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## Different survival of three populations of European sea bass (*Dicentrarchus labrax*) following challenge with two variants of nervous necrosis virus (NNV)

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### ABSTRACT

Viral Nervous Necrosis (VNN, also called viral encephalo- and retinopathy (VER)), is a widespread disease of marine aquaculture caused by betanodavirus (or nervous necrosis virus - NNV), a segmented positive sense RNA virus, member of the nodaviridae family. VNN affects predominantly marine fish and cause significant losses to the Mediterranean fish farming industry, including the production of European sea bass (*Dicentrarchus labrax*). Of the four circulating genotypes of betanodavirus, red-spotted grouper NNV (RGNNV) and the reassortant genotype red-spotted grouper/striped jack NNV (RG/SJNNV) are most prevalent in the Mediterranean. Inheritable resistance against VNN has been detected in sea bass, and selective breeding could be a mean to limit this untreatable disease. In the current study, we compare resistance to disease among three populations from the Atlantic Ocean (AT), Eastern Mediterranean (EM) and Western Mediterranean (WM), by challenge trials using both a highly pathogenic isolate of RGNNV and a lower pathogenic reassortant isolate of RG/SJNNV. The survival of the three populations were modelled with a logistic regression, and the odds ratio (OR) of surviving was calculated. The challenge with RG/SJNNV reduced the odds of surviving three-fold (OR = 0.29 [0.07-0.87]), whereas the challenge with RGNNV reduced the odds of surviving 100-fold (OR = 0.01 [0.00-0.03]). Overall, the EM population had 3.32 (1.92–5.86) times higher odds of surviving the challenge than the AT and WM stocks. All survivors were harboring viral RNA in the brain, as demonstrated by RT-qPCR. However, viral RNA levels were in average lower in survivors from the EM population in both challenges, though only significantly lower in the challenge with RG/SJNNV ( $p < 0.01$ ). The survival results combined with the RT-qPCR results indicate that the EM sea bass population has a natural resistance to disease caused by RGNNV, possibly associated with limited viral entry into and/or replication in the brain.

### 1. Introduction

Viral Nervous Necrosis (VNN) causes huge losses in the Mediterranean fish farming industry every year, both in terms of loss of fish and reduced growth of surviving fish (Le Breton et al., 1997; Muniesa et al., 2020). VNN is caused by an RNA virus called nervous necrosis virus (NNV), or betanodavirus, belonging to the family of *Nodaviridae*. NNV has more than 70 host species, including farmed and wild fish, and in the Mediterranean in particular, both sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) are affected (Bandín and Souto, 2020). Lack of

effective treatments and vaccines strengthens the need for alternative measures to limit the losses caused by the virus.

NNV is a small icosahedral, non-enveloped RNA virus (30 nm). The genome consists of two single-stranded, positive-sense RNA molecules. RNA1 encodes for the polymerase and RNA2 encodes the capsid protein. Finally, a subgenomic segment of RNA1, named RNA3 encodes for protein B2 which is involved in intracellular viral RNA accumulation (Fenner et al., 2006). Based on the T4 region of the RNA2 segment the virus can be clustered into four different genotypes; Red-spotted Grouper Nervous Necrosis Virus (RGNNV), Striped Jack Nervous

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Necrosis Virus (SJNNV), Tiger Puffer Nervous Necrosis Virus (TPNNV) and Barfin Flounder Nervous Necrosis Virus (BFNNV) (Nishizawa et al., 1997; OIE, 2019). These genotypes have different geographical distribution, optimal growth temperature and host species (Mori et al., 2003; Panzarin et al., 2014). The RGNNV is more prevalent in warmer waters, such as the Mediterranean basin and waters around Asia, USA and Australia (Chérif et al., 2009; Panzarin et al., 2012; Thiery et al., 2004). SJNNV was originally only found in Japanese waters, although it recently appeared in Mediterranean waters as well (Nishizawa et al., 1997; OIE, 2016; Panzarin et al., 2012; Thiery et al., 2004). BFNNV mainly affects cold-water fish species in North America, Norway, Japan and France and TPNNV is prevalent in Japan (Nishizawa et al., 1997). However, the continuing discovery of new genotypes, such as turbot-, Atlantic Halibut-, and grey mullet-NNV, suggests that the host-specificity is low and that the hosts are merely the fish species living in the water temperature, optimal for the specific viral genotype (Johansen et al., 2004, 2002; Korsnes et al., 2017; Thiery et al., 2004). Movement of cultured fish between regions and countries as well as wild fish stock migrations induced by changes in the environment could be ways of spreading exotic genotypes between distinct geographical regions and allowing the virus to encounter new hosts (Lampert et al., 2020; Munday et al., 2002; Nishizawa et al., 1997).

Sequence analysis of both the RNA1 and RNA2 segment has demonstrated the existence of reassortant strains, with RNA1 from RGNNV and RNA2 from SJNNV (RG/SJNNV) or opposite (SJ/RGNNV). They have been isolated from sea bream, senegalese sole (*Solea senegalensis*), and sea bass (Oliveira et al., 2009; Panzarin et al., 2012; Toffan et al., 2017; Toffolo et al., 2007). These reassortments could be the result of co-infection with both genotypes in asymptomatic hosts (Lopez-Jimena et al., 2010).

Sea bass are naturally distributed in the eastern Atlantic and in the Mediterranean seas, with a strong genetic structure, equivalent to a subspecies division between these two areas because of reproduction isolation during the last glaciation (Duranton et al., 2018). A lower level of subdivision is described within the Mediterranean, separating the Mediterranean in an eastern and western population, with a gradient of Atlantic genes from the West to the East, due to a secondary contact after the last glaciation. Selecting fish stocks with a natural resistance to infection has proven to be efficient in limiting other diseases in aquaculture (reviewed by Gjedrem, 2015; Ødegård et al., 2011). Recently, resistance to infection by NNV in sea bass was associated with moderate heritability (0.26–0.27) expected to be sufficient to be included in selective breeding programs (Doan et al., 2017b; Palaiokostas et al., 2018). Quantitative trait loci associated with resistance against NNV and explaining up to 9.21 % of the additive genetic variance has been identified (Griot et al., 2021; Palaiokostas et al., 2018). Crosses involving broodstock from different natural populations of sea bass have further suggested that large differences in NNV resistance between populations exist, with expected highest resistance in fish from the Eastern Mediterranean, intermediate resistance in fish from the Western Mediterranean and lowest resistance in fish from the Atlantic (Doan et al., 2017a). However, this remains to be investigated with fish from the original populations.

In the present study, we investigated the resistance of three natural populations of sea bass, which alone or in combination form the basis of farmed populations of sea bass, to experimental infection with NNV. The three populations originated from wild fish caught in the Atlantic Ocean (AT), the Eastern Mediterranean (EM), and the Western Mediterranean (WM) (Vandeputte et al., 2014). The EM population was a backcross (75 % EM-25 % WM) while the other two were pure WM and AT. All fish groups were challenged with either an RGNNV isolate or a reassortant RG/SJNNV isolate. Both isolates were derived from outbreaks of VNN in farmed sea bass around Italy (Panzarin et al., 2012), and have previously been shown to be high and low pathogenic, respectively (Vendramin et al., 2014). The resistance to VNN was evaluated as end-survival, and presence of virus in the brain of survivors was determined and

quantified with reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR).

## 2. Materials and methods

### 2.1. Fish

Sea bass originating from three natural populations from the Atlantic Ocean (AT), Eastern Mediterranean (EM) and Western Mediterranean (WM) (Vandeputte et al., 2014), were produced by artificial mating at the Ifremer Experimental Aquaculture Research Station in Palavas-les-Flots (France). Nine females from AT, 22 from WM and 13 from an EMxWM F1 hybrid population were stripped on the 5th of February 2018 and *in vitro* fertilized with cryopreserved sperm from respectively 26 males from AT, 40 males from WM and 39 males from EM as described by Doan et al. (2017a). Thus, we produced pure AT and WM offspring, and EM offspring as a backcross containing 75 % EM genes and 25 % WM genes, which was the best possibility as no wild EM females were available. Offspring from the different origins were reared in triplicate following the same rearing protocol described by Doan et al. (2017a) until shipping from Ifremer to DTU at 103 days post hatching (dph). Upon arrival at the DTU facilities (Denmark), fingerlings were kept at 16 °C in 1 % artificial saltwater in 180 L cylindrical acrylic aquaria for three weeks to acclimatize. They were fed INICIO plus size 0.8 mm at 2 % body weight (Biomar, Denmark). Temperature was slowly increased to 22 °C over two days prior to initiation of the experiment. The fish were 6.0 ± 3.6 g and 7.6 ± 1.4 cm (fork length, AT); 5.1 ± 1.6 g and 7.5 ± 0.8 cm (EM); 5.2 ± 1.7 g and 7.4 ± 0.9 cm (WM) at the start of the experiment. The fish were too small for sexing, though later sexing of the stock from which they originate revealed balanced and similar sex-ratios (50.1 % females in AT, 53.0 % in EM and 49.7 % in WM).

The sea bass were reared in an NNV free facility, yet prior to starting the experiment three pools of 10 fish per population were tested with RT-qPCR for nodavirus RNA1 (as described in 2.6) and found negative.

### 2.2. Ethics approval

All experimental challenges were carried out according to European guidelines (Directive 2010/63/EU) and Danish legislation. The experiment was approved by the Danish Animal Experiments Inspectorate under experimental animal license no. 2013-15-2934-00976, held by Prof., DVM Niels Jørgen Olesen.

### 2.3. Virus strains

Virus strain 283.2009 belonging to RGNNV and strain 367.2.2005 belonging to the reassortant RG/SJNNV was kindly provided by Dr. A. Toffan, (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy). Both were isolated from farmed European sea bass in Italy in the previous published work by Panzarin et al. (2012). The virulence of the two isolates was earlier compared in a bath challenge with European sea bass (mean weight 0.2 g, unspecified population), resulting in 36.25 % and 10 % mortality, respectively (Vendramin et al., 2014).

### 2.4. Virus production

Virus was propagated and titrated on striped snake head cells (SNN-1)(Frerichs et al., 1996) with L-15 medium (Leibovitz 15 medium enriched with 5 % fetal calf serum, 2 % L-Glutamin, 0.1 % Gentamicin (50 mg/ml) and 0.8 % 2-mercaptoethanol) and incubated at 25 °C. Cell monolayers with approx. 90 % confluency were inoculated with resuspended lyophilized virus solution in 75 cm<sup>2</sup> primary cell culture flasks (Falcon). The replicated virus was harvested when the cell layer was destroyed completely due to cytopathic effect (CPE). Following freezing at -80 °C over night, and thawing the following day, cell debris was

removed by centrifugation (15 min at 4000 RPM/4110 G, Hettich Rotanta 460R centrifuge) and the supernatant stored at  $-80^{\circ}\text{C}$ . The two virus strains were titrated on a 96 well plate with a monolayer of SNN-1 cells in 10-fold dilutions made with L15 media in six replicates. CPE was evaluated regularly, and final read was performed on day 10. The titer was calculated as 50 % tissue culture infective dose (TCID<sub>50</sub>) with the Reed-Muench method (Reed and Muench, 1938).

## 2.5. In vivo challenge trial

Approximately thirty fish were transferred to each of 21 10-liter plastic bowls as shown in Table 1. Each combination of virus and fish population was performed in triplicate. In addition, one control bowl not challenged with virus was included per fish population. Artificial 1 % saltwater was flowing in at  $12^{\circ}\text{C}$  at a rate of approximately 2 L/h. The bowls were fitted with individual heaters raising the temperature to  $25.0 \pm 1.5^{\circ}\text{C}$ . The temperature was monitored daily during peak mortality (until day 14, thereafter sporadically) and adjusted, if found out of the acceptable interval. The fish were anaesthetized in 100 mg/L benzocaine (Sigma-Aldrich, Brøndby, Denmark) and injected intramuscularly (IM) with 50  $\mu\text{L}$  of either sterile L15 medium (controls) or virus-containing L15 medium from cell culture (supernatant). The titer of both virus strains was  $8.6 \times 10^5$  TCID<sub>50</sub>/mL resulting in injection of  $4.3 \times 10^4$  TCID<sub>50</sub> per fish.

## 2.6. Sampling

Fish reaching the humane end-point, defined as spiral swimming pattern, were collected three times a day during the experimental period. Moribund fish were euthanized in an overdose of benzocaine (300 mg/L) and was sampled by splitting the head in the sagittal plane with a scalpel and transferring one half of the brain into a round-bottom 2 mL Eppendorf tube with RLT buffer (Qiagen, Germany) for detection of the virus by RT-qPCR. The samples were placed at  $-80^{\circ}\text{C}$  until further processing. The experiment was terminated at day 28, when clinical signs had been absent for more than a week. All surviving fish were sampled as described above.

## 2.7. RNA extraction and RT-qPCR

To each tube with thawed sampled brain in RLT buffer 1 % of 2-mercaptoethanol was added together with one steel bead before the tissue was disrupted at 25 Hz for 2 min. in a tissuelyser (TissueLyser II, Qiagen). 620  $\mu\text{L}$  lysate were transferred to 2 ml screw-cap tubes for further RNA extraction on the QIA-cube robot (Qiagen) using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol.

The multiplex TaqMan one step RT-qPCR reaction had a final volume of 25  $\mu\text{L}$  with 1x Quantitect Probe RT-PCR master mix (Qiagen), 5  $\mu\text{L}$  purified sample diluted 1:10 as well as primers and probes found in Table 2 (Baud et al., 2015; Rocha et al., 2009). The primers for betanodavirus target the RNA1 sequence, which is similar in both RGNNV and RG/SJNNV, allowing the same primers to be used for both strains. The other target was the elongation factor 1 $\alpha$  (Ef-1 $\alpha$ ) which has

**Table 1**  
Experimental setup.

Strain/Population	AT	WM	EM	Total
RGNNV	92 (30;31;31)	95 (31;33;31)	90 (30;30;30)	277
RG/SJNNV	90 (30;30;30)	94 (30;34;30)	90 (30;31;29)	274
Control	30	31	30	91
Total	212	220	210	642

AT = Atlantic, WM = Western Mediterranean, EM = Eastern Mediterranean. RG/SJNNV = Red-spotted Grouper/Striped Jack Nervous Necrosis Virus, RGNNV = Red-spotted Grouper Nervous Necrosis Virus. The numbers in brackets indicate the number of fish in the three replicate tanks.

**Table 2**  
Primers and probes for multiplex RT-qPCR.

Target gene	5'-3' sequence	nM	Average Efficiency
Ef-1 $\alpha$ (Rocha et al., 2009)	F: GGAGTGAAGCAGCTCATCGTT	200	109.2 %
	R: GCGGGCCCTGGCTGTAAG	300	
RNA1 (Baud et al., 2015)	P: [5HEX]AGTCAA[ZEN] CAAGATGGACTCCACTGAGCCC[3IABkFQ]	50	93.5 %
	F: TCCAAGCCGGTCTAGTCAA R: CACGAACGKCGCATCTCGT	600 600	
	P: [6FAM]CGATCGATC[ZEN] AGCACCTSGTC[3IABkFQ]	400	

previously been evaluated as a constant expressed housekeeping gene in sea bass brain tissue suitable for normalization (Alvarado et al., 2013; Mitter et al., 2009; Paria et al., 2016). The samples were amplified according to Baud et al. (2015), briefly 30 min at  $50^{\circ}\text{C}$  followed by 15 min at  $95^{\circ}\text{C}$ ; 50 cycles of 15 s at  $94^{\circ}\text{C}$  and 60 s at  $60^{\circ}\text{C}$ . The amplifications were either performed on a Stratagene MX3000 or MX3005 (Agilent Technologies). Two standard curves, one for each target, were included in every run. The betanodavirus standard curve was made from a 336 bp synthetic double stranded DNA (gblock, Integrated DNA technologies) diluted 10 fold 7 times successively. The Ef-1 $\alpha$  standard curve was based on a control fish sample not infected with betanodavirus. The sample standard was analyzed undiluted and at 3 times successive 10 fold dilutions. The efficiency of the primers were calculated as an average of the efficiency in each run in the range of quantification cycle (Cq) 19–29 (Table 2).

### 2.7.1. Relative quantification of virus

The  $2^{-\Delta\Delta\text{Cq}}$  method was used to calculate the relative amount of virus in the brain of sampled sea bass, as described by Livak and Schmittgen (2001) for relative gene expression analysis, although altered to match viral quantitation. Prior to exporting the Cq values from MxPro to Microsoft Excel, the threshold was normalized between the different runs by manually setting the threshold for both RNA1 and Ef1 $\alpha$  so that the Cq value in the lowest dilutions of the two standard curves (described in 2.7), respectively, was the same in each run. The Cq value of Ef1 $\alpha$  was used as internal control to normalize for the amount of RNA in the different samples as  $\Delta\text{Cq} = \text{Cq}_{\text{RNA1}} - \text{Cq}_{\text{Ef1}\alpha}$ .

After normalization, the sample with the highest  $\Delta\text{Cq}$  value, and thus the lowest amount of virus, was used as calibrator to calculate the  $\Delta\Delta\text{Cq}$  for each sample. Thereby all samples were expressed as fold increase relative to this calibrator as in  $\Delta\Delta\text{Cq} = \Delta\text{Cq}_{\text{sample}} - \Delta\text{Cq}_{\text{calibrator}}$ . The amount of target was calculated using the formula:  $\text{relative amount of target} = 2^{-\Delta\Delta\text{Cq}}$ . The calibrated amount of virus was converted into logarithmic values to minimize inter-sample variation.

## 2.8. Statistical analysis

### 2.8.1. Survival

Statistical analysis was performed in R (ver. 3.6.3.)

Survival curves were made with the R-packages "survival" and "survminer".

The probability of surviving until the end of the experiment (day 28) for the different combinations of fish populations and challenge virus was modelled with a logistic regression with the glm-function; Logit ( $P_{\text{surv}} = \alpha + \beta_{\text{virus}} + \beta_{\text{population}}$ ). The significance of each level of the factors were evaluated with a chi-square test in the drop1 function in R.

The random effect of tank was evaluated in a likelihood ratio test comparing the difference in log-likelihood from the glm model (above) and a more complex glmer model with tank as a random effect (R-package "lme4"), as recommended by Bolker et al. (2009) for binomial

data with few random effects (<4).

Interaction between population and virus was evaluated by adding virus\*population to the glm function and running a chi-square-test in the drop1 function again.

From the estimated values of  $\beta$  the odds ratio (OR) of surviving for the individual factors until day 28 p.c. was calculated as  $OR = \exp(\beta)$ . Since the factors were found to be without interactions, the combined OR for the combinations of population and challenge virus can be calculated;  $OR_{population} \times OR_{virus} = OR_{pop,vir}$ . The variance ( $\sigma^2$ ) of the  $OR_{pop,vir}$  is

$$\sigma^2(OR_{pop,vir}) = OR_{pop}^2 \times \sigma_{vir}^2 + OR_{vir}^2 \times \sigma_{pop}^2 + 2 \times OR_{pop} \times OR_{vir} \times OR_{pop,vir}$$

from this the CI was calculated;

$$CI = OR_{pop,vir} \mp 1.96 \times \sqrt{\sigma^2(OR_{pop,vir})}$$

### 2.8.2. Comparison of level of virus in the brain of survivors

The relative amount of virus in the brain of the survivors for the two virus strains were log-transformed to achieve linearity and compared by an analysis of variance (ANOVA) on a 95 % significance level in R (version 3.6.3). Model control was performed with Levene's test.

## 3. Results

### 3.1. In vivo challenge trial

The infected fish started showing clinical signs on day 5, and the number of cases peaked on day 5–7 depending on population and virus (Fig. 1). The most prominent clinical sign was spiraling swimming pattern (Video 1 (online version only)). The control groups all had high survival (0, 1 and 2 fish died in the three control tanks).

The survival percentage at day 28 of the different populations and the 95 % confidence interval is summarized in Table 3 and the cumulative survival for each day is illustrated in Fig. 1. The survival% and measured temperature from each replicate tank are available in the Supplementary Material 1.

**Table 3**

Survival percentage after experimental challenge.

Population/Virus	L15 media	RG/SJNNV	RGNNV
Atlantic	100.0	94.4 [89.7–99.2]	14.2 [7.0–21.2]
Western Mediterranean	93.3	83.3	20.2
Eastern Mediterranean	[84.9–100]	[75.4–90.9]	[12.0–28.0]
	96.7	92.1	52.2
	[90.2–100]	[86.7–97.8]	[41.9–62.5]

All values are % survivors. AT = Atlantic, WM = Western Mediterranean, EM = Eastern Mediterranean, RG/SJNNV = Red-spotted Grouper/Striped Jack Nervous Necrosis Virus, RGNNV = Red-spotted Grouper Nervous Necrosis Virus. [95 % Confidence interval] calculated as for a proportion with a binomial distribution.

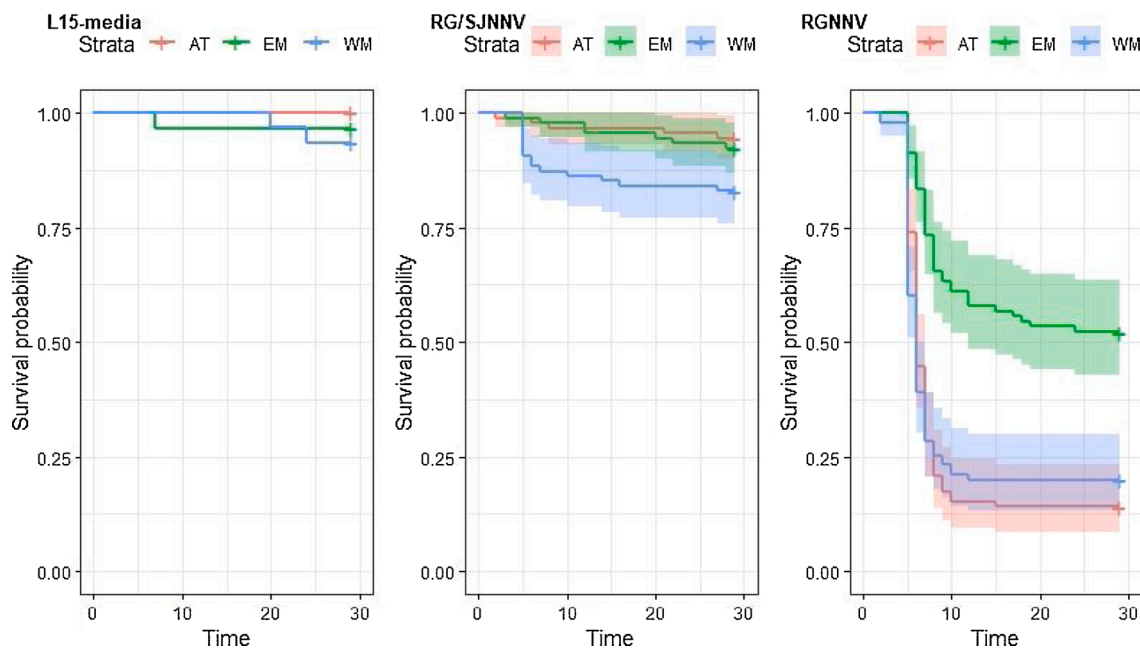
The chi-square test of the logistic regression revealed that both population and virus had a significant influence on the probability of survival. However, adding a random effect of the tanks did not improve the accuracy of the model ( $p = 0.19$ ) and there was no interaction between the factors “population” and “virus”. Therefore, the final model could be determined as:

$$\text{Logit}(p_{\text{surv}}) = \alpha + \beta_{\text{virus}} + \beta_{\text{population}}$$

Being from the EM population positively influence the  $p(\text{surv})$  ( $p < 0.001$ ), and increase the odds of surviving 3.3 times ( $OR = 3.32$  [1.92;5.86] (Table 5)). The lack of interaction between virus and population indicate that this increased odds of survival of the EM population apply to challenge with both virus. On the contrary, being challenged with either RGNNV or RG/SJNNV significantly reduces the  $p(\text{surv})$  ( $p < 0.001$  and  $p = 0.050$ , respectively), with an almost 100-fold reduction in the odds of surviving if challenged with RGNNV ( $OR = 0.01$  [0.00;0.03]) and a 3 fold reduction when challenged with RG/SJNNV (0.29 [0.07;0.87]).

### 3.2. Detection of virus with RT-qPCR and relative quantification

Thirty brain-samples from fish that were euthanized on day 5 post challenge (p.c.) (peak mortality) were chosen for further analysis with



**Fig. 1.** Survival probability at days post challenge (Time) for the three different populations of European sea bass (Atlantic (AT), Eastern Mediterranean (EM) and Western Mediterranean (WM)) when injected IM with either sterile L15 media, RG/SJNNV (red-spotted grouper/striped jack nervous necrosis virus) or RGNNV (red-spotted grouper nervous necrosis virus). The shaded area indicate 95 % confidence interval.

**Table 5**  
Odds ratio (OR) of survival depending on population and challenge-virus.

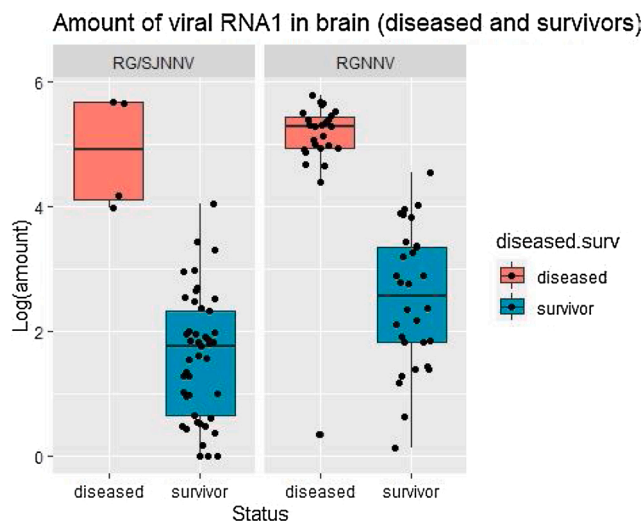
Virus	OR <sub>Population</sub>	OR <sub>RG/SJNNV</sub>	OR <sub>RGNNV</sub>
Atlantic	1	0.29 [0.07–0.87] *	0.01 [0.00–0.03] *
Western Mediterranean	0.77 [0.44–1.32]	0.22 [0.00–1.57] *	0.01 [0.00–0.25] *
Eastern Mediterranean	3.32 [1.92–5.86] *	0.96 [0.00–4.07]	0.03 [0.00–0.54] *

OR (odds ratio) of survival when challenged with either RG/SJNNV or RGNNV compared to the survival in the control, extracted from the logistic regression. OR<sub>Population</sub> indicate the overall modelled OR of surviving in the trial depending on population, and this value is used to calculate OR<sub>RG/SJNNV</sub> and OR<sub>RGNNV</sub>, as described in 2.7.1. AT = Atlantic, WM = Western Mediterranean, EM = Eastern Mediterranean, RG/SJNNV = Red-spotted Grouper/Striped Jack Nervous Necrosis Virus, RGNNV = Red-spotted Grouper Nervous Necrosis Virus. \* Indicate significant OR (where 1 is not in the CI).

RT-qPCR. Most of these were from the RGNNV-challenge because very few of the RG/SJNNV-challenged fish became diseased. From the survivors (euthanized on day 28 post challenge), 15 samples from each population/challenge combination were chosen for RT-qPCR as well (total 120). Unfortunately, the samples from the very few fish from the AT population surviving the RGNNV challenge were lost during storage in the freezer and thus not analyzed. Both fish euthanized because of reaching the humane endpoint of spiraling swimming pattern (from now on referred to as “diseased”), and surviving fish were found positive for RGNNV. However, a significant difference ( $p < 0.001$ ) was observed in the viral load of diseased fish compared to survivors (Fig. 2). The survivors from the EM population had a significantly lower amount of virus in the brain than the survivors from AT and WM populations, when comparing the RG/SJNNV-survivors ( $p < 0.01$ , Table 4). Although not significant, a similar pattern was seen for the RGNNV-survivors from the EM versus the WM population ( $p = 0.35$ ). When comparing the amount of virus across the viral type, EM challenged with RG/SJNNV had a significantly lower amount of virus than EM challenged with RGNNV ( $p < 0.001$ ). The same pattern was seen with the WM population, though not significant ( $p = 0.08$ ) (Fig. 3 and Table 4).

#### 4. Discussion

Natural resistance to disease is a desired trait helping to ensure a

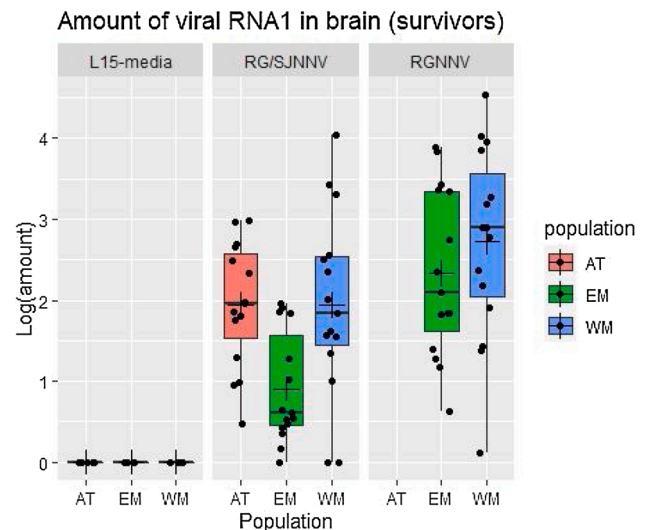


**Fig. 2.** Log transformed relative amount ( $\text{Log}(2^{-\Delta\Delta Cq})$ ) of virus in the brain of diseased fish (day 5 post challenge) and survivors (day 28 post challenge), challenged IM with either RG/SJNNV or RGNNV, detected by RT-qPCR on brain tissue. The horizontal line indicates the median value.

**Table 4**  
Relative amount of viral RNA1 in the brain of survivors.

Population/Virus	L15 media	RG/SJNNV	RGNNV
Atlantic	0.0 <sup>a</sup>	1.94 [1.55–2.33] <sup>b</sup>	-* <sup>d</sup>
Western Mediterranean	0.0 <sup>a</sup>	1.94 [1.36–2.52] <sup>b</sup>	2.72 [2.12–3.32] <sup>d</sup>
Eastern Mediterranean	0.0 <sup>a</sup>	0.90 [0.56–1.25] <sup>c</sup>	2.33 [1.81–2.86] <sup>d</sup>

All values are Log10 (relative amount of virus), determined as described in 2.7.1. AT = Atlantic, WM = Western Mediterranean, EM = Eastern Mediterranean, RG/SJNNV = Red-spotted Grouper/Striped Jack Nervous Necrosis Virus, RGNNV = Red-spotted Grouper Nervous Necrosis Virus. Different letters indicate statistically significantly different values ( $p \leq 0.05$ ). \* There were only few survivors from the AT/RGNNV challenge, and these were not analyzed.



**Fig. 3.** Log-transformed relative amount ( $\text{Log}(2^{-\Delta\Delta Cq})$ ) of virus in the brain of survivors from the three populations (Atlantic (AT), Eastern Mediterranean (EM) and Western Mediterranean (WM)) challenged IM with either RG/SJNNV, RGNNV or non-challenged controls (L15-media) detected with RT-qPCR on brain tissue. The horizontal line indicates the median value and the “+” indicates the mean ( $n = 15$  fish pr. population, except AT, where there were only few survivors which were not analyzed).

healthy production and lowering the disease-associated costs, such as loss of animals, reduced weight gain, cost of medicines etc. (Gjedrem, 2015). In the current study, we show that the EM backcross population of sea bass has a significantly higher resistance to experimental infection with RGNNV with a survival of 52.5 % [42.3–62.2] compared to 14.2 % [4.3–24.1] and 20.2 % [10.3–30.2] for the AT and WM populations, respectively and an overall 3.32 [1.92–5.86] times higher odds of surviving than the other two populations (Table 5). These findings support the previous studies by Doan et al. (2017a) who challenged and compared animals from WM, ATxWM hybrids and EMxWM hybrids. However, they could only estimate the possible resistance of the pure AT and EM populations by assuming a hypothetical full additive genetic contribution of the sires (carrying the AT and EM origin) and the dams (coming from WM only), thus their estimates may have been over-estimated in case there is heterosis (superior performance of crosses) between the WM and the AT and EM populations. Here, we compared the pure AT and WM breeds and an EMxEM-WM backcross (75 % EM). When comparing with the results of Doan et al. (2017a), the present RGNNV challenge resulted in much lower survival in the WM population with only 20.2 % survivors compared to 62 % in Doan et al. (2017a). Since they also injected virus into the fish (IP, we injected it IM) and at a much higher dose than we did ( $2 \times 10^7$  TCID<sub>50</sub>/fish), this difference is probably due to lower pathogenicity of the virus strain. To compare both experiments, we normalized results with a probit threshold model (see Supplementary Materials 2) and compared the normalized survival of

the three AT, WM and EM base populations. Although they are not subject to heterosis for AT, and much less than Doan's for EM, our estimates for normalized survival are almost exactly proportional with theirs (1.02 regression coefficient,  $r^2 = 0.97$ ), showing that the additive model is correct. This means that there is no heterosis to expect by crossing EM or AT with WM for resistance to VNN, contrary to what was shown for sex-ratio, for which crosses between AT and Mediterranean fish produced more females than the parental populations (Guinand et al., 2017).

Challenge with RG/SJNNV significantly reduced the odds of surviving 3 times (OR = 0.29 [0.07;0.87]) for the AT population, compared with the unchallenged controls. The same was the case for the other two populations, although not significant because 1 is within the CI for the OR (OR<sub>WM,RG/SJNNV</sub> = 0.22 [0–1.57], OR<sub>EM,RG/SJNNV</sub> = 0.96 [0–4.07]). This is despite the high survival rate of the AT population challenged with RG/SJNNV (AT = 94.4 [89.7–99.2]) compared to the WM and EM (83.3 [75.4–90.9] and 92.1 [86.7–97.8]), respectively (Table 3)). A low reduction in the survival rate was observed in the EM and WM control groups (2 and 1 specimen, respectively) which increase the statistical uncertainty of the survival percentage thus demanding a larger sample size to determine if the observed difference in survival is in fact due to the RG/SJNNV challenge. Nonetheless, the virus was detected in the brain of both diseased and survivors from all populations, indicating that the virus replicated in the fish and moved from the intramuscular injection site to the brain, though without causing cases enough to demonstrate a significant reduction in survival. Due to ethical considerations and the animal health legislation, we were obliged to euthanize all fish reaching the humane end point of clinical signs of VNN. It is not excluded that some of these fish would have survived the infection if we had not intervened, although previous studies have shown that clinically affected fish that survives are likely to succumb in a chronic phase of the disease. The survival rate therefore reflect the proportion of fish *not* developing clinical disease and the proportion of fish surviving the infection might have been higher.

There was a significantly higher load of viral RNA in the brain of the challenged sea bass euthanized/dead on day five p.c. compared to survivors euthanized on day 28 p.c., irrespective of virus strain. Due to the low number of euthanized fish on day five challenged with RG/SJNNV (n = 4), no further comparison between the two strains were made at this point. Specimens sampled on day five displayed clinical signs of disease and had high NNV RNA1 loads, suggesting a correlation between the appearance of clinical signs and the NNV RNA1 load in brain tissue. In parallel the detection of lower viral RNA in most of the survivors despite lack of clinical disease further support this correlation. Previous studies have demonstrated a relatively stable level of RNA1 in the brain of infected live sea bass without clinical signs, euthanized during the first month after experimental IM infection (Lopez-Jimena et al., 2011). In the same study, fish succumbing from the infection had a higher level of RNA1 in the brain, supporting our findings. In a study by Chaves-Pozo et al. (2012), nodavirus-resistant sea bream maintained a stable level of virus in the brain at day 7, 15 and 30 after IM injection of RGNNV. The level was lower than the lowest level measured in susceptible sea bass in the same study. In the infected sea bass, viral RNA was present in the brain already at day 1 and increased until day 15 accompanied by development of clinical disease. These findings, in addition to ours, suggest that development of clinical disease is related to reach of a threshold load of virus in brain tissue. Furthermore, once the threshold level of viral load in the brain is reached and clinical disease is triggered there is a high risk of the fish not surviving the infection. Viral replication and thus viral RNA1 load is enhanced at higher temperatures (25–30 °C) for RGNNV (and the RG/SJNNV harboring the RNA1 of RGNNV) (Panzarin et al., 2014; Toffan et al., 2016), why possibly this threshold is easier reached at higher temperatures and may never be reached at lower temperatures, although the fish is infected.

In a large study with different *Drosophila* species and virus either naturally occurring in the species or not, Duxbury et al. (2019)

demonstrate that coevolution of a host and a pathogen increase the genetic variation in susceptibility to infection most likely due to selection induced by the pathogen. The low reduction in survival, and relatively lower load of viral RNA in the brain of the EM population could reflect a natural selection of resistance genes in the coevolution of this population and the virus strain, since RGNNV is native to the warmer Mediterranean waters such as the eastern part. A similar population-dependent resistance is seen with infectious pancreatic necrosis virus (IPNV)-infection in Atlantic salmon (*Salmo salar*). In these studies, IPNV-resistant populations show the same ability to keep a low level of virus and low mortality after experimental challenge with IPNV (Reyes-López et al., 2015; Robledo et al., 2016).

The high-virulent RGNNV strain reached a higher level of virus in the brain of infected survivor-fish from the same population than RG/SJNNV, as well as a higher reduction in survival. A similar direct correlation between high levels of virus and high mortalities or high pathogenicity is classical for many host-virus interactions and is also seen e.g. in experimental challenge of Atlantic salmon with different IPNV isolates (Skjesol et al., 2011) and in rainbow trout (*Oncorhynchus mykiss*) and sockeye salmon (*O. nerka*) experimentally infected with different genotypes of infectious hematopoietic necrosis virus (IHNV) (Peñaranda et al., 2011, 2009; Purcell et al., 2011, 2009). Interestingly, the opposite host-pathogen relationship was seen in a recent study by Tapia et al. (2020) with rainbow trout infected with IPNV genotype 1 and 5, the first being more pathogenic, however resulting in lower viral load. Genotype 5 was less pathogenic but both survivors and diseased fish had higher viral load. This demonstrates that the mechanism of disease and susceptibility is determined by the unique interactions between host and pathogen and may vary with both host and pathogen.

In terms of epidemiological aspects, it is noteworthy that most survivors in our study had the viral RNA in their brain. Assuming that at least some of this comes from viable virus particles, it should be expected that all the populations of sea bass, irrespective of resistance to VNN, has the potential to act as carriers of virus. Previous experimental cohabitation challenges investigating the transmission from infected individuals have shown opposed results; Péducasse et al. (1999) was successful in transferring VNN by co-habitation, while this did not happen in a challenge experiment described in Hodneland et al. (2011). Nevertheless a key difference from experimental challenge to farm condition to be considered is that, under experimental conditions clinical fish are removed as soon as they reach humane end point, whereas in farm condition, fish can remain in the farm environment for hours prior to removal, and this allow decay of tissues that enhance release of viral particles in the environment. Further more cannibalistic behavior has to be considered in these circumstances, so although carrier fish are not actively shedding sufficient viral dose to transfer pathogen to naïve fish they might release the virus in the farm environment and thereby. This could pose a threat to younger and more susceptible sea bass or other species on or around the farm. Sea bream appear to be more susceptible to the reassortant RG/SJNNV than sea bass (Toffan et al., 2017) and sea bass acting as carriers of RG/SJNNV on a mixed species farm with both sea bass and sea bream, causing diseases in sea bream, was recently seen on a farm in Italy (Volpe et al., 2020). Potentially, VNN resistant sea bass could also harbor multiple NNV infections and foster new reassortments, as hypothesized in other species (Lopez-Jimena et al., 2010).

In this study, we have demonstrated a statistically significant difference between the EM sea bass population and the AT and WM populations in terms of disease resistance when challenged with RGNNV. Although the genetic background and molecular mechanisms remain to be determined, the results support earlier indications of the EM population harboring resistance traits allowing the fish to keep the virus replication in the brain at a low level. Including this population in future breeding programs for farmed sea bass could thus benefit the Mediterranean aquaculture, which often suffers from great losses due to VNN. This population also has satisfactory growth rates compared to the WM

and AT populations, and lower fat content than the AT population (Vandeputte et al., 2014). However, although this population has interesting characteristics, farmed sea bass have been bred for growth and morphology for several generations (Vandeputte et al., 2014). Thus, coming back to wild populations may negatively impact other economically important traits. It will be thus of high interest to dissect the genetic architecture of resistance to VNN in the EM population, which may lead to the possibility to introgress their resistance in populations already selected for other traits. However, it should be kept in mind that despite increased resistance to the disease, the virus replicate in resistant fish, as demonstrated by the detection of viral RNA in brain of survivors. Thus, resistant fish can potentially harbor the virus and possibly be a source of an outbreak in younger naïve fish or other species. Other preventive measures should therefore still be implemented such as vaccination, biosecurity measures and reassurance of disease-free status in newly introduced fish stocks. The population-related resistance is also important to keep in mind when comparing different experimental studies of NNV virulence and pathogenesis in sea bass.

### CRedit authorship contribution statement

**Sofie Barsøe:** Methodology, Data curation, Formal analysis, Writing - original draft, Visualization, Writing - review & editing, Project administration. **François Allal:** Resources, Visualization, Writing - review & editing, Funding acquisition, Conceptualization. **Alain Vergnet:** Resources, Conceptualization, Writing - review & editing. **Marc Vandeputte:** Resources, Funding acquisition, Conceptualization, Writing - review & editing. **Niels Jørgen Olesen:** Supervision, Funding acquisition. **Jacob Günther Schmidt:** Investigation, Data curation, Writing - original draft, Writing - review & editing. **Cathrine Agnete Larsen:** Investigation, Data curation, Writing - original draft, Writing - review & editing. **Argelia Cuenca:** Supervision, Methodology, Writing - review & editing. **Niccolò Vendramin:** Conceptualization, Investigation, Supervision, Project administration, Funding acquisition, Writing - review & editing.

### Declaration of Competing Interest

The authors report no declarations of interest.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.aqrep.2021.100621>.

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