Instituto Politécnico de Leiria
Escola Superior de Turismo e Tecnologia do Mar

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Master Degree in Aquaculture

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A thesis submitted to the School of Tourism and Maritime Technology, Polytechnic Institute of Leiria as partial fulfilment for the requirements for the Master's Degree in Aquaculture, held under the scientific supervision of Doctor Kimberly Thompson (Moredun Research Institute), Doctor Janina Costa (Moredun Research Institute) and specialist Teresa Baptista (School of Tourism and Maritime Technology, Polytechnic Institute of Leiria).

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Resumo

Atualmente, uma das principais preocupações com a indústria aquícola está relacionada com os surtos de Encefalopatia e Retinopatia Viral (ERV), uma vez que esta doença está associada a níveis elevados de mortalidade (até 90-100% de mortalidade), especialmente em peixes juvenis. Mais de 120 espécies cultivadas e selvagens são suscetíveis a esta patologia. O agente etiológico desta patologia é designado por Betanodavirus, um vírus constituído por duas cadeias simples de RNA sem envelope (ssRNA).

Recentemente, a técnica de RT-PCR em tempo real tem vindo a adquirir mais importância, como método confiável para deteção de Betanodavirus. O órgão preferencial para detetar Betanodavirus é o cérebro, porém, para analisar reprodutores, este método de amostragem letal não é adequado. Os métodos de amostragem não-letal, como utilização de barbatanas, brânquias ou serologia, têm sido investigados numa sociedade cada vez mais preocupada com o bem-estar animal. O objetivo deste estudo consistiu em avaliar a viabilidade destes tecidos para a deteção de uma infeção de Betanodavirus em robalo legítimo (*Dicentrarchus labrax*).

Foram analisados quatro tecidos distintos, o cérebro (órgão preferencial para deteção de Betanodavirus), brânquias, barbatana caudal e sangue como tecidos provenientes de amostragem não-letal para avaliar a sua adequabilidade ao método de deteção RT-PCR em tempo real. Juvenis de robalo foram infetados com o vírus por injeção intramuscular ou imersão, e amostras de tecido, sangue e serum foram recolhidas nos dias 7, 15 e 30 após infeção experimental.

O método ELISA permitiu a deteção de anticorpos específicos para Betanodavirus aos 7 dias após a infeção experimental. Concentrações mais elevadas de anticorpos foram detetadas nos dias 15 e 30 após infeção experimental, para ambos os métodos de infeção. A presença de anticorpos indica que este é um método adequado para avaliar o estado serológico dos peixes em estágios de infeção precoces até pelo menos um mês após infeção.

Foi possível detetar o RNA viral em todos os tecidos analisados com RT-PCR em tempo real. A carga viral presente nos tecidos provenientes de amostragem não letal foi sempre mais reduzida do que a apresentada pelo cérebro em todos os dias de amostragem. Vírus foi detetado nos tecidos de amostragem não letal a partir do dia 7

após infeção experimental. A carga viral na barbatana caudal apresentou diferenças estatisticamente significativas entre dias de amostragem, enquanto que as brânquias apresentaram diferenças entre tratamentos e dias de amostragem. Como esperado, o cérebro foi o único tecido que registou a presença do vírus independentemente do método de infeção ou do dia de amostragem.

A presença de vírus ao longo do período de amostragem não foi constante nos tecidos de amostragem não letal (brânquias, barbatana caudal e sangue) e a carga viral foi sempre mais reduzida dos que a registada para o cérebro. Cargas virais elevadas foram detetadas no sangue de peixes infetados por injeção intramuscular desde dia 15 até ao final do ensaio.

A presença do vírus detetada em todos os dias de amostragem e em quantidade mais elevada, e o reconhecido tropismo deste vírus para tecidos do sistema nervoso, permitem aconselhar que o cérebro permaneça como órgão preferencial para o diagnóstico e triagem de peixes infetados com Betanodavirus. Porém, os resultados apontam para o facto de que são necessários mais estudos sobre o uso destes tecidos de amostragem não letal de forma aceitar ou rejeitar, sem dúvidas, a possibilidade de utilizar estes tecidos no diagnóstico de uma infeção em peixes por Betanodavirus.

Palavras-chave: Encefalopatia e Retinopatia Viral (ERV), Betanodavirus, amostragem não letal, deteção, ELISA, RT-PCR em tempo real.

Abstract

Currently, one of the major concerns to the industry in the Mediterranean is related with Viral Encephalopathy and Retinopathy (VER) outbreaks, since this disease is associated with high levels of mortality (up to 90-100% mortality), especially in juvenile fish. More than 120 farmed and wild species are susceptible to this disease. The etiological agent of VER is Betanodavirus, a non-enveloped two single-stranded RNA virus (ssRNA).

In recent years, the real time RT-PCR has been gaining importance as a reliable method of detection for Betanodavirus. The preferred organ for Betanodavirus detection is the brain, however, this method of screening is unsuitable for valuable broodstock. Non-lethal sampling methods, such as the use of fin or gill clips or serology, are being investigated as society becomes more concerned about animal welfare. The aim of this study was to evaluate the suitability of using these non-lethally sampled tissues as a method to detect Betanodavirus infection in European sea bass (*Dicentrarchus labrax*).

Four different tissues were analysed, the brain (standard organ for detection of Betanodavirus) and gills, caudal fin and blood as non-lethal tissues to access their suitability for detecting Betanodavirus infection using real time RT-PCR. European sea bass juveniles were infected with the virus, by either intramuscular injection or immersion, and tissue samples, blood and serum were collected on 7, 15 and 30 days post infection (dpi).

The ELISA allowed the detection of antibodies against Betanodavirus as early as 7 dpi. However, higher mean antibody titres were detected at 15 and 30 dpi for both infection route. The presence of Betanodavirus specific antibodies indicates that this is a suitable method to evaluate the serological status of the fish from early stages of infection and up to at least one-month post-infection.

It was possible to detect virus RNA in all tissues analysed with the real time RT-PCR. The viral load present in the non-lethal tissues was always lower than that measured in the brain tissue at all sampling points. Virus was detected in the non-lethal tissues from 7 dpi. The viral load detected in the caudal fin presented statistical significant differences between sampling days, while the gills presented differences between treatments and sampling days. As expected, the brain was the only tissue that

showed the presence of the virus, independent of the route of infection or the sampling day.

Presence of virus in non-lethal tissues (gills, fin clips and blood) was inconsistent and always at lower values of viral load than in the brain. Nevertheless, higher viral loads were detected in the blood of fish infected by intramuscular injection at 15 dpi until the end of the challenge.

Due to the constantly higher viral load displayed in the brain at all sampling points, reflecting the virus' tropism for nervous tissue, it is suggested that the brain should remain as the target organ for screening fish for the detection of Betanodavirus. However, the results reveal the necessity to perform further studies regarding the use of non-lethal tissues, before undoubtedly confirming or rejecting the possibility of using them to determine a Betanodavirus infection in fish.

Key-words: Viral Encephalopathy and Retinopathy (VER), Betanodavirus, non-lethal sampling, detection, ELISA, Real Time RT-PCR.

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AGK – Asian Grouper Kidney;

CPE – Cytopathic Effect;

Dpi – Days post infection;

ELISA – Enzyme-Linked Immunosorbent Assay;

HBSS – Hank's Balanced Salt Solution;

HSWB – High Salt Washing Buffer;

IM – Intramuscular injection;

LSD – Least Significance Test;

LSWB – Low Sal Washing Buffer;

MAb – Monoclonal Antibodies;

NBF – Neutral Buffered Formalin;

NNV – Nervous Necrosis Virus;

OD – Optical density;

PBS – Phosphate Buffered Saline Solution;

RGNNV – Red spotted grouper nervous necrosis virus;

RT – Room Temperature;

RT−PCR – Reverse Transcriptase Polymerase Chain Reaction;

SSN 1 – Fish cell line obtained from *Channa* (Ophicephalus) *striatus* suitable for virus isolation (piscine nodaviruses).

$TCID_{50}$ – 50% Tissue Culture Infective Dose;

VER – Viral Encephalopathy and Retinopathy;

VTM – Viral Transport Medium;

1. Introduction

Currently, capture fisheries have been fully exploited, and therefore, there is no potential for the fisheries sector to grow. However, the aquaculture sector presents an alternative for providing seafood, finfish and shellfish, to feed the increasing global population (Brummett, 2013; FAO, 2016).

World aquaculture production of fish has contributed 44.1% of total food production (including non-food uses) in 2014. The aquaculture sector was responsible for the production of 73.8 million tonnes of aquatic animals in 2014 (FAO, 2016). There is a general trend of increasing the total fish production through aquaculture production (Subasinghe, 2005; FAO, 2016), and globally, the aquaculture sector can be seen as the fastest growing food-production sector (Subasinghe *et al*., 2009; Gjedrem *et al*., 2012). Marine aquaculture has also increased its production in the last decades, because of the economic value of reared marine species and their higher demand (Shetty *et al*., 2012). As aquaculture production increases, farming intensification also increases (Naylor *et al*., 2000). The intensification of production, is achieved with high stocking densities and several diseases have emerged as significant problems in probably all aquaculture species, due most likely to the high stress levels induced in the fish (Murray & Peeler, 2005).

1.1. Production of the species *Dicentrarchus labrax* in aquaculture

The first documented intensive rearing for *Dicentrarchus labrax* was made in 1970's, when the reproductive cycle was closed with several spawning experiments for controlling reproduction performed on the Mediterranean coast (Barnabé & Tournamille, 1972; Volckaert *et al*., 2008). In the following years, numerous studies were made for improving the rearing/husbandry conditions, nutrition and larval survival of the stock (Volckaert *et al*., 2008). These studies made possible the exploitation of the European sea bass in aquaculture and its current commercialization (Volckaert *et al*., 2008). The European sea bass aquaculture industry has grown considerably during the last 15 years producing a few thousand tonnes in 1990 to 156.000 tonnes in 2014, demonstrating that this is one of the most successful and important species reared in aquaculture (FAO, 2016) (Fig. 1.1). Europe is the major producer of sea bass with 80% of worldwide production (Fisheries and Aquaculture in Europe, 2012). Europe and Turkey are the main producers with a share of 65.500 and 42.500 tonnes in 2012 (Hillen *et al*., 2014). This fish species is considered high-value, since its monetary value ranking is higher than the

weight ranking, however, with the development of the European sea bass aquaculture industry the over-production has been contributing to the decline of fish prices; that might explain the overall decrease in the total production between 2000 and 2002 (FAO, 2016). Currently, the price for 1 kg of a medium-size European sea bass reared in aquaculture can range from 4 to 6 euros (FAO, 2016). Different processes can be applied for rearing European sea bass, depending on the final value desired for the annual production.

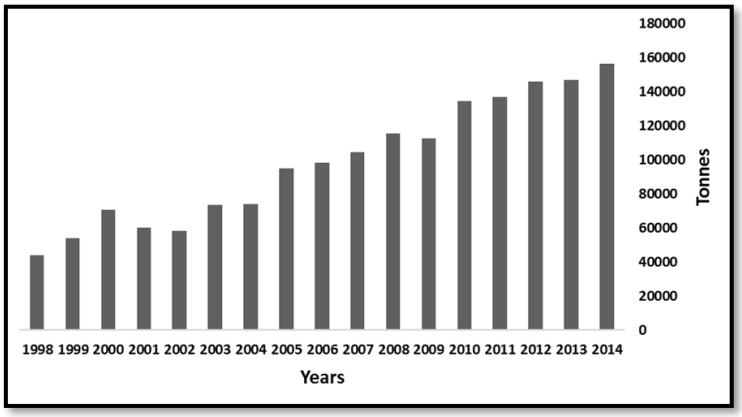


Figure 1.1: Aquaculture total production of *Dicentrarchus labrax* (Linnaeus, 1758) (Adapted from: FAO, 2016).

Traditionally, rearing methods involve the management of appropriate costal lagoons, where the juveniles are captured during their migration to the open sea. These individuals are reared in polyculture systems and the total production reaches a value of 50-150 kg/ha/year (FAO, 2016; Ortega, 2013). However, the most common type of production is rearing sea bass in sea-cages in coastal areas with annual production levels as high as 500-700 kg/ha/year (FAO, 2016; Ortega, 2013).

The reproduction of European sea bass is fully controlled, and the major aquaculture facilities maintain their own improved broodstock, and selective breeding for this species has been receiving increasing interest (Vandeputte *et al*., 2009). As European sea bass is one of the most important seawater species for the Southern Europe aquaculture, studies about the tissue distribution and diagnosis of Viral Encephalopathy and Retinopathy (VER) are very important to avoid outbreaks and optimize the rearing process (Valero *et al*., 2015; Carballo *et al*., 2016).

1.2. Biology, life cycle and distribution of *Dicentrarchus labrax* (Linnaeus, 1758)

European sea bass belongs to the Moronidae family and is related phylogenetically with the Serranidae family (Volckaert *et al*., 2008). The reproduction season for the European sea bass takes place during winter, from December/January to

March/June, after the adults have completed their migration to the spawning areas (Pawson *et al*., 2007; Vázquez & Muñoz-Cueto, 2015). Reproduction occurs at sea (Pickett & Pawson, 1994). Spawning takes place in groups in mid-water, and the pelagic eggs hatch and are transported inshore by currents to shallow coastal areas and estuaries, where they develop into juveniles and spend their first year of life (Bento *et al*., 2016). The European sea bass is a gonochoric species that reaches maturity about 35 cm for males and 42 cm for females, approximately when they reach a weight of 300-400g and 500-600g, respectively (Pawson & Pickett, 1996; Ortega, 2013). The European sea bass life cycle can be separated in different phases: planktonic eggs and larvae, juveniles, adolescent and adults (Carroll, 2014).

European sea bass is a demersal species, with a distribution range from the coast of Senegal, Morocco and the Canary Islands up to the Norwegian coastlines, the Mediterranean Sea and Black Sea (Fig. 1.2) (Fritsch *et al*. 2007; Ortega, 2013; Vázquez & Muñoz-Cueto, 2015). It also has been reported near the waters of Iceland (Jonsson, 1992). This wide geographical distribution is related with the physiological adaptations exhibited by this species, which allows the animal to survive in a diverse range of temperatures (from 2°C up to 32°C) and salinity (Barnabé, 1990; Vázquez & Muñoz-Cueto, 2015).

Figure 1.2: *Dicentrarchus labrax* (Linnaeus, 1758) global geographic distribution (Adapted from: FAO, http://www.fao.org/fishery/species/2291/en).

This species habits deep waters in coastal areas (as low as 100 meters) (Volckaert *et al*., 2008), being observed in gravelly zones or rocky bottoms, or the open sea and can enter in estuaries, lagoons, and occasionally, rivers or salt marshes (Pickett *et al*., 2004; Ortega, 2013). The fully mature individuals go through seasonal migrations between the feeding areas and the spawning territories, dispersing during the winter months (Carroll, 2014). The adults of this species feed mostly on crustaceans, molluscs and some species of fish, polychaetes, aquatic insects, snails and seagrasses or

seaweeds (Laffaille *et al*., 2001; Spitz *et al*., 2013; Vázquez & Muñoz-Cueto, 2015), fishing during the night and using ambushes, catching their prey against the currents and hiding in inlets (Vázquez & Muñoz-Cueto, 2015). The European sea bass is a species with a key role in the littoral food web, since it allows the transference of organic matter between different levels and environments, serving as a basic prey for several species of fish, aquatic birds and mammals (Laffaille *et al*., 2001).

1.3. Betanodavirus

One of the major constrains to European sea bass aquaculture sector is the economic losses that result due to outbreaks of disease caused by various infectious agents (Subasinghe, 2005; Qi *et al*., 2009; OIE, 2016). Viral diseases are considered to be the ones that pose major risk to the aquaculture production due to the high mortalities associated with these worldwide (Shetty *et al*., 2012).

One of the diseases that is recognized as a major treat to Mediterranean and Asian marine aquaculture is VER, due to the high mortalities registered in affected species (Gomez *et al*. 2009; Vendramin *et al*. 2013; Kara *et al*. 2014; Costa & Thompson, 2016). This disease has a worldwide distribution, including the Mediterranean region, UK, Norway, North America, Caribbean, Australia, and South and East Asia (OIE, 2016). VER affects more than 120 species of wild and farmed fish, some invertebrate species and especially marine fish (Costa & Thompson, 2016). The most affected species are striped jack (*Pseudocaranx dentex*), European sea bass (*Dicentrarchus labrax*), groupers and soles (Table I) (Munday *et al*., 2002; OIE, 2016).

1.3.1. Biology and epidemiology of Betanodavirus

The first record of this disease was an event of mass mortality of European sea bass in French Martinique (Bellance and Gallet de Saint-Aurin, 1988). In 1990 a viral agent was described that was responsible for affecting the central nervous system and the retina of fish in a hatchery-reared aquaculture of parrotfish (*Oplegnathus fasciatus*) in Japan and in barramundi larvae (*Lates calcarifer*) in Australia (Glazebrook *et al*., 1990; Yoshikoshi & Inoue, 1990). Several observations under light microscopy and electron microscopy revealed “conspicuous vacuolation and pyknosis in the spinal cord, spinal ganglia and brain of affected fish, and non-enveloped icosahedral particles in the tissues” and “picorna like viral particles” (Glazebrook *et al*., 1990; Yoshikoshi & Inoue, 1990).

Table I: List of wild and farmed fish species in which Betanodavirus has been detected or isolated (is not fully comprehensive of all the affected species).

Species	Common name	Order	Countries	Reference
Anguilla anguilla	European eel	Anguilliformes	Taiwan, Spain	Lai *et al*., 2001 Chi *et al*., 2003 Bandín *et al*., 2014
Dicentrarchus labrax	European sea bass	Perciformes	Martinique, France, Greece, Malta, Italy, Spain, Portugal	Bellance & Gallet, 1988 Skliris *et al*., 2001 Bovo *et al*., 1996 Cutrin *et al*., 2007
Diplodus sargus sargus	White seabream	Perciformes	Spain	García-Rosado *et al*., 2007 Toffolo *et al*., 2007
Epinephelus akaara	Redspotted grouper	Perciformes	Taiwan, China	Chi *et al*., 1997 Lin *et al*., 2001
Epinephelus coioides	Orange spotted Grouper	Perciformes	China, Philippines, Australia	Lin *et al*., 2001 Maeno *et al*., 2002 Moody *et al*., 2009
Epinephelus marginatus	Dusky grouper	Perciformes	Tunisia, Algeria	Haddad-Boubaker *et al*., 2014 Kara *et al*., 2014
Epinephelus septemfasciatus	Sevenband grouper	Perciformes	Japan	Fukuda *et al*., 1996 Kokawa *et al*., 2008
Gadus morhua	Atlantic cod	Gadiformes	U.K., Canada, Norway	Starkey *et al*., 2001 Johnson *et al*., 2002 Nylund *et al*., 2008
Hippoglossus hippoglosus	Atlantic halibut	Pleuronectiformes	Norway, U.K.	Grotmol *et al*., 1997 Starkey *et al*., 2000

Lates calcarifer	Asian sea bass/Barramundi	Perciformes	Israel, Taiwan, Malaysia, India	Azad *et al*., 2005 Banerjee *et al*., 2014 Ransangan *et al*., 2010 Ucko *et al*., 2004
Oplegnathus fascinatus	Japanese parrotfish	Perciformes	Japan	Yoshikoshi & Inoue, 1990
Oreochromis niloticus	Nile tilapia	Perciformes	France, Thailand	Bigarré *et al*., 2009 Keawcharoen *et al*., 2015
Paralichthys olivaceus	Japanese flounder	Pleuronectiformes	Japan	Kang *et al*., 2003 Nguyen *et al*., 1994
Poecilia reticulata	Guppy	Cyprinodontiformes	Singapore	Hedge *et al*., 2002 Nazari *et al*., 2014
Pseudocaranx dentex	Striped jack	Perciformes	Japan, Australia	Mori *et al*., 1992 Arimoto *et al*., 1993 Moody *et al*., 2004
Scophtalmus maximus	Turbot	Pleuronectiformes	Denmark, Norway	Bloch *et al*., 1991 Johansen *et al*., 2004
Solea senegalensis	Senegalense sole	Pleuronectiformes	Spain, Portugal	Thiéry *et al*., 2004 Cutrin *et al*., 2007 Oliveira *et al*., 2009
Sparus aurata	Gilthead seabream	Perciformes	France, Spain, Portugal, Tunisia	Thiéry *et al*., 2004 Cutrin *et al*., 2007 Oliveira *et al*., 2009 Chérif *et al*., 2009

The disease was designated as viral nervous necrosis (VNN) in 1990 (Yoshikoshi & Inoue, 1990), and then as encephalomyelitis (Bloch *et al*, 1991), or viral encephalopathy and retinopathy (VER) (Munday *et al*., 1992). Based on the characteristics of Striped Jack NNV (SJNNV), such as the genome and the virion size, this virus was identified as belonging to the Nodavirus family (Mori *et al*., 1992). The Nodavirus family has two genera, but just one (Betanodavirus) affects fish (Costa & Thompson, 2016).

Betanodavirus are icosahedral non-enveloped virus, with a spherical form and have approximately 25-30 nm in diameter; their genome consists in two single-stranded positive-sense RNA molecules: RNA1 with 1.01×10^6 Da, responsible for encoding the catalytic part of the RNA-dependent RNA polymerase (RdRp) that allows the replication of the genome, and it is also involved in the regulation of the virus sensitivity to temperature (Hata *et al*., 2010; Panzarin *et al*., 2014); RNA2 with a weight of 0.49×10^6 Da, encodes the coat protein (Nishizawa *et al*., 1995).

According to the variable region (T4-region) in the RNA2 molecule, Betanodavirus can be classified into four different genotypes: red spotted grouper nervous necrosis virus (RGNNV) (*Epinephelus akaara*), striped jack nervous necrosis virus (SJNNV) (*Pseudocaranx dentex*), barfin flounder nervous necrosis virus (BFNNV) (*Verasper moseri*) and tiger puffer nervous necrosis virus (TPNNV) (*Takifugu rubripes*) (Nishizawa *et al*., 1997). The designations for the different genotypes are related with the fish species where they were first isolated (Chi *et al*., 2001).

The ability of Betanodavirus to infect different fish species depends on the Betanodavirus coat protein that differs according to the RNA2 gene (genotype), and the water temperature (RNA1) (Iwamoto *et al*., 2004). The distinct genotypes have different optimal growth temperatures, RGNNV 25-30°C; SJNNV 20-25°C; TPNNV 20°C; BFNNV 15-20°C) (Iwamoto *et al*., 2000; OIE, 2016).

The BFNNV is found in cold-water species such as the Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), Atlantic halibut (*Hippoglossus hippoglossus*), Pacific cod (*Gadus macrocephalus*), Dover sole (*Solea solea; Solea vulgaris*), turbot (*Scophthalmus maximus*), barfin flounder (*Verasper moseri*) and was isolated in Norway, Scotland, France, Japan and along the east coast of the North America (Bloch *et al*., 1991; Watanabe *et al*.,1998; Grotmol *et al*., 2000; Gagné *et al*., 2004; Starkey *et al*., 2000; Starkey *et al*., 2001; Shetty *et al*., 2012). RGNNV has a wider

geographic distribution compared to the other genotypes since it can be found in Japan, French Polynesia, Asia, Australia, Mediterranean Sea, North of France and the USA (Nishizawa *et al*., 1995; Nishizawa *et al*., 1997; Skliris *et al*., 2001; Johansen *et al*. 2004) and infects many fish species such as European sea bass (*Dicentrarchus labrax*), Asian sea bass (*Lates calcarifer*), humpback grouper (*Chromileptes altivelis*), red spotted grouper (*Epinephelus akaara*) and gilthead seabream (*Sparus aurata*) (Carballo *et al*., 2016; Nakai *et al*., 2009; Nishizawa *et al*., 1997; Toffan *et al*., 2017; Yuasa *et al*., 2007). The SJNNV genotype is known to affect Striped jack (*Pseudocaranx dentex*), Atlantic halibut (*Hippoglossus hippoglossus*) and greasy grouper (*Epinephelus tauvina*), and it is restricted to the japanese waters (Tan *et al*., 2001; Nagai *et al*., 1999; Costa & Thompson, 2016). The TPNNV genotype affects a single fish species, the tiger puffer (*Takifugu rubripes*) (Costa & Thompson, 2016).

The existence of reassortment between RNA1 and RNA2 genes was confirmed in Betanodavirus isolates (Toffolo *et al*., 2007; Panzarin *et al*., 2012). In Italy and Croatia, a reassortment virus was isolated from European sea bass, with an RNA1 segment originated from the SJNNV genotype and RNA2 segment from the RGNNV (SJNNV/RGNNV) (Toffolo *et al*., 2007). Another reassortment was reported in the Iberian Peninsula with a RNA1 segment derived from the RGNNV genotype and the RNA2 segment from the SJNNV genotype (RGNNV/SJNNV) and was isolated from European sea bass, gilthead sea bream and Senegalese sole (Cutrin *et al*., 2007; Toffolo *et al*., 2007). *In vivo* studies performed with both Betanodavirus reassortments shown that they can induce the development of a VER infection (Olveira *et al*., 2009; Toffan *et al*., 2017). In a study published in 2010, it was showed that SJNNV and RGNNV genotypes coexist in wild meagre (*Argyrosomus regius*), which can explain the existence of genetic reassortments between both genotypes (Lopéz-Jimena *et al*., 2010).

Mass mortalities occur depending on the life stage and the fish species (Munday & Nakai, 1997; Péducasse *et al*., 1999). Studies have been shown that mortality in the infected animals is aged related, with younger fish more susceptible to the disease (Munday & Nakai, 1997; Péducasse *et al*., 1999). This disease frequently occurs in larval and juvenile stages of marine fish species (Nguyen *et al*., 1996; Munday & Nakai, 1997; Péducasse *et al*., 1999; Lin *et al*., 2007), nevertheless, significant mortalities have been observed in more advanced life stages, severely affecting production, particularly with European sea bass (Breton *et al*., 1997; Munday *et al*., 2002).

1.3.2. Clinical signs

The clinical signs caused by Betanodavirus can differ between the fish species, nevertheless there are several symptoms that characterize this disease, such as abnormal coloration (dark or pale coloration), lethargy, reduced appetite, gas accumulation, anorexia, opacity of the cornea or a bilateral exophthalmia, slow or inactive behaviour, floating upside down at the water surface at rest or staying in the bottom quietly, swim bladder hyperinflation and altered swimming behaviour: spinning, rotating, horizontal looping, uncoordinated darting, corkscrew swimming (Munday & Nakai, 1997; Breton *et al*., 1997; Péducasse *et al*., 1999; Munday *et al*., 2002; Parameswaran *et al*., 2008; Shetty *et al*., 2012; Costa & Thompson, 2016).

1.3.3. Transmission of Betanodavirus

As a disease, VER can be transmitted both vertically and horizontally (Glazebrook *et al*., 1990; Arimoto *et al*. 1993; Nguyen *et al*. 1996; Grotmol *et al*., 1999; Skliris & Richards, 1999; Grotmol & Totland, 2000; Castric *et al*., 2001; Breuil *et al*., 2002; Azad *et al*., 2006; Kai *et al*., 2010).

Betanodavirus has been detected in ovarian tissues, sperm, fertilized eggs and hatched larvae, confirming its vertical transmission (Dalla Valle *et al*., 2000; Valero *et al*., 2015). It is also possible that the virus can be carried in the gonads of broodstock and in other organs (due to the stress originated during spawning) (Valero *et al*., 2015). Other results suggested Betanodavirus could be spread into the fish germ cells and in the gonadal fluid (Dalla Valle *et al*., 2000; Mushiake *et al*., 1994; Valero *et al*., 2015; Costa & Thompson, 2016). The virus has the ability of colonize and replicate in the testis of gilthead sea bream and European sea bass. Also, it has the capability of modulating their reproductive function, since the presence of the virus can change the sensitivity of the brain and testis to specific hormones in both species (Valero *et al*., 2015). The horizontal transmission of this virus was confirmed in numerous cohabitation assays and it was proved that the viral particles could survive in water (OIE, 2016).

Some species of invertebrates can act as vectors and become natural reservoirs for the virus, aiding in its transmission. Some species have been described as vectors, such as the sand worms, from the genus Nereis (spreading the Betanodavirus through their commercialization) (OIE, 2016), bivalves *Mytilus galloprovincialis*, spiny lobster *Pamulirus versicolor*, the crustacean humpback shrimp *Pandalus hypsinotus*, the

copepod *Tigriopus japonicus*, the brine shrimp *Artemia salina* and the rotifer *Brachionus plicatilis* (Sudhakaran *et al*., 2006; Gomez *et al*., 2008; Costa & Thompson, 2016). Also, fish or mollusc species, such as the Japanese mackerel (*Trachurus japonicus*) and the Japanese squid (*Todarodes pacificus*) are used for feeding carnivorous species in aquaculture and these can be infected with VER and transmit the virus to the reared species (Nuñez-Ortiz *et al*., 2016a). Due to its ability to spread through the water in the rearing systems and between generations of fish, that can be asymptomatic carriers of the virus, VER represents a major treat to aquaculture.

1.3.4. Diagnosis/detection methods

The clinical signs displayed by the animals infected with Betanodaviruses are very characteristic, but it is necessary to access a rapid preclinical state of the infection to prevent the manifestation of symptoms. There are several methods for diagnosing VER such as histology, immunohistochemistry, isolation of virus in a susceptible cell line (e.g. SSN-1 or E11), *in situ* hybridization, reverse transcription polymerase chain reaction (RT-PCR), quantitative PCR (real time or q-PCR), nested PCR, antibody-based identification, like enzyme linked immunosorbent assay (ELISA), indirect florescent antibody tests (IFAT) and LAMP (Valle *et al*., 2005; Goswami *et al*., 2012 ; Shetty *et al*., 2012; Costa & Thompson, 2016; Doan *et al*., 2016). Also, histopathological analysis is a major tool in Betanodavirus diagnostic and findings revealed abnormalities present in the nervous central system, characterized by vacuolation and necrosis in these tissues in all the susceptible fish species (Munday *et al*., 2002; Shetty *et al*., 2012; Costa & Thompson, 2016). The anterior brain is one of the most affected organs, when compared to the posterior brain and the spinal cord (Munday *et al*., 2002). Vacuolation of the neurone soft encephalic ganglia and the spinal ganglia was observed in Atlantic halibut juveniles (Grotmol *et al*., 1997). Other described lesions include shrinkage, basophilia, granularity of the neuropil, the presence of mononuclear cell infiltration, pyknosis and cell lysis (Yoshikoshi & Inoue, 1990; Munday *et al*., 2002; Costa & Thompson, 2016). The endothelial cells of the blood brain vessels can be congested due to an inflation of the endothelial lining. Vacuolated cells were detected in the bipolar and ganglionic nuclear layer of the retina in infected European sea bass (Breton *et al*., 1997).

For diagnosis purposes, it is usual to collected tissue samples from fish using lethal sampling techniques, except for broodstock, from which seminal and ovarian fluids can be collected and analysed (Burbank *et al*., 2017). Non-lethal sampling methods

become more desirable for sampling valuable individuals, such as broodstock or rare species, or repetition of the sampling procedure. Also, the sacrifice of animals for research purposes is becoming more restricted due to ethical considerations regarding animal welfare (Drennan *et al*., 2007; Blessing *et al*., 2010; Henderson *et al*., 2016). Non-lethal sampling methods also have other advantages, since they can be more easily applied in the field for sample collection and the fish sexual fluids are not always available for collection (Burbank *et al*., 2017). Non-lethal sampling methods for diagnosis purposes are gaining more importance, since they can be a reliable alternative to invasive diagnosis methods. However, it is necessary to evaluate and establish if the non-lethal sampling methods are as suitable for diagnosis purposes as lethal sampling methods. The comparison between these methods is required to understand the most suitable sampling method to be implemented for diagnosis and avoid the spreading of an infection (Burbank *et al*., 2017). Several different methods have been used for detecting viral (Monaghan *et al*., 2014; Collet *et al*., 2015; Lopez-Vázquez *et al*., 2006), bacterial (Elliot *et al*., 2015; Monte *et al*., 2016; Tavares *et al*., 2016) and parasite (Fox *et al*., 2000; Ek-Huchim *et al*., 2012; Krkošek *et al*., 2016) infections in fish with the target tissues being selected according to the infection route. Different types of non-lethal material can be collected, including sampling blood by venipuncture, biopsy, faeces collection and mucus swabs, for detecting infectious disease in fish (Tavares *et al*., 2015). The most common non-lethal methods for analysing the presence of Betanodavirus in fish included taking samples in eggs, ovary and seminal fluids by RT-PCR and the detection of specific antibodies in collected fish serum (ELISA) (Krishnan *et al*., 2016).

1.3.5. Treatment and prevention of VER

To avoid an outbreak of this disease, it is necessary to apply and develop a set of management practices such as, screening the broodstock, better husbandry practices, good quality water, quality of the fish's feed, use of immunostimulants (probiotics, prebiotics, etc.), or good sanitation (Costa & Thompson, 2016).

Betanodavirus is a very stable virus, and because of this can be very difficult to eradicate from an aquaculture facility, since the virus is resistant to exposure of pH 2-9 for 30 min and to heat treatment at 50°C for 1 h (Arimoto *et al*., 1996; Frerichs *et al*., 1996; Frerichs *et al*., 2000). Chemicals have been studied to verify their efficacy in eliminating or inactivating Betanodavirus, including epinecidin-1 or hepcidin 1-5, cyclic shrimp anti-lipopolysaccharide factor (cSALF), furan-2yl-acetate, gymnemagenol,

dasyscyphin C (Wang *et al*. 2010; Khanna *et al*., 2011; Ichinose *et al*., 2013). The virus can be inactivated at pH 12 and with sodium hypochlorite, calcium hypochlorite, benzalkonium chloride or iodine. The use of heat treatment, ultra-violet light (UV) and ozone also proved to be effective (Chia *et al*., 2010; Krishnan *et al*., 2010; Suthindhiran *et al*., 2010; Wang *et al*., 2010; Khanna *et al*., 2011).

Another method for preventing a VER outbreak is related with fish immunization. Currently, there are numerous studies for evaluating the efficiency of vaccines against Betanodavirus, however there is still no commercial vaccine available except for one RGNNV vaccine available for the seven-band grouper (*Epinephelus septemfasciatus*) (OIE, 2016). The vaccination of the broodstock would be another measure to prevent a VER outbreak, since it would stop the virus from spreading to the eggs and prevent the adults from being affected (Frerichs *et al*., 2000; Kai *et al*., 2010; Costa & Thompson, 2016).

1.4. Aim and objectives

The aim of this study was to verify if it was possible to detected Betanodavirus with non-lethal sampling methods for diagnosis purposes.

2. Materials and Methods

2.1. Fish husbandry

European sea bass (n=700, average size= 6.6g±0.01) were obtained from a hatchery in Portugal (Atlantik Fish Lda., Castro Marim). The juveniles were acclimatized for four weeks in a 2000 L (2 m^3) fiberglass tank and fed with a commercial pellet *ad libitum* three times per day. On arrival ten fish were randomly selected and euthanized to screen for the presence of Betanodavirus by RT-PCR. During acclimation the fish were kept at a water temperature of 22.2°C±1 with a salinity of 32.6±1 and density of 2.45 kg m^{-3}.

In order to evaluate the growth of the fish, one hundred fish were anaesthetised with 2-phenoxyethanol (Sigma-Aldrich, USA) (0.5 mL L^{-1}) and weighted.

Once the fish reached 10.6g±0.01, the fish were transferred to the pathology suite to be challenged, as described in Section 2.3.

2.2. Pathogen

The Betanodavirus (ARG/VIR/2016-02) belonging to the RGNNV genotype used for challenging the fish was produced at the Moredun Research Institute. The virus was cultured in AGK cells (Asian Grouper Kidney) at 26°C until a full cytopathic effect (CPE) was observed.

The concentration of the virus was determined by calculating the virus titre (number of infectious units per unit volume) as 50% tissue culture infective dose per mL ($TCID_{50}$ mL^{-1}). Infectivity titrations were performed with SSN-1 cell lines. The virus titres were calculated by the method of Spearman-Karber (Hierholzer & Killington, 1996).

2.3. Experimental design and virus challenge

Once the fish reached 10.6g±0.01, they were allocated into 8 tanks (16 L) with 30 fish added per tank, with a density of 18kg m^{-3}. The water was maintained with a salinity of 33.3 and water temperature of 26.5°C±1.

Each tank had an independent recirculating system with an UV light (TMC®, Des Moines, USA) and a protein skimmer (TMC® Aquarium, Chorleywood, UK) for ensuring the maintenance of the water quality throughout the experimental period. During the challenge, the salinity was measured daily with a refractometer (Hanna Instruments Inc, Rhode Island, USA), and temperature and dissolved oxygen were controlled daily with a HI9147-04 multiparameter probe (Hanna Instruments Inc, Romania). Once a week, the concentration of ammonia and nitrites were determined with the use of commercial kits (API®, Pennsylvania, USA). During the challenge, the tanks were cleaned daily and 30% of the water changed daily. The fish were fed with a commercial pellet, three times a day *ad libitum*.

Two different trials were performed at the same time: **Study 1 –** examined **mortality**; and **Study 2 –** for the **non-lethal sampling**. In both studies the *in vivo* challenges were performed with two different infection routes: immersion or intramuscular injection (IM) (see Table II). Each study and infection route had a virus infected group and a non-infected control group.

Table II: Experimental design for *in vivo* challenge.

Designation	Treatment	Tanks	Number of fish	Sampling per tank	Sampling days post infection (dpi)
Study 1	Immersion	Control	30	4 fish per sampling day	30
		Infected	30		
	IM	Control	30		
		Infected	30		
Study 2	Immersion	Control	30	4 fish per sampling day	7,15 and 30
		Infected	30		
	IM	Control	30	5 fish per sampling day	
		Infected	30		

For the immersion infection, 60 fish were transferred to a tank containing a 10^6 $TCID_{50}$ mL^{-1} of Betanodavirus, at a density of 80 kg m^{-3}, where they were kept for one hour. For both the immersion infected and control tanks, the fish were submitted to the same conditions, but the water in control tanks did not contained the virus. After one hour, the fish were transferred to the challenge tanks system.

For the IM infection, the fish were anaesthetized with 2-phenoxyethanol (0.5 mL L^{-1}) and injected with 100 µL of 10^7 $TCID_{50}$ mL^{-1} of Betanodavirus near the caudal fin. The control fish were injected with phosphate buffered saline solution (PBS 1x). After this procedure, the fish were carefully placed in the designated tanks. The tanks were

monitored twice a day for any clinical signs, and dead or moribund fish were removed from the tanks.

Moribund fish were sampled with the brain been collected into RNAlater (Thermo Fisher Scientific, Vilnius, Lithuania) and viral transport medium (VTM) (Becton, Dickinson and Company, Maryland, USA) and stored at −80°C until analysed. The trials lasted for 4 weeks and mortalities were recorded daily.

2.4. Sampling of fish

The fish were euthanized with an overdose of 2-phenoxyethanol (0.5 mL L^{-1}) and the sampling process was performed immediately after. On day zero pre-challenge, the organs (brain, gills and caudal fin) and blood of ten stock fish were sampled. From Study 1, four fish per tank were sampled 30 days post infection (dpi). In Study 2, five fish per tank were sampled for the IM infected individuals and four fish per tank were sampled for the immersion infected individuals, on 7, 15 and 30 dpi.

The fish were anaesthetised, and the first to be sampled was the blood. The blood samples were collected from the caudal vein with a 22-gauge needle and 1 mL syringe (both sterile). After collecting the blood, the samples were transferred into 1.5 mL tubes and the blood was allowed to clot at 4°C overnight. The following day the samples were centrifuged at 7000g for 7 min (Eppendorf AG, Hamburg, Germany). Serum was collected, aliquoted and stored at −80°C. RNAlater was then added to the blood clot, which stored at −80°C.

After the blood collection, the targeted organs were removed. The caudal fin, gills and brain were collected into RNAlater (1 vial per organ per individual). The tissues were cut into smaller pieces and for each organ a different sterile scalpel was used. Each cryovial was pre-filled with 1 mL RNAlater and the weight was recorded. After adding the samples, the RNAlater cryotubes were weight again, left overnight at 4°C, and on the following day the RNAlater cryotubes with tissues were stored at −80°C. During the sampling procedure, the fish from control tanks were always sampled first, and the brain was always the first organ to be collected. For the cell culture, the brains were collected and placed into viral transport medium (VTM) vials and stored at −80°C. All the dissection instruments were disinfected between individuals with 70% ethanol.

2.5. Isolation of Betanodavirus by cell culture

The brain samples collected from IM infected and immersion infected fish, and respective controls, (n=4 from stock, n=3 from IM control, n=9 from IM infected, including moribunds, n=3 from immersion control and n=3 from immersion infected) were homogenised with mortar and pestle in Hanks' Balanced Salt Solution supplemented with Penicillin (300 IU mL^{-1}), Streptomycin (300 µg mL^{-1}) and Kanamycin Sulphate (300 µg mL^{-1}) (HBSS/PenStrepKan). Each sample was diluted in the appropriated volume of HBSS/PenStrepKan in order to have a final sample diluted 1:10 (tissue weight: diluent volume). The homogenised samples were centrifuged at 2600g, for 15 min at 4°C (Sigma 4K15). After centrifugation the supernatant was collected and filtered (0.45 µm filter, Sartorius) for reducing the bacterial load.

The samples were inoculated in triplicate (100 µL $well^{-1}$) into 24 well plates with SSN-1 cells (passage 1 of the virus). The samples were inoculated by simultaneous inoculation and onto pre-formed monolayers (at a confluence of 70%). The plates were incubated at 26°C, observed regularly and the CPE recorded. After 7 days, 100 µl of each well were transferred (blind passage, passage 2) into a well with fresh SSN-1 cells (24 well plates) and incubated for another 7 days. The cells were observed for CPE and after the 7 days period, the cell culture supernatant from passage 2 wells was collected and used for extracting viral RNA to confirm the virus growth observed by CPE. All the cell culture supernatants were collected and stored at -80°C.

All the cell culture reagents were acquired from Gibco, Paisley, Scotland.

2.6. ELISA (enzyme-linked immunosorbent assay)

ELISA plates (Greiner Bio-one, Germany) were coated with 50 µL per $well^{-1}$ of 0.01% poly-L-lysine and incubated during 60 min at room temperature (RT). After the adsorption of poly-L-lysine the wells were washed 3 times with LSWB (Low Salt Washing Buffer, pH 7.3) and 100 µL of antigen (virus supernatant, 10^6 $TCID_{50}$ mL^{-1}) was added to each well and incubated at 4°C overnight. The next day, 50 µL $well^{-1}$ of 0.05% glutaraldehyde was added to each well and incubated for 20 min at RT. Then, the plates were washed 3 times with Low Salt Washing Buffer (LSWB), was added 250 µL $well^{-1}$ of blocking buffer (3% skimmed milk) (Premier Foods, London, UK) and blocked for 2 h at 22°C. Meantime, doubling dilutions of fish serum in PBS (1x) (starting with 1:32 dilution) were prepared for each serum sample. The blocking buffer was tapped off, 100 µL of

diluted fish serum was added to each well, and the plates were incubated overnight at 4°C. PBS (1x) was used as negative control and as positive control European sea bass serum with anti-Betanodavirus was used (1:128 in PBS 1x). The wells were washed 5 times with High Salt Washing Buffer (HSWB) and soaked during 5 min on the last wash. Anti-European sea bass IgM MAb (Aquatic Diagnostics Ltd, Stirling Scotland), diluted at 1:150 in antibody buffer was added to each well (100 µL $well^{-1}$) and incubated for 60 min at RT. The plates were washed 5 times with HSWB and soaked for 5 min on the last wash, before adding 100 µL of goat anti-mouse IgG-conjugated with horseradish peroxidase (HRP) to each well, and incubated for 60 min at 22°C. The washing procedure with HSWB was repeated. After this step, substrate was added to each well at 100 µL $well^{-1}$ and incubated during 10 min at 22°C. The reaction was stopped by adding 50 µL $well^{-1}$ of stop solution. The plates were read at 450 nm in the ELISA reader (Promega, Southampton, UK).

The serum samples from both infected and non-infected fish were analysed in duplicate. The average of the negative control was calculated. A sample was considered positive when its value was 3 times higher than the negative control average.

With the 2-fold dilutions used in each ELISA assay (starting in 1:32), it was possible to calculate the antibody titres expressed as a $-Log_2+1$ value. The $-Log_2+1$ values were obtained with the corresponding optical density (OD at 450 nm) values considering the last dilution in which it was possible to record a positive OD value.

Except when mention all the reagents used in the ELISA were purchased from Sigma-Aldrich, UK.

All the reagent formulations are described in Appendix I.

2.7. RNA Extractions

The sampled organs were placed in a 1.5mL sterile tube and homogenized with a mortar and pestle and the appropriated buffer provided in the RNeasy Mini Kit (Qiagen, Hilden Germany). The homogenised tissue was then placed into the kit columns and the procedure was performed according to the manufacture instructions.

The blood clot samples preserved in RNALater were centrifuged at 12000 g and the blood clot was retrieved from the RNAlater. The blood clot was weighted, and the

RNA extraction was performed with a QIAamp RNA Blood Mini Kit (Qiagen, Hilden Germany). As suggested by the manufacturer's instructions, the homogenized cell lysates were stored at −80°C for 24 h and the extraction process was then continued. All extractions were performed according to the manufacture instructions.

A QIAamp Viral Mini Kit (Qiagen, Hilden Germany) was used to extract viral RNA from the cell culture supernatant. A 140 µL volume was collected from all cell culture wells and the viral RNA was extracted following the extraction kit instructions.

All the RNA samples were stored at −21°C until used.

The Nanodrop Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) was used for evaluating the RNA concentration from all the samples. The RNA purity was assessed by the ratios 260/280 nm and 260/230 nm.

2.8. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The RT-PCR was performed with SuperScript One Step RT-PCR with Platinum Taq Kit (Invitrogen, Carlsbad USA). To prepare the PCR reaction solution with a final volume of 25 µL, each sample contained 12.5 µL of Reaction Mix (1x), 0.5 µL of 10 µM of sense and anti-sense primers (F2 and R3) (Nishizawa *et al*., 1994), 0.5 µL of RT-Platinum Taq Mix. The PCR reaction solution was briefly mixed by vortexing and to each 0.2 mL micro-centrifuge tube (Axygen, USA) 22.5 µL of the Reaction Mix was added and then 2.5 µL of the desired extracted RNA sample. The microcentrifuge tubes were briefly centrifuged and placed in the Biometra Tone Analytik Jena thermal cycling for RT-PCR following the reverse transcriptase and amplification conditions described in Table III.

Table III: Conditions for the RT-PCR.

Conditions (for F2 and R3 primers)		
Temperature (˚C)	**Time (minutes/seconds)**	
42	30 min	
95	5 min	
94	15 s	
55	30 s	35 cycles
72	1 min	
72	5 min	

After amplification of the viral RNA, the samples were loaded onto a 1.5% agarose gel (Bioline, London, UK) in TAE buffer (Sigma Aldrich, Missouri, USA) stained with Gel Red (Biotium, Fremont, USA). A DNA ladder Hyper Ladder 100 bp (Promega,

Southampton, UK) was added to each gel to verify the product size. The gel was electrophoresed at 150 V for 100 min (Bio-Rad Laboratories Ltd., Hertfordshire, UK). The viral RNA fragments were visualized using a UV Transilluminator (Alpha imager 2200).

2.9. Real Time RT-PCR

Real-time RT-PCR was performed using a SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen, California USA). The final volume for each one of the reactions was 20 µL, with 5 µL of sample and 15 µL of Master Mix. The primers used were the Noda Taq1-FW: CAACTGACARCGAHCACAC and Noda Taq1-RV: CCCACCAYTTGGCVAC, while the probe was Noda Taq1-probe: CARGCRACTCGTGGTGCVG (Panzarin *et al*., 2010). The primers and target probe (Sigma-Aldrich, USA) were used with a final concentration of 10 µM. The master mix was prepared according to the Table IV and was added to MicroAmp optical 96 well reaction plate (Applied Biosystems, California USA).

Table IV: Preparation of the reaction mix for the Real Timer RT-PCR using SuperScript III Platinum One-Step qRT-PCR Kit.

Reagent	Volume (per reaction)
Superscript II	0,4
2 x Reaction Mix	10
Noda Taq I Forward	0,9
Noda Taq I Reverse	0,9
Probe Noda Taq I	0,6
ROX (1/10 diluted in distilled water)	0,4
Water	1,8
Template	5
Total	20 µL

The templates were loaded into the respective wells (5 µL), the plate was sealed with the plate sealer, and briefly centrifuged. The plate was placed in an Applied Biosystems 7500 Real-Time PCR System and the assay performed following the conditions described in Table V. Once the data was acquired, it was exported and analysed with the 7500 Applied Biosystems Software version 2.3.

Table V: Thermal cycler profile for Real Time RT-PCR.

Thermal Cycler Profile			
Stage	**Repetitions**	**Temperature (°C)**	**Time (min)**
1	1	55.0	10:00
2	1	95.0	05:00
3	40	95.0	00:15
-	-	60.0	01:00

For each real-time RT-PCR assay a standard curve was prepared, in triplicates, with 10-fold serial dilutions of RNA extract from a Betanodavirus supernatant with a known concentration of infective particles ($10^{7.75}$ $TCID_{50}$ mL^{-1}). The results of four real-time RT-PCR standard curves, efficiencies from 90 to 97.05 and R^2 higher than 0.994 (automatically calculated with the 7500 Software for Real Time PCR systems version 2.3) were used to calculate the average of the mean of the Ct value for each viral dilution. The results were plotted as a linear regression with the mean Ct values on the Y-axis and 10-fold Betanodavirus dilution on the X axis. This allowed to obtain the equation of the line (y=mx+b) that permits to calculate the $TCID_{50}$ mL^{-1} of unknown samples.

2.10. Statistical analysis

To analyse the endpoint antibody titres for the mortality study (Study 1), a t-student test (Zar, 2010) was performed in order to verify statistical differences between the infection routes (IM and immersion) at 30 days post infection (dpi). For analysing the endpoint antibody titres for the non-lethal study (Study 2), an analysis of variance two-way (ANOVA-2 Way) with replication, followed by a Least Significance Difference (LSD) test (Zar, 2010) was performed to evaluate statistical significant differences between treatments (IM and immersion) and sampling days (7,15 and 30 dpi). In order to study differences in the qPCR results for the analysed tissues (brain, gills, caudal fin and blood) (Study 2) an ANOVA 2-Way (with replication) was applied (Zar, 2010). Also, when applicable a LSD test was performed to evaluate statistical significant differences (Zar, 2010) between treatments (IM and immersion) and days (7, 15 and 30 dpi), and to determine differences between sampling days, a Tukey HSD was performed. All the assumptions inherent to the performance of the methods (namely, normality of data and homogeneity of variances) were validated.

For all statistical tests the significance level was set at 0.05 (that is, $p\text{-}value \leq 0.05$). All calculations and statistical analysis were performed with IBM SPSS Statistics 24 software. When applicable, results were presented with mean±standard deviation (S.D) or mean±standard error of the mean (SEM). All graphs were designed with the Graph Pad Prism 7 Software.

3. Results

3.1. Challenge *in vivo*

Before performing the *in vivo* challenge, a sample of 100 individuals was weighted and the mean weight of the fish determined. At the time of transferring the individuals to the challenge tanks, mean weight was 10.6g±0.01.

The animals were kept in a controlled environment and the water parameters were measured daily, always assuring the animal welfare during this period. The water temperature was maintained in a range of values suitable for the replication of Betanodavirus in fish tissues. The water physico-chemical parameters of the water are presented in Table VI for both studies.

Table VI: Mean values for water physico-chemical parameters obtained during *in vivo* challenge.

Study 1	**Temperature (°C)**	**DO (%)**	**DO (mg/L)**	**Salinity**
IM Control	26,8	92,4	6,2	33,1
IM Infected	26,4	95,6	6,4	33,6
Immersion Control	26,8	88,2	6,1	33,2
Immersion Infected	26,3	95,2	6,4	33,5
Study 2	**Temperature (°C)**	**DO (%)**	**DO (mg/L)**	**Salinity**
IM Control	26,9	94,3	6,3	32,9
IM Infected	26,1	95,4	6,4	33,9
Immersion Control	26,7	91,7	6,2	33,0
Immersion Infected	26,2	96,4	6,5	33,6

Note: DO - Dissolved Oxygen in percentage (%) or mg L^{-1}.

3.2. Study 1 - *mortality study*

The mortality study was performed in order to evaluate the Betanodavirus strain virulence and capacity of inducing disease in healthy fish, and to confirm that the experimental infection with the virus was responsible for the animals' death.

In the IM infected fish, clinical signs became evident after 5 dpi, with the fish starting to display dark coloration, abnormal swimming behaviour (spinning, rotating, and corkscrew swimming). Also, the first mortalities were recorded at 6 dpi, in the IM infected individuals, and mortality reached a plateau at 18 dpi (Fig. 3.1). The mortalities for the immersion infected fish began later and were all recorded at 14 dpi. The highest

cumulative mortality value obtained during the challenge was 40% for the IM Infected individuals, and 3.3% for the immersion infected individuals. Neither treatment controls had mortalities occurring during the challenge period.

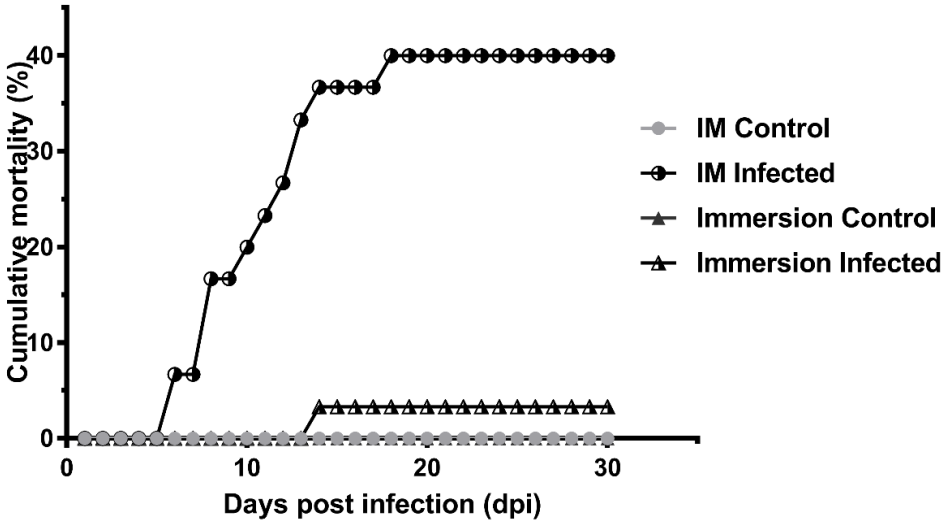


Figure 3.1: Cumulative mortality (%) observed during challenge with Betanodavirus for Study 1 after experimental infection (n=30 per group).

At the end of the trial (30 dpi) it was possible to detect Betanodavirus antibodies in the individuals infected by both challenge methods (Fig.3.2). The mean endpoint antibody titres for the IM infected group were higher than that obtained for the immersion infected individuals at 30 dpi, with 10 $-Log_2+1$ and 6 $-Log_2+1$ respectively (Fig. 3.2).

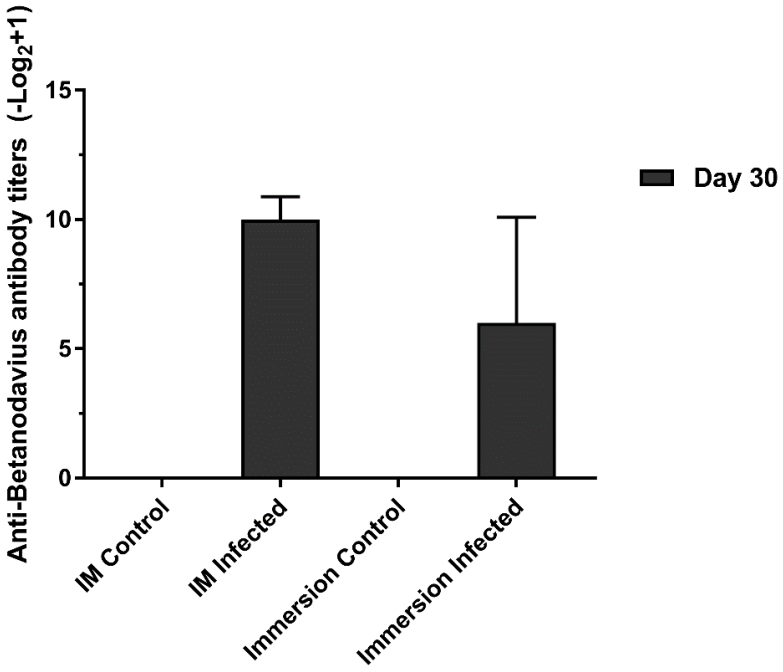


Figure 3.2: Endpoint antibody titres ($-Log_2+1$) at 30 dpi for the different infection methods. The results are presented as mean±SD (t-student, *p-value*>0.05).

Control fish from both IM and immersion challenged groups had no specific antibodies against Betanodavirus. There were no statistical differences in antibody titres at 30 dpi when the two treatments (IM and the immersion challenge groups) were compared (t-student, $t_{(6)}$=1.433, *p-value*=0.202>0.05; Fig. 3.2).

The individual endpoint antibody titres for the IM infected fish were higher than the immersion infected fish for all the individuals analysed (Fig. 3.3). Controls from both infection methods had no specific antibodies against Betanodavirus.

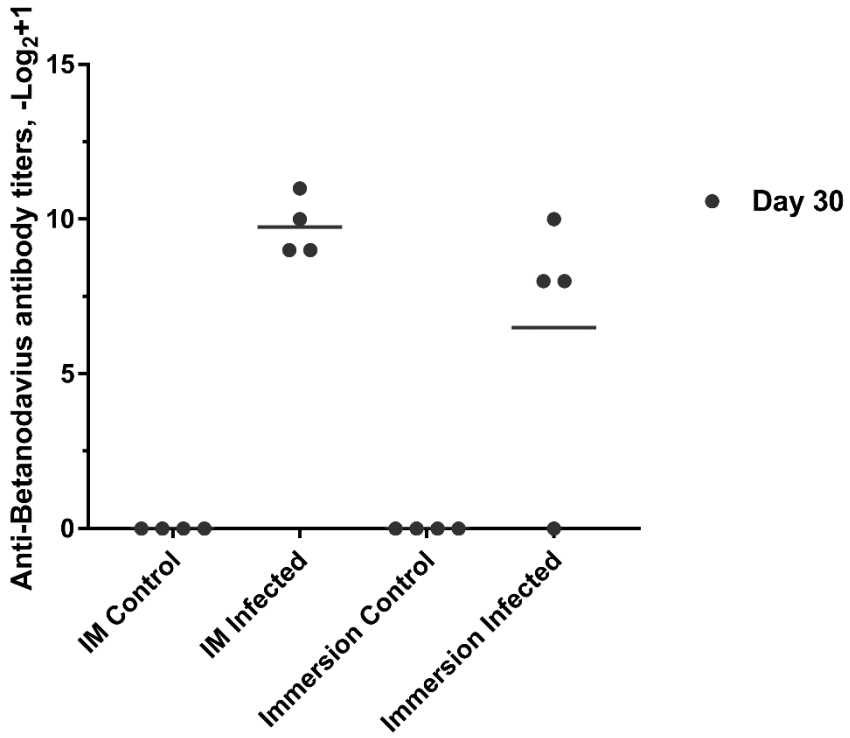


Figure 3.3: Individual endpoint antibody titre ($-Log_2+1$) at 30 dpi for the two infection routes used in Study 1 (n=4).

At the end of the mortality study, 30 dpi, random individuals were selected as mentioned above, and their brains were sampled for virus isolation by cell culture. Throughout the study, moribund individuals (n=6) were also sampled for cell culture.

No CPE was observed from samples taken from the stock fish or control fish, analysed by cell culture. As presented in Table VII, 8 out of 9 infected brains from IM infected and 2 out of 3 infected brains from immersion infected individuals, inoculated into SSN-1 cell line, produced a CPE after the incubation period.

Table VII: Results for the cell culture samples and the RT-PCR. The results are shown as positive or negative. The moribund collected individuals are represented with (M).

Sample number	Treatment	Cell Culture	RT-PCR
1	Stock	Negative	Negative
2	Stock	Negative	Negative
3	Stock	Negative	Negative
4	Stock	Negative	Negative
5	IM Infected (M)	Positive	Positive
6	IM Infected (M)	Positive	Positive
7	IM Infected (M)	Positive	Positive
8	IM Infected (M)	Positive	Positive
9	IM Infected (M)	Positive	Positive
10	IM Infected (M)	Positive	Positive
11	Immersion Control	Negative	Negative
12	Immersion Control	Negative	Negative
13	Immersion Control	Negative	Negative
14	IM Control	Negative	Negative
15	IM Control	Negative	Negative
16	IM Control	Negative	Negative
17	Immersion Infected	Positive	Positive
18	Immersion Infected	Positive	Positive
19	Immersion Infected	Negative	Positive
20	IM Infected	Positive	Positive
21	IM Infected	Positive	Negative
22	IM Infected	Negative	Negative

The cell monolayer inoculated with the infected tissue supernatant presented CPE 7 days after inoculation. The infected cells shrunk and aggregated, and the SSN-1 cell monolayer began to present signs of disruptions. The CPE of the Betanodavirus can be observed in Figure 3.4.

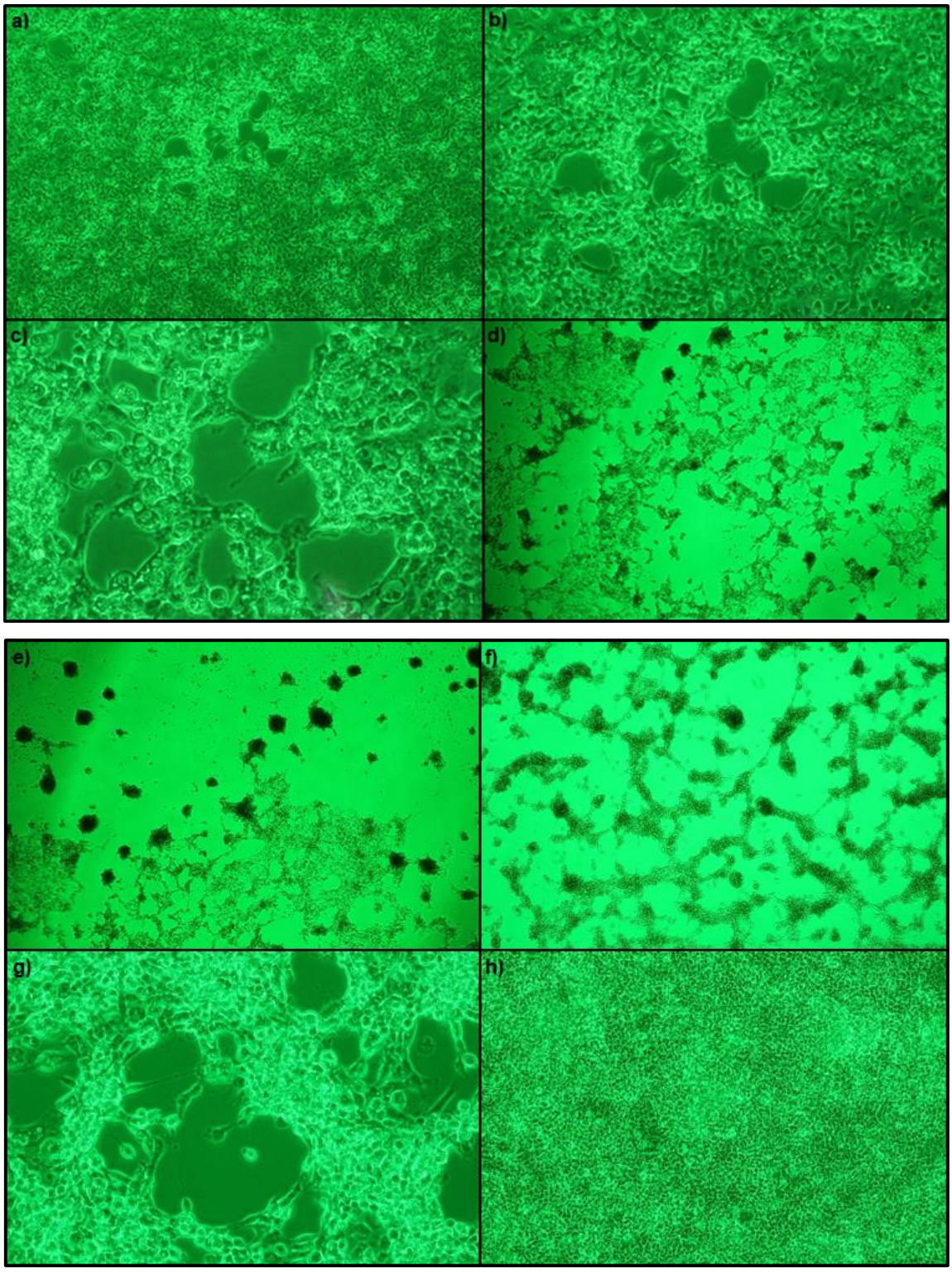


Figure 3.4: Cytopathic effects (CPE) were seen in cell line in cell line SSN-1 after it was inoculated with tissue from Betanodavirus from infected and non-infected fish. a), b) and c) Infected fish showing signs of early CPE (amplification of 100x, 200x and 400x, respectively); d), e) and f) cells inoculated with infected individual at 14 days after incubation and total destruction of the cell line can be observed (40x, 100x and 100x, respectively); g) corresponds to infected cells at day 7 post inoculation (amplification of 400x); h) cells inoculated with non-infected individual at 14 days after incubation (100x).

The CPE observed on the cell culture were confirmed by RT-PCR. As displayed in the electrophoresis gel photographs (Fig. 3.5) all the samples from the stock and controls had no bands specific for the presence of Betanodavirus. The cell culture supernatants of the individuals infected by both challenges showed the presence of 430 bp product, that is specific for Betanodavirus. The amplified product resulting from the RT-PCR performed in all the cell culture supernatants is showed in Figure 3.5.

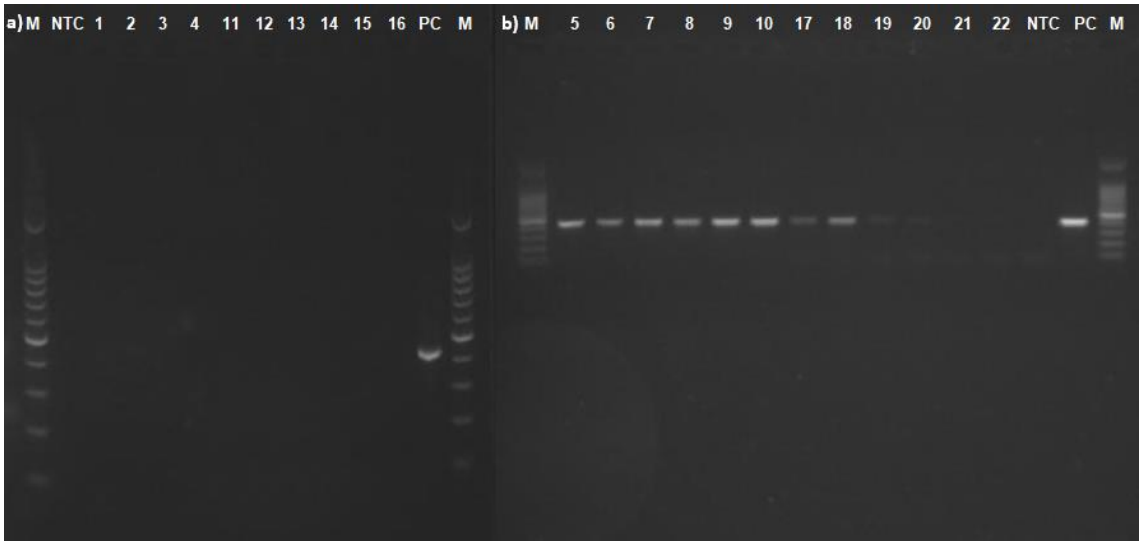


Figure 3.5: Results obtained for the RT-PCR for the cell culture samples using a standard PCR with the F2-R3 primer pair (Nishizawa *et al*. 1994): a) the non-infected samples (1, 2, 3, 4 – stock; 11,12, 13 – immersion Control; and IM Control – 14, 15, 16) and b) infected samples with Betanodavirus (5, 6, 7, 8,9,10 – IM Infected moribunds; 17,18,19 – immersion Infected; 20, 21, 22 – IM Infected). NTC – no template control; PC – positive control; M = molecular size marker (100 bp ladder).

3.3. Study 2 - *non-lethal sampling study*

The non-lethal study was performed with the main objective of accessing the suitability of blood, gills and caudal fin, as a non-lethal sampling tissues for diagnosis purposes. The brain as the main infected tissue was collected to confirm the Betanodavirus infection. The viral load was determined in all the samples. Serum was also collected to determine the development of the immune response (antibodies) of the challenged individuals.

The first and only mortalities for the non-lethal study were recorded at 6 dpi in the IM infected individuals, with the cumulative mortality at the end of the study of 5%. First mortalities for the immersion infected individuals occurred later, at 12 dpi, and at the end of the challenge a value of 3.3% was recorded. For the Immersion treatment, a plateau

was reached at 18 dpi. Both treatment controls registered no mortality during the challenge. The cumulative mortality (%) results are presented in Figure 3.6.

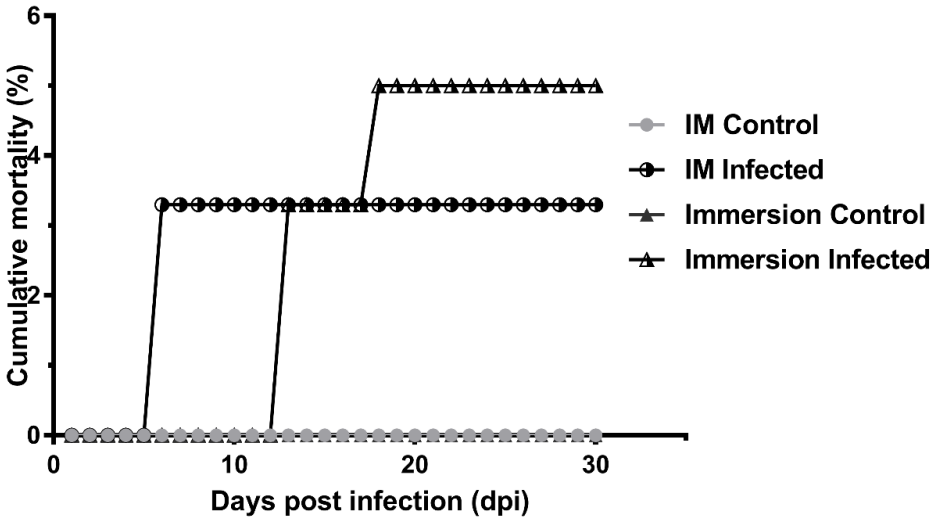


Figure 3.6: Cumulative mortality (%) observed during challenge with Betanodavirus for Study 2 after experimental infection (n=30 per group).

Antibodies against Betanodavirus were detected in both treatment groups at 15 and 30 dpi. After just 7 dpi it was possible to detect specific antibodies against Betanodavirus in the IM infected individuals, with a mean value of 7 $-Log_2+1$, with the antibodies titre raised up to 8 $-Log_2+1$ at 15 dpi (Fig. 3.7). The lower value was recorded for the immersion treated group, 2 $-Log_2+1$ at 15 dpi (Fig. 3.7). At 30 dpi, both treatments had the same endpoint antibody titres, 8 $-Log_2+1$ (Fig. 3.7). Control fish from both the IM and immersion challenge groups had no specific antibodies against Betanodavirus.

The levels of antibodies revealed that there were no statistical significant differences between sampling days 7, 15 and 30 dpi for the IM infected group (ANOVA-2way, $F_{(2.12)=}0.325$, *p-value*=0.729>0.05). Statistical significant differences were observed between sampling days for the immersion treated group (t-student, $t_{(6)}=-2.524$, *p-value*=0.045<0.05). When the infection methods were compared at both sampling days (15 and 30 dpi), statistical significant differences were observed (ANOVA-2way, $F_{(1.14)=}7.622$, *p-value*=0.015<0.05). More specifically, the statistical significant differences were due to the differences observed at 15 dpi (LSD, *p-value*=0.002<0.05; Fig. 3.7 labelled *). These results confirmed the statistical significant differences observed for the immersion group at 15 and 30 dpi when compared (LSD, *p-value*= 0.006<0.05; Fig. 3.7, labelled with different letters).

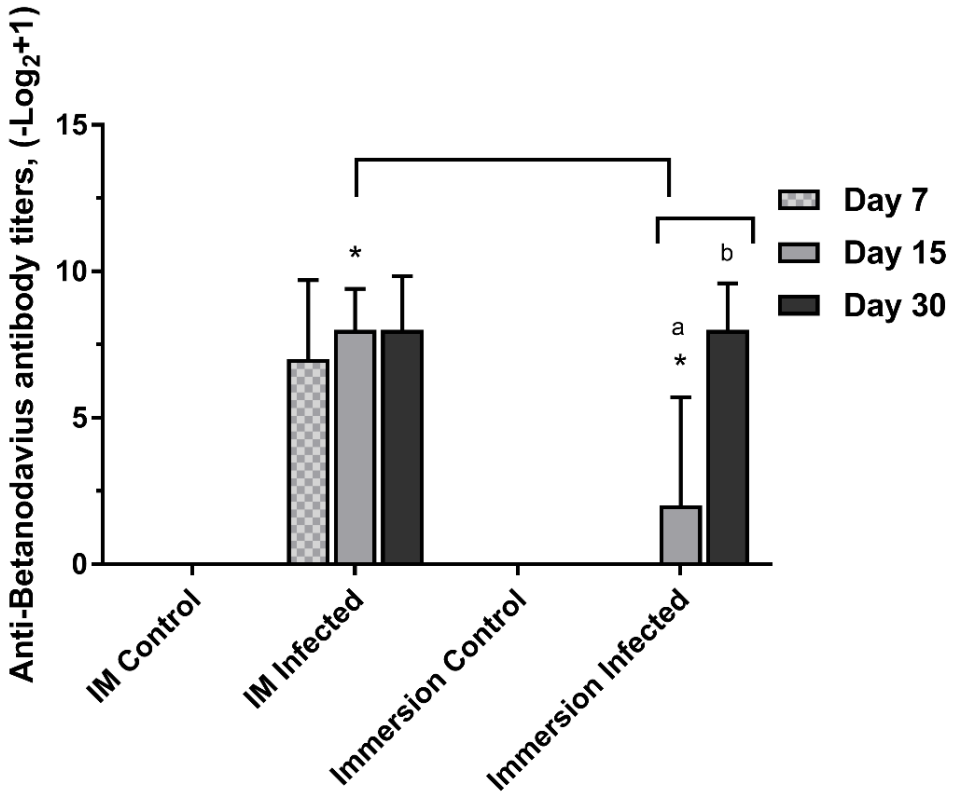


Figure 3.7: Mean endpoint antibody titres ($-Log_2+1$) for 7, 15 and 30 days post infection for the different infection methods, IM and immersion, for Study 2 (n= 4 per immersion group and n=5 per IM group). The results are presented as mean±SD. Different letters/symbols represent statistical differences between treatments and days (ANOVA-2-Way, LSD, p-value<0.05).

When analysing the individual endpoint antibody titres (Fig. 3.8), it was possible to observe that all the IM infected individuals were responsive with most of the individuals having antibody titres levels between 7 and 10 $-Log_2+1$. However, it was always possible to observe that at each sampling point one individual presents lower antibody levels than the remain fish analysed on that day. With the immersion challenged 75% of the individuals did not revealed the presence of antibodies at 15 dpi, however by 30 dpi all the individual analysed have antibodies and at similar level as presented in the IM infected fish. Control fish from both infection routes showed no specific antibodies against Betanodavirus.

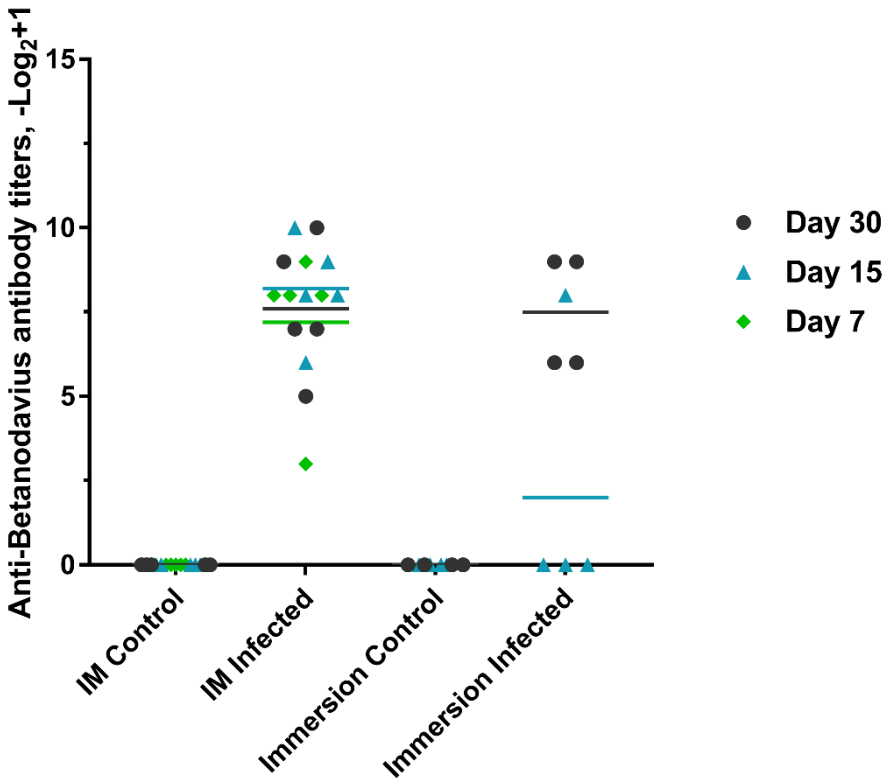


Figure 3.8: Individual endpoint antibody titres ($-Log_2+1$) for 7, 15 and 30 days post infection for the different infection methods, IM and immersion, for Study 2. Each dot represents an individual (n= 4 per immersion group and n=5 per IM group).

The mean Ct values obtained with the standard curves prepared and analysed in each real time RT-PCR established a linear regression between the mean Ct values and the viral titre of a Betanodavirus supernatant with a known $TCID_{50}$ mL^{-1}. The linear regression equation achieved was: $y = -3.438(x) + 36.39$, $R^2 = 0.9988$ (*p-value*<0.0001) (Fig. 3.9).

This standard curve can be seen as simple method to obtain an indication of the viral load present in the infected tissues. With a known mean Ct value, the viral load can be extrapolated from the curve.

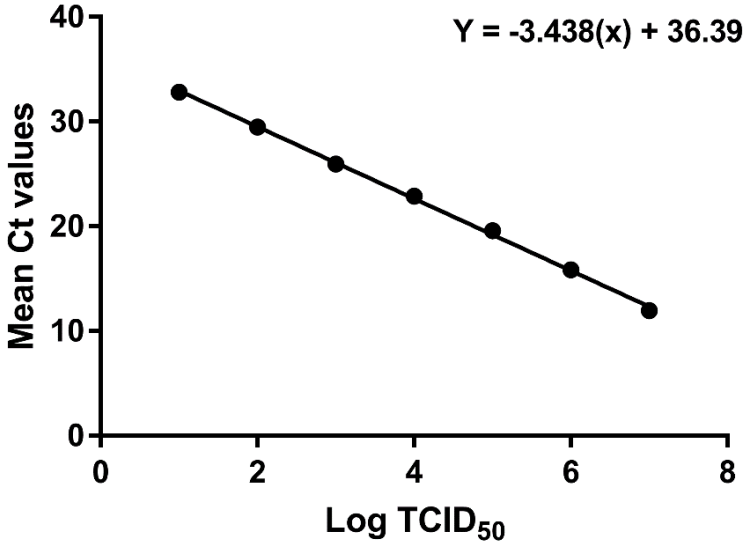


Figure 3.9: Standard curve of the mean Ct values vs 10-fold dilutions of a Betanodavirus with a known $TCID_{50}$ concentration (y =−3.438(x) + 36.39, R^2 = 0.9988).

Positive results were obtained for both infection methods in the analysed tissues by qPCR. It is possible to observe that 7 dpi was the sampling day that registered the higher mean Ct values for all the analysed organs, indicating a lower viral load in these. Depending on infection method and analysed tissue, the mean Ct values varied between the sampling days (Fig. 3.10). In the immersion infected fish, a general higher viral load was registered at 15 dpi than 7 dpi and by 30 dpi the non-lethal tissues presented no signs of Betanodavirus infection. With this infection route it was possible to detect virus in the brain at 30 dpi, with a higher mean Ct value, of 10.5, when compared to 15 dpi that showed, a Ct value of just 5.2. All the organs analysed for IM infected fish presented positive results at 7 and 30 dpi with higher viral load registered at the end of the challenge. However, at 15 dpi the caudal fin and the gills showed no signs of infection. Independent of the route of infection, the brain was the only tissue that presented positive results at all sampling points. When this tissue was compared to the non-lethal tissues, differences were statistical significant for Ct values (ANOVA-2Way, $F_{(2,21)}$=13.12, *p-value*=0.000<0.05). In addition, it was possible to observe that the values were lower at 7 dpi, which indicates higher viral load for the brain (Tukey HSD, *p-value*=0.001<0.05 represented by “A” and *p-value*=0.000, represented by “B”, for the caudal fin and gills, respectively, Fig. 3.10). The lower and higher viral loads, for the gill tissue were recorded in immersion infected individuals at 7 dpi (mean Ct value of 29.0) and 15 dpi (mean Ct value of 6.8), respectively, and by 30 dpi the gills showed no signs of virus infection. The gills of IM infected individuals revealed the higher viral load on 30 dpi (mean Ct value of

19.0) after been absent at 15 dpi. The gills revealed statistical differences between treatments and sampling days (ANOVA-2Way, $F_{(2,21)}$=5.312, *p-value*=0.014<0.05). For the IM infected individuals, differences were detected between 15 and 30 dpi (LSD, *p-value*=0.004<0.05, represented by the symbol #, Fig. 3.10), and 15 and 7 dpi (LSD, *p-value*=0.001<0.05, represented by the symbol *, Fig. 3.10). For the immersion infected fish differences were also observed between 7 and both 15 and 30 dpi (LSD, *p-value*=0.003<0.05 represented by the symbol &, and *p-value*=0.000<0.05, represented by the symbol + respectively, Fig. 3.10). At 30 dpi there were statistical differences between both treatment groups for the gills (LSD, *p-value*=0.007<0.05; represented by the symbol "»", Fig. 3.10). For the caudal fin of IM infected fish, the higher mean Ct value was obtained at 7 dpi with a value of 27.1, and the lower mean Ct value of 12.6 was observed at 30 dpi and 15 dpi showed no signs of viral infection. For the Immersion infected group, the lowest value was registered at 15 dpi with a mean Ct of 7.7 and by 30 dpi it was not possible to detect viral genome. For the caudal fin, statistical significant differences were detected when the sampling days were compared between the challenge model groups (ANOVA 2Way, $F_{(2,21)}$=9.095, *p-value*=0.001<0.05). Therefore, statistical significant differences were observed between 7 dpi and the other two sampling days (15 and 30 dpi) (LSD, *p-value*=0.002<0.05, represented by the letter "a" and *p-value*=0.007<0.05, represented by letter "b", respectively; Fig. 3.10). The blood was compared at 15 and 30 dpi. No statistical differences were registered for the blood (*p-value*>0.05). However, it was possible to detect viral genome on 15 dpi with both infected methods. Nevertheless, at 30 dpi it was possible to detect viral particles in the IM infected fish blood, registering the lower mean Ct value with 6.0. The higher mean Ct value was recorded at 15 dpi for the immersion infected fish with a mean Ct value of 13.7. The results showed that was not possible to detect the presence of virus particles in any of the control individuals tested for either infection route in any of the analysed organs.

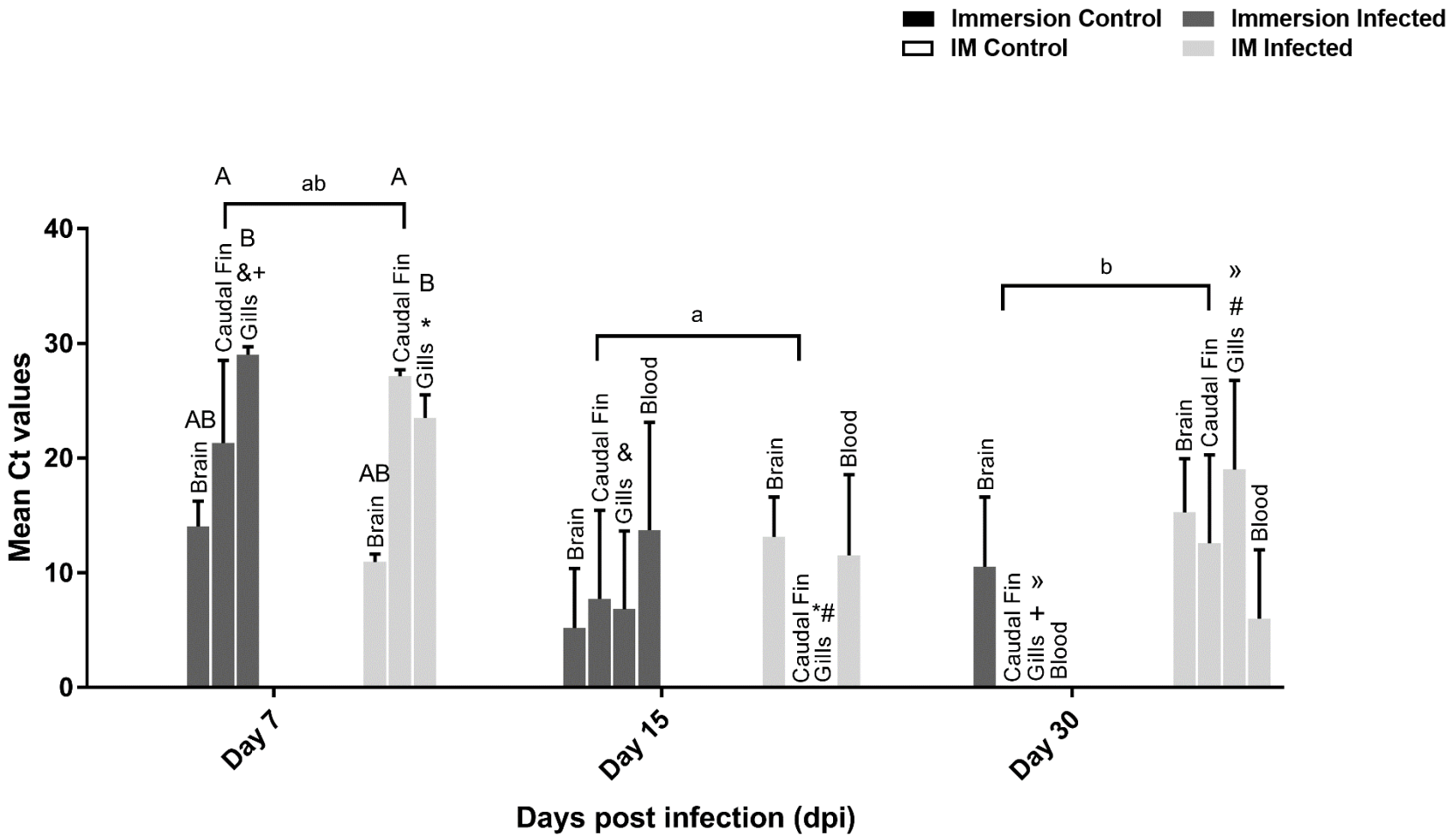


Figure 3.10: Results of the qPCR analysis presented as Ct mean values for each organ during Study 2 for sampling days 7, 15 and 30 (n=4 per group for Immersion and n=5 per group for IM). The results are present as mean±SEM. Different symbols represent statistical differences between treatments and days (in the gills) (ANOVA-2 Way, LSD, *p-value*<0.05), small letters represent statistical differences between days (in the caudal fin) (ANOVA-2 Way, Tukey HSD, *p-value*<0.05), and capital letters represent statistical differences between tissues (brain) (ANOVA-2 Way, Tukey HSD, *p-value*<0.05).

4. Discussion

VER is described as one of the major threats for global aquaculture, since it has had devastating effects in farmed fish, especially in the Mediterranean region (Costa & Thompson, 2016; Doan *et al*., 2017). With increasing water temperatures due to climate change, it is predictable that more outbreaks of VER will occur globally, resulting mainly from the RGNNV genotype (Doan *et al*., 2017). Therefore, it is important to have viable and accurate methods to detect this disease in the early stages of infection, before the manifestation of clinical signs in fish. Through reliable sampling and diagnosis methods, it is possible to better control the spreading of the VER infectious agent, Betanodavirus, in aquaculture systems.

Dicentrarchus labrax is one of the most important aquaculture species in the Atlantic Ocean and the Mediterranean region. In addition, it is considered as a highly susceptible marine fish to VER, being a suitable model to perform experimental infections with Betanodavirus (RGNNV genotype) (Haddad-Boubaker *et al*., 2013; Nuñez Ortiz *et al*., 2016a; Toffan *et al*., 2016).

The main purpose of the present work was to ascertain if it was possible to detect Betanodavirus with non-lethal sampling methods, through the collection of non-lethal tissues, such as the blood, gills and caudal fin. Two studies were carried out to accomplish this purpose: a mortality study (Study 1) and a non-lethal sampling study (Study 2). In the mortality study levels of specific fish mortality were evaluated to confirm that Betanodavirus used was pathogenic, was responsible for the clinical signs observed and the challenge model was correct. In the non-lethal sampling study four different tissues, brain, gills, caudal fin and blood, were analyse by Real Time RT-PCR to access their suitability for Betanodavirus detection.

During both studies the water temperature was maintained at 26°C±1, a suitable water temperature for Betanodavirus replication in fish tissues, contributing to infection development. These range of temperature values are within the optimal temperature for RGNNV Betanodavirus replication that is between 25-30°C (Iwamoto *et al*., 2000). These water temperatures are also suitable rearing temperatures for *D. labrax*, assuring best animal welfare of the reared fish, since this species can survive in temperatures from 2°C up to 32°C (Barnabé, 1990; Vázquez & Muñoz-Cueto, 2015).

For the mortality study, the cell culture method was performed to fulfil the Koch's postulates, since this method allows the isolation of the pathogenic agent and confirm its identity. Cytopathic effects (CPE) were observed in the cell monolayers inoculated with the brain homogenates collected from infected individuals by both infection methods at the end of Study 1. The CPE was visible in the SSN-1 cell monolayer 5 days after the blind passage, revealing that the observed CPE were originated from the virus pathogenicity and were not caused by fish tissue toxicity (Landry & Leland, 2016). These CPE results were also observed in other studies like, Chua *et al*. (1995), Ciulli *et al*. (2004), Qin *et al*. (2006), Babu *et al*. (2013) and John *et al*. (2014) for Betanodavirus. Therefore, the CPE on the cell monolayer confirmed the presence of a virus infection in the brains collected from the infected fish during this experimental infection. Although the observation of CPE in cell culture is considered as a useful and simple diagnosis method, the infectious agent must be confirmed by other methods, such as the RT-PCR or neutralization assay. The Betanodavirus infection in the SSN-1 cell line was confirmed by RT-PCR, utilizing the set of primers (F2-R3) developed by Nishizawa *et al*. (1994). The absence of CPE and negative results by RT-PCR for the non-infected fish and the positive results (CPE and RT-PCR) for the experimentally infected fish in both infection methods, indicates that the fish, were in fact, infected with Betanodavirus.

The clinical signs observed for Study 1 for the intramuscular injection (IM), which included a dark coloration, abnormal swimming behaviour and lack of swim bladder control, started at 5 dpi and led to the cumulative mortality recorded, for the intramuscular infected fish. Skliris and Richards (1999) and Souto *et al*. (2015a) had similar results in fish infected via IM, since the clinical signs were identical and started at 4 and 3 dpi, respectively. The clinical signs for the immersion infected fish were much less evident when compared to the IM infected fish, which is corroborated by the lower cumulative mortality recorded. The observed symptoms are typical of VER infection, as recorded for numerous fish species (Curtis *et al*., 2001; Sommer *et al*., 2004; Vendramin *et al*. 2014; Souto *et al*., 2015a; Souto *et al*., 2015b; Carballo *et al*., 2016; Jaramillo *et al*., 2017). During the challenge, 40% of cumulative mortality was registered for the IM infected fish, which indicates an acute Betanodavirus infection. The cumulative mortality registered in the mortality study is within the expected values, since it was used the same infection route and observed the same level of cumulative mortality as previously reported by Skliris and Richards (1999) and Lopéz-Jimena *et al*. (2011), for Betanodavirus infection in *D. labrax*. Also, similar cumulative mortalities for *D. labrax* (15 g juveniles) were obtained previously with the same isolate, virus dose and infection method (Costa, personal comment). Although this route of infection is an artificial infection route, IM

injection is considered to be the most effective infection method for this virus, to achieve its transmission in experimental infections (Lopéz-Jimena *et al*., 2011; Kim *et al*., 2018), being associated with high mortality rates in sea bass (Péducasse *et al*., 1999; Skliris & Richards, 1999). This infection route has also been proven to be effective for transmitting Betanodavirus in other fish species (Amundsen & Sommer, 1999; Aranguren *et al*., 2002; Furusawa *et al*., 2006; Kim *et al*., 2018). However, in the non-lethal sampling study (Study 2), the recorded cumulative mortality values were much lower when compared to those obtained in the mortality study (Study 1), since a value of just 3.3% was recorded for the IM infected fish. Such lower mortality can be related with the changes in the disease dynamics due to the sampling process. The infected fish shed viral particles to the environment creating the possibility of reinfection. With sampling the tanks, the number of fish in the tanks are reduced, and consequently, a lower number of virus are shed by the infected fish, leading to a lower amount of viral particles available to reinfect them, and inducing to the lower cumulative mortality values observed.

In the case of the immersion infected fish for Study 1, the cumulative mortality values were lower than those infected with the virus by IM injection, with a resulting value of just 3.3%, corresponding to a subclinical infection. For the non-lethal sampling study (Study 2), 5% was the cumulative mortality values recorded for the immersion treated group after 30 dpi. These cumulative mortality values can be considered as an indication of a subclinical Betanodavirus infection, capable of being detected by diagnosis methods, but with no manifestations of clinical disease in the fish (Binesh, 2013). The lower cumulative mortality values and the less significant symptoms observed in the fish, shows that it is a much less effective method of infection. Immersion is an infection method that implies that virus present in the water needs to enter the host during the pre-determined exposure period, surpass the hosts immune barriers (such as mucus and other immune responses), before been able of invading the target tissues, in this case the fish central nervous system (CNS) (Kim *et al*., 2018). It has already been proven that Betanodavirus can spread through the surrounding water (Grotmol *et al*., 1999; Breton *et al*., 1997; Péducasse *et al*., 1999; Castric *et al*., 2001; Hick *et al*., 2011; Manin & Ransangan, 2011), consequently immersion is an infection route that simulates this horizontal transmission of Betanodavirus, as occurs naturally (Kim *et al*., 2018). Therefore, this is an interesting method to infect the fish and study the natural ways Betanodavirus enters its host.

The non-lethal sampling methods are acquiring more importance, since the legislation regarding animal welfare for experimentation purposes is becoming more

restricted (Levenda, 2013; Oidtmann *et al*., 2013; Henderson *et al*., 2016). Also, non-lethal sampling is a welfare friendly method of sampling at farm sites without having to kill the fish. This is an important consideration, especially for market size fish and valuable broodstock.

Caudal fin and gill clippings have been used as effective non-lethal sampling methods in numerous studies (Sanderson *et al*., 2009; Cornwell *et al*., 2013; Baker *et al*., 2004; Burbank *et al*., 2017), proving to be a reliable alternative for traditionally used lethal sampling methods. Fin and gill biopsies have been utilized for contaminant monitoring, genetic analysis and measure physiological responses in the fish (Martinelli-Liedtke *et al*., 1999; Sanderson *et al*., 2009). Also, blood collection is frequently used in studies to evaluate plasma indicators of stress, such as haemoglobin, haematocrit, glycemia and cortisol, to determine specific hormone levels or metal/chemical contaminants in fish and to evaluate immune responses (antibody detection against specific infectious agents) (Sumpter *et al*., 1991; Roche *et al*., 1996; Brumbaugh *et al*., 2005; Barsiene *et al*., 2015; Adel *et al*., 2016; Tan *et al*., 2018). The sampling process to obtain such tissues have, generally, minimal consequences for the sampled individual (Martinelli-Liedtke *et al*., 1999; Sanderson *et al*., 2009), revealing its usefulness. In the present study these three different tissues were examined for Betanodavirus detection.

Nowadays molecular methods, mainly the real-time RT-PCR are gaining more importance due its reliability and sensitivity in the diagnosis of Betanodavirus infections (Munday *et al*., 2002; Hodneland *et al*., 2011; Shetty *et al*., 2012; Doan *et al*., 2017). A real time RT-PCR assay is also less time consuming than other used diagnosis methods (Doan *et al*., 2017) and can detect low viral copy numbers, being a useful method to screen broodstock and potential asymptomatic carriers (Hodneland *et al*., 2011). The levels of the pathogen in each tissue were traced throughout the challenge period using this method. Positive results were observed in the non-lethal sampling tissues, blood, gills and caudal fin, and in the brain analysed by real time RT-PCR. The Ct levels obtained for the brain tissue were generally lower when compared with the other tissues analysed for fish infected by both of the two infection methods. This is explained by the fact that Betanodavirus is a virus that has as its target the organs of the central nervous system (CNS) (Glazebrook *et al*., 1990; Nakai *et al*., 2009; Lopéz-Jimena *et al*., 2011). The study developed by Lopéz-Jimena *et al*. (2011) confirmed that the viral replication starts in brain tissues being detected after 3 dpi, from where the virus starts to spread in the direction of the eyes (optical nerves) and the internal organs in general, through the circulatory system. The Ct values obtained for the brain were statistically different from

those obtained in the non-lethal tissues at 7 dpi. In this same sampling point, the Ct levels in the brain registered 14.0 and 10.9 for the immersion and the IM infected fish, respectively. The brain Ct levels at 15 dpi for the immersion individuals were half of the Ct levels at 7 dpi with a value of 5.2, showing a high increase in the viral load. However, in the case of the IM infected fish the viral load in the brain reduced throughout all the study with a Ct levels of 13.1 at 15 dpi, and 15.3 at 30 dpi. The same decreased in viral load was observed on 30 dpi for the immersion infected fish (Ct mean value of 10.5). These reductions of the Ct values in the brain of fish infected for both routes of infection, reveal that the virus was still replicating in the brain up to the end of the study (30 dpi) and the infection was active in the target organ. Although this was considered as a subclinical infection, the observed decrease in the Ct values at 15 dpi indicates that the virus was replicating (active infection) in the target tissue and the infection levels were successively increasing in the non-lethal tissues at 30 dpi (IM infected fish).

Several studies have been described in which the VER infection activates B lymphocytes, expression and infiltration of immunoglobulins in the CNS, generating a powerful adaptive humoral response by the host (Valero *et al*., 2018), allowing the host to cope with the infection and clear the virus. The presence of such specific immune response and the ability to control and clean up the Betanodavirus infection can be reason behind the slightly increase in the Ct values from 15 dpi to 30 dpi, and the low mortalities observed. Hodneland *et al*. (2011) correlated Ct levels with mortality data in an experimental infection and demonstrated that European sea bass individuals can die with a Betanodavirus infection when a mean Ct level lower than 11.7 is reached, independent of the infection route or dose. These authors also verified that asymptomatic sea bass individuals had Ct values ranging from 11.6 to 31.0, corresponding to different infection levels. The data registered in the present experiment shows that the relatively low cumulative mortality recorded with both infection methods in the non-lethal study (Study 2) may have been related with high Ct levels, as Hodneland *et al*. (2011) verified. A later sampling day would have been useful to confirm the brain's viral load is decreasing and if no late mortality would be registered.

Generally, the non-lethal tissues (gills, caudal fin and blood) registered higher mean Ct values compared to the sampled brains, indicating a lower viral load. Since these are not target organs for Betanodavirus, a lower quantity of viral particles is expected (Lopéz-Jimena *et al*., 2011; Costa & Thompson, 2016). The blood presented higher mean Ct values at 15 dpi, with 13.7 and 11.5 mean Ct values, for the immersion and IM infected fish, respectively. A decrease in the blood Ct values was seen at 30 dpi

when compared to 15 dpi for the IM infected individuals while the immersion infected individuals revealed no virus was present in the blood on 30 dpi. There is no data available for blood at 7 dpi due to technical issues. The increase of viral particles on 30 dpi in the IM infected fish shows that the virus was still replicating, was active and the fish were virahemic, what might explain the presence of viral particles in the gills and caudal fin after been absent in 15 dpi sampled tissues. The absence of viral particles in the blood of immersion infected fish at the end of the study may be explained by the development of an immune response as mentioned before. For the gills of immersion infected individuals, a peak viral load was noticed at 15 dpi, with significant lower mean Ct values of 6.8. Also, for the caudal fin, the lower mean Ct value was registered at 15 dpi, with a value of 7.7 for the same infection group, with significant differences between treatments and sampling days. At 30 dpi no viral genome was detected for the immersion group non-lethal tissue samples.

Studies have established that mucosal surfaces, such as the gills and skin, constitute physical barriers, preventing the access of infectious agents and perform a fundamental role in the fish immune system (Kim *et al*., 2018). These tissues can, therefore, constitute an obstacle for Betanodavirus to enter the host. Also, Lopéz-Jimena *et al*. (2011), claimed that the Betanodavirus viral replication in these non-nervous tissues does not produce mature virions and observed that the expected increase in viral titre was not produced in their experiment. As well, the specific immune response appeared on 15 dpi and reached its peak on 30 dpi. Consequently, it is possible that the lack of non-lethal tissues samples with virus genome observed in the immersion infected fish in the last sampling day may be due to the fish immune system activity and the replication mechanism of Betanodavirus, resulting in such a lower viral load that is not detectable in these not Betanodavirus targeted tissues or even a completely clean out tissues. The caudal fin and gills of IM infected fish registered a decrease in Ct values from 7 to 30, meaning higher viral load, but no values were recorded at 15 dpi. The dynamics of the viral load in these fish might be explained by the presence of specific antibodies that reach its peak on 15 dpi when the non-lethal tissues shown no presence of viral particles. The results obtained confirmed the presence of Betanodavirus viral genome in non-lethal tissues in both infection methods, although suggest that these are not target organs for the viral replication.

The real-time RT-PCR data can correlate with a wide-range of viral titres quantified by endpoint titration ($TCID_{50}$ mL^{-1}) and was successfully applied for the estimation of picornavirus titres (Jonsson *et al*., 2008). The regression analysis of the

four independent standard curves shows that the assay is highly repeatable and can be used with a standard curve generated with mean Ct values to accurately estimate viral titres. This standard curve can be used for example in future studies to indicate the viral titre present in an unknown fish tissue sample or to easily determine the titre of cell culture supernatant replacing titration assays that are highly time demanding.

The ELISA method was used to determine the endpoint of anti-Betanodavirus specific antibody titres in fish at the end of the mortality study (Study 1) and, to establish that Betanodavirus was the infectious agent causing a specific immune response. During Study 2, the antibodies dynamic was analysed with the sequential samples been analysed. In both infection methods, Betanodavirus antibodies were detected, indicating successful infection of the fish, and control fish had showed no antibody responses. During Study 1, the mean endpoint antibody titres for the IM infected fish were higher than those of the immersion infected fish at 30 dpi. Also, the individual endpoint antibody titres obtained for the IM infected fish, in all the individuals, were higher when compared to the immersion infected ones. The lower antibody responses detected in the immersion infected fish are explained by the infection method itself, since, as mentioned above, the less effective immersion method requires that the virus surpass physical and chemical protective barriers to entry the host and cope with the produced fish immune response (Kim *et al*., 2018). Consequently, with the potential for a later fish humoral immune response, a subsequent sampling point to the ones carried out here may be necessary to observe a higher endpoint antibody titre in the immersion infected individuals. Also, as showed by the cumulative mortality values, the Betanodavirus immersion dose may not have been sufficient to cause higher levels of mortality in fish, and therefore to produce a more intense immune response to induce higher levels of antibody production. As described by Workenhe *et al*. (2010) and Chen *et al*. (2014), the increase in antibody production against specific viruses, like Betanodavirus, is related with an increase in survival probability for the infected fish. Therefore, the fish immune system was able to react against the Betanodavirus infection, as demonstrated by the relatively low cumulative mortality values for this experimental infection. For Study 1 the mean endpoint antibody titre measured at 30 dpi for the IM infected fish, was 10^{-Log_2+1} (OD_{450nm}= 0.797) and for the immersion infected fish a value of 6^{-Log_2+1} (OD_{450nm}= 0.573) was recorded, with no statistical differences between these values. Studies for Betanodavirus such as that performed by Grove *et al*. (2003) lower OD values for antibody responses were obtained for Atlantic halibut (*Hippoglossus hippoglossus*), at 30 dpi, for immersion infected individuals (OD_{450nm}= 0.750), when compared to the

intraperitoneal infected fish (OD_{450nm}= 0.100), revealing a more reduced antibody activity in the plasma of the immersion infected fish.

The corresponding OD values in Study 1 - mortality study (see Appendix II), were similar to the study performed by Grove *et al*. (2003) for the intramuscular infected fish. The immersion infected individuals had a higher mean OD value, when compared to the Grove *et al*. (2003) OD values for the bath challenge infected fish. Nuñez Ortiz *et al*. (2016b) performed an immunization study in *D. labrax* with Betanodavirus inactivated with formalin, β-propiolactone and heat treatment. The intraperitoneal immunization with formalin-inactivated, presented OD individual values between 0.500 and 0.800, 30 dpi, while the immersion immunization recorded individual OD values were lower than 0.100. In the present mortality study (Study 1), similar results were recorded for the individual endpoint antibody titres.

The mean endpoint antibody titres of the collected serum samples were also determined by ELISA for the non-lethal study (Study 2). However, in this case, samples were collected throughout the challenge, at 7, 15 and 30 dpi, that allowed to evaluate the development of the fish antibody response. The observed mean endpoint antibody titres for the IM infected fish were, again, higher compared to the immersion infected fish at 15 and 30 dpi. There is no data available of the 7-dpi immersion infected, due to technical issues. The mean endpoint antibody titres for the immersion infected individuals had a statistical significant increase at 30 dpi, when compared to 15 dpi for the same infection route. In the IM infected fish, it is possible to detect an increase in the mean antibody titres throughout the study, with values of 7, 8 and 8 $-Log_2+1$, for 7, 15 and 30 dpi respectively. For the immersion infected fish an antibody production peak was registered at 30 dpi, with a value of 8 $-Log_2+1$. Cheng *et al*. (2016) obtained antibody titres against Betanodavirus in the sera of vaccinated groupers slightly higher than those here displayed, although the groupers antibody levels were determined at 24 dpi after vaccination, while were determined at 30 dpi for the present study. In a study by Buonocore *et al*. (2017), sera from sea bass individuals were collected 30 days post intraperitoneal immunization with Betanodavirus, showed OD values for the infected fish similar to the values recorded in the present study for both infection methods at 30 dpi. During the present study was possible to observe a delay in the immune response of the immersion infected fish and the lower antibody titres registered that can be explained by the infection method itself. As previously discussed in that an increase in time is required for the virus to enter the host with the same viral load as the one registered for the IM

injected fish, and a lower viral load is reflected in a lower fish immune response against Betanodavirus.

The levels of antibodies detected in this study, are indicative of a good immune response in the infected fish, with a considerable fish immune response detected as early as 7 dpi, in what, as discussed above, its considered as a subclinical infection. Consequently, antibody responses measured by ELISA can be used as an early indirect indicator of Betanodavirus infection and may be a useful non-lethal sampling method and diagnosis test for fish, allowing a regular screening method for VER in aquaculture facilities (Doan *et al*., 2017), preventing the spreading of this problematic disease.

A similar trend was observed for the individual endpoint antibody titres, in the IM infected fish, having higher mean values in comparison with the immersion infected fish during the challenged. The highest recorded individual values were obtained at 15 and 30 dpi, with 10 $-\text{Log}_2+1$, and the lowest registered at 7 dpi for the IM infected fish, with a value of 3 $-\text{Log}_2+1$. These lower registered values at 30 dpi may indicate that the fish immune system is responding to the subclinical Betanodavirus infection and cope against it, becoming asymptomatic carries. The higher viral loads detected by real-time RT-PCR (lower Ct values) were obtained at 15 dpi for both infection methods, which corresponded to one of the highest mean antibody titres for the IM infected fish. Teleosts have an immune system that can produce complex responses against Betanodavirus, since antibodies can be directly responsible for the virus neutralization and for protecting the host from damage (Chen *et al*., 2014). This humoral response can be verified in both acute and subclinical infections, by an upregulation of the immunoglobulin gene (Wu *et al*., 2012). Therefore, these results suggest that after the Betanodavirus infection of the host, and consequent humoral immune response, with a high antibody level production at 15 dpi, the number of viral particles present in the host are diminishing at 30 dpi, resulting in lower antibody production. The immune response is known to be faster in warm water fish species than in cold water, with antibody production for a cold-water species been usually not detected by diagnosis methods until at least 4 to 6 weeks after the exposure to the infectious agent (Sommerset *et al*., 2005). It is interesting to conclude that the antibody production in this case, starter much earlier, with high levels of antibody titres being detected at 7 dpi. These such high values can be explained by the water temperature where the fish were challenged, since the temperature was placed in the optimal temperature values for the replication of the RGNNV genotype (Doan *et al*., 2017). Also, the fish metabolism is known to be accelerated by high water temperature (Clarke & Fraser, 2004; Ruyet *et al*., 2004), inducing a faster and higher immune

response to VER. A sampling day before 7 dpi should be considered to stablish if antibody production against Betanodavirus can occur earlier in European sea bass.

Non-lethal tissues can have several applications in an aquaculture environment, since they can be used in the regular screening of broodstock fish and restocking fish without any harm to the analysed individuals, or for accessing the prevalence of Betanodavirus in wild populations, always considering the welfare of the animals and without having to sacrifice the sampled individuals. The present study demonstrated that although the Betanodavirus viral genome was detected in the non-lethal tissues, the brain remains as the most suitable tissue for detecting Betanodavirus infections in fish. Therefore, the brain should continue to be the sampled organ to detect this infection, since the obtained Ct values were high or were not detected in non-lethal samples, and the brain was always positive independent of the infection method or sampling point. Consequently, a decisive diagnosis of a Betanodavirus infection should be performed using nervous tissues as target organs by the real-time RT-PCR diagnosis method, since Betanodavirus viral replication occurs in the CNS organs, and these organs are more probable to obtain positive and accurate results for a correct diagnosis. Another factor to be considered, regarding the non-lethal tissues, is that the immersion infection method may have allowed the attachment of viral particles in the gills and caudal fin, facilitating the Betanodavirus detection by this diagnosis method. The unclear results suggest that more studies should be developed in order to confirm if the gills, caudal fin and blood can be suitable tissues for the diagnosis of Betanodavirus.

The ELISA is a valuable method to evaluate the host response to a previous Betanodavirus infection, and these results suggest that can be implemented to detect fish immune responses against this infectious agent in early stages of the disease. The studies regarding these non-lethal sampling methods are important to find out viable new ways to assure a regular screening in aquaculture facilities to avoid the spreading of VER. Further studies should be performed to access the onset of the immune response (earlier than 7dpi) and until when it is possible to detect antibodies in the fish.

5. Conclusion

Currently, non-lethal sampling methods are acquiring more importance due to the concerns regarding animal welfare and because of the more restricted legislation for animals utilized for experimental scientific purposes. Also, the amount of fish species that are becoming endangered and tagged/released, involves the development of non-lethal sampling techniques (Williams *et al*., 2015). Therefore, there is an increasing necessity of searching for new tissues/sampling methods that can prevent animal suffering or sacrifice. However, the suitability of these non-lethal sampled tissues requires evaluation, since these could not be the desirable target tissues, leading to dubious diagnosis results.

Although the brain and eyes are considered as the target tissues for VER diagnosis, as described by the World Organization for Animal Health (OIE, 2016), RGNNV viral genome was detected in the non-lethal sampling tissues (gills, caudal fin and blood) collected in this experiment. Even though the brain and all nervous tissues are regarded as the more adequate diagnosis organs, this study demonstrates that there may be potential in non-lethal tissues to become suitable for VER diagnosis in fish. Further studies should be developed in order to access if these tissues collected through a non-lethal sampling method, are appropriate for an accurate Betanodavirus infection diagnosis, when compared with the lethal sampling methods.

The ELISA method was able to detect the antibodies against Betanodavirus infection as early as 7 dpi in *Dicentrarchus labrax*, and throughout the challenge, being a reliable indirect diagnosis to validate a previous infection through a non-lethal sampling. The obtained results propose that this is a good non-lethal procedure to evaluate the fish serological status from early stages of a Betanodavirus infection, presenting a possible good alternative to screen fish regularly and avoid an outbreak of this major aquaculture treat in an aquaculture system.

VER is a very difficult disease to eradicate from an aquaculture facility, and therefore, the acquirement of new stock or broodstock individuals should be done with caution to avoid outbreaks of this lethal disease. There are no effective commercialized vaccines or treatments to prevent its spreading between facilities, so nowadays, the control of VER basically depends on reliable diagnosis and sampling methods to detect its presence on early stages of the infection.

6. References

Adel, M., Nayak, S., Lazado, C.C., Yeganeh, S.J., 2016. Effects of dietary prebiotic GroBiotic-A on growth performance, plasma thyroid hormones and mucosal immunity of great sturgeon, *Huso huso* (Linnaeus, 1758). Appl. Ichthyol., 32: 825-831.

Allan, G., Burnell, G., 2013. Advances in Aquaculture Hatchery Technology. Woodhead Publishing Limited. Woodhead Publishing Series in Food Science, Technology and Nutrition.

Amundsen, M., Inger-Sommer, A., 1999. Viral Nervous Necrosis in spotted wolfish (*Anarhicas minor*), Norwegian institute of fisheries and aquaculture Ltd. Tromso Norway.

Aranguren, R., Tafalla, C., Novoa, B., Figueras, A., 2002. Experimental transmission of encephalopathy and retinopathy induced by nodavirus to sea bream, *Sparus aurata* L., using different infection models. J. Fish Dis., 25: 317-324.

Arimoto, M., Maruyama, K., Furusawa, I., 1993. Epizootiology of Viral Nervous Necrosis (VNN) in Striped Jack. Fish Pathol. 29: 19-24.

Arimoto, M., Mori, K., Nakai, T., Muroga, K., Furusawa, I., 1993. Pathogenicity of the causative agent of viral nervous necrosis disease in striped jack, *Pseudocaranx dentex*. J. Fish Dis., 16: 461-469.

Arimoto, M., Sato, J., Maruyama, K., Mimura, G., Furusawa, I.,1996. Effect of chemical and physical treatments on the inactivation of Striped Jack Nervous Necrosis Virus (SJNNV). Aquaculture, 143: 15-22.

Azad, I.S., Shekhar, M.S., Thirunavukkarasu, A.R., Jithendran, K.P., 2006. Viral nerve necrosis in hatchery-produced fry of Asian sea bass, *Lates calcarifer* sequential microscopic analysis of histopathology. Dis. Aquat. Org., 73: 123-130.

Babu, V.S., Majeed, S.A., Nambi, K.S.N., Taju, G., Madan, N., Raj, N.S., Hameed, A.S.S., 2013. Comparison of Betanodavirus replication efficiency in ten Indian fish cell lines. Arch. Virol., 158: 1367-1375.

Baker, R.F., Blanchfield, P.J., Paterson, M.J., Flett, R.J., Wesson, L., 2004. Evaluation of Nonlethal Methods for the Analysis of Mercury in Fish Tissue, Trans. Am. Fish. Soc., 133: 568-576.

Bandin, I., Souto, S., Cutrin, J.M., Lopez-Vazquez, C., Olveira, J.G., Esteve, C., Alcaide, E., Dopazo, C.P., 2014. Presence of viruses in wild eels *Anguilla anguilla* L., from the Albufera Lake (Spain). J. Fish Dis., 37: 597-607.

Banerjee, D., Hamod, M.A., Suresh, T., Karunasagar, I., 2014. Isolation and characterization of a nodavirus associated with mass mortality in Asian sea bass (*Lates calcarifer*) from the west coast of India. Virus Dis., 25: 425-429.

Bang, J.D., Kim, J.W., Lee, S.D., Park, I., Chun, S., Jeong, C., Park, J., 1996. Humoral immune response of flounder to *Edwardsiella tarda*: the presence of various sizes of immunoglobulins in flounder. Dis. Aquat. Org., 26: 197-203.

Barnabé, G., Tournamille, J.C., 1972. Expériences de reproduction artificielle du loup *Dicentrarchus labrax* (Linné, 1758). Rev. Trav. Inst. Pèches mart., 36: 185-189.

Barnabé G., 1990. Rearing bass and gilthead bream, in: Barnabé, G. (Ed.), Ellis Horwood, New York. Aquaculture 647-686.

Barsiene, J., Butrimaviciene, L., Michailovas, A., Grygiel, W., 2015. Assessing the environmental genotoxicity risk in the Baltic Sea: frequencies of nuclear buds in blood erythrocytes of three native fish species. Environ. Monit. Assess., 187: 40-78.

Bellance, R., Gallet de Saint-Aurin, D., 1988. L'encephalite virale du loup de mer Caraibes Medical., 2: 105-44.

Bento, E.G., Grilo, T.F., Nyitrai, D., Dolbeth, M., Pardal, M.E., Martinho, F., 2016. Climate influence on juvenile European sea bass (*Dicentrarchus labrax*, L.) populations in an estuarine nursery: A decadal overview. Marine Environ. Res., 122: 93-104.

Bigarre, L., Cabon, J., Baud, M., Heimann, M., Body, A., Lieffrig, F., Castrid, J., 2009. Outbreak of Betanodavirus infection in Tilapia, *Oreochromis niloticus* (L.) in fresh water. J. Fish Dis., 32: 667-673.

Binesh, C.P., 2014. Elevation of temperature and crowding trigger acute viral nervous necrosis in zebra fish, *Brachydanio rerio* (Hamilton-Buchanan), subclinically infected with Betanodavirus. J. Fish Dis., 37: 279-282.

Blessing, J.J., 2010. Marshall JC, Balcombe SR. Humane killing of fishes for scientific research: a comparison of two methods. Fisheries Society of the British Isles, J. Fish Bio., 76: 2571-2577.

Bloch, B., Gravningen, K., Larsen, J.L., 1991. Encephalomyelitis among turbot associated with a picornavirus-like agent. Dis. Aquat. Organ., 10: 65-70.

Bovo, G., Borghesan, F., Mutinelli, F., Montesi, F., Comuzzie, M., 1996. Viral Encephalo-Retinopathy of cultured sea bass: first detection in Italy. Boll. Soc. It. Patol. Ittica., 19: 52-64.

Breton, A.L., Grisez, L., Sweetman, J., Ollevier, F., 1997. Viral nervous necrosis (VNN) associated with mass mortalities in cage-reared sea bass, *Dicentrarchus labrax*. J. Fish Dis., 20: 145-151.

Breuil, G., Pepin, J.F., Castric, J., Fauvel, C., Thiery, R., 2000. Detection of Serum Antibodies Against Nodavirus in Wild and Farmed Adult Sea Bass: Application to the Screening of Broodstock in Sea Bass Hatcheries. Bull. Eur. Ass. Fish Pathol., 20: 95.

Breuil, G., Pépin, J.F.P., Boscher, S., Thiéry, R., 2002. Experimental vertical transmission of nodavirus from broodfish to eggs and larvae of the sea bass, *Dicentrarchus labrax* (L.). J. Fish Dis., 25: 697-702.

Breuil, G., Romestand, B., 1999. A rapid ELISA method for detecting specific antibody level against nodavirus in the serum of the sea bass, *Dicentrarchus labrax* (L.): application to the screening of spawners in a sea bass hatchery. J. Fish Dis., 22: 45-52.

Brumbaugh, W.G., Schmitt, C.J., May, T.W., 2005. Concentrations of Cadmium, Lead, and Zinc in Fish from Mining-Influenced Waters of Northeastern Oklahoma: Sampling of Blood, Carcass, and Liver for Aquatic Biomonitoring. Arch. Environ. Contam. Toxicol., 49: 76-88.

Brummett, R., 2013. Growing Aquaculture in Sustainable Environments. Agriculture and Environmental Services Department Notes.

Buonocore, F., Nuñez-Ortiz, N., Picchietti, S., Randelli, E., Stocchi, V., Guerra, L., Toffan, A., Pascoli, F., Fausto, A.M., Mazzini, M., Scapigliati, G., 2017. Vaccination and immune responses of European sea bass (*Dicentrarchus labrax* L.) against Betanodavirus. Fish Shellfish Immunol.

Burbank, D.R., Fehringer, T.R., Chiaramonte, L.V., 2017. Comparison of Selected Nonlethal Samples from Adult Steelhead for Detection of Infectious Hematopoietic Necrosis Virus Using Cell Culture. J. Aquat. Anim. Health, 29: 67-73.

Cabral, H., Costa, M.J., 2001. Abundance, feeding ecology and growth of 0-group sea bass, *Dicentrarchus labrax*, within the nursery areas of the Tagus estuary. J. Marine Biol. Assoc. of the UK, 81: 679-682.

Carballo, C., Garcia-Rosado, E., Borrego, J.J., Alonso, M.C., 2016. SJNNV down-regulates RGNNV replication in European sea bass by the induction of the type I interferon systems. Vet. Res., 47: 6.

Castric, J., Thiéry, R., Jeffroy, J., Kinkelin, P., Raymond, J.C., 2001. Sea bream *Sparus aurata*, an asymptomatic contagious fish host for nodavirus. Dis. Aquat. Org., 47: 33-38.

Chen, Y-M., Wang, T-Y., Chen, T-Y., 2014. Immunity to Betanodavirus infections of marine fish. Dev. Comp. Immunol., 43: 174-183.

Cheng, Y-K., Wu, Y-C., Chi, S-C., 2017. Humoral and cytokine responses in giant groupers after vaccination and challenge with Betanodavirus. Dev. Comp. Immunol., 67 385-394.

Chérif, N., Thiéry, R., Castric, J., Biacchesi, S., Brémont, M., Thabti, F., Limem, L., Hammami, S., 2009. Viral encephalopathy and retinopathy of *Dicentrarchus labrax* and *Sparus aurata* farmed in Tunisia. Vet. Res. Commun., 33: 345-353.

Chi, S-C., Lo, C-F., Kou, G-H., Chang, P-S., Peng, S-E., Chen, S-N., 1997. Mass mortalities associated with viral nervous necrosis (VNN) disease in two species of hatchery-reared grouper *Epinephelus fuscogutatus* and *Epinephelus akaara* (Temminck & Schlegel). J Fish Dis., 20:185-93.

Chi, S-C., Lo, C-F., Lin, S-C., 2001. Characterization of grouper nervous necrosis virus. J. Fish Dis., 24: 3-13.

Chi, S-C., Shieh, JR., Lin, S-C., 2003. Genetic and antigenic analysis of Betanodaviruses isolated from aquatic organisms in Taiwan. Dis. Aquat. Organ., 55: 221-8.

Chia, T-J., Wu, Y-C., Chen J-Y., Chi SC., 2010. Antimicrobial peptides (AMP) with antiviral activity against fish nodavirus. Fish Shellfish Immunol., 28: 434-439.

Chua, F.H.C., Loo, J.J., Wee, J.Y., 1995. Mass mortality in juvenile greasy grouper, *Epinephelus tauvina*, associated with vacuolating encephalopathy and retinopathy. Dis. Asian Aquacult., 235-241.

Ciulli, S., Di Marco, P., Natale, A., Galleti, E., Battilani, M., Scagliarini, A., Purpari, G., Cannella, V., Castiglione, F., Guercio, A., 2006. Detection and characterization of Betanodavirus in wild fish from Sicily, Italy. Ittiop., 3: 101-112.

Ciulli, S., Ostanello, F., Battilani, M., Prosperi, S., Hedrick, R.P., 2004. Effect of Temperature on Betanodavirus Infection in SSN-1 Cell Line. Vet. Res. Communications, 28: 283-286.

Clarke, A., Fraser, K.P.P., 2004. Why does metabolism scale with temperature? Funct. Ecol., 18: 243-251.

Collet, B., Urquhart, K., Monte, M., Collins, C., Garcia Perez, S., Secombes, C.J., 2015. Hall M. Individual Monitoring of Immune Response in Atlantic Salmon *Salmo salar* following Experimental Infection with Infectious Salmon Anaemia Virus (ISAV). PLoS ONE 10(9).

Cornwell, E.R., Bellmund, C.A., Groocock, G.H., Wong, P.T., Hambury, K.L., Getchell, R.G., Bowser, P.R., 2013. Fin and gill biopsies are effective nonlethal samples for detection of Viral haemorrhagic septicaemia virus genotype IVb. J. Vet. Diagn. Invest., 25: 203-209.

Costa, J.Z., Thompson, K.D., 2016. Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. Fish Shellfish Immunol., 53: 35-49.

Curtis, P.A., Drawbridge, M., Iwamoto, T., Nakai, T., Hedrick, R.P., Gendron, A.P., 2001. Nodavirus infection of juvenile white sea bass, *Atractoscion nobilis*, cultured in southern California: First record of viral nervous necrosis (VNN) in North America. J. Fish Dis., 24: 263-271.

Cutrin, J.M., Dopazo, C.P., Thiery, R., Leao, P., Olveira, J.G., Barja, J.L., Bandín, I., 2007. Emergence of pathogenic Betanodaviruses belonging to the SJNNV genogroup in farmed fish species from the Iberian Peninsula. J. Fish Dis., 30: 225-32.

Dalla Valle, L., Zanella, L., Patarnello, P., Paolucci, L., Belvedere, P., Colombo, L., 2000. Development of a sensitive diagnostic assay for fish nervous necrosis virus based on RT-PCR plus nested PCR. J. Fish Dis., 23: 321-327.

Doan, Q.K., Vandeputte, M., Chatain, B., Morin, T., Allal, F., 2017. Viral encephalopathy and retinopathy in aquaculture: a review. J. Fish Dis., 40: 717-742.

Drennan, J.D., LaPatra, S.E., Samson, C.A., Ireland, S., Eversman, K.F., Cain, K.D., 2007. Evaluation of lethal and non-lethal sampling methods for the detection of white sturgeon iridovirus infection in white sturgeon, *Acipenser transmontanus* (Richardson). J. Fish Dis., 30: 367-379.

Edholm, E., Bengten, E., Wilson, M., 2011. Insights into the function of IgD. Dev. Comp. Immunol., 35: 1309-1316.

Ek-Huchim, J.P., Jiménez-Garcia, I., Pérez-Vega, J.A., Rodríguez-Canul, R., 2012. Non-lethal detection of DNA from *Cichlidogyrus spp*. (Monogenea, Ancyrocephalinae) in gill mucus of the Nile tilapia *Oreochromis niloticus*. Dis. Aquat. Org., 98: 155-162.

Elliott, D.G., McKibben, C.L., Conway, C.M., Purcell, M.K., Chase, D.M., Applegate, L.J., 2015. Testing of candidate non-lethal sampling methods for detection of *Renibacterium salmoninarumin* juvenile Chinook salmon *Oncorhynchus tshawytscha*. Dis. Aquat. Org., 114: 21-43.

FAO, 2016. Fisheries and Aquaculture Department: Species Fact Sheet (*Dicentrarchus labrax*). http://www.fao.org/fishery/species/2291/en (accessed in: 17/11/16 - FAO).

FAO. 2016. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.

Fox, M.D., Palenzuela, O., Bartholomew, J.L., 2000. Strategies for the diagnosis of *Ceratomyxa shasta* using the polymerase chain reaction: comparison of lethal and nonlethal sampling with microscopic examination. J. Aquat. Anim. Health, 12: 100-106.

Frerichs, G.N., Rodger, H.D., Peric, Z., 1996. Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*. J. Gen. Virol., 77: 2067-2071.

Frerichs, G.N., Tweedie, A., Starkey, W., 2000. Richards R. Temperature, pH and electrolyte sensitivity, and heat, UV and disinfectant inactivation of sea bass (*Dicentrarchus labrax*) neuropathy nodavirus. Aquaculture, 185: 13-24.

Fritsch, M., Morizur, Y., Lambert, E., Bonhomme, F., Guinand, B., 2007. Assessment of sea bass (*Dicentrarchus labrax*, L.) stock delimitation in the Bay of Biscay and the English Channel based on mark-recapture and genetic data. Fish Res., 83:123-132.

Fukuda, Y., Nguyen, H.D., Furuhashi, M., Nakai, T., 1996. Mass mortality of cultured sevenband grouper, *Epinephelus septemfascinatus*, associated with Viral Nervous Necrosis. Fish Pathol., 31:165-70.

Furusawa, R., Okinaka, Y., Nakai, T., 2006. Betanodavirus infection in the freshwater model fish medaka (*Oryzias latipes*). J. Gen. Virol., 87: 2333-2339.

Gagné, N., Johnson, S.C., Cook-Versloot, M., MacKinnon, A.M., Olivier, G., 2004. Molecular detection and characterization of nodavirus in several marine fish species from the northeastern Atlantic. Dis. Aquat. Organ., 62:181-9.

García-Rosado, E., Cano, I., Martín-Antonio, B., Labella, A., Manchado, M., Alonso, M.C., Castro, D., Borrego, J.J., 2007. Co-occurrence of viral and bacterial pathogens in disease outbreaks affecting newly cultured sparid fish. Int. Microbiol., 10:193-9.

Georgalas, V., Malavasi, S., Franzoi, P., Torricelli, P., 2007. Swimming activity and feeding behaviour of larval European sea bass (*Dicentrarchus labrax* L.): Effects of ontogeny and increasing food density. Aquaculture, 264: 418-427.

Glazebrook, J.S., Heasman, M.P., De Beer, S.W., 1990. Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* Bloch. J. Fish Dis., 13: 245-249.

Gomez, D.K., Baeck, G.W., Kim, J.H., Choresca Jr., C.H., Park, S.C., 2008. Molecular detection of Betanodavirus from apparently healthy wild marine invertebrates. Pathology, 97: 197-202.

Gomez, D.K., Matsuoka, S., Mori, K., Okinaka, Y., Park, S.C., Nakai, T., 2009. Genetic analysis and pathogenicity of Betanodavirus isolated from wild red spotted grouper *Epinephelus akaara* with clinical signs. Virol., 154: 343-346.

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

Goswami, J., Mondal, S., Mondal, A., Ganguly, S., Paul, I., Mukhopadhayay, S.K., 2012. Immunohistochemistry: A Novel Tool for the Diagnosis of Animal Disease. Int. J. Bio-resource Stress Manage., 3: 109-115.

Grotmol, S., Berghl, O., Totland, G.K., 1999. Transmission of viral encephalopathy and retinopathy (VER) to yolk-sac larvae of the Atlantic halibut *Hippoglossus hippoglossus*: occurrence of nodavirus in various organs and a possible route of infection. Dis. Aquat. Org., 36: 95-106.

Grotmol, S., Nerland, A.H., Biering, E., Totland, G.K., Nishizawa, T., 2000. Characterisation of the capsid protein gene from a nodavirus strain affecting the Atlantic halibut *Hippoglossus hippoglossus* and design an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay. Dis. Aquat. Org., 39: 79-88.

Grotmol, S., Totland, G.K., Kryvi, H., 1997. Detection of a nodavirus-like agent in heart tissue from reared Atlantic salmon *Salmo salar* suffering from cardiac myopathy syndrome (CMS). Dis. Aquat. Org., 29:79-84.

Grotmol, S., Totland, G.K., Thorud, K., Hjeltnes, B.K., 1997. Vacuolating encephalopathy and retinopathy associated with a nodavirus-like agent: a probable cause of mass

mortality of cultured larval and juvenile Atlantic halibut *Hippoglossus hippoglossus*. Dis. Aquat. Org., 29: 85-97.

Grotmol, S., Totland, G.K., 2000. Surface disinfection of Atlantic halibut *Hippoglossus hippoglossus* eggs with ozonated sea-water inactivates nodavirus and increases survival of the larvae. Dis. Aquat. Org., 36: 95-106.

Grove, S., Faller, R., Soleim, K.B., Dannevig, B.H., 2006. Absolute quantitation of RNA by a competitive real-time RT-PCR method using piscine nodavirus as a model. J. Virol. Methods, 132: 104-112.

Grove, S., Johansen, R., Dannevig, B.H., Reitan, L.J., Ranheim, T., 2003. Experimental infection of Atlantic halibut *Hippoglossus hippoglossus* with nodavirus: tissue distribution and immune response. Dis. Aquat. Org., 53: 211-221.

Gutiérrez, E., Morales-Nin, B., 1986. Time series analysis of daily growth in *Dicentrarchus labrax* L. otoliths. J. Exp. Mar. Biol. Ecol., 103: 163-179.

Haddad-Boubaker, S., Bigarré, L., Bouzgarou, N., Megdich, A., Baud, M., Cabon, J., Chéhida, N.B., 2013. Molecular epidemiology of Betanodaviruses isolated from sea bass and sea bream cultured along the Tunisian coasts. Virus Genes., 46: 412-422.

Haddad-Boubaker, S., Boughdir, W., Sghaier, S., Souissi, J.B., Megdich, A., Dhaouadi, R., Amara, A., Panzarin, V., Fakhfakh, E., 2014. Outbreak of viral nervous necrosis in endangered fish species *Epinephelus costae* and *E. marginatus* in Northern Tunisian coasts. Fish Pathol., 49: 53-6.

Haffray, P., Tsigenopoulos, C.S., Bonhomme, F., Chatain, B., Magoulas, A., Rye, M., Triantafyllidis, A., Triantaphyllidis, C., 2007. European sea bass - *Dicentrarchus labrax*. Genimpact final scientific report 40-46.

Hata, N., Okinaka, Y., Iwamoto, T., Kawato, Y., Mori, K., Nakai, T., 2010. Identification of RNA regions that determine temperature sensitivities in Betanodaviruses. Arch. Virol., 155: 1597-606.

Hedge, A., Chen, C.L., Qin, Q.W., Lam, T.J., Sin, Y.M., 2002. Characterization, pathogenicity and neutralization studies of a nervous necrosis virus isolated from grouper, *Epinephelus tauvina*, in Singapore. Aquaculture, 213: 55-72.

Henderson, C.J., Stevens, T.F., Lee, S.Y., 2016. Assessing the suitability of a non-lethal biopsy punch for sampling fish muscle tissue. Fish. Physiol. Biochem., 1-6.

Henderson, P.A., Seaby, R.M.H., Somes, J.R., 2011. Community level response to climate change: The long-term study of the fish and crustacean community of the Bristol Channel. J. Exp. Biol. Ecol., 400: 78-89.

Hick, P., Schipp, G., Bosmans, J., Humphrey, J., Whittington, R., 2011. Recurrent outbreaks of viral nervous necrosis in intensively cultured barramundi (*Lates calcarifer*) due to horizontal transmission of Betanodavirus and recommendations for disease control. Aquaculture, 319: 41-52.

Hick, P., Tweedie, A., Whittington, R.J., 2011. Optimization of Betanodavirus culture and enumeration in striped snakehead fish cells. J. Vet. Diagn. Invest., 23: 465-475.

Hick, P., Whittington, R.J., 2010. Optimisation and validation of a real-time reverse transcriptase-polymerase chain reaction assay for detection of Betanodavirus. J. Virol. Methods, 163: 368-377.

Hillen, J., Coscia, I., Volckaert, F., 2014. European sea bass (*Dicentrarchus labrax*) - AquaTrace species leaflet.

Hodneland, K., Garca, R., Balbuena, J.A., Zarza, C., Fouz, B., 2011. Real-time RT-PCR detection of Betanodavirus in naturally and experimentally infected fish from Spain. J. Fish Dis., 34: 189-202.

Huang, B., Tan, C., Chang, S.F., Munday, B., Mathew, J.A., Ngoh, G.H., Kwang, J., 2001. Detection of nodavirus in barramundi, *Lates calcarifer* (Bloch), using recombinant coat protein-based ELISA and RT–PCR. J. Fish Dis., 24: 135-141.

Hwang, J., Suh, S., Park, M., Oh, M., Kim, J., Lee, S., Lee, T., 2016. Detection of coat protein gene of nervous necrosis virus using loop-mediated isothermal amplification. Asian Pac. J. Trop. Med., 9: 235-240.

Ichinose, T., Musyoka, T.M., Watanabe, K., Kobayashi, N., 2013. Evaluation of antiviral activity of Oligonol, an extract of *Litchi chinensis*, against Betanodavirus. Drug Discov. Ther., 7: 254-260.

Iwamoto, T., Mori, K., Arimoto, M., Nakai, T., 1999. High permissivity of the fish cell line SSN-1 for piscine nodaviruses. Dis. Aquat. Organ., 39: 37-47.

Iwamoto, T., Nakai, T., Mori, K., Arimoto, M., Furusawa, I., 2000. Cloning of fish cell line SSN-1 for piscine nodavirus. Dis. Aquat. Organ., 43: 81-89.

Jaramillo, D., Hick, P., Deece, K., Tweedie, A., Kirkland, P., Arzey, E., Whittington, R.J., 2016. Comparison of ELISA formats for detection of antibodies specific for nervous necrosis virus (Betanodavirus) in the serum of immunized barramundi *Lates calcarifer* and Australian bass *Macquaria novemaculeata*. Aquaculture, 451: 33-38.

Jaramillo, D., Hick, P., Whittington, R.J., 2017. Age dependency of nervous necrosis virus infection in barramundi *Lates calcarifer* (Bloch). J. Fish Dis., 40: 1089-1101.

Jia, P., Jia, K., Yi, M., 2015. Complete genome sequence of a fish nervous necrosis virus isolated from sea perch (*Lateolabrax japonicus*) in China. Genome Announce 3: e00048-15.

Johansen, R., Sommerset, I., Torud, B., Korsnes, K., Hjortaas, M.J., Nilsen, F., Nerland, A.H., Dannevig, B.H., 2004. Characterization of nodavirus and viral encephalopathy and retinopathy in farmed turbot, *Scophthalmus maximus* (L.). J. Fish Dis., 27: 591–601.

John, K.R., George, M.R., Jeyatha, B., Saravanakumar, R., Sundar, P., Jithendran, K.P., Koppang, E.O., 2014. Isolation and characterization of Indian Betanodavirus strain from infected farm-reared Asian sea bass *Lates calcarifer* (Bloch, 1790) juveniles. Aquaculture Res., 45: 1481-1488.

Johnson, S.C., Sperker, S.A., Leggiadro, C.T., Groman, D.B., Griffiths, S.G., Ritchie, R.J., Cook, M.D., Cusack, R.R., 2002. Identification and Characterization of a Piscine Neuropathy and Nodavirus from Juvenile Atlantic Cod from the Atlantic Coast of North America. J. Aquat. Anim. Health, 14: 124-33.

Jonsson, G., 1992. Islenskir fiskar. Fiolvi, Reykjavik.

Jonsson, N., Gullberg, M., Lindberg, A.M., 2009. Real-time polymerase chain reaction as a rapid and efficient alternative to estimation of picornavirus titers by tissue culture infectious dose 50% or plaque forming units. Microbiol. Immunol., 53: 149-154.

Kai, Y., Chi, S., 2008. Efficacies of inactivated vaccines against Betanodavirus in grouper larvae (*Epinephelus coioides*) by bath immunization. Vaccine, 26: 1450-1457.

Kai, Y., Su, H., Tai, K., Chi, S., 2009. Vaccination of grouper broodfish (*Epinephelus tukula*) reduces the risk of vertical transmission by nervous necrosis virus. Vaccine, 28: 996-1001.

Kamil, A., 2013. Analysis of IgM sub-variants related to ancestral tetraploidy in salmonid fish. Dissertation for the degree of philosophy doctor (PhD) University of Bergen.

Kang, M.S., Oh, M.J., Kim, Y.J., Kawai, K., Jung, S.J., 2003. Establishment and characterization of two new cell lines derived from flounder, *Paralichthys olivaceus* (Temminck & Schlegel) J. Fish Dis., 26: 657-665.

Kara, H.M., Chaoui, L., Derbal, F., Zaidi, R., de Boisseson, C., Baud, M., Bigarré, L., 2014. Betanodavirus-associated mortalities of adult wild groupers *Epinephelus marginatus* (Lowe) and *Epinephelus costae* (Steindachner) in Algeria. J. Fish Dis., 37: 273-278.

Keawcharoen, J., Techangamsuwan, S., Ponpornpisit, A., Lombardini, E.D., Patchimasiri, T., Pirarat, N., 2015. Genetic characterization of a Betanodavirus isolated from a clinical disease outbreak in farm-raised tilapia *Oreochromis niloticus* (L.) in Thailand. J. Fish Dis., 38: 49-54.

Kelley, D.F., 1987Food of bass in U.K. waters. J. Mar. Biol. Assoc., 67:275.

Khanna, V.G., Kannabiran, K., Babu, V.S., Hameed, A.S.S., 2011. Inhibition of fish nodavirus by gymnemagenol extracted from *Gymnema sylvestre*. J. Ocean. Uni. China, 10: 402-408.

Kim, J-O., Kim, S-J., Kim, J-O., Kim, W-S., Oh, M-J., 2018. Distribution of nervous necrosis virus (NNV) in infected sevenband grouper, *Hyporthodus septemfasciatus* by intramuscular injection or immersion challenge. Aquaculture, 489: 1-8.

Kokawa, Y., Takami, I., Nishizawa, T., Yoshimizu, M., 2008. A mixed infection in sevenband grouper *Epinephelus septemfasciatus* affected with viral nervous necrosis (VNN). Aquaculture, 284: 41-45.

Krishnan, A.N., Bhuvaneswari, T., Praveena, P.E., Jithendran, K.P., 2016. Paper-based archiving of biological samples from fish for detecting Betanodavirus. Archives of Virology Official J. Virol. Div. Inter. Union Microbiol. Soc., 161: 2019-2024.

Krishnan, K., Khanna, V.G., Hameed, S., 2010. Antiviral Activity of Dasyscyphin C Extracted from *Eclipta prostrata* Against Fish Nodavirus. J. Antivir. Antiretrovir., 1: 29-32.

Krkošek, M., Morton, A., Volpe, J.P., 2005. Nonlethal Assessment of Juvenile Pink and Chum Salmon for Parasitic Sea Lice Infections and Fish Health. Trans. Am. Fish. Soc., 134: 711-716.

Labarca, C.C., Makhutu, M., Lumsdon, A.E., Thompson, K.D., Jung, R., Kloas, W., Knopf, K., 2015. The adjuvant effect of low frequency ultrasound when applied with an inactivated *Aeromonas salmonicida* vaccine to rainbow trout (*Oncorhynchus mykiss*). Vaccine, 33: 1369-1374.

Laffaille, P., Lefeuvre, J.C., Schricke, M.T., Feunteun, E., 2001. Feeding Ecology of 0-Group Sea Bass, *Dicentrarchus labrax*, in Salt Marshes of Mont Saint Michel Bay (France). Estuaries, 24: 116-125.

Lai, Y-S., Murali, S., Chiu, H-C., Ju, H-Y., Lin, Y-S., Chen, S-C., Guo, I-C., Fang, K., Chang, C-Y., 2001. Propagation of yellow grouper nervous necrosis virus (YGNNV) in a new nodavirus-susceptible cell line from yellow grouper, *Epinephelus awoara* (Temminck & Schlegel), brain tissue. J. Fish Dis., 24: 299-309.

Landry, M., Leland, D., 2016. Primary Isolation of Viruses. In Loeffelholz M, Hodinka R, Young S, Pinsky B, Clinical Virology Manual, Fifth Edition, 79-93. ASM Press, Washington, DC.

Léon, F.J.G., Chikhi, L., Bonhomme, F., 1997. Microsatellite polymorphism and population subdivision in natural populations of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758). Mol. Eco., 6: 51-62.

Levenda, K., 2013. Legislation to protect the welfare of fish. Animal Law 20: 110.

Lin, C., Lin, J.H., Chen, M., Yang, H., 2007. An oral nervous necrosis virus vaccine that induces protective immunity in larvae of grouper (*Epinephelus coioides*). Aquaculture, 268: 265-273.

Lin, L., He, J., Mori, K-I., Nishioka, T., Wu, J.L., Weng, S., Mushiake, K., Arimoto, M., Nakai T., 2001. Mass mortalities associated with viral nervous necrosis in hatchery-reared groupers in the People's Republic of China. Fish Pathol., 36: 186-8.

Lopéz-Jimena, B., Chérif, N., Garcia-Rosado, E., Infante, C., Cano, I., Castro, D., Hammami, S., Borrego, J.J., Alonso, M.C., 2010. A combined RT-PCR and dot-blot hybridization method reveals the coexistence of SJNNV and RGNNV Betanodavirus genotypes in wild meagre (*Argyrosomus regius*). J. Appl. Microbiol., 109: 1361-1369.

Lopéz-Vázquez, C., Dopazo, C.P., Olveira, J.G., Barja, J.L., Bandín, I., 2006. Journal Development of a rapid, sensitive and non-lethal diagnostic assay for the detection of viral haemorrhagic septicaemia virus. J. Virol. Methods, 133: 167-174.

Manin, B.O., Ransangan, J., 2011. Experimental evidence of horizontal transmission of Betanodavirus in hatchery-produced Asian sea bass, *Lates calcarifer* and brown-marbled grouper, *Epinephelus fuscoguttatus* fingerling. Aquaculture, 321: 157-165.

Maeno, Y., De la Peña, L., Cruz-Lacierda, E., 2002. Nodavirus infection in hatchery-reared orange-spotted grouper *Epinephelus coioides*: first record on viral nervous necrosis in the Philippines. Fish Pathol., 37: 87-89.

Martinelli-Liedtke, T.L., Shively, R.S., Holmberg, G.S., Sheer, M.B., Schrock, R.M., 1999. Nonlethal Gill Biopsy Does Not Affect Juvenile Chinook Salmon Implanted with Radio Transmitters. North Am. J. Fish. Manage., 19: 856-859.

Monaghan, S.J., Thompson, K.D., Adams, A., Bergmann, S.M., 2014. Sensitivity of seven PCRs for early detection of koi herpesvirus in experimentally infected carp, *Cyprinus carpio* L., by lethal and non-lethal sampling methods. J. Fish Dis., 38: 303-319.

Monte, M.M., Urquhart, K., Secombes, C.J., Collet, B., 2016. Individual monitoring of immune responses in rainbow trout after cohabitation and intraperitoneal injection challenge with *Yersinia ruckeri*. Fish Shellfish Immunol., 55: 469-478.

Moody, N.J., Horwood, P.F., McHardy, S., 2004. Aquatic animal health subprogram: development of diagnostic tests for the detection of nodavirus. Fisheries Research and Development Corporation Project 2001/626 Final Report. 133.

Moody, N.J., Horwood, P.F., Reynolds, A., Mahony, T.J., Anderson, I.G., Oakey, H.J., 2009. Phylogenetic analysis of Betanodavirus isolates from Australian finfish. Dis. Aquat. Org., 87: 151-60.

Mori, K-I., Nakai, T., Muroga, K., Arimoto, M., Mushiake, K., Furusawa, I., 1992. Properties of a new virus belonging to nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. Virol., 187: 368-71.

Munday, B.L., Kwang, J., Moody, N., 2002. Betanodavirus infections of teleost fish: a review. J. Fish Dis., 25: 127-142.

Munday, B.L., Langdon, J.S., Hyatt, A., Humphrey, J.D., 1992. Mass mortality associated with a viral-induced vacuolating encephalopathy and retinopathy of larval and juvenile barramundi, *Lates calcarifer*. Aquaculture, 103: 197-211.

Munday, B.L., Nakai, T., 1997. Nodaviruses as pathogens in larval and juvenile marine finfish. World J. Microb. Biot., 13: 375-381.

Mushiake, K., Nishizawa, T., Nakai, T., Furusawa, I., Muroga, K., 1994. Control of VNN in striped jack: selection of spawners based on the detection of SJNNV gene by polymerase chain reaction (PCR). Fish Pathol., 3: 177-182.

Nagai, T., Nishizawa, T., 1999. Sequence on the non-structural protein gene encoded by RNA 1 of striped jack nervous necrosis virus. J. Gen. Virol., 80: 3019-3022.

Nakai, T., Mori, K., Sugaya, T., Nishioka, T., Mushiake, K., Yamashita, H., 2009. Current Knowledge on Viral Nervous Necrosis (VNN) and its Causative Betanodaviruses. Israeli J. Aqua., 61: 198-207.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge M.C.M., Cay, J., Folke, C., Lubchenco, J., Mooney, H., Troell, M., 2000. Effect of aquaculture on world fish supplies. Nature, 405: 1017-1024.

Nazari, A., Hassan, M.D., Bovo, G., Zorriehzahra, M.J., Azmi, T.I., Arshad, S.S., 2014. Pathogenicity of viral nervous necrosis virus for guppy fish, *Poecilia reticulata*. Iran. J. Fish. Sci., 13: 168-177.

Nerland, A.H., Skaar, C., Eriksen, T.B., Bleie, H., 2007. Detection of nodavirus in seawater from rearing facilities for Atlantic halibut *Hippoglossus hippoglossus* larvae. Dis. Aquat. Org., 73: 201-205.

Nguyen, H.D., Mekuchi, T., Imura, K., Nakai, T., Nishizawa, T., Muroga, K., 1994. Ocurrence of viral nervous necrosis (VNN) in hatchery-reared juvenile japanese flounder *Paralichthys olivaceus*. Fisheries Sci., 60: 551-554.

Nguyen, H.D., Nakai, T., Muroga, K., 1996. Progression of striped jack nervous necrosis virus (SJNNV) infection in naturally and experimentally infected striped jack *Pseudocaranx dentex* larvae. Dis. Aquat. Org., 24: 99-105.

Nishizawa, T., Furuhashi, M., Nagai, T., Nakai, T., Muroga, K., 1997. Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. Appl. Environ. Microbiol., 63: 1633–1636.

Nishizawa, T., Mori, K., Furuhashi, M., Nakai, T., Furusawa, I., Muroga, K., 1994. Polymerase chain reaction (PCR) 1016 amplification of RNA of striped jack nervous necrosis virus (SJNNV). Dis. Aquat. Organ., 18: 103-107.

Nishizawa, T., Mori, K., Furuhashi, M., Nakai, T., Furusawa, I., Muroga, K., 1995. Comparison of the coat protein genes of five fish nodaviruses, the causative agents of viral nervous necrosis in marine fish. J. Gen. Virol., 76: 1563–1569.

Nishizawa, T., Takami, I., Kokawa, Y., Yoshimizu, M., 2009. Fish immunization using a synthetic double-stranded RNA Poly (I:C), an interferon inducer, offers protection against RGNNV, a fish nodavirus. Dis. Aquat. Org., 83: 115-122.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic. Acids Res., 28: 63-63.

Nuñez-Ortiz, N., Stocchi, V., Toffan, A., Sood, N., Buonocore, F., Picchietti, S., Papeschi, C., Taddei, A.R., Thompson, K.D., Scapigliati, G., 2016a. Quantitative immunoenzymatic

detection of viral encephalopathy and retinopathy virus (Betanodavirus) in sea bass *Dicentrarchus labrax*. J. Fish Dis., 39: 821-831.

Nuñez-Ortiz, N., Pascoli, F., Picchietti, S., Buonocore, F., Bernini, C., Toson, M., Scapigliati, G., Toffan, A., 2016b. A formalin inactivated immunogen against viral encephalopathy and retinopathy (VER) disease in European sea bass (*Dicentrarchus labrax*): immunological and protection effects. Vet. Res., 47: 89.

Nylund, A., Karlsbakk, E., Nylund, S., Isaksen, T.E., Karlsen, M., Korsnes, K., Handeland, S., Martinsen, R., Pedersen, T.M., Ottem, K.F., 2008. New clade of Betanodaviruses detected in wild and farmed cod (*Gadus morhua*) in Norway. Arch. Virol., 153: 541-547.

O.I.E., 2016. Chapter 2.3.12. Viral Encephalopathy and Retinopathy Manual of Diagnostic Tests for Aquatic Animals.

Oidtmann, B., Peeler, E., Lyngstad, T., Brunb, E., Jensen, B.B., Stark, K.D.C., 2013. Risk-based methods for fish and terrestrial animal disease surveillance. Prevent. Vet. Med., 112: 13-26.

Olveira, J.G., Souto, S., Dopazo, C.P., Thiery, R., Barja, J.L., Bandin, I., 2009. Comparative analysis of both genomic segments of Betanodaviruses isolated from epizootic outbreaks in farmed fish species provides evidence for genetic reassortment. J. Gen. Microbiol., 90: 2940-2951.

Panzarin, V., Cappellozza, E., Mancin, M., Milani, A., Toffan, A., Terregino, C., Cattoli, G., 2014. In vitro study of the replication capacity of the RGNNV and the SJNNV Betanodavirus genotypes and their natural reassortants in response to temperature. Vet. Res., 45: 56.

Panzarin, V., Patarnello, P., Mori, A., Rampazzo, E., Cappellozza, E., Bovo, G., Cattoli, G., 2010. Development and validation of a real-time Taq-man PCR assay for the detection of Betanodavirus in clinical specimens. Arch. Virol., 155: 1193-1203.

Parameswaran, V., Kumar, S.R., Ahmed, V.P.I., Hameed, A.S.S., 2008. A fish nodavirus associated with mass mortality in hatchery-reared Asian sea bass, *Lates calcarifer*. Aquaculture, 275: 366-369.

Pawson, M.G., Pickett, G.D., Leballeur, J., Brown, M., Fritsch, M., 2007. Migrations, fishery interactions, and management units of sea bass (*Dicentrarchus labrax*) in Northwest Europe – ICES. J. Mar. Sci., 64: 332-345.

Pawson, M.G., Pickett, G.D., 1996. The annual pattern of condition and maturity in bass (*Dicentrarchus labrax* L.) in waters around the UK. J. Mar. Biol. Assoc. U.K, 76: 107-126.

Pawson, M.G., 1992. Climatic influences on the spawning success, growth, and recruitment of bass (*Dicentrarchus labrax* L.) in British waters. - ICES mar. Sei. Symp. 195: 388-392.

Péducasse, S., Castric, J., Thiery, R., Jeffroy, J., Le Ven, A., Laurencin, F.B., 1999. Comparative study of viral encephalopathy and retinopathy in juvenile sea bass *Dicentrarchus labrax* infected in different ways. Dis. Aquat. Org., 36: 11-20.

Pickett, G.D., Kelley, D.F., Pawson, M.G., 2004. The patterns of recruitment of sea bass, *Dicentrarchus labrax* L. from nursery areas in England and Wales and implications for fisheries management. Fisheries Res., 68: 329-342.

Pickett, G.D., Pawson, M.G., 1994. Sea bass Biology, exploitation and conservation. Fish and Fisheries Series 12, Chapman and Hall, London. First edition.

Qin, Q.W., Wu, T.H., Jia, T.L., Hegde, A., Zhang, R.Q., 2006. Development and characterization of a new tropical marine fish cell line from grouper, *Epinephelus coioides* susceptible to iridovirus and nodavirus. J. Virol. Methods, 131: 58-64.

Ransangan, J., Manin, B.O., 2010. Mass mortality of hatchery-produced larvae of Asian sea bass, *Lates calcarifer* (Bloch), associated with viral nervous necrosis in Sabah, Malaysia. Vet. Microbiol., 145:153-157.

Ravi, M., Hameed, A.S.S., 2015. Experimental transmission of Macrobrachium rosenbergii nodavirus (MrNV) and extra small virus (XSV) in *Macrobrachium malcolmsonii* and *Macrobrachium rude*. Aquaculture Int., 23: 195-201.

Roche, H., Bogé, G., 1996. Fish Blood Parameters as a Potential Tool for Identification of Stress Caused by Environmental Factors and Chemical Intoxication. Mar. Environ. Res., 41: 27-43.

Ruyet, J.P.L., Mahé, K., Bayon, N.L., Delliou, H.L., 2004. Effects of temperature on growth and metabolism in a Mediterranean population of European sea bass, *Dicentrarchus labrax*. Aquaculture, 237: 269-280.

Sanderson, B.L., Tran, C.D., Coe, H.J., Pelekis, V., Steel, E.A., Reichert, W.L., 2009. Nonlethal Sampling of Fish Caudal Fins Yields Valuable Stable Isotope Data for Threatened and Endangered Fishes. Trans. Am. Fish. Soc., 138: 1166-1177.

Shetty, M., Maiti, B.S., Santhosh, K., Venugopal, M.N., Karunasagar, I., 2012. Betanodavirus of Marine and Freshwater Fish: Distribution, Genomic Organization, Diagnosis and Control Measures. Indian J. Virol., 23:114-123.

Skliris, G.P., Kondiris, J.V., Sideris, D.C., Shinn, A.P., Starkey, W.G., Richards, R.H., 2001. Phylogenetic and antigenic characterization of the new fish nodavirus isolates from Europe and Asia. Virus Res., 75: 59-67.

Skliris, G.P., Richards, R.H., 1999. Induction of nodavirus disease in sea bass, *Dicentrarchus labrax*, using different infection models. Virus Res., 63: 85-93.

Sommer, A-I., Strand, M.A., Rasmussen, E., Mennen, S., 2004. Susceptibility of spotted wolffish *Anarhichas minor* to experimental infection with nodavirus and infectious pancreatic necrosis virus. Dis. Aquat. Org., 59: 101-108.

Sommerset, I., Krossoy, B., Biering, E., Frost, P., 2005. Vaccines for fish in aquaculture. Future Drugs Ltd. Review.

Souto, S., Lopéz-Jimena, B., Alonso, M.C., García-Rosado, E., Bandín, I., 2015a. Experimental susceptibility of European sea bass and Senegalese sole to different Betanodavirus isolates. Vet. Microb., 177: 53-61.

Souto, S., Oliveira, J.G., Bandín, I., 2015b. Influence of temperature on Betanodavirus infections in Senegalese sole (*Solea senegalensis*). Vet. Microb., 179: 162-167.

Spitz, J., Chouvelon, T., Cardinaud, M., Kostecki, C., Lorance, P., 2013. Prey preferences of adult sea bass *Dicentrarchus labrax* in the north eastern Atlantic: implications for bycatch of common dolphin Delphinus delphis – ICES. J. Mar. Sci. 70: 452-461.

Starkey, W.G., Ireland, J.H., Muir, K.F., Jenkins, M.E., Roy, W.J., Richards, R.H., Ferguson, H.W., 2001. Nodavirus infection in Atlantic cod and Dover sole in the UK. Vet Rec., 149: 179-81.

Starkey, W.G., Ireland, J.H., Muir, K.F., Shinn, A.P., Richards, R.H., Ferguson, H.W., 2000. Isolation of nodavirus from Scottish farmed halibut, *Hippoglossus hippoglossus* (L.). J. Fish Dis., 23: 418-22.

Su, Y., Xu, H., Ma, H., Feng, J., Wen, W., Guo, Z., 2015. Dynamic distribution and tissue tropism of nervous necrosis virus in juvenile pompano (*Trachinotus ovatus*) during early stages of infection. Aquaculture, 440: 25-31.

Subasinghe, R.P., Soto, D., Jia, J., 2009. Global Aquaculture and its role in sustainable development. Reviews in Aquaculture, 1: 2-9.

Subasinghe, R.P., 2005. Epidemiological approach to aquatic anima health management: opportunities and challenges for developing countries to increase aquatic production through aquaculture. Prevent. Vet. Med., 67: 117-124.

Sudhakaran, R., Yoganandhan, K., Ishaq Ahmed, V.P., Sahul Hameed, A.S., 2006. Artemia as a Possible Vector for *Macrobrachium rosenbergii* Nodavirus (MrNV) and Extra Small Virus Transmission (XSV) to *Macrobrachium rosenbergii* Post-Larvae. Dis. Aquat. Org., 70: 161-166.

Sugaya, T., Mori, K., Nishioka, T., Masuma, S., Oka, M., Mushiake, K., Okinaka, Y., Nakai, T., 2009. Genetic heterogeneity of Betanodaviruses in juvenile production trials of Pacific bluefin tuna, *Thunnus orientalis* (Temminck & Schlegel). J. Fish Dis., 32: 815-823.

Sumpter, J.P., Lincoln, R.F., Bye, V.J., Carrgher, J.F., Le Bail, P.Y., 1991. Plasma growth hormone levels during sexual maturation in diploid and triploid rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endo., 83: 103-110.

Sunyer, J.O., 2013. Fishing for mammalian paradigms in the teleost immune system. Nature Immunol., 14: 320-326.

Suthindhiran, K., Babu, V.S., Kannabiran, K., Ahmed, V.P.I., Hameed, A.S.S., 2011. Anti-fish nodaviral activity of furan-2-yl acetate extracted from marine Streptomyces spp. Nat. Prod. Res., 25: 834-843.

Tan, C., Huang, B., Chang, S.F., Ngoh, G.H., Munday, B., Chen, S.C., Kwang, J., 2001. Determination of the complete nucleotide sequences of RNA1 and RNA2 from greasy grouper (*Epinephelus tauvina*) nervous necrosis virus, Singapore strain. J. Gen. Virol., 82: 647-653.

Tan, C., Sun, D., Tan, H., Liu, W., Luo, G., Wei, X., 2018. Effects of Stocking Density on Growth, Body Composition, Digestive Enzyme Levels and Blood Biochemical Parameters of *Anguilla marmorata* in a Recirculating Aquaculture System. Turk. J. Fish. Aquat. Sci., 18: 9-16.

Tavares, G.C., Costa, F.A.A., Santos, R.R.D., Barony, G.M., Leal, C.A.G., Figueiredo, H.C.P., 2015. Nonlethal sampling methods for diagnosis of *Streptococcus agalactiae* infection in Nile tilapia, *Oreochromis niloticus* (L). Aquaculture, 454: 237-242.

Thiéry, R., Cozien, J., de Boisséson, C., Kerbart-Boscher, S., Névarez, L., 2004. Genomic classification of new Betanodavirus isolates by phylogenetic analysis of the coat protein gene suggests a low host-fish species specificity. J Gen. Virol., 85: 3079-3087.

Thiéry, R., Raimond, J.C., Cástric, J., 1999. Natural outbreak of viral encephalopathy and retinopathy in juvenile sea bass, *Dicentrarchus labrax*: study by nested reverse transcriptase-polymerase chain reaction. Virus Res., 63: 11-17.

Toffan, A., Panzarin, V., Toson, M., Cecchettin, K., Pascoli, F., 2016. Water temperature affects pathogenicity of different Betanodavirus genotypes in experimentally challenged *Dicentrarchus labrax*. Dis. Aquat. Org., 119: 231-238.

Toffan, A., Pascoli, F., Pretto, T., Panzarin, V., Abbadi, M., Buratin, A., Quartesan, R., Gijón, D., Padrós, F., 2017. Viral nervous necrosis in gilthead sea bream (*Sparus aurata*) caused by reassortant Betanodavirus RGNNV/SJNNV: an emerging threat for Mediterranean aquaculture. Sci. Rep., 7: 46755.

Toffolo, V., Negrisolo, E., Maltese, C., Bovo, G., Belvedere, P., Colombo, L., Valle, L., 2007. Phylogeny of Betanodaviruses and molecular evolution of their RNA polymerase and coat proteins. Mol. Phylogenet. Evol., 43: 298-308.

Ucko, M., Colorni, A., Diamant, A., 2004. Nodavirus infections in Israeli mariculture. J. Fish Dis., 27: 459-469.

Valero, Y., Arizcun, M., Esteban, M.Á., Bandín, I., Olveira, J.G., Patel, S., Cuesta, A., Chavez-Pozo, E., 2015. Nodavirus Colonizes and Replicates in the Testis of Gilthead Seabream and European Sea Bass Modulating Its Immune and Reproductive Functions. PLoSONE10, 12: e0145131.

Valero, Y., Awad, E., Buonocore, F., Arizcun, M., Esteban, M.A., Meseguer, J., Chavez-Pozo, E., Cuesta, A., 2016. An oral chitosan DNA vaccine against nodavirus improves transcription of cell-mediated cytotoxicity and interferon genes in the European sea bass juveniles gut and survival upon infection. Dev. Comp. Immunol., 65: 64-72.

Valero, Y., Boughlala, B., Arizcun, M., Patel, S., Fiksdal, I.U., Esteban, M.A., Juan, J., Meseguer, J., Chaves-Pozo, E., Cuesta, A., 2018. Genes related to cell-mediated cytotoxicity and interferon response are induced in the retina of European sea bass upon intravitreal infection with nodavirus. Fish Shellfish Immunol., 74: 627-636.

Valle, L.D., Toffolo, V., Lamprecht, M., Maltese, C., Bovo, G., Belvedere, P., Colombo, L., 2005. Development of a sensitive and quantitative diagnostic assay for fish nervous necrosis virus based on two-target real-time PCR. Vet. Microbiol., 110: 167-179.

Vandeputte, M., Dupont-Nivet, M., Haffray, P., Chavanne, H., Cenadelli, S., Parati, K., Vidal, M., Vergnet, A., Chatain, B., 2009. Response to domestication and selection for growth in the European se bass (*Dicentrarchus labrax*) in separate and mixed tanks. Aquaculture, 286: 20-27.

Varsamos, S., Connes, R., Diaz, J.P., Barnabé, G., Charmantier, G., 2001. Ontogeny of osmoregulation in the European sea bass, *Dicentrarchus labrax* L. Mar. Bio., 138: 909-915.

Vendramin, N., Patarnello, P., Toffan, A., Panzarin, V., Cappellozza, E., Tedesco, P., Terlizzi, A., Terregino, C., Cattoli, G., 2013. Viral Encephalopathy and Retinopathy in groupers (*Epinephelus* spp.) in southern Italy: a threat for wild endangered species? Vet. Res., 9: 20.

Vendramin, N., Toffan, A., Mancin, M., Cappellozza, E., Panzarin, V., Bovo, G., Cattoli, G., Capua, I., Terregino, C., 2014. Comparative pathogenicity study of ten different Betanodavirus strains in experimentally infected European sea bass, *Dicentrarchus labrax* (L.). J. Fish Dis., 37: 371-383.

Volckaert, F.A.M., Batargias, C., Canario, A., Chatzipli, D., Chistiakov, D., Haley, C., Libertini, A., Tsigenopoulos, C., 2008. European Sea Bass, (Chapter 5): 234-147.

Wang, Y., Kung, C., Chen, J., 2010. Antiviral activity by fish antimicrobial peptides of epinecidin-1 and hepcidin 1-5 against nervous necrosis virus in medaka. Peptides, 31: 1026-1033.

Wang, Y., Kung, C., Chi, S., Chen, J., 2010. Inactivation of nervous necrosis virus infecting grouper (*Epinephelus coioides*) by epinecidin-1 and hepcidin 1–5 antimicrobial peptides, and downregulation of Mx2 and Mx3 gene expressions. Fish & Shellfish Immunol., 28: 113-120.

Watts, M., Munday, B.L., Burke, C.M., 2001. Immune responses of teleost fish. Aust. Vet. J., 79: 570-574.

Watts, M., 2000. Immunology of southern Bluefin tuna [PhD Thesis]. University of Tasmania, Australia.

Wen, C.M., Lee, C.W., Wang, C.S., Cheng, Y.H., Huang, H.Y., 2008. Development of two cell lines from *Epinephelus coioides* brain tissue for characterization of Betanodavirus and megalocytivirus infectivity and propagation. Aquaculture, 278: 10.

Workenhe, S.T., Rise, M.L., Kibenge, M.J.T., Kibenge, F.S.B., 2010. The fight between the teleost fish immune response and aquatic viruses. Mol. Immunol., 47: 2525-2536.

Wu, M.S., Chen, C.W., Lin, C.H., Tzeng, C.S., Chang, C.Y., 2012. Differential expression profiling of orange-spotted grouper larvae, *Epinephelus coioides* (Hamilton), that survived a Betanodavirus outbreak. J. Fish Dis., 35: 215-225.

Yoshikoshi, K., Inoue, K., 1990. Viral necrosis in hatchery reared larvae and juveniles of Japanese parrotfish, *Oplegnatus fasciatus* (Temminck & Schlegel). J. Fish Dis., 13: 69-77.

Yuasa, K., Koesharyani, I., Mahardika, K., 2007. Effect of high water temperature on Betanodavirus infection of fingerling humpback grouper *Cromileptes altivelis*. Fish Pathol., 42: 219-21.

Zar, J.H, (2010). Biostatistical analysis, (5th ed.). Upper Saddle River, N.J.: Prentice-Hall/Pearson.

7. Appendix

Appendix I

ELISA reagent formulation

Poly-L-lysine (Solution at 0.01%)

Add 1.5 mL of Poly-L-lysine solution dissolved in 13.5 mL of coating buffer.

Coating Buffer (Carbonate-bicarbonate solution, pH 9.6)

This solution must be made fresh. For preparing the coating buffer dissolve one tablet of carbonate-bicarbonate buffer (Sigma) in 100 ml of distilled water.

Glutaraldehyde (Solution at 0.05%)

Add 10 µl of glutaraldehyde solution to 10 ml of PBS (1x).

Low Salt Wash Buffer *(LSWB)* (0.02 M Tris; 0.38 M NaCl; 0.05 % Tween 20; pH 7.3)

For preparing	1000 mL	500 mL
Trizma base	24.2 g	12.1 g
Sodium chloride	222.2 g	111.1 g
Tween 20	5 mL	2.5 mL

Dissolve in distilled water and adjust pH to 7.3 with HCl.
This solution is 10x concentrate (must be diluted in distilled water).

Blocking buffer (3% casein)

This solution must be made fresh. Add 3 g of skimmed milk (Marvel) to 100 mL of distilled water.

Phosphate Buffered Saline *(PBS)* (0.02 M phosphate; 0.15 M NaCl)

For preparing	1000 mL	500 mL
Sodium chloride	8.77 g	4.385 g
Sodium phosphate monobasis dehydrate	0.876 g	0.438 g
Sodium phosphate dibasic dehydrate	2.56 g	1.28 g

Dissolve in distilled water and adjust pH to 7.2 with HCL.
This solution is 10x concentrate (must be diluted in distilled water).

Antibody Buffer (solution at 1%)

This solution must be made fresh. Add 1 g of BSA to 100 ml of PBS (1x).

Conjugate Buffer (solution at 1%)

This solution must be made fresh. Add 1 g of BSA to 100 mL of LSWB (1x).

High Salt Wash Buffer *(HSWB)* (0.02M Tris; 0.5 M NaCl; 0.1% Tween 20)

For preparing	1000 mL	500 mL
Trizma base	24.2 g	12.1 g
Sodium chloride	292.2 g	146.1 g
Tween 20	10 mL	5 mL

Dissolve in distilled water and adjust pH to 7.7 with HCL.
This solution is 10x concentrate (must be diluted in distilled water).

Substrate

To 15 mL of substrate buffer add 150 µL of substrate solution and 5 µL of hydrogen peroxidise.

Substrate buffer (0.1 M Citric acid; 0.1 M Sodium acetate)

For preparing	1000 mL	500 mL
Citric acid monobasic	21 g	10.5 g
Sodium acetate	8.2 g	4.1 g

Dissolve in distilled water and adjust the pH to 5.4 with NaOH.

Substrate Solution (42 mM TMB, 3'3'5'5' – tetramethylbenzidine dihydrochloride)

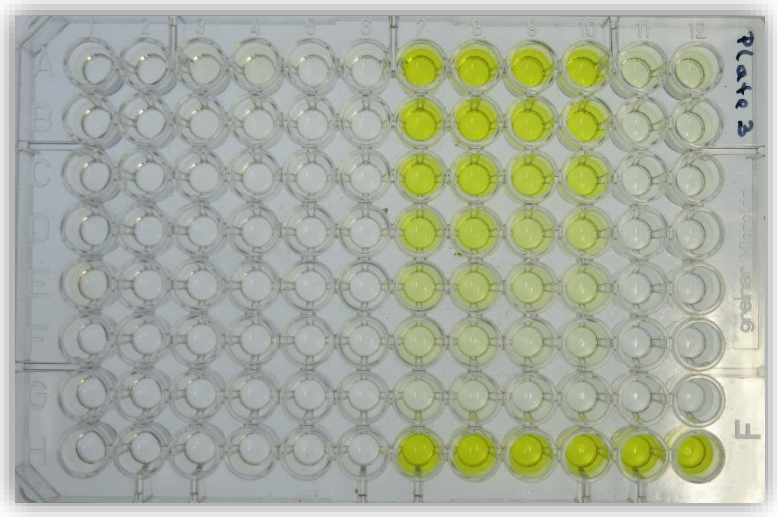
TMB	Final Volume
0.07896 g	6 mL
0.0658 g	5 mL
0.03948 g	3 mL

This solution must be made fresh.
Prepare the TMB solution in distilled water. To the TMB solution add acetic acid in a proportion 2:1 (water:acid).

Stop Reagent (H_2SO_4 2 M)

For 50 ml of solution add 5.5 ml of sulphuric acid (98%) to 44.5 ml of distilled water.

ELISA Photo (with controls and infected samples):



Appendix II

Table I: Individual values obtained with the ELISA assay for Study 2 (non-lethal study).

Study 2

Treatment	IM Infected		Immersion Infected		IM Control		Immersion Control	
Days	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**
7	8	0,415			0	0		
7	3	0			0	0		
7	9	0,854			0	0		
7	8	0,571			0	0		
7	8	0,881			0	0		
15	8	0,661	0	0	0	0	0	0
15	6	0,104	0	0	0	0	0	0
15	10	0,903	0	0	0	0	0	0
15	8	1,126	8	0,743	0	0	0	0
15	9	0,590			0	0		
30	7	0,392	6	0,797	0	0	0	0
30	7	0,324	6	0,775	0	0	0	0
30	5	0	9	0,008	0	0	0	0
30	9	0,783	9	0,191	0	0	0	0
30	10	0,866			0	0		

Individual values

Table II: Individual values obtained with the ELISA assay for Study 1 (mortality study).

Study 1

Treatment	IM Infected		Immersion Infected		IM Control		Immersion Control	
Days	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**
30	11	0,762	10	0,875	0	0	0	0

Individual values

30	9	0,670	8	0,836	0	0	0	0
30	9	0,802	0	0	0	0	0	0
30	10	0,955	8	0,582	0	0	0	0

Table III: Mean values obtained with the ELISA assay for Study 2 (non-lethal study).

Study 2

Treatment	**IM Infected**		**Immersion Infected**		**IM Control**		**Immersion Control**	
Days	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**
7	7	0,544			0	0		
15	8	0,677	2	0,186	0	0	0	0
30	8	0,473	8	0,443	0	0	0	0

Means

Table IV: Mean values obtained with the ELISA assay for Study 1 (mortality study).

Study 1

Treatment	**IM Infected**		**Immersion Infected**		**IM Control**		**Immersion Control**	
Days	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**	**Log_2+1**	**OD 450nm**
30	10	0,797	6	0,573	0	0	0	0

Means

Appendix III

Table I: Statistical analysis for ELISA Study 1 (mortality Study) data utilizing a T-student test.

Teste de amostras independentes

		Teste de Levene para igualdade de variâncias		Teste-t para Igualdade de Médias						
		F	Sig.	t	gl	Sig. (bilateral)	Diferença média	Erro padrão da diferença	95% Intervalo de Confiança da Diferença	
									Inferior	Superior
- Log_2+1	Variâncias iguais assumidas	4,348	0,082	1,433	6	**0,202**	3,250	2,268	-2,301	8,801
	Variâncias iguais não assumidas			1,433	3,279	0,240	3,250	2,268	-3,634	10,134

Table II: Statistical analysis for ELISA Study 2 (non-lethal Study) utilizing a LSD Test.

Comparações por Método Pairwise

Variável dependente:	**-Log_2+1**				
Treatment		**Diferença média (I-J)**	**Erro**	**Sig.[b]**	**95% Intervalo de Confiança para Diferença[b]**

						Limite inferior	Limite superior
IM Infected	15	30	0,600	1,521	0,699	-2,663	3,863
	30	15	-0,600	1,521	0,699	-3,863	2,663
Immersion Infected	15	30	-5,500*	1,701	0,006	-9,148	-1,852
	30	15	5,500*	1,701	0,006	1,852	9,148

Baseado em médias marginais estimadas

*. A diferença média é significativa no nível ,05.

b. Ajustamento para diversas comparações: Diferença Menos Significativa (equivalente a nenhum ajustamento).

Table III: Statistical analysis for ELISA Study 2 (non-lethal Study) utilizing a LSD Test;

Comparações por Método Pairwise

Variável dependente - Log_2+1

Days			**Diferença média (I-J)**	**Erro**	**Sig.[b]**	**95% Intervalo de Confiança para Diferença[b]**	
						Limite inferior	**Limite superior**
15	**IM Infected**	**Immersion Infected**	6,200*	1,614	0,002	2,739	9,661
	Immersion Infected	**IM Infected**	-6,200*	1,614	0,002	-9,661	-2,739
30	**IM Infected**	**Immersion Infected**	0,100	1,614	0,951	-3,361	3,561
	Immersion Infected	**IM Infected**	-0,100	1,614	0,951	-3,561	3,361

Baseado em médias marginais estimadas

*. A diferença média é significativa no nível ,05.

b. Ajustamento para diversas comparações: Diferença Menos Significativa (equivalente a nenhum ajustamento).

Table IV: Statistical analysis for qPCR data collected in Study 2 (non-lethal study) for the blood.

Testes de efeitos entre sujeitos[a]

Variável dependente:	**Ct Values**				
Origem	**Tipo III Soma dos Quadrados**	**gl**	**Quadrado Médio**	**F**	**Sig.**
Modelo corrigido	564,626[b]	3	188,209	0,962	0,435
Intercepto	1220,841	1	1220,841	6,237	0,024
Day	462,113	1	462,113	2,361	0,144
Treat	18,126	1	18,126	0,093	0,765
Day * Treat	84,386	1	84,386	0,431	0,521
Erro	3131,717	16	195,732		
Total	4917,184	20			
Total corrigido	3696,343	19			

a. Organs = Blood

b. R Quadrado = ,153 (R Quadrado Ajustado = -,006)

Table V: Statistical analysis for qPCR data collected in Study 2 (non-lethal study) for the brain.

Testes de efeitos entre sujeitos[a]

Variável dependente:	**Ct Values**				
Origem	**Tipo III Soma dos Quadrados**	**gl**	**Quadrado Médio**	**F**	**Sig.**
Modelo corrigido	260,963[b]	5	52,193	0,742	0,601

Intercepto	3415,170	1	3415,170	48,551	0,000
Day	73,527	2	36,764	0,523	0,601
id_treat	65,700	1	65,700	0,934	0,345
Day * id_treat	142,809	2	71,405	1,015	0,380
Erro	1406,832	20	70,342		
Total	5144,850	26			
Total corrigido	1667,794	25			
a. Organs = Brain					

Table VI: Statistical analysis for qPCR data collected in Study 2 (non-lethal study) for the caudal fin utilizing a Tukey HSD Test.

Comparações múltiplas[a]

Variável dependente

Tukey HSD

Day	**Diferença média (I-J)**		**Erro**	**Sig.**	**Intervalo de Confiança 95%**	
					Limite inferior	**Limite superior**
7	15	21,1160*	5,18388	**0,002**	8,0497	34,1824
	30	17,5620*	5,18388	**0,007**	4,4957	30,6284
15	7	-21,1160*	5,18388	**0,002**	-34,1824	-8,0497
	30	-3,5540	5,18388	0,774	-16,6203	9,5123
30	7	-17,5620*	5,18388	**0,007**	-30,6284	-4,4957
	15	3,5540	5,18388	0,774	-9,5123	16,6203

Com base em médias observadas.
O termo de erro é Quadrado Médio (Erro) = 120,927.

*. A diferença média é significativa no nível ,05.

a. Organs = Caudal Fin

Table VII: Statistical analysis for qPCR data collected in Study 2 (non-lethal study) for the gills utilizing a LSD Test.

Comparações por Método Pairwise[a]

Variável dependente: **Ct Values**

Treatment			Diferença média (I-J)	Erro	Sig.[c]	95% Intervalo de Confiança para Diferença[c]	
						Limite inferior	Limite superior
IM Infected	7	15	23,516*	5,950	**0,001**	11,143	35,889
		30	4,518	5,950	0,456	-7,855	16,891
	15	7	-23,516*	5,950	**0,001**	-35,889	-11,143
		30	-18,998*	5,950	**0,004**	-31,371	-6,625
	30	7	-4,518	5,950	0,456	-16,891	7,855
		15	18,998*	5,950	**0,004**	6,625	31,371
Immersion Infected	7	15	22,194*	6,652	**0,003**	8,361	36,028
		30	29,023*	6,652	**0,000**	15,189	42,857
	15	7	-22,194*	6,652	**0,003**	-36,028	-8,361
		30	6,829	6,652	0,316	-7,005	20,662
	30	7	-29,023*	6,652	**0,000**	-42,857	-15,189
		15	-6,829	6,652	0,316	-20,662	7,005

Baseado em médias marginais estimadas

*. A diferença média é significativa no nível ,05.

a. Organs = Gills

c. Ajustamento para diversas comparações: Diferença Menos Significativa (equivalente a nenhum ajustamento).

Table VIII: Statistical analysis for qPCR data collected in Study 2 (non-lethal study) for the brain utilizing a Tukey HSD Test.

Comparações múltiplas[a]

Variável dependente

Tukey HSD

Organs		Diferença média (I-J)	Erro	Sig.	Intervalo de Confiança 95%	
					Limite inferior	Limite superior
Brain	**Caudal Fin**	-12,2172*	2,88182	**0,001**	-19,4810	-4,9534
	Gills	-13,6304*	2,88182	**0,000**	-20,8942	-6,3666
Caudal Fin	**Brain**	12,2172*	2,88182	**0,001**	4,9534	19,4810
	Gills	-1,4132	2,88182	0,877	-8,6770	5,8506
Gills	**Brain**	13,6304*	2,88182	**0,000**	6,3666	20,8942
	Caudal Fin	1,4132	2,88182	0,877	-5,8506	8,6770

Com base em médias observadas.
O termo de erro é Quadrado Médio (Erro) = 37,372.

*. A diferença média é significativa no nível ,05.

a. Day = 7

Appendix IV

Part of this work was accepted and presented as poster in the Aquaculture International Conference & Exposition that took place in Dubrovnik, Croatia in October of 2017 with the title “Is possible to detect Betanodavirus with non-lethal sampling methods?”.

INTERNATIONAL CONFERENCE
& EXPOSITION

October 17-20, 2017

Dubrovnik, Crotia

ABSTRACTS

IS POSSIBLE TO DETECT BETANODAVIRUS WITH NON-LETHAL SAMPLING METHODS?

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Introduction

Since the first outbreak of Viral Encephalopathy and Retinopathy (VER) in the mid 80's, outbreaks of the disease have been associated with mortalities of up to 100%, especially in post-hatch larvae, fingerlings and juvenile fish. The etiological agent of VER is Betanodavirus, a non-enveloped dsRNA virus. Over 120 species belonging to 30 families from 11 different orders are susceptible to VER infection, and this infection can occur in both farmed and wild fish, and in marine and freshwater fish (Costa and Thompson, 2016). Betanodavirus transmission can occur vertically or horizontally. The use of adequate biosecurity measures can prevent the horizontal transmission, but cannot control the spread of the virus by asymptomatic carriers and vertical transmission. Fish broodstock can carry the virus particles in their gonads, liver, kidney, stomach and intestine. The physiological stress associated with reproduction promotes virus replication and shedding of the virus from the gonads and other organs, and in turn can infect eggs, sperm or larvae (Valero et al. 2015). It has been demonstrated that the Betanodavirus present in the sperm is capable of infecting eggs during fertilization (Breuil et al. 2002). One measure for reducing the probability of vertical transmission is screening broodstock for the presence of the virus. Usually Betanodavirus detection is performed by virus isolation on cells or with molecular techniques such as RT-PCR or qRT-PCR, screening brain as the target organ. However, screening valuable broodstock must be performed using non-lethal methods such as serology or molecular detection of virus particles in tissues such as fin or gill clips. The aim of this study was to access the suitability of using these tissues for molecular diagnostics and serum to assess antibodies levels against the virus in fish that had been experimentally infected with Betanodavirus by immersion and intramuscular injection.

Material and Methods

European sea bass (*Dicentrarchus labrax*, 10.6g) were challenged by intramuscular injection (IM, $TCID_{50}=10^4$ per fish) or immersion ($TCID_{50}=10^6$ l^{-1}, for 1h at a stocking density of 80 kg m^{-3}). Control fish were challenged with PBS (IM or immersion). The challenge lasted 30 days and the fish (30 per route of infection/control) were kept at 26°C±1 with a stocking density of 18 kg m^{-3}. The fish were monitored daily and samples were collected at 0, 7, 15 and 30 days post-infection (dpi). Fin and gill clips and brain tissue were collected into RNAlater for the detection of Betanodavirus by qRT-PCR and fish serum was collected for detection of Betanodavirus-specific antibodies by ELISA.

Antibody detection by ELISA were performed according to Costa (2005). Plates were coated with virus supernatant ($TCID_{50}$ = 10^6 ml^{-1}), and the presence of fish antibodies was detected with an anti-sea bass IgM MAb (Aquatic Diagnostic Ltd) and a conjugated anti-mouse-IgG HRP antibody (Sigma). Endpoint antibodies titres were expressed as $-\log_2+1$ for a two-fold serial dilution.

Fish RNA samples was extracted with an RNeasy Mini Kit (Qiagen). The qRT-PCR used was based on the method developed by Panzarin et al. (2010) with the pair of primers and probe acquired from Sigma. The qRT-PCR was performed in an Applied Biosystems 7500 Real-Time PCR System using a SuperScript III Platinum One-Step qRT-PCR Kit (55°C for 10min; 95°C for 5min; 40 cycles of 95°C for 15 seconds and 60°C for 1min). Quantification of Betanodavirus was performed with a standard curve prepared with Betanodavirus RNA extracted from infected supernatant (QIAamp Viral Mini Kit, Qiagen). The qRT-PCR results were expressed in copy numbers per mg of tissue and were log-transformed. Standard curves were constructed using triplicate samples of 10 fold serial dilutions of extracted positive control supernatant. The standard curve and qRT-PCR efficiencies were determined using the Applied System Software version 2.3. The efficiency of each RT-PCR was accessed according to:

$$E \text{ (amplification efficiency \%)} = (10 \text{ ^ } (-1/\text{slope})-1) \times 100\%.$$

Results and Discussion

The mortality levels recorded during the trial were very low in the immersion-infected tanks, with 3.3% mortalities obtained and no mortalities were obtained in the IM-infected fish, indicative of a subclinical infection.

The fish from 0 dpi and the control fish from both the IM and the immersion challenge groups had no specific antibodies against Betanodavirus. The presence of anti-Betanodavirus antibodies in the IM infected fish peaked at 15 dpi and 30 dpi in the immersion group.

As well as being able to detect the presence of the Betanodavirus in the brains of both the IM and the immersion challenge groups, the virus could also be detected in the non-lethal samples collected from the gills and fins of the fish.

To answer our question if it is possible to detect Betanodavirus in non-lethal samples, we compare the viral load present in fin and gill with the target tissue, brain. The usefulness of serology relative to the viral loads in the non-lethal samples will also be discussed.

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References

Arimoto M., K. Mushiake, Y. Mizuta, I. Furusawa. 1992. Detection of Striped Jack Nervous Necrosis Virus (SJNNV) by Enzyme-Linked Immunosorbent Assay (ELISA). Gyobyo Kenkyo, 27:191-195.

Breuil, G., J.-F. Pépin, S. Boscher, R. Thiéry. 2002. Experimental vertical transmission of nodavirus from broodfish to eggs and larvae of the sea bass, *Dicentrarchus labrax* (L.). Journal Fish Diseases, 25:697-702.

Costa, J.Z. (2005). B-cell Epitopes in Fish Nodavirus. PhD thesis submitted to University of Stirling, Stirling, UK.

Costa, J.Z. & K.D. Thompson. 2016. Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. Fish and Shellfish Immunology: 53:35-49.

Valero Y., M. Arizcun, M.A. Estéban, I. Bandin, J.G. Oliveira, S. Patel. 2015. Nodavirus colonizes and replicates in the testis of Gilthead seabream and European sea bass modulating its immune and reproductive functions. PloS One, 10: e0145131.