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Interspecies transmission between *Solea senegalensis* and *Sparus aurata* of reassortant Nervous Necrosis Virus (NNV) strains and effect of stress on the outcome of the infection

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A B S T R A C T

Viral Encephalopathy and Retinopathy (VER) episodes in Southern Europe have led to the isolation of several reassortant NNV strains from different fish species, including Senegalese sole and gilthead seabream. Polyculture of both species is being developed to optimize available resources. However, this farming technique can be threatened by NNV horizontal transmission between diseased fish or asymptomatic carriers and non-infected individuals from both fish species, which could lead to a VER outbreak in the facility and seriously affect fish production. Therefore, in this study we have assessed the susceptibility of gilthead seabream and Senegalese sole to two reassortant NNV strains isolated from each of the two fish species, and the possibility of interspecies transmission by cohabiting infected and naïve individuals. Our results showed that both NNV isolates caused moderate mortality rates and replicated in both fish species. In the cohabitation challenges, infective NNV particles were recovered from naïve cohabitants, demonstrating interspecies transmission from infected individuals that shed NNV into the water column. In addition, cumulative mortality in sole cohabitants was significantly higher, presumably due to the stress provoked by the aggressiveness of gilthead seabream. This is supported by the analysis of the *hsp70* gene, a stress biomarker overexpressed in the sole cohabitants, especially in those that died on the first day of cohabitation. Therefore, despite the numerous advantages of polyculture, the risk of VER outbreaks represents a serious constraint for the implementation of this technique in Mediterranean aquaculture.

1. Introduction

The Nervous Necrosis Virus (NNV) -G. *Betanodavirus*- is the causative agent of Viral Encephalopathy and Retinopathy (VER), a neurological pathology affecting a great number of fish larvae and juveniles worldwide. This non-enveloped virus contains two molecules of positive-sense ssRNA, RNA 1 and RNA2, encoding the viral polymerase and the capsid protein, respectively (Mori et al., 1992). Based on a partial sequence of RNA2, namely the T4 region, the NNV is clustered into 4 genotypes: BFNNV (Barfin Flounder Nervous Necrosis Virus), RGNNV (Red-spotted Grouper Nervous Necrosis Virus), SJNNV (Striped Jack Nervous Necrosis Virus) and TPNNV (Tiger Puffer Nervous Necrosis Virus) (Nishizawa et al., 1997). Moreover, reassortant strains RGNNV/SJNNV and SJNNV/RGNNV have been isolated from sea bass (*Dicentrarchus labrax*), Mediterranean mackerel (*Trachurus mediterraneus*), sole (*Solea solea* and *S. senegalensis*) and gilthead seabream (*S. aurata*), both farmed and in the wild in Southern Europe (Bitchava et al., 2019; Olveira et al., 2009; Panzarin et al., 2012; Toffan et al., 2017; Toffolo et al., 2007; Volpe et al., 2020).

VER-infected fish shed NNV into the water environment probably through the skin and gills (Souto et al., 2018). This water-borne virus enters the new hosts, targeting the central nervous system and retina, where it causes the histological damage responsible for the alteration of swimming patterns and loss of appetite (Bandín and Souto, 2020).

Abbreviations: NNV, nervous necrosis virus; SJNNV, striped jack nervous necrosis virus; RGNNV, redspotted grouper nervous necrosis virus; TPNNV, tiger puffer nervous necrosis virus; BFNNV, barfin flounder nervous necrosis virus; VER, viral encephalopathy and retinopathy; CPE, cytopathic effect; M.O.I, multiplicity of infection; bp, base pair; HSP, heat shock protein; PP, posterior probability; pi, post-infection; dpi, days post-infection; dpc, days post-cohabitation; pc, post-cohabitation.

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Horizontal transmission commonly occurs among diseased and healthy fish belonging to the same species (Hick et al., 2011; Péducasse et al., 1999; Souto et al., 2015c), but has also been observed between different species. Interspecies transmission has been reported between European sea bass and gilthead seabream both on farms and under experimental conditions (Castric et al., 2001; Volpe et al., 2020), Asian sea bass (*Lates calcarifer*) and brown-marbled grouper (*Epinephelus fuscoguttatus*) (Manin and Ransangan, 2011) and Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) (Korsnes et al., 2012).

The increasing worldwide demand for fish has led to the development of new aquaculture strategies, such as polyculture, to optimize and maximize available resources. This technique, which consists of rearing different invertebrate and/or fish species by cohabiting the same area, provides both economic benefits and improved product quality. However, it can also constitute a serious threat to production due to pathogens spreading through susceptible individuals (FAO, 2020) and because of the stress induced by rearing species with different behavioural patterns, since coping with stress deteriorates fish health and worsens the outcome of a possible infection (Kristiansen et al., 2020). Under stress conditions, teleost fish activate the hypothalamus – pituitary gland – interrenal gland (HPI) axis (Tort et al., 2019) that leads to the release of cortisol into the bloodstream and enhances the synthesis of heat shock proteins (HSP), a warning signal for the immune system (Mazeaud et al., 1977; Pockley, 2003; Sopinka et al., 2016). Overexpression of hsp70 gene has been reported in NNV-infected European seabass (*D. labrax*) and Asian seabass (*L. calcarifer*) (Lama et al., 2020; Liu et al., 2016).

Gilthead seabream and Senegalese sole are two finfish species cultivated using this approach (Ferreira et al., 2010), with the highest production rate in Mediterranean aquaculture (FAO, 2020). Therefore, the aim of this work was to assess the susceptibility of gilthead seabream and Senegalese sole to two different reassortant (RGNNV/SJNNV) NNV strains isolated from each fish species and to investigate the possibility of interspecies transmission between cohabiting infected and naïve individuals as well as to analyse the effects of cohabitation stress on the outcome of the infection.

2. Methods

2.1. Viral isolates and propagation

Two NNV isolates were used in this study: SpSs_IAusc160.03 (hereafter 160.03) isolated from diseased Senegalese sole (Olveira et al., 2009) and SpSa_IAusc382.17 (hereafter 382.17) isolated from gilthead seabream juveniles. These strains were propagated at a MOI of 0.1 in semiconfluent E-11 cell monolayers (Iwamoto et al., 2000) grown on Leibovitz's L-15 medium (Gibco, Fisher Scientific, Scotland, UK) supplemented with 5% fetal bovine serum (FBS; Corning, NY, USA). Infected cell cultures were maintained at 25 °C with L-15 supplemented with 2% FBS until cytopathic effect (CPE) was extensive and then crude viruses were clarified by centrifugation at 3000 x g for 15 min at 4 °C. The supernatants were titrated in 96-well plates for 10 days at 25 °C, expressing the titre as the 50% tissue culture infection dose per ml ($TCID_{50}$ ml^{-1}) (Reed and Muench, 1938).

2.2. Genetic characterisation and phylogenetic analysis of SpSa382.17

Both genomic segments of the 382.17 isolate were sequenced with the primers described by Olveira et al. (2009), and the nucleotide sequence was submitted to GenBank with accession numbers MT707973 for RNA1 and MT707974 for RNA2. Partial sequences (994 bp in RNA1 and 632 bp in RNA2) edited using DNASTAR Lasergene® v.7.1 (DNASTAR, Madison, USA) were subjected to phylogenetic analysis.

The fitting model was estimated with JModelTest (Darriba et al., 2012), comparing the edited sequence of both RNA molecules with those of the four genotypes (4 RGNNV, 2 SJNNV, 4 BFNNV and 1 TPNNV) and 17 reassortant (RGNNV/SJNNV) strains deposited in GenBank (Table 1). The phylogenetic relationship was established by Bayesian inference employing the HYK + G model using BEAST 1.10.4. Four Markov chains were run for 1000,000 generations and, to obtain a consensus tree, the first 10,000 trees were discarded and then sampled every 1000. The robustness of the phylogenetic representation was indicated by the Bayesian Posterior Probability (PP), considering significative values higher than 70%. Trees were rooted using the sequence of Nodamura Virus [NoV; GenBank accession no. NC_002690 (RNA1), NC_0026911 (RNA2)] and Black Bettle Virus [BBV; NC_001411 (RNA1), NC_002037 (RNA2)] as an outgroup.

To determine the pairwise identity of the coding sequence, the 382.17 isolate was subjected to multiple alignment using MegAlign (DNASTAR) with the sequences listed in Table 1. In addition, the amino acid identity percentage was calculated with regard to the Italian and Iberian reassortant isolates using Geneious (Geneious v. 4.5.6, Biomatters, http://www.geneious.com/).

2.3. Fish infection challenges

Senegalese sole and gilthead seabream juveniles (mean weight 1 g) were infected by bath and cohabitation challenges according to the European Guidelines (Directive 2010/63/UE) for the protection of animals used for scientific purposes. The infection protocol was approved by the Galician Committee for experimental animal welfare and by the Xunta de Galicia (Permit Id. 15,010/2020/004). A total of 846 individuals (423 of each fish species) fed ad libitum with commercial fish feed were kept in opaque 50-l tanks containing sea water (salinity 33 g l^{-1}) and acclimated for 14 days at 25 °C (L12:D12) at the fish facilities of University of Santiago de Compostela. Prior to the challenges, 3 fish of each species were killed with an MS-222 anaesthetic overdose (Sigma-Aldrich, Missouri, USA) and tested for the presence of viral and bacterial pathogens. Viral detection was accomplished by RT-PCR using specific primers for infectious pancreatic necrosis virus (IPNV), viral haemorrhagic necrosis virus (VHSV) and NNV as previously described (Olveira et al., 2013). For bacteriological analysis, internal organs were inoculated in tryptone soy agar supplemented with 1% NaCl (TSA-1).

Infection trials were performed as following:

- Bath infection: a total of 210 Senegalese sole and 210 gilthead seabream were bath exposed to a viral concentration of 10^5 $TCID_{50}$ ml^{-1} for 5 h with strong aeration with either the 160.03 or 382.17 strains. Triplicate groups (35 fish/tank) were established for each experimental infection. Challenges were terminated after 35 days of infection.
- Cohabitation challenges (Fig. 1): a total of 360 fish (180/fish species) were used. For each cohabitation experiment 90 Senegalese sole and 90 gilthead seabream were bath infected for 5 h with the 160.03 and 382.17 strain, respectively, at 10^5 $TCID_{50}$ ml^{-1}. Fish belonging to each species were distributed into 3 tanks (30 fish/tank). At 48 h post infection, 30 naïve sole or gilthead seabream were introduced in each of the tanks to cohabit with infected gilthead seabream and Senegalese sole, respectively. Challenges were terminated after 33 days of cohabitation.

In addition, in order to reduce the number of fish used in this study, one control group per species ($N = 15$) challenged with L-15 medium and one cohabitation control (15 fish of each species) were set up and handled like the infected groups, following the conditions described in the bath infection. Clinical signs and mortalities were recorded daily. Dead fish were removed from the tanks and stored at −20 °C until virological analysis. Surviving fish at the end of the experiment were euthanized using an MS-222 overdose.

Table 1
Reference strains used for the phylogenetic analysis and the genetic characterisation.

Virus isolate (genotype)	Year	Species	Country	Reference	GenBank accession no.	
					RNA1	RNA2
SaIAUSC61_05 (RGNNV/SJNNV)	2005	Gilthead seabream	Portugal	(Olveira et al., 2009)	FJ803912	FJ803918
SaIAUSC74_05 (RGNNV/SJNNV)	2005	Gilthead seabream	Portugal	(Olveira et al., 2009)	FJ803913	FJ803919
SsIAUSC573_04 (RGNNV/SJNNV)	2004	Senegalese sole	Portugal	(Olveira et al., 2009)	FJ803914	FJ803920
SaIAUSC156_03 (RGNNV/SJNNV)	2003	Gilthead seabream	Spain	(Olveira et al., 2009)	FJ803916	FJ803921
SsIAUSC160_03 (RGNNV/SJNNV)	2003	Senegalese sole	Spain	(Olveira et al., 2009)	FJ803911	FJ803923
SsIAUSC1974_08 (RGNNV/SJNNV)	2008	Senegalese sole	Spain	(Olveira et al., 2009)	FJ803917	FJ803922
SpSaIAUSC382_17 (RGNNV/SJNNV)	2017	Gilthead seabream	Spain	This report	MT707973	MT707974
G9508KS (RGNNV)	2002	Redspotted grouper	Japan	(Lee et al. 2002)	AY690597	AY690596
SpPmIAUSC1586_10 (RGNNV)	2010	Turbot	Spain	(Olveira et al., 2013)	KC696563	KC696562
SGWak97 (RGNNV)	1997	Redspotted grouper	Japan	(Iwamoto et al., 2004)	NC_008040	NC_008041
DlIAUSC1688_08 (RGNNV)	2008	Sea bass	Spain	(Olveira et al., 2013)	FJ803915	FJ829452
SJNNV (SJNNV)	1993	Striped jack	Japan	(Nagai & Nishizawa 1999)	AB025018	–
SJ_G91 (SJNNV)	1993	Striped	Japan	(Nishizawa et al. 1995)	–	D30814
SJ93Nag (SJNNV)	1993	Striped jack	Japan	(Iwamoto et al. 2001)	AB056571	AB056572
BF93Hok (BFNNV)	1993	Barfin flounder	Japan	(Nishizawa et al. 1995)	EU826137	EU826138
AH99NorA (BFNNV)	1995	Atlantic halibut	Norway	(Grotmol et al. 2000; Sommerset & Nerland 2004)	AJ401165	AJ245641
Ac06NorT (BFNNV)	2007	Atlantic cod	Norway	Patel and others (Unpublished data)*	EF617330	EF617329
GmMR11_06 (BFNNV)	2008	Atlantic cod	Norway	(Nylund et al. 2008)	EF433472	EF433468
TPKag93 (TPNNV)	1993	Tiger puffer	Japan	(Nishizawa et al. 1995; Toffolo et al., 2007)	AM085332	D38637
430.2004 (RGNNV/SJNNV)	2004	Senegalese sole	Italy	(Panzarin et al., 2012)	JN189911	JN189932
477.2004 (RGNNV/SJNNV)	2004	Senegalese sole	Italy	(Panzarin et al., 2012)	JN189913	JN189938
17.1C.2004 (RGNNV/SJNNV)	2004	Sea bass	Italy	(Panzarin et al., 2012)	JN189900	JN189934
367.2.2005 (RGNNV/SJNNV)	2005	Sea bass	Italy	(Panzarin et al., 2012)	JN189909	JN189936
82.4.2007 (RGNNV/SJNNV)	2007	Gilthead seabream	Italy	(Vendramin et al., 2014)	JX290516	JX290518
169-4/Mar2009 (RGNNV/SJNNV)	2009	Gilthead seabream	Italy	(Toffan et al., 2017)	KY354683	KY354695
203-3/May2010 (RGNNV/SJNNV)	2010	Gilthead seabream	Italy	(Toffan et al., 2017)	KY354685	KY354697
461-1/Nov2014 (RGNNV/SJNNV)	2014	Gilthead seabream	Italy	(Toffan et al., 2017)	KY354688	KY354702
127-1/Mar2015 (RGNNV/SJNNV)	2015	Gilthead seabream	Italy	(Toffan et al., 2017)	KY354692	KY354704
575/Nov2015 (RGNNV/SJNNV)	2015	Gilthead seabream	Italy	(Toffan et al., 2017)	KY354691	KY354705
165-6/Mar2016 (RGNNV/SJNNV)	2016	Gilthead seabream	Italy	(Toffan et al., 2017)	KY354693	KY354706

* Unpublished data; direct submission to GenBank

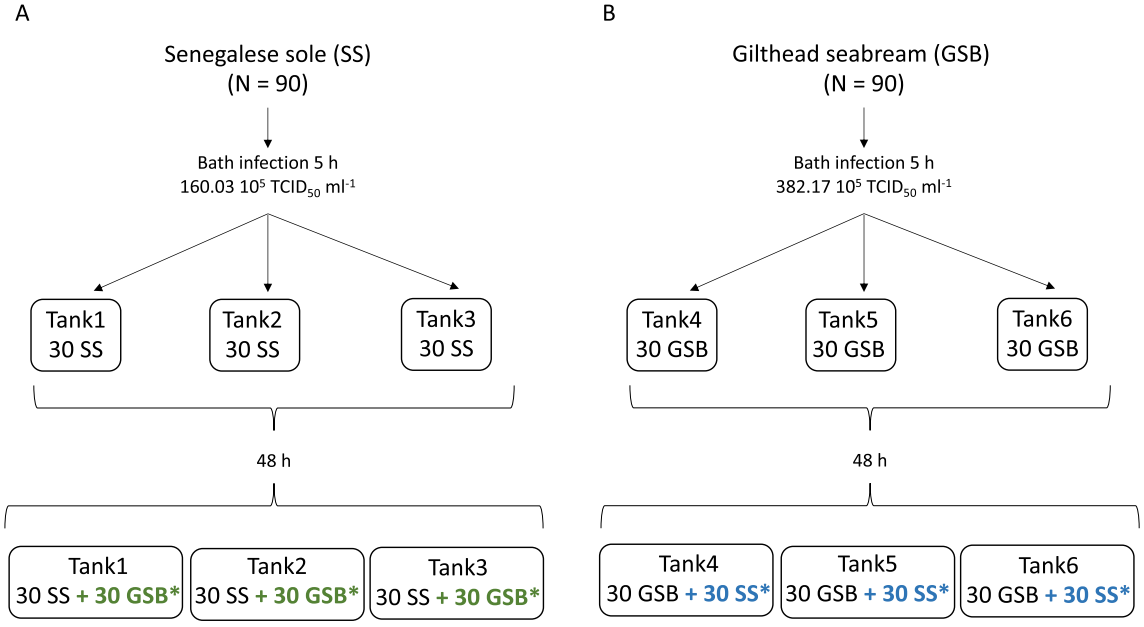

Fig. 1. Schematic representation of the cohabitation challenges. **A**) Cohabitation 1: 160.03-infected Senegalese sole (SS) + naïve gilthead seabream (GSB) co-habitants and **B**) cohabitation 2: 382.17-infected GSB + naïve SS. Naïve cohabitants are represented by an asterisk (*).

2.4. Virological analysis and NNV quantification

Individual head samples (including eye and brain tissue) were homogenized (1:10 w v^{-1}) in Earle's balanced salt solution (Hyclone Laboratories Inc., Logan, Utah, USA) supplemented with antibiotics (amphotericin B 200 μg ml^{-1}, gentamycin 500 μg ml^{-1}, penicillin 1000 units ml^{-1} and streptomycin 1000 units ml^{-1}). After centrifugation at 4000 x g for 20 min at 4 °C, the resulting supernatants were split into two aliquots; one was stored at −80 °C for later use in RT-qPCR and the other incubated for 24 h at 4 °C, and then inoculated in duplicate onto E-11 cells in 48-well plates (dilutions 10^{-1} and 10^{-2}). The plates were incubated at 25 °C and monitored for CPE development for 10 days. A subcultivation was then performed by inoculating 100 μl of the scrapped cell suspension onto new cultures for a further 10 days. Samples from

survivor fish (S) and control tanks were also analysed.

For NNV quantification, RNA was extracted using NucleoSpin ® RNA (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. Next, reverse transcription was performed with a RevertAid First Strand cDNA Synthesis Kit (Thermoscientific Inc., Vilnius, Lithuania) in a thermocycler MyCycler™ (Bio-Rad, Hercules, California, USA) by incubating the viral RNA with random primers for 5 min at 95 °C and with the reverse transcription mixture for 1 h at 42 °C. Finally, the enzyme was inactivated at 70 °C for 10 min. The resulting cDNA was amplified by qPCR in a CFX-96 Real-Time PCR Detection System (Bio-Rad) by mixing 2 μl of viral cDNA, with 10 μl of iQ ™ SYBR ® Green Supermix (Bio-Rad) and 200 nM of SnodR1 F/R primers (Olveira et al., 2013). Briefly, after a denaturation/activation step at 95 °C for 3 min, cDNA was subjected to 40 denaturation (15 s at 95 °C) and annealing-extension (20 s at 59 °C) cycles. Total viral RNA content was estimated using a standard curve generated from 20-fold serial dilutions of a DNA plasmid containing SpSsIAusc160.03 RNA1 in the range of 10^1 to 10^7 RNA copies ml^{-1}.

2.5. Transcription of the hsp70 gene

Relative transcription of the heat stock protein (*hsp*) 70 gene was quantified in fish subjected to cohabitation assays because HSP70 is considered a biomarker/indicator of several types of stress in fish (Sopinka et al., 2016). In addition, two dead Senegalese sole and two sacrificed seabream individuals from the cohabitation control were also analysed. To this end, the cDNA obtained in the previous section was subjected to qPCR using 200 nM of specific *hsp*70 primers for both Senegalese sole (Salas-Leiton et al., 2010) and gilthead seabream (Bildik et al., 2019), following the same protocol as described above, except for the annealing-extension step, performed at 70 °C for 30 s and at 55 °C for 20 s, for sole and seabream primers, respectively. The expression of the *hsp70* gene was normalised following the 2-ΔΔCt method (Livak and Schmittgen, 2001) using ribosomal 18S RNA as the endogenous reference gene (F: 5'-GTCTGGTTAATTCCGATAA-3'; R: 5'-ACCTGTTATTGCTCAATC-3'; López-Vázquez et al., 2016).

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.1 (San Diego, California, USA). Cumulative mortality was analysed using survival curves and the Kaplan-Meyer test, and significant differences among groups were evaluated with the Log-Rank (Mantel-Cox) test. Significance of the viral load and gene expression was assessed using Two-way ANOVA and Tukey correction. $P < 0.05$ was considered statistically significative.

3. Results

3.1. Genetic characterisation and phylogenetic analysis

The phylogenetic representation of the gilthead seabream isolate 382.17 revealed that the RNA1 molecule clustered with the RGNNV strains (Fig. 2A), whereas the RNA2 molecule was grouped within the SJNNV genotype (Fig. 2B), indicating that it is an RGNNV/SJNNV reassortant. As both trees show, 382.17 diverged from the same speciation event (PP = 0.9) like other Italian seabream reassortants (203-3/May2010, 127-1/Mar2015, 575/Nov/2015 or 165-6/Mar2016) and other RGNNV/SJNNV isolates obtained from sea bass and Senegalese sole in the Iberian Peninsula and Italy. Agreeing with the phylogenetic analysis, the nucleotide sequence of the RNA1 segment showed a 96.4% identity with the SGWak97 strain (RGNNV), and RNA2 98.4% with regard to the SJNag93 strain (SJNNV). The homology with the Italian and the Iberian reassortants was 98.1–99.5 and 99.1–99.2%, respectively, in RNA1, and 98.3–99.6% and 97.8–99.4% in RNA2.

As for the amino acid sequence, the 382.17 isolate shows 6 substitutions in the capsid protein with respect to the SJNag93 strain, three of them located on the N-terminal side of the protein ($aa13_{Pro \rightarrow Ala}$, $aa20_{Ser \rightarrow Pro}$, $aa27_{Thr \rightarrow Ile}$) and the remaining 3 on the C-terminal ($aa247_{Ala \rightarrow Ser}$, $aa270_{Asn \rightarrow Ser}$, $aa298_{Phe \rightarrow Tyr}$). Whereas the changes in positions 13 and 20 are shared by the Iberian isolates and the 247 and 270 substitutions were observed in both the Iberian and Italian reassortants, positions 27 and 298 seem to be exclusive to 382.17, although there is no data available for the Italian seabream isolates.

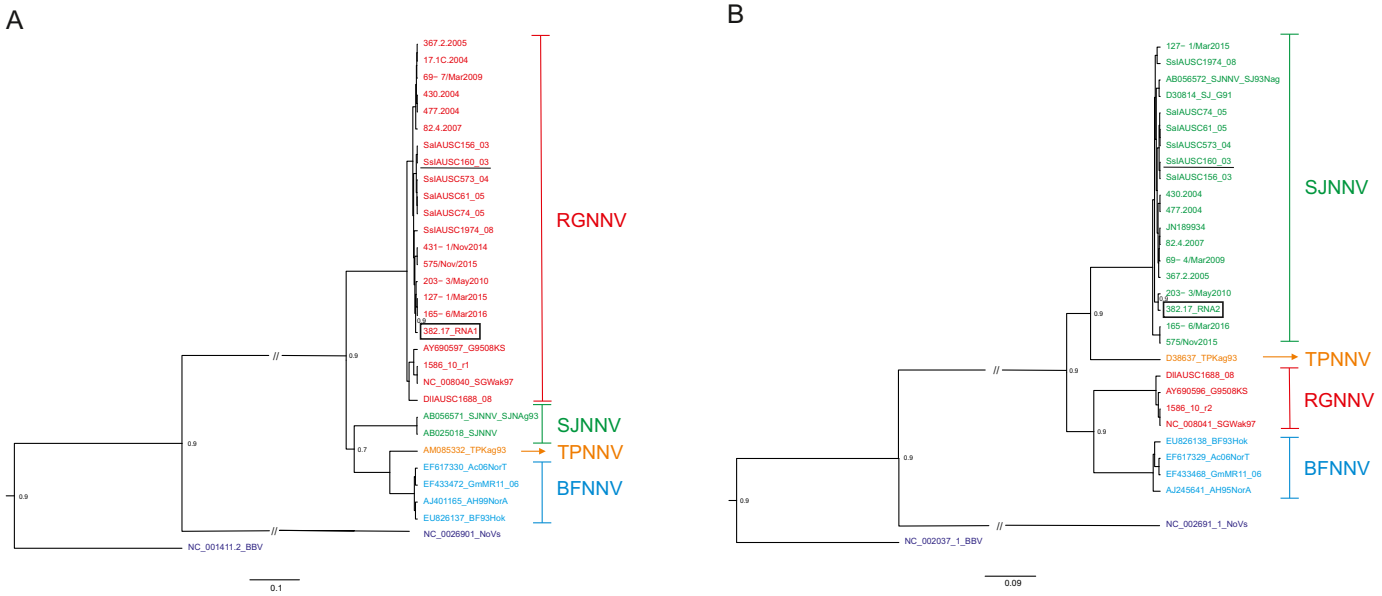

Fig. 2. A) Phylogenetic relationships of 160.03 (underlined) and 382.17 (framed in a box) isolates based on RNA 1 and **B)** RNA2. Numbers indicate Bayesian PPs obtained from the majority rule consensus of trees sampled every 1000 generations after discarding the first 10,000. Bars, (**A**) 0.1 and (**B**) 0.09 nucleotide substitutions per site.

3.2. Gilthead seabream and Senegalese sole susceptibility challenges

Moderate mortality was observed in both species regardless of the source of the viral strain used. Fish infected with strain 160.03 (isolated from *S. senegalensis*) showed the highest mortality levels at 35 dpi (55 and 52% in gilthead seabream and sole, respectively) (Fig. 3A), whereas the challenge with strain 382.17 (isolated from *S. aurata*) caused slightly lower mortality (approximately 47% in gilthead seabream and 40% in Senegalese sole) (Fig. 3B). Furthermore, cumulative mortality recorded in NNV-challenged groups was significantly higher ($p = 0.01$) than in the control groups (CG). First mortalities occurred in gilthead seabream at 8 days post infection (dpi) whereas in Senegalese sole they were not observed until 14 and 15 dpi (fish challenged with 382.17 and 160.03, respectively). In both fish species, mortalities were recorded up to 35 dpi when challenge was terminated because of the worsening of the water conditions. Regarding signs of disease, abnormal swimming behaviour, namely looping and resting belly up, was observed in the infected sole from 7 dpi. However, the gilthead seabream succumbed to death with no obvious VER signs.

Head samples of individual dead fish were grouped ($N = 9$) according to the mortality phase (T1 - initial mortality, T2 – acute mortality and T3 – late mortality). As shown in Fig. 3C and Fig. 3D, both NNV strains replicated to a higher extent in Senegalese sole than in gilthead seabream (a significantly higher number of viral RNA copies, p value <0.05). Infected sole showed an initial average viral load around 10^9 NNV RNA copies per fish gram (7.76×10^8 and 1.48×10^9, in fish infected with 160.03 and 382.17, respectively) that decreased to 3.4×10^7 at T3 (3.55×10^7 and 3.24×10^7, in 160.03 and 382.17 infected sole, respectively). On the other hand, infected gilthead seabream contained a similar NNV load in all the phases analysed, with an average of 2.1×10^5 RNA copies g^{-1} regardless of the strain (T1 average values: 5.25×10^5 and 3.24×10^4; T2: 1.26×10^5 and 3.02×10^5; T3: 1.32×10^5 and 6.76×10^4 RNA copies g^{-1} in fish infected with 160.03 and 382.17, respectively). In both fish species, survivors carried a similar number of NNV copies to dead fish (p value >0.05). NNV infective particles were recovered in cell culture from all samples obtained in all 4 challenges. No virus was detected in the control fish.

3.3. Interspecies transmission challenges

3.3.1. Cohabitation 1: infected Senegalese sole and naïve gilthead seabream

Senegalese sole infected with strain 160.03 exhibited an altered behaviour after naïve gilthead seabream were added to the tanks. Calmly resting on the bottom of the tanks (normal behaviour for flat fish) turned into agitated and quick swimming, trying to escape from the seabream that became aggressive and attacked the resting sole, although no external wounds were observed. The onset of mortality in sole occurred just 1 day post cohabitation (dpc), 3 dpi, reaching 50% at 10 dpc (12 dpi) and no fish survived the challenge beyond 21 dpc (23 dpi). Erratic swimming behaviour was observed from day 6 pc, 8 dpi, onwards. No disease signs were observed throughout the experiment in cohabitant gilthead seabream and the first deaths (2%) were observed on day 6 pc. At 28 dpc, 50% of the individuals had died and at the end of the challenge (33 dpc), mortality reached 78% (Fig. 4A). These data indicated that the cohabitation challenge led to an earlier onset of mortality than the immersion challenges in both species, and to a significantly higher cumulative mortality ($p < 0.01$) with regard to the susceptibility challenges and the cohabitation control (CC). Most of the

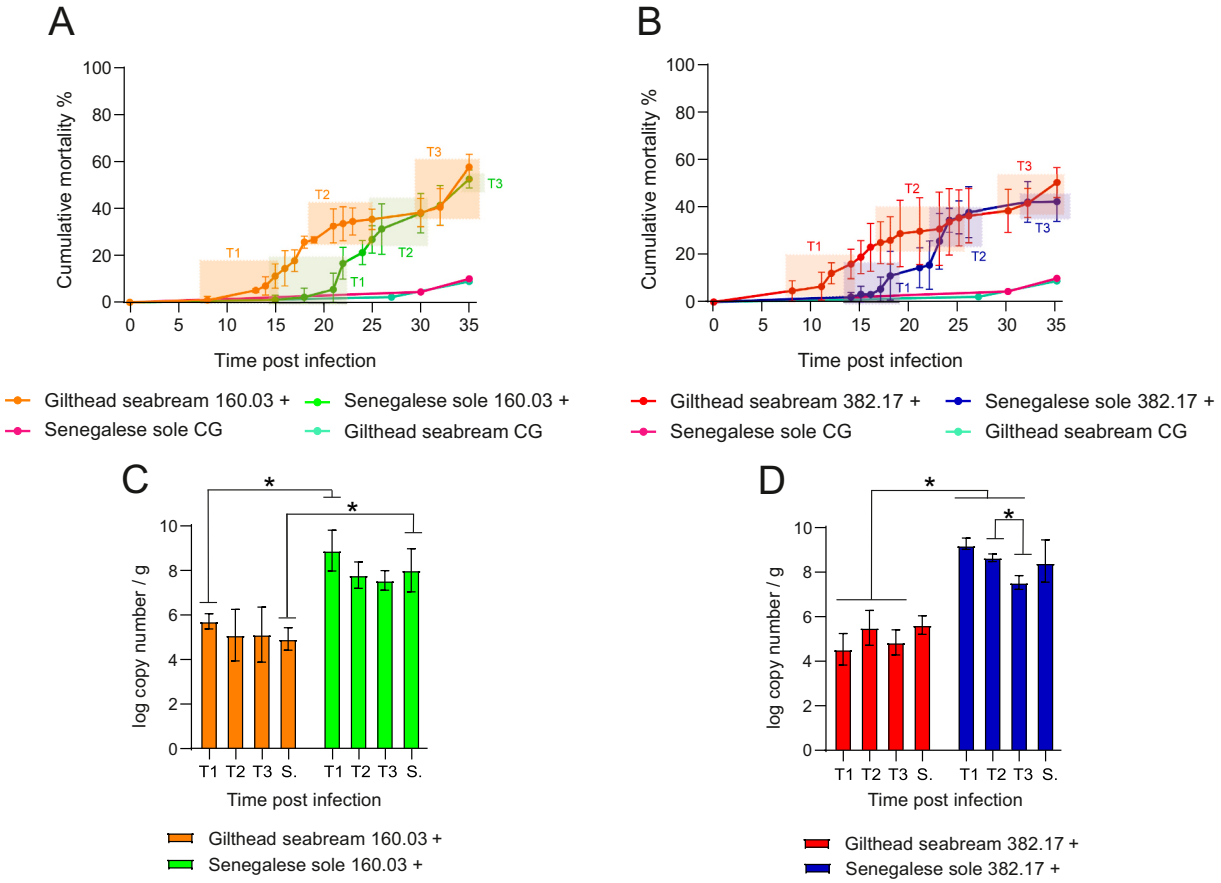

Fig. 3. Susceptibility challenges of gilthead seabream and Senegalese sole. Cumulative mortality in fish challenged with **A)** 160.03 and **B)** 382.17 strains. CG, control group mock infected with L-15. Number of NNV copies (log10 RNA) per fish gram detected in fish challenged with **C)** 160.03 and **D)** 382.17 strains at initial (T1), acute (T2) and final (T3) mortality stages. S, survivors. Each bar represents the mean ± SD ($N = 9$). Asterisks * represent significative differences (p value <0.05).

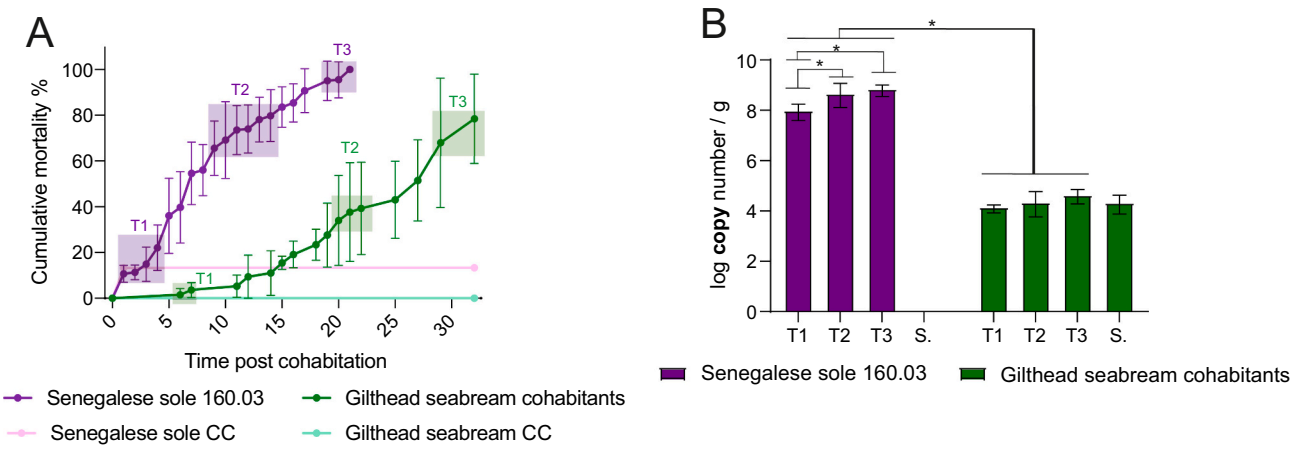

Fig. 4. Interspecies transmission challenge, cohabitation 1. **A)** Cumulative mortality in 160.03-infected Senegalese sole and naïve gilthead seabream cohabitants, and fish from cohabitation controls (CC). **B)** Viral load detected in head samples of 160.03-infected Senegalese sole and naïve gilthead seabream cohabitants at the three time points analysed as well as in seabream survivors (S). Each bar represents the mean ± SD (N = 9). Asterisks * indicate significative differences (p value <0.05).

dead individuals showed partially empty skull and ocular cavities because tissues were eaten by surviving seabream. Regarding the viral load, at the initial phase (T1), the number of RNA copies in infected sole was 9.77×10^7 and increased significantly in the following phases to 4.57×10^8 at T2 and 7.08×10^8 at T3 (p value <0.05). Significant differences of around 3 logs were observed with regard to gilthead seabream cohabitants (Fig. 4B), which yielded average values of 8.18×10^4.

NNV was recovered in cell culture from all challenged fish, confirming that the 160.03 strain was transmitted from infected Senegalese sole to naïve gilthead seabream. No virus was isolated from the control group.

3.3.2. *Cohabitation 2: infected gilthead seabream and naïve sole*

Infected gilthead seabream attacked and chased naïve Senegalese sole immediately after their introduction as cohabitants, which brought about an agitated and quick swimming pattern in the latter. Although seabream did not show typical VER signs, mortality was first recorded at 4 dpc (6 dpi); on day 18 pc (20 dpi) cumulative mortality yielded 50% and reached 80% at the end of the experiment (Fig. 5A). As occurred in the previous cohabitation experiment, Senegalese sole started to die at 1 dpc and erratic swimming and looping was observed from day 6 pc. Cumulative mortality increased to 50% on day 16 pc and reached similar values to seabream (82%) at the end of the challenge. A comparison with the immersion challenges indicated that the mortality onset was earlier and final cumulative values were significantly higher in both species than in the susceptibility challenges and in the cohabitation control ($p < 0.01$). As in the previous cohabitation experiment, although cohabitants did not show injuries, the smooth tissues of dead fish were partially absent.

The quantification of the viral load (Fig. 5B) showed differences between both species. Thus, whereas gilthead seabream individuals provided similar RNA values throughout the experiment (around 10^5 RNA copies), cohabitant sole showed significantly (p value 0.03) lower initial RNA values (1.45×10^4 at phase T1) but increased progressively to 8.32×10^4 and 4.25×10^5 (T2 and T3, respectively). In the survivors, the number of NNV copies was comparable to that of dead fish in the acute phase of mortality (4.47×10^5). Infective viral particles were recovered in cell culture from both infected and cohabitant fish samples, supporting the interspecies transmission from seabream to sole, but not from the control group.

The gilthead seabream's aggressive behaviour towards the Senegalese sole was observed also in the cohabitation control group. In addition, a 13.3% mortality was recorded in the cohabitant sole on day 1 pc, although no further deaths occurred in either species, and neither were any wounds observed.

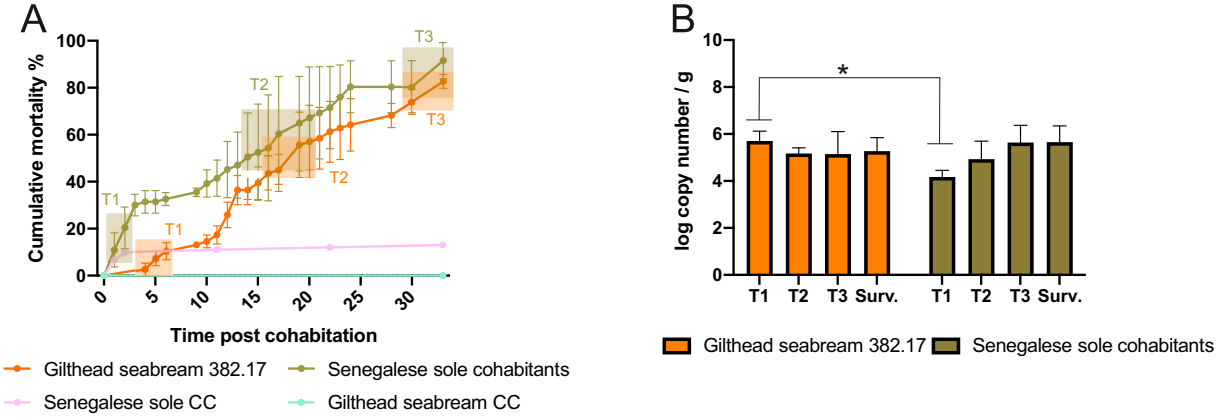

Fig. 5. Interspecies transmission challenge, cohabitation 2. **A)** Cumulative mortality in 382.17-infected gilthead seabream and naïve sole cohabitants, and fish from the cohabitation control (CC). **B)** Viral load detected in head samples of 382.17-infected gilthead seabream and naïve sole cohabitants, at the three time points analysed as well as in survivors (S). Each bar represents the mean ± SD (N = 9). Asterisk * show significative differences (p value <0.05).

3.4. Transcription of the hsp70 gene

As indicated above, in the cohabitation experiments, Senegalese sole no longer rested on the bottom of the tank and tried to escape from the attacks of the gilthead seabream. Therefore, transcription of the *hsp70* gene, a well-known stress biomarker, was quantified in cohabitant fish. In the cohabitation experiment of infected sole with naïve seabream, a significant induction of the relative expression of *hsp70* was observed in the sole that died at 1 dpc (fold change values of 26.44), while this value decreased to around 1–1.5-fold change in the sole individuals that succumbed to death later (Fig. 6A). In the case of the Senegalese sole introduced as cohabitants with the infected seabream, *hsp70* was also overexpressed, although to a lower extent (fold change value at the beginning of mortalities was 7.40), but as mortality progressed, transcription values were maintained slightly higher than in the other cohabitation experiment (5-fold change for T2, and 2-fold change for T3 and survivors) (Fig. 6B). Sole individuals from the cohabitation control ($N = 2$, mortalities recorded at 1 dpc) also showed an increased *hsp*70 expression (average fold change value 6.43) with respect to the non-cohabiting fish (Fig. 6C). Regarding the gilthead seabream, the *hsp70* transcription remained at similar levels throughout both challenges (fold change values between 1 and 2) and non-significant differences were observed with respect to the cohabitation control (Fig. 6A and Fig. 6B).

4. Discussion

In the early 2000s a number of reassortant RGNNV/SJNNV strains were obtained from VER episodes affecting farmed Senegalese sole and gilthead seabream in the Iberian Peninsula (Olveira et al., 2009). More recently, different mass mortality episodes affecting gilthead seabream in the Mediterranean area were also reported to have been caused by RGNNV/SJNNV strains (Toffan et al., 2017; Volpe et al., 2020). Later outbreaks in the Iberian Peninsula led to the isolation of additional strains and among them 382.17 has been phylogenetically characterized and demonstrated to also be an RGNNV/SJNNV reassortant. Although sequencing of the capsid protein showed high homology with reassortant strains isolated so far, two substitutions were detected, one in the N-terminal region, position 27 (Thr ➔ Ile), and the other substitution, aa298 (Phe ➔ Tyr), in the C-terminal region, which is involved in host cell recognition (Ito et al., 2008; Iwamoto et al., 2004) and in virulence for fish (Moreno et al., 2019; Souto et al., 2015a) Therefore, the aim of this study was to evaluate the potential for interspecies transmission of strains isolated from each of the two species and to assess the consequences of cohabitation stress on the outcome of the infection.

First, the susceptibility to the NNV isolates of juvenile fish (mean weight 1 g) from both species was studied. Fish were challenged by immersion, given that waterborne infection provides a more accurate approach of NNV spreading in nature (Kim et al., 2018). Although VER outbreaks in farmed gilthead seabream can occur at temperatures below 20 °C (Toffan et al., 2017) and that the sole-derived isolate (160.03) causes higher mortality rates in Senegalese sole at 22 °C (Souto et al., 2015c), these experimental infections were conducted at 25 °C because it is a temperature commonly reached in the Mediterranean and in the South European Atlantic coastal waters during the summer, when most VER episodes are reported. Although both NNV strains caused moderate-high mortalities in both fish species, the 160.03 isolate was responsible for the highest. However, mortality in sole at 25 °C was not as high as at 22 °C, as already reported (Souto et al., 2019). The results obtained for gilthead seabream can only be compared to a natural outbreak affecting juveniles (Volpe et al., 2020). Although the mortality observed in that report was higher than that observed in our challenge, the fact that the fish were survivors from a previous outbreak and the higher temperature (28 °C) could account for these differences. Besides, none of the challenged seabream showed signs of infection, which is in agreement with previous findings in larvae and juveniles during disease outbreaks (Toffan et al., 2017; Volpe et al., 2020) and under experimental conditions (Toffan et al., 2021), whereas erratic swimming was exhibited in infected sole from the first days pi. Infective NNV particles were recovered in cell culture from all infected individuals, demonstrating that both NNV isolates can cause infection and replicate in gilthead seabream and sole. Therefore, our results support the threat that the RGNNV/SJNNV reassortant may represent to gilthead and sole farming as previously reported (Souto et al., 2015b; Toffan et al., 2017; Volpe et al., 2020).

The ability of the sole-derived strain to infect other species such as turbot and sea bass has already been demonstrated in experimental infections, although mortality caused in these species was significantly lower than that observed in sole (Souto et al., 2015b, 2016). Moreover, we have previously shown that the seabream-derived strain can be transmitted to sole larvae through infected *Artemia* nauplii causing high mortalities (Vázquez-Salgado et al., 2020). On the other hand, experimental infections of sea bass with different seabream reassortant RGNNV/SJNNV strains have been reported, although low mortalities were recorded (Vendramin et al., 2014). Nevertheless, cross transmission of a reassortant strain from infected to naïve fish had not yet been demonstrated. Our results indicate not only that naïve sole and seabream cohabitants were infected with either of the two NNV isolates but also that cohabitation caused earlier and higher mortality than the bath challenges.

The onset of mortality in sole at just 1 dpc (in both infected and naïve cohabitants) and their behavioural changes in response to the continuous attacks from gilthead seabream, a dominant pattern already described (Kudoh and Yamaoka, 2004), prompted us to measure stress

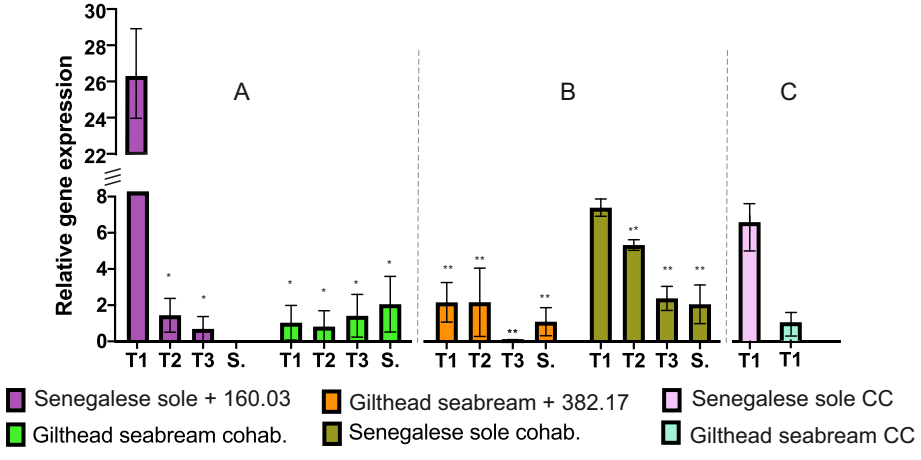

Fig. 6. Relative *hsp*70 expression in head samples of dead individuals from cohabitation challenges at 3 mortality stages. **A**) 160.03-infected Senegalese sole (T1: 1 dpc, T2: 9 dpc, T3: 21 dpc) and gilthead seabream cohabitants (T1: 6 dpc, T2: 20 dpc, T3: 29 dpc). **B)** 382.17-infected seabream (T1: 4 dpc, T2: 19 dpc, T3: 30 dpc) and sole cohabitants (T1: 1 dpc, T2: 14 dpc, T3: 30 dpc). **C)** Fish from the cohabitation control (CC) (T1: 1 dpc). Each bar represents the mean ± SD (N = 9). Significative differences (p value <0.05) with respect to the T1 of 160- infected Senegalese sole* and cohabitant Senegalese sole**.

levels in the fish. Therefore, the relative expression of the *hsp70* gene, a well-known stress biomarker (Pockley, 2003; Tort et al., 2019), was quantified in the fish subjected to cohabitation experiments. Gene transcription in the naïve sole individuals cohabiting with infected seabream was enhanced at 1 dpc (7.40-fold change) which seems to indicate that cohabitation is a stress factor. This hypothesis is supported by the similar *hsp70* transcription values observed in the sole from the cohabitation control group and by previous reports showing an increase in *hsp70* expression in the optic and vagal lobes of goldfish (*Carassius auratus*) after cohabiting with bluegills (*Lepomis macrochirus*), a potential predator (Kagawa et al., 1999). The overexpression of *hsp*70 was significantly higher in the cohabitant infected sole which died 1 dpc (26.44-fold change), suggesting that NNV infection could also be involved in the induction of this gene, as observed in other NNV-infected fish (Lama et al., 2020; Liu et al., 2016). This overexpression could be caused by the activation of the gene promotor, as demonstrated in *Ctenopharyngodon idellus* kidney (CIK) cells infected with the Grass Carp Reovirus (GCRV) (Yu et al., 2020). Although an apparently stressed behaviour was observed in the sole throughout the cohabitation experiment, the dead fish analysed in the acute and late mortality phases showed significantly lower *hsp70* transcription levels. This finding suggests that after a severe increase, the stress response in sole in terms of gene expression can be modulated, as reported in rainbow trout or tilapia (Basu et al., 2001; Iwama et al., 2004).

On the other hand, it is interesting that the viral load in naïve sole cohabitants was significantly lower than that observed in the infected individuals (from both the bath and cohabitation challenges), possibly due to the low viral load in the water, as it depends on the shedding from infected gilthead seabream. This finding, together with the aforementioned increase in *hsp70* gene expression, suggests that both stress and viral infection could be involved in the early mortalities observed in the naïve cohabitant sole and could have contributed to the increased mortality observed in the cohabitation experiments. The effect of stress on the correct functioning of the immune system and its consequences on the severity of fish infections has already been reported (Tort, 2011).

Regarding gilthead seabream, NNV horizontal transmission from infected sole could have occurred not only through the water column, as observed in striped jack or Senegalese sole (Nguyen et al., 1997; Souto et al., 2018), but also because of the ingestion of NNV infected nervous tissue from dead fish. Gilthead seabream display a cannibalistic behaviour (Castric et al., 2001) especially observed with soft tissues of dead fish. Cannibalism was more evident in the case of dead congeners, which showed the skull and eye cavity to be almost empty, whereas in sole the brain was partially eaten, probably because of their head morphology. The low viral quantification achieved in this study could be due to an underestimation of the viral load. However, considering that NNV replication in gilthead seabream larvae was demonstrated to be around 2 logs above to that detected in our study (Toffan et al., 2021), it could be due to the low replication efficiency displayed by NNV reassortants in seabream individuals beyond the larval stage, as suggested by these authors. Alternatively, it could be explained by the neutralization of the NNV particles by the strong immune response in the early stages of viral infection as observed in the brain of adult seabream individuals (Chaves-Pozo et al., 2012).

On the other hand, the *hsp70* expression levels in this fish species were not significantly enhanced after the cohabitation with the Senegalese sole, indicating that, as a species with an intrinsic nervous and aggressive behaviour, the cohabitation with sole did not increase their stress levels. Therefore, other factors rather than stress must be involved in the increase in mortality observed in both cohabitation challenges.

To conclude, this study has demonstrated that reassortant NNV strains isolated from gilthead seabream and Senegalese sole can infect both fish species causing similar levels of mortality. In addition, NNV horizontal transmission between both fish species was recorded, highlighting the threat that interspecies transmission can represent for Mediterranean aquaculture. It is also worth mentioning the serious impact of stress on the outcome of the infection in Senegalese sole, which led to a significant increase in mortality. Therefore, rearing NNV susceptible species in cohabitation, showing dominant and submissive behaviour, constitutes a serious risk of VER outbreaks that could lead to large production and economic losses.

Author statement

IB, LVS, conceived and designed the experiments, LVS, JGOH performed the experiments; IB, LVS, JGOH, CPD analysed the data; LVS and IB wrote the paper; CPD revised the paper. All authors declared to read and accepted the manuscript.

Declaration of Competing Interest

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

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