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Title: VACCINATION AND IMMUNE RESPONSES OF EUROPEAN SEA BASS
(*Dicentrarchus labrax* L.) AGAINST BETANODAVIRUS

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Keywords: betanodavirus; sea bass; *Dicentrarchus labrax* L.; vaccination;
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Abstract: This review summarizes the available knowledge on the immune defences of European sea bass against antigenic preparations derived from the viral encephalopathy and retinopathy virus (betanodavirus), which represents a major threat to the health of this fish species. The nodavirus is widely present and differentiates into several strains that infect invertebrates (in insects, alphanodavirus) and teleost fish, and thus may represent a great problem for farmed fish species. Many efforts have been directed to discovering new immunizations to induce protection in sea bass, especially at young stages, and these efforts have included employing diverse betanodavirus strains, antigen preparation, vaccination routes, and the addition of adjuvants and/or immunostimulants. The obtained results showed that inactivated preparations of betanodavirus that were administered intraperitoneally may induce both immune recognition and protection. Attempts at performing mucosal immunization by immersion and/or oral administration, which is a vaccination route that is highly preferred for sea bass, have shown intriguing results, and more studies are necessary for its improvement. Overall, the objective of identifying a reliable vaccine that also cross-protects against different genotypes or reassortant viruses for use in European sea bass against betanodavirus appears to be an attainable goal in the near future.

Dear Editor,

I am submitting the revision of the review: “**VACCINATION AND IMMUNE RESPONSES OF EUROPEAN SEA BASS (*Dicentrarchus labrax* L.) AGAINST BETANODAVIRUS**”, author team: Francesco Buonocore, Noelia Nuñez-Ortiz, Simona Picchietti, Elisa Randelli, Valentina Stocchi, Laura Guerra, Anna Toffan, Francesco Pascoli, Anna Maria Fausto, Massimo Mazzini, Giuseppe Scapigliati for the publication in the Special Issue: “Targeting Fish Vaccination”.

All the comments of the reviewers has been taken into consideration and, during the text, the differences from the first version has been highlighted in **bold**.

Best regards

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Reviewers' comments:

Reviewer #1: I enjoyed reading the manuscript submitted by Buonocore et al entitled "Vaccination and Immune Responses of European Sea Bass (*Dicentrarchus labrax* L.) against Betanodavirus" (FSIM-D-17-00519) and found it to be a useful contribution to the betanodavirus research.

The manuscript does require extensive language editing throughout before being published, however.

The language has been edited.

I have some general comments for the authors.

Sometimes the authors use betanodavirus and other times VERv - I think betanodavirus should be used throughout.

I have used only betanodavirus during all the text.

Highlights

change to 1) Betanodavirus is one of the main concerns for European sea bass aquaculture
Ok.

2) An effective vaccination protocol procedure is the best method for controlling response to betanodavirus - are my changes what you meant - I'm not sure what was exactly meant here
Yes, your indication is OK and related to what we would like to mean with this highlight.

3) Indication on sea bass immune responses after betanodavirus infection are mandatory - Clarify what is meant here - is it "should be mandatory"? I didn't see any mention of this in the review?

I have changed the highlight to better clarify it.

Abstract

Line 35 - "The betanodavirus is widely present differentiated in several strains infecting invertebrates and teleost fish, and thus may represent a great problem for farmed species" I believe betanodavirus infects fish - and alphanodavirus infects insects, mammals and fish?

I have changed the sentence.

Line 39...and the use of adjuvants/immunostimulants - Do you mean in combination with vaccination or as an alternative?

I have clarified in the text.

Line 40 antigenic against in sea bass??? Is this what you meant?

I have changed the sentence.

There is no mention of vaccines to cross-protect against different genotypes or reassortants?

A specific paper on the lack of cross-protection of different VNN viral species was recently made in collaboration with our group and it is in press in the same Special Issue where this manuscript will be published, so we prefer to not repeat the same

information on this manuscript to avoid repetitions. We added a reference in the manuscript named as "Pascoli et al., in press".

Introduction

Line 93 - "...and at different water temperatures comparing diverse virus genotypes [11] - Clarify what is meant here -do you mean different genotypes have different temperature ranges?

I have clarified in the text.

Line 120 - "Moreover, in the same paper a new polyclonal antibody against the 283.2009 VERv strain has been tested to detect the virus uptake in gills by IHC showing high immunoreactivity [19] 48 h after the immersion immunization," what is meant here immunoreactivity of the Mab or the host?

I have clarified in the text.

Conclusion

Line 301 - "Recently, an autogenous viral nervous necrosis vaccine has been released 302 (Pharmaq, 2014)": autogenous vaccines are made with isolates from individual farming systems, but the way it is worded implies it is on general release in Spain or Greece - please clarify?

I have clarified in the text. We agree with the reviewer that autogenous vaccines should be made from a single isolate obtained from the addressed farm, but in the Pharmaq VNN vaccine datasheet the product is named "Autogenous VNN vaccine" and indicated as "available in Spain and Greece". So we just reported the informations given by the manufacturer, no other informations are available at the moment. For more informations on the vaccine, please refer to the manufacturer website at <https://www.pharmaq.no/products/injectable/mediterranean/>.

There is a very nice supplementary table in "Nuñez-Ortiz, N., Pascoli, F., Picchietti, S., Buonocore, F., Bernini, C., Toson, M., Scapigliati, G., & Toffan, A. (2016). A formalin-inactivated immunogen against viral encephalopathy and retinopathy (VER) disease in European sea bass (*Dicentrarchus labrax*): immunological and protection effects. 47, 89. Veterinary research" showing the vaccine studies performed for betanodavirus entitled "A formalin-inactivated immunogen against viral encephalopathy and retinopathy (VER) disease in European sea bass (*Dicentrarchus labrax*): immunological and protection effects" I think the authors should refer to this - or use it if they can?

I cannot use the same table but I have added the citation in the text.

I think the authors should cite other reviews relating to Betanodavirus vaccines/immune response and relate how their review complements these e.g. including new data, new methodologies etc.

I have added in the Background section a sentence to evidence the focus of this review, which was not clearly indicated during the text. I have cited some other reviews.

More information should be given about the lack of cross protection between genotypes and the presence of reassortants and how this can impact on a vaccination regime, maybe mentioning the current patents for betanodavirus.

We thank the reviewer for the prompt observation, but, as for the previous comment, a specific paper on the lack of cross-protection of different VNN viral species is in press

by our group in this Special Issue.

Reviewer #3: This is a review summarizing knowledge on the immune response of European sea bass against the viral encephalopathy and retinopathy virus (VER virus). Nodavirus infection in sea bass is important and the review can be justified.

The manuscript should be more worked through. There are many linguistic mistakes; from the abstract: "nodavirus can be highly antigenic against sea bass,"

I have changed this sentence and edited the text with regard to the linguistic mistakes.

abbreviaton of VER virus with VERv (a small v for virus, it should be VERV (would the authors use HIv or HIV?);

I have changed the abbreviation and used mainly betanodavirus as asked be reviewer 1.

L96 "the hosh-pathogen interaction"

Ok.

L100-101 "The virus is present and still infectious for a long time in sub-clinically infected fish" This is the definition of subclinically infected!

I have changed the sentence.

L315 "that stills remain"

Ok.

L318-19 "because the betanodavirus is an intracellular pathogen" All viruses are intracellular pathogens

I have removed this sentence.

The authors need to explain better why injection vaccine is not considered to be a good option. Here we get the impression that the authors own work with mucosal immunization is one reason.

I have explained this aspect in the "Conclusions" section.

Highlights

- 1) **Betanodavirus** is one of the main **concerns** for European sea bass aquaculture
- 2) An effective vaccination procedure is the best **method for controlling response to betanodavirus**
- 3) Indication on sea bass immune responses after betanodavirus infection **are fundamental for producing a reliable vaccine**

ABSTRACT

This review summarizes the available knowledge on the immune defences of European sea bass against antigenic preparations derived from the viral encephalopathy and retinopathy virus (betanodavirus), which represents a major threat to the health of this fish species. The nodavirus is widely present and differentiates into several strains that infect invertebrates (**in insects, alphanodavirus**) and teleost fish, and thus may represent a great problem for farmed fish species. Many efforts have been directed to discovering new immunizations to induce protection in sea bass, especially at young stages, and these efforts have included employing diverse betanodavirus strains, antigen preparation, vaccination routes, and the **addition** of adjuvants **and/or** immunostimulants. The obtained results showed that inactivated preparations of **betanodavirus** that were administered intraperitoneally may induce both immune recognition and protection. Attempts at performing mucosal immunization by immersion and/or oral administration, which is a vaccination route that is highly preferred for sea bass, have shown intriguing results, and more studies are necessary for its improvement. Overall, the objective of identifying a reliable vaccine **that also cross-protects against different genotypes or reassortant viruses** for use in European sea bass against betanodavirus appears to be an attainable goal in the near future.

Keywords: **betanodavirus**; sea bass; *Dicentrarchus labrax* L.; vaccination; immune response.

VACCINATION AND IMMUNE RESPONSES OF EUROPEAN SEA BASS (*Dicentrarchus labrax* L.) AGAINST BETANODAVIRUS

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ABSTRACT

This review summarizes the available knowledge on the immune defences of European sea bass against antigenic preparations derived from the viral encephalopathy and retinopathy virus (betanodavirus), which represents a major threat to the health of this fish species. The nodavirus is widely present and differentiates into several strains that infect invertebrates (**in insects, alphanodavirus**) and teleost fish, and thus may represent a great problem for farmed fish species. Many efforts have been directed to discovering new immunizations to induce protection in sea bass, especially at young stages, and these efforts have included employing diverse betanodavirus strains, antigen preparation, vaccination routes, and the **addition** of adjuvants **and/or** immunostimulants. The obtained results showed that inactivated preparations of **betanodavirus** that were administered intraperitoneally may induce both immune recognition and protection. Attempts at performing mucosal immunization by immersion and/or oral administration, which is a vaccination route that is highly preferred for sea bass, have shown intriguing results, and more studies are necessary for its improvement. Overall, the objective of identifying a reliable vaccine **that also cross-protects against different genotypes or reassortant viruses** for use in European sea bass against betanodavirus appears to be an attainable goal in the near future.

Keywords: **betanodavirus**; sea bass; *Dicentrarchus labrax* L.; vaccination; immune response.

1. Background

The betanodavirus is a small, non-enveloped icosahedral RNA virus that is a member of the Nodaviridae family, genus *Betanodavirus*, and it causes a severe infection — viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) — in several fish species [1; 2]. The **betanodavirus (VERV)** genome is composed of 2 single-stranded linear RNA segments: RNA1 (approximately 3 kb in length), which is responsible for the production of the RNA-dependent RNA polymerase and of the subgenomic RNA3 and RNA2 (approximately 1,4 kb in length), which encodes the capsid protein precursor [3; 4; 5]. In fish, taking into consideration the variability of the RNA2 T4 region, four **betanodavirus** genotypes have been isolated: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) [6; 7]. **The antigenic properties of the four genotypes have been recently described, generating new data that will be crucial for the development of serological tests for their detection [8].**

Several studies have shown that RGNNV is most common in the Mediterranean region, and this genotype shows very high infectivity in European sea bass (*Dicentrarchus labrax* L.) [2; 9]. **In this review, we will focus on the immune responses of sea bass after different vaccination strategies were performed to prevent the betanodavirus infection in this important aquaculture species.**

2. Betanodavirus pathology and epidemiology

The central nervous system of the fish is the main target for betanodavirus, especially the brain, the spinal cord and the retina [1; 10; 11]. Clinical signs are related to the appearance of neurological damage, and they consist of abnormalities in movement, tonic spasms, lethargy, change in pigmentation and loss of appetite. Sea bass is particularly infected both in the larval and juvenile stages, and, eventually, adults are infected, with high rates of mortality [9; 12]. The ability of the **betanodavirus** to infect sea bass has been studied in different water salinities [13], which demonstrated that changes in this parameter do not influence the capacity of the virus to induce clinical signs and mortality, and in studies with different water temperatures that compared diverse virus genotypes [14]. In this case, the data indicate that both the water temperature and the genetic features related to the virus strain that was used influenced both the mortality and the virus load in the brain. Moreover, the immune response of the fish and, therefore, the **host-pathogen interactions**, are also modified by these factors. **More specifically, it has been demonstrated that clinical disease and mortality**

due to the RGNNV strain infection appears only at high temperatures, while for the SJNNV strain, virus replication was detected only at 20°C and, finally, for reassortant strains (RGNNV/SJNNV and SJNNV/RGNNV; the first possesses the polymerase gene of RGNNV and the coat protein gene of SJNNV, and the opposite is true for the other strain), low mortality was observed independent of temperature; however, the viral load was influenced by the water temperature and by the genetic type of the polymerase gene.

The transmission of the virus has been widely studied, with evidence of both vertical, which means from broodstock to offspring via the eggs or sperm [15], and horizontal, which means from virus-contaminated water and from infected fish or feed. **The virus is present for a long time** in sub-clinically infected fish [16], and it has been demonstrated that contaminated trash fish could also be responsible for its transmission [17]. Moreover, it was demonstrated that it is not easy to inactivate the virus, even in extreme environmental conditions [18].

3. *Betanodavirus* diagnosis

The presence of the **betanodavirus** in fish can be diagnosed with different methods: histology, investigating the presence of the characteristic lesions in the brain and/or in the retina; tissue culture of the virus; identification of virions by electron microscopy; biomolecular methods, such as PCR and real-time PCR; and detection of specific antibodies in sera or body fluids [7]. The **betanodavirus** can be cultivated using specific cell lines [19; 20], such as the striped snakehead cell lines E-11 and SSN-1. The identification of viral antigens by immunohistochemistry (IHC) depends on the available antibodies; for example, in sea bass, mAb 4C3, a mouse monoclonal IgG2a antibody specific for the peptides corresponding to the residues 141-162 and 181-202 of the virus capsid protein [21], has been used to show the presence of the virus in gills after immersion immunization trials with the isolate 283.2009 RGNNV that was inactivated by formalin with very good results [22]. In particular, at 48 h post-immersion, mAb 4C3 recognized scattered cells, with a strong positive signal, distributed in the gill filaments and parallel secondary lamellae. In addition, a weak reaction was homogeneously detected in the gill mucosal tissue. Moreover, in the same paper, a new polyclonal antibody against the 283.2009 **betanodavirus** strain was tested by IHC to detect the virus uptake in gills, showing a high presence of immunoreactive cells in the fish [22] 48 h after the immersion immunization, and a strong reaction both in the thin epithelium covering the secondary lamellae and in the basal cells localized between the

secondary lamellae on gill filaments. The RT-PCR amplification of viral RNA is one of the main diagnostic methods for fish nodaviruses, as it can be used to find small quantities of the viral genome in any tissue. The first approach was to amplify a 430-bp target sequence of SJNNV RNA2 that was useful to diagnose infections related to different virus strains [23]. Successively, as the sensitivity of the assay was dependent on the nodavirus strain, more specific primers and nested PCRs were developed [24]. More recently, a new PCR-based methodology has been validated; because it uses degenerate external primers and two genotype-specific internal primers in a single PCR, it provides the ability to rapidly assess the nervous necrosis virus genotype in quite a simple and affordable way [25]. Finally, numerous sensitive and efficient real-time PCR procedures have also been developed [26; 27; 28]. Another important diagnostic method is the screening of specific virus antibodies in blood or body fluids with the ELISA technique [29; 30]. In sea bass, this method has been used in experimentally infected larvae [31], and, recently, a sensitive assay for the detection of the RGNNV genotype by indirect and capture-based ELISA has been developed and used in samples where PCR analysis is not possible [32]. However, sometimes the ELISA results may be difficult to understand, as the difference in the titre of the antibodies from infected and non-infected individuals is not always very high [33; 34].

4. Vaccination against betanodavirus

Due to its high influence on the loss of aquaculture marine fish species, the possible vaccination procedures against **betanodavirus** have been extensively studied in different teleost species (**for a summary of the current available vaccine technology in different fish species, see the Supplementary Table present in [22]**). Vaccination with a recombinant capsid protein was one of the first attempts, and it has shown a good level of protection in turbot that were vaccinated at a weight of approximately 5 g and challenged after approximately two months [35; 36]. A similar procedure was used in Asian sea bass (*Lates calcarifer*), with the immunization of fish with body weights of 10-15 g showing antibody induction against **betanodavirus**; **additionally**, a good level of protection was observed in sevenband grouper (*Epinephelus septemfasciatus*) [38] and humpback grouper (*Cromileptes altivelis*) [39] after a challenge was attempted with the intramuscular injection of live virus [37]. A DNA vaccination based on the capsid protein has been performed in turbot [36], with no induction of protection, while a DNA vaccine based on the envelope G-protein from a fish rhabdovirus was effective with high, but short-term, protection against nodavirus infection [40]. The use of inactivated **betanodavirus** has been reported in grouper

larvae (*Epinephelus coioides*), with the indication that the bath immunization with nano-encapsulated formalin-inactivated or BEI-inactivated vaccine can be a promising strategy [41] and that bath and oral vaccinations with BEI-inactivated nodavirus triggered the gene expression of humoral and cellular immunity [42]. A formalin-inactivated **betanodavirus** has been tested in the vaccination of sevenband grouper, *Epinephelus septemfasciatus*, alone and in combination with an aquabirnavirus, with the indication that the vaccine is able to induce the production of neutralizing antibodies in the fish [43; 44; 45] and that the combined inoculation of the two viruses conferred both rapid non-specific and delayed specific protection against nodavirus [46]. In Asian sea bass (*Lates calcarifer*), after a single injection of the formalin-inactivated RGNNV, a rapid clearance of the virus was observed in the brains and kidneys of vaccinated fish, followed by a significant increase in neutralizing-antibody titres [47]. Live nodavirus vaccines have been evaluated in sevenband grouper, *Epinephelus septemfasciatus*, at low rearing temperatures, with the indication that in fish reared at 17°C, the nodavirus onset was easier to control [48]; it was also evaluated in natural seawater temperatures, which demonstrated that a protective immune response to **betanodavirus** was mounted [49]. Finally, virus-like particles (VLPs) of grouper nervous necrosis virus have been used in the vaccination of *Epinephelus lanceolatus*, with the evidence that a dosage of 1 µg/g per fish body weight was enough to stimulate a full-scale immune response [50].

5. Vaccination and immune responses of European sea bass against betanodavirus

The European sea bass (*Dicentrarchus labrax* L.) is the most important fish species for Mediterranean aquaculture, and all stages of this teleost are highly sensitive to **betanodavirus**, with larval and juvenile most affected, showing a mortality rate up to 100% in some cases. The first attempt to find a vaccination strategy for **betanodavirus** in sea bass was performed with four synthetic peptides of 15 amino acids that corresponded to partial sequences of the virus RNA2 molecules [51]. Serum specific antibodies against nodavirus that were produced in fish after the immunization were determined by ELISA, and the obtained protection was investigated by challenging the sea bass specimens with a virulent viral suspension. The results showed that one of the selected peptides, named N-ter, and the heat-inactivated nodavirus are both protective for the fish. Another vaccination was performed with intramuscular injection of **betanodavirus** malabaricus grouper nervous necrosis virus (MGNNV) VLPs [52]. The serum of the vaccinated sea bass specimens was analysed to investigate the presence of anti-**betanodavirus** antibodies with neutralizing activities, and, successively, a challenge with the live virus provided evidence of the

protective effects caused by the used methodology. These results demonstrated that both the immune response and the protection are dose-dependent (the used VLP concentrations were approximately between 0,1 µg to 20 µg per fish) and that the higher vaccine doses reduced the number of sea bass that contained detectable **betanodavirus** RNA, as determined by PCR. Recently, a DNA vaccine encapsulated in chitosan (Sigma) nanoparticles was obtained using the plasmid pcDNA3.1/V5-His-TOPO, which contains the entire open reading frame of the RNA2 gene (genotype RGNNV, strain It/411/96) [53]. The optimal chitosan formulation was composed of 0.04% (w/v) chitosan and 200 µg/ml plasmid DNA; the vaccine was prepared by spreading the chitosan-DNA solution onto commercial feed and allowing the obtained pellet to dry; the feed was then administered orally to sea bass specimens (approximately 6 g body weight, 125 days post-hatching). Unfortunately, the analysis of the fish serum indicated that the oral vaccine failed to induce circulating or neutralizing specific antibodies. In contrast, the expression of genes related to cell-mediated cytotoxicity (the T cell receptor β -chain and the CD8 α) and the interferon pathway (type I interferon, Mx protein and interferon- γ) was up-regulated in the posterior gut of vaccinated sea bass. Finally, a challenge of the fish by intramuscular injection of **betanodavirus** performed 3 months after the vaccination procedure showed complete protection until day 21, when mortality started and reached 55%; in contrast, mortality started on day 4 in the control group and reached 100% by day 22. In another recent paper, two different vaccination routes were investigated, intra-peritoneal and immersion, using an isolate (283.2009 RGNNV) and three different types of inactivation: formalin, β -propiolactone and heat treatment [22]. The obtained results indicated that intra-peritoneal immunization with the formalin-inactivated virus induced, as indicated by ELISA and serum neutralization assays, a significant and reliable specific IgM production. Interestingly, these experiments also tested the effect of a commercial adjuvant (Seppic Montanide ISA763A) administered together with formalin-inactivated **betanodavirus**, and the ELISA results, shown in Figure 1 (for Materials and Methods, see Supplementary files), suggest an increase in serum specific IgM. Moreover, the expression of transcripts related to antiviral response, such as the MxA gene and ISG12, was up-regulated at different time points in the head kidney and gut of vaccinated sea bass, as determined by real-time PCR. Finally, a challenge with the live virus (strain RGNNV) indicated that the immunization without the adjuvant was able to induce a significant increase (81.9%) in the relative survival rate compared to the control fish.

To better understand the physiological mechanisms involved in fish antiviral responses, other investigations have focused on cellular and molecular immune responses after the experimental infection of **betanodavirus**; therefore, these results lead to the identification of improved vaccination procedures. In the paper by Scapigliati et al. (2010) [54], sea bass juveniles were intramuscularly infected with a sub-lethal dose of **betanodavirus** isolate 378/103, followed by a boosting immunization under the same conditions after 43 days. Different cellular and molecular parameters were analysed. The results showed an increase of T and B cell proliferation in the peripheral blood leukocytes (PBL) and an increase in cell viability upon the addition of inactivated viral particles in the head kidney and gill leukocytes after the infection. Low specific antibody titres were detected in fish sera after infection, in addition to a strong up-regulation of immune-related genes, such as type I IFN, Mx, IgM after infection and boosting and COX-2, TGF- β and IL-10 after boosting. Chaves-Pozo et al. (2012) [55] focused their paper on the cell-mediated cytotoxicity (CMC) induced in sea bass after intramuscular injection of the **betanodavirus** strain 411/96 produced in SSN-1 cell lines. The head kidney leukocyte CMC increased significantly 7 days after infection, and in the same samples, there was a significant up-regulation of the non-specific cytotoxic cell receptor transcripts (NCCRP-1), while the respiratory burst activity decreased at day 7 and day 15. The presence of replicating nodavirus cells in the brain was observed, and the significant up-regulation of the Mx gene in these tissues was also demonstrated. In another paper, a comprehensive transcriptomic profiling of four cDNA libraries from different tissues (liver, spleen, head kidney and brain) obtained from sea bass specimens intramuscularly infected with nodavirus strain 475-9/99 was performed [56]. The tissues were sampled at 4 and 24 h after infection, and the cDNA libraries were analysed by the BigDye 3.1 sequencing method. The resulting transcripts that up-regulated after the infection were transferrin and ferritin, probably due to the ability of the enzymatically cleaved forms of these proteins to activate fish macrophages, apolipoprotein A1 and 14 kDa apolipoprotein. Real-time PCR analysis confirmed the results obtained by in silico expression data; therefore, these genes were considered as putative markers of the nodavirus infection in sea bass. The innate immune response against viruses is mediated by type I IFN in fish; therefore, the IFN pathway of European sea bass specimens infected by intramuscular injection with **betanodavirus** strain 411/96, genotype RGNNV, was studied in the brain, the main viral target tissue, and the gonad, which are used to vertically transmit the virus [57]. The expression of different genes, such as melanoma differentiation-associated gene 5 (MDA5), TANK-binding kinase 1 (TBK1), IFN regulatory factor 3 (IRF3), IFN, Mx,

dsRNA-dependent protein kinase receptor (PKR), Laboratory of Genetics and Physiology 2 (LGP2), mitochondrial antiviral signalling (MAVS), TNF receptor-associated factor 3 (TRAF3), TRAF family member-associated NF- κ B activator (TANK) and IFN regulatory factor 7 (IRF7), was up-regulated in the gonad. In contrast, only some transcripts, i.e., MDA5, LGP2, IRF3, Mx and PKR, increased in the brain. These results are in agreement with the knowledge that sea bass are unable to clear the live virus from the brain and that the gonads are an important tissue for controlling the dissemination of the nodavirus to progeny. The presence of **betanodavirus** in sea bass testes after intramuscular infection was demonstrated in another paper [58] that also investigated the immune responses in this reproductive organ and in the brain. All the cytokine genes (TNF- α , IL-6 and IL-1 β) analysed by real-time PCR were up-regulated at a minimum of one sampling time-point in the testes; in contrast, with regard to the lymphocyte marker genes, IgM was up-regulated at day 7, and TcR β expression remained un-modified. The antimicrobial response in the testes after **betanodavirus** infection was the focus of another study [59] that showed an up-regulation of the expression of complement factor 3 (c3), hepcidin (hamp) and dicentracin (dic) and a down-regulation of lysozyme (lyz) in this organ at different sampling time-points; in contrast, no differences were found in piscidin and β -defensin (b-def).

Finally, various papers have investigated the involvement of different immune-relevant genes in the antiviral sea bass tissue response after infection with **betanodavirus**: TNF- α , IL-1 β and Mx in the brain and head kidney [60]; galectin-1 and two C-type lectins in the head kidney [61]; complement factor 3 (c3) [62] in the head kidney; CD83 in the head kidney [63]; natural killer enhancing factor (NKEF)-A and NKEF-B (also known as peroxiredoxins-1 and peroxiredoxins-2) in the brain and head kidney [64]; MxA and MxB in the brain and head kidney [65]; histone 1 and histone 2b in the brain and head kidney [66]; and IgT in the gills and spleen [67].

Considering that **betanodavirus** infection in sea bass occurs primarily at young stages, when an intraperitoneal vaccination cannot be performed, immersion immunization is undoubtedly the optimal vaccination route. However, the results obtained from diverse laboratories note that a single immersion immunization does not induce measurable specific antibody titres. We have performed a series of experiments to test the effects of immersion immunization of sea bass (2.6 ± 1.2 grams) with formalin-inactivated **betanodavirus** (10^6 TCID₅₀/ml) on the production of specific Ig and on the transcription of immunoregulatory genes after 24, 48, and 72 h. The obtained results have confirmed a lack of measurable anti-

betanodavirus serum IgM (data not shown), but from gene transcription data, some interesting considerations can be drawn. The transcription of genes that code for IFN, MxA, ISG-12, IL-8, MHC-I, IgM, IgT, and IgD (results shown in Figure 2, for Materials and Methods, see Supplementary files) was tested in the gills, where an effective uptake of **betanodavirus** has been shown [22], as well as in the spleen, where lymphocyte responses are present. Interestingly, it appears evident that tissue-dependent antigen processing is occurring; however, in most cases, the statistical significance (due to the high individual variability) was too low. Indeed, in the gills where the first antigen encounter occurs, the selected genes showed an up-regulation at 24 h. On the other hand, the antigen(s) reaches the spleen after passing through mucosal tissues, and hence, a delayed response in gene transcription is expected, and can actually be observed in the data of Figure 2, where investigated genes are more up-regulated after 48 and 72 h. Overall, this set of experiments showed that, despite the lack of an antibody response, immersion immunization might be effective in modulating antiviral responses, and more investigation is needed for its optimal improvement. **Recently, the in vivo cross-protection of the two main betanodavirus species (RGNNV and SJNNV) has been investigated by intraperitoneally immunizing juvenile sea bass with formalin-inactivated RGNNV and SJNNV vaccines, followed by a challenge with RGNNV [68].**

6. Conclusions

The infection caused by **betanodavirus** is one of the main concerns in sea bass aquaculture. Recently, an autogenous viral nervous necrosis vaccine was released (Pharmaq, 2014); it is composed of a formaldehyde-inactivated culture of the virus and mineral oil (paraffin light liquid) as adjuvant, and it indicated that the recommended administration route is by intraperitoneal injection. **Unfortunately, as an autologous vaccine, it is not easily exploitable, and it is currently useful only against betanodavirus strains isolated in Spain and Greece.** Some actions should be undertaken to avoid spreading this disease; this can be done by selecting **VERV**-free broodstock, screening any food that contains fish formulations, disinfecting eggs by ozonation, and disinfecting inlet water by UV-treatment [2; 34]. However, for long-term control of the disease, the main strategy should be the identification of a proper oral or immersion vaccine; this is especially important considering that, as reported above, interesting results have been recently obtained only with an intraperitoneal vaccination using formalin-inactivated betanodavirus [22]. **In fact, a problem that still needs to be solved is the delivery of the vaccine through mucosal routes to**

avoid the stress caused by injection handling or the inability to inject small fish [45], a practice that is labour intensive and expensive. Mucosal vaccination increases specific antibodies in the mucosal tissue, and it potentially induces local immunity [69]. Moreover, although information concerning the sea bass immune responses after **betanodavirus** infection is available, there are some points that **still** remain to be fully elucidated, specifically in terms of the best administration routes for a possible vaccine, the use of adjuvants and boosting protocols, and the best effective vaccine dose and duration of the induced protection in the fish. The adjuvant added to the vaccine should stimulate both B- and T-cell response, as both types of immune responses are needed to provide effective protection. Finally, it seems that there is no cross-protection between genotypes; therefore, fish can be infected with more than one genotype at the same time, meaning that a multivalent vaccine may be required [68; 70] in some particular situations. Moreover, recent results have directed attention to possible procedures that are linked to selective breeding for improving the resistance of European sea bass to betanodavirus disease [71].

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FIGURE LEGENDS

Figure 1. ELISA of VERV-intraperitoneal immunization. Sera from different groups (n=10 per group) were collected 30 days post-immunization and were tested by capture-based ELISA using a capture polyclonal antiserum against sea bass IgM, as well as a revealing monoclonal antibody anti-VERV. Assays were performed in triplicate wells, and background values (no viral preparation added) were automatically subtracted from samples.

Figure 2. Transcription of some immunoregulatory genes after immersion immunization. The expression level of genes that code for IFN, MxA, ISG12, MHC-I, IgM, IgT, IgD, and IL-8 was determined in gills and spleen leukocytes 24, 48 and 72 h after immunization. Transcription values were expressed as a ratio relative to 18S rRNA in the same samples; the calibrator was the muscle from an untreated sea bass specimen. The quantitative PCR amplification was performed in PCR arrays, and each point represents the mean + SD of cells from 4 individual fish.

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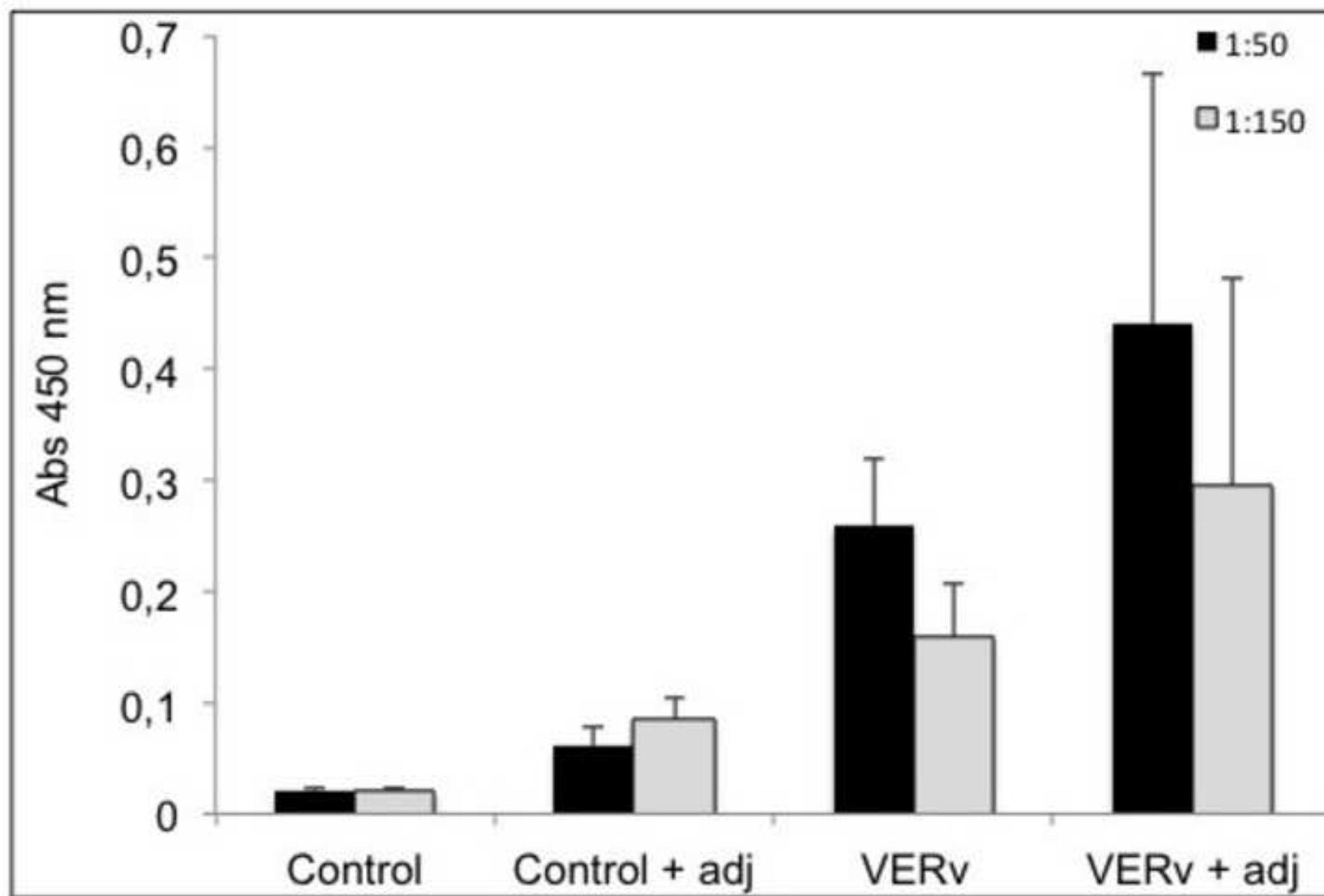
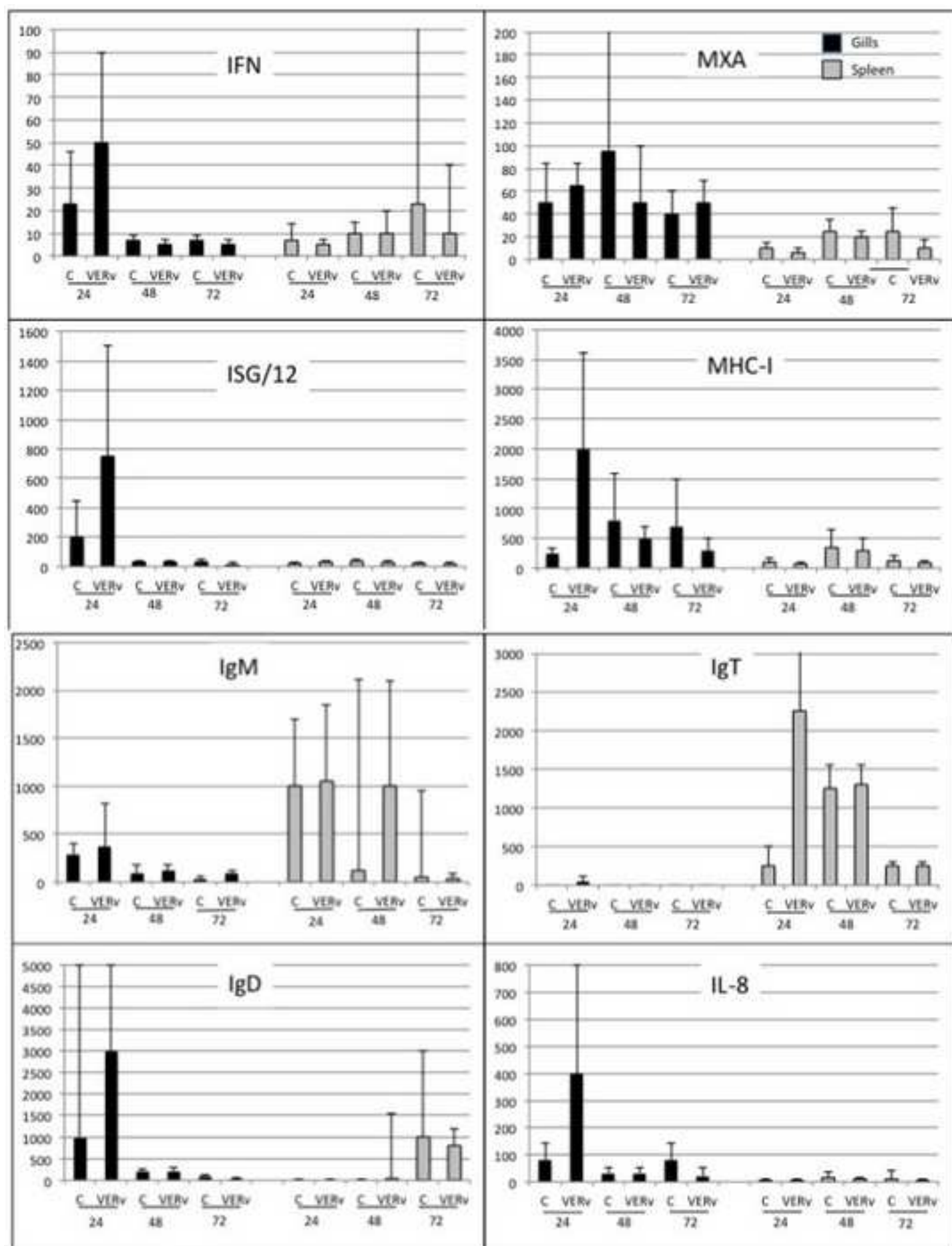


Figure 2
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