Contents lists available at ScienceDirect

Aquaculture

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Short communication

Viral nervous necrosis outbreaks caused by the RGNNV/SJNNV reassortant betanodavirus in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*)

E. Volpe[a], A. Gustinelli[a], M. Caffara[a], F. Errani[a], F. Quaglio[b], M.L. Fioravanti[a], S. Ciulli[a,*]

[a] *Department of Veterinary Medical Sciences, Alma Mater Studiorum University of Bologna, Ozzano Emilia (BO), Italy*
[b] *Department of Comparative Biomedicine and Food Science, University of Padua, Legnaro, Italy*

A B S T R A C T

Mediterranean marine aquaculture has suffered significant economic losses due to viral nervous necrosis (VNN) outbreaks mainly caused by different RGNNV betanodavirus strains. In recent years, the marine aquaculture sector has experienced the emergence of the RGNNV/SJNNV reassortant betanodavirus, harbouring the RNA1 segment of RGNNV genotype and the RNA2 segment of SJNNV genotype. So far, the reassortant strains caused massive mortality outbreaks in gilthead sea bream (*Sparus aurata*) larvae sparing the European sea bass (*Dicentrarchus labrax*). In this study, multiple mortality outbreaks occurred in one Italian marine hatchery involving both European sea bass and gilthead sea bream at different life stages were investigated through a complete microbiological and molecular analysis. Gilthead sea bream larvae and juveniles have recorded the highest mortality rates, however, both European sea bass and gilthead sea bream incurred a RGNNV/SJNNV reassortant betanodavirus persistent infection, able to act as asymptomatic carriers and viral source for susceptible fish. These new epidemiological data on nervous necrosis virus (NNV) reassortant infection provide precious advice on how to manage fish to reduce VNN spread in Mediterranean aquaculture. Evidence of interspecies transmission of RGNNV/SJNNV reassortant strains and the persistent infection in both European sea bass and gilthead sea bream, point out the importance to enforce a wide surveillance and a strict biosecurity programme addressing both RGNNV and reassortant RGNNV/SJNNV betanodaviruses in Mediterranean European sea bass and gilthead sea bream farms. Furthermore, the presence assessment of betanoviruses in all newly-introduced fish batches in the farm, regardless of the species and a strict segregation between European sea bass and gilthead sea bream batches within farms can significantly reduce the risk of NNV transmission. Finally, surviving fish can act as carrier fish, and thereby must be segregated from other batches and protected from stress conditions that could trigger a new clinical phase.

1. Introduction

Viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER), represents one of the main causes of economic losses in Mediterranean aquaculture. VNN is caused by a naked positive-sense single-stranded RNA virus belonging to the genus *Betanodavirus*, family *Nodaviridae* (Thiéry et al., 2012). The genome of the nervous necrosis virus (NNV) consists of two segments named RNA1 (3.1 Kb) and RNA2 (1.4 Kb) which code for the RNA-dependent RNA polymerase and the coat protein, respectively. Based on the phylogenetic analysis of the T4 variable region within the RNA2 segment, betanodaviruses have been clustered into four genotypes, currently accepted by the International Committee on Taxonomy of Viruses (ICTV)

as official species of this genus: Striped jack nervous necrosis virus (SJNNV), Tiger puffer nervous necrosis virus (TPNNV), Barfin flounder nervous necrosis virus (BFNNV) and Redspotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997; Thiéry et al., 2012). A large number of fish species have proven to be susceptible to the NNV, including economically-relevant farmed species such as European sea bass (*Dicentrarchus labrax*, ESB) and gilthead sea bream (*Sparus aurata*, GSB) and several wild fish species (Ciulli et al., 2007; Toffan et al., 2016; Doan et al., 2017).

The presence of a reassortant betanodavirus strain containing the RNA1 segment from the SJNNV genotype and the RNA2 segment from the RGNNV-type (SJNNV/RGNNV) was first reported in ESB in 2007 (Toffolo et al., 2007). More recently, a new reassortant strain (RGNNV/

SJNNV), whose genome is composed of RGNNV-type RNA1 and SJNNV-type RNA2, responsible for severe outbreaks has been detected in GSB, Senegalese sole (*Solea senegalensis*) and common sole (*Solea solea*) from the Mediterranean basin (Olveira et al., 2009; Toffan et al., 2017). In particular, GSB seems to be the most susceptible Mediterranean species to the RGNNV/SJNNV reassortant betanodavirus. The susceptibility of ESB to RGNNV/SJNNV reassortant betanodavirus was demonstrated via experimental infection even if the mortality rates were lower (< 25%) than those reported for parental RGNNV strains (Vendramin et al., 2014). Despite these studies, few data are available on the pathogenicity and epidemiology of the RGNNV/SJNNV reassortant strains under field conditions where several factors such as host, environment and virus features contribute to the course of the infection. As a result, the increase in the occurrence of VNN outbreaks due to reassortant betanodavirus strains represents a new challenge for Mediterranean aquaculture.

In this study, multiple mortality outbreaks that occurred in one Italian marine hatchery involving both ESB and GSB at different life stages were described. These outbreaks were studied through a complete microbiological and molecular analysis, which enabled us to identify a RGNNV/SJNNV reassortant betanodavirus strain as the responsible agent of the mortalities and to expand the knowledge on the epidemiology of this infection in both ESB and GSB.

2. Materials and methods

2.1. Case history and sampling

In December 2017, an Italian ESB and GSB hatchery reported a mortality outbreak involving larvae of both species. Larvae of 20–25 days post hatch (dph) were kept in recirculation systems supplied with marine water (density 150/200 larvae per litre, salinity 37‰, temperature at 19 °C and 20 ± 1 °C for GSB and ESB respectively). ESB larvae showed irregular swimming while GSB larvae rested near the surface due swim bladder hyperinflation. The cumulative mortality rate during the outbreak, lasting 3–4 days, attained 10% and around 95% in ESB and GSB respectively. Six months later (May 2018), the ESB batch (mean weight 4 g) survived the first outbreak in 2017 started to exhibit anorexia, lethargy and redness of the snout. ESB juveniles were kept in recirculation systems supplied with marine water (density 15 kg/m^3, salinity 37‰, temperature at 20 ± 1 °C). Similarly to the previous outbreak the mortality rate reached 10%.

In August 2018, a third severe mortality outbreak hit the batch of GSB (mean weight 6.8 g) survived from the first outbreak. No clinical signs were observed except for mortality reaching 100%. GSB juveniles were kept in recirculation systems supplied with marine water (density 15 kg/m^3, salinity 37‰). Tanks were provided with marine water, not refrigerated, so the water temperature was 28 °C during the August outbreak consistent with seasonal sea water temperature.

Samples from each species involved in the different outbreaks were collected and sent refrigerated to the laboratory for microbiological investigations. Affected GSB larvae from the first outbreak were fixed in 10% buffered formalin on site and sent to the laboratory for histological analysis.

2.2. Parasitological analysis

Fish from all batches were subjected to parasitological examination by microscopical observation of fresh mounts from skin, gills and visceral organs.

2.3. Bacteriological analysis

Pooled larvae and brain of juveniles were inoculated in Tryptone Soy Agar (TSA, Oxoid, United Kingdom) supplemented with 1.5% NaCl and Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Oxoid). Plates

were incubated at 25 ± 1 °C for 1 week (Austin, 2016).

2.4. Virological analysis

2.4.1. Virus isolation on cell culture and viral titration

Samples of whole larvae (100 mg) and brain (*n* = 5) of specimens were collected during the outbreaks and subjected to viral isolation onto SSN-1 cells (Frerichs et al., 1996). The incubation was carried out at 20 °C and 25 °C. Monolayers were observed daily by microscopy for cytopathic effects (CPE) for one week. In case that no CPE was observed two subcultures were carried out. Samples negative to the third subculture were tested by RT-nested PCR for betanodavirus presence as described below (Section 2.4.2). Cell culture supernatants with CPE were collected and stored at −80 °C.

The viral titrations for all viral strains isolated at 25 °C (Sa-416-Dec17, Dl-417-Dec17, Dl-484-May18, Sa-528-Aug18) and for Sa-416-Dec17 isolated at 20 °C and 25 °C were performed in order to quantify the viral infectious capacity and the influence of the isolation temperature on viral replication. The titrations were performed in triplicate in 96-well plates incubated at 20 °C or 25 °C (Volpe et al., 2017). The titre was expressed as $TCID_{50}$ ml^{-1} and calculated using Spearman-Karber method (Hierholzer and Killington, 1996). Viral titres obtained at different temperatures, after testing for normality, were analyzed by 1-way ANOVA followed by Tukey's tests to determine statistically the differences among virus titres detected (Prism version 6.0 software, GraphPad Software). Throughout, the level for accepted statistical significance was $p < 0.05$.

2.4.2. Molecular investigations, genetic characterization and phylogenetic analysis

Whole larvae (20 mg) of GSB and ESB involved in the first outbreak were processed, while brain samples (*n* = 5) were collected from affected fish during the second and third outbreak. The tissues were homogenized and the RNA was extracted according to the manufacturer's instructions with NucleoSpin RNA (Macherey-Nagel, Duren, Germany). The presence of betanodavirus was considered by two RT-PCR methods previously described (Volpe et al., 2018).

The amplification step was conducted through a one-step RT-PCR assay with primers VNNV5 (5′-GTTGAGGATTATCGCCAACG-3′) and VNNV6 (5′-ACCGGCGAACAGTATCTGAC-3′; Toffolo et al., 2007) or S6 (5′-ATGGTACGCAAAGGTGATAAGAAA-3′) and S7 (5′-GTTTTCCGAGT CAACACGGGT-3′; Ciulli et al., 2006) targeting the RNA1 and RNA2 viral genome respectively.

PCR products were purified with ExoSAP-IT® (Affymetrix, Santa Clara, USA) according to the manufacturer's instructions and then sequenced at Bio-Fab Sequencing Service (Rome, Italy).

RNA1 and RNA2 nucleotide sequences were aligned and compared with parental betanodavirus sequences previously obtained from isolates detected in finfish and aquatic invertebrates, a number of reassortant RGNNV/SJNNV strain sequences, and the betanodavirus reference strains (Table S1) available in GenBank (www.ncbi.nlm.nih.gov) using Clustal W implemented in the BioEdit software (http://bioedit.software.informer.com/). To infer the phylogenetic relationships among the betanodavirus strains, the maximum likelihood (ML) method, with the general time-reversible (GTR) nucleotide substitution model (Darriba et al., 2012), available in MEGA 7 software (www.megasoftware.net) was used. One thousand bootstrap replicates were performed to assess the robustness of individual nodes, and only values ≥70% were considered significant. The percentage of pairwise nucleotide and amino acid identity among the RNA1 and RNA2 were calculated with the BioEdit software comparing sequences of the strain characterized in this study with betanodavirus reference strain sequences and betanodavirus sequences used for the phylogenetic analysis (Table S1).

2.5. Histology

During December 2017 outbreak, whole GSB larvae were fixed in 10% neutral buffered formalin at the hatchery and then processed for histology. Samples were dehydrated and embedded in paraffin wax. Cross-sections of 4 μm have been stained with Hematoxyline-Eosine (HE).

3. Results

3.1. Parasitological analysis

All the samples were negative for parasites.

3.2. Bacteriological analysis

No fish-pathogenic bacteria were isolated from larvae and juveniles of ESB and GSB examined during the three different outbreaks.

3.3. Virological analysis

3.3.1. Virus isolation on cell culture and viral titration

The presence of betanodavirus in the specimens collected during the three outbreaks was confirmed by virus isolation. Accordingly, all the samples presented betanodavirus-typical CPE on SSN-1 cells incubated at 25 °C consisting of wide vacuolization of the monolayer. The sample Sa-416-Dec17 and Dl-417-Dec17 obtained from GSB and ESB larvae collected during the first outbreak showed CPE when incubated both at 20 °C and 25 °C (Table 1).

The viral titration analysis in SSN-1 cells showed that the $TCID_{50}$ ml^{-1} values for all strains titrated at 20 °C were statistically lower than the values obtained at 25 °C (Fig. 1).

3.3.2. Molecular investigations, genetic characterization and phylogenetic analysis

All batches collected during the survey resulted positive for betanodavirus by both RT-PCR methods targeting RNA1 and RNA2 fragments. The molecular assay allowed us to obtain both RNA1 and RNA2 partial sequences of the betanodavirus genome from the four samples (Sa-416-Dec17, Dl-417-Dec17, Dl-484-May18, Sa-528-Aug18). The sequences were submitted to GenBank database (Accession number MN896006-MN896012). The sequences obtained from samples from the different outbreaks and host species showed 100% nucleotide and amino acid identities, demonstrating the circulation of the same viral strain during the different outbreaks.

Regarding RNA1, the virus showed the highest nucleotide and amino acid identities with the RGNNV genotype (96.6% and 98%, respectively). By contrast, the nucleotide and amino acid identities with other genotypes (BFNNV, SJNNV, TPNNV) were always < 82% and < 90.2% respectively (Table 2). Regarding RNA2, the virus detected in
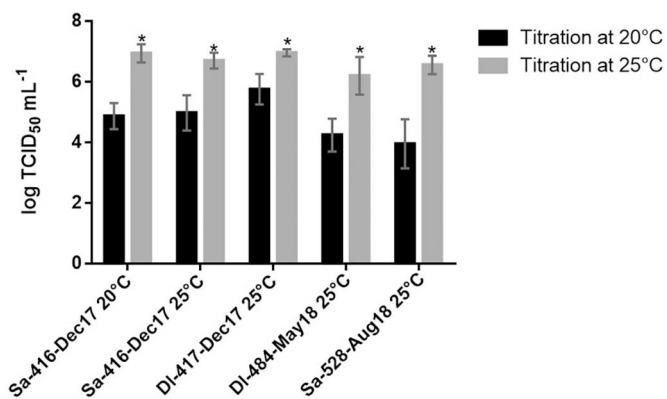

Fig. 1. Viral titration assays in SSN-1 cells performed at 20 °C and 25 °C of strains Sa-416-Dec17 isolated at 20 °C and 25 °C and strain Dl-417-Dec17, Dl-484-May18 and Sa-528-Aug18 isolated at 25 °C. Bars represent viral loads expressed as $TCID_{50}$ ml^{-1} obtained at different titration temperatures. Asterisk (*) indicates significantly differences between $TCID_{50}$ ml^{-1} values obtained at 20 °C and 25 °C ($p < .05$).

Table 2
Comparisons of nucleotide and amino acid sequences of the RNA1 and RNA2 fragments of the strain characterized in this study (Sa-416-Dec17) with betanodavirus reference strains (RNA1: RGNNV AY324869; BFNNV EU826137; SJNNV AB056571; TPNNV EU236148; RNA2: RGNNV AY324870; BFNNV EU826138; SJNNV AB056572; TPNNV EU236149). Percentages of pairwise nucleotide and amino acid identities are shown.

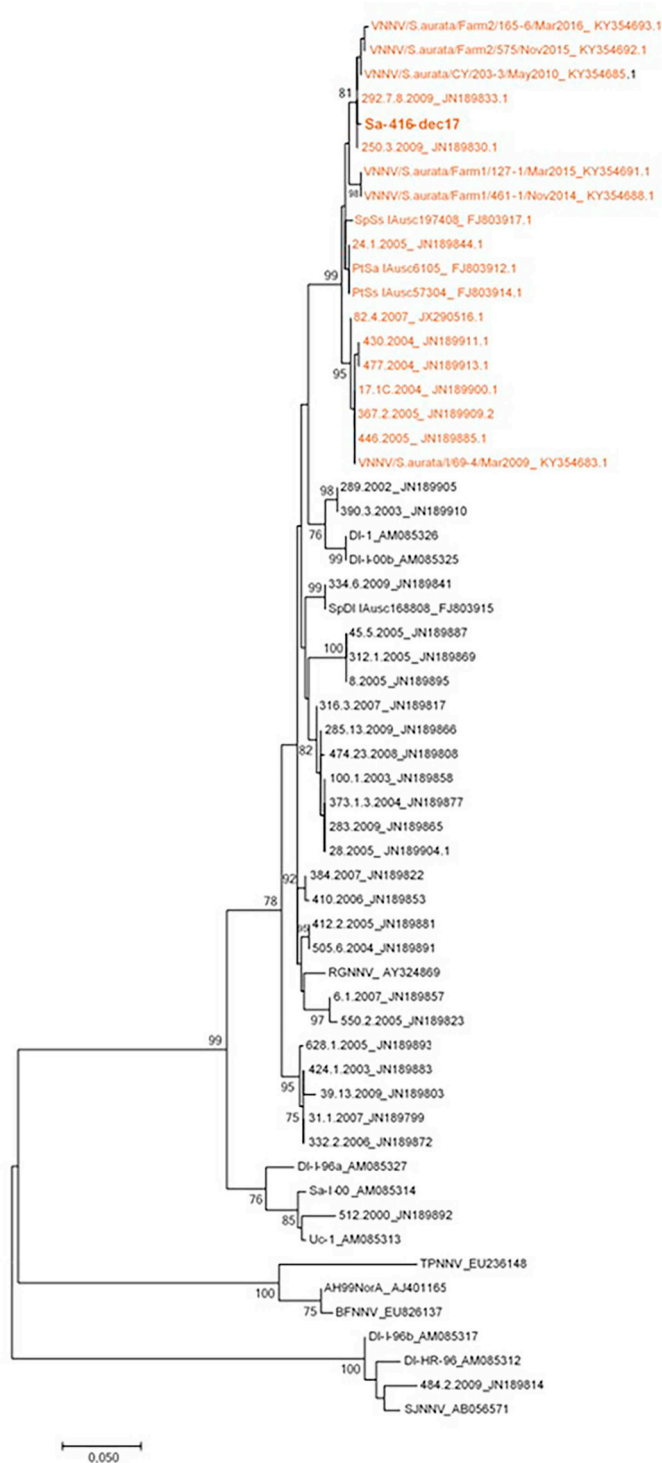|	TPNNV		BFNNV		RGNNV		SJNNV	
	aa	nt	aa	nt	aa	nt	aa	nt
Sa-416-Dec17_RNA1	89.9	81.0	90.2	82.0	98.0	96.6	90.2	81.1
Sa-416-Dec17_RNA2	81.6	80.4	81.3	75.9	81.9	78.3	99.0	98.3

this study showed the highest nucleotide and amino acid identities with the SJNNV genotype (98.3% and 99%, respectively). Contrarily, the nucleotide and amino acid identities with other genotypes (BFNNV, RGNNV, TPNNV) were always < 80.4% and < 81.9%, respectively (Table 2). Accordingly, the percentage values of nucleotide and amino acid identities of strain characterized in this study with the four betanodavirus genotypes suggest it might be a RGNNV/SJNNV reassortant betanodavirus.

The comparison of RNA1 sequence of viral strain detected in the studied outbreaks with those of selected sequences of reassortant and parental betanodaviruses showed the highest nucleotide and amino acid identities with all other reassortant strains (95.9–99.7% and 95.7–100% at nucleotide and amino acid level respectively) and particularly with two reassortant strains (99.7% and 100% at nucleotide and amino acid level respectively) isolated from *Artemia salina* and *Sparus aurata* (250.3.2009) and *Dicentrarchus labrax* (292.7.8.2009) (Table S2).

The ML phylogenetic trees of RNA1 viral fragment divided the analyzed strains in different clusters corresponding to the four betanodavirus genotypes. Notably, within the RGNNV cluster, one subgroup was composed exclusively of sequences originated from RGNNV/SJNNV reassortant strains (bootstrap 99%) isolated from both finfish and aquatic invertebrates throughout 2004–2016 in different countries from the Mediterranean basin. Consequently, the phylogenetic analysis of the RNA1 sequences showed that the detected betanodavirus clusters in the reassortant subgroup within the RGNNV group (Fig. 2).

Conversely, the phylogenetic analysis based on the RNA2 showed that the betanodavirus strain collected in this study clusters within SJNNV genotype group (Fig. 3).

Therefore, the strain detected in both GSB and ESB harbors the

Table 1
Results of virus isolation on cell culture conducted at different temperatures.

Sample ID	Isolation temperature	Cultures		
		1st	2nd	3rd
Sa-416-Dec17	20 °C	+	nd	nd
	25 °C	+	nd	nd
Dl-417-Dec17	20 °C	−	+	+
	25 °C	+	+	nd
Dl-484-May18	20 °C	−	−	−[a]
	25 °C	−	+	+
Sa-528Aug18	20 °C	−	−	−[a]
	25 °C	+	+	+

+: CPE detected; −: no CPE detected; nd: not determined.
[a] Supernatants were tested for NNV presence by RT-nested PCR.

Fig. 2. Maximum likelihood phylogenetic tree based on RNA1 nucleotide sequences. The strain characterized in this study is shown in bold. Sequences retrieved from GenBank are reported with the isolate name and accession number. RGNNV/SJNNV reassortant strains are labeled in orange. Bootstrap values > 70% are shown. Branch lengths are scaled according to the number of nucleotide substitutions per site. The scale bar is reported.

RNA1 and RNA2 fragments belonging to RGNNV genotype and SJNNV genotype respectively, demonstrating its reassortant nature.

3.4. Histological examination

Histological analysis of GSB larvae revealed the presence of

degenerative and vacuolated lesions in the central nervous system (CNS) and in the eye (Fig. 4A). In some larvae hyperinflation of the swim bladder was observed (Fig. 4B). Generalized and multifocal vacuolations defined by empty areas of 5–13 μm diameter were observed in the retina (Fig. 4C) and optic nerve, in the grey matter of the olfactory bulb, telencephalon, diencephalon, mesencephalon (Fig. 4D), cerebellum, medulla oblongata and spinal cord (Fig. 4E). The vacuoles were in the external and internal nuclear layers of the retina. Neuronal degeneration characterized by pyknosis and karyorrhexis was evidenced in all the nervous tissues of the fish (Fig. 4F). No histopathological changes were observed in other organs.

4. Discussion

This study reports a thorough investigation of three mortality outbreaks caused by a RGNNV/SJNNV reassortant betanodavirus strain in one marine hatchery. In the first outbreak, both GSB and ESB larvae exhibited clinical signs and mortalities caused by this viral strain. To the best of our knowledge, this is the first reported case of field mortality due to RGNNV/SJNNV reassortant betanodavirus strain infection involving both GSB and ESB simultaneously.

Both ESB and GSB are frequently infected by betanodavirus, but clinical signs and mortalities are generally described for only one species at a time because of different species' sensitivity towards various betanodavirus strains. RGNNV outbreaks are typically described in ESB, while, in general, no clinical signs are reported for GSB reared close to ESB tanks suffering high mortality rates, despite the fact that the virus was detected in both fish species (Chérif et al., 2009). Conversely, dramatic outbreaks caused by RGNNV/SJNNV betanodavirus strains were reported in GSB larvae and postlarvae (< 70 dph). Field trials carried out on 80–100 g ESB and GSB kept in cohabitation with surviving GSB showed their resistance to this strain since neither signs of disease nor virus were detected (Toffan et al., 2017). Several hypotheses were proposed to explain the lack of transmission, including the reduced infectivity of the GSB survivors after the acute phase and the expected resistance of the ESB to the reassortant RGNNV/SJNNV (Toffan et al., 2017).

In our study, interspecies RGNNV/SJNNV reassortant betanodavirus strain transmission between ESB and GSB was observed. In the first outbreak described in this study, ESB and GSB were exposed to each other during the acute phase of infection probably via contaminated equipment or water, imposing a high viral load in the aquatic environment. Furthermore, this outbreak involved ESB at larval stage, which are reported to be particularly susceptible to betanodavirus infection. This early stage infection had led us to hypothesise a vertical transmission. Furthermore, the broodstock consisted in both wild and own farmed fish, and the examination of the farm biosecurity program, pointed out the lack of surveillance towards NNV of broodstock, making the vertical transmission the primary suspect as the source of infection.

The pathogenic potential of the RGNNV/SJNNV reassortant strains in ESB has been demonstrated by experimental infections (Vendramin et al., 2014; Souto et al., 2015; Toffan et al., 2016). These studies showed ESB to be sensitive to a broad range of betanodaviruses including the RGNNV/SJNNV reassortant strains isolated from different host species including GSB and *Senegalese sole*. In most of the experimental trials, mortality induced by reassortant strains (1.5–25%) was lower than that obtained with an RGNNV parental genotype (10–36.25%; Souto et al., 2015; Toffan et al., 2016). However, high variability was observed when comparing mortalities induced by different strains belonging to the same genotype suggesting that other factors than the genotype of the virus can influence the course of the disease and the kinetics of mortality (Vendramin et al., 2014; Souto et al., 2015; Toffan et al., 2016).

The ESB mortality rate (10%) reported in the first and second natural outbreaks described in our study was consistent with those recorded after experimental infection in ESB with RGNNV/SJNNV
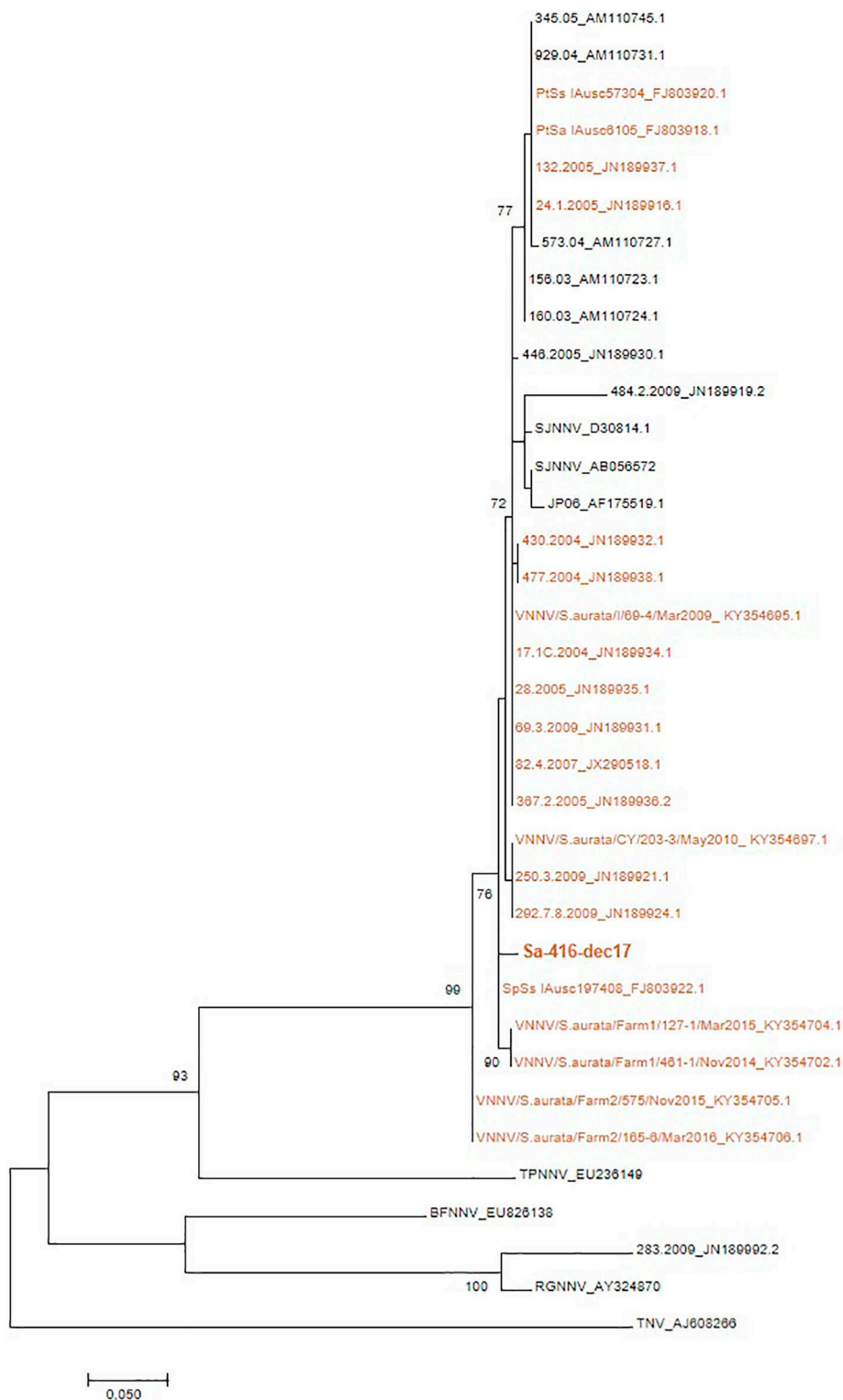
Fig. 3. Maximum likelihood phylogenetic tree based on RNA2 nucleotide sequences. The strain characterized in this study is shown in bold. Sequences retrieved from GenBank are reported with the isolate name and accession number. RGNNV/SJNNV reassortant strains are labeled in orange. Bootstrap values > 70% are shown. Branch lengths are scaled according to the number of nucleotide substitutions per site. The scale bar is reported.

reassortant strains (Vendramin et al., 2014). The same strain was responsible for a 95% mortality rate in GSB larvae, confirming previous reports that showed age-related sensitivity of this species with most of the mortality outbreaks in larvae < 70 dph (Toffan et al., 2017). In this regard, the mortality observed in the GSB juveniles (6.8 g) appears to be quite unusual, although different environmental conditions recorded

during the third outbreak may have strongly conditioned the outcome of the infection. Particularly, temperature can greatly influence the course of both natural and experimental infections (Vendramin et a. 2014; Toffan et al., 2016; Doan et al., 2017). In fact, betanodavirus replication in vivo is a composite process influenced by both the genetic features of the viral strain and water temperatures (Toffan et al., 2016).
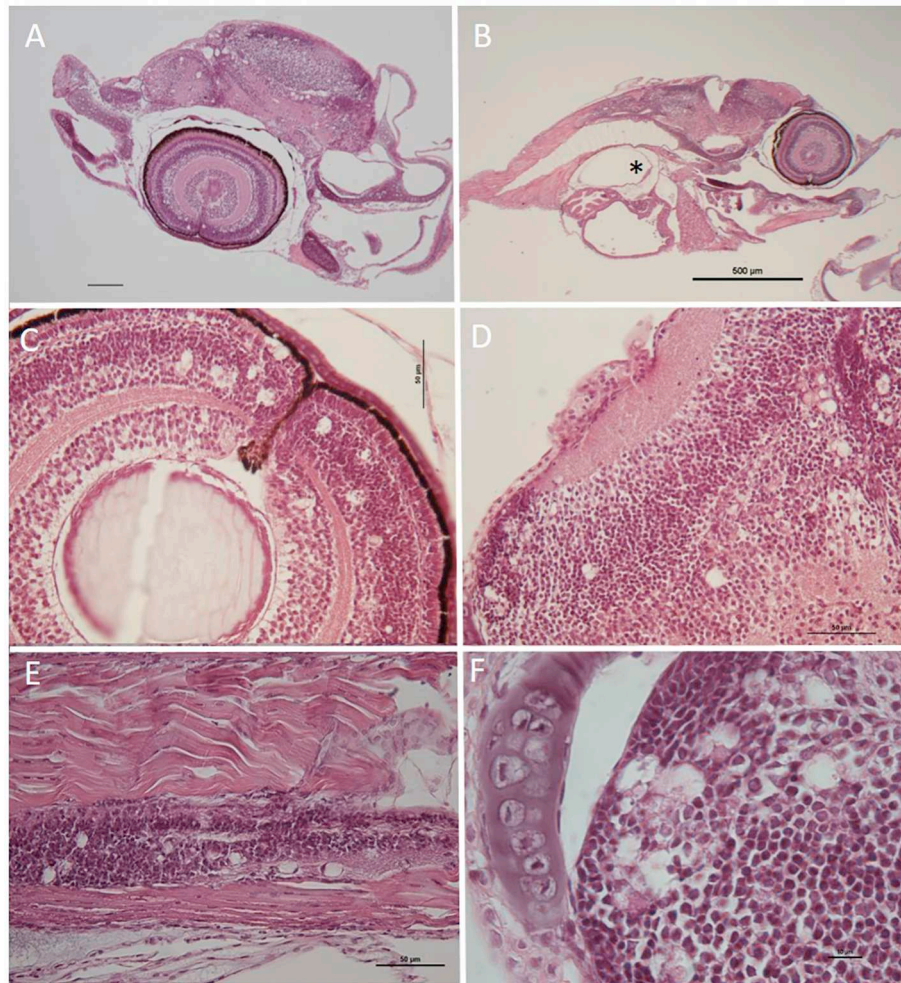
Fig. 4. Sections of the gilthead sea bream larvae at 20–25 dph affected by VNN. (A) Vacuolar lesions in the brain and the retina, (B) overinflated swim bladder (asterisk), (C) vacuoles in the external and internal nuclear layers of the retina; (D) vacuolated mesencephalon; (E) vacuolar degeneration of the spinal cord; (F) multifocal vacuolations with necrosis characterized by pyknosis and karyorrhexis. (Hematoxyline-Eosine).

Natural outbreaks induced by RGNNV/SJNNV reassortant strains in GSB larvae described so far occurred at temperature between 19 °C and 21 °C (Beraldo et al., 2011; Toffan et al., 2017). Accordingly, the first outbreak described in this study involving GSB was observed at 19 °C. Similarly, ESB outbreaks occurred at 21 °C both at larval and juvenile stages. This temperature is rather lower than the temperature generally associated with RGNNV outbreaks. However, in vitro analysis of the RGNNV/SJNNV reassortant strain isolated in this outbreak showed a higher replication activity at 25 °C than at 20 °C producing a significantly higher viral titre. This result was observed independently from the fish species and size, the virus was isolated from. In fact, all the viral isolates obtained from ESB and GSB showed a better fitness replication at 25 °C than at 20 °C. Previous studies showed that replication capacity can be affected by viral RNA polymerase coded by RNA1 and particularly, RGNNV-RNA1 has been associated with a better replication activity at higher temperature compared to SJNNV-RNA1 (Panzarin et al., 2014). Conversely, in vivo experimental trials showed that mortality rates associated with genotypes other than RGNNV are not significantly affected by temperature (Toffan et al., 2016), suggesting that host factors can affect the course of the disease and the mortality kinetics as well.

The August outbreak observed in GSB occurred at an anomalous temperature (28 °C). Furthermore, this outbreak involved 6.8 g GSB, significantly older than those usually affected by NNV-associated mortality (Comps and Raymond, 1996; Beraldo et al., 2011; Toffan et al., 2017). Only one previous report of NNV-associated mortality has

been described in GSB older than larval stage during the summer period at 28 °C (Bitchava et al., 2007). Both water temperature and bigger fish size made the third outbreak described in our study similar to that observed by Bitchava et al. (2007). Unfortunately, no genotyping was carried out on the strain responsible for the outbreak described by these authors, making comparison impossible. Nevertheless, in the outbreak we observed in 6.8 g GSB the high temperature might have acted as a stressor by itself, reducing the ability of GSB to face NNV infection.

Therefore, depending on several environmental and host factors, the infection with RGNNV/SJNNV reassortant strains can lead to variable rates of mortality in both ESB and GSB causing economic losses in marine aquaculture.

Furthermore, along with low ESB mortality records during the first outbreaks, the surviving ESB could have acted as asymptomatic viral carriers, leading to a further mortality outbreak in 4 g ESB with an increased cumulative mortality of the batch. Even though the overall losses due to ESB mortality induced by the reassortant strain were moderate in both outbreaks, the persistence of the virus in ESB may represent a relevant risk factor for the maintenance and spread of the infection to other susceptible fish.

A persistent infection was prompted by the RGNNV/SJNNV reassortant strain in surviving GSB as already suspected (Toffan et al., 2017). The finding of the same viral strain in GSB involved in the third outbreak may suggest that the GSB that survived the first outbreak showed a long-lasting infection with occurrence of mortality triggered by stressful conditions such as high temperature.

The observation of these natural outbreaks in a marine hatchery rearing both ESB and GSB, confirmed that both ESB and GSB can act as a viral reservoir of RGNNV/SJNNV betanodavirus able to transmit the infection to susceptible fish. Due to interspecies transmission of RGNNV/SJNNV reassortant strains and the persistent infection in both ESB and GSB, wide surveillance and a strict biosecurity enforcement programme addressing both RGNNV and reassortant RGNNV/SJNNV betanodaviruses are strongly recommended in Mediterranean ESB and GSB farms. Regarding diagnostics, 25 °C has been showed as the most successful temperature to isolate both RGNNV/SJNNV reassortant and RGNNV parental strains regardless of the temperature at which the outbreaks occur. Concerning the direct prophylaxis, it is mandatory to assess the presence of betanoviruses in all newly-introduced fish batches in the farm, regardless of species. Furthermore, strict segregation between ESB and GSB batches within farms can significantly reduce the risk of NNV transmission considering the possibility of viral transmission during both clinical and asymptomatic phases. Finally, surviving fish can act as carrier fish, and therefore must be segregated from other batches and protect from stress conditions that could trigger a new clinical phase.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735155.

Declaration of Competing Interests

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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