Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
www.hcmr.gr
DOI: http://doi.org/10.12681/mms.34425

Correlations of betanodavirus load in the brain of experimentally infected sea bass (*Dicentrarchus labrax*, L.) with varying levels of resistance to viral nervous necrosis

Vasileios BAKOPOULOS[1], Anastasia PANAGIOTAKI[1], Michail-Aggelos VALSAMIDIS[1], Daniella-Mari WHITE[1], Leonidas PAPAHARISIS[2], Arkadios DIMITROGLOU[3] and Elli KAKAVA[4]

[1] Department of Marine Sciences, School of The Environment, University of The Aegean, University Hill, Mytilene 81100, Greece
[2] Natural & Technological Resources Consultancy, Filyra, Trikala, Greece
[3] Department of Animal Science, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
[4] Avramar Aquaculture S.A., 19.3Km of Markopoulo-Paiania Avenue, Attica, Greece

Corresponding author: Vasileios BAKOPOULOS; v.bakopoulos@marine.aegean.gr

Contributing Editor: Stelios SOMARAKIS

Received: 08 May 2022; Accepted: 18 August 2023; Published online: 08 September 2023

Abstract

The red-spotted grouper nervous necrosis virus (RGNNV) genotype of Betanodavirus causes significant losses to the European sea bass aquaculture industry. The lack of widely available vaccines against this genotype has led researchers and the industry to investigate other avenues for reducing mortality caused by the disease, such as the selection of resistant stocks. The objectives of this study were: a) to assess the presence of natural resistance in sea bass families propagated in the genetic improvement program of Nireus S.A., Greece and, b) to test an ELISA method able to detect viral coat antigen and measure viral load in the brain of tested fish. The second objective was to investigate virus antigen fluctuations during the course of the infection and its correlation with disease development, mortality and resistance. Further aspects of disease pathogenesis were investigated. A population of sea bass consisting of 89 families was experimentally infected with an RGNNV genotype of Betanodavirus; mortalities were recorded and brain samples from dead and survived fish were collected. Experimental infection of fish resulted in a typical pattern of mortality development that reached 56%. Different levels of natural resistance between families were found with cumulative mortality ranging from 20% to 86.2%. There was no statistical difference between the weight of dead fish and the resistant and susceptible families, except when extreme phenotypes were tested, indicating that the weight of fish that died was not a significant factor of final mortality. Brain virus load in the population as a whole increased sharply until D6 post-infection and then gradually dropped until the end of the experiment. When the viral load in the population (dead and survived fish) was tested against time of death, no correlation was found. In fish that survived the infection, the virus load remained high. The viral load per mg of brain tissue in samples taken from dead fish was not a factor that influenced family cumulative % mortality. Brain samples from survived fish reproduced the disease after infection of healthy fish. This study revealed a natural resistance of sea bass to Betanodavirus infection. This important finding can be used as an additional tool in reducing the mortality of cultured stocks. Additionally, there was clear evidence that fish surviving infection become subclinical carriers of the pathogen. Apparently, survival may not be associated with mechanisms of viral clearance but to other mechanisms that may suspend viral replication or aid infected cell endurance. These findings may assist in developing future vaccines and stock selection. A combination of effective vaccination and resistant stocks could be the way ahead.

Keywords: Betanodavirus; RGNNV genotype; *Dicentrarchus labrax*; virus load; disease resistance.

Introduction

Viral nervous necrosis or viral encephalopathy and retinopathy (VNN or VER, respectively, hereafter) is caused by the genus Betanodavirus that includes viruses affecting fish. Typical signs of VNN include dilation of the abdomen, lateral, spiral, vertical swimming, redness and ulceration of the snout and blindness (Costa & Thompson, 2016; Bakopoulos *et al.*, 2018a). Necropsy of fish brains provides evidence of encephalitis with intense inflammation (Breuil *et al.*, 1991; Comps *et al.*, 1994; Munday & Nakai, 1997; Munday *et al.*, 2002).Various genotypes of betanodaviruses have been reported to cause VNN in many commercially important fish species worldwide, including European sea bass (*Dicentrarchus labrax*, L.), which is affected by the red-spotted grouper nervous necrosis virus (RGNNV, hereafter) genotype (Frerichs *et al.*, 1996; Skliris *et al.*, 2001; Montes *et al.*, 2010). Beta-

nodaviruses are small icosahedral non-enveloped viruses containing two single-stranded RNA molecules (RNA1 encoding viral RNA-dependent RNA polymerase and RNA2 encoding capsid protein). The main viral antigen (Ag, hereafter) employed to induce specific antibodies and activate a protective immune memory is the capsid protein, largely shown to be immunogenic in fish (Coeurdacier *et al.*, 2003; Costa & Thompson, 2016). Regarding prevention of VNN, various approaches have been tested experimentally, such as DNA vaccines (Sommerset *et al.*, 2005), heat-inactivated viral particles (Breuil & Romestand, 1999), recombinant capsid protein vaccines (Yuasa *et al.*, 2002) and virus-like particles (Thiery *et al.*, 2006), to name a few, with or without success. Finally, an anti-RGNNV genotype Betanodavirus commercial formulation, that has recently been granted marketing authorization for commercial use in Greece, Spain, Italy and Croatia, could contribute significantly to the protection of sea bass against the disease in these countries.

The lack of commercially licensed, worldwide available, vaccines against this genotype (and other genotypes affecting other important for aquaculture fish species), has led researchers to investigate alternative ways to reduce mortality caused by the disease, namely, the quest for naturally resistant wild stocks of sea bass and/or genetic company-based selection programs for this trait among their sea bass brood stock. Indeed, Khanh *et al.* (2017) demonstrated differences in the survival of offspring originating from different sea regions and found survival rates of 99%, 94%, 62%, and 44% for South-East Mediterranean, North-East Mediterranean, Western Mediterranean and Northern Atlantic stocks, respectively. The authors calculated a moderate intrapopulation heritability of VNN resistance (0.26 ± 0.11), which is a good value for selection of this trait. Similar heritability was also calculated in another study on *D. labrax* by Faggion *et al.* (2021). Finally, variation in survival and the existence of naturally resistant offspring has been demonstrated by our laboratory as well, using offspring of European sea bass families, propagated in the selection program of Nireus S.A., Greece (Bakopoulos *et al.*, 2018a) for this trait. The combination of naturally resistant populations and vaccination with an effective anti-VNN vaccine, could offer even higher survival rates for cultured stocks.

Scarce information is available in the scientific literature on a comprehensive quantitative analysis of Betanodavirus fate in the brain of infected fish. Furthermore, no data is available on virus load in combination with different levels of natural resistance to VNN. Thus, the objectives of this study were: a) to assess the presence of natural resistance (and its level) in sea bass families propagated within the framework of the genetic improvement program of Nireus S.A., Greece and, b) to measure viral load in the brain of tested fish, in an effort to investigate virus quantity fluctuations during the course of the trial and correlate it with disease development, mortality and resistance.

Materials & Methods

Fish

For the purposes of this study, the term family is used to describe specific parents and their offspring used in the genetic improvement program from which experimental fish were taken. Specifically, the term "family" in the experimental population describes only the offspring. A total of 2,516 sea bass individuals (approximately 30 fish per family and 89 families, 5-month old) with an average initial weight of 12g were used in this study. These fish represented a whole year class of fish originating from Nireus S.A breeding nucleus of sea bass. The commercial family-based breeding program operates with overlapping generations and the experimental population used in this study represents offspring of year class 2012. Families in the breeding program are produced by artificial stripping of mature females and males and subsequent controlled crossings in family design including both maternal and paternal half-sibs. Each fish carried an electronic ID (pit-tag inserted in the abdominal cavity by staff of Nireus S.A.) allowing the allocation of each fish to a certain family.

Experimental facilities and physicochemical parameters

Fish were randomly placed in two $2m^3$ cylindroconical tanks in the wet laboratory of the Department of Marine Sciences, University of The Aegean. The wet laboratory is part of the Laboratory of Ichthyology, Aquaculture and Aquatic Animal Health (ICHTHYAI), and the facilities are licensed to carry out experiments on aquatic animals (permit EL83 BioExp 01, according to Presidential Decree 56/2013 in compliance with Directive 2010/63/EC; Decision No 2700/11-3-2022 of the competent Regional Veterinary Authority). The system housing the experimental population is a closed recirculated sea water system with a total capacity of $16m^3$, consisting of eight $2m^3$ cylindroconical tanks. The quality of water was maintained by solids filtration, disinfection (UV-C 200W), biological filtration and aeration. Water temperature was kept at 22.5°C during the acclimatization period (1 week) and at 27±0.1°C during the experimental challenge (with the help of water heaters). The temperature for the challenge test was elevated at a rate of 0.5°C/day and fish were acclimatized for a further week when the challenge temperature was reached. The photoperiod was kept at 12h light:12h dark, salinity 3.8-3.9%, dissolved O_2 >4.8mg/l, pH 8, total ammonia nitrogen and nitrite <0.05ppm and <0.5ppm, respectively, and nitrate levels <40mg/l. The fish were fed a commercial diet at 1% of body weight, with the feed administered every 8h. The fish were fasted for 24h upon arrival, before and after the experimental infection and on the termination of the experiment.

Experimental infection

For the efficacy studies, a strain of Betanodavirus of the RGNNV genotype isolated from a cultured European sea bass outbreak in 2012 in Western Greece, was used (kind donation of Dr E. Karagouni, Hellenic Pasteur Institute). A vial containing the necessary quantity of the betanodavirus stock suspension was retrieved from -85°C and placed at 4°C overnight to thaw gradually. The stock suspension was diluted with sterile phosphate-buffered saline, pH 7.2 (PBS), down to a betanodavirus concentration of 10^6 $TCID_{50}$/ml. This dilution was shown, in two pre-trial tests, to produce a 53.3-60% cumulative mortality in a sea bass mixed family population. The fish were anesthetized with 0.2% phenoxyethanol and then injected intramuscularly (i.m.) (Sommerset *et al.*, 2005; Vimal *et al.*, 2014) with 100µl of the 10^6 $TCID_{50}$/ml suspension. The fish were then randomly distributed in three $2m^3$ tanks. For positive control, 30 fish were also injected with the same virus suspension in order to validate the level of cumulative mortality; for negative controls, another 30 fish were injected with 100µl of sterile PBS. Both control populations originated from the mixed population used in pre-trials.

In order to investigate the condition (i.e. infective or not) of the virus retrieved from brain samples of the fish that survived the infection (after treatment and dilution as described in the following section), samples with purified high viral Ag content were i.m injected in the dorsal muscle of naïve fish (100µl/fish, n=3 per sample). The experimental infection protocol has been approved by Decision No 5690/11-5-2022, of the competent Regional Veterinary Authority.

Monitoring, sampling and treatment of samples

The fish were monitored every 4h post-infection (p.i.) for development of disease symptoms and mortality. Dead fish were collected, individually weighed, their electronic ID scanned. Their brains were excised, placed on pre-weighed sterile eppendorfs and weighed, prior to placement at -85°C for future use. Recently died fish were microbiologically sampled to eliminate the cause of death, other than VNN, every second day of the trial. Briefly, aseptically collected cephalic kidney samples were inoculated into brain heart infusion agar plates (with 2% NaCl), incubated for 48h at 22°C and inspected for bacterial growth.

The trial ended when no mortalities were recorded for two consecutive days. Surviving fish were euthanized with an overdose of anesthetic and then the weight of each fish was recorded, their electronic ID scanned and the brain of 10% of the surviving fish population was excised and processed as reported above. All measurements and records were saved in an excel file for later processing. When all brain samples were collected, they were thawed and frozen consecutively three times in order for the brain cells to brake and release VNNv particles (adopted by Poisa-Beiro *et al.*, 2008). After the last thawing, sterile PBS was added to each brain sample at 1:166.67 dilution, w:v, respectively, in order for all samples to be diluted by the same factor and provide sufficient sample volume for further tests. Then, using sterile 1ml pipette tips, each sample was completely homogenized in sterile PBS and centrifuged at 10,000rpm for 5min in order to spun down cells and cell debris. Each separate vial's supernatant was collected, filtered through a 0.45µm filter and placed in a separate sterile eppendorf and stored at -85°C for future testing.

Rabbit anti-viral nervous necrosis virus (VNNv) polyclonal serum

Rabbit anti-VNNv polyclonal serum was prepared at the premises of the Hellenic Pasteur Institute (Athens, Greece) (HPI) with prior approval from the Animal Bioethics Committee of the HPI according to the EC Directive 1986/609 and the National Law 1992/2015. The rabbits were reared in institutional facilities under specific pathogen-free conditions, receiving a diet of commercial food pellets and water ad libitum.

Stock suspension of betanodavirus was concentrated using polyethylene glycol (PEG) (MW 8,000) and benzoylated cellulose membrane (12kDa MWCO), at 4°C until sufficient concentration was achieved. The concentrated suspension was then dialyzed against PBS, pH 7.2, over 24h at 4°C, with three changes of buffer. Virus particles were then heat-inactivated for 1h at 70°C and stored at -85°C for subsequent use. The protein content of the concentrate was measured using the BIORAD protein assay kit, following the instructions of the manufacturer.

New Zealand white female rabbits were subcutaneously injected thrice with 200µl per injection, on days 0, 21 and 35. The immunogen was an emulsion containing 2.62mg/ml of genotype RGNNV Betanodavirus particles (measured as protein Ag) mixed 1:1 with Freund's complete for the first injection and Freund's incomplete adjuvant for the second. The last injection was performed without adjuvant. Blood was collected 14 days after the last boost, allowed to clot for 10min at room temperature and then overnight at 4°C and then it was centrifuged at 750 x g for 10min to separate the serum. Serum was titrated against Betanodavirus Ag using a simple indirect ELISA and then aliquoted and stored at -20°C for future use. The main steps of the titration ELISA included: a) coating with the Ag, b) adding dilutions of the rabbit anti-Betanodavirus serum, c) adding anti-rabbit IgG HRP conjugate, d) reading the reactions after addition of tetramethyl benzidine in substrate buffer.

ELISA for the detection of VNNv Ag

A simple indirect ELISA was developed that was a modification of the Bakopoulos *et al.* (1997; 2003) method. Briefly, 96-well plates were coated with 80µl/well of 0.001% poly_L_lysine in carbonate/bicarbonate buffer, pH 9.6 for 1h at r.t. After washing, they were then filled

with either a betanodavirus suspension (10-fold dilutions 0.0262 to 0.00000262mg/ml serving as positive controls and the creation of standard curve) or the samples to be tested, in triplicate, at 50µl/well for 8h at 4°C, followed by the addition of 25µl/well of 0.05% glutaraldehyde in 0.02M phosphate buffered saline (PBS), pH 7.4, for 20min at r.t. The plates were washed and 15% bovine serum albumin (BSA) in Low Salt Wash Buffer (LSWB), 100µl/well, was added to block free-sites in the wells, overnight at 4°C. Then, after washing, rabbit anti-betanodavirus serum at dilution 1:1000 in LSWB+1% BSA, 50µl/well for 1h at r.t. was added. The negative control included wells where instead of rabbit anti-Betanodavirus serum, 5% goat serum in LSWB was added. After another wash, anti-rabbit IgG conjugated to biotin, diluted 1:1,500 in 5% goat serum in LSWB, 50µl/well was added and the plates were incubated for 30min at r.t. Next, Extravidin® conjugated to HRP and diluted 1:1000 in 5% goat serum in LSWB was added at 50µl/well for 30min at r.t. Reactions developed after the addition of chromogen for 5min and further development stopped with the addition of 2M H_2SO_4. Results were read at 450nm. The viral protein in brain samples was calculated from the standard curve of the OD reading versus the known concentrations of viral protein concentrate (10-fold dilutions) used in the positive control wells of the assay. Then, using the dilution factor of brain samples and the weight of each brain sample, the viral protein concentration per brain weight unit was calculated.

Statistics

Data collected from the measurement of betanodavirus Ag in brain samples were statistically processed using the SPSS Statistics software (v. 20). Normal distribution of values was checked with the Shapiro-Wilk test. In addition to the correlations performed according to Pearson's test, statistical differences were checked with the student t-test, Wilcoxon test and ANOVA. In all cases, comparisons were considered significant when $p<0.05$. For the purpose of this study, data analysis focused on designated (according to results) resistant and susceptible families.

Results

Experimental Infection (whole population data)

The trial lasted a total of 26 days. During the course of the experimental infection, no mortality (or symptoms related to the disease) was observed in the negative control group, while a cumulative mortality of 56% was recorded for the positive control group (data not shown). In the population tested, some mortality was recorded from day 2 post-infection (D2 p.i.) without development of symptoms. Immediately after, and as mortality rose, classical symptoms of VER were observed, such as abdomen distension, lateral, spiral, vertical and erratic (bursts) swimming. As the infection progressed, further to the latter symptoms, congestion, haemorrhage and ulceration of the snout as well as unilateral or bilateral blindness were observed. Brain necropsy of recently died fish revealed brain congestion and intense inflammation, while individuals with distended abdomen had their swim bladder inflated. All these findings are in agreement with previously reported classical symptoms of VNN (Breuil *et al.*, 1991; Comps *et al.*, 1994; Munday & Nakai, 1997; Munday *et al.*, 2002; Bakopoulos *et al.*, 2018a). Microbiological sampling of head kidney from fish that died recently did not reveal the presence of bacteria. In total 1,464 individuals died and 1,052 fish survived. Figure 1 illustrates the progress of daily and cumulative mortality.

As it is obvious from Figure 1, the experimental infection caused acute mortality between days 4-6 (maxi-

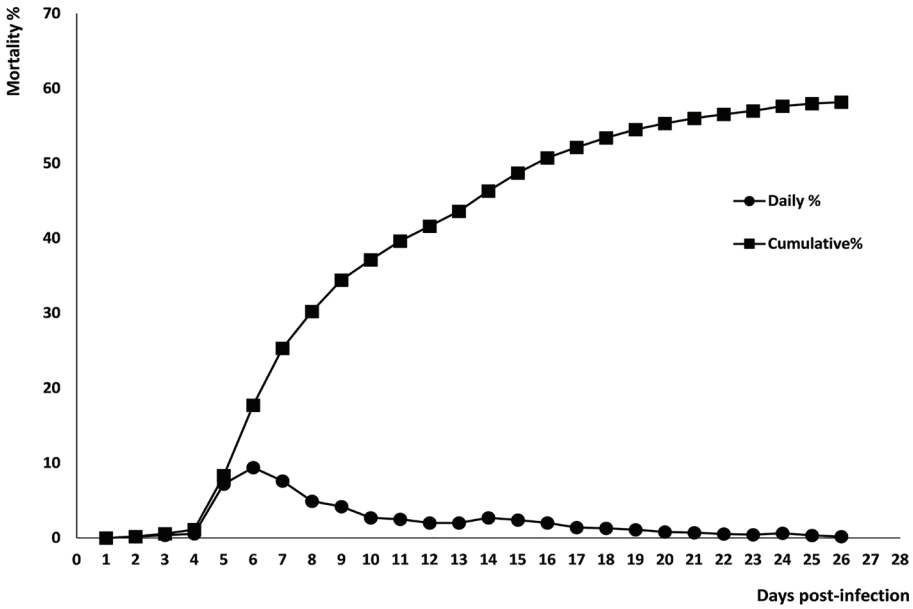

Fig. 1: Development of daily and cumulative mortality (data presented as percentages). Cumulative mortality is shown with the line with square points; daily mortality is shown with the line with circular points.

mum daily mortality was recorded on D6) with a sharp increase from D4 onwards and then deaths dropped gradually from D6 to D10. The rate of daily mortality drop was then normalized and finally deaths stopped after D26 p.i. Cumulative mortality of this trial was 58.19%. The remaining 41.81% of the population survived but many fish had permanent eye lesions.

Regarding the assessment of virulence of brain samples taken from fish that survived the infection but carried viral Ag, all samples tested caused mortality over a period of 24 days post-infection.

The average weight of dead fish in the whole population was 13.93±4.3g, whereas that of the fish that survived was 22.23±6.9g. The weight of dead fish differed significantly from the weight of fish that survived challenge ($t=-35.76$; $p<0.001$).

Figure 2 illustrates the development of average daily weight of dead fish during the course of the trial.

There has been a clear tendency, in the whole population, for smaller-sized fish to die during the first days of the trial. The size of dead fish increased gradually until D18 of the trial, then dropped until D22 and then fluctuated until trial termination. Until D18, the weight of dead fish during the first week of the trial was significantly lower compared to those in the following week. This is supported by the positive correlation ($r=0.31$; $p<0.05$) between weight of dead fish and time of death.

Brain Samples (whole population data)

There was a positive correlation between brain weight (samples of both dead and survived fish) and body weight (samples of both dead and survived fish) ($r=0.616$, $p<0.05$). This positive correlation was maintained when samples of either dead or survived fish were tested ($r=0.55$ and $r=0.65$, respectively; data not illustrated.

ELISA detection of betanodavirus protein Ag (whole population data)

The ELISA method developed for measuring betanodavirus Ag was able to detect as low as 2.62ng/ml of Betanodavirus protein Ag and this sensitivity limit was always statistically different from the negative control of the assay.

Figure 3 illustrates the Betanodavirus Ag load (expressed as mg Ag/mg brain) as measured for each individual sampled during the course of the trial.

As shown in Figure 3, an increasing viral load is apparent from D3 to D6, then a downward trend from D6 to D11 and then the viral load fluctuated at lower levels until the end of the trial. However, there was no statistical difference between time-points.

After measuring betanodavirus Ag in brain samples, the results were correlated with brain weight. No correlation was found between brain weight and viral load (mg of viral Ag / mg of brain tissue), when either all samples or samples from dead fish or samples from fish that survived were tested. This indicates an even distribution of the virus in brain tissue. Furthermore, when mg of viral Ag/mg brain of dead fish was compared to that of fish that survived, no significant difference was found ($t=-0.69$; $p=0.48$).

Resistance and susceptibility to infection (family-wise data)

Each fish that took part in the trial could be allocated to a certain family. Supplementary Table S1, summarizes records per family. Cumulative % mortality per family ranged from 20% to 86.2%, average weight of dead individuals within families ranged from 8.72±2.69 to 20.54±3.66g, average weight of survived individuals within families ranged from 13.10±2.96 to 29.70±11.30g and the average time of death per family ranged from

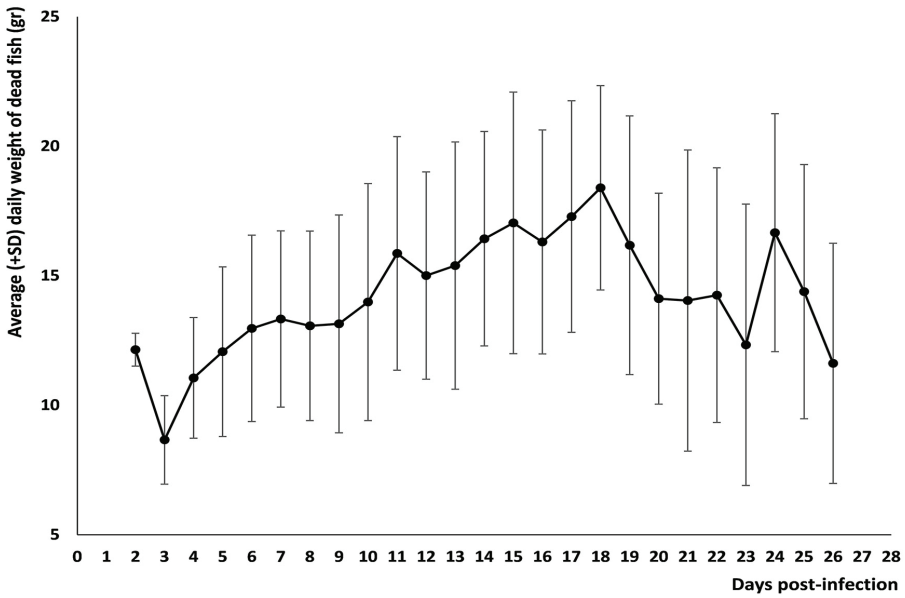

Fig. 2: Average weight (grams) of dead fish during the course of infection. Average values ± standard deviation SD).

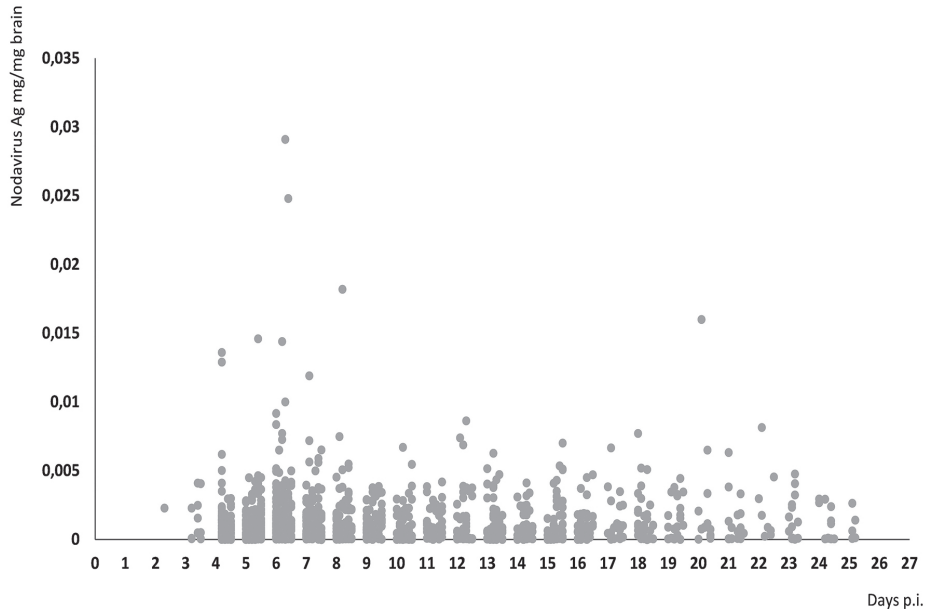

Fig. 3: Betanodavirus antigen concentration (milligrams per milligram of brain tissue) in the brain of fish that died during the course of infection.

142.6±61.5 to 278.9±152.8 hours, with high SD values (Table S1) characterizing large time gaps between recorded death time. Obviously, certain families showed resistance to the disease, mortality in other families ranged close to total population mortality, while others reached higher cumulative mortality, indicating certain susceptibility to VNN. For classification reasons, families were arbitrarily distinguished into resistant (cumulative mortality ≤45%), of average susceptibility (45.01-65%) and susceptible (mortality ≥65.01%), representing 21.3%, 44.9% and 33.7%, respectively, of the total number of families (89) participating in this trial.

For the purposes of this study, comparisons between families focused on only resistant (19 families) and susceptible families (30 families), as classified above; comparison of cumulative mortality between these two groups of families indicated that it was significantly different.

Individual data per family were used in various correlations, in order to identify the presence of any patterns of responses. There was no correlation between cumulative % mortality per family and the average weight of dead fish in each family (r=-0.097). In addition, as regards the weight of dead fish, no statistical difference was found between resistant and susceptible families (data not illustrated), except only extreme phenotypes were tested (five most resistant versus five more susceptible families). In the latter case, dead fish weight of resistant families was significantly higher compared to susceptible families. In all other cases, weight of dead fish was not a significant factor influencing final mortality. In contrast, a negative correlation was found between cumulative % mortality per family and the average weight of surviving fish in each family (r=-0.36, $p<0.05$); the lower the final mortality in the family, the higher the average body weight of fish that survived (Fig. 4).

No correlation was found when family cumulative % mortality was tested against family average time of death p.i. (r=0.088, $p<0.05$) (data not illustrated). This indicates that deaths were distributed almost evenly p.i. irrespective of the resistance/ susceptibility of the family to the infection. Furthermore, there was no statistical difference in time of death when resistant families were compared with susceptible families (both, either all families or extreme phenotypes). Concerning the whole population, average body weight of dead fish within a family was positively correlated with family average time of death p.i. (r=0.36, $p<0.05$), as illustrated in Figure 5. Smaller-sized fish died earlier.

ELISA detection of betanodavirus protein Ag (family-wise data)

Supplementary Table S2, presents all betanodavirus Ag measurements per family. The average brain weight of dead fish in the families ranged from 47.39±12.99 to 82.69±18.40mg and of survived fish between 42.00±0.00 and 123±0.00mg. The average concentration of viral Ag in samples from dead fish (mg viral Ag/ mg brain tissue x 10^{-3}) ranged from 0.42±0.59 to 3.56±6.54 and from fish that survived between 0.01±0.00 and 11.16±13.77.

No correlation was found between average viral load per mg of brain tissue of dead fish and cumulative % family mortality. In fact, when the brain viral load of resistant and susceptible families was compared, no statistical difference was found and this includes tests on extreme phenotypes. A low negative correlation was found between average viral load per mg of brain tissue of fish that survived and cumulative % family mortality (r=-0.19; $p<0.05$); this means that there was a trend individuals that survived from the families with high cumulative% mortality, to have lower viral load in the brain. Correlation was even lower (r=0.1) when average viral load per mg of brain tissue of dead fish was tested against cumulative % family mortality. A low

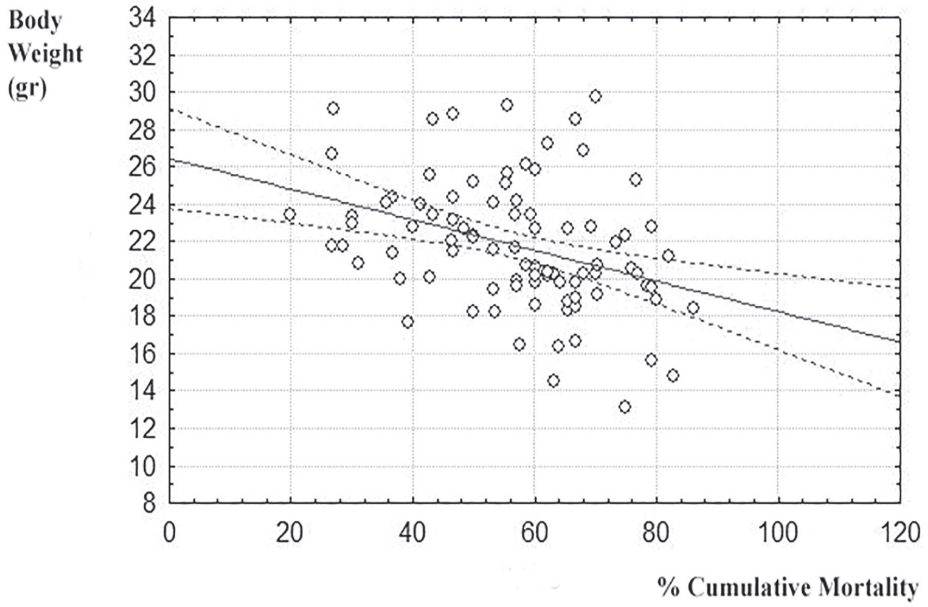

Fig. 4: Correlation between family cumulative mortality (data presented as percentages) with average body weight (grams) of surviving fish.

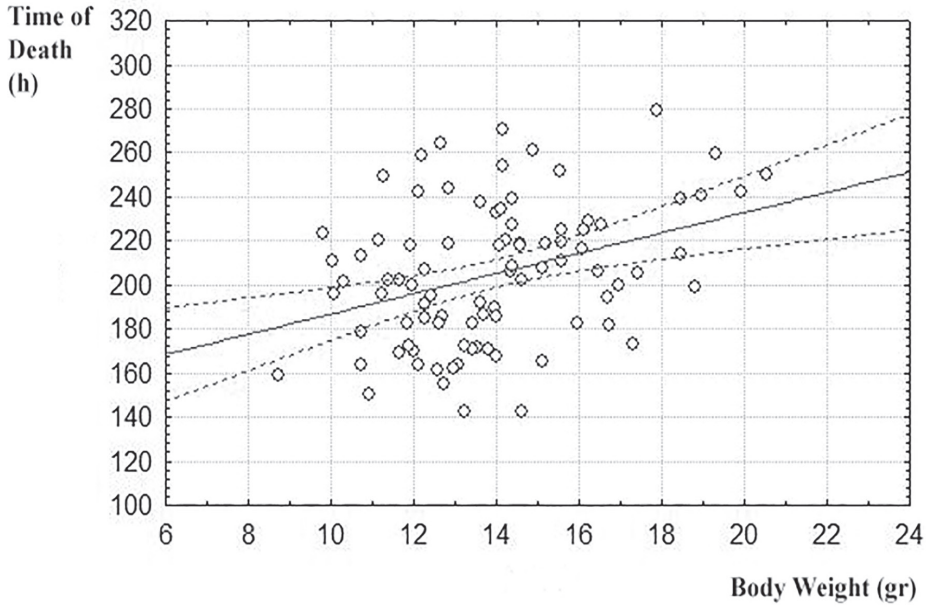

Fig. 5: Correlation between family average body weight (grams) of dead fish and family average time of death (hours).

negative correlation was also found when the average viral load/mg brain tissue was tested against the average time of death of each family (r=-0.21; p<0.05); this signifies that there was a tendency for late average death time of families to correlate with lower concentrations of betanodavirus Ag. Figure 6 illustrates viral Ag average quantities at various time-points post-infection in resistant (6a) and susceptible (6b) families.

As it is evident, in these distinct populations, brain viral load differed during the course of the disease, with the resistant population showing two extreme peaks at around 300 and 380 hours post-infection (Fig. 6a), whereas in the susceptible population extreme peaks were found at approximately 490 hours post-infection (Fig. 6b).

Similarly for the whole population, when average viral concentration/mg tissue per family of dead fish were tested against brain weight, a very low negative correlation was found (r=-0.17) while the findings for surviving fish were similar (r=-0.18).

Supplementary Table S3 presents the Pearson correlations of viral Ag concentration (viral Ag mg/mg tissue) with time of death in each family, in an attempt to assess whether viral Ag concentration decreased or increased with time. Therefore, negative R values suggest a gradual drop of concentration and the opposite holds for positive R values. These R values were then correlated with cumulative % mortality in each family. These tests revealed a low positive correlation between viral Ag concentration development in time p.i. in dead fish with cumulative % mortality of each family (r=0.2). Thus, in families with low final mortality there seems to be a tendency for the viral Ag concentration to decrease or remain stable over time with the opposite happening in families with higher final mortality.

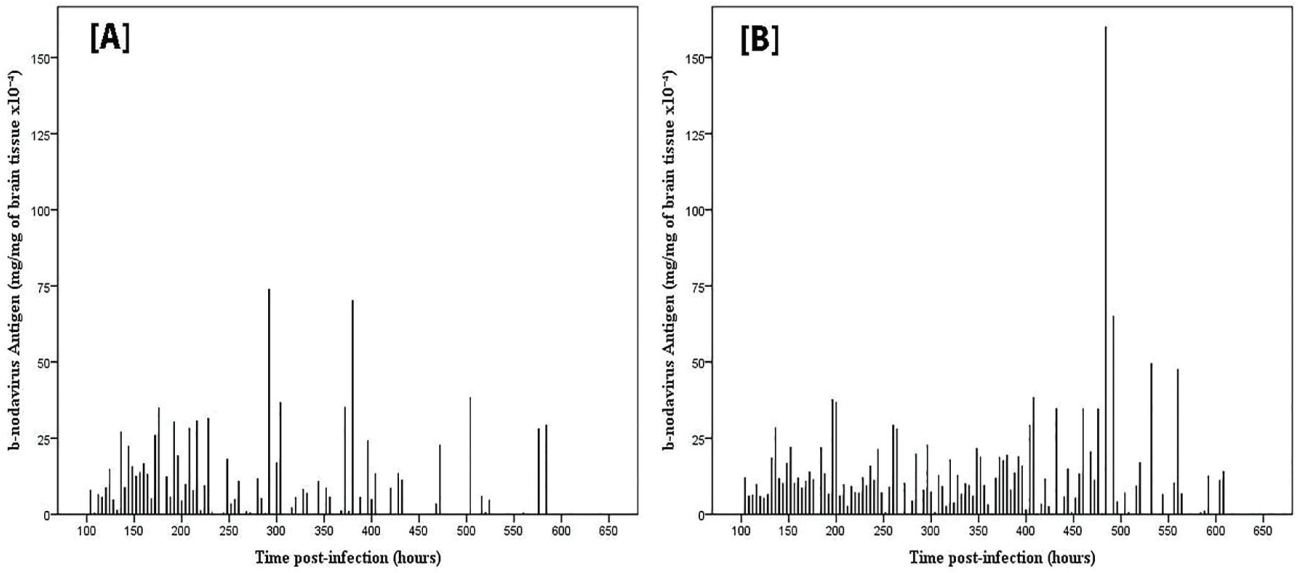

Fig. 6: Concentration of betanodavirus antigen (milligrams x 10^{-4} per milligram of brain tissue) in brain samples of dead fish in relation to time of death (hours): (a) resistant families and (b) susceptible families.

Discussion

This study attempted to confirm and measure the natural resistance of the offspring of sea bass brood stock crossings of a commercial company against VNN. In addition, it attempted to identify any correlations between VNN Ag quantity in the brain of infected fish (dead or survived) and various parameters such as cumulative mortality, weight of fish and time of death post-infection. For the later, an ELISA method for measuring VNN Ag was developed and utilized. Experimental infection of fish resulted in a typical pattern of mortality as demonstrated previously by other researchers and our group (Thiery *et al.*, 2006; Bakopoulos *et al.*, 2018a, 2018b; Faggion *et al.*, 2021). In sea bass populations, similar infection doses have caused either 43-55% cumulative mortality, depending on experimental temperature, (Chaves-Pozo *et al.*, 2012) or 57.3% (Bakopoulos *et al.*, 2018a) or 55-81.7% (Bakopoulos *et al.*, 2018b) or 80.5% cumulative mortality (Thiery *et al.*, 2006). These previous studies support the data presented in this work, with the differences noted in final mortality attributed to the experimental animals used or differences in the physicochemical characteristics of each experiment.

Data from the whole population showed that the average weight of dead fish was significantly lower compared to the fish that survived. This could, at a first glance, be attributed to the inability of affected fish to feed; however, the duration of the experiment was not that long and the feeding rate (1% of body mass) was not high enough to account for this difference. It is widely accepted that smaller-sized fish are more susceptible to VNN (Costa & Thompson, 2016 and references therein) and, most probably, this explains the recorded significant difference. Furthermore, the findings of this study support the latter since a positive correlation was found when weight of dead fish was tested against time of death, with smaller-sized fish dying earlier p.i.

When individual fish data were allocated to each family, a great variation was noted in the final mortality recorded per family, which ranged from 20% to 86.2%. This finding is a clear indication of different levels of natural resistance to the pathogen between the families used. Moreover, the presence of natural resistance among different stocks of European sea bass (Khanh *et al.*, 2017; Faggion *et al.*, 2021) and among different stocks of Atlantic cod (Bangera *et al.*, 2011) has been demonstrated previously.

Although, as stated above, the average weight of dead dish in the whole population was significantly lower than the average weight of survived fish, no correlation was found between cumulative % mortality of each family and the average weight of dead fish in each family. This indicates that apparently the weight of fish that died was not a significant factor influencing final mortality. Nevertheless, this trait was significantly different when extreme phenotypes (either resistant or susceptible) were tested. On the other hand, and as expected, a negative correlation was found between cumulative % mortality of each family and the average weight of surviving fish in each family (r=-0.36). This suggests that the lower the final mortality in a family, the higher the average body weight of the fish that survived. The former finding was unexpected and may indicate a great variation in the contribution of this trait to final mortality. Regarding the latter finding, our results are supported by other recent studies on *D. labrax* reporting a high probability of a negative genetic correlation between mortality and body weight (Faggion *et al.*, 2021). Interestingly, both this and the study of Khanh *et al.* (2017) calculated the same moderate negative correlation between resistance to VNN and body weight, indicating that body weight is a maintained and important survival trait.

The ELISA method developed is characterised by high sensitivity as it can detect as low as 2.62ng/ml of Betanodavirus protein Ag. This detection limit proved sufficient, allowing us to measure viral Ag in all the samples tested. Unfortunately, a bibliographic search did not reveal

scientific articles applying immunological methods for detecting betanodavirus. Instead, the majority of articles consulted, applied molecular methods for virus detection and quantification (Grove *et al.*, 2006; Korsnes *et al.*, 2009; Chaves-Pozo *et al.*, 2012; Costa & Thompson, 2016 and references therein) or calculation of the $TCID_{50}$/ml from brain homogenates (Pakingking Jr *et al.*, 2009; 2010; Lopez-Jimena *et al.*, 2011). Quantitative molecular methods detect numbers of RNA copies that are present in the sample irrespective of their functionality (i.e. whether they originate from a complete and functional gene). Thus, an overestimation of functional / virulent virus quantities may be calculated and there is no indication of the amount of viral Ag present. Similarly, an immunological technique detects Ag produced by the virus but it does not provide any information on the functionality / virulence of viral particles (i.e. viral particles may not be virulent, may be incomplete or not contain RNA). However, it does provide additional information on the levels of Ag present in the target tissue, which may be of interest for developing an immune response. In contrast, calculating the $TCID_{50}$/ml from brain homogenates is probably the best way of assessing functional viral load, but the method may be limited by the number of samples needed to be tested. For the aforementioned reasons and because the ELISA method developed had a very good detection limit, it was decided to use it in this study.

In the whole population, virus load increased sharply until D6 p.i and then gradually dropped and the observed pattern coincides with the daily mortality rate illustrated in Figure 1, supporting the hypothesis that there may be a correlation between virus load increase in the brain and mortality.

However, when in the whole population (dead and survived fish) viral load was tested against recorded time of death, no correlation was found, indicating the involvement of more factors that determine the fate of infected fish and not only virus load. Two previous studies were found using the same infectious dose of Betanodavirus and European sea bass but differed as regards the experimental temperature. Both studies support our finding that virus quantity increases during the exponential phase of mortality development and then gradually decreases as daily mortality rate drops and decreases even more when daily mortality is stabilized (Lopez-Jimena *et al.*, 2011; Chaves-Pozo *et al.*, 2012). Certain differences were noted in the pattern of mortality in relation to time p.i. but this is attributed to the different experimental temperature. For the same reason, there were differences in the virus load detected in these two studies in relation to time p.i. Furthermore, studies on other fish species are in agreement with the reported virus load pattern over time p.i., such as the studies of Pakingking Jr *et al.* (2009; 2010) on Asian sea bass, Grove *et al.* (2006) on halibut and Korsnes *et al.*, (2009) on Atlantic cod.

The low positive correlations found between brain weight and total viral Ag load indicate that brain weight (size) is not a significant parameter influencing betanodavirus Ag load. In particular, the lower positive correlation between total viral load in samples from fish that survived and brain weight may indicate the aggregation of infection in certain locations of the brain, which may not increase in size at the same rate as other brain regions in larger fish. Indeed, Renault *et al.* (1991) reported infection of cells in certain regions of the brain such as the optic tectum, cerebellum, tegmentum, vagal lobes and medulla oblongata in Asian sea bass larvae.

As reported in the results section, when viral load was expressed per brain weight unit (i.e. mg of viral Ag / mg of brain tissue), no correlation was found between brain weight and viral load in any case (whole population tested or dead or live individuals). These results indicate that even in fish that survived the infection with no clinical symptoms of the disease, the viral load remains high. This finding is supported by numerous studies on European sea bass and other fish species, which showed that although Betanodavirus brain load decreases over time p.i. as mortality and symptoms remit, infectious viral particles and viral RNA are still detected sometimes in high numbers, with fish being asymptomatic carriers of the pathogen (Thiery *et al.*, 2006; Korsnes *et al.*, 2009; Pakingking Jr *et al.*, 2009; 2010; Lopez-Jimena *et al.*, 2011; Chaves-Pozo *et al.*, 2012). Our results are supported further by Grove *et al.* (2006), with the authors reporting that experimentally infected halibut showed no significant changes in viral load even at D81 p.i., remaining at high levels despite that fish being asymptomatic, after reaching the highest levels at D18 p.i.

Population descriptive statistics provided a preview on the findings of correlations at family level. Interestingly, average viral load per mg of brain tissue from dead fish was not a factor influencing family cumulative % mortality. In contrast, as regards the families with high cumulative % mortality, in the individuals that survived, the viral load in the brain was lower, albeit marginally. Moreover, late average death time of a family correlated marginally with lower concentration of Betanodavirus Ag in the brain, indicating that lower Ag concentration in the brain of surviving fish and late average time of death in families are somewhat correlated with lower cumulative mortality. Pearson correlations of the angle of brain viral load development with time p.i. (negative angle drop of viral load and vice versa), showed that, in agreement with previous reports, there is a tendency for viral Ag concentration to drop or remain stable over time p.i. in families with lower cumulative mortality (Thiery *et al.*, 2006; Korsnes *et al.*, 2009; Pakingking Jr *et al.*, 2009; 2010; Lopez-Jimena *et al.*, 2011; Chaves-Pozo *et al.*, 2012).

All the above findings and correlations indicate that brain viral load over time post-infection and survival or resistance are not directly correlated, and that other factors seem to play an important role in the final outcome. In addition, previous studies have demonstrated that the brain homogenates of fish that survived the infection remain infective for eukaryotic cell cultures (Pakingking Jr *et al.*, 2010; Lopez-Jimena *et al.*, 2011; Toffan *et al.*, 2019). This study also demonstrated the above infectivity in fish. Therefore, surviving fish employ mechanisms to control the development of the disease, at least for a

period of up to 81 or 130 days post-infection (Grove *et al.*, 2006 or Korsnes *et al.*, 2009, respectively). Studies have also been carried out on different aquaculture species where quantitative trait loci (QTL) were detected for traits of economic and welfare importance using medium to high density SNP arrays. For example, the major QTLs detected in Atlantic salmon for different traits include resistance against infectious pancreatic necrosis virus (Houston *et al.*, 2008; Moen *et al.*, 2009) and resistance against piscine myocarditis virus (Boison *et al.*, 2019; Hillestad & Moghadam, 2019), etc. QTLs have also been detected in European sea bass with moderate to low impact. The main targeted traits in sea bass for QTL detection included body weight, resistance against VNN disease and stress resistance (Chatziplis *et al.*, 2007; Palaiokostas *et al.*, 2018; Chatziplis *et al.*, 2020; Griot *et al.*, 2021). QTLs for resistance of European sea bass against VER have been detected at loci LG3, LG8, LG12, LG15, LG19 and LG20 and, interestingly, most of the genes that may be involved in resistance are associated with normal functions, multiplication, stress management and protection of nervous cells and of the endothelium. The majority of genes reported does not seem to be directly involved in the function of the immune system, but rather in the metabolic pathways of infected cells. Therefore, it can be assumed that survival after infection by the virus may be based on both supportive mechanisms for infected cells, reduced susceptibility and the development of a specific immune response. This hypothesis is supported by the study of Bangera *et al.*, (2013) on Atlantic cod (*Gadus morhua*). The authors investigated whether observed time-until-death in challenge tests with Betanodavirus were due to differences in susceptibility or increased endurance (individual hazard, considering that the animal is susceptible). Differences between estimated susceptibility and recorded cumulative mortality after challenge, were attributed to endurance of the fish and in addition, quoting from their report "susceptibility and endurance are apparently distinct genetic traits". Further research could, therefore, focus on the underlying mechanisms of resistance and robustness of sea bass against VNN.

Conclusions

This study confirmed the presence of natural resistance of European sea bass commercial stocks to viral nervous necrosis caused by the RGNNV genotype of Betanodavirus, as shown for wild stocks.

Although smaller-sized fish died earlier post-infection when the whole population is concerned, no correlation was found between mortality level and weight of dead fish, when families were tested. In contrast, cumulative % mortality per family was apparently negatively correlated with the average weight of surviving fish in each family; the lower the final mortality in the family, the higher the average body weight of fish that survived.

Deaths were distributed almost evenly p.i. irrespective of the resistance/ susceptibility of the family to the infection. There was no statistical difference in time of death when resistant families were compared with susceptible families.

A sensitive Elisa method was developed for the detection of Betanodavirus coat protein Ag in brain tissue extracts and it was successfully used for measuring this Ag in infected brains.

Apparently, there were no significant differences in the amount of viral Ag found in the brain of dead fish or fish that survived the infection, even when resistant and susceptible families were compared.

For individuals that survived compared to those with high cumulative % mortality, a lower viral load in the brain was observed.

In families with low final mortality there seems to be a tendency for the viral Ag concentration to drop or remain stable over time with the opposite happening in families with higher final mortality.

Survival seems to be related not so much with clearance of the virus but with mechanisms that provide endurance of the infected brain cells to the virus.

Acknowledgements

This work has been funded by Nireus S.A. (Research Committee of University of The Aegean, project No.: 70088). We would like to thank Dr E. Karagouni for the provision of the betanodavirus RGNNV genotype isolate.

References

Bakopoulos, V., Volpatti, D., Papapanagiotou, E., Richards, R.H., Galeotti, M. *et al.*, 1997. Development of an ELISA to detect *Pasteurella piscicida* in culture and in "spiked" fish tissue. *Aquaculture*, 156 (3/4), 359-365.

Bakopoulos, V., Volpatti, D., Gusmani, L., Galeotti, M., Adams, A. *et al.*, 2003. Vaccination trials of sea bass, *Dicentrarchus labrax* (L.), against *Photobacterium damsela*e subsp. *piscicida*, using novel vaccine mixtures. *Journal of Fish Diseases,* 26 (2), 77-90.

Bakopoulos, V., Palaiologos, A., Kantham, P., Kottaras, L., Kyvelou, V. *et al.*, 2018a. Selective breeding and disease resistance of sea bass, *Dicentrarchus labrax*, against viral nervous necrosis. In: *3rd International Congress on Applied Ichthyology & Aquatic Environment, Hydromedit 2018, Volos, Greece, 8-11 November 2018*.

Bakopoulos, V., Palaiologos, A., Poulos, K., Malatestas, K., Dimitrakopoulou, E. *et al.*, 2018b. Efficacy testing of anti-VNN vaccine formulations against the RGNNV b-nodavirus genotype in European sea bass (*Dicentrarchus labrax*). In: *3rd International Congress on Applied Ichthyology & Aquatic Environment, Hydromedit 2018, Volos, Greece, 8-11 November 2018*.

Bangera, R., Odegard, J., Praebel, A.K., Mortensen, A., Nielsen, H.M., 2011. Genetic correlations between growth rate and resistance to vibriosis and viral nervous necrosis in Atlantic cod (*Gadus morhua* L.). *Aquaculture*, 317, 67-73.

Bangera, R., Odegard, J., Nielsen, H.M., Gjoen, H.M.,

Mortensen, A., 2013. Genetic analysis of vibriosis and viral nervous necrosis resistance in Atlantic cod (*Gadus morhua* L.) using a cure model. *Journal of Animal Science, 91*, 3574-3582.

Boison, S., Ding, J., Leder, E., Gjerde, B., Bergtun, P.H. *et al.*, 2019. QTLs Associated with Resistance to Cardiomyopathy Syndrome in Atlantic salmon. *Journal of Heredity*, 110, 727-737.

Breuil, G., Bonami, J.R., Pepin, J-F., Pichot, Y., 1991. Viral infection (picorna-like virus) associated with mass mortalities in hatchery-reared sea-bass (*Dicentrarchus labrax*) larvae and juveniles. *Aquaculture*, 97, 109-116.

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. *Journal of Fish Diseases,* 22, 45-52.

Chaves-Pozo, E., Guardiola, F.A., Meseguer, J., Esteban, M.A., Cuesta, A., 2012. Nodavirus infection induces a great innate cell-mediated cytotoxic activity in resistant, gilthead seabream, and susceptible, European sea bass, teleost fish. *Fish & Shellfish Immunology*, 33, 1159-1166.

Chatziplis, D., Batargias, C., Tsigenopoulos, C.S., Magoulas, A., Kollias, S. *et al.*, 2007. Mapping quantitative trait loci in European sea bass (*Dicentrarchus labrax*): The BASSMAP pilot study. *Aquaculture*, 272, S172-S182.

Chatziplis, D., Oikonomou, S., Loukovitis, D., Tsiokos, D., Samaras, A., *et al.*, 2020. QTL for Stress and Disease Resistance in European Sea Bass, *Dicentrarhus labrax* L. *Animals* (Basel) 10, 1668.

Coeurdacier, J-L., Laporte, F., Pepin, J-F., 2003. Preliminary approach to find synthetic peptides from nodavirus capsid potentially protective against sea bass viral encephalopathy and retinopathy. *Fish & Shellfish Immunology*, 14, 435-447.

Comps, M., Pepin, J.-F., Bonami, J.R., 1994. Purification and characterization of two fish encephalitis viruses (FEV) infecting *Lates calcarifer* and *Dicentrarchus labrax*. *Aquaculture*, 123, 1-10.

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 Immunology*, 53, 35-49.

Faggion, S., Bertotto, D., Babbucci, M., Rovere, G.D., Bovolenta, M. *et al.*, 2021. Resistance to viral nervous necrosis in European sea bass (*Dicentrarchus labrax* L.): heritability and relationships with body weight, cortisol concentration, and antibody titer. *Genetics Selection Evolution,* 53, 32-44.

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

Griot, R., Allal, F., Phocas, F., Brard-Fudulea, S., Morvezen, R. *et al.*, 2021. Genome-wide association studies for resistance to viral nervous necrosis in three populations of European sea bass (*Dicentrarchus labrax*) using a novel 57k SNP array DlabChip. *Aquaculture*, 530, 735-745.

Grove, S., Johansen, R., Reitan, L.J., McL. Press, C. *et al.*, 2006. Quantitative investigation of Ag and immune response in nervous and lymphoid tissues of Atlantic halibut (*Hippoglossus hippoglossus*) challenged with nodavirus. *Fish & Shellfish Immunology*, 21, 525-539.

Hillestad, B., Moghadam, H. K., 2019. Genome-Wide Association Study of Piscine Myocarditis Virus (PMCV) Resistance in Atlantic Salmon (*Salmo salar*). *Journal of Heredity,* 110, 720-726.

Houston, R.D., Gheyas, A., Hamilton, A., Guy, D.R., Tinch, A.E. *et al.*, 2008. Detection and confirmation of a major QTL affecting resistance to infectious pancreatic necrosis (IPN) in Atlantic salmon (*Salmo salar*). *Developmental Biology* (Basel), 132, 199-204.

Khanh, D.Q., Vandeputte, M., Chatain, B., Haffray, P., Vergnet, A. *et al.*, 2017. Genetic variation of resistance to Viral Nervous Necrosis and genetic correlations with production traits in wild populations of the European sea bass (*Dicentrarchus labrax*). *Aquaculture*, 478, 1-8.

Korsnes, K., Karlsbakk, E., Devold, M., Nerland, A.H., Nylund, A., 2009. Tissue tropism of nervous necrosis virus (NNV) in Atlantic cod, *Gadus morhua* L., after intraperitoneal challenge with a virus isolate from diseased Atlantic halibut, *Hippoglossus* (L.). *Journal of Fish Diseases,* 32, 655-665.

Lopez-Jimena, B., Alonso, M.d.C, Thompson, K.D., Adams, A., Infante, C. *et al.*, 2011. Tissue distribution of Red Spotted Grouper Nervous Necrosis Virus (RGNNV) genome in experimentally infected juvenile European seabass (*Dicentrarchus labrax*). *Veterinary Microbiology,* 154, 86-95.

Moen, T., Baranski, M., Sonesson, A. K., Kjøglum, S., 2009. Confirmation and fine-mapping of a major QTL for resistance to infectious pancreatic necrosis in Atlantic salmon (*Salmo salar*): population-level associations between markers and trait. *BMC Genomics*, 10, 368.

Montes, A., Figueras, A., Novoa, B., 2010. Nodavirus encephalopathy in turbot (*Scophthalmus maximus*): Inflammation, nitric oxide production and effect of anti-inflammatory compounds. *Fish & Shellfish Immunology*, 28, 281-288.

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

Munday, B.L., Nakai, T., 1997. Special topic review: nodaviruses as pathogens in larval and juvenile marine finfish. *World Journal of Microbiology and Biotechnology*, 13, 375-381.

Pakingking, Jr R., Seron, R., dela Pena, L., Mori, K., Yamashita, H. *et al.*, 2009. Immune responses of Asian sea bass, *Lates calcarifer* Bloch, against an inactivated betanodavirus vaccine. *Journal of Fish Diseases,* 32, 457-463.

Pakingking, Jr R., Bautista, N.B., de Jesus-Ayson, E.G., Reyes, O., 2010. Protective immunity against viral nervous necrosis (VNN) in brown-marbled grouper (*Epinephelus fuscogutattus*) following vaccination with inactivated betanodavirus. *Fish & Shellfish Immunology,* 28, 525-533.

Palaiokostas, C., Cariou, S., Bestin, A., Bruant, J.-S., Haffray, P. *et al.*, 2018. Genome-wide association and genomic prediction of resistance to viral nervous necrosis in European sea bass (*Dicentrarchus labrax*) using RAD sequencing. *Genetics Selection Evolution*, 50, 30.

Poisa-Beiro, L., Dios, S., Montes, A., Aranguren, R., Figueras, A. *et al.*, 2008. Nodavirus increases the expression of Mx and inflammatory cytokines in fish brain. *Molecular Immunology,* 45, 218-225.

Renault, T., Haffner, P., Baudin-Laurencin, F., Breuil, G., Bona-

mi, J.R., 1991. Mass mortalities in hatchery-reared sea bass (*Lates calcarifer*) larvae associated with the presence in the brain and retina of virus-like particles. *Bulletin of the European Association of Fish Pathologists,* 11, 68-73.

Skliris, G.P., Krondiris, J.V., Sideris, D.C., Shinn, A.P., Starkey, W.G. *et al.*, 2001. Phylogenetic and Agic characterization of new fish nodavirus isolates from Europe and Asia. *Virus Research*, 75, 59-67.

Sommerset, I., Skern, R., Biering, E., Bleie, H., Fiksdal, I.U. *et al.*, 2005. Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. *Fish & Shellfish Immunology*, 18, 13-29.

Thiery, R., Cozien, J., Cabon, J., Lamour, F., Baud, M. *et al.*, 2006. Induction of a protective immune response against Viral Nervous Necrosis in the European sea bass *Dicentrarchus labrax* by using Betanodavirus Virus-Like Particles. *Journal of Virology*, 80 (20), 10201-10207.

Toffan, A., De Salvador, M., Scholz, F., Pretto, T., Buratin, A. *et al.*, 2019. Lumpfish (*Cyclopterus lumpus*, Linnaeus) is susceptible to viral nervous necrosis: Result of an experimental infection with different genotypes of *Betanodavirus*. *Journal of Fish Diseases*, 42 (12), 1667-1676.

Vimal, S., Abdul Majeed, S., Nambi, K.S.N., Madan, N., Farook, M.A. *et al.*, 2014. Delivery of DNA vaccine using chitosan–tripolyphosphate (CS/TPP) nanoparticles in Asian sea bass, *Lates calcarifer* (Bloch, 1790) for protection against nodavirus infection. *Aquaculture*, 420-421, 240-246.

Yuasa, K., Koesharyani, I., Roza, D., Mori, K., Katata, M. *et al.*, 2002. Immune response of humpback grouper, *Cromileptes altivelis* (Valenciennes) injected with the recombinant coat protein of betanodavirus. *Journal of Fish Diseases*, 25, 53-56.

Supplementary Data

The following supplementary information is available online for the article:

Table S1. Data recorded for each separate family.

Table S2. Measurements of viral load in each family.

Table S3. Family ranking (lower to higher cumulative % mortality) and correlation between viral Ag concentration and time of death per family.