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Water temperature affects pathogenicity of different betanodavirus genotypes in experimentally challenged *Dicentrarchus labrax*

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ABSTRACT: Betanodaviruses are the causative agents of a highly infectious disease of fish known as viral nervous necrosis (VNN). To date, 4 different nervous necrosis virus (NNV) genotypes have been described, but natural reassortant viruses have also been detected, which further increase viral variability. Water temperature plays an important role in determining the appearance and the severity of VNN disease. We assessed the effect of temperature (20°, 25° and 30°C) on mortality and virus load in the brain of European sea bass *Dicentrarchus labrax* experimentally infected with 4 genetically different betanodaviruses, namely red-spotted grouper NNV (RGNNV), striped jack NNV (SJNNV) and the reassortant strains RGNNV/SJNNV and SJNNV/RGNNV. The RGNNV/SJNNV virus possesses the polymerase gene of RGNNV and the coat protein gene of SJNNV, and vice versa for the SJNNV/RGNNV virus. The obtained results showed that the RGNNV strain is the most pathogenic for juvenile sea bass, but clinical disease and mortality appeared only at higher temperatures. The SJNNV strain is weakly pathogenic for *D. labrax* regardless of the temperature used, while virus replication was detected in the brain of survivors only at 20°C. Finally, reassortant strains caused low mortality, independent of the temperature used, but the viral load in the brain was strongly influenced by water temperature and the genetic type of the polymerase gene. Taken together, these data show that nodavirus replication *in vivo* is a composite process regulated by both the genetic features of the viral strain and water temperatures.

KEY WORDS: Viral nervous necrosis · European sea bass · Reassortant · RGNNV · SJNNV · Infection

INTRODUCTION

Fish are poikilothermic animals and, as a consequence, their pathogens (parasites, bacteria, viruses) need to adapt to variations in water temperature, which becomes one of the key factors for disease onset and development. This is particularly true for viral infections, where the effect of water temperature acts mainly through 2 primary mechanisms: by driving viral fitness (e.g. replication capacity) and by triggering or inhibiting the immune response of the host. Such a phenomenon has been described for some of the most important viral fish pathogens, including viral haemorrhagic septicaemia virus (Avunje et al. 2012, Vo et al. 2015), koi herpesvirus (Rakus et al. 2013), spring viraemia of carp virus (Ahne et al. 2002) and betanodavirus (Le Breton et al. 1997, Chi et al. 1997, Maltese & Bovo 2007, Vendramin et al. 2013).

Betanodavirus is the causative agent of a highly infectious disease of fish, known as viral nervous necrosis (VNN). VNN is considered one of the most important threats to global aquaculture, particularly Mediterranean mariculture, affecting fish belonging

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to 44 species and 24 families worldwide (Sano et al. 2011). Betanodaviruses are small, spherical, non-enveloped RNA viruses with a genome made of 2 genetic segments: RNA1 (3.1 Kb) encoding the RNA-dependent RNA-polymerase (RdRp), and RNA2 (1.4 Kb) encoding the capsid protein (Mori et al. 1992). An additional sub-genomic transcript, called RNA3 (0.4 Kb), originates from the 3′ terminus of the RNA1 molecule, and encodes the non-structural proteins B1 and B2 (Fenner et al. 2006, Chen et al. 2009). Betanodaviruses are classified into 4 different genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer NNV (TPNNV), barfin flounder NNV (BFNNV) and red-spotted grouper NNV (RGNNV) (Nishizawa et al. 1997). Natural reassortment events can occur between the RGNNV and SJNNV genotypes, and to date, 2 types of reassortants have been detected. The RGNNV/SJNNV harbours the polymerase gene of the RGNNV and the coat protein gene of the SJNNV, whereas the SJNNV/RGNNV possesses the RNA1 segment of the SJNNV and the RNA2 gene of the RGNNV (Toffolo et al. 2007, Olveira et al. 2009, Panzarin et al. 2012).

Genetically different betanodaviruses show diverse optimal growth temperatures in cell culture. The most psychrophilic betanodavirus genotypes include the BFNNV and the TPNNV species, whose optimal culture temperature ranges between 15 and 20°C (Iwamoto et al. 2000). The SJNNV genotype shows its best replication fitness *in vitro* at 25°C. Notably, the RGNNV species shows the widest tolerance to temperature *in vitro* (15 to 35°C; Ciulli et al. 2006, Hata et al. 2010), with an optimum between 25 and 30°C (Iwamoto et al. 1999). The *in vitro* replication capacity at different incubation temperatures of the reassortant strains RGNNV/SJNNV and SJNNV/RGNNV depends on their RNA1-type, leading to the conclusion that temperature dependency of fish nodaviruses is mainly regulated by the RNA-dependent RNA polymerase gene. In particular, the RGNNV-RNA1 confers the capacity of efficiently replicating at higher temperatures (30°C; Hata et al. 2010, Panzarin et al. 2014). Taken together, these data suggest that betanodavirus multiplication is a temperature-sensitive process, resulting from viral adaptation to different environmental conditions. In agreement with this hypothesis, we can infer that distinctive temperature preferences of different betanodavirus genotypes reflect their geographic distribution. While BFNNV occurs only in the cold waters of the northern Atlantic and Japan, the RGNNV genotype causes mortalities worldwide, including the warm waters of the Mediterranean Basin, the Indian Ocean and the Asian side of the Pacific Ocean. Notably, the SJNNV genotype seems to be restricted to Spain and Japan, while VNN outbreaks resulting from reassortant strains have been described only in the Mediterranean Sea (Cutrín et al. 2007, He & Teng 2015).

Water temperature influences the betanodavirus course of infection and plays an important role in determining the severity of the diseases (Fukuda et al. 1996, Le Breton et al. 1997, Chi et al. 1999, Maltese & Bovo 2007, Vendramin et al. 2013, Souto et al. 2015b). Here we evaluated the effect of different temperatures on the mortality rate and viral load in brains of surviving European sea bass *Dicentrarchus labrax* juveniles experimentally infected with RGNNV, SJNNV, RGNNV/SJNNV and SJNNV/RGNNV strains.

MATERIALS AND METHODS

Fish

Juvenile sea bass (2–3 g, approximately 3 mo old, n = 2000) were purchased from an Italian commercial hatchery with no history of VNN disease. Fish were housed in a 2500 l tank at 22°C and gradually adapted to 25‰ salinity (1 ppm d^{-1} starting from 33‰ salinity in the hatchery). Commercial feed was supplied once a day (1% of live weight estimation), and artificial light was provided for 10 h d^{-1}.

In order to further ascertain the absence of betanodavirus in the batch, 50 fish were randomly selected, euthanized at arrival and tested by real-time reverse transcription (RT)-PCR (Panzarin et al. 2010) and virus isolation (Frerichs et al. 1996).

Viruses

Four isolates of *Betanodavirus* with diverse genomes (283.2009: RGNNV; 367.2.2005: RGNNV/SJNNV; 389/I96: SJNNV/RGNNV; 484.2.2009: SJNNV) were used for the experimental challenge of sea bass juveniles (Table 1). They were fully sequenced, and their growth kinetics on cell culture in response to temperature had already been thoroughly investigated by Panzarin et al. (2014).

Viral strains were multiplied in E-11 cells (European Collection of Authenticated Cell Cultures, ECACC no. 01110916; Iwamoto et al. 2000) at 25°C, with Leibovitz medium (L-15) (Sigma) containing 10% fetal bovine serum (FBS), L-glutamine (2 mM)

and antibiotics (100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B). Infected monolayers were checked regularly for the presence of cytopathic effect (CPE), and cell supernatant was collected upon disruption of cell monolayers. The supernatant was clarified by centrifugation at 4000 × *g* (15 min at 4°C) and then titrated. Titration was performed (in quadruplicate) on 1 d old E-11 cells seeded in 96-well plates (Corning) with L-15, supplemented with 10% FBS, L-glutamine (2 mM) and antibiotics. Plates were observed after 10 d for CPE appearance, and viral titre was calculated according to the Spearman and Karber formula (Finney 1978). All isolates used in this study were at seventh passage on E-11 cells.

Table 1. Nervous necrosis virus (NNV) strains used in this study. Genotype refers to the source of the polymerase / capsid protein genes (RNA1/ RNA2, respectively). SJNNV: striped jack NNV; RGNNV: red-spotted grouper NNV

Virus strain	Origin		Genotype		GenBank accession no.	
	Species	Country	RNA1	RNA2	RNA1	RNA2
283.2009	*Dicentrarchus labrax*	Italy	RGNNV	RGNNV	JN189865	JN189992
367.2.2005	*Dicentrarchus labrax*	Italy	RGNNV	SJNNV	JN189909	JN189936
389/I96	*Dicentrarchus labrax*	Italy	SJNNV	RGNNV	KF386163	KF386164
484.2.2009	*Solea senegalensis*	Spain	SJNNV	SJNNV	JN189814	JN189919

Experimental design

Fish were divided into 15 groups of 130 fish each, and kept in 300 l fibreglass tanks. Groups of 5 tanks were gradually adapted to different water temperatures (1°C d^{-1}): 20, 25 and 30°C. Once fully adapted to the selected water temperature, 4 groups (tanks) of fish were infected by bath immersion with theoretical 10^5 $TCID_{50}$ ml^{-1} of the 4 different betanodaviruses. Infection was performed by moving fish to separate 25 l tanks with aeration, and by adding different volumes of each challenge virus, calculated according to the inoculum viral titre. After 4 h, fish were moved back to their former 300 l tanks. The fifth group was used as negative uninfected control, 1 for each incubation temperature. Control fish were mock infected following the above described procedure, by using minimum essential medium instead of the viral inoculum. After infection, water samples collected from each tank were back-titrated in cell culture according to the same procedure used for viruses.

Fish were checked twice a day, and animals showing severe clinical signs, as well as moribund and dead fish, were collected and subjected to necropsy, and brain specimens were sampled. The experiment was terminated at 30 d post challenge (dpc), and all surviving fish were euthanized by an overdose of anaesthetic (MS-222). Real-time RT-PCR targeting the RNA2 gene (Panzarin et al. 2010) was performed on brains collected from dead fish in order to confirm the cause of death. A proportion of randomly selected live fish at the end of the experiment (n = 13 $tank^{-1}$, corresponding to 10% of total infected fish $group^{-1}$) was tested by RNA2 real-time RT-PCR to establish viral load in brains of survivors. Cumulative mortality was calculated based on the number of dead fish counted at the end of the experiment.

The experiment protocol was evaluated and approved by the Internal Ethic Commission (Opinion CE.IZSVE.4/2012) and the Italian Ministry of Health (Law decree 237/2012 of 10/10/2012).

Statistical analysis on cumulative mortality

Statistical analyses were performed using STATA 12.1 software. The number of fish per experimental group was established taking into account the following factors: (1) the expected mortality, estimated between 10 and 30%, as previously reported by Vendramin et al. (2014), (2) a low estimated increase of mortality (15%) due to temperature variation and (3) setting α error (1-sided) at 0.10 and power at 80% ($1 - \beta = 0.80$).

To estimate the survival function from lifetime data, the non-parametric Kaplan-Meier method was used, which allowed the survival curve to be drawn as a step curve for each experimental group, measuring the length of time the fish survives the infection (van Belle et al. 2004). To compare survival curves under different experimental conditions, the Wilcoxon nonparametric test was used.

For each challenge group, the prevalence at the end of the experiment and the relative binomial exact 95% confidence interval were calculated. Fisher's exact test was used to compare the cumulative mortality at the end of the experiment between 2 or more experimental groups infected with the same virus at different temperatures, because one of the

Table 2. Viral titres obtained from water collected at the end of sea bass *Dicentrarchus labrax* challenge (4 h). Viral titres are expressed as $TCID_{50}$ ml^{-1}. NNV genotypes are described in Table 1

Virus strain	NNV genotype	Titre at temperature 20°C	25°C	30°C
283.2009	SJ/SJ	$10^{5.55}$	$10^{4.3}$	$10^{4.8}$
367.2.2005	RG/SJ	$10^{5.3}$	$10^{5.3}$	$10^{5.55}$
389/I96	SJ/RG	$10^{5.55}$	$10^{5.55}$	$10^{5.05}$
484.2.2009	RG/RG	$10^{5.55}$	$10^{4.55}$	$10^{4.3}$

values obtained applying a 2 × 2 contingency table was below 5, while a 2-sample test of proportions was used for pairwise comparisons. One-sided tests with $p < 0.10$ were considered significant.

RESULTS

Experimental challenge

In total, 1929 fish were used for the experimental testing; 21 fish were lost during the trial at 20°C due to technical problems, and were therefore excluded from the study.

Back titration results of water samples collected at the end of the challenge confirmed the expected infectious titres, as reported in Table 2. Mock-infected fish remained healthy throughout the duration of the experiment. Only 2 specimens were found dead in control tanks, one in the 20°C tank and the second in the 30°C tank, respectively at 19 and 9 dpc, without showing clinical signs before dying. Both samples tested negative by real-time RT-PCR.

All dead fish collected in the infected tanks showed typical VNN clinical signs before death. Affected fish showed lethargy, anorexia, darkening and typical abnormal swimming behaviour characterised by whirling, circular movements alternated with long periods of lethargy, abnormal bathymetry and anomalous vertical positions in the water column. The appearance of anomalous swimming behaviours always led to the death of the infected fish, while specimens showing only lethargy and skin darkening were able to recover. Clinical signs appeared from 5 to 9 dpc. All brains sampled from dead fish yielded positive results by real-time RT-PCR for betanodavirus, confirming VNN as the cause of death. Strain 283.2009 (RGNNV) showed the earliest appearance (5 dpc) and the most serious clinical signs at 25 and 30°C, with mortality rates of 30 and 30.8%, respectively. Cumulative mortality at 20°C was 3.7%. On the other hand, reassortant viruses, as well as strain 484.2.2009 (SJNNV), showed fewer signs than those listed above, a delayed time of onset (7 to 9 dpc) and a lower mortality rate compared to the RGNNV (1.5–5.2%). These viral strains displayed slightly higher mortality rates at 20°C, in comparison with the other incubation temperatures (SJNNV: 5.2%; RGNNV/SJNNV: 3.3%; SJNNV/RGNNV: 4%). Detailed results on the cumulative mortality data and percentages per tank and temperature are reported in Table 3.

Real-time RT-PCR performed on fish brains collected at the end of the trial (30 dpc) confirmed the presence of betanodavirus in survivors (Table 4). Notably, all survivors appeared healthy. In the RGNNV-infected groups, brains of survivors always yielded positive results independently from the water temperature. On the other hand, SJNNV virus was detected in brains of almost 50% of surviving fish (6 out of 13) only at 20°C, while no positive signal was observed at 25 and 30°C. Intriguingly, RNA of

Table 3. Raw data of cumulative survival and mortality of sea bass *Dicentrarchus labrax*, mortality ratio and binomial exact 95% confidence intervals (95%CI) for each experimental tank, according to viral strain (see Table 1) and water temperature

Tank	Virus strain (NNV genotype)	Total	Dead fish	Mortality (%)	95%CI Min.	Max.
20°C						
1	283.2009 (RG/RG)	134	5	3.7	1.2	8.5
2	367.2.2005 (RG/SJ)	120	4	3.3	0.9	8.3
3	389/I96 (SJ/RG)	126	5	4.0	1.3	9.0
4	484.2.2009 (SJ/SJ)	116	6	5.2	1.9	10.9
5	Control	133	1	0.8	0	4.1
Total		629	21	3.3	2.1	5.1
25°C						
1	283.2009 (RG/RG)	130	39	30	22.3	38.7
2	367.2.2005 (RG/SJ)	130	2	1.5	0.2	5.4
3	389/I96 (SJ/RG)	130	2	1.5	0.2	5.4
4	484.2.2009 (SJ/SJ)	130	4	3.1	0.8	7.7
5	Control	130	0	0	–	–
Total		650	47	7.2	5.4	9.5
30°C						
1	283.2009 (RG/RG)	130	40	30.8	23.0	39.5
2	367.2.2005 (RG/SJ)	130	3	2.3	0.5	6.6
3	389/I96 (SJ/RG)	130	2	1.5	0.2	5.4
4	484.2.2009 (SJ/SJ)	130	5	3.8	1.3	8.7
5	Control	130	1	0.8	0	4.2
Total		650	51	7.8	5.9	10.2

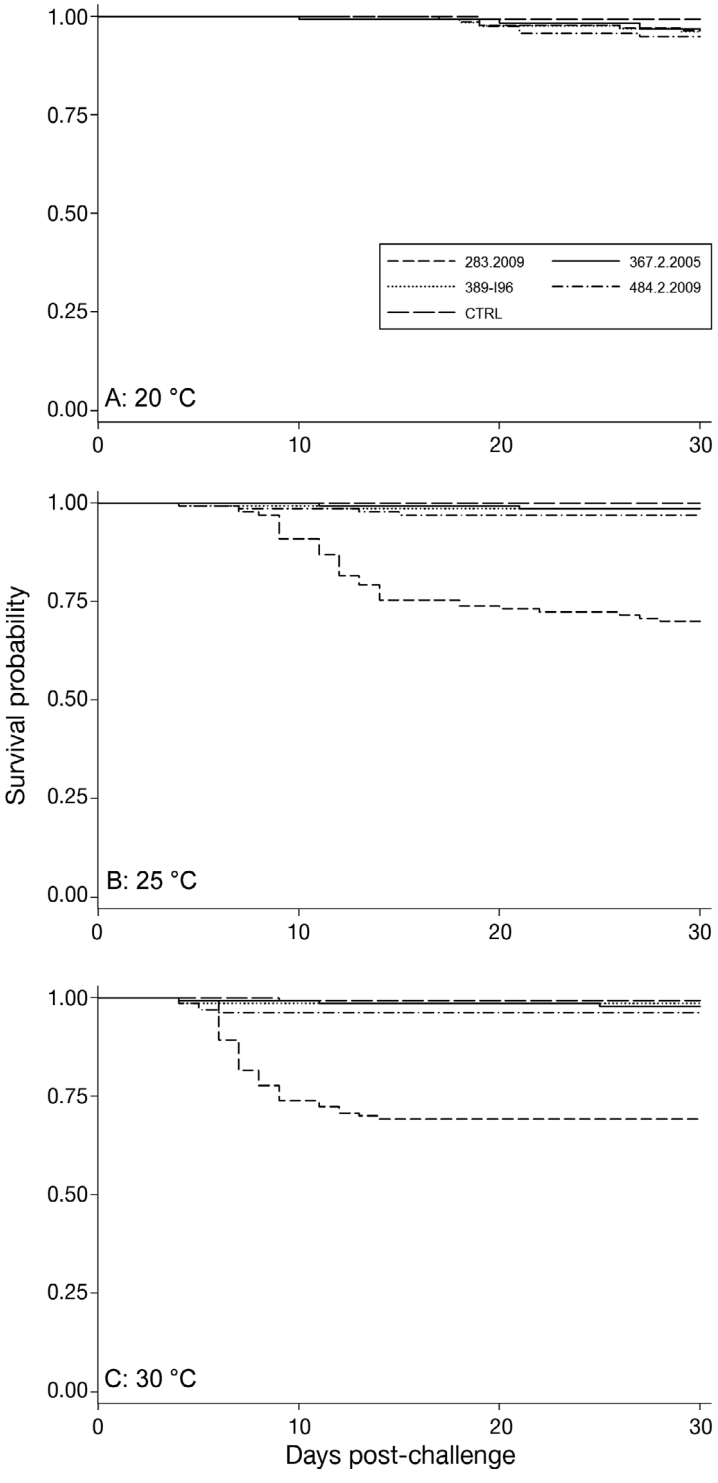

Fig. 1. Kaplan-Meier survival describing the survivor function over time of sea bass *Dicentrarchus labrax* surviving post challenge at (A) 20°C, (B) 25°C and (C) 30°C. Each step curve represents an experimental group infected with a different betanodavirus genotype (see Table 1), and the uninfected control group (CTRL). Stain numbers (genotype): 283.2009 (RGNNV); 484.2.2009 (SJNNV); 367.2.2005 (RGNNV/SJNNV); 389/I96 (SJNNV/RGNNV)

Table 4. Results of real-time RT-PCR performed on brain samples collected from apparently healthy sea bass *Dicentrarchus labrax* at the end of the experiment (30 d post infection). See Table 1 for details of virus strains

Virus strain (NNV genotype)	Temp. (°C)	Positive/ Tested
283.2009 (RG/RG)	20	13/13
	25	13/13
	30	13/13
367.2.2005 (RG/SJ)	20	13/13
	25	13/13
	30	13/13
389/I96 (SJ/RG)	20	13/13
	25	13/13
	30	0/13
484.2.2009 (SJ/SJ)	20	6/13
	25	0/13
	30	0/13

strain 389/I96 (SJNNV/RGNNV) was detectable in brains of all survivors tested at 20 and 25°C but not at 30°C; on the other hand, RNA of strain 367.2.2005 (RGNNV/SJNNV) was detected at all incubation temperatures (Table 4).

Statistical analysis of cumulative mortality

At 20°C, significant differences in the survival curves were not observed across the viral strains used for the experimental infection (Wilcoxon test: $p = 0.3863$; Fig. 1). When evaluating the association between housing temperature and mortality rate by virus through Fisher's exact test, no significant differences between mortalities across temperatures were noticed, except for the RGNNV virus (RGNNV: $p < 0.001$; RGNNV/SJNNV: $p = 0.644$; SJNNV/RGNNV: $p = 0.444$; SJNNV: $p = 0.757$; control group: $p = 1.000$).

Interestingly, significant differences in the survival curves were recorded at 25 and 30°C (Wilcoxon test: $p < 0.001$), with strain 283.2009 (RGNNV) showing a significantly higher mortality than the other strains (1-sided 2-sample test of proportions: $p < 0.01$). Of note, the cumulative mortality at the end of the experiment of strain 283.2009 at 25°C did not differ from that observed at 30°C (2-sided 2-sample test of proportions: $p = 0.89$; Wilcoxon test: $p = 0.1963$), but both were significantly higher than the mortality at 20°C (1-sided 2-sample test of proportions: $p < 0.01$). Kaplan-Meier survival curves at 25 and 30°C are shown in Fig. 1.

By comparing survival curves with a Wilcoxon nonparametric test, no statistical differences were recor-

ded among viral strains 484.2.2009 (SJNNV), 367. 2.2005 (RGNNV/SJNNV) and 389/I96 (SJNNV/ RGNNV) at all incubation temperatures tested (20°C, $p = 0.24$; 25°C, $p = 0.26$; 30°C, $p = 0.35$).

DISCUSSION

Water temperature strongly influences the severity of clinical signs and the mortality caused by VNN in field conditions (Chi et al. 1999, Le Breton 1999, Maltese & Bovo 2007). For this reason, VNN in European sea bass was originally called 'summer disease', as outbreaks are generally more frequent during the warm season (Bellance & Gallet de Saint Aurin 1988, Fukuda et al. 1996, Chi et al. 1999, Vendramin et al. 2013, OIE 2014).

A recent study by Vendramin et al. (2014) compared the pathogenicity of 10 different betanodavirus strains (including the 4 viral strains used in the present study) in sea bass juveniles at 20°C, gradually increasing the housing water temperature from 23 to 25°C. Overall, mortality increased after the rise in water temperature, with particular reference to strains RGNNV and RGNNV/SJNNV. While that study was designed to compare the pathogenicity amongst different viral strains despite the environmental conditions, the aim of the present study was to compare the pathogenicity of different betanodavirus strains at different water temperatures. In agreement with Vendramin et al. (2014), our results confirmed that European sea bass is susceptible to all VNN strains tested at all temperatures, given the mortality and the virus replication in nervous tissue of dead fish. However, only the RGNNV strain was able to cause significant mortality at higher temperatures (25 and 30°C). Notably, this strain showed no significant differences in mortality when compared to the SJNNV, the RGNNV/SJNNV and the SJNNV/ RGNNV isolates at 20°C. This finding further confirms the influence of water temperature on the pathogenicity of the RGNNV genotype. On the other hand, in our experiment, SJNNV and the 2 reassortant viruses caused very low mortality despite the water temperature, and this finding is supported by statistical analysis. The highest mortality rates observed by Vendramin et al. (2014) for the SJNNV, RGNNV/SJNNV and the SJNNV/RGNNV strains can be ascribed to the long period of observation post challenge (60 d vs 30 d in our study), as well as to the progressive increase in water temperature.

The low pathogenicity of the SJNNV strain for *Dicentrarchus labrax* has also been reported in a recent study, suggesting a different host specificity of this strain (Souto et al. 2015a). The RNA2 nucleotide region spanning positions 695 to 1061 determines the host specificity of the SJNNV genotype (Ito et al. 2008). The low virulence of SJNNV to European sea bass could therefore be due to poor adaptation of SJNNV to this host species. Indeed, to the best of our knowledge, VNN field outbreaks caused by SJNNV have never been reported in this species. Notably, the SJNNV strain used in the present study was originally isolated from Senegalese sole *Solea senegalensis*. Both reassortant strains, RGNNV/SJNNV and SJNNV/RGNNV, caused very low mortality in sea bass regardless of the water temperature. On one hand we observed a lower virulence for the RGNNV/SJNNV likely due to the poor adaptation of its RNA2 and/or encoded protein to sea bass; on the other, we hypothesize that the low pathogenicity of the SJNNV/RGNNV might be imputable to the lower replication fitness at higher temperatures given by the SJNNV-type RNA1.

The productive viral infection in apparently healthy experimentally infected fish has been described in other fish species, such as Atlantic halibut *Hippoglossus hippoglossus*, Atlantic cod *Gadus morhua* and Senegalese sole (Grove et al. 2003, Korsnes et al. 2009, Souto et al. 2015a). In the present study, viral replication in surviving sea bass was detected by real-time RT-PCR in all challenged groups and, differently from mortality data, results were strongly influenced by the water temperature and the genetic features of the viral isolates. *In vitro* studies proved that at a high temperature (30°C), the RGNNV and its RNA1-derived reassortant (i.e. RGNNV/SJNNV) have a better replication fitness, while multiplication of the SJNNV and the SJNNV/RGNNV is suboptimal and their growth kinetics are slower (Panzarin et al. 2014). These results are consistent with our findings. When tested by real-time RT-PCR, a positive signal was detected in the brains of the survivors belonging to the SJNNV-infected group only at 20°C, while specimens infected with strain SJNNV/RGNNV tested positive at 20 and 25°C but not at 30°C. On the other hand, viral genome of the RGNNV and the RNNV/SJNNV was detected in all surviving fish tested at all water temperatures.

The RNA1 molecule determines the temperature dependency of betanodavirus *in vitro* (Iwamoto et al. 2000, Ciulli et al. 2006, Hata et al. 2010, Panzarin et al. 2014). However, we should highlight that cell cultures are a much simpler model than a whole-animal model, and they do not take into account a number of factors that might influence betanodavirus virulence,

such as the immune response of the host and viral host-specificity and adaptation. Testing the immunity response of betanodavirus-infected fish at different conditions will be the subject of further study. Therefore, although the real-time RT-PCR results on survivors clearly reflect the temperature dependency of different betanodaviruses, the low cumulative mortality data recorded can be attributed to the combination of temperature sensitivity (determined by RNA1) and host specificity (determined by RNA2) of the different viruses, as already postulated for the SJNNV genotype. This means that, even if facilitated by warm temperatures, the RGNNV/SJNNV could have a lower pathogenicity due RNA2 poorly adapted to sea bass; conversely, the SJNNV/RGNNV virus should be better adapted to the host but could be impaired by the low water temperature preference determined by RNA1.

As far as we know, this is the first study aimed at investigating *in vivo* the effect of temperature on the pathogenicity of different betanodavirus strains in European sea bass, and the results confirmed in principle the temperature dependency of genetically different betanodaviruses. Further testing on the remaining genotypes, such as BFNNV and TPNNV, as well as additional research on the host specificity of the different genotypes, for example replicating this study in a different fish species such as Senegalese sole, would be of interest to increase our knowledge concerning betanodavirus pathogenicity.

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