POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Passive immunisation of European seabass (*Dicentrarchus labrax*) against Nervous Necrosis Virus (NNV)

Catarina Rebelo Abrantes

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Dissertation for a Master's Degree in Aquaculture

Dissertation carried out under the supervision of Specialist Professor Teresa Baptista

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Escola Superior de Turismo e Tecnologia do Mar – Peniche

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Abstract

Viral infections in aquaculture are one of the main obstacles to aquaculture development, as they cause high economic losses. Viral encephalopathy and retinopathy (VER), caused by nervous necrosis virus (NNV), significantly impacts marine and freshwater aquaculture, causing high mortality in larval and juvenile populations with mortality rates up to 100%. There is no effective treatment against this infection nor commercial vaccine development. This study aimed to assess the applicability of a polyclonal antibody with antiviral potential for supplementing the diet of European seabass (*Dicentrarchus labrax*) in the larval stage after contact with NNV of the RGNNV genotype, as well as to evaluate how this antibody treatment could influence both fish survival rates and the reduction of viral replication over time. A neutralisation protocol was established using rabbit anti-NNV polyclonal antibodies as a positive control. Results demonstrate that PAb can be effectively administered to fish through both immersion and dietary routes, with findings suggesting potential protective effects when delivered via the diet. It was found that NNV infection is more efficient via injection (20%) compared to immersion (0%). Mortality was monitored and analysed throughout the trial, along with viral load, to gain a comprehensive understanding of the potential benefits associated with the administration of this polyclonal antibody following NNV infection. A reduction in mortality was observed in the treatment in which the feed was supplemented with rabbit serum (33.2%) compared to the treatment where the fish were infected with NNV (41.7%). A reduction in viral load was also observed between these treatments. Thus, supplementation with antiviral immunostimulants could be a promising strategy to prevent irreversible damage. However, more studies are still needed to understand how the immune system works in the presence of NNV with subsequent supplementation with polyclonal antibodies.

Key-words: Aquaculture; Betanodavirus; Larvae; Polyclonal antibody; Rabbit serum; Feed supplementation.

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List of abbreviations

BFNNV: *Barfin flounder nervous necrosis virus*

bp: Base Pair

CM: Cumulative mortality rate

CNS: Central nervous system

CPA-LFD: *Isothermal cross-priming amplification with lateral flow dipstick*

Ct: Cycle Threshold

DGAV: Direção Geral da Alimentação e Veterinária

DNA: *Deoxyribonucleic Acid*

dph: Days post-hatch

dpi: Days post-infection

ELISA: *Enzyme-Linked Immunosorbent Assay*

EPPO: Estação Piloto de Piscicultura de Olhão

EU: European Union

FAO: *Food and Agriculture Organization*

Ig: Immunoglobulins

IgD: Immunoglobulin D

IgM: Immunoglobulin M

IgT: Immunoglobulin T

IgZ: Immunoglobulin Z

IPMA: Instituto Português do Mar e da Atmosfera

LAMP: Loop-mediated isothermal amplification

LD50: Lethal dose 50%

LFB: *Lateral flow paper biosensors*

LoQ: Limit of quantification

MRI: Moredun Research Institute

NASBA: *Nucleic acid sequence-based amplification*

NNV: Nervous Necrosis Virus

OD: Optical Density

PAbs: Polyclonal antibodies

PBS: Phosphate-buffered saline

RGNNV: Red-spotted grouper nervous necrosis virus

rpm: Revolutions Per Minute

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

RT-qPCR; qPCR: Real-time Quantitative polymerase chain reaction

SJNNV: Striped jack nervous necrosis virus

TAE: Tris-Acetate-EDTA

TCID50: 50% Tissue Culture Infective Dose

TMB: Tetramethylbenzidine

TNV: Turbot nodavirus strain

TPNNV: Tiger puffer nervous necrosis virus

T-TBS: Tris- Buffered Saline with Tween 20

UV: Ultraviolet Radiation

VER: Viral Encephalopathy and Retinopathy

VNN: Viral Nervous Necrosis

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I.Introduction

The exponential growth of the world's population in recent decades and the pressing need to ensure food security have driven the development of aquaculture systems to sustain resources (Finegold, 2009). Aquaculture is the controlled production of plants and animals in marine, brackish, or freshwater environments for human consumption. This practice supports the recovery of wild resources (Barnaby, 2006). In 2022, global production of aquatic animals reached a record high of 185 million tonnes (live weight equivalent), marking, a 4 per cent increase compared to 2020. Aquaculture contributed approximately 94 million tonnes (51% of fish consumption), surpassing capture fisheries for the first time, which produced 91 million tonnes (49% of fish consumption) (Figure 1). Meanwhile, the global fishing fleet has declined from its 2019 peak of 5.3 million vessels to an estimated 4.9 million vessels in 2022, two-thirds of which are motorized (FAO, 2024).

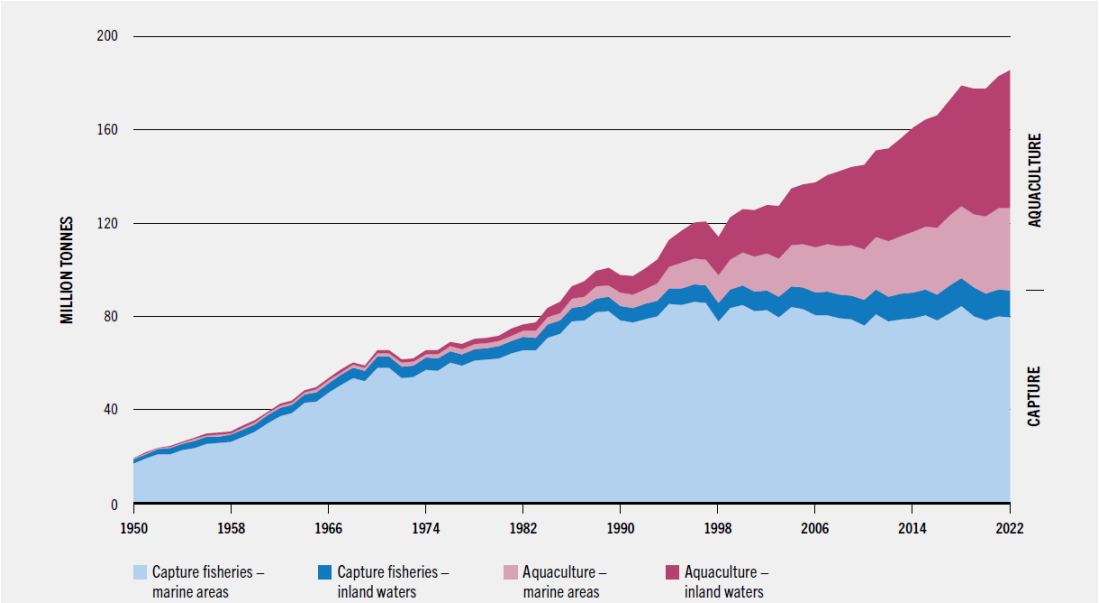

Figure 1. World capture fisheries and aquaculture production (Source: FAO, 2024 - The State of World Fisheries and Aquaculture).

Aquaculture development is closely associated with large-scale production facilities, where stocking densities are typically high. As a result, organisms are constantly exposed to stressful conditions, creating an ideal environment for the emergence and spread of infectious diseases (van Strijp, & Bitter, 2014).

Viral infectious diseases pose one of the greatest threats to aquaculture, mainly due to the limited availability of effective vaccines and antiviral treatments. These viruses are naturally present in the environment and evolve alongside their host species, challenging infection control efforts (Valero & Cuesta, 2023). The aquatic environment further complicates these efforts; the continuous water flow and high population densities make it difficult to detect and isolate infected individuals, allowing diseases to spread rapidly. As a result, viral infections have become a global concern, causing substantial economic losses in the sector (FAO, 2022) and negatively affecting the health and

welfare of farmed organisms. Furthermore, the spread of these diseases increases the risk of infection in wild populations, potentially impacting broader aquatic ecosystems (Kibenge, 2019).

Dicentrarchus labrax, Linnaeus 1758, commonly known as European seabass, is one of the most prized and widely cultivated species in the Northeast Atlantic. This species thrives in the Mediterranean region, requires relatively low production costs, and can be farmed alongside other species. European seabass are eurythermic (tolerant of wide temperature variations) and euryhaline (tolerant of large salinity variations) teleosts, which is why they are commonly found in estuaries and lagoons between spring and autumn, particularly during their juvenile stage. As opportunistic predators, they feed on plankton during the larval stage and shift to fish and crustaceans in the juvenile and adult stages. From an economic point of view, European seabass is a valuable aquaculture species, with production steadily increasing. In 2023, aquaculture production of sea bass in the European Union reached approximately 54,000 tonnes (European Commission, 2022).

However, fish larval production, faces high mortality rates, often due to pathogens or environmental factors (Teiba et al., 2020; Lieke et al., 2021; El-Dahhar et al., 2024). In most aquatic species, only about 1% of organisms survive to sexual maturity, with the highest mortality rates occurring during early life stages (Vadstein et al., 2013; El-Dahhar et al., 2024). To address this challenge, developing immunological stimulants, especially for early life stages, has become crucial (Schar et al., 2021; El-Dahhar et al., 2024).

Viral nervous necrosis (VNN) is a serious viral disease affecting many marine fish species, including European seabass (Kaplan & Karaoğlu, 2021). The causative agent, nervous necrosis virus (NNV), causes severe symptoms such as viral encephalopathy and retinopathy (VER) by damaging the fish´s nervous system (Bandín & Souto, 2020). First identified in the late 1980s, the disease has led to high mortality rates (up to 100%) and significant economic losses in aquaculture worldwide, affecting both marine and freshwater species (Bandín & Souto, 2020).

VNN primarily affects post-spawning larvae, fry, and juvenile fish. Survivors of the infection often show reduced performance even after recovery (Arimoto et al., 1994; Johansen et al., 2004; Costa & Thompson, 2016). In 2000, viruses from the family *Nodaviridae* were reclassified into two genera: Alphanodavirus, which infects insects, and Betanodavirus, which infects fish (Costa & Thompson, 2016). The causative agent of VNN is classified as a betanodavirus within the family *Nodaviridae* (Kaplan & Karaoğlu, 2021).

Betanodaviruses, like VNN, are non-enveloped, spherical viruses approximately 25 nm in diameter. Their genome comprises two positive-sense single-stranded RNA segments, RNA1 and RNA2, each containing three open reading frames (ORFs), (Johnson et al., 2003; Kaplan & Karaoğlu, 2021). RNA1 encodes an RNA-dependent RNA polymerase (110 kDa) that carries all the information required for autonomous replication and regulates the virus´s temperature dependence (Gallagher & Rueckert, 1988; Nagai & Nishizawa, 1999; Kaplan, & Karaoğlu, 2021). RNA2 (1.4 kb) encodes a capsid protein responsible for host tropism and immunoreactivity (Nishizawa et al., 1995; Johnson, 2003; Iwamoto et al., 2004; Ito et al., 2008; Kaplan& Karaoğlu, 2021). The

RNA2 segment also includes the T4 multivariable region, which is used to classify genotypes (Nishizawa et al., 1997; Kaplan & Karaoğlu, 2021).

Betanodavirus genotypes are classified based on the RNA sequence of the T4 variable region of the capsid protein, with each genotype named after the fish species from which it was first identified (Costa & Thompson, 2016). Currently, five recognized genotypes of betanodavirus exist: BFNNV (barfin flounder nervous necrosis virus), SJNNV (striped jack nervous necrosis virus), TPNNV (tiger puffer nervous necrosis virus), TNV (turbot nodavirus) and RGNNV (red-spotted grouper nervous necrosis virus) (Nishizawa et al., 1995; Nishizawa et al., 1997; Skliris et al., 2001; Costa and Thompson, 2016).

The geographical distribution of betanodaviruses is extensive, which may be associated with the thermotolerance of each genotype, Figure 2 (Bandín & Souto, 2020). More than 120 species of fish and invertebrates, both wild and farmed, have been reported to be infected with betanodaviruses. Larval and juvenile fish are particularly susceptible, with mortality rates reaching up to 100% in some cases (Costa & Thompson, 2016; Kaplan & Karaoğlu, 2021). Consequently, age is a critical risk factor for disease susceptibility (Hick et al., 2011; Kaplan, & Karaoğlu, 2021).

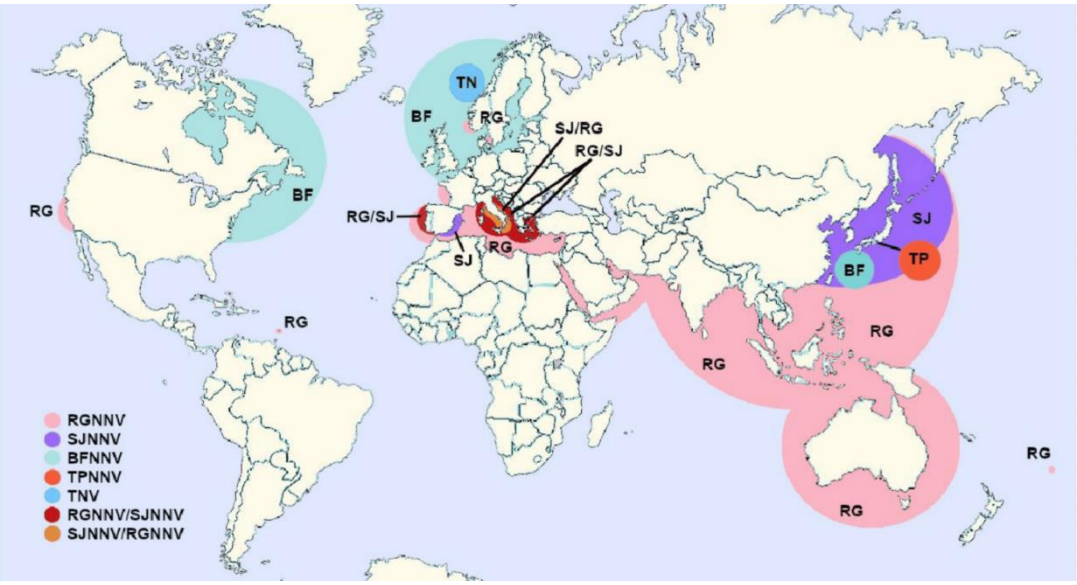

Figure 2. Distribution of Nervous necrosis virus (NNV) genotypes (Source: Bandín, & Souto, 2020).

Viral evolution can lead to recombination events, which occur when two distinct viral genomes infect a single host cell. During co-infect, their genomic components can rearrange, creating a new virus with a unique genome, different from the parental strains. The genetic diversity of the parent viruses increases the potential impact of this recombinant offspring on viral evolution (Lowen, 2018; Valero & Cuesta, 2023).

The BFNNV genotype has been detected in wild fish in Scandinavia (Norway, Sweden, and Denmark) and Canada, though it is most prevalent in cold-water farmed fish in Japan, northern Europe, and North America. It affects species such as Atlantic and Pacific cod (*Gadus macrocephalus*), haddock (*Melanogrammus aeglefinus*), Atlantic halibut (*Hippoglossus hippoglossus*), and barfin flounder (*Verasper moseri*), wrasses (ballan, corkwing, and goldsinny wrasses, *Labrus bergylta*, *Symphodus melops,* and

Ctenolabrus rupestri) as well as winter flounder (*Pseudopleuronectes americanus*) (Korsnes et al., 2017; Bandín & Souto, 2020).

The RGNNV genotype is the only one associated with outbreaks in freshwater species across Europe, Asia, and Australia. Due to its adaptation to different temperature ranges, RGNNV has the broadest geographic distribution and affects both tropical and temperate species (Panzarin et al., 2014). Infections have been reported in farmed and wild fish throughout the Mediterranean, along the coasts of Asia and Australia (Bandín & Souto, 2020). It has also been isolated from Californian-farmed white seabass (*Atractoscion nobilis*) (Curtis et al., 2001).

The TPNNV genotype has only been identified in one Japanese species (Nishizawa et al., 1997; Bandín & Souto, 2020), while the SJNNV genotype has been found in farmed Senegalese sole and Gilthead seabream on the Iberian Peninsula, despite being long considered limited to Japanese waters (Bandín & Souto, 2020). Natural reassortants of SJNNV and RGNNV have been isolated from sea bass along the Italian coast (Athanassopoulou et al., 2003; Bandín & Souto, 2020). The opposite reassortment form (RGNNV/SJNNV) is widely distributed across Southern Europe (Figure 2) infecting Gilthead seabream (Olveira et al., 2009; Panzarin et al., 2012; Toffan et al., 2017; Bandín & Souto, 2020), and most recently, wild Mediterranean horse mackerel (*Trachurus mediterraneus)* (Bitchava et al., 2019; Bandín & Souto, 2020).

Pathogen-host interactions and environmental factors influence infectious disease outbreaks. Temperature is particularly significant, as fish are ectothermic and thus highly susceptible to water temperature changes. Temperature affects the immune response of fish and virus-host interactions, especially in betanodaviruses, each genotype of which has a distinct optimal growth range: 15–20°C for BFNNV, 20 °C for TPNNV, 20–25°C for SJNNV, and 25–30°C for RGNNV (Bandín & Souto, 2020). Consequently, global warming could increase infectious disease outbreaks by weakening the immune systems, making them more susceptible to infections (Niedbalski & Fitzner, 2018). In addition to temperature, poor rearing practices such as low-quality nutrition, inadequate environmental conditions, high stocking densities, and injuries during transport can weaken fish immunity and increase disease susceptibility (Yang et al., 2022). Like other vertebrates, fish rely on both innate and adaptative immune systems to combat pathogens like NNV. The innate immune system provides a rapid, non-specific response, while the adaptive immune system, involving lymphoid tissues such as the thymus, head kidney, spleen, and skin, offers long-term, pathogen-specific protection through functions like immune surveillance, hematopoiesis, antibody production, and responses against pathogens (Stambas & Tripp, 2020; Chia et al., 2024).

Detection of NNV infection relies on several methods, including optical observation, microscopy, molecular techniques (e.g. RT-PCR), and cell culture. Common symptoms include central nervous system damage (brain, spinal cord, and retina) (Faggion et al., 2021), and abnormal behaviours such as spiral swimming, spinning, lying on the bottom, skin darkening, and loss of appetite (Munday & Nakai, 1997; Faggion et al., 2021). Histologically, NNV infection is characterized by vacuolation in the central nervous system (CNS), widespread necrosis in larval and juvenile stages, and occasionally, lesions in the liver and spleen (Munday et al., 2002; Yang et al., 2022).

While optical microscopy can identify some signs of infection, full confirmation typically requires molecular methods such as RT-PCR, which detects viral RNA (Grotmol

et al., 2000; Yang et al., 2022). Other molecular techniques for NNV detection include loop-mediated isothermal amplification (LAMP) (Notomi et al., 2015; Yang et al., 2022), nucleic acid sequence-based amplification (NASBA) (Starkey et al., 2004; Yang et al., 2022), lateral flow paper biosensors (LFB) (Toubanaki et al., 2015; Yang et al., 2022), and isothermal cross-priming amplification with lateral flow dipstick (CPA-LFD) (Su et al., 2015; Yang et al., 2022). Immunohistochemistry (IHC) has also been used to detect viral antigens in fixed tissue samples, providing a complementary approach to histological analysis (Arimoto et al., 1992; OIE, 2019). Since 2005, quantitative real-time RT-PCR (qRT-PCR) has become a highly sensitive tool for quantifying betanodaviruses, with detection limits as low as 10 TCID50 /mL (Panzarin et al.,2010).

NNV infection begins when the virus enters host cells via micropinocytosis, followed by capsid protein removal and the release of the viral genome for replication (Liu et al., 2005; Yang et al., 2022).

Viral nervous necrosis disease spreads through both horizontal and vertical transmission routes. Horizontal transmission occurs between infected and healthy fish via contaminated water, food, or direct contact. Cannibalistic behaviour further increases risk, as infected fish may be consumed, facilitating viral spread. The virus can enter through epithelial cells on the skin, fins, gills, and oral and nasal cavities (Souto et al., 2018; Bandín & Souto, 2020).

Vertical transmission occurs when the virus is passed from infected broodstock to their offspring through contaminated gametes (eggs and sperm), leading to infected larvae at birth (Yang et al., 2022). This highlights the importance of selecting virus-free broodstock in aquaculture breeding programs to prevent perpetuating disease in future generations. Effective VNN control in aquaculture requires strict biosecurity measures, including regular monitoring and testing, quarantining of new fish, and implementing vaccination programs when possible (Costa & Thompson, 2016). Also, live feeds for larvae, such as brine shrimp (*Artemia salina*) and rotifers (*Brachionus plicatilis*), can act as vectors of NNV, facilitating transmission to susceptible fish populations during feeding (Costa & Thompson, 2016; Volpe et al., 2018; Vázquez-Salgado et al., 2020; Vázquez-Salgado et al., 2022). Early detection is essential to prevent the viral spread within aquaculture facilities and mitigate economic losses from outbreaks.

Once VNN is established within a fish population, eradication becomes exceedingly difficult, emphasizing the importance of prevention. Effective strategies include maintaining strict biosecurity protocols, such as regular testing of fish stocks, quarantining new animals, and, where applicable, vaccination (Frerichs et al., 1996; Costa & Thompson, 2016). Additional inactivation methods, such as extreme pH levels, disinfectants (e.g., sodium hypochlorite, calcium hypochlorite, benzalkonium chloride), and physical treatments (UV light, heat), show varying success across different NNV genotypes (Costa & Thompson, 2016). Preventing vertical transmission is another key component of VNN management. Screening broodstock via RT-PCR on ovarian biopsies, eggs, and sperm, and antibody detection through ELISA, helps ensure infected broodstock does not transmit the virus to offspring (Costa & Thompson, 2016). Vaccination of broodstock with inactivated vaccines, such as Icthiovac®VNN (Hipra) and Alpha ject micro®1Noda (Pharmaq), has shown promise in reducing virus spread (Nuñez-Ortiz et al., 2016).

In fish, including European seabass, immunoglobulins (antibodies) are central to the adaptive immune response, playing a key role in neutralizing pathogens and signalling foreign structures within the organism (Bunnoy et al., 2020; Vaz, 2021; Yang et al., 2022). Fish produce several types of antibodies, each serving different functions within the immune system. In marine teleosts, the most significant types are IgM, IgD, and IgT/IgZ (Buonocore et al., 2020). IgT is specialized for mucosal immunity, protecting the gills, skin, and gastrointestinal tract (Salinas et al., 2021), and elicits antigen-specific responses (Zhang et al., 2010). Meanwhile, IgM and IgD are the primary antibody classes (Fillatreau et al., 2013). IgD is mainly associated with B cell activation, although its exact functions are still not fully understood (Amendt et al., 2021). Immunoglobulin M (IgM) is the most prevalent antibody in fish blood and is essential for the primary immune response. When macrophages present antigens to T and B lymphocytes, IgM is produced to neutralize these antigens, targeting viruses, bacteria and other pathogens. Once the infection clears, memory cells (T and B lymphocytes) remain, allowing for a faster and more effective immune response if the fish is exposed to the same pathogen again (Chia et al., 2024).

However, as ectothermic animals, fish develop their immune systems more slowly than endothermic animals. This slower immune development in sea bass, particularly in larvae and juveniles, results in their reliance mainly on innate immunity during early life stages. Adaptative immunity (or acquired immunity) is responsible for recognizing and specifically responding to pathogens (such as viruses, bacteria, or infected cells) that the organism encounters. Unlike innate immunity, which is an immediate and general response, adaptive immunity is more specific, flexible, and includes a memory component, allowing for a faster and more efficient response to subsequent infections (Vaillant et al., 2022). In fish, the development of adaptative immunity, including immunoglobulin production, is gradual and may take several months to fully mature (Yu et al., 2020) making juvenile fish more vulnerable to infections until their immune systems become fully functional. In sea bass, juveniles often exhibit a stronger immune response around 61 days post-hatch (dph), marking a transition to a more mature immune system capable of effectively managing infections (Bols et al., 2001).

Passive immunisation involves the administration of non-self-antibodies to an organism to induce temporary therapeutic protection against a pathogen (Rajan et al., 2017). Currently, passive immunisation in aquaculture is a topic of great interest, as it may serve to prevent infectious diseases (Hedegaard & Heegaard, 2016; Rajan et al., 2017). Passive immunity can also occur naturally through vertical transmission from parent to offspring. This transfer of antibodies can happen through the egg yolk or the yolk sac shortly after hatching (Swain & Nayak, 2009; Vadstein et al., 2013; Rajan et al., 2017). Several studies on passive immunisation in fish have demonstrated that immunity can be provided through dietary supplementation or intraperitoneal injection (Rajan et al., 2017). To bolster immune defences during the early life stages of fish development in aquaculture, researchers are exploring antibody-enriched feeds, which provide enhanced protection during critical developmental periods when the adaptative immune system is still maturing (Toranzo et al., 2005).

Studies on betanodavirus infections indicate that surviving fish develop neutralizing antibodies, emphasizing the importance of adaptative immunity in controlling pathogens such as NNV (Tanaka et al., 2001; Costa & Thompson, 2016). During passive

immunisation, an interaction occurs between the pathogen and the administered antibody. When the pathogen enters through the intestine (via contaminated water and/or food), passive immunisation can work in three main ways: neutralization of the pathogen at the mucosal surface (Figure 3, A), blocking pathogen adhesion to host cells (Figure 3, B), and defence mediated by effector cells (Figure 3, C).

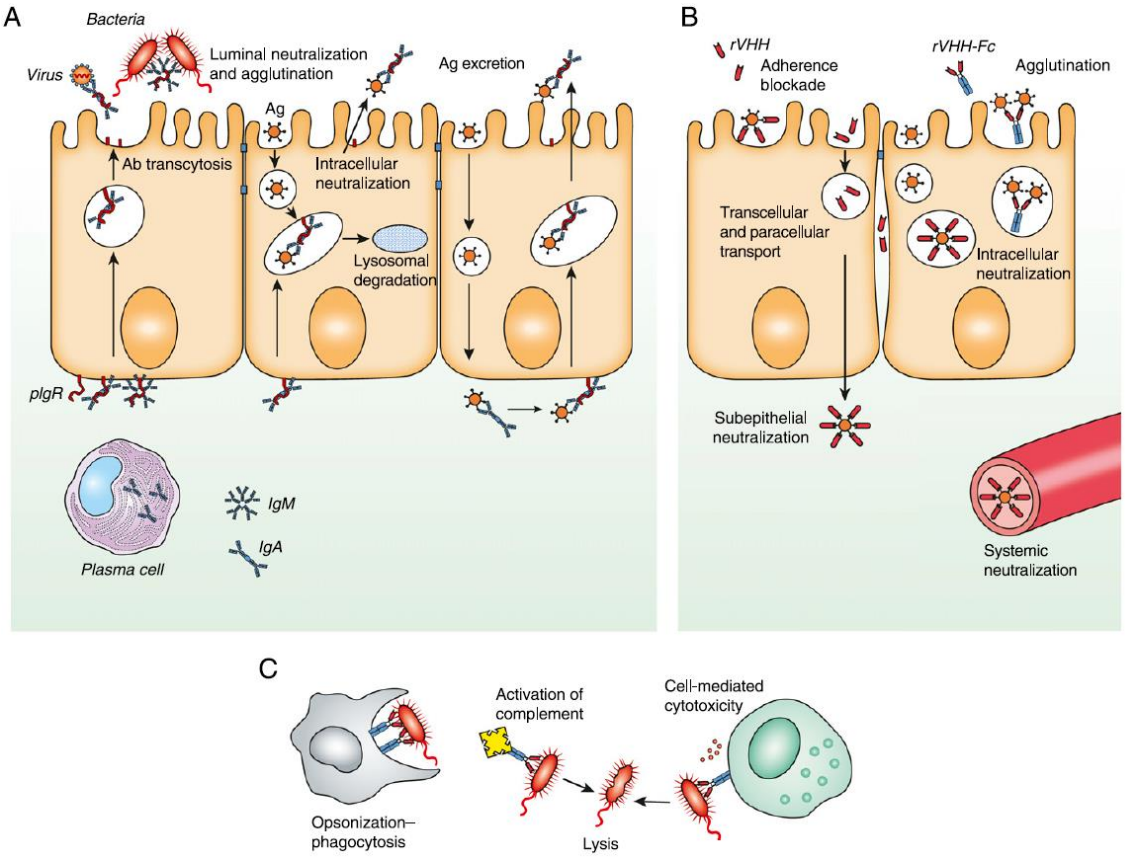

Figure 3. Mucosal actions of antibodies. (Source: Rajan et al., 2017).

Pathogen neutralization begins once antibodies are administered, transported through the epithelial cells lining the intestine, and into the lumen where they bind to the pathogen, neutralizing its infectious ability. Additionally, antibodies can agglutinate pathogens, causing their movement difficult and facilitating elimination (Virdi et al., 2013; Rajan et al., 2017). Another mechanism of action is adherence blockade in which antibodies prevent the pathogen from binding to the host cell, thereby neutralizing it (Rajan et al., 2017). Effector cell-mediated defence occurs when antibodies activate immune cells like macrophages and neutrophils, marking the pathogen for elimination through opsonization (promoting phagocytosis (Rajan et al., 2017). The three mechanisms of passive immunisation are critical in the early life stages of fish, such as in larvae, whose adaptive immune system is not yet fully developed, and thus they rely on external protection for survival.

Polyclonal antibodies (pAbs) are produced by multiple clones of B cells, each targeting a specific epitope of the pathogen, which enhances the immune response by attacking different regions of the pathogen, increasing the chance of neutralisation (Lipman et al., 2005; Murphy et al., 2016). A novel strategy to control VER is passive immunisation. It involves administering pre-formed antibodies from immunised donor

animals, often rabbits, who are exposed to a specific pathogen, like the viral nervous necrosis virus (Lipman et al., 2005). Over time, the rabbits produce antibodies against the VNN antigen, which accumulate in the blood serum. Periodic blood sampling allows researchers to collect serum containing these polyclonal antibodies for therapeutic use.

This study was included in the EU's Horizon Europe-funded Cure4Aqua project, with a budget of €4.8 million over 4.5 years, involving 31 partners from 16 countries. The project´s primary objectives include developing innovative approaches to disease prevention in fish by introducing and validating innovative technologies for early disease detection and advancing alternative treatments to reduce reliance on pharmaceuticals in disease control.

This study focuses on evaluating the effectiveness of passive immunisation in European seabass larvae (*Dicentrarchus labrax*) through the administration of polyclonal antibodies, to determine if this approach could reduce mortality and the severity of symptoms caused by viral nervous necrosis (NNV), more specifically, the RGNNV genotype.

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II.Methodology

All the trials conducted in this dissertation adhered to the ethical guidelines for the protection of animals used for scientific purposes, as outlined in European Directive 2010/63/EU. The experimental work was carried out under the supervision of an expert accredited by the Directorate-General for Food and Veterinary (DGAV), in Laboratory Animal Sciences. The trials were performed at CETEMARES, Marine Sciences R&D, Education, and Knowledge Dissemination Centre, located in Peniche, Portugal.

The virus was produced at the Moredun Research Institute (MRI) using SSN-1 cells. These cells were cultured in T25 flasks at 25°C without CO_2 using Leibovitz's L-15 medium with GlutaMAX, Earl's EMEM, and 5% fetal bovine serum (FBS). Cells were subcultured every 1-2 weeks at a 1:3 ratio by detaching them with Trypsin EDTA, followed by resuspension in fresh medium. For infection, SSN-1 cells were maintained at 28°C for 6-7 days or split into a 1:3 ratio, mixed with the virus, and incubated at 25°C. In infections using pre-formed monolayers, cells were grown to 70% confluence before virus adsorption at 25ºC for 2 hours. The harvested virus was subsequently used for in vivo studies.

1. Neutralization of NNV in water using a rabbit anti-NNV polyclonal Ab (PAb)

An experiment was carried out to determine the optimal dilution of rabbit anti-NNV serum for use in *in vivo* infection trials and to establish protocols for neutralization assays involving fish, the design of which is shown in Figure 4. The experimental setup included 600 ml beakers arranged in five boxes, each one containing triplicates of all treatments, and maintained under constant aeration. The RGNNV genotype provided by Moredun Research Institute (NNV ERV283 P6 105.83) was added to the beakers to achieve a final concentration of 10^5 TCID50/ml.

The treatments were as follows:

- Treatment 1 (T1) as the negative control with only 250 ml seawater.
- Treatment 2 (T2) as the positive control with 225 ml seawater plus 25 ml of the virus.
- Treatment 3 (T3) with a 1:500 dilution of rabbit serum consisting of 222.5 ml seawater, 0.5 ml rabbit serum, and 25 ml virus.
- Treatment 4 (T4) with a 1:1000 dilution of 224.75 ml seawater, 0.25 ml rabbit serum, and 25 ml virus.
- Treatment 5 (T5) with a 1:5000 dilution including 224.95 ml seawater, 0.05 ml rabbit serum, and 25 ml virus.

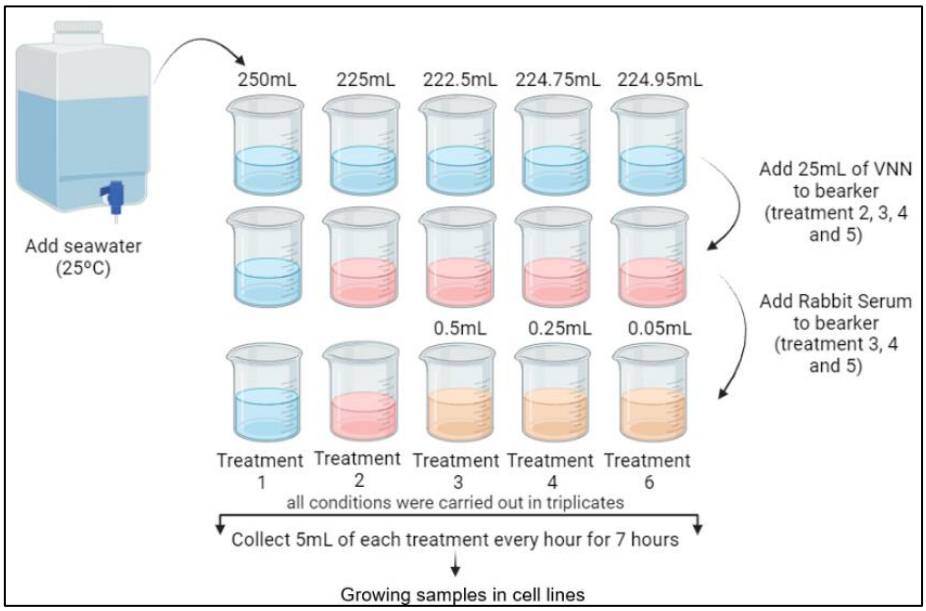

Figure 4. Schematic representation of the neutralization trial.

The experiment was conducted over a 7 h period at 25°C to assess virus neutralisation in water samples treated with rabbit anti-NNV polyclonal antibody serum. Samples were collected at the start and then hourly for 7 hours. Each sample was transferred into a 15 mL Falcon tube and stored at -20°C. After the final sampling, the tubes were transferred to a -80°C cooler. The samples were sent to MRI to evaluate the level of virus neutralisation by culturing the water samples on E11 cells and observing cytopathic effect (CPE) development as an indicator of active virus. This method allowed for the quantification of viral activity and the determination of neutralisation effectiveness. The anti-NNV PAb serum, derived from immunised rabbits, was tested for its neutralizing efficiency by observing virus culture outcomes on SNN-1 cells.

Results indicate that a 1/500 dilution of the rabbit serum achieved complete neutralization, of the virus, as no CPE was observed. Conversely, a 1/1000 dilution of the serum resulted in minimal CPE. Based on these findings, a 1/500 dilution ratio was selected as the optimal concentration for use in forthcoming *in vivo* experiments.

2. Juvenile infection trial to calculate Lethal Dose 50% (LD50)

As part of the Cure4Aqua project, a trial will be conducted to evaluate the effect of recombinant monoclonal VLRB derived from hagfish against NNV in European seabass juveniles. Therefore, it is necessary to establish an infection protocol, to determine the LD50. A total of 80 juvenile European seabass (*Dicentrarchus labrax*), sourced from a commercial hatchery and previously vaccinated against *Photobacterium damselae subsp. piscicida*, with an average initial weight of 5 g, were used to determine

the Median Lethal Dose (LD50) for viral nervous necrosis (VNN). The juveniles were acclimatized for one week in the aquaculture facility, and the infection trials were conducted in a containment room to ensure biosecurity. The study involved two infection methods of viral exposure: immersion and intramuscular injection, as illustrated in Figure 5. For the immersion infection, three tanks were designated to test varying viral concentrations of NNV: 10^3, 10^4, and 10^5 TCID50/mL. Immersion exposure was performed in 4 buckets, one for each viral concentration, with 1.5 litres of seawater per bucket. The virus was diluted in phosphate-buffered saline (PBS) (Sigma Aldrich, EUA). The immersion period lasted 2 hours, after which the fish were transferred back to their respective aquaria for monitoring.

In the intramuscular injection group: four tanks were used, each with a different viral concentration: 10^3, 10^4, 10^5, and 10^6 TCID50/mL. The fish were anaesthetized using 0.5 mL/L of 2-phenoxyethanol (VWR Chemicals, Portugal) and injected intramuscularly with 0.1 mL of the viral solution corresponding to the respective concentration before being returned to their aquaria.

The purpose of this LD50 trial was to establish the viral concentration required to induce 50% mortality in juvenile sea bass, providing crucial data for understanding the virulence of the VNN virus under these two different infection methods.

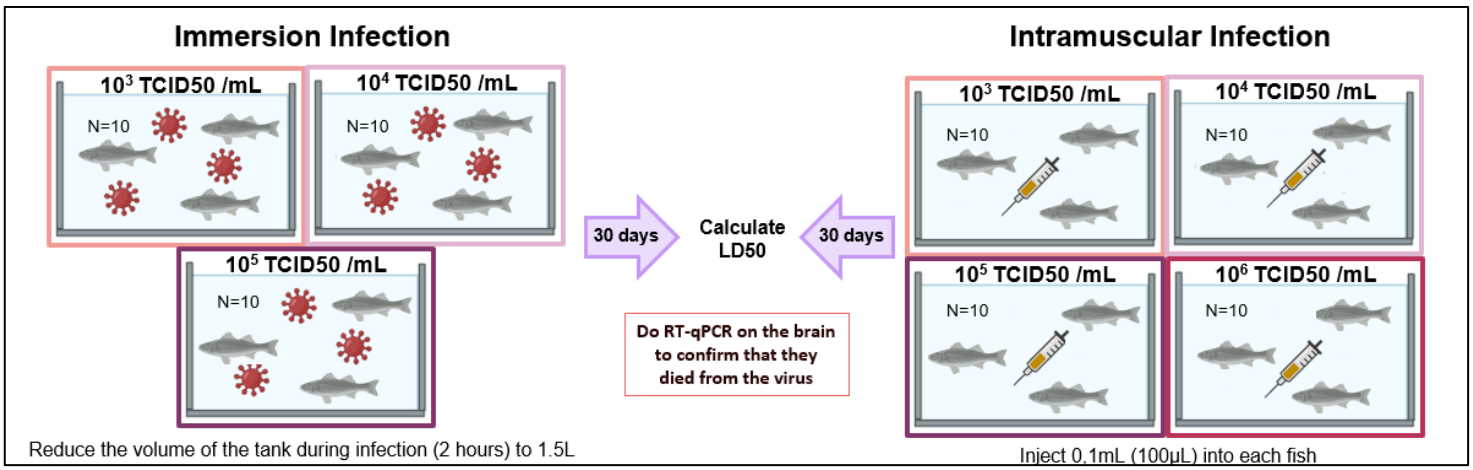

Figure 5. Schematic representation of the juvenile's trial.

Each of the seven treatments was maintained in an independent recirculating aquaculture system (RAS), which included mechanical and biological filters. Each aquarium had a capacity of 20 litres, and 10 fish were used per treatment. The trial lasted 14 days, during which mortality rate and behavioural changes were recorded several times daily. Mortality throughout the trial was analysed as a cumulative mortality rate (CM), calculated using the following equation:

$$CM\ (\%) = \frac{number\ of\ deaths\ for\ the\ day\ * 100}{initial\ number\ of\ fish\ in\ the\ aquarium} + CM\ from\ the\ previous\ day$$

The experiment was conducted at a temperature of 25.13 ± 0.49°C, while daily monitoring of key water parameters: dissolved oxygen (6.60 ± 0.38 mg/L) at an average saturation level of 96.47%, salinity (33.06 ± 0.24), ammonia (0.70 ± 0.32 mg/L), and nitrites (0.00± 0.00 mg/L). Water quality parameters were measured using an OxyGuard Handy Polaris multiparameter probe (Farum, Denmark) for dissolved oxygen and temperature, a seawater refractometer, H196822 (Hanna Instruments, Portugal) for salinity, and a Salifert NH_3 Profi Test kit (Salifert, Netherlands) for ammonia.

To maintain water quality, each aquarium was siphoned daily to remove uneaten food and faeces. All treatments were fed *ad libitum* twice daily (morning and afternoon) with a commercial feed.

At the end of the trial, fourteen days after infection, all fish were euthanized. From each treatment, three fish were randomly selected for brain sample collection to quantify the viral load using RT-qPCR. Additionally, blood samples were collected for antibody detection through Enzyme-Linked Immunosorbent Assay (ELISA). The brain samples were placed in 1.5 mL Eppendorf tubes with 1000 µL RNAlater (Sigma Aldrich, USA) and stored at 4°C for 24 hours before being transferred to - 80°C for long-term storage. Blood samples were collected using syringes without heparin to allow clotting and serum separation. After refrigeration overnight, the samples were centrifuged at 3000 g (or 3000 rcf) for 7 minutes in a refrigerated centrifuge (Eppendorf, Germany) at 4°C to obtain serum. The serum was then collected in sterile Eppendorf tubes and stored at -80°C until subjected to ELISA for antibody detection. The remaining clots in the original Eppendorf tubes were preserved by adding 750 µL of RNA later to maintain RNA integrity. These clots, like the serum, were stored at -80°C until further analysis.

3. Passive immunisation trial of larvae testing rabbit anti-NNV PAb

A total of 1,192 European seabass larvae (*D. labrax*), approximately 58 days post-hatch (dph), were used to conduct the passive immunisation experiment. These larvae were sourced from the Portuguese Institute for the Sea and Atmosphere (IPMA) at the Olhão Pilot Fish Farming Station (EPPO) and were transported to the CETEMARES facilities. Upon arrival, the larvae were randomly distributed into 10 aquariums, each with a volume of 25 litres, and acclimatized for 3 days before the infection trial commenced. The trial was conducted in an open system using two independent systems, set up in duplicate. Each system consisted of 5 aquariums, along with 2 tanks (200 L and 500 L) to supply the required water volume, ensuring an ideal water renewal rate of 30% per hour for the larvae´s life stage. A thermostat maintained a stable water temperature of 23°C, and a UV filter ensured biological decontamination of the incoming water. Aeration was provided through serological pipettes to avoid excessive agitation, with larger aeration stones used in the tanks. To maintain water quality, the aquariums were siphoned twice daily (morning and evening) to remove uneaten food, faeces, and dead larvae. Siphoning was performed using a 500 µm sieve placed inside a bucket, along with a serological pipette, allowing for accurate mortality counts. The aim of this trial was to understand whether passive immunisation through

the administration of rabbit serum with polyclonal antibodies causes a reduction in viral load, leading to a reduction in mortality during the larval life stage.

The trial included five treatments:

1. Negative infection control: Immersion in PBS (aquariums A and F).
2. Rabbit serum treatment: Immersion in rabbit serum (aquariums B and G).
3. Positive infection control): Immersion in virus (aquariums C and H).
4. Combined treatment: Joint immersion in rabbit serum and virus (aquariums D and I).
5. Supplemented feed: Immersion in virus with larvae fed a diet supplemented with rabbit serum (aquariums E and J).

All treatments were fed commercial feed (WFAST 500, Sparus). The rabbit serum-supplemented feed was prepared at CETEMARES by adding 25 µL of rabbit serum per gram of commercial feed pellets. The mixture was manually stirred and placed in a Universal Oven (Binder, Germany) for 2 hours at 35°C. The supplemented feed was stored in a refrigerator to prevent degradation and prepared weekly to maintain its quality.

The infection trial was conducted by immersion using VNN ERV283 P6 and P7 viral stocks, produced at MRI. The viral concentrations used for immersion included $10^{5.83}$ TCID50/mL, $10^{6.25}$ TCID50/mL, and $10^{5.375}$ TCID50/mL, selected based on findings by Vázquez-Salgado et al. (2022), who successfully infected 12dph sea bass larvae with 10^5 $TCDI_{50}$/mL. Immersion with rabbit serum was performed at a 1:500 dilution ratio. This dilution was previously shown to effectively neutralize NNV in preliminary trials, thereby providing passive immunization against the virus. Each immersion was conducted in a separate 1-litre goblet containing 580 mL of the respective treatment mixture, along with aeration to ensure optimal conditions. Before the immersion, the appropriate treatment mixtures were prepared as follows:

- For immersion in PBS: 58 mL PBS and 522 mL seawater.
- For immersion in rabbit serum: 1.16 mL rabbit serum and 578.84 mL seawater.
- For immersion in the virus: 58 mL NNV and 522 mL seawater.
- For immersion in rabbit serum and virus: 58 mL NNV, 1.16 mL rabbit serum, and 520.84 mL seawater.

Larvae were collected from their respective aquariums using a 500 µm sieve and placed into the goblets containing the treatment mixtures. Each immersion lasted four hours, after which mortality was recorded. After the immersion, the larvae were returned to their corresponding aquaria, as illustrated in Figure 6.

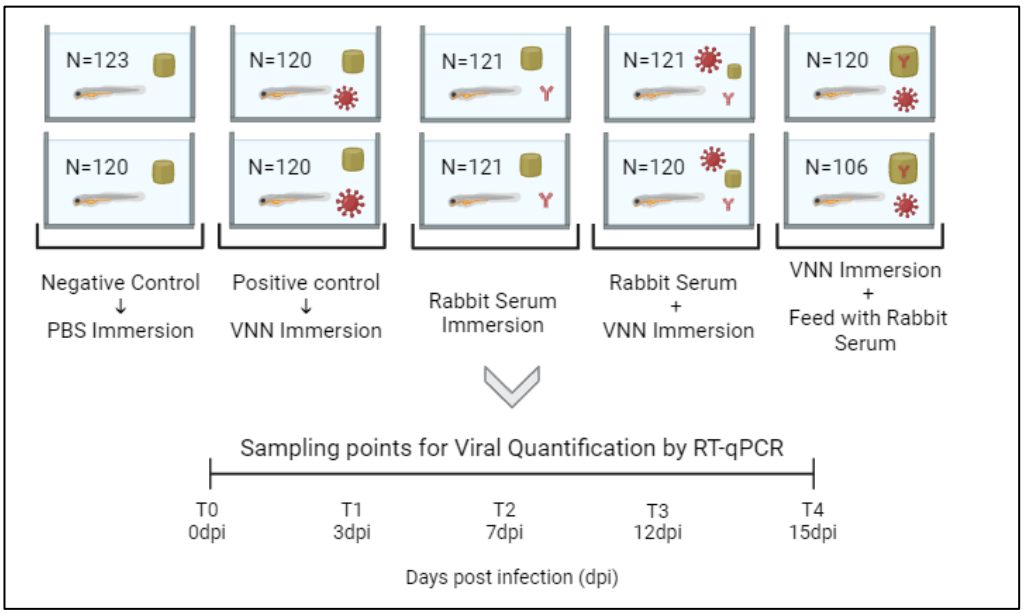

Figure 6. Schematic representation of the larvae trial. A and F- Negative control, C and H- Positive control B and G- Immersion in rabbit serum, D and I- Immersion in rabbit serum and virus, E and J- Immersion in virus and supplemented feed with rabbit serum.

The trial lasted 15 days, during which mortality was recorded daily. The mortality data from this experiment were treated similarly to that of the Juvenile infection trial conducted to calculate the LD50, specifically by calculating the cumulative mortality rate. To maintain water quality, each aquarium was siphoned daily to remove uneaten food and faeces. All treatments were fed *ad libitum* six times daily with commercial feed. Additionally, behavioural changes were documented when observed (Attached, Table I). The trial was conducted at a temperature of 22.80 ± 0.46°C, with the following water quality parameters monitored daily: dissolved oxygen (6.58 ± 0.24 mg/L, with an average saturation level of 92.76%), salinity (33.01 ± 0.32), ammonia (0.00 ± 0.02 mg/L), and nitrites (0.00± 0.00 mg/L). Water quality parameters were measured using an OxyGuard Handy Polaris multiparameter probe (Farum, Denmark) for dissolved oxygen and temperature, a seawater refractometer, H196822 (Hanna Instruments, Portugal) for salinity, and a Salifert NH_3 Profi Test kit (Salifert, Netherlands) for ammonia.

During the trial, sampling was conducted at four time points (days 3, 7, 12, and 14) following immersions in the respective treatments. At each sampling point, five larvae per aquarium were sampled, totalling 10 larvae per treatment (since each treatment was in duplicate). Larvae were collected for sampling using a 500 µm sieve, euthanized with an overdose of anaesthetic (1 mL/L), and then transferred to their respective Eppendorf tubes. They then were placed in 1 mL of RNAlater (Sigma Aldrich, USA) at a ratio of 1mL/5 larvae and refrigerated for 24 hours before being transferred to a -20°C freezer for an additional 24 hours. Subsequently, they were stored at -80°C until further analysis via RT-qPCR to quantify the viral concentration in each treatment. Upon completing the 14-day trial, all remaining larvae were collected and treated identically to earlier samples.

4. Viral quantification (RT-qPCR)

The RNA samples collected from both larvae and juvenile trials were subjected to RT-qPCR (Reverse transcription-quantitative polymerase chain reaction) to quantify and monitor the viral loads over the course of the study. Before RT-qPCR, RNA extraction was performed to prepare the samples.

RNA was extracted using the NZY Total RNA Isolation kit (NZYTech, Portugal). The extraction process began by adding 500 µL of NZYol reagent (NZYTech, Portugal) to lyse the cells, followed by homogenization using a Precellys homogenizer (Bertin Technologies, France) for two 20 seconds cycles each at 6000 rpm with beads. Following homogenization, 150 µL of chloroform was added to separate the nucleic acids from other cellular components. After another round of homogenization, the clear aqueous phase was collected and mixed with 70% ethanol. At this stage, NZYSpin Binding columns (NZYTech, Portugal) were utilized according to the manufacturer´s instructions. The extracted RNA was quantified using the NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, USA). RNA purity was assessed by measuring absorbance ratios at 260/280 nm and 260/230 nm. To verify RNA quality, electrophoresis was performed on a 2% agarose gel (Lonza, Switzerland), which was prepared using 1 µL of GreenSafe Premium dye (NZYTech, Portugal) and 500 µL of commercial bleach (Aranda et al., 2012). Before loading, 2 µL of loading dye was added to each sample to increase its density, ensuring that the sample would sink into the gel wells and prevent dispersion in the running buffer (TAE). The samples were then loaded into the wells, and the gel was run on a Wide Mini-Sub Cell GT system (Bio-Rad, USA) at 100 V for 50 minutes. After electrophoresis, the gel was removed, and RNA integrity was analyzed using the ChemiDocTM MP Imaging System (Bio-Rad, USA).

Viral quantification was conducted with the SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen, Carlsbad, USA). To minimize contamination risks, all reactions and plate preparations were conducted in a laminar flow chamber (Telstar, Bio II Advance 4). The reaction mixture was prepared according to the specifications outlined in Table I, ensuring to account for the total number of reactions. Each sample was analyzed in triplicate, with primers specific for Betanodaviruses (Noda Taq1-FW: CAACTGACARCGAHCACAC and Noda Taq1-RV: CCCACCAYTTGGCVAC) and probe (Noda Taq1-probe: CARGCRACTCGTGGTGCVG) obtained from Stabvida, (Caparica, Portugal) added at a concentration of 10 µM (Panzarin et al., 2010).

Table I- Components and respective volume needed for the reaction mixture in the qPCR protocol.

Components	Volume per reaction (µL)
RNase-free water	1.1
2x Reaction Mix (Buffer)	5
Noda Taq I Forward	0.45
Noda Taq I Reverse	0.45
Probe Noda Taq I	0.3
Superscript II	0.2
Sample (RNA)	2.5
Final volume = 10 µL	

The prepared reaction mixture was distributed into the wells of a 96-well PCR plate (Bio-Rad, California, USA). After filling the wells, the plate was sealed using a plate sealer. It was then placed in the CFX Connect™ Real-Time System (Bio-Rad, California, USA). The specific qPCR cycling conditions are detailed in Table II.

Table II- RT-qPCR Cycling Conditions.

Step	Cycles	Temperature (°C)	Time
Reverse transcription (RNA → cDNA)	1	55	10 minutes
Taq polymerase activation and DNA denaturation	1	95	5 minutes
Denaturation	40 cycles	95	15 seconds
Annealing and extension		60	1 minute

The standard curve was established using successive dilutions of a plasmid containing the specific VNN target sequence. This plasmid provided by Dr Kim Thompson, from the MRI, is a circular DNA molecule derived from a bacterium. It can be genetically manipulated to include a portion of the genome or a specific gene, in this case, the VNN gene, facilitating the identification of the virus. This plasmid is introduced into bacterial cells, typically *Escherichia coli*, which replicate the plasmid along with their DNA, allowing for large-scale production of the plasmid for further use in quantification assays.

5. Quantification of antibodies

Serum samples collected during the juvenile infection trial were analysed using ELISA (Enzyme-linked immunosorbent assay) to quantify total antibodies (IgM Total) as well as specific antibodies against NNV (IgM Specific).

a. Total IgM Measurement

Total IgM in the serum of infected fish was quantified using an ELISA protocol. Serum samples (100 µL) diluted 1:100 in Na_2CO_3 (50 mM), were distributed in triplicate across a 96-well plate. For the negative control, 100 µL of buffer (Na_2CO_3) was added to designated wells. The serum samples (antigens) were incubated at 25°C for 1 hour to facilitate adherence to the antigen-coated plate, after which the liquid was removed. Following incubation, 300 µL of blocking buffer (5% skimmed milk powder in T-TBS (0.1% Tween 20) was added to each well and incubated for 1 hour at 25°C. The blocking buffer

was then removed, followed by three consecutive washes with 300 µL of T-TBS. After washing, 100 µL of primary Anti-European seabass IgM monoclonal antibody (Aquatic Diagnostics, UK), diluted 1:100 in blocking buffer, was added to each well and incubated for 1 hour at 25°C. Following removal of the primary antibody, three additional washes were performed as previously described. Subsequently, 100 µL of secondary mouse anti-IgG-HRP antibody, diluted 1:1000 in blocking buffer, was added and incubated for 1 hour at 25°C. The plate was then washed three times. Next, 100 µL of the TMB substrate solution for ELISA (BioLegend) was added to each well and incubated for 5 minutes. The colour change reaction was stopped by adding 100 µL of 2 M sulphuric acid, and the optical density was read at 450 nm using the Synergy HT microplate reader (Biotek, SynergyHT).

b. Specific IgM Measurement against NNV

Specific IgM against NNV in serum of infected fish was measured using the following protocol: The plate was first coated with 100 µL of the virus at a concentration of $10^{7.25}$ TCID50/mL from the SNN-1 cell line (RGNNV 378/03 P6-D4) and incubated at room temperature for 24 hours. It was then washed three times with 300 µL of washing buffer (T-TBS). Next, 300 µL of blocking buffer was added to each well and incubated for an additional 24 hours. After this incubation, the plate was washed again with T-TBS as described above. Subsequently, 100 µL of serum, diluted 1:100 in Na_2CO_3 (50 mM), was added in triplicate to a 96-well plate, with 100 µL of buffer (Na_2CO_3) serving as a negative control. The samples (antigen) were incubated at 25°C for 24 hours to adhere to the antigen plate, after which they were removed. Immediately afterwards, 300 µL of blocking buffer (5% skimmed milk powder in T-TBS) was added to each well and left to incubate for 1 hour at 25°C. The blocking buffer was then removed, followed by three consecutive washes with 300 µL of T-TBS. After washing, 100 µL of primary Anti-European seabass IgM monoclonal antibody (Aquatic Diagnostics, UK), diluted 1:100 in blocking buffer, was added to each well and incubated for 1 hour at 25°C. Following removal of the primary antibody, three consecutive washes were performed as described above. Next, a secondary mouse anti-IgG-HRP antibody, diluted 1:1000 in blocking buffer, was then added and incubated for 1 hour at 25°C. The plate was then washed three times. Finally, 100 µL of the TMB substrate solution for ELISA (BioLegend) was added to each well and incubated for 5 minutes. The colour change reaction was stopped by adding 100 µL of 2 M sulphuric acid, and the optical density was read at 450 nm using the Synergy HT microplate reader (Biotek, SynergyHT).

6. Statistical analysis

Mortality, viral quantification, and antibody quantification data are presented as median values with interquartile ranges (if not normally distributed) or mean ± standard deviation (if normally distributed). Normality was assessed using the Shapiro-Wilk test ($N < 50$). Although viral load data passed the normality test, they did not meet homogeneity of variances; therefore, a Welch ANOVA with Holm-Sidak post-hoc was

performed. Viral load and total/specific IgM levels against NNV that did not meet normality assumptions were analysed using the Kruskal-Wallis test. For mortality data with two factors (days post-infection, treatment), a non-parametric two-way analysis was applied due to normality violations. All analyses were conducted using SigmaPlot (version 12.0, Systat Software, Inc., USA).

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III. Results

1. Juvenile infection trial to calculate LD50

Mortality was recorded from the onset of infection (day 0) through to the end of the trial (day 14) (Table III), with 10 fish in each tank. No mortality was observed in the immersion treatment groups. In contrast, the intramuscular injection route led to higher mortality rates, especially at the viral concentration of 10^5 TCID50/mL.

Table III- Mortality recorded during the trial (IM3- intramuscular injection 10^3 TCID50/mL; IM4- intramuscular injection 10^4 TCID50/mL; IM5- intramuscular injection 10^5 TCID50/mL; IM6- intramuscular injection 10^6 TCID50/mL).

Treatment	Total Individuals	Observed Mortality	Cumulative Mortality (%)	Mortality (dpi)
IM3	10	1	10	4
IM4	10	1	10	8
IM5	10	2	20	5 and 8
IM6	10	1	10	12

The cumulative mortality rate was calculated based on the initial number of fish (N=10) and the mortalities recorded throughout the trial. No mortality was observed in the immersion treatments, resulting in a cumulative mortality rate of zero. In treatments IM3, IM4, and IM6, the cumulative mortality rate was 10%, whereas in treatment IM5, it was 20%. No statistically significant differences were found among the immersion or intramuscular injection treatments, except between treatment IM5 and the other injection treatments.

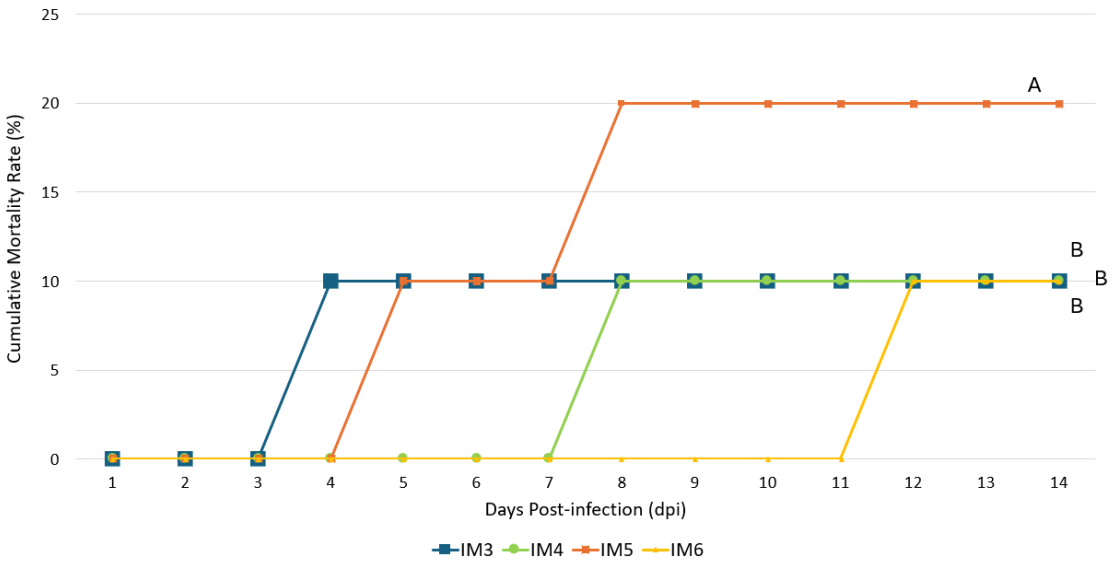

Figure 7. Cumulative mortality rate throughout the juvenile infection trial. (IM3- intramuscular injection 10^3 TCID50/mL; IM4- intramuscular injection 10^4 TCID50/mL; IM5- intramuscular injection 10^5 TCID50/mL; IM6- intramuscular injection 10^6 TCID50/mL). Different letters indicate statistically significant differences ($p < 0.05$).

Throughout the study, various behavioural changes were observed in fish exposed to different concentrations of VNN infection, with significant variation depending on the treatment. In the intramuscular injection groups, treatment IM6 (10^6 TCID50/mL) was the first to exhibit symptoms, including rapid swimming, which persisted from day 2 to day 13 post-infection. Starting on day 8, two fish in this group showed darkened skin, and one developed exophthalmia on day 9. In treatment IM5 (10^5 TCID50/mL), fish displayed rapid and vertical swimming during the initial days post-infection, and by day 8, darkened skin was observed, with one fish developing mouth lesions and reduced appetite. These symptoms persisted until the end of the observation period. In treatment IM4 (10^4 TCID50/mL), one fish exhibited uncontrolled swimming from day 4 to day 7, which was one of the more notable symptoms in this group. In the immersion-infected groups (B), symptoms appeared later, starting on day 8 post-infection. In the B4 group (10^4 TCID50/mL), one fish showed agitation and mouth lesions, swimming against the aquarium walls until the end of the observation period. In the B5 group (10^5 TCID50/mL), vertical swimming was observed on day 8, with reduced appetite noted in the subsequent days. Overall, intramuscular infections caused more severe and visible symptoms, such as darkened skin and exophthalmia, while immersion infections led to milder symptoms, such as reduced appetite and vertical swimming. These behavioural changes suggest a correlation between viral concentration and symptom severity, with intramuscular infections generally associated with more intense and visible symptoms.

2. Detection of antibodies in the juvenile infection trial

a. Total IgM Measurement

The total antibody levels were measured 14 days post-infection across all treatments and are shown graphically in Figure 8, where the Y-axis represents optical density (OD) at 450 nm.

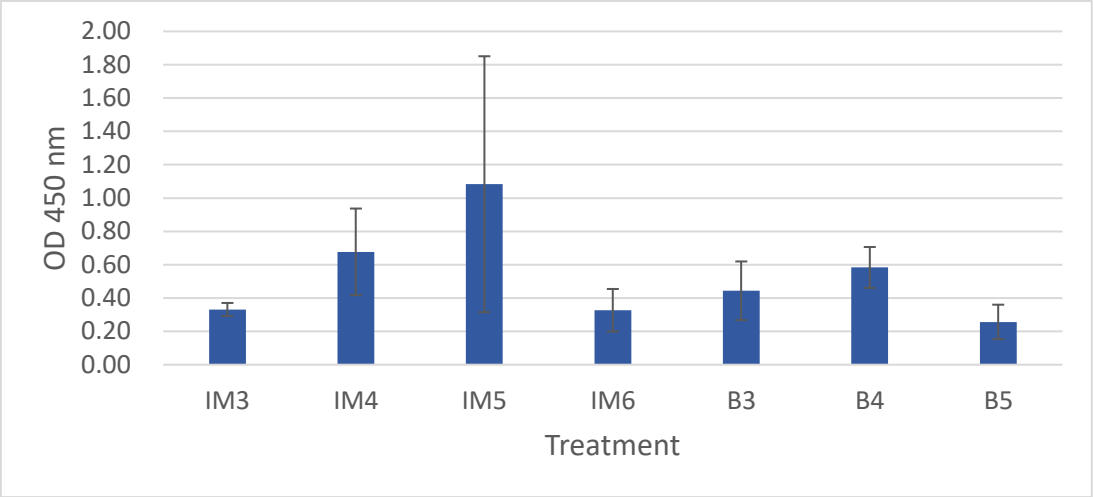

Figure 8. Total antibodies production in the serum of infected juveniles at the end of the trial 450nm (mean ± standard deviation) (n = 3) in each treatment. (IM3- intramuscular injection 10^3 TCID50/mL; IM4- intramuscular injection 10^4 TCID50/mL; IM5- intramuscular injection 10^5 TCID50/mL; IM6- intramuscular injection 10^6 TCID50/mL; B3- Immersion 10^3 TCID50/mL; B4- Immersion 10^4 TCID50/mL; B3- Immersion 10^3 TCID50/mL). There are no statistically significant differences (p › 0.05).

All treatments showed the presence of antibodies (Figures 8 and 9), with treatment IM5 exhibiting the highest total antibody level (1.083 ± 0.768 OD at 450 nm), followed by IM4 (0.677 ± 0. 260 OD at 450 nm), B4 (0.584 ± 0.123 OD at 450 nm), B3 (0.444 ± 0.176 OD at 450 nm), IM3 (0.332 ± 0.768 OD at 450 nm), and B5 (0.257± 0.104 OD at 450 nm), which had the lowest antibody level. However, no statistically significant differences were observed among the treatments.

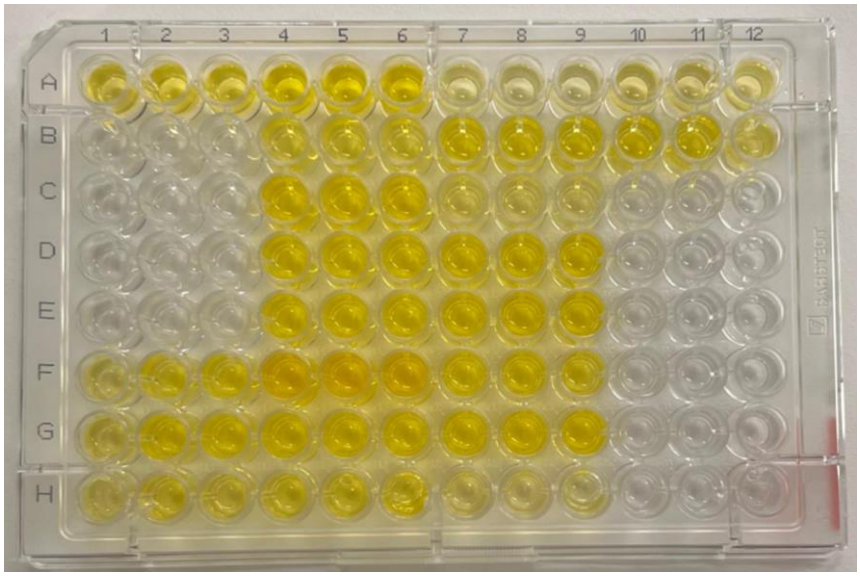

Figure 9. ELISA plate for the quantification of total antibodies, in triplicate. Wells A1-A3 contain a mix of sample, primary antibody, and secondary antibody; wells B1-B3 contain a mix of sample and secondary antibody; wells C1-C3 contain a mix of sample and primary antibody; wells D1-D3 contain primary and secondary antibodies only (no sample); wells E1-E3 (blank) contain Na_2CO_3 and both antibodies. Wells F1-F3, G1-G3, and H1-H3 contain samples from fish 1, fish 2, and fish 3 of the IM3 treatment, respectively. Wells A4-A6, B4-B6, and C4-C6 contain samples from fish 1, fish 2, and fish 3 of the IM4 treatment, respectively. Wells D4-D6, E4-E6, and F4-F6 contain samples from fish 1, fish 2, and fish 3 of the IM5 treatment, respectively. Wells G4-G6, H4-H6, and A7-A9 contain samples from fish 1, fish 2, and fish 3 of the IM6 treatment, respectively. Wells B7-B9, C7-C9, and D7-D9 contain samples from fish 1, fish 2, and fish 3 of the B3 treatment, respectively. Wells E7-E9, F7-F9, and G7-G9 contain samples from fish 1, fish 2,

and fish 3 of the B4 treatment, respectively. Wells H7-H9, A10-A12, and B10-B12 contain samples from fish 1, fish 2, and fish 3 of the B5 treatment, respectively.

b. Specific IgM Measurement against NNV

The quantity of specific antibodies against NNV was measured 14 days post-infection across all treatments, as shown graphically in Figure 10, with the Y-axis representing optical density (OD) at 450 nm.

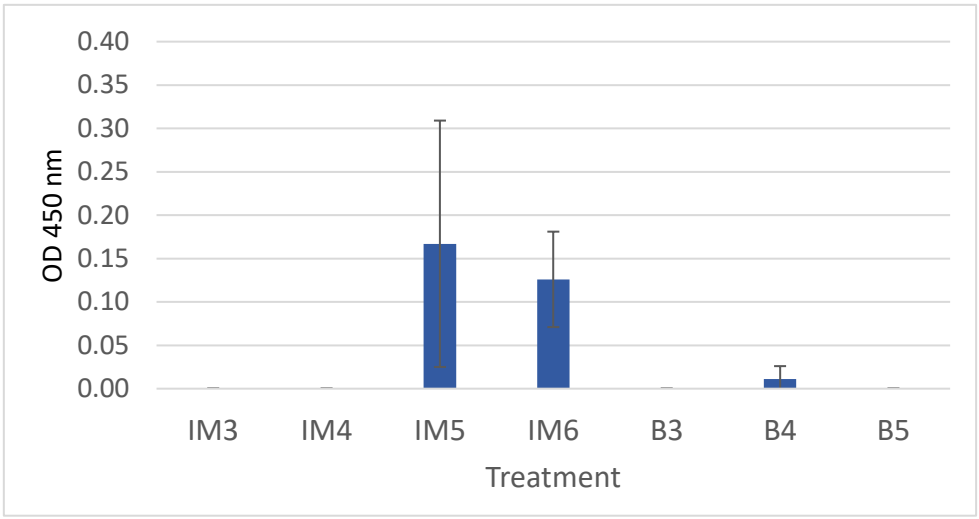

Figure 10. Production of specific antibodies against NNV in the serum of infected juveniles at the end of the trial measured at 450nm (mean ± standard deviation) (n = 3) for each treatment. (IM3- intramuscular injection 10^3 TCID50/mL; IM4- intramuscular injection 10^4 TCID50/mL; IM5- intramuscular injection 10^5 TCID50/mL; IM6- intramuscular injection 10^6 TCID50/mL; B3- Immersion 10^3 TCID50/mL; B4- Immersion 10^4 TCID50/mL; B3- Immersion 10^3 TCID50/MI). There are no statistically significant differences (p › 0.05).

Only treatments IM5 (0.167 ± 0.142 OD at 450 nm), IM6 (0.126 ± 0.055 OD at 450 nm), and B4 (0.011 ± 0.015 OD at 450 nm) produced specific antibodies against NNV (Figures 10 and 11). Treatment IM5 had the highest antibody production, followed by IM6 and then B4. Specific antibodies against NNV were not detected in the other treatments, which recorded optical density values of zero at 450 nm. However, no statistically significant differences were observed among the treatments.

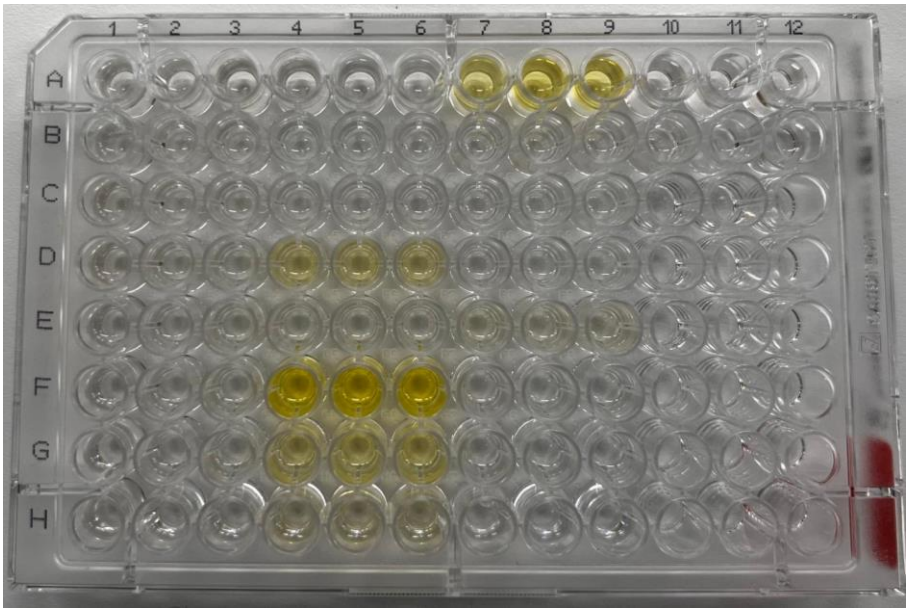

Figure 11. ELISA plate for the quantification of specific antibodies against VNN, in triplicate. Wells A1-A3 contain a mix of sample, primary antibody, and secondary antibody; wells B1-B3 contain a mix of sample and secondary antibody; wells C1-C3 contain a mix of sample and primary antibody; wells D1-D3 contain primary and secondary antibodies only (no sample); wells E1-E3 (blank) contain Na_2CO_3 and both antibodies. Wells F1-F3, G1-G3, and H1-H3 contain samples from fish 1, fish 2, and fish 3 of the IM3 treatment, respectively. Wells A4-A6, B4-B6, and C4-C6 contain samples from fish 1, fish 2, and fish 3 of the IM4 treatment, respectively. Wells D4-D6, E4-E6, and F4-F6 contain samples from fish 1, fish 2, and fish 3 of the IM5 treatment, respectively. Wells G4-G6, H4-H6, and A7-A9 contain samples from fish 1, fish 2, and fish 3 of the IM6 treatment, respectively. Wells B7-B9, C7-C9, and D7-D9 contain samples from fish 1, fish 2, and fish 3 of the B3 treatment, respectively. Wells E7-E9, F7-F9, and G7-G9 contain samples from fish 1, fish 2, and fish 3 of the B4 treatment, respectively. Wells H7-H9, A10-A12, and B10-B12 contain samples from fish 1, fish 2, and fish 3 of the B5 treatment, respectively.

3. Viral load by RT-qPCR

Viral loads in the samples (brains and larvae) were quantified using RT-qPCR, with the plasmid pjET÷NNV2 serving as a calibration standard. A standard curve was created by serially diluting the plasmid to plot a linear regression between NNV copy numbers and the dilutions. The viral concentration in the larvae was calculated using the regression equation y = -2.7762x + 33.941, which demonstrated a strong correlation with an R^2 value of 0.9804, as shown in Figure 12.

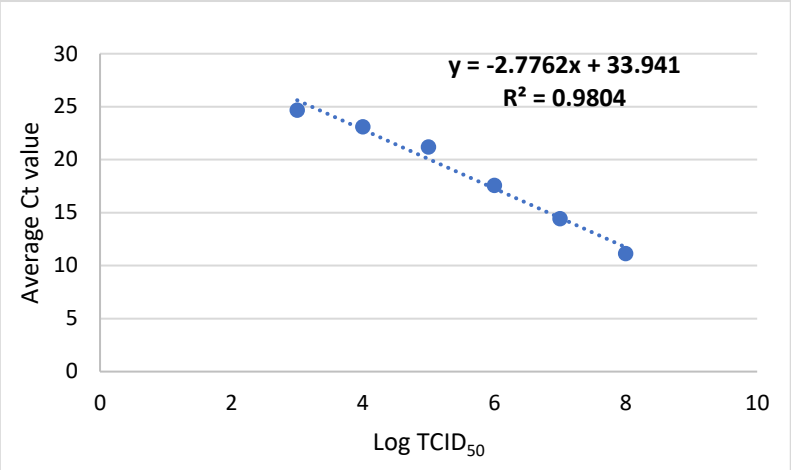

Figure 12. Standard curve of the average Ct values for each sample, based on the plasmid dilutions.

All RNA samples were analysed in real time to quantify the viral load, with samples from each treatment exhibiting average Ct values within the range of the standard curve and therefore considered positive for VNN.

After RNA extraction, the quantity and quality of each sample were assessed. The RNA concentration in brain tissue varied among the treatments (Attachment, Table II): IM3 ranged from 474.8 ng/µL to 286.3ng/µL; IM4 from 702.3 ng/µL to 277.7 ng/µL; IM5 from 610. 3 ng/µL to 282.2 ng/µL; IM6 from 615.5 ng/µL to 338.2 ng/µL; B3 from 311.1 ng/µL to 237.8 ng/µL; B4 from 490.1 ng/µL to 359.2 ng/µL; and B5 from 349.2 ng/µL to 288.8 ng/µL.

The 260/280 ratio was used to assess RNA purity, indicating the absence of proteins, phenol, and other contaminants that absorb close to 280 nm, with an ideal value of 2.0. Ratios between 1.9 and 2.4 are considered acceptable, and all samples were within this range. The 260/230 ratio served as a secondary purity measure, with values outside the range of 2.0 to 2.3 indicating the presence of organic contaminants (such as phenol and TRIzol) that absorb below 230 nm. The ideal value for this ratio is 2.2. After extraction, several samples did not fall within this range, including two samples from IM3, IM5, and IM6, as well as one sample from IM4, B3, and B5.

RNA quality and integrity were further assessed by 2% agarose gel electrophoresis, which confirmed RNA integrity and the absence of degradation. The 2% agarose gel electrophoresis was also employed to detect the presence of viruses. A NZYTech Ladder V (NZYTech, Lisbon, Portugal) (Bandín and Souto, 2020) (Figure 13).

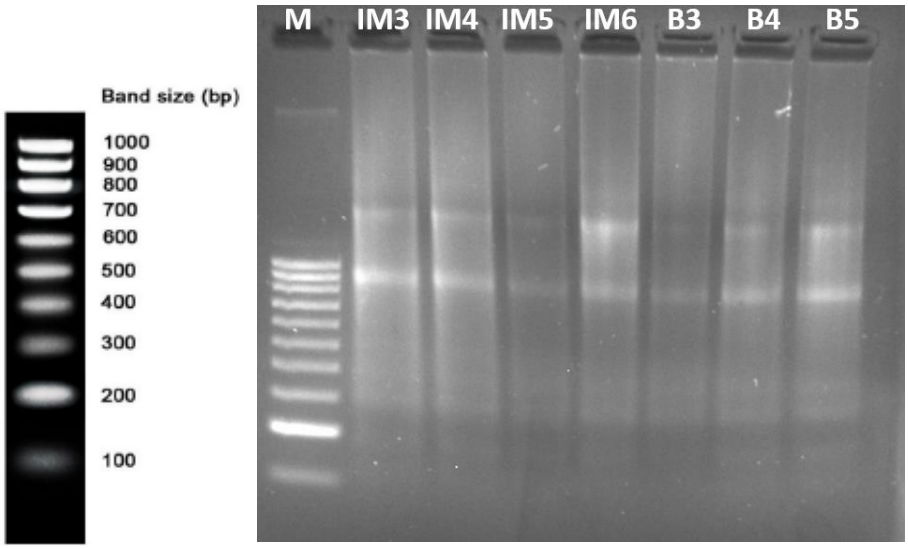

Figure 13. Electrophoresis of the RNA extraction from the juvenile infection test samples. Agarose gel 2%: M- NZYTech Ladder V marker; IM3- intramuscular injection 10^3 TCID50/mL; IM4- intramuscular injection 10^4 TCID50/mL; IM5- intramuscular injection 10^5 TCID50/mL; IM6- intramuscular injection 10^6 TCID50/mL; B3- Immersion 10^3 TCID50/mL; B4- Immersion 10^4 TCID50/mL; B3- Immersion 10^3 TCID50/mL).

At the end of the trial, viral load quantification in each treatment (Figure 14) revealed that the treatment IM3 had the highest viral concentration ($10^{5.79}$ TCID50/mL), followed by B3 ($10^{5.29}$ TCID50/mL), IM6 ($10^{5.28}$ TCID50/mL), B4 ($10^{5.23}$ TCID50/mL), IM4 ($10^{5.04}$ TCID50/mL), and B5 ($10^{3.99}$ TCID50/mL). The treatment IM5 had the lowest viral concentration ($10^{2.78}$ TCID50/mL). These results indicate that treatments IM3, B3, B4, and IM4, experienced an increase in the number of viral copies from the beginning to the end of the trial., while treatments IM6, B5, and IM5 showed a reduction in viral copies. However, no statistically significant differences (p › 0.05) were observed among the treatments.

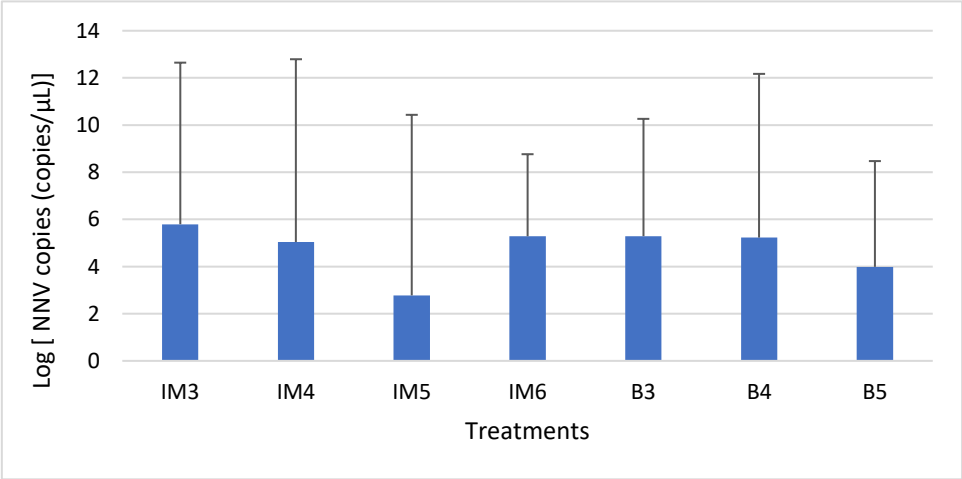

Figure 14. Virus quantification in the brain of infected juveniles at the end of the trial (mean ± standard deviation) (n = 3) in each treatment. (IM3- intramuscular injection 10^3 TCID50/mL; IM4- intramuscular injection 10^4 TCID50/mL; IM5- intramuscular injection 10^5 TCID50/mL; IM6- intramuscular injection 10^6 TCID50/mL; B3- Immersion 10^3 TCID50/mL; B4- Immersion 10^4 TCID50/mL; B3- Immersion 10^3 TCID50/mL). Bars represent mean ± standard deviation. There are no statistically significant differences (p › 0.05).

4. Passive immunisation trial of larvae testing rabbit anti-NNV PAb

Mortality was monitored daily, revealing distinct patterns across the five treatments as depicted in Figure 15. The highest mortality rate (59.3%) was observed in the group where the virus and rabbit serum were administered simultaneously via immersion (VNN+RS) ($p \leq 0.05$). This was followed by the positive control group, which received immersion infection with VNN (VNN) (41.7%). In contrast, the negative control group, which was immersed in PBS (PBS), showed the lowest mortality rate (16.9%) ($p \leq 0.05$). The group that received rabbit serum through immersion (RS) (30%) showed no statistically significant differences in mortality compared to the group that received rabbit serum through feeding after infection (VNN+ Feed RS) (33.2%), unlike the other treatments. In particular, the VNN+ Feed RS group exhibited the lowest mortality among the infected treatments. Mortality rates in all groups increased from the first to the seventh day post-infection (dpi), with a peak between the fourth and seventh days. After day thirteenth, mortality rates plateaued with no further deaths recorded until the end of the trial. Statistically significant differences were observed among all treatments, except between the immersion rabbit serum treatment and the group where the fish were infected and fed rabbit serum-supplemented feed.

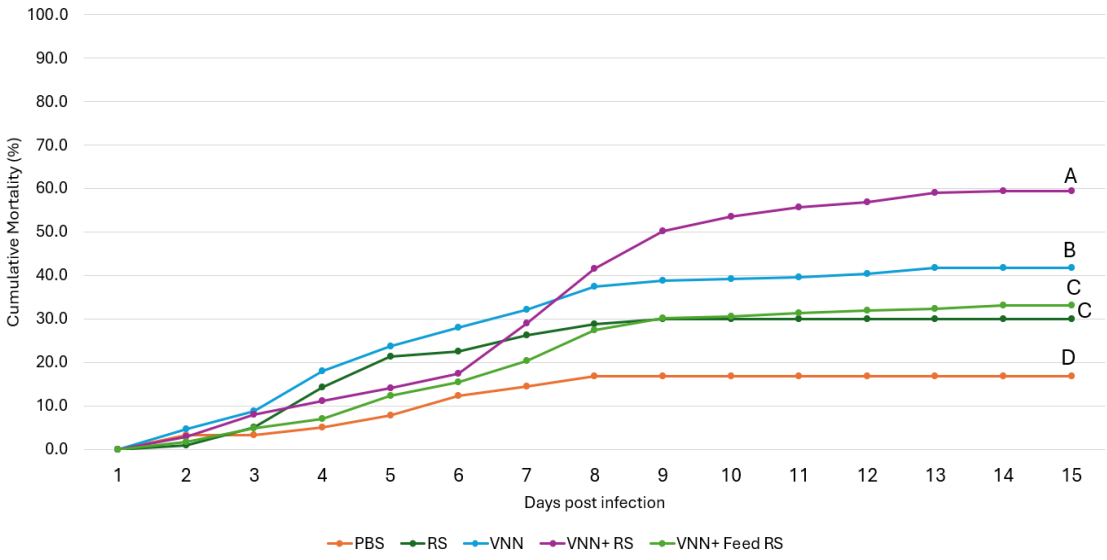

Figure 15. Mortality kinetics for passive immunisation trial. (PBS - immersion in PBS; RS - immersion in rabbit serum; VNN - immersion in virus; VNN + RS - immersion in virus and rabbit serum; VNN + Feed RS- immersion in virus and supplementary feeding with rabbit serum). Different letters indicate statistically significant differences ($p < 0.05$).

The viral loads of the samples were quantified by RT-qPCR using the same method applied to the brains of the juveniles. The viral concentration in the larvae was calculated using the regression equation $y = -2.7762x + 33.941$, which showed a strong correlation with an R^2 value of 0.9804, as shown in Figure 13, just like for the juvenile brains. Before infection, the virus was shown to be absent in the larvae by RT-qPCR. After nucleic acid extraction, the quantity and quality of the samples were assessed. The

concentration of nucleic acids varied among the different treatments (Attachment, Table III). In the PBS treatment, concentrations ranged from 476.3 ng/µL to 37.9 ng/µL over the days 0, 3, 7, 12, and 14. The RS treatment showed concentrations ranging from 579.2 ng/µL to 23.4 ng/µL. For the VNN treatment, concentrations varied from 278.8 ng/µL to 1030.5 ng/µL. In the VNN+RS treatment, values ranged from 284.9 ng/µL to 971.1 ng/µL, while the VNN+Feed RS treatment exhibited concentrations between 55.2 ng/µL and 605.5 ng/µL.

The 260/280 ratio was used to assess nucleic acid purity, with an ideal value of 2.0. Values between 1.9 and 2.4 are considered acceptable, and all values obtained for the different samples fell within this range, except for some samples such as VNN+Feed RS on day 7 (1.50) and PBS on the same day (1.53). The 260/230 ratio served as a secondary measure of purity, with ideal values ranging from 2.0 to 2.3. Samples that did not conform to this range included PBS on day 7 (0.60), RS on the same day (0.64), VNN+Feed RS on day 7 (0.62), and on day 12 (1.73). The VNN+RS sample on day 14 presented the lowest 260/230 ratio (1.33). The RNA concentrations extracted from these samples ranged between 200 ng/µL to 800 ng/µL, although a few samples, particularly those from 7 days post-infection (dpi), registered below 100 ng/µL. The quality and purity of the RNA, reflected in the 260/280 and 260/230 ratios, generally fell within acceptable levels, except in samples with lower RNA concentrations

The quality and integrity of the RNA in each sample were assessed by agarose gel electrophoresis, in the same way as for the samples obtained in the juvenile infection trial. (Figure 16).

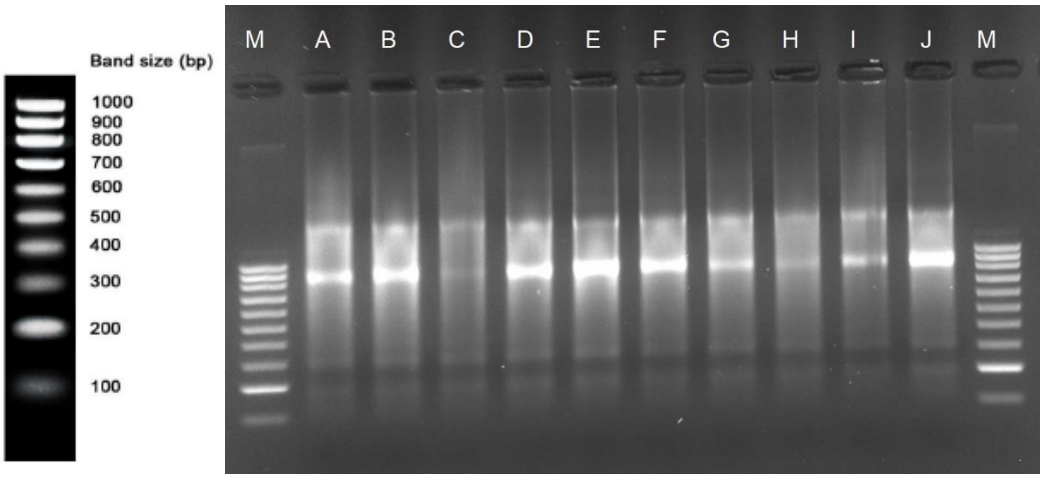

Figure 16. Electrophoresis of RNA extracted from samples of the passive immunisation test on larvae, *T1 Sample Point (3 days post-infection).* Agarose gel 2%: M- NZYTech Ladder V marker; A and F- PBS Immersion; B and G- Rabbit Serum Immersion; C and H- VNN Immersion; D and I- VNN and Rabbit Serum Immersion; E and J- VNN Immersion and Feed with Rabbit Serum.

Five samples of larvae were collected from each treatment before the start of the trial and subjected to viral quantification to confirm that all larvae were negative for NNV a. An average Ct value of 37.41 was obtained, indicating no positive larvae at the beginning, as this value falls outside the limits of the standard curve. During the trial, all RNA samples were analysed in real time to quantify the viral load. The uninfected fish samples, specifically, those from treatments where the larvae were immersed in PBS and those immersed in rabbit serum, showed extremely high average Ct values (Ct >

25), which were outside the range of Ct values obtained from the standard line. Therefore, all these samples were considered negative.

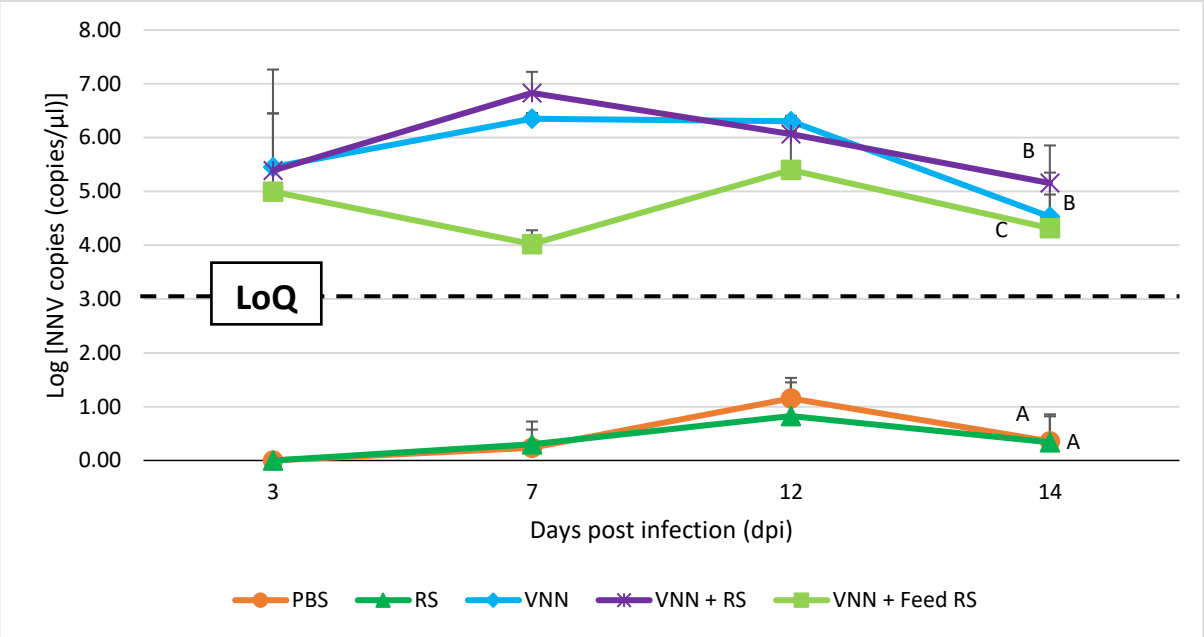

Figure 17. Evolution of virus quantification in larvae throughout the trial (mean ± standard deviation) (n = 5) for each treatment. Limit of Quantification (LoQ) =3 copies/µL. Different letters indicate statistically significant differences ($p < 0.05$).

The infected fish samples in each treatment showed average Ct values within the range of the standardized line and were therefore considered positive for NNV. The lowest level of viral load that can be quantified with precision and accuracy by quantitative PCR (qPCR) is defined as the limit of quantification (LoQ). This value was established using the previously obtained standard straight line, with the minimum limit of quantification corresponding to the highest viral dilution, set at 3 copies/µL.

After quantifying the viral load in each treatment (Figure 17), a reduction in viral copies was observed from the third-day post-infection until the fourteenth day. However, peaks in copy number were noted in all three treatments. For the NNV-infected treatments and the treatment where fish were fed supplemented feed, the peak occurred on day 12, reaching 6.30 copies/µL and 5.40 copies/µL, respectively. In the VNN+RS treatment, the peak occurred on day 7, reaching 6.83 copies/µL. After these peaks, there was a significant reduction in the number of viral copies, especially following the peak on day 12. Statistically significant differences were observed only between the twelfth and fourteenth days. As shown in Figure 17, there was a similar pattern in copy numbers between the VNN and VNN+RS treatments, as well as between the PBS and RS treatments. This similarity was statistically supported, with significant differences noted among all treatments, except between the VNN and VNN+RS pairs, and the PBS and RS pairs.

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IV.Discussion

Viral Nervous Necrosis (VNN) is one of the most concerning diseases in aquaculture, causing high mortality rates in several fish species, including European seabass. It poses a significant threat to fish producers (Bandín & Souto, 2020). Larvae and juveniles are particularly susceptible, with outbreaks leading to mortality rates as high as 100% in larval stages and around 20% in more developed juveniles. In addition, surviving fish often experience reduced growth, resulting in significant economic losses to producers (Munday, et al., 2002). This study aimed to evaluate the effectiveness of passive immunisation in European seabass larvae (*Dicentrarchus labrax*) through the administration of polyclonal antibodies and to determine whether this approach could reduce mortality and the severity of symptoms caused by viral nervous necrosis (VNN).

The LD50 is an essential parameter for selecting an appropriate dose to ensure infection and is needed for subsequent trials. Therefore, determining the LD50 of the viral culture before its use is essential. The mortality data obtained during the LD50 trial reflect minimal losses between treatments. Mortality occurred in the intramuscular infection treatments, while no mortality was observed in the immersion treatments. The highest mortality (20%) occurred in the IM5 treatment, while cumulative mortalities of 10% were recorded in the other treatments. Behavioural changes across all treatments showed that the IM5 treatment presented the most severe clinical signs. These symptoms included vertical swimming and darkened skin, both associated with neurological damage caused by betanodaviruses (Munday et al., 2002). Accelerated swimming suggests increased stress or neurological impairment, a hallmark of NNV infection (Munday et al., 2002). Specific behaviours, such as spinning and vertical swimming with the head up, indicate severe neurological symptoms linked to direct viral damage to the central nervous system (Costa & Thompson, 2016). The fact that these behaviours were more pronounced in IM treatments compared to the immersion treatments suggests that intramuscular injection may be a more efficient method of infection. Kim et al. (2018), explain that intramuscular injection is an artificial viral infection method that requires a lower viral dose than immersion infection. Additionally, the virus reaches its primary replication organ, the brain, more quickly via injection. In contrast, immersion infection, which is a more natural route, involves the virus overcoming multiple barriers to establish an infection (Kim et al., 2018). The mucosal surfaces of fish, such as the skin and gills, serve as protective physical barriers between the fish and the external environment. They play an important role in the complex immune system (Gomez et al., 2013; Kim et al., 2018), acting as a first line of defence. When the virus crosses them, it triggers the activation of innate immune cells, such as macrophages and neutrophils, which respond rapidly in a generalized manner.

In addition, immersion infection can lead to a slower adaptive immune response, with the gradual production of virus-specific antibodies after the viral antigens have been recognized by the adaptive immune system cells (B and T cells). Therefore, this route of infection activates both non-specific (first line of defence) and specific immune responses (specific antibodies against the virus, as in the case of NNV), but with a slower and less direct initial response (Pakingking et al., 2021).

Considering that obtaining the LD50 requires inducing mortality of 50% of the individuals, and in this trial, only 20% of the fish died, we conclude that this virus strain is not suitable for subsequent studies aimed at evaluating immune responses or the efficacy of prophylactic treatments/vaccines. This is because, at its initial concentration of 10^6 TCID/mL, the virus did not induce sufficient mortality. The lack of relevant results for determining the LD50 prompted us to focus on assessing the presence of total and specific antibodies against NNV, as well as viral quantification at the end of the experiment.

Antibody analysis provides valuable insights into the dynamics of infection and the immune response of the fish, complementing the mortality data obtained and clarifying the relationship between the virus and the host (Vanalli et al., 2022; Kelley et al., 2023). Antibody analysis focused on immunoglobulin M (IgM), as it is the most prevalent antibody in teleost fish, including European seabass. Although the highest mortality rate recorded in this study was relatively low (20%), this does not imply that the fish did not mount an immune response. Antibody production is part of the adaptive immune system´s reaction to pathogen presence, meaning the organism recognises the virus and responds to it. By assessing antibody levels, we can evaluate whether a humoral immune response occurred, even in the absence of severe clinical signs or high mortality rates (Jackson & Elsawa, 2015).

The non-specific antibody response was measured using the optical density (OD) at 450 nm as an indicator of antibody concentration. The IM treatments showed higher OD values compared to the immersion treatments (B). Specifically, the IM5 treatment showed the highest antibody concentration, with an average OD close to 1.5, followed by the IM4 and IM6, which showed lower antibody concentrations. In contrast, the immersion treatments had lower OD values, indicating a weaker or delayed immune response.

Regarding the presence of specific antibodies against NNV, OD values were significantly lower in all treatments compared to the non-specific response. Consistent with previous studies, the presence of specific antibodies against NNV was higher in fish infected by injection than by immersion. For example, Grove et al. (2003) reported lower OD values for Atlantic halibut (*Hippoglossus hippoglossus*) infected via immersion (OD450 nm = 0.750) compared to those infected via intraperitoneal injection (OD450 nm = 1.000), indicating reduced antibody activity in serum from immersion-infected fish. Similarly, Nuñez Ortiz et al. (2016) found that immunisation of European seabass (*Dicentrarchus labrax*) with formalin-inactivated Betanodavirus, β-propiolactone, or heat treatment resulted in OD values ranging from 0.500 to 0.800 for intraperitoneal infection, compared to around 0.100 for immersion infection. Ferreira et al. (2019) also noted that fish infected via injection exhibited a higher mean titre of specific antibodies against NNV compared to those infected by immersion.

The IM5 treatment consistently showed the highest concentration of specific antibodies, though these values were significantly lower than those for total antibodies. This suggests that while the fish produced antibodies, their specificity for VNN was not strong. This may indicate an inability of the immune system to develop a robust and specific response, potentially due to the virus losing virulence after multiple passages. In the present study, it was observed that fish infected with NNV via intramuscular (IM) injection mounted a more pronounced immune response, although it was not as strong

as expected. This could be due to the virus strain used, which had undergone seven serial passages. Multiple viral passages often lead to attenuation, where the virus loses its ability to cause disease (Zhang et al., 2024). This attenuation typically occurs due to mutations in genes related to virulence or viral surface protein, reducing the virus's ability to infect its host cells (Témoin et al., 2008)

Immersion-infected groups did not show a robust immune response or the presence of specific antibodies against the virus, unlike the IM groups. This difference is consistent with existing scientific literature, which suggests that the intramuscular injection route is more efficient in activating the adaptive immune system, leading to a more rapid and pronounced production of specific antibodies (Costa & Thompson, 2016). In contrast, immersion infection requires the virus to overcome several physical barriers, leading to a slower immune response and lower antibodies (Thompson et al., 2018).

The IM5 group had the highest mortality, which correlates with the higher antibody response observed in this group. This suggests that the viral dose of 10^5 TCID50/mL induced a stronger immunological response, leading to higher mortality. The results obtained are consistent with a study by Mould et al. (2017), which indicate that injection-based infections can induce a faster and more specific immune response, resulting in higher production of specific antibodies against the pathogen. In contrast, immersion infections, conversely, can induce both specific and non-specific immune responses, but to a lesser extent. A study in pond-raised orange-spotted grouper (*Epinephelus coioides*) also showed that viral infection by injection-induced higher levels of neutralizing antibodies against viral nervous necrosis (VNN) compared to immersion infection, supporting the findings of the present study (Pakingking et al., 2021).

To complement the immune response analysis, the viral load present in the fish was also quantified, and better understand the relationship between the observed immune response and virus replication. The viral load quantification was not significantly different among treatments. The low variation in viral concentration may suggest that the infection routes (immersion and IM) and the viral concentration did not positively affect viral replication. Additionally, the viral concentration data showed a high standard deviation, indicating large variability in viral load between individuals, even within the same treatment group. This high variability may suggest either inefficiency in the infection process or differences in the individual fish's immune response to infection.

Hodneland et al. (2011), correlated Ct values with mortality levels in European seabass experimentally infected with betanodavirus, finding that fish with Ct values below 15.0 (range: 11.7-20.4) could not survive the infection, regardless of infection dose or transmission route, and that mortality was certain with median Ct values below 11.7. The data obtained in the present study are consistent with these findings, as the Ct values observed at the study´s end ranged from 18.35 to 26.72, with no recorded mortality. Similarly, the viral quantification data from Ferreira et al. (2019) which used the same viral culture, also supports these findings, as fish that survived showed Ct values lower than 11.7. In addition, the virus was detected in the brain of surviving fish, indicating that while the virus replicated and migrated from the injection site to the brain, it did not cause sufficient mortality to impact survival significantly (Barsøe et al., 2021).

Although it was not possible to determine the LD50 for 5-gram juveniles, this 10^5 TCID50/mL was used in a passive immunisation trial on larvae, as larvae at 60 dph are more immunologically sensitive than 5-gram juveniles (García-Álvarez et al., 2022).

Vázquez-Salgado et al. (2022) previously infected 12 dph larvae via immersion with a viral concentration of 10^5 TCID50/ml, a concentration chosen for this study as well.

Passive immunisation has been shown to play a crucial role in protecting farmed fish, such as European seabass, against infectious diseases, particularly in early life stages (Toranzo et al., 2005; Hedegaard & Heegaard, 2016; Rajan et al., 2017; Chettri et al., 2019). This protection is immediate, which is essential since larvae have underdeveloped immune systems. Based on the ontogeny of the seabass immune system where the thymus and head kidney become functionally active between 30 and 44 dph (Scapigliati et al., 2002), and full immunocompetence for antibody production is reached around 50 dph, we chose larvae at 58 dph for our trials to ensure more mature immune system (Munday & Nakai, 1997). The administration of antibodies, i.e., passive immunisation, can support immune development while offering infection protection (Raja et al., 2017). In aquaculture, reducing mortality and improving survival rates are key. Previous studies show that dietary antibody supplementation has a positive effect on the antiviral response. For instance, De Lima et al. (2019) demonstrated that polyclonal antibodies, like those in the rabbit serum used here, were effective against bovine viruses. Likewise, Engle and Diamond (2003) found that passive antibody administration helped prevent West Nile virus infection in mice, highlighting antibody-based therapies potential. Gye et al. (2024) reported that the rabbit serum used in their study reduced NNV infectivity but did not completely neutralise the virus, which could regain infectivity upon protein dissociation. Introducing these antibodies to fish via injection, immersion, or feed imparts temporary immunity without an active immune response. Accordingly, we use rabbit serum containing anti-NNV antibodies for larval passive immunisation. Cumulative mortality showed a significant increase in the VNN-exposed treatments, while the PBS treatment displayed low and stable mortality, representing baseline mortality for uninfected fish. Baseline mortality highlights that environmental factors and treatment methods also contribute to mortality (Oliveira et al., 2021), emphasizing the importance of infection control (Shi et al., 2021). The RS treatment, exposed only to rabbit serum, showed a mortality pattern similar to PBS, confirming that the serum is non-toxic and does not induce NNV-related mortality. In the VNN treatment, mortality increased from day 7, reaching 41.7% by the study´s end, a typical pattern for betanodavirus infections in sea bass, (Vendramin et al., 2014; Bakopoulos et al., 2023). However, given the post-larvae stage, cumulative mortality is expected to reach around 100% (Costa & Thompson, 2016), due to viral encephalopathy and retinopathy. Mortality was significantly higher (59.3%) in the VNN + RS treatment, suggesting that although rabbit serum was intended for immunotherapy, it might not have effectively reduced mortality. Higher antibody concentrations may enhance better protection but can also cause excessive immune responses, potentially leading to immunopathology and increased mortality (Okon et al., 2023). The rabbit serum might have exacerbated the fish's immune response, resulting in immune hyperactivation and increased mortality. The virus can exploit non-neutralizing antibodies, which bind to its surface proteins, to facilitate more efficient entry into target cells, thereby enhancing viral infection. This effect, known as antibody-dependent enhancement (ADE), occurs when sub-neutralizing or non-neutralizing antibody levels increase viral infectivity rather than neutralizing it, leading to immune system hyperactivation and worsening of the disease (Hohdatsu et

al., 1991; Thomas et al., 2022). However, antibody research is necessary to confirm this, as no antibody-specific research was conducted in this study.

In the VNN+Feed RS treatment, mortality was lower (33.2%) than in the VNN+RS treatment, suggesting that the rabbit serum supplementation may have provided some protection against viral infection. Although this protection was not enough to completely prevent mortality, it does suggest a beneficial effect when the serum is administered continuously through feed. While oral antibody administration is challenging in early life stages due to the small size of the fish, antibodies administered orally enter the gastrointestinal tract, where they may neutralize the viral particles (Rajan et al., 2017). However, the supplemented feed has a limited shelf life (Winkelbach et al., 2015), and gastric acid must be considered as it can denature the antibodies. Thus, antiacids might be needed to prevent the degradation of antibody-enriched feed (Lavelle & Harris, 1997; Raja et al., 2017). However, raising pH levels can affect feed digestibility (Arasteh et al., 2004) complicating culture optimization for these fish. Therefore, microencapsulation could offer a more effective method for dietary supplementation by protecting polyclonal antibodies from gastric enzymes and allowing for controlled release into the intestine (Wu et al., 2011; Encina et al.,2016; Hashim et al., 2021).

The behavioural symptoms observed (vertical swimming, uncoordinated movements, and rapid death) in the VNN+RS treatments are consistent with symptoms of severe betanodavirus infection, which targets the central nervous system, leading to abnormal behaviour and sudden death. Such symptoms have been well documented in the study of viral encephalopathy in fish (Costa & Thompson, 2016), highlighting the rapid disease progression in these cases.

One of the main findings of this study is the potential immunomodulatory effect of rabbit serum, though its efficacy appears to be strongly dependent on the administration route. Antibody administration via the feed (VNN+Feed RS) resulted in lower mortality compared to simultaneous immersion with the virus (VNN+RS), suggesting that the route of administration may be critical in determining serum efficacy. Overall, these results highlight the complexity of the fish immune response to viral infection and suggest the dual role of rabbit serum as an immunomodulator. To optimize rabbit serum immunotherapy further studies on different dosages, timing, and delivery methods are needed to maximize its efficacy without compromising fish survival.

Throughout the experiment, Betanodavirus (NNV) quantification was conducted on the larvae to monitor infection dynamics and correlate viral load with observed mortality, providing insights into infection progression and impact over time. Whole larvae were used for viral quantification to assess systemic viral load, acknowledging that the virus may spread beyond the brain to other organs and tissues. However, this approach produced RNA of lower purity after extraction, likely due to gut proteases that could degrade viral material or interfere with RNA extraction (Wilde et al., 1990). While this whole larva method aimed for a comprehensive infection assessment, future studies may benefit from focusing on specific tissues like the brain or immune-related organs, where protease activity is lower. Additionally, careful dissection to remove the gastrointestinal tract before preservation could also improve viral quantification accuracy by reducing interference from digestive enzymes, highlighting a methodological improvement for future studies. Optimizing the RNA extraction protocol to achieve purer RNA could involve proportionally increasing NZYol reagent doses for cell lysis, improving

homogenization, and adjusting chloroform quantities to effectively separate nucleic acids (Martínez-Martínez et al., 2011). It was observed during initial homogenization that larvae were not fully homogenized, potentially affecting extraction efficiency and quality.

Viral quantification in the PBS treatment (negative control), consistently showed low viral levels over time, as expected, since these fish were not exposed to the virus. This control establishes a baseline for natural mortality during the study. Similarly, the RS treatment (rabbit serum immersion) showed no significant viral load increase, indicating that rabbit serum alone does not cause infection in immersed fish confirming that the rabbit serum does not contain viral particles or NNV-causing components. Both PBS and RS treatments remained below the LoQ (limit of quantification), affirming that these fish were not infected with NNV. However, the VNN immersion groups showed different results. The VNN treatment showed a high viral load, particularly on day 7 post-infection, suggesting active viral replication in the fish at that point, consistent with typical betanodavirus activity, which peaks in replication before declining (Costa and Thompson, 2016). In the VNN+RS and VNN+Feed RS groups, the viral load followed a similar trend but showed a more pronounced decrease in copy numbers from day 12 post-infection in the VNN + Feed treatment group. This pattern may indicate that dietary supplementation with rabbit serum helped modulate the fish´s immune response, allowing for more efficient viral elimination.

V.Conclusion

The data from this study, along with existing literature, highlight the value of passive immunization as a complementary strategy to active immunization, such as vaccination, in aquaculture. By providing immediate protection, passive immunization reduces the need for antibiotics and chemical treatments, thereby minimizing antibiotic resistance and supporting sustainable practices. Integrating passive immunization with traditional vaccination can enhance fish health and survival rates, promoting environmentally responsible approaches in aquaculture.

In this study, we established an effective neutralization protocol using rabbit polyclonal serum against NNV (PAb) as a positive control. We demonstrated that PAb can be efficiently administered to European seabass larvae (*Dicentrarchus labrax*) through immersion and dietary methods, with evidence suggesting that dietary administration may help protect against Betanodavirus (NNV) infections. The effectiveness of this approach was validated using RT-qPCR assays to quantify viral loads, alongside mortality data, marking a significant advancement in developing robust methodologies for future trials.

Given that fish in aquaculture are often kept at high densities under stressful conditions, their susceptibility to viral outbreaks such as NNV is significantly increased. Passive immunization offers early protection during vulnerable stages, thereby improving growth rates, feed efficiency, and overall production performance. By reducing reliance on antibiotics, passive immunization supports food safety and decreases the risk of antimicrobial resistance.

Future research should explore the use of monoclonal antibodies targeting multiple NNV strains, evaluate antibody concentrations in feed, and assess the duration of the protective effect. To improve the accuracy of viral quantification, more rigorous RNA extraction methods, such as targeted brain dissection, are recommended to prevent RNA degradation by intestinal enzymes. Preserving samples in antiprotease solutions may further maintain RNA integrity. Additional studies should examine passive immunization under varying environmental conditions.

VI.References

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VII.Appendix

Table IV- Alterations in the juveniles' behaviour during the trial

Treatment	Days post-infection	Changes in behaviour
IM6	2	Speed Swimming
IM4	4	1 fish spinning out of control
IM5		Speed swimming; 1 fish swimming vertically (head up)
IM6		Speed Swimming
IM4	5	1 fish spinning out of control
IM5		1 fish swimming vertically (head down)
IM6		Speed Swimming
IM4	6	1 fish spinning out of control
IM5		Speed Swimming
IM6		Speed Swimming
IM4	7	1 fish spinning out of control
IM5		Speed Swimming
IM6		Speed Swimming
IM3	8	2 fish with darkened skin
IM4		1 fish spinning out of control
IM5		1 fish with darkened skin and slight mouth sores
IM6		Speed Swimming; 1 fish with darkened skin and mouth sores
B4		1 agitated fish with a mouth sore
B5		1 fish swimming vertically (head down)
IM5	9	1 fish swimming vertically (head down)
IM6		Speed Swimming; 1 fish with exophthalmos
B4		1 fish with a mouth sore (swimming against aquarium walls)
IM5	10	1 fish with darkened skin; low appetite
IM6		2 fish with darkened skin; low appetite
B4		1 fish with a mouth sore (swimming against aquarium walls)
B5		low appetite
IM5	11	1 fish with darkened skin; low appetite
IM6		2 fish with darkened skin
B4		1 fish with a mouth sore (swimming against aquarium walls)
IM5	12	1 fish with darkened skin; low appetite
IM6		2 fish with darkened skin
B4		1 fish with a mouth sore (swimming against aquarium walls)
IM5	13	1 fish with darkened skin; low appetite
IM6		2 fish with darkened skin

B4		1 fish with a mouth sore (swimming against aquarium walls)

Table II - Quantity and quality of RNA extracted from juvenile brains (3 fish from each treatment)

Treatment	Concentration of nucleic acids (ng/µL)	260/280 (nm)	260/230 (nm)
IM3	474.8	2.12	2.27
	328.9	2.11	2.45
	286.3	2.10	1.86
IM4	702.3	2.13	2.32
	403.9	2.10	2.43
	277.7	2.09	2.34
IM5	610.3	2.11	2.14
	282.2	2.10	2.49
	300.1	2.09	2.46
IM6	401.3	2.12	2.26
	615.5	2.12	2.73
	338.2	2.10	2.42
B3	237.8	2.10	2.35
	295.3	2.08	2.26
	311.1	2.10	2.29
B4	490.1	2.12	2.19
	359.2	2.09	2.29
	365.6	2.09	2.21
B5	349.2	2.13	2.27
	288.8	2.09	2.19
	334.8	2.10	1.96

Table III- Quantity and quality of RNA extracted from larvae (media ± standard deviation)

Treatment	Day post-infection	Concentration of nucleic acids (ng/Ml)	260/280 (nm)	260/230 (nm)
Sample 1	0	476.3	2.12	2.16
Sample 2		834.3	2.13	2.38
PBS (A)	3	490.8	2.05	1.9
PBS (F)		442.1	2.13	2.06
PBS (A)	7	484.8	2.11	1.86
PBS (F)		37.9	1.53	0.60
PBS (A)	12	66.6	1.62	0.73
PBS (F)		430.8	2.02	1.87
PBS (A)	14	469.9	2.11	2.25
PBS (F)		491.6	2.11	1.86
RS (B)	3	579.2	2.11	2.03
RS (G)		386.2	2.11	2.25
RS (B)	7	1186.0	2.12	2.35

RS (G)		23.4	1.68	0.64
RS (B)	12	537.1	2.08	2.22
RS (G)		221.6	2.02	1.87
RS (B)	14	970.8	2.11	2.25
RS (G)		737.8	2.13	2.25
VNN (C)	3	278.8	2.1	2.12
VNN (H)		309.8	2.06	1.8
VNN (C)	7	520.5	2.09	2.31
VNN (H)		405.8	2.11	1.90
VNN (C)	12	792.4	2.09	2.29
VNN (H)		345.4	2.05	1.99
VNN (C)	14	453.0	2.09	2.24
VNN (H)		1030.5	2.10	2.30
VNN+RS (D)	2	430.9	2.11	2.12
VNN+RS (I)		284.9	2.07	2.1
VNN+RS (D)	7	286.8	2.10	2.11
VNN+RS (I)		619.8	2.14	2.36
VNN+RS (D)	12	617.8	2.01	1.79
VNN+RS (I)		353.9	2.02	1.79
VNN+RS (D)	14	971.1	2.10	2.29
VNN+RS (I)		10.2	2.11	1.33
VNN+Feed RS (E)	3	605.5	2.11	2.09
VNN+Feed RS (J)		391.1	2.08	2.2
VNN+Feed RS (E)	7	55.2	2.01	1.81
VNN+Feed RS (J)		78.7	1.50	0.62
VNN+Feed RS (E)	12	381.8	2.06	2.09
VNN+Feed RS (J)		146.5	1.99	1.73
VNN+Feed RS (E)	14	548.2	2.10	2.31
VNN+Feed RS (J)		293.4	2.12	2.04