4 °C. After washing and incubating the membranes with an anti-rabbit IgG peroxidase conjugated (Sigma-Aldrich) diluted 1:2500 in TBT with 0.1% BSA for 1 h, the reaction was developed by the ECL system (ThermoFisher Scientific).

2.5. Light microscopy and immunohistochemical methods

Half of the brain and one eye ($n = 5$ fish/group) were fixed in Bouin's solution for 16 h at 4 °C, dehydrated with increasing concentration of ethanol in water (70%, 90 min; 96%, 60 min; and twice with 100%, 60 min each), washed twice in n-isoamyl-acetate for 30 min each and embedded in paraffin (Paraplast Plus, Sherwood Medical) overnight. The sections were performed at 5 μm. After dewaxing and rehydration, some sections were stained with haematoxylin-eosin to determine morphological changes, while others were subjected to an indirect immunohistochemical method (IHC). The cells containing the capsid protein of the NNV were stained using a commercial antiserum against NNV particles at the optimal dilution of 1:500 as the primary antibody and an anti-rabbit IgG peroxidase conjugated at the optimal dilution of 1:100 as the secondary antibody. The reaction was developed by incubation with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in PBS containing 0.05% of H_2O_2 for 15 min at room temperature. The sections were lightly counterstained with haematoxylin. The specificity of the reactions was determined by omitting the first antiserum in the sections from infected fish and using sections from mock-infected control fish.

2.6. Isolation of NNV infective particles from the brain

Brain fragments from mock- or NNV-infected fish ($n = 4$/group) were weighted and independently homogenised in 1:10 (w:v) of 0.01 M PBS. Homogenates (n = 4/group) were then tested for the NNV presence by titration on E-11 cells as above, at either 25 or 20 °C depending on the viral genotype. In the case the CPE was not evident after 10 days, the culture was then tested by a second passage for titration.

2.7. Statistical analysis

Survival percentage was represented for all treatments using Graphpad Prism 5.01 software, and curve comparison was performed using the Log-rank (Mantel-Cox) test and the Log-rank test for trend ($P \leq 0.05$). Weight was represented as mean ± SEM and comparisons between groups were analysed by the Student-*t*-test ($P \leq 0.05$).

3. Results

3.1. Shi drum juveniles are susceptible to all NNV genotypes

Mock-infected fish showed completely normal behaviour, and no mortalities were recorded. In sharp contrast, all NNV genotypes triggered clinical signs (Supplementary videos 1–5, Table 1) and mortalities (Fig. 1A, Table 1) and altered the weight (Fig. 1B) of shi drum specimens. Interestingly, the first signs of disease were observed at day 3 for RGNNV- and TPNNV-infected fish, day 4 for BFNNV-infected fish and day 7 for SJNNV-infected fish, while the first mortalities were recorded at day 10 for BFNNV, day 13 for RGNNV, day 16 for SJNNV and day 18 for TPNNV (Fig. 1A, Table 1). Also, although all the clinical signs were compatible with the VER disease, some differences among the different NNV genotypes were recorded (Supplementary videos 1–5, Table 1).

Thus, fish infected with RGNNV showed vertical swimming with twister movement as the first sign of infection (Supplementary video 2), while TPNNV-infected fish failed to swim and kept still at the bottom upside down with great limitations to recover their normal position and suffering from muscle spasms (Supplementary video 3). The BFNNV-infected shi drum remained lying on the bottom of the tanks without being able to swim in the water column but without suffering from muscle spasms (Supplementary video 4). Interestingly, scarce and very light lateral swimming was observed in SJNNV-infected shi drums (Supplementary video 5). Moreover, from day 16 in TPNNV-infected and day 19 in BFNNV-infected fish, some fish were unable to see correctly (Table 1). In fact, on day 21, BFNNV- and TPNNV-infected fish showed exophthalmia and opaque eyes, while in SJNNV-infected fish, exophthalmia appeared from day 25 of infection onwards (Supplementary Fig. S1; Table 1). Although dark skin is usually considered a clinical sign, we did not relate this to the VER disease because i) shi drum larvae have dark-coloured skin in rearing conditions, which becomes white when growing, and ii) fish quickly become dark upon small perturbations such as feeding, being covered by shadows or experiencing water vibrations (Basurco and Abellán, 1999; Koumoundouros et al., 2005; Zaiss et al., 2006). Therefore, although the percentage of dark fish in infected groups seemed to be a bit higher than in the control group (Supplementary videos 1–5), we did not account for this as a disease sign.

Regarding fish survival, shi drum specimens infected with RGNNV showed the highest survival percentage (71%), followed by TPNNV (54%), SJNNV (53%) and BFNNV (44%) (Fig. 1A). Survival curves were

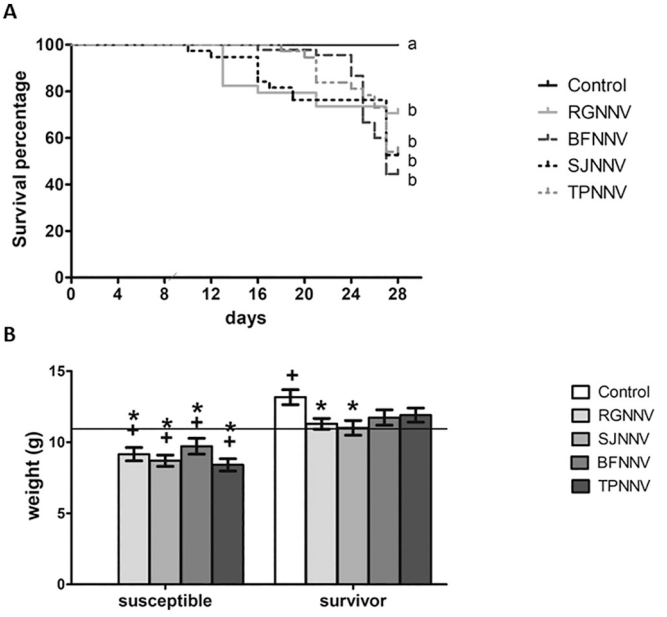

Fig. 1. Survival percentage (A) and mean body weight of susceptible and survivor (B) shi drum specimens after 28 days of intramuscular injection with 10^7 $TCID_{50}$/fish of RGNNV or 10^8 $TCID_{50}$/fish of SJNNV, BFNNV or TPNNV. The control was a mock-infected group. (A) Survival percentage. Different letters denote statistical differences according to the Log-rank (Mantel-Cox) test and the Log-rank test for trend ($P \leq 0.05$). (B) Mean value ± S.E.M. of the weight of susceptible and survivor fish in each experimental group. The horizontal line corresponds to the initial fish body weight. Asterisks denote statistical differences with the mean weight of mock-infected fish (control), and crosses denote differences with the initial mean weight of each group according to a Student-*t*-test ($P \leq 0.05$).

Table 1
Summary of the different clinical signs and mortalities in shi drum specimens infected with different NNV genotypes expressed in days post-infection (dpi). ND, not detected.

	Mock	RGNNV	SJNNV	BFNNV	TPNNV
First signs of disease	ND	3dpi	7dpi	4dpi	3dpi
First mortalities	ND	13dpi	16dpi	10dpi	18dpi
Vision problems	ND	ND	ND	19dpi	16dpi
Exophthalmia/opaque eyes	ND	ND	25dpi	21dpi	21dpi
Survival rate (%)	100	71	53	44	54
Optimal viral replication temperature (Bandín and Souto, 2020)	–	25–30 °C	20–25 °C	15–20 °C	20 °C

significantly different with *P* values of 0.0026 and 0.0041 in the Log-rank (Mantel-Cox) test and the Log-rank test for trend, respectively. All groups infected with NNV genotypes showed similar survival curves among them but were significantly different from those of the control group.

Very interestingly, as expected, mock-infected fish gained weight during the trial. However, although all fish were fed *ad libitum*, NNV-infected fish failed to gain weight (Fig. 1B). In fact, all the susceptible fish showed a statistically significant decrease in their weight compared to the initial body (Fig. 1B). Also, at the end of the trial, the final weight of RGNNV- and SJNNV-survivor fish was significantly reduced compared to the mock-infected group, while for the fish infected with TPNNV or BFNNV, the reduction did not reach significance (Fig. 1B).

3.2. Brain lesions were evidenced and NNV was immunodetected and isolated

First, we demonstrate that the commercial antiserum against NNV was able to bind and recognise all the NNV genotypes, being more sensitive for the RGNNV and SJNNV genotypes than for BFNNV or TPNNV (Fig. 2). This allows the use of this antibody to detect the presence and distribution of all the NNV genotypes, while considering that cells with a low amount of virus will not be stained in BFNNV- or TPNNV-infected fish and no comparisons might be performed.

Regarding the brain (Fig. 3), vacuolation was greatly observed in fish

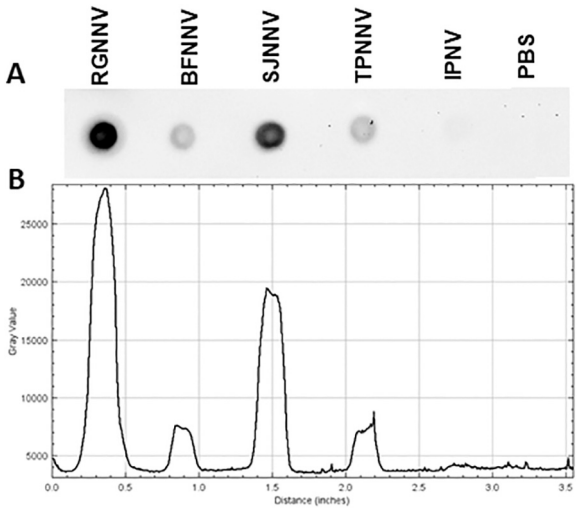

Fig. 2. Detection of the different genotypes by a commercial antiserum against NNV. (A) A Ten-fold dilution of RGNNV, BFNNV, SJNNV or TPNNV stocks were blotted on nitrocellulose membranes and immunostained with the anti-NNV antiserum. PBS or the infectious pancreatic necrosis virus (IPNV) were blotted as controls. (B) Quantification of the reaction was done using ImageJ software.

infected with RGNNV (Fig. 3B), slightly observed in TPNNV (Fig. 3E) and hardly observed in those with SJNNV and BFNNV (Fig. 3C, D). However, NNV-positive cells were detected by IHC in all infected fish (Fig. 3F–J). Thus, brain cells and nerves appeared extensively stained with an anti-NNV serum in RGNNV-infected fish (Fig. 3G), while in the other infected groups, only scattered cells were immunostained (Fig. 3H–J). No lesions or immunostaining were evidenced in the brain from any of the mock-infected specimens.

The isolation of NNV-infective particles from the brain of infected shi drums was possible only in three experimental groups (Table 2). Thus, E-11 cell cultures directly infected with brain homogenates from all BFNNV- or TPNNV-infected specimens resulted in a titre of 3.2×10^6 $TCID_{50}/g$ and 3.2×10^5 $TCID_{50}/g$, respectively. Interestingly, in the brains of RGNNV-infected shi drum specimens, the CPE was evident only after two passages on E-11 culture cells, resulting in a high variability between specimens that showed titres from 10^3 to 10^5 $TCID_{50}/g$ (Table 2). Strikingly, we were unable to observe any CPE in E-11 cells upon two passages with brain homogenates from SJNNV-infected or control specimens.

3.3. NNV infection resulted in histopathological alterations in the retina

Vacuolation and virus detection was evidenced in layers of the retina of shi drum specimens after 8 days of infection with different NNV genotypes (Fig. 4). The most affected retinas were observed in the RGNNV-infected fish, which showed a clear vacuolation and disruption of different layers, with the outer plexiform layer (OPL), the inner nuclear layer (INL), and the inner plexiform layer (IPL) being the most affected (Fig. 4B). Also, these layers, as well as the ganglion layer (GL), showed high numbers of anti-NNV positive cells by IHC (Fig. 4G). In sharp contrast, no vacuolation was observed in the retinas of SJNNV-infected fish (Fig. 4C), although the viral protein was immunodetected in some cells of the INL (Fig. 4H and inset). In fish infected with BFNNV, a high degree of vacuolation and some NNV-positive cells were observed in the GL (Fig. 4D). Similarly, TPNNV-infected fish showed the GL greatly vacuolated, but a certain degree of vacuolation was also observed in the INL and IPL (Fig. 4E). Accordingly, in the TPNNV-infected fish, these three layers showed NNV-positive cells (Fig. 4J). No lesions or NNV immunodetection were evidenced in the retina from mock-infected specimens.

4. Discussion

NNV is able to infect many fish and several invertebrate species (Bovo et al., 2016; Gómez-Casado et al., 2011; Gomez et al., 2008; Munday et al., 2002). Among the very susceptible European sea bass and grouper species in the Mediterranean area, the shi drum seems to be another susceptible species (Comps et al., 1996; Dalla Valle et al., 2001; Katharios et al., 2010; Pavoletti et al., 1998). As far as we are concerned, only the RGNNV genome has been isolated from diseased shi drum specimens, although susceptibility to other NNV genotypes is unknown. Considering that in the Mediterranean basin, different NNV genotypes have been detected, as well as a certain occurrence of genetic reassortment (Panzarin et al., 2012), it is mandatory to ascertain if the shi drum is susceptible to NNV genotypes if we want to diversify the aquaculture sector with this fish species.

Our data show that the four NNV genotypes are able to produce signs of VER disease and mortalities in shi drum specimens. As far as we are concerned, this is the first study in which the four genotypes are proven to be infective in the same fish species. Most studies focus on the impact of different strains of the same genotype or in no more than two different genotypes. Regarding this issue, some studies have analysed the infective features of RGNNV and SJNNV, and their reassortants, in the same fish species (Bandín and Dopazo, 2011; Souto et al., 2015; Vendramin et al., 2014). A striking issue observed in this study is that TPNNV is able to infect and produce mortalities in the shi drum, although this genotype

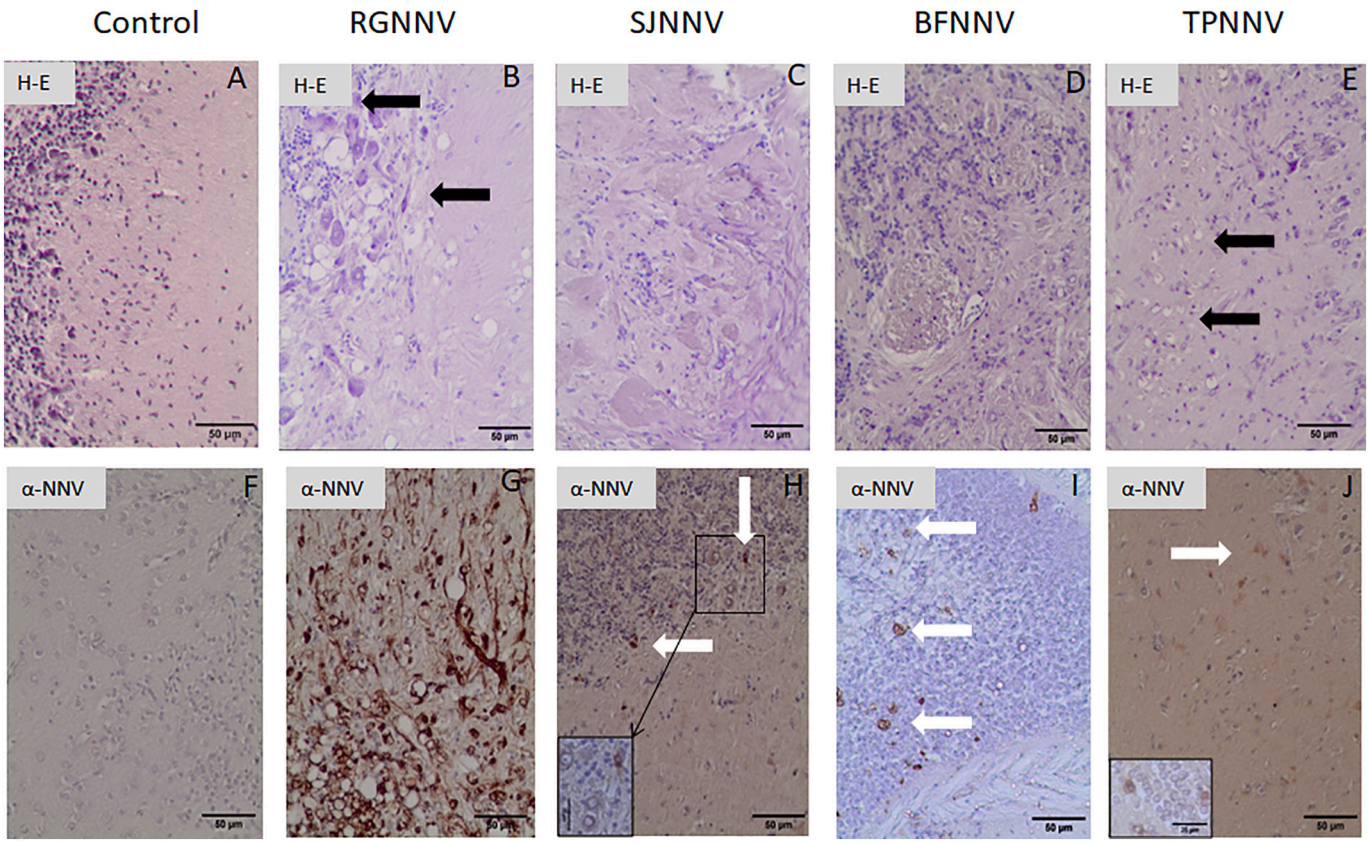

Fig. 3. Representative micrographs of the shi drum brain after 8 days of intramuscular injection with PBS (Control, A, F); 10^7 $TCID_{50}$/fish of RGNNV (B, G); 10^8 $TCID_{50}$/fish of SJNNV (C, H); BFNNV (D, I); TPNNV (E, J); stained with haematoxilin-eosin (H-E) (A–E) or subjected to indirect immunohistochemistry to detect the NNV capsid protein (α-NNV) (F–J). Scale bar = 50 µm (A–J); 25 µm (inset J); 20 µm (inset H). Black arrows denote cell degradation and vacuolisation; white arrows denote cells stained with the antibody against the viral capsid protein.

Table 2
Viral load ($TCID_{50}$/g tissue) in the brain of shi drum specimens infected with different NNV genotypes. ND, not determined.

Group/Genotype	Fish specimens	1st passage	2nd passage
Control/Mock	N = 4	All negative	All negative
RGNNV	N = 4	Negative	3.2×10^5 3.2×10^5 1.5×10^4 1.4×10^3
SJNNV	N = 4	All negative	All negative
BFNNV	N = 4	All 3.2×10^6	ND
TPNNV	N = 4	All 3.2×10^5	ND

has been naturally described in only one species that inhabits Japanese waters (Bandín and Souto, 2020; Munday et al., 2002). This fact highlights the need to increase the constraint measures to limit the spread of different NNV genotypes from their actual locations.

It has been demonstrated that the strains of BFNNV and TPNNV used in this work belong to serotype B, and a certain grade of cross-reaction between some members of RGNNV, SJ/RGNNV, TPNNV and BFNNV genotypes has been suggested (Panzarin et al., 2016). Moreover, the cross-reactivity of some antibodies between RGNNV and SJNNV has been described (Iwamoto et al., 2004). These data would explain why our antibody cross-reacted with all the strains used and allowed us to demonstrate that viral protein is produced in shi drum specimens infected with all the NNV at 8 dpi. Also, the IHC technique is not good enough for quantitative determinations, which could partly account for the great differences in the immunodetection of NNV-positive cells. Therefore, we cannot correlate the intensity of the staining in the brain and retina with viral recovery load and disease signs.

As our data demonstrate, the different NNV genotypes produce different clinical signs in the shi drum, but all are compatible with the typical signs (Chen et al., 2014). Thus, the typical altered swimming in a corkscrew fashion from the VER disease was detected only in RGNNV-infected animals, while BFNNV- and TPNNV-infected fish showed the failure of swimming as the first sign of infection. Only specimens infected with SJNNV showed a very slight lateral swimming ability. The ability of shi drum juveniles to rapidly darken their body colour, as described previously, explains why there are some dark-coloured fish in the mock-infected group video.

Also, we found that all susceptible fish, as well as survivors from the RGNNV- and SJNNV-infected groups, significantly decreased their body weight. These findings are compatible with the description of appetite-loss (Chen et al., 2014) or growth problems (Vendramin et al., 2014) as clinical signs of VER disease. Our observations might be due to the NNV infection and not stress or suboptimal culture conditions, since mock-infected fish were able to grow as expected (Shetty et al., 2012). Perhaps this growth alteration is due to the abnormal swimming and vision that hampers diet-detection and proper feeding.

Independent of the behaviour signs, the shi drum was clearly susceptible to all NNV genotypes under laboratory conditions. RGNNV, BFNNV and TPNNV genotypes produced the first signs of VER disease at 3 or 4 dpi, which is like other studies in the literature (Bandín and Souto, 2020), while the SJNNV genotype provoked them later. Strikingly, the first mortalities appeared 6, 9, 10 or 15 days later for BFNNV, SJNNV, RGNNV or TPNNV, respectively, which is more than the normal established values observed in the literature that usually range from the same day, in which disease signs appeared too few days later. This could be due to several factors, such as virus–host interactions, different viral infectivity or temperature. In fact, temperature greatly influences viral

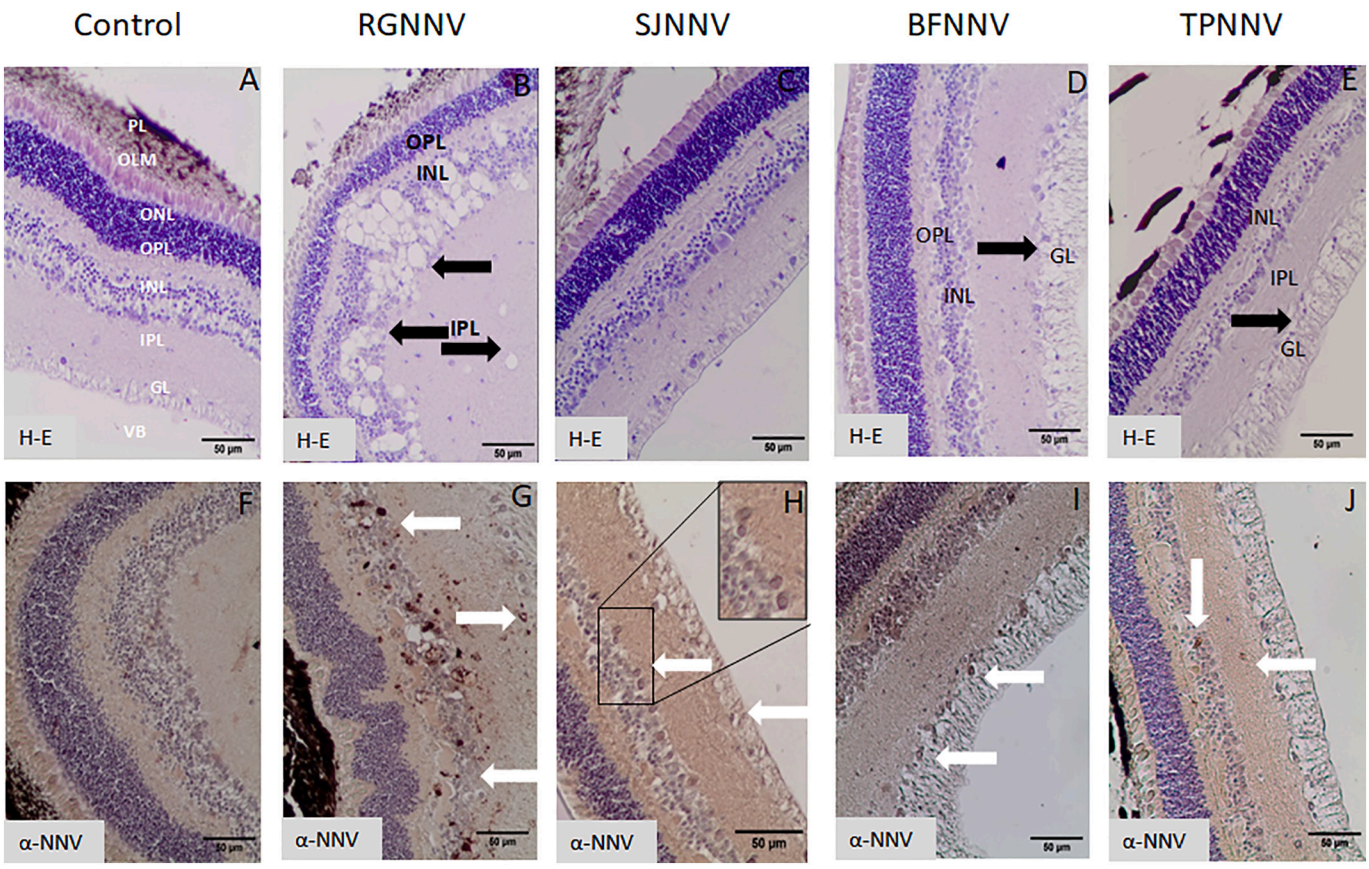

Fig. 4. Representative micrographs of the shi drum retina after 8 days of intramuscular injection with PBS (Control, A, F); 10^7 $TCID_{50}$/fish of RGNNV (B, G); 10^8 $TCID_{50}$/fish of SJNNV (C, H); BFNNV (D, I); or TPNNV (E, J); stained with haematoxilin-eosin (H–E) (A–E) or subjected to indirect immunohistochemistry to detect the NNV capsid protein (α-NNV) (F–J). Scale bar = 50 μm (A–J). Pigment layer (PL), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GL), vitreous body (VB). Black arrows denote cell degradation and vacuolisation; white arrows denote cells stained with the antibody against the viral capsid protein.

replication (Bandín and Souto, 2020). The optimal temperature for BFNNV and TPNNV genotypes is 15–20 °C and 20 °C, respectively (Iwamoto et al., 2000), closer to the temperature used in this study, 19 ± 1 °C, than the optimal temperature for RGNNV (25–30 °C). In addition to water temperature, NNV replication *in vivo* has been reported to be regulated by genetic features of the viral strain in European sea bass (Toffan et al., 2016). This could explain why the survival rates in the BFNNV- and TPNNV-infected groups were the lowest, which is also concomitant with the highest recovery of infective particles from the shi drum brain.

When we focused on the RGNNV, infected shi drum specimens showed very low recovery of infective particles from the brain at 8 dpi, as we needed two blind passages to titre them. In contrast, we observed high immunostaining in the brain and a high vacuolisation of the brain and retina. It has been demonstrated that the amount of viral infective particles depends on several factors, such as the evading anti-viral host immune response, successful viral RNA production and encapsulation and maturation of virions (Yong et al., 2017). Moreover, it has been described that up-regulation of some apoptotic genes significantly inhibits RGNNV replication (Xiang et al., 2019). Although further studies are needed to demonstrate this issue in shi drum specimens infected with RGNNV, the promotion of cell apoptosis to repress viral replication would explain our contradictory data in the RGNNV group. Although the NNV load in the brain is low, perhaps their infectivity is very high, or the host is very susceptible, producing devastating effects at the tissue and organism levels.

On the other hand, the SJNNV genotype produced very light signs of VER disease (from day 7) much later than any other genotype. Although SJNNV produced mortality (from day 16 onwards) and the virus was detected in some brain cells, no brain histopathological lesions were observed or infective particles isolated at day 8 of infection, that is, the first day after the apparition of signs. This could agree with the observations done in cell lines infected with SJNNV where the cytopathic effect and the viral dose were not correlated (Panzarin et al., 2014). It is also possible that 8 days was not enough for SJNNV to complete the production of infective particles in our experimental conditions, although viral protein production was detected with the antibody. Also, and similar to our data, in a natural outbreak of RGNNV in cultured shi drum in Greece, no vacuolisation of the brain was observed in any of the brains sampled, though mortality was recorded and the virus was identified by PCR (Katharios et al., 2010). So, we can conclude that 8 days of infection is enough for BFNNV, TPNNV and RGNNV to produce infective particles at different loads, but not for SJNNV. However, our data cannot discard the production of infective particles by SJNNV in the shi drum later on, when mortalities are occurring. Therefore, further studies will be needed to clarify this issue.

The histological diagnosis of VER includes vacuolisation of the retina (see for review Costa and Thompson (2016)); however, in this work, we observed that not all NNV genotypes affected the same layers of the retina at 8 dpi. As in the brain, RGNNV produced high vacuolisation in the OPL, INL and IPL, while in a natural outbreak of RGNNV, the vacuoles were observed in the ONL, INL and IPL (Katharios et al., 2010). Our data also showed that viral capsid protein was detected in the OPL, INL and IPL, and in some cells of the GL, RGNNV being the only genotype in which the viral protein was detected in the OPL. SJNNV- or BFNNV-positive cells were detected only in the INL or GL, respectively,

and the TPNNV genotype was located in the INL, IPL and GL, but not in the OPL. Interestingly, for these last three genotypes, opaque eyes and exophthalmia were observed later during the infection period.

As previously described in European sea bass, at different times of infection, the virus progressively infects different layers, from the outer layer to deeper layers, reaching the optic nerve and the medullar regions after 6 days of infection, when alterations of the different layers and the microglia are evident (Valero et al., 2018). Although this kinetic study should be performed in the shi drum to determine the disruption of different parts of the eye, the progressive time-dependent effect of the retina layers might explain why SJNNV, BFNNV and TPNNV produce opaque eyes and exophthalmia in some fish after long-term infections (25, 21 or 21 dpi, respectively), even when the alterations observed in the retina at 8 dpi were less abundant than those produced by RGNNV. These alterations will not allow fish to see, as previously suggested for European sea bass (Valero et al., 2018).

5. Conclusions

Although the infection performed in this work cannot reflect natural infections because of the route of infection and the viral concentration used, this work demonstrates that shi drum juveniles are susceptible to all four genotypes of NNV and represent the first step in studying host–NNV interactions and immune responses in this species. However, we cannot make comparisons among the NNV genotypes since the experimental conditions were the same and not optimal for each one. All NNV genotypes produced signs of disease compatible with VER disease, including brain and retina histopathological lesions and mortalities in the shi drum at 19 ± 1 °C. The NNV capsid protein was immunodetected in the brain and retina from shi drum specimens infected with all the NNV genotypes, using a commercial antibody that cross-reacts with the different genotypes. Interestingly, except for SJNNV, we were able to isolate infective particles from the brain of NNV-infected specimens at 8 dpi, earlier than the appearance of the first mortalities. Host-NNV interactions in the case of the shi drum need to be investigated to include it in aquaculture diversification in the Mediterranean area.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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