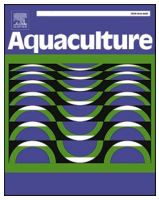
Aquaculture 579 (2024) 740211

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Nervous necrosis virus (NNV) vaccination of carrier Senegalese sole *(Solea senegalensis)*

Sandra Souto [a,*], Jose G. Olveira [a], Carmen López-Vázquez [a], Carlos P. Dopazo [a], Alejandro Labella [b], Isabel Bandín [a]

[a] Departamento de Microbiología y Parasitología, Instituto de Acuicultura, Universidade de Santiago de Compostela, Santiago de Compostela 15782, Spain
[b] Departamento de Microbiología, Instituto de Biotecnología y Desarrollo Azul (IBYDA), Universidad de Málaga, Málaga 29071, Spain

ARTICLE INFO

Keywords:
Senegalese sole
Nervous necrosis virus
NNV carrier
Immune response
Vaccination

ABSTRACT

Viral encephalopathy and retinopathy (VER), caused by nervous necrosis virus (NNV), is one of the most threatening diseases affecting marine farmed fish, especially in early developmental stages. In addition, subclinical infections are commonly detected in both farmed and wild fish. In recent years numerous efforts have been made to achieve effective vaccines for the protection of different fish species against NNV infection. Vaccination experiments are always conducted on pathogen-free animals, but subclinically infected individuals may respond differently to immunization. In this study, we have observed a different response between experimentally-induced-NNV carrier Senegalese sole and NNV-free animals when immunized with a BEI-inactivated vaccine, whereas mock-vaccinated carrier fish behaved like the NNV-free group. Analysis of the immune response after vaccination showed specific antibody production as well as up-regulation of the genes coding for the proinflammatory cytokines IL-6 and IL-8, the antiviral protein HERC4 and the cytotoxic T lymphocyte (CTL) and T helper markers CD8 and CD4 in vaccinated NNV-free and mock-vaccinated carrier individuals. However, no significant antibody production was detected and only *cd4* up-regulation was recorded in the vaccinated carrier fish. After challenge, viral replication was similar in the vaccinated groups up to 15 days post-challenge (dpc), although thereafter the viral load in the vaccinated carrier fish reached values similar to the control fish, whereas in the vaccinated NNV-free sole remained 1.5–2 log lower. At 2 dpc 10% mortality was recorded in the vaccinated carrier group that did not correlate with a higher viral titre, but with an over-expression of inflammation-related genes. These results suggest that an acute inflammatory reaction could have been the cause of the fish death. Excepting the values recorded in this episode, poor modulation of the immune response was observed at the early days post-challenge. However, at 30 dpc the IgM levels in the vaccinated carrier fish were significantly higher than those of the NNV-free sole. Longer post-vaccination studies would be necessary to clarify if this antibody increase may be relevant for fish protection.

1. Introduction

Among viral diseases that affect fish, viral encephalopathy and retinopathy (VER) is one of the most prevalent and threatening. The disease is caused by nervous necrosis virus (NNV) a small non-enveloped single-stranded RNA virus, member of the genus *Betanodavirus* within the family *Nodaviridae*. NNV genome is composed of two segments named RNA1 and RNA2, which encode the RNA-dependent polymerase (RdRp), and the capsid protein (CP), respectively. Sequence analysis of the genomic segments leaded to the classification of the different NNV isolates into four genotypes: tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV), striped jack nervous necrosis virus (SJNNV), and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997), that have recently been recognized as different viral species (Sahul Hameed et al., 2019). Moreover, NNV isolates with reassortment of genome segments between different genotypes have been reported in Southern Europe (Olveira et al., 2009; Toffan et al., 2017; Volpe et al., 2020).

NNV has been detected worldwide and has a wide range of hosts including marine and freshwater fish (Bandín and Souto, 2020). VER

* Corresponding author.
E-mail addresses: sandra.souto@usc.es (S. Souto), Jose.olveira@usc.es (J.G. Olveira), mdelcarmen.lopez.vazquez@usc.es (C. López-Vázquez), carlos.pereira@usc.es (C.P. Dopazo), amlabella@uma.es (A. Labella), isabel.badin@usc.es (I. Bandín).

https://doi.org/10.1016/j.aquaculture.2023.740211
Received 30 May 2023; Received in revised form 6 September 2023; Accepted 9 October 2023
Available online 10 October 2023

epizootic outbreaks have been reported in a variety of economically important farmed species including groupers (*Epinephelus* sp), Asian sea bass (*Lates calcarifer*) and European sea bass (*Dicentrarchus labrax*). In addition, the rearing of species such as Senegalese sole (*Solea senegalensis*) and gilthead seabream (*Sparus aurata*) is compromised by highly virulent natural RGNNV/SJNNV reassortant strains (Olveira et al., 2009; Toffan et al., 2017; Vázquez-Salgado et al., 2022; Volpe et al., 2020).

Two commercial inactivated vaccines are available for the protection of European sea bass (Bandín and Souto, 2020) and numerous experimental vaccination approaches, including DNA-, protein- and virus like particles (VLPs)-based vaccines as well as physically and chemically inactivated vaccines, have been reported in recent years for use in different fish species (Chen et al., 2015; Chien et al., 2018; Cho et al., 2017; Gonzalez-Silvera et al., 2019; Lin et al., 2016; Thwaite et al., 2020; Valero et al., 2016, 2018). The experimental vaccination assays are always performed with pathogen-free animals. However, asymptomatic NNV-infected individuals have been reported frequently among farmed fish species (Gomez et al., 2011; Gomez et al., 2004; Haddad-Boubaker et al., 2013; Olveira et al., 2013; Panzarin et al., 2012; Wang et al., 2019). These carrier fish can be subjected to vaccination programs in field conditions but, to the best of our knowledge, no evaluation of the vaccine protection or possible side-effects in these fish has been conducted up to date. Thus, the aim of the present study was to analyze the performance of a previously reported binary ethylenimine (BEI)-inactivated vaccine (Valero et al., 2021) in experimentally induced NNV-carrier Senegalese sole juveniles in comparison with NNV-free animals. In addition, humoral immune response, transcription of relevant immune- related genes after vaccination and challenge, as well as viral replication, have been analyzed.

2. Material & methods

2.1. Viral propagation and vaccine preparation

NNV strain SpSs-IAusc160.03 (hereafter Ss160.03), a RGNNV/SJNNV reassortant isolated from farmed Senegalese sole during a NNV outbreak (Olveira et al., 2009), was used for the vaccine preparation and for the experimental infection.

Ss 160.03 was propagated in E-11 cells, clone-derived form the SSN-1 cells (Iwamoto et al., 1999), with L-15 medium (Lonza) containing 2% foetal bovine serum (FBS, Lonza), penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}) at 25 °C. Viral titration was performed in triplicate by the endpoint dilution method and the viral titre was expressed as $TCID_{50}$ ml^{-1} according to the method described by Reed and Müench (1938).

The inactivated vaccine was prepared as previously described (Valero et al., 2021). Briefly, Ss 160.03 strain (5×10^8 $TCID_{50}$ ml^{-1}) was mixed with 1 mM binary ethylenimine (BEI) for 72 h at 25 °C. The inactivation was confirmed by the absence of cytopathic effects in E-11 cells after three 10-day blind passages.

2.2. Fish

A total of 410 Senegalese sole juveniles (average weight 2.5 ± 0.1 g) were obtained from a commercial fish farm and maintained at the fish facilities of the Universidade de Santiago de Compostela. Fish were placed in two opaque 200 l-tanks containing sea water (salinity 33 g l^{-1}) at 18 °C, the temperature used at the farming site, to resemble putative field vaccination conditions (Fig. 1). Prior to the experiments 10 fish

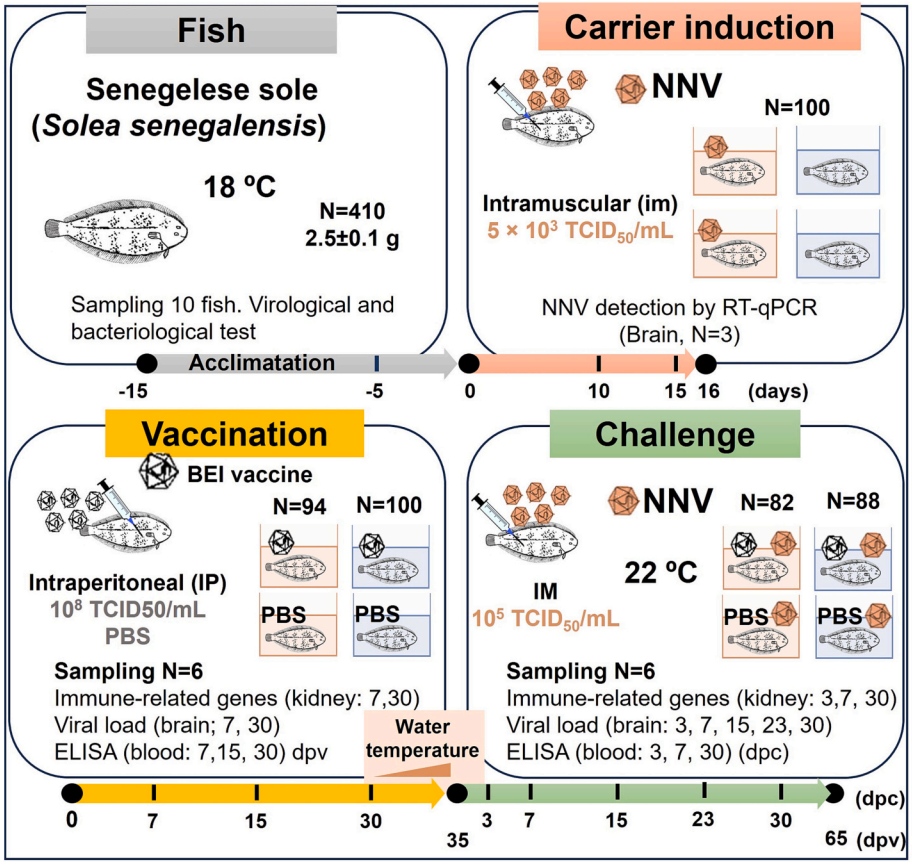


Fig. 1. Experimental setup to test immunogenicity and the protective efficacy of BEI-vaccine against a lethal NNV challenge in NNV-carrier and non-carrier Senegalese sole specimens. Infections were performed by injecting 5×10^3 $TDID_{50}$ mL^{-1} (carrier induction) and 1×10^5 $TDID_{50}$ mL^{-1} (challenge). BEI-inactivated vaccine was administered intraperitoneally. Dpv: days post vaccination; dpc: days post-challenge. The sticks on the timelines represent sampling points and the numbers below the time after treatments.

were killed with tricaine methanesulfonate (MS-222; Sigma-Aldrich, Missouri, USA) overdose to confirm the absence of viral and bacterial pathogens. RT-PCR tests using specific primers were carried out for viral haemorrhagic necrosis virus (VHSV), infectious pancreatic necrosis virus (IPNV), and NNV as previously described (Olveira et al., 2013). Besides, internal organs were inoculated in tryptone soy agar supplemented with 1% NaCl (TSA-1) for bacteriological analysis. The fish were fed *ad libitum* with dry commercial pellets daily and all efforts were made to minimize animal suffering. Experimental protocols were approved by the Bioethics and Experimental Animal Welfare Committees of the University of Santiago de Compostela and Xunta de Galicia (Permit Id. 15,010/2020/004).

2.3. Induction of the carrier state

Prior to the vaccination assay, fish were distributed in four 100 l tanks ($N = 100$) and individuals from two of the tanks were infected by intramuscularly injection (im) with 100 μl of a Ss160.03 suspension at 5×10^3 $TCID_{50}$ ml^{-1}, as this viral concentration was proven to induce asymptomatic infection in sole in previous *in vivo* experiments (unpublished data). Infected fish were monitored daily for disease signs and samples from brain tested by quantitative reverse transcription polymerase chain reaction (RT-qPCR) to confirm the carrier state ($N = 3$ per time point) at 10 and 15 days post infection (dpi), using SnodR1 primers as described earlier (Olveira et al., 2013).

2.4. Vaccination

For the vaccination assay four experimental groups (each located in a separate tank) were established (Fig. 1): 1) vaccinated NNV-free fish, 2) mock-vaccinated NNV-free fish (control group), 3) vaccinated carrier fish, and 4) mock-vaccinated carrier fish. Sole individuals were anaesthetized with 75–100 mg l^{-1} of MS-222(Sigma-Aldrich) and intraperitoneally injected (ip) with 100 μl of the BEI-inactivated vaccine (10^8 $TCID_{50}$ ml^{-1}) or PBS.

2.5. NNV challenge

After 30 days post vaccination (dpv) fish were acclimated for 5 days at increasing temperatures to reach 22 °C, the optimal replication temperature for the reassortant NNV strain (Souto et al., 2015), and at 35 dpv NNV carrier ($N = 82$) and non-carrier ($N = 88$) sole were anaesthetized as previously described and im injected with Ss160.03 strain (10^5 $TCID_{50}$ ml^{-1}) (Fig. 1).

2.6. Fish sampling

Six individuals per group were sampled at each time point. Blood samples were collected with a 30G needle at 7, 15 and 30 dpv and at 3, 7 and 30 days post-challenge (dpc) (Fig. 1). After collecting the serum was clarified by centrifugation at 10,000 ×*g* for 10 min at 4 °C and stored at −20 °C until needed. Head-kidneys were extracted under sterile conditions at 7 and 30 dpv and at 3, 7 and 30 dpc for immunological analysis. Additionally, brain tissues ($n = 3$) were sampled at 7 and 30 dpv and at 3, 7, 15, 23 and 30 dpc for viral load quantification.

2.7. Specific antibody levels

Antibody detection was accomplished by an indirect enzyme-linked immunosorbent assay (ELISA) as previously described (Valero et al., 2021). All assays were repeated twice. Briefly, 96-well plates were coated with 20 μg of total proteins from serum samples overnight at 4 °C, washed with PBST [PBS with 0.2% of Tween-20] and blocked with 5% skimmed milk in PBST. Afterwards, incubation with the NNV suspension, the rabbit anti-NNV (Abcam, Inc.; 1:10,000), and the anti-rabbit IgG-HRP (Sigma Aldrich; 1:25,000) was performed at room temperature. The reaction was revealed with 3,3′,5,5′-tetramethylbenzidine single solution (ThermoFisher, Vilnius, Lithuania) and the absorbance was read at 450 nm with an iMark™ Microplate Absorbance Reader (BioRad, Hercules, USA). ELISA results are provided as the optical density (OD) at 450 nm. Positive and negative controls were included.

2.8. Quantification of immune-related gene transcription

RNA extraction, reverse transcription and SYBR green real-time PCR procedures are referred to a previous assay performed with the BEI-inactivated vaccine (Valero et al., 2021). Briefly, total RNA was isolated from head-kidney using Nucleospin RNA II kit (Macherey-Nagel), reverse transcription was carried out using Superscript IV (Invitrogen™) with Random Hexamers (ThermoFisher) and mRNA transcriptional levels of sole immune-related genes were analyzed by RT-qPCR in an iCycler iQ CFX96™ Real Time System (BioRad, Hercules, USA). Reaction mixtures (containing 20 μl of iQ™ SYBR Green supermix with 0.2 μM of the specific primers and 2 μl of cDNA template) were incubated for 3 min at 95 °C as an activation/denaturation step, followed by 40 cycles of 15 s at 95 °C and 30 s at 55 or 58 °C. Negative controls were always included in the reactions. The analyzed genes were the following: *cd8, cd4, herc4, il6* and *il8*. In addition, to assess a putative inflammatory response in the challenged fish, the transcription of toll-like receptor 7 (*tlr7*), tumor necrosis factor alfa (*tnfα*) and *il10* genes were quantified. The specific primers used are shown in Table 1. The relative expression of all genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the β actin (*actb*) coding gene as the endogenous reference.

2.9. Viral quantification

NNV RNA1 extraction and amplification from brain samples was accomplished as described above using SnodR1 primers (Olveira et al., 2013). All samples were tested in triplicate. The corresponding standard curve was generated using 20-fold dilutions (from 10^1 to 10^7copies/μl) of a plasmid DNA containing the full-length RNA1 of strain Ss160. Viral load data were calculated as RNA1 copies per gram of fish tissue.

2.10. Calculations and statistics

The cumulative mortalities were used to calculate the relative survival percentage (RPS) according to the following formula:

Table 1
Primer sequence used for gene transcription analysis.

Gene	Sequence 5′-3′	Accesion No[a], Reference or Unigene ID[b]
cd4	F: GACCTCAGGCTGCAATGGT R: TGAGCAGAGTGATGGACAGACT	Montero et al., 2015
cd8a	F: GTCGCAGTTCTGCTCTCCGC R: TCGGTTGCAGTAGAGGACGG	López-Vázquez et al., 2023
herc4	F: GCCAAAACACTGGCATGGTT R: AACGCCAAACAGGAAGTACCT	Gémez-Mata et al., 2021
Il6	F: GTGCGTGACATCTACAACCC R: GCCGTCTTCTCCTTTCCCTT	solea_v4.1_UNIGENE631474
Il8	F: AAGGTCCTTACTGCGCAAAC R: TGCTCTTCCCTGCTGATGAA	López-Vázquez et al., 2023
Il10	F: ACTCCACAGTCTCTCCTCCT R: GAAACCCTCCACGAAGCGA	solea_v4.1_unigene343952a
tnfα	F: TGTGTACATGGGAGCTGTGT R: CACAGAGCGAACACACCAAA	XM_044052089
tlr7	F: GGGAGTGAGGTCAAAGTGGA R. CGTGGAAGGAGGAGGAGTTT	XM_044052250
actb	F GACGACATGGAGAAGATC R GGTGTTGAAGGTCTCAAA	DQ485686

[a] https://www.ncbi.nlm.nih.gov/nuccore
[b] https://www.scbi.uma.es/soleadb

$$RPS = \left[1 - \frac{\%mortality\ of\ vaccinated\ group}{\%mortality\ control\ group}\right] x\ 100\%$$

All statistical analyses were conducted using GraphPad Prism 8 and the data are shown as the mean ± standard error of the mean (SEM). Variations between different time points and groups were analyzed by a two-way ANOVA followed by Tukey's post-hoc analysis. Survival curves were compared using the Kaplan Meier test.

3. Results

3.1. Induction of the carrier state

NNV-infected sole did not show any sign of disease, but low viral loads were detected in all fish analyzed at 10 and 15 dpi. At 15 dpi RNA1 copy number ranged from 1.5×10^2 to 1.0×10^4 per gram of fish tissue (average 3.5×10^3 RNA1 copy number/g).

3.2. Vaccine performance and viral load in fish

After vaccination no mortalities were recorded, and all fish groups showed normal behavior. In carrier fish, the viral load at 7 and 30 dpv showed an average value of 8.1×10^2 and 3.02×10^3 for mock-vaccinated fish, and 1.41×10^3 and 4.04×10^3 RNA1 copy number/g fish tissue for vaccinated fish.

After challenge, clinical signs (abnormal swimming behavior and anorexia) were observed in the control group at 5 dpc and mortality onset was at 7 dpc, whereas in the vaccinated NNV-free individuals VER signs and mortality were slightly delayed until 8 and 11 dpc, respectively. In the vaccinated carrier fish, an unexpected mortality (8 out of 82, 9.8%) was recorded at 2 dpc, whereas in the mock-vaccinated carrier group 2 individuals died. However, VER signs were not observed in these animals and were not recorded in the carrier groups until 11 dpc. Both vaccinated groups showed a better survival rate than the control fish, but significant improvement was only observed in the vaccinated NNV-free group ($p = 0.018$, Fig. 2A) which showed a relative percent of survival (RPS) of 51.6. The RPS of the vaccinated carrier fish was 29.24, but, if the sudden mortality observed at 2 dpc is not considered it would increase up to 42.71, whereas that of the mock-vaccinated carrier fish was 16.6.

The analysis of the viral load revealed that at 3 dpc the number of RNA1 copies was similar in both vaccinated and non-vaccinated groups. However, in the following time points a significant difference was observed between the vaccinated NNV-free fish and the control fish. Thus, from 7 to 23 dpc the viral load detected in the vaccinated NNV-free sole was >2 logs lower than in the control, whereas at 30 dpc the difference was reduced to around 1.5 log (Fig. 2B). The vaccinated carrier fish also showed a significantly lower viral load than the control fish at 7 and 15 dpc, but no differences were recorded afterwards (Fig. 2B).

3.3. Immune response in vaccinated fish

3.3.1. Humoral response

Significant IgM production was recorded only in the vaccinated NNV-free group at 30 dpv, whereas antibody production was detected in all vaccinated and non-vaccinated animals after viral challenge (Fig. 3). However, significant antibody levels when compared with control fish were only detected at 30 dpc in both vaccinated groups, the increase being much higher in the vaccinated carrier fish (Fig. 3).

3.3.2. Immune-related genes transcription

The transcription of immune-related genes was tested at 7 and 30 dpv. A significant up-regulation of all 5 genes was only recorded at the end of immunization assay in vaccinated NNV-free and mock-vaccinated carrier individuals. Thus, the expression of *cd8* was significantly induced in both groups when compared not only with control fish but also with vaccinated carrier fish (Fig. 4A). The transcriptional levels of *cd4*, *herc4*, *il6* and *il8* were also modulated in a similar extent in both groups (Fig. 4B, C, D, E). However, after challenge only significant over-expression of *cd8* and *il6* was recorded at 3 dpc and in different fish groups. Thus, *cd8* up-regulation was observed in both vaccinated groups (Fig. 4A), whereas *il6* was only up-regulated in mock-vaccinated carrier fish (Fig. 4D).

Additional analysis of immune-related genes putatively related with an inflammatory response was performed in the vaccinated and mock-vaccinated carrier individuals that died at 2 dpc. For comparative purposes, transcriptional analyses were also performed in 3 control and 3 vaccinated NNV-free fish sacrificed in the same day and in fish from all experimental groups sampled at 30 dpv. All four genes analyzed were overexpressed in the dead fish belonging to the vaccinated carrier group when compared not only with fish from the control group but also with vaccinated NNV-free fish and with the dead mock-vaccinated individuals. As shown in Fig. 5A transcriptional levels of *tlr7* were significantly higher in the 8 dead vaccinated carrier individuals with respect to the control fish ($p = 0.0036$), the mock-vaccinated carriers and the vaccinated NNV-free fish ($p = 0.0102$). Likewise, significant *tnfα* and *il10* up-regulation was observed in the vaccinated carriers when compared with the other three groups (Fig. 5B, D). Transcription of *il6* was the most highly modulated (18-fold) with respect to the control fish, but a moderate up-regulation was also recorded in the mock-vaccinated carrier individuals (Fig. 5C). Moreover, when the results of the dead vaccinated carrier fish were compared with those obtained before

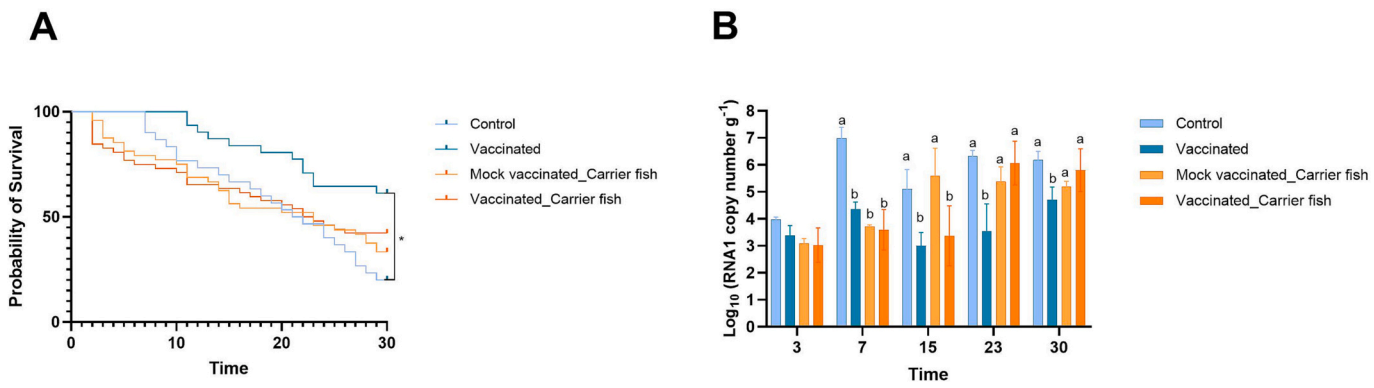


Fig. 2. Protection conferred by the vaccine in NNV-carrier and non-carrier *Senegalese sole* specimens and viral load in the fish brains during 30 days of *in vivo* infection. (**A**) Percent of survival and (**B**) viral load ($n = 3$ fish/group and time) in brain. Fish were vaccinated by intraperitoneal injection and infected by intramuscular injection with 10^5 $TCID_{50}$ mL^{-1}. Survival rates were compared between groups using the Kaplan Meier and Log-rank tests (*$p = 0.0012$). Letters denote statistical differences between groups at the same time point according to the two-way ANOVA test ($p < 0.01$).

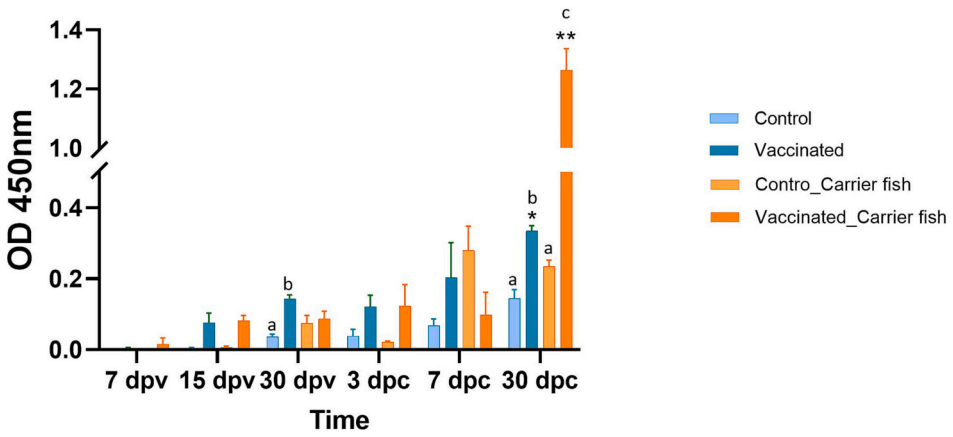


Fig. 3. IgM levels in the serum of carrier and non-carrier fish at 7 and 30 days post-vaccination and 3, 7 and 30 days post-challenge. Data represent the mean ± standard error of the mean (SEM) ($n = 6$ fish/group and time). Letters denote significant differences between groups at each time point ($p < 0.05$), whereas asterisks denote differences between time points in the same group (*$p < 0.005$, **$p < 0.0001$).

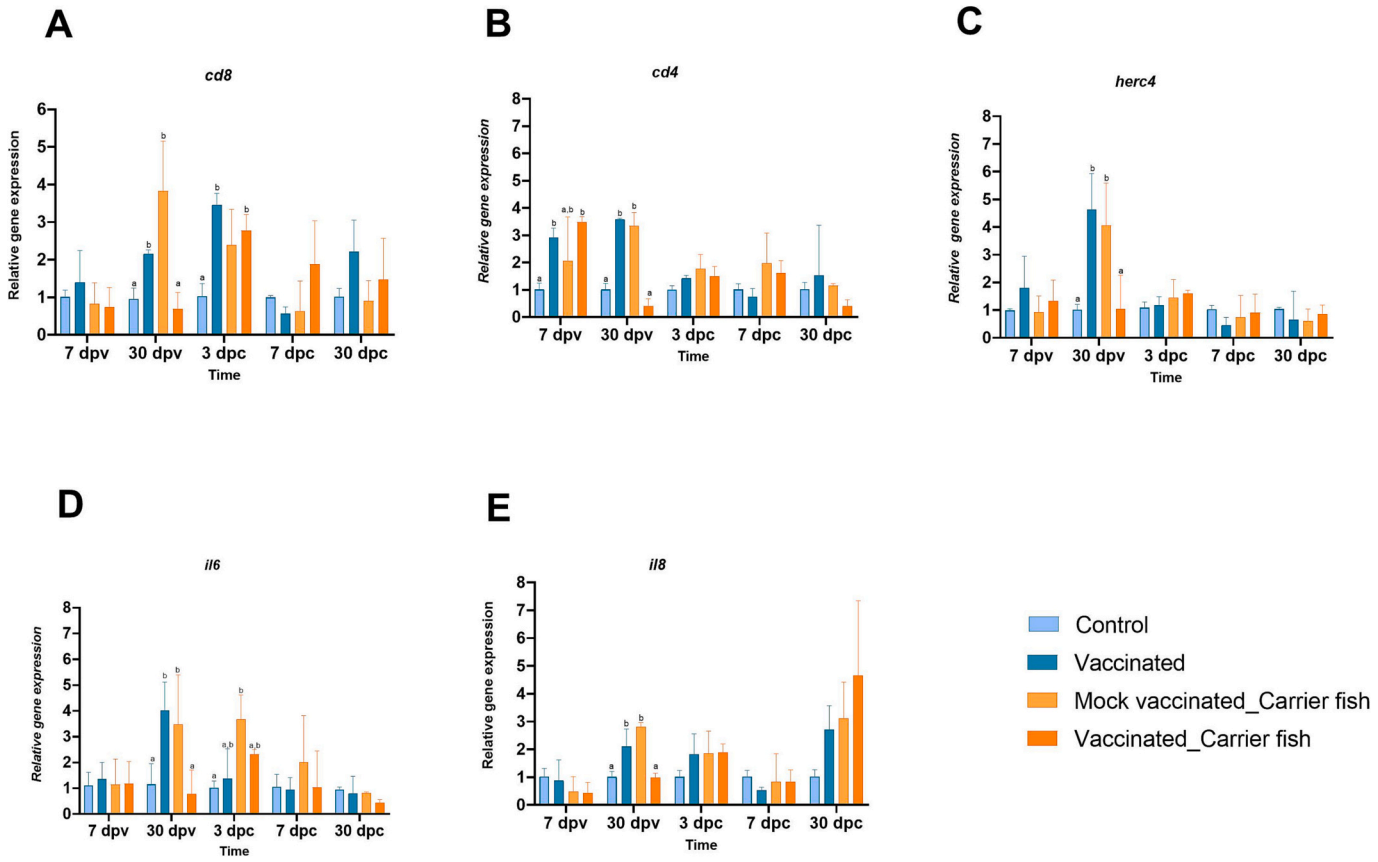


Fig. 4. Expression of immune-related genes in the head-kidney of carrier and non-carrier Senegalese sole specimens after vaccination and challenge. (A) *cd8a*, (B) *cd4*, (C) *herc4* (D) *il6* and (E) *il8*. Fish were vaccinated intraperitoneally using a BEI-inactivated vaccine or PBS (control groups) and sampled at 7 and 30 days post-vaccination. After an intramuscular challenge, fish were sampled at 3, 7 and 30 dpc. Data represent the mean ± standard error of the mean (SEM) (n = 6 fish/group and time). Letters denote statistical differences between groups at a same time-point ($p < 0.05$).

challenge (30 dpv) a significant induction was observed in the 4 genes (Fig. 5 A, B, C, D).

4. Discussion

After a VER outbreak survivors can become NNV asymptomatic carriers and it has been demonstrated that they can transmit the virus to their progeny and cohabitant fish (Costa and Thompson, 2016; Munday et al., 2002). However, to date no data is available on the response of subclinically infected animals to NNV vaccination. Therefore, the aim of this study was to assess the vaccination performance and immune response in experimentally induced NNV-carrier sole in comparison with NNV-free individuals.

After vaccination no mortalities were recorded, and no apparent differences were observed in the fish belonging to the four experimental groups. However, the analysis of the immune response elicited by the vaccine in carriers and NNV-free animals showed some differences. Significant anti-NNV IgM production, as well as an overexpression of the

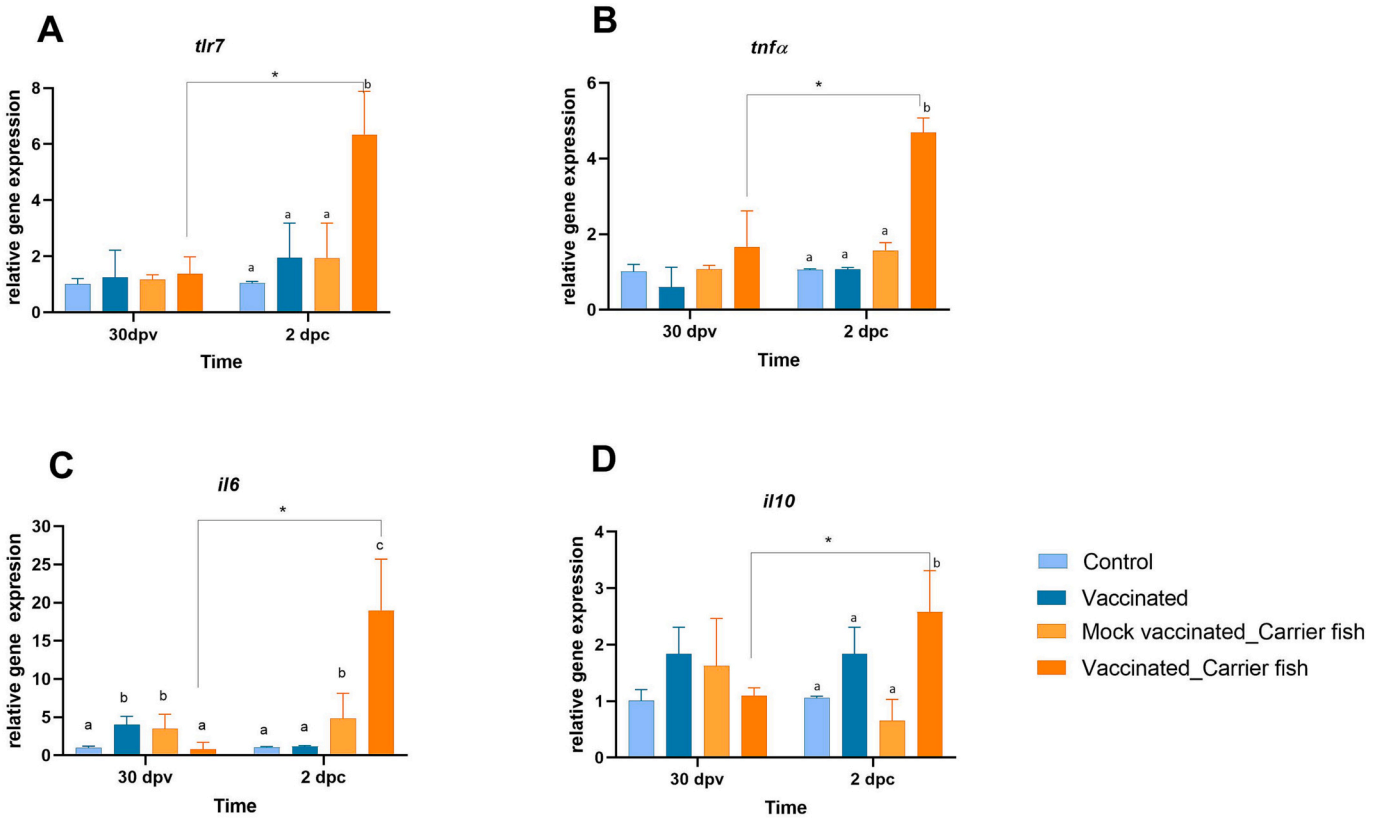


Fig. 5. Expression of genes related with an inflammatory reaction in the head-kidney of carrier and non-carrier Senegalese sole specimens at 2 days post-challenge. (A) *tlr7*, (B) *tnfα*, (C) *il6* and (D) *il10*. Samples from the carrier groups (vaccinated and mock-vaccinated) were obtained from dead animals, whereas samples from the non-carrier groups were obtained from sacrificed individuals. Data represent the mean ± standard error of the mean (SEM). Dead vaccinated carrier fish ($n = 8$); dead mock-vaccinated carrier fish ($n = 2$); sacrificed non-carrier vaccinated and control individuals (n = 3, each). Letters denote statistical differences between groups at the same time-point ($p < 0.005$), whilst asterisks indicate those between time-points in the same group (*$p < 0.0001$).

genes coding for the proinflammatory cytokines IL-6 and IL-8, the cytotoxic T lymphocytes (CTL) and T helper markers CD8 and CD4 and the antiviral protein HERC4, was observed in NNV-free individuals. However, no significant antibody production and only *cd4* up-regulation were detected in the carrier fish. A transient inflammatory response has been regarded as a key step for the vaccination-induced protective immunity (Tang and Nguyen, 2014). Moreover, CTLs are considered to play a major role in fish antiviral defense (Somamoto et al., 2014) and fish CD4 + T-helper cells are thought to coordinate the immune response by acting either as effector cells or as memory cells as their mammalian counterparts (Ashfaq et al., 2019). Finally, HERC4 is a ligase that regulates the Hedgehog (HH) signaling pathway (Jiang et al., 2019) which can positively or negatively influence the immune response in a context-dependent manner (Zhou et al., 2022). Both CTL-related genes and *herc4* have been previously demonstrated to be up-regulated by NNV vaccination (Cheng et al., 2017; López-Vázquez et al., 2023; Valero et al., 2021). The poor gene modulation observed in the carrier fish after vaccination suggests that asymptomatic NNV infection may affect the vaccine efficacy contrarily to what has been reported in children receiving the rhesus rotavirus vaccine in which an enhanced immunogenicity was reported because of prior subclinical infection (Pichichero and Losonsk, 1993). In this regard, the time between viral infection and vaccination may be a relevant factor. An experimentally induced carrier state has been demonstrated to last for at least two months in sole and more than four in Altantic salmon (*Salmo salar*) (Souto et al., 2015; Korsnes et al., 2005), but no information was provided about the immunological status of those animals. By contrast, the immune response in mock-vaccinated carrier fish was very similar to that of vaccinated NNV-free animals which suggests that subclinical infection may act as a protective factor against a subsequent viral infection as it has been reported in sevenband grouper, *Epinephelus septemfasciatus* (Nishizawa et al., 2012; Oh et al., 2013).

After challenge with the homologous strain a sudden mortality (10% of the fish) was recorded at 2 dpc in the vaccinated carrier group. Average viral load in the dead animals was 4×10^3 $TCID_{50}$/g fish which was very similar to that detected in this fish group before challenge and in vaccinated NNV-free and control fish which were sacrificed for comparative purposes. Therefore, it can be concluded that the observed mortality was not due to the viral replication in the brain. Vaccination should induce a highly specialized inflammatory profile which ensures that the most appropriate effector functions are elicited (Alter and Sekaly, 2015). However, this inflammatory response may have also undesired effects such as an acute phase reaction (APR) (Khalil and Al-Humadi, 2020). Vaccination induces the production of inflammatory and proinflammatory cytokines which triggers the synthesis of acute phase proteins (APP). APPs have a direct impact in the maintenance of homeostasis in vertebrates, particularly in the kidney and the liver (Mills et al., 1998), and disruption of this homeostasis can negatively affect the animal (Cray et al., 2009). As already discussed, after vaccination, no up-regulation of genes related with inflammatory response (*il6 and il8*) was observed in the vaccinated carrier fish. However, in the fish which died at 2 dpc a significant increase in the transcription of several genes related with an inflammatory reaction as *tlr7*, *tnfα*, *il6,* and *il10* was observed. Toll-like receptors are an important family of pattern recognition receptors (PRRs) which can recognize viral nucleic acids and different bacterial products as well as endogenous danger molecules released from dying cells and damaged tissues (Tanekhy, 2016). In our study TLR7 was chosen because it can detect single stranded RNA as the NNV genome (Zou and Secombes, 2011). TLRs participate in the inflammatory response by activating macrophages to produce pro-

inflammatory cytokines which coordinate local and systemic inflammatory reactions (Medzhitov, 2007). TNFα is involved in triggering the expression of other genes associated with inflammation as *il-8*, *il-1* and *il-7c* and therefore *tnfα* is one of the early immune genes expressed in infected fish (Zou and Secombes, 2016). TNFα can also show pro-apoptotic activity and has been associated with pathogenesis of several chronic diseases of fish (Zou and Secombes, 2016). Moreover, in rats it has been demonstrated that the release of proinflammatory molecules including TNFα, IL-I and IL-6 from the peripheral immune system, produces physiopathological outcomes in brain (Lacroix and Rivest, 1998). Although IL-10 is an anti-inflammatory cytokine that helps to moderate the immune responses (Zou and Secombes, 2016), anti-inflammatory cytokines are also involved in APR (Khalil and Al-Humadi, 2020). APR has been described in some fish species as rainbow trout (*Oncorhynchus mykiss*) and cod (*Gadus morhua*) (Gerwick et al., 2002; Magnadottir et al., 2011). It is therefore reasonable to assume that an APR may have been the cause of the early mortality observed in vaccinated carrier fish. This hypothesis is also supported by the fact that no modulation of *il6* or *il8* was recorded in the vaccinated animals which were sampled at 3, 7 and 30 dpc. In fact, *il6* up-regulation was only recorded at 3dpc in the mock-vaccinated carrier fish. However, we cannot rule out alternative explanations such as a transitory immunodepression associated to a high stress response after the challenge as observed in NNV experimentally infected Atlantic sea bass (Lama et al., 2020). In this study the stress response was reported to be activated at 1 dpi and the authors suggest that the associated immunodepression could be due to the fact that most of the available resources may be diverted to cope with the stressor. However, according to these authors alternative regulatory routes would allow the immune system to react and, therefore, an immune response is observed afterwards.

The previous subclinical infection did not seem to affect viral replication after challenge because viral load was very similar in all groups at 3 dpc. At 7 dpc the RNA1 copy number in vaccinated and mock-vaccinated carrier fish was significatively lower than that recorded in the control fish, indicating the subclinical infection has induced a protective effect on the fish as previously reported in grouper (Nishizawa et al., 2012; Oh et al., 2013). However, this protective effect seems to be limited because from 15 dpc onwards the viral load in the mock-vaccinated carrier fish reached similar values to the control fish. Similarly, the effect of the vaccine on viral replication in carrier fish was also of limited duration and the viral load was not significantly different to the control fish from 15 dpc, whereas that of the vaccinated non-carrier remained significantly lower than the control. The increased viral load in the vaccinated carriers at the end of the experimental infection could be the main factor accounting for the differences in survival observed between vaccinated carrier and NNV-free fish as, if the sudden mortality observed at 2 dpc is not considered, most deaths in the vaccinated carrier group were recorded from 15 to 26 dpc. However, other factors must be involved in the deaths recorded in this group from 3 to 15 dpc. On the other hand, the immune response was similar in both vaccinated groups, being poorly modulated in the early days post-challenge in agreement with previous assays performed by our group (López-Vázquez et al., 2023). The antibody production was not significantly different to that of the control fish and only *cd8* up-regulation was recorded. However, at 30 dpc the IgM levels in the vaccinated carrier fish were 8-fold higher than those in the NNV-free sole, and much higher than those obtained after prime or booster immunization with the same inactivated vaccine (López-Vázquez et al., 2023; Valero et al., 2021). Unfortunately, because the experiment was finished at 30 dpv we cannot figure out if this increase in IgM could be related with a long-term protection.

In conclusion, the inactivated vaccine conferred a lower protection in NNV-carrier individuals than in NNV-free sole, probably because a weaker immune response was elicited in the vaccinated carrier fish and viral replication was controlled for a shorter time. Strikingly, 10% of vaccinated carrier fish died 2 days after challenge. The up-regulation of genes related with an inflammatory response observed only in these individuals suggests that an acute inflammatory reaction could have been involved in the mortality. These findings indicate that caution should be taken when vaccinating fish suspected of having been previously infected or in contact with infected fish.

Author statement

IB, CPD: Conceptualization. SS, JGO, CLV: Methodology, Investigation. IB, SS and AL: Data curation. IB and SS: Writing- Original draft preparation, visualization. CPD and AL: Writing- Reviewing and Editing. 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.

Data availability

Data will be made available on request.

Acknowledgments

This research was funded by Ministerio de Ciencia, Innovación y Universidades (MICIU), the Agencia Estatal de Investigación (AEI) and FEDER, grant number RTI2018-094687-B-C21. Dr. Sandra Souto was funded with a postdoctoral grant from Consellería de Cultura, Educación y Universidad, Xunta de Galicia (postdoctoral grant ED481D-2022/024). The authors are grateful to Stolt Sea Farm for kindly providing the fish, J. Franqueira for technical assistance and Native English School of Languages for the English revision.

References

Alter, G., Sekaly, R.P., 2015. Beyond adjuvants: antagonizing inflammation to enhance vaccine immunity. Vaccine 33, B55–B59. https://doi.org/10.1016/j.vaccine.2015.03.058.

Ashfaq, H., Soliman, H., Saleh, M., El-Matbouli, M., 2019. CD4: a vital player in the teleost fish immune system. Vet. Res. 50, 1–11. https://doi.org/10.1186/s13567-018-0620-0.

Bandín, I., Souto, S., 2020. Betanodavirus and VER disease: a 30-year research review. Pathogens. https://doi.org/10.3390/pathogens9020106.

Chen, S.P., Peng, R.H., Chiou, P.P., 2015. Modulatory effect of CpG oligodeoxynucleotide on a DNA vaccine against nervous necrosis virus in orange-spotted grouper (*Epinephelus coioides*). Fish Shellfish Immunol. 45, 919–926. https://doi.org/10.1016/j.fsi.2015.06.013.

Cheng, Y.K., Wu, Y.C., Chi, S.C., 2017. Humoral and cytokine responses in giant groupers after vaccination and challenge with betanodavirus. Dev. Comp. Immunol. 67, 385–394. https://doi.org/10.1016/j.dci.2016.08.013.

Chien, M.H., Wu, S.Y., Lin, C.H., 2018. Oral immunization with cell-free self-assembly virus-like particles against orange-spotted grouper nervous necrosis virus in grouper larvae, *Epinephelus coioides*. Vet. Immunol. Immunopathol. 197, 69–75. https://doi.org/10.1016/j.vetimm.2018.01.012.

Cho, H.S., Seo, J.Y., Park, S.I., Kim, T.G., Kim, T.J., 2017. Oral immunization with recombinant protein antigen expressed in tobacco against fish nervous necrosis virus. J. Vet. Med. Sci. 80, 272–279. https://doi.org/10.1292/jvms.16-0408.

Costa, J.Z., Thompson, K.D., 2016. Understanding the interaction between *Betanodavirus* and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. Fish Shellfish Immunol. 53, 35–49. https://doi.org/10.1016/j.fsi.2016.03.033.

Cray, C., Zaias, J., Altman, N.H., 2009. Acute phase response in animals: a review. Comp. Med. 59, 517–526.

Gémez-Mata, J., Labella, A.M., Bandín, I., Borrego, J.J., García-Rosado, E., 2021. Immunogene expression analysis in betanodavirus infected-Senegalese sole using an OpenArray® platform. Gene 774. https://doi.org/10.1016/j.gene.2021.145430.

Gerwick, L., Steinhauer, R., Lapatra, S., Sandell, T., Ortuno, J., Hajiseyedjavadi, N., Bayne, C.J., 2002. The acute phase response of rainbow trout (*Oncorhynchus mykiss*) plasma proteins to viral, bacterial and fungal inflammatory agents. Fish Shellfish Immunol. 12, 229–242. https://doi.org/10.1006/fsim.2001.0367.

Gomez, D.K., Okinaka, Y., Nakai, T., Sato, J., Mushiake, K., Isshiki, T., 2004. PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. J. Fish Dis. 27, 603–608.

Gomez, D.K., Lim, D.J., Baeck, G.W., Youn, H.J., Shin, N.S., Youn, H.Y., Hwang, C.Y., Park, J.H., Park, S.C., 2011. Detection of betanodaviruses in apparently healthy

aquarium fishes and invertebrates. J. Vet. Sci. 7, 369. https://doi.org/10.4142/jvs.2006.7.4.369.
Gonzalez-Silvera, D., Guardiola, F.A., Espinosa, C., Chaves-Pozo, E., Esteban, M.Á., Cuesta, A., 2019. Recombinant nodavirus vaccine produced in bacteria and administered without purification elicits humoral immunity and protects European sea bass against infection. Fish Shellfish Immunol. 88, 458–463. https://doi.org/10.1016/j.fsi.2019.03.013.
Haddad-Boubaker, S., Bigarré, L., Bouzgarou, N., Megdich, A., Baud, M., Cabon, J., Chéhida, N. Ben, 2013. Molecular epidemiology of betanodaviruses isolated from sea bass and sea bream cultured along the Tunisian coasts. Virus Genes 46, 412–422. https://doi.org/10.1007/s11262-012-0869-8.
Iwamoto, T., Mori, K., Arimoto, M., Nakai, T., 1999. High permissivity of the fish cell line SSN-1 for piscine nodaviruses. Dis. Aquat. Org. 39, 37–47. https://doi.org/10.3354/dao039037.
Jiang, W., Yao, X., Shan, Z., Li, W., Gao, Y., Zhang, Q., 2019. E3 ligase Herc4 regulates hedgehog signalling through promoting smoothened degradation. J. Mol. Cell Biol. 11, 791–803. https://doi.org/10.1093/jmcb/mjz024.
Khalil, R.H., Al-Humadi, N., 2020. Types of acute phase reactants and their importance in vaccination (review). Biomed. Reports 12, 143–152. https://doi.org/10.3892/br.2020.1276.
Korsnes, K., Devold, M., Nerland, A.H., Nylund, A., 2005. Viral encephalopathy and retinopathy (VER) in Atlantic salmon Salmo salar after intraperitoneal challenge with a nodavirus from Atlantic halibut *Hippoglossus hippoglossus*. Dis. Aquat. Org. 68, 7–15. https://doi.org/10.3354/dao068007.
Lacroix, S., Rivest, S., 1998. Effect of acute systemic inflammatory response and cytokines on the transcription of the genes encoding cyclooxygenase enzymes (COX-1 and COX-2) in the rat brain. J. Neurochem. 70, 452–466. https://doi.org/10.1046/j.1471-4159.1998.70020452.x.
Lama, R., Pereiro, P., Muñoz, V.V., Escárate, C.G., Tort, L., Figueras, A., Novoa, B., 2020. RNA - Seq analysis of European sea bass (*Dicentrarchus labrax* L .) infected with nodavirus reveals powerful modulation of the stress response. Vet. Res. 1–22. https://doi.org/10.1186/s13567-020-00784-y.
Lin, K., Zhu, Z., Ge, H., Zheng, L., Huang, Z., Wu, S., 2016. Immunity to nervous necrosis virus infections of orange-spotted grouper (*Epinephelus coioides*) by vaccination with virus-like particles. Fish Shellfish Immunol. 56, 136–143. https://doi.org/10.1016/j.fsi.2016.06.056.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
López-Vázquez, C., Souto, S., Olveira, J.G., Riaza, A., González, Ó., Brea, C., Labella, A. M., Castro, D., Bandín, I., 2023. Nervous necrosis virus (NNV) booster vaccination increases Senegalese sole survival and enhances Immunoprotection. Animals 13. https://doi.org/10.3390/ani13010051.
Magnadottir, B., Audunsdottir, S.S., Bragason, B.T., Gisladottir, B., Jonsson, Z.O., Gudmundsdottir, S., 2011. The acute phase response of Atlantic cod (*Gadus morhua*): humoral and cellular response. Fish Shellfish Immunol. 30, 1124–1130. https://doi.org/10.1016/j.fsi.2011.02.010.
Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. Nature 449, 819–826. https://doi.org/10.1038/nature06246.
Mills, P.C., Auer, D.E., Kramer, H., Barry, D., Ng, J.C., 1998. Effects of inflammation-associated acute-phase response on hepatic and renal indices in the horse. Aust. Vet. J. 76, 187–194. https://doi.org/10.1111/j.1751-0813.1998.tb10126.x.
Montero, D., Benitez-Dorta, V., Caballero, M.J., Ponce, M., Torrecillas, S., Izquierdo, M., Zamorano, M.J., Manchado, M., 2015. Dietary vegetable oils: effects on the expression of immune-related genes in Senegalese sole (*Solea senegalensis*) intestine. Fish Shellfish Immunol. 44, 100–108. https://doi.org/10.1016/j.fsi.2015.01.020.
Munday, B.L., Kwang, J., Moody, N., 2002. Review article Betanodavirus infections of teleost: a review. J. Fish Dis. 127–142 https://doi.org/10.1046/j.1365-2761.2002.00350.x.
Nishizawa, T., Furuhashi, M., Nagai, T., Nakai, T., Muroga, K., 1997. Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. Appl. Environ. Microbiol. 63, 1633–1636.
Nishizawa, T., Gye, H.J., Takami, I., Oh, M.J., 2012. Potentiality of a live vaccine with nervous necrosis virus (NNV) for sevenband grouper *Epinephelus septemfasciatus* at a low rearing temperature. Vaccine 30, 1056–1063. https://doi.org/10.1016/j.vaccine.2011.12.033.
Oh, M.J., Gye, H.J., Nishizawa, T., 2013. Assessment of the sevenband grouper *Epinephelus septemfasciatus* with a live nervous necrosis virus (NNV) vaccine at natural seawater temperature. Vaccine 31, 2025–2027. https://doi.org/10.1016/j.vaccine.2013.02.049.
Olveira, J.G., Souto, S., Dopazo, C.P., Thiéry, R., Barja, J.L., Bandín, I., 2009. Comparative analysis of both genomic segments of betanodaviruses isolated from epizootic outbreaks in farmed fish species provides evidence for genetic reassortment. J. Gen. Virol. 90 https://doi.org/10.1099/vir.0.013912-0.
Olveira, J.G., Souto, S., Dopazo, C.P., Bandín, I., 2013. Isolation of betanodavirus from farmed turbot *Psetta maxima* showing no signs of viral encephalopathy and retinopathy. Aquaculture 406–407, 125–130. https://doi.org/10.1016/j.aquaculture.2013.05.007.
Panzarin, V., Fusaro, A., Monne, I., Cappellozza, E., Patarnello, P., Bovo, G., Capua, I., Holmes, E.C., Cattoli, G., 2012. Molecular epidemiology and evolutionary dynamics of betanodavirus in southern Europe. Infect. Genet. Evol. 12, 63–70. https://doi.org/10.1016/j.meegid.2011.10.007.
Pichichero, M.E., Losonsk, G.A., 1993. Asymptomatic infections due to wild-type rotavirus may prime for a heterotypic response to vaccination with rhesus rotavirus. Clin. Infect. Dis. 16, 86–92.
Reed, L., Müench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27, 493–497.
Sahul Hameed, A.S., Ninawe, A.S., Nakai, T., Chi, S.C., Johnson, K.L., 2019. ICTV virus taxonomy profile: Nodaviridae. J. Gen. Virol. 100, 3–4. https://doi.org/10.1099/jgv.0.001170.
Somamoto, T., Koppang, E.O., Fischer, U., 2014. Antiviral functions of CD8+ cytotoxic T cells in teleost fish. Dev. Comp. Immunol. 43, 197–204. https://doi.org/10.1016/j.dci.2013.07.014.
Souto, S., Olveira, J.G., Bandín, I., 2015. Influence of temperature on Betanodavirus infection in Senegalese sole (Solea senegalensis). Vet. Microbiol. 179, 162–167. https://doi.org/10.1016/j.vetmic.2015.07.004.
Tanekhy, M., 2016. The role of toll-like receptors in innate immunity and infectious diseases of teleost. Aquac. Res. 47, 1369–1391. https://doi.org/10.1111/are.12607.
Tang, D.C.C., Nguyen, H.H., 2014. The yin-Yang arms of vaccines: disease-fighting power versus tissue-destructive inflammation. Expert Rev. Vaccines 13, 417–427. https://doi.org/10.1586/14760584.2014.882775.
Thwaite, R., Berbel, C., Aparicio, M., Torrealba, D., Pesarrodona, M., Villaverde, A., Borrego, J.J., Manchado, M., Roher, N., 2020. Nanostructured recombinant protein particles raise specific antibodies against the nodavirus NNV coat protein in sole. Fish Shellfish Immunol. 99, 578–586. https://doi.org/10.1016/j.fsi.2020.02.029.
Toffan, A., Pascoli, F., Pretto, T., Panzarin, V., Abbadi, M., Buratin, A., Quartesan, R., Gijon, D., Padros, F., 2017. Viral nervous necrosis in gilthead sea bream (*Sparus aurata*) caused by reassortant betanodavirus RGNNV/SJNNV: an emerging threat for Mediterranean aquaculture. Sci. Rep. 7, 1–12. https://doi.org/10.1038/srep46755.
Valero, Y., Awad, E., Buonocore, F., Arizcun, M., Esteban, M.Á., Meseguer, J., Chaves-Pozo, E., Cuesta, A., 2016. An oral chitosan DNA vaccine against nodavirus improves transcription of cell-mediated cytotoxicity and interferon genes in the European sea bass juveniles gut and survival upon infection. Dev. Comp. Immunol. 65, 64–72. https://doi.org/10.1016/j.dci.2016.06.021.
Valero, Y., Mokrani, D., Chaves-Pozo, E., Arizcun, M., Oumouna, M., Meseguer, J., Esteban, M.Á., Cuesta, A., 2018. Vaccination with UV-inactivated nodavirus partly protects European sea bass against infection, while inducing few changes in immunity. Dev. Comp. Immunol. 86, 171–179. https://doi.org/10.1016/j.dci.2018.05.013.
Valero, Y., Olveira, J.G., López-Vázquez, C., Dopazo, C.P., Bandín, I., 2021. Bei inactivated vaccine induces innate and adaptive responses and elicits partial protection upon reassortant betanodavirus infection in senegalese sole. Vaccines 9. https://doi.org/10.3390/vaccines9050458.
Vázquez-Salgado, L., Olveira, J.G., Dopazo, C.P., Bandín, I., 2022. 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. Aquaculture 547. https://doi.org/10.1016/j.aquaculture.2021.737519.
Volpe, E., Gustinelli, A., Caffara, M., Errani, F., Quaglio, F., Fioravanti, M.L., Ciulli, S., 2020. Viral nervous necrosis outbreaks caused by the RGNNV/SJNNV reassortant betanodavirus in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). Aquaculture 523, 735155. https://doi.org/10.1016/j.aquaculture.2020.735155.
Wang, L., Tian, Y., Cheng, M., Li, Z., Li, S., Wu, Y., Zhang, J., Ma, W., Li, W., Pang, Z., Zhai, J., 2019. Transcriptome comparative analysis of immune tissues from asymptomatic and diseased *Epinephelus moara* naturally infected with nervous necrosis virus. Fish Shellfish Immunol. 93, 99–107. https://doi.org/10.1016/j.fsi.2019.07.020.
Zhou, Y., Huang, J., Jin, B., He, S., Dang, Y., Zhao, T., Jin, Z., 2022. The emerging role of hedgehog signaling in viral infections. Front. Microbiol. 13, 1–13. https://doi.org/10.3389/fmicb.2022.870316.
Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Dev. Comp. Immunol. 35, 1376–1387. https://doi.org/10.1016/j.dci.2011.07.001.
Zou, J., Secombes, C.J., 2016. The function of fish cytokines. Biology (Basel). 5 https://doi.org/10.3390/biology5020023.