Characterization of the IFN pathway in the teleost fish gonad against vertically transmitted viral nervous necrosis virus

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One of the most powerful innate immune responses against viruses is mediated by type I IFN. In teleost fish, it is known that virus infection triggers the expression of ifn and many IFN-stimulated genes, but the viral RNA sensors and mediators leading to IFN production are scarcely known. Thus, we have searched for the presence of these genes in gilt-head sea bream (Sparus aurata) and European sea bass (Dicentrarchus labrax), and evaluated their expression after infection with viral nervous necrosis virus (VNNV) in the brain, the main viral target tissue, and the gonad, used to transmit the virus vertically. In sea bream, a fish species resistant to the VNNV strain used, we found an upregulation of the genes encoding MDA5 (melanoma differentiation-associated gene 5), TBK1 (TANK-binding kinase 1), IRF3 (IFN regulatory factor 3), IFN, Mx [myxovirus (influenza) resistance protein] and PKR (dsRNA-dependent protein kinase receptor) proteins in the brain, which were unaltered in the gonad and could favour the dissemination by gonad fluids or gametes. Strikingly, in European sea bass, a very susceptible species, we also identified transcripts coding for LGP2 (Laboratory of Genetics and Physiology 2), MAVS (mitochondrial antiviral signalling), TRAF3 (TNF receptor-associated factor 3), TANK (TRAF family member-associated NFκB activator) and IRF7 (IFN regulatory factor 7), and found that all the genes analysed were upregulated in the gonad, but only mda5, lgp2, irf3, mx and pkr were upregulated in the brain. These findings supported the notion that the European sea bass brain innate immune response is unable to clear the virus and pointed to the importance of gonad immunity to control the dissemination of VNNV to the progeny – an aspect that is worth investigating in aquatic animals.

INTRODUCTION

The innate immune response against virus infections uses different mechanisms such as interferon (IFN), the complement system or cytotoxic cells (Ellis, 2001); the IFN response is the most well-characterized in fish. Mammalian IFNs have been classified as type I (α, β, ω, ε, and κ), type II (γ) and type III (λ) IFNs (Sadler & Williams, 2008). In fish, apart from type II, genome sequencing projects have detected different IFN genes ranging from one gene in fugu (Takifugu rubripes) or medaka (Oryzias latipes) to 11 genes in Atlantic salmon (Salmo salar) belonging to types I and III (Sun et al., 2009; Zou & Secombes, 2011). Evolutionary and phylogenetic studies have demonstrated the problems in fish ifn gene nomenclature. In fact, fish IFNs share characteristics with mammalian type I and III IFNs, and act as co-orthologues; it has been suggested they be renamed as IFN-φ (Hamming et al., 2011; Levraud et al., 2007). Fish IFNs can be divided into two groups: two cysteine-containing group I IFNs and four cysteine-containing group II IFNs (Zou et al., 2007). In addition, group I can be subdivided into subgroup a and subgroup d IFNs, and group II into subgroup c and subgroup b IFNs. Group I ifn genes are found in all the fish species, whilst group II ifn genes are only found in the most primitive fish such as salmonids and cyprinids (Sun et al., 2009;
Zhang & Gui, 2012; Zou et al., 2007). Therefore, several names have been proposed for fish IFNs: type I IFNs, virus-induced IFNs, IFN-α, IFN-β or even simply IFNs (Langevin et al., 2013). Although it has been demonstrated that fish virus-induced IFNs are structurally type I IFNs, a consensus about a consistent nomenclature for these cytokines has still to be reached. Apart from the controversies in IFN nomenclature, all of these fish type I IFNs have been shown to be induced by virus infections and mediate a type I IFN response by the use of the Jak–Stat (Janus kinase–signal transducer and activator of transcription) pathway. Their activation in cells produces an antiviral state through the induction of many IFN-stimulated genes (ISGs), including genes such as that for the antiviral molecule myxovirus (influenza) resistance protein (Mx), with direct antiviral activity (Verrier et al., 2011). Thus, most of the studies in fish use the expression of mx genes as an indicator of viral infection and activation of the type I IFN response; the cellular components sensing the viral genomes and leading to the IFN response have already been characterized (Aoki et al., 2013; Zou et al., 2009).

Pathogen-associated molecular patterns (PAMPs) are detected by germline-encoded pattern recognition receptors (PRRs) and amongst them the most studied are the Toll-like receptors (TLRs), followed by retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain-like receptors. In the case of fish viruses, TLR3 and TLR22 are induced by dsRNA viruses (Matsuo et al., 2008), whilst TLR7 and TLR8 are induced by ssRNA viruses (Crozet & Beutler, 2004), which in both cases induce a type I IFN-mediated response. To date, the involvement of RLRs in the induction of the type I IFN response is the best characterized (Hansen et al., 2011). This family has three members: RIG-I (also known as DDX58), melanoma differentiation-associated gene 5 (MDA5, also known as IFI16) and Laboratory of Genetics and Physiology 2 (LGP2, also known as DHX58). These sensors are upregulated by Viral hemorrhagic septicemia virus (VHSV), Spring viremia of carp virus, Grass carp reovirus (GCRV; Aquareovirus C), Viral nervous necrosis virus (VNNV; members of the genus Betanodavirus) and Infectious pancreatic necrosis virus (IPNV), as well as by polyinosinic acid (polyI: C; a synthetic analogue of viral dsRNA), leading to an increase in the IFN-mediated antiviral response (Chen et al., 2015; Peng et al., 2011; Rise et al., 2008, 2010; Skjæsul et al., 2011; Su et al., 2010; Yang et al., 2011). However, further studies are needed to definitely define their roles in the antiviral response, and to identify and characterize their mediators in the molecular pathway leading to IFN activation.

In all vertebrates, the gonad is considered an immunologically privileged site, as with the brain and retina, where the immune response proceeds in a different manner in order to avoid cell damage (Chaves-Pozo et al., 2005; Helder, 2002), and therefore it is used by some pathogens to hide and escape immunological control. VNNVs, bipartite and positive ssRNA betanodoviruses viruses, are known vertically and horizontally transmitted pathogens (Arimoto et al., 1992; Kuo et al., 2012) able to infect >50 marine fish species, some of them especially sensitive, e.g. the European sea bass (Dicentrarchus labrax), and others only susceptible to some strains, e.g. the gilt-head sea bream (Sparus aurata) (Castric et al., 2001; Frerichs et al., 1996). Interestingly, although the main target tissues of VNNV are the brain and the retina (Castric et al., 2001; Frerichs et al., 1996), both immune-privileged tissues, as with the gonad, the virus has also been detected in the European sea bass liver, spleen and caudal fin (López-Jimena et al., 2012), and more recently we have also found VNNV in, and isolated from, the gonad (Valero et al., 2014). Previous studies have documented that VNNV infection induces the immune response with special emphasis in the type I IFN response. Thus, expression of ifn and/or mx genes was greatly upregulated in the brain or immune-relevant tissues of gilt-head sea bream, orange-spotted grouper (Epinephelus coioides) or Atlantic halibut (Hippoglossus hippoglossus), but only slightly in the European sea bass (Chaves-Pozo et al., 2012; Chen et al., 2014; López-Muñoz et al., 2012; Overgård et al., 2012; Poisabeiro et al., 2008; Scapigliati et al., 2010). In addition, mda5 and Ifg2 transcription was also upregulated in the brain of gilt-head sea bream (Dios et al., 2007) and Atlantic cod (Gadus morhua) (Rise et al., 2010) by VNNV infection.

Bearing this in mind, in this study we aimed to further characterize the type I IFN pathway of European sea bass and gilt-head sea bream, and its involvement upon infection with VNNV, as well as in their respective cell lines, focusing on the gonad, and compared with that found in the brain, i.e. the target tissue for VNNV.

## RESULTS

### Identification of genes involved in the IFN pathway

We have identified most of the genes known to be involved in the RLR activation pathway of IFN (Fig. 1). In gilt-head sea bream and European sword fish species, ifn and mx genes have already been characterized (Casani et al., 2009; Fernández-Trujillo et al., 2011; Scapigliati et al., 2010). Searching the expressed sequence tag (EST) databases, we found partial or full-length sequences of sea bream mda5, tbk1, irf3 and pkr as well as European sea bass mda5, lgp2, irf3 and pkr, which were expanded to mavs, traf3, tank and irf7 by searching a sea bass gill transcriptome obtained by RNA sequencing (Nuñez Ortiz et al., 2014). However, we did not investigate the presence of multiple gene copies or alternative splicing forms. As previously demonstrated (Zou et al., 2009), we also failed to find any rig1 mRNA sequences in the sea bream and sea bass, both belonging to the modern teleosts. The predicted length, homology and E values obtained from the gene sequences were compared with their zebra-fish orthologues (Table 1), resulting in bona fide sequences, which was further confirmed by the analysis of the
predicted protein domains and their conservation (Table S1, available in the online Supplementary Material). These domains include: helicase in MDA5 and LGP2, CARD in the RLR adaptor protein mitochondrial antiviral signalling (MAVS, also known as IFN-β promoter stimulator 1), RING and MATH_TRAF3 in TNF receptor-associated factor 3 (TRAF3), TBD in RAF family member-associated NFκB activator; TBK1, TANK-binding kinase 1; IRF3/7, IFN regulatory factor 3/7; Mx, myxovirus (influenza) resistance protein; PKR, dsRNA-dependent protein kinase receptor; ISRE, IFN-stimulated response element; ISG, IFN-stimulated gene. This figure contains the molecules found and analysed in this study, and is inspired by the literature (Aoki et al., 2013; Hansen et al., 2011; Takeuchi & Akira, 2008; Verrier et al., 2011; Zhang et al., 2014).

**Genes of the IFN pathway are constitutively expressed**

Before determining the effects of any of the stimuli on the levels of expression of the different IFN pathway genes, we determined the constitutive levels of expression of these genes in the brain and gonad of naive gilt-head sea bream and European sea bass specimens and cell lines (Fig. 2). In gilt-head sea bream, all genes were similarly expressed in the brain and gonad, whilst the transcription levels in SAF-1 cells were much lower for pkr, ifn and mx. In European sea bass, all the genes were constitutively expressed with little variation between the tissues and usually lower in the DLB-1 cell line, derived from sea bass brain.

**Most of the genes were upregulated in vitro by polyI: C and VNNV infection**

In the gilt-head sea bream SAF-1 cell line, mda and irf3, but not tbk1, transcription levels were similarly induced by polyI: C or VNNV, except in the case of ifn transcription levels, which were unaffected by polyI: C and greatly upregulated by VNNV infection (Fig. 3). However, whilst mx gene expression was greatly induced, pkr transcription...
was downregulated by both stimuli. In a similar manner, both polyI: C and VNNV induced most of the genes related to the IFN production pathway in the sea bass DLB-1 cell line, although polyI: C usually provoked a greater induction (Fig. 3). Interestingly, VNNV failed to induce mda5 and lgp2 transcription, although the downstream genes were significantly upregulated. Moreover, in the sea bass DLB-1 cell line, tbk1 expression was unaltered with both polyI: C and VNNV, whilst pkr was increased only with polyI: C treatment.

**Sensors of viral dsRNA are upregulated in the gonad of VNNV-infected European sea bass**

We evaluated the expression of the two identified RLRs, mda5 and lgp2, which are sensors for dsRNA, after VNNV infection (Fig. 4). In sea bream, mda5 transcription was increased in the brain, but unaffected in the gonad. However, in the sea bass, both mda5 and lgp2 were similarly regulated upon VNNV infection in both tissues. Thus, in the brain, they were downregulated at 1 and 7 days post-infection (p.i.), to be later upregulated. In contrast, these genes were upregulated in the gonad at 1 and 7 days p.i., and then remained unchanged.

**Adaptor and intermediaries are triggered by VNNV infection in the gonad of European sea bass**

In gilt-head sea bream, we only identified the tbk1 and irf3 intermediaries (Fig. 5). Transcription of tbk1 was unaltered by VNNV infection in any tissue, whilst irf3 gene expression was induced by VNNV at 7 and 15 days p.i. in the brain, and only at 1 day p.i. in the gonad. In European sea bass, the RLR adaptor mavs and most of the IFN production pathway intermediary genes were identified. As with the receptors, all the studied genes were downregulated in the brain of sea bass infected with VNNV, except for irf3 that was induced at 15 days p.i. (Fig. 4). In contrast, in the gonad, all of the genes (mavs, traf3, tank, tbk1, irf3 and irf7) were upregulated at different time points, mainly at 1 and 7 days p.i.

**VNNV greatly induces ifn, mx and pkr gene expression in the European sea bass gonad**

Finally, ifn was unaltered upon VNNV infection in the gilt-head sea bream brain and reduced its expression in the European sea bass brain (Fig. 6). However, in the gonad, ifn transcription was decreased in sea bream at 15 days p.i., but induced in sea bass at 1 and 7 days p.i. After IFN production, we evaluated the transcription of two ISGs, which are responsible for the antiviral response, in our case mx and pkr. Thus, in sea bream, both genes were upregulated upon VNNV infection in the brain,

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*Sequences with predicted full length.
increasing their levels along with infection, but were unaltered in the gonad (Fig. 6). In contrast, sea bass brain mRNA levels of mx were greatly increased at 1 day p.i. and decreased thereafter at 7 days p.i., whilst pkr was only induced at 15 days p.i. (Fig. 6). In the gonad, however, mx was greatly induced at 7 and 15 days p.i., but undetected at 1 day p.i. Nevertheless, pkr transcription was always induced; the highest levels were reached at 1 day p.i. and decreased thereafter.

**DISCUSSION**

Gilt-head sea bream and European sea bass are the most important fish species in Mediterranean aquaculture. So far, single ifn genes, belonging to type I IFNs, have been documented and partially characterized together with the IFN-induced mx gene (Casani et al., 2009; Fernández-Trujillo et al., 2011; Scapigliati et al., 2010). Focusing on VNNV, the two viral genes, coding for the capsid and RNA-dependent RNA polymerase, were found at very low levels in the brain of sea bream specimens and increased up to 10^7-fold in the brain of sea bass (Chaves-Pozo et al., 2012). Strikingly, it has been recognized that VNNV infections induce a major type I IFN response in the main target tissue, the brain, and that this activation might be responsible for the viral clearance in the resistant fish species gilt-head sea bream, whilst low activity is observed in those susceptible species, such as European sea bass (Chaves-Pozo et al., 2012; Chen et al., 2014; López-Muñoz et al., 2012; Overgaard et al., 2012; Poisabeiro et al., 2008; Scapigliati et al., 2010). However, very little is known about the molecular mechanisms leading to virus-induced type I IFN activation in fish, particularly by VNNV (Dios et al., 2007; Rise et al., 2010). Moreover, none of these studies have looked at the gonad immune response in these species – an issue that is highlighted when taking into account that this tissue is used to vertically transmit VNNV to the progeny (Arimoto et al., 1992; Kuo et al., 2012). Concretely, although we failed to detect any viral gene expression by conventional and real-time qPCR, we have already shown that VNNV is able to replicate in the gonad of gilt-head sea bream and European sea bass by *in situ* PCR, immunohistochemistry and viral recovery using cell culture (Valero et al., 2014). In addition, and most strikingly, the activity of antimicrobial
peptides, and their transcription, was greatly upregulated in the gonad of VNNV-infected sea bass specimens, but this was not the case in the sea bass brain and in the gonad of sea bream specimens (Valero et al., 2015). These data point to the importance of gonad immunity in VNNV establishment and dissemination, and prompted us to carry out this study.

We have searched ESTs databases of gilthead sea bream and European sea bass as well as the European sea bass gill transcriptome for RLR genes and mediators leading to IFN production. We found some RNA sensors such as mda5 sequences in both fish species and lgp2 in only sea bass, but failed to detect any rgl1 mRNA (Fig. 1). In a similar manner, mda5 and lgp2 genes have been identified in all teleost fish studied so far, although the presence of rgl1 is limited to ancient fish and has never been identified in modern fish (class Acanthopterygii) (Aoki et al., 2013), in which our fish species are included. Our data showed that the expression level of mda5 was upregulated in the SAF-1 cell line, which supports VNNV replication (Bandin et al., 2006), in a similar way to the zebrafish ZF-4 cell line, which also supports VNNV replication, in which rgl1, mda5 and lgp2 transcription was upregulated by VNNV infection (Chen et al., 2015). However, neither mda5 nor lgp2 was altered in the newly obtained sea bass DLB-1 cells, in contrast to the situation with polyI:C stimulation. This could indicate that VNNV is not able to replicate in sea bass DLB-1 cells, although this needs to be further confirmed. Moreover, upregulation of the transcription of mda5 and lgp2 after VNNV infection in vivo suggests that their production is induced upon viral infection, and that they may recognize viral RNA and induce the IFN response. The induction is of particular importance in sea bream brain and in sea bass gonad, indicating that these tissues exert a high antiviral response. Similar upregulation has been documented in the brain of sea bass and Atlantic halibut exposed to VNNV (Dios et al., 2007; Rise et al., 2010), and this supports our data. Moreover, these sensors are also upregulated by several fish RNA viruses or polyI:C in several tissues of fish, such as spleen, head-kidney, liver or intestine, as well as in some fish cell lines, leading to an increase in the type I IFN-mediated antiviral response (Feng et al., 2011; Rise et al., 2008, 2010; Skjesol et al., 2011; Su et al., 2010; Yang et al., 2011). Moreover, fish rgl1 and mda5 transient overexpression leads to the induction of ifn expression and confers an antiviral state (Biachesi et al., 2009; Sun et al., 2011). Very recently, in addition, rgl1 knock-down in ZF-4 cells has demonstrated the importance of the group II type I IFN pathway in VNNV infections (Chen et al., 2015). However, lgp2 overexpression can produce both inducing and inhibitory effects on ifn expression, as evidenced in fish and mammals (Komuro & Horvath, 2006; Ohtani et al., 2012; Sun et al., 2011), probably due to the lack of the CARD domain, which is only present in RIG-I and MDA5 proteins.

We also investigated the presence and regulation of genes between the RLRs and IFN (Fig. 1). Thus, we looked for and found in the gilthead sea bream ESTs databases sequences two intermediate molecules, tbk1 and irf3 transcripts, and in the European sea bass we successfully obtained sequences for most of the molecules involved in the INF-induced pathway: mavs, traf3, tank, tbk1, irf3 and irf7 mRNA. Although most of them are only partial sequences, analysis of the predicted proteins resulted in bona fide orthologues to the expected proteins. Their expression in naive conditions and upon VNNV infection in brain and gonad correlated with the expression of ifn and two ISGs, i.e. mx and pkr. Regarding these genes, our results showed that VNNV was able to increase the expression of genes related to the RLR adaptor mavs and intermediaries of the pathway leading to the IFN production. Strikingly, these genes were usually downregulated in the brain of sea bass specimens infected with VNNV, but upregulated in the gonad. This fact would suggest a high

Fig. 4. In vivo VNNV infection modifies the expression of (a) mda5 and (b) lgp2 in the brain and/or gonad. Gene expression was studied by qPCR at 1, 7 and 15 days p.i. (10^6 TCID_{50} per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n=4–6) mRNA fold increase with respect to control samples. Significant differences (ANOVA, P<0.05) compared with controls at each sampling time are denoted by an asterisk.
IFN or antiviral response in the sea bass gonad and a very low response in the brain, which could explain the low resistance of this fish species, but this needs to be confirmed at functional level. These results are in agreement with other studies in fish showing the upregulation of most of these genes after virus infection in several tissues or their antiviral function after cell line overexpression (Biacchesi et al., 2009; Chen et al., 2015; Feng et al., 2011; Rise et al., 2008, 2010; Skjesol et al., 2011; Su et al., 2010; Sun et al., 2011; Xiang et al., 2011; Yang et al., 2011), and support the fact that the sequences identified in our study mediate the IFN activation cascade. In the case of tbk1, which is also activated by the TLR response, it is only upregulated in sea bass specimens infected with VNNV. However, fish tbk1 has been shown to be activated by virus, polyI: C, peptidoglycan and/or lipopolysaccharide, indicating that this molecule can be activated by both viral and bacterial pathogens (Chi et al., 2011; Feng et al., 2011, 2014; Zhang et al., 2014). Moreover, some data indicate the activation of tbk1 and the antiviral response without the major involvement of IRF3/7, pointing to the existence of other activation pathways in fish (Feng et al., 2014). Our data showed that in the case of gilt-head sea bream, which is able to clear VNNV

**Fig. 5.** *In vivo* VNNV infection modifies the expression of (a) tbk1 and irf3 in gilt-head sea bream and (b) mavs, traf3, tank, tbk1, irf3 and irf7 in European sea bass. Gene expression was studied by qPCR at 1, 7 and 15 days p.i. (10⁶ TCID₅₀ per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n=4–6) mRNA fold increase with respect to control samples. Significant differences (ANOVA, P<0.05) compared with controls at each sampling time are denoted by an asterisk.
infection (Chaves-Pozo et al., 2012). tbk1 expression was not upregulated, suggesting that this molecule is not essential to the gilt-head sea bream antiviral immune response.

Finally, this cascade leads to the activation of the IFN response (Fig. 1). Our data showed that ifn transcription in gilt-head sea bream was not achieved through the downstream activation of ISGs such as mx and pkr was mainly observed in the brain of VNNV-infected specimens. This could be explained by the different induction times, as ifn expression is usually very fast and lasts for a short period, or to the presence of different ifn forms and splicing variants, which is unknown so far and deserves further attention. In contrast, in the European sea bass, inhibition of brain expression of ifn, as with most of those genes involved in the induction cascade, was concomitant with an increase in the transcription of mx and pkr.

All the data point to the existence of other activation pathways in fish, as suggested previously (Feng et al., 2014) and demonstrated in ZF-4 cells in which the involvement of the TLR activation pathway is evidenced after VNNV infection (Chen et al., 2015). In addition, pkr is designed as an ISG, but it is able to directly recognize and bind to viral RNA, and therefore might be considered as another PRR. This could be supported by the finding that ZF-4 cells knocked down in rig1 and infected with VNNV showed upregulated pkr expression (Chen et al., 2015). Interestingly, in the gonad of VNNV-infected sea bass specimens, ifn, mx and pkr genes were also upregulated, as also occurred with the sensors and intermediary genes. In previous studies, the induction of the IFN pathway after viral infection has been evaluated in several immune-relevant tissues (Chaves-Pozo et al., 2010; Feng et al., 2011, 2014), but never in the fish gonad. This is important as it is known that gonad immunity is tissue-specifically regulated in fish (Chaves-Pozo et al., 2005) and used by pathogens for their dissemination (Arimoto et al., 1992; Kuo et al., 2012). The upregulation of the antiviral response in the gonad of European sea bass specimens surviving VNNV infection could be a mechanism for which fighting the pathogen is more important than maintaining the functionality of the gonad for reproductive purposes. However, in the gilt-head sea bream, specimens which overcome the infection, the tight regulation of the gonad immune response could avoid germ cell damage, but at the same time allow the transmission of the virus through the gonad fluids and gametes. This hypothesis is supported by the fact that when other immune molecules such as antimicrobial peptides are studied, their expression patterns in the brain and gonad of VNNV-infected sea bass are similar (Valero et al., 2015). However, the antiviral immune response in the reproductive organs deserved further investigation, as in immature rainbow trout (Oncorhynchus mykiss) females, VHSV infection provoked an upregulation of type I IFN genes (ifn1, ifn2, ifn3/4, mx1, mx2 and mx3) in the ovary (Chaves-Pozo et al., 2010). In addition, recombinant IFN1 and IFN2 were able to induce the expression of mx genes and confer antiviral activity against VHSV in vitro; mx3 showed the highest upregulation (Chaves-Pozo et al., 2010). This points to the importance of the gonad IFN response to control the dissemination of viral pathogens in fish – an aspect that has not been considered in the past.

In conclusion, this study represents one of the most complete characterizations of the genes leading to the IFN response after viral infection by RLRs in fish. Thus, for the first time, to the best of our knowledge, we have identified several molecules of gilt-head sea bream and European sea bass involved in the activation cascade of IFN,
including viral RNA receptors (MDA5 and LGP2), the RLR adaptor (MAVS) and intermediaries (TRIF3, TANK, TBK1, IRF3 and IRF7). We also reported their simultaneous regulation upon VNNV infection. Thus, in sea bream, we found that mda5, irf3, mx and pkr were upregulated in the brain, but not in the gonad. However, in the susceptible European sea bass, the expression levels of most of the genes were downregulated in the brain, but significantly upregulated in the gonad, which resulted in an enhanced transcription of ifn, mx and pkr in this tissue. This is the first time that a study has covered a broad view of the fish IFN pathway after viral infection, and has also included the gonad as an important tissue where the virus might be hidden and transmitted to the progeny.

METHODS

Animals and cell lines. Adult specimens of the marine teleost gilt-head sea bream (Sparus aurata) and European sea bass (D. labrax) (125 ± 25 and 305 ± 77 g body weight, respectively) were bred at the Centro Oceanográfico de Murcia (IEO) under natural conditions of photoperiod, temperature, salinity and aeration, and transferred to the University of Murcia aquaria. Fish were kept in 450–500 l running seawater (28 ‰ salinity) aquaria at 24°C, following a dark photoperiod and daily fed with 1 g per fish of a commercial pellet diet (Skretting). Animals were acclimatized for 15 days prior to the experiments. All animal studies were carried out in accordance with the Guidelines of the European Union Council (2010/63/UE), the Bioethological Committee of the University of Murcia (Spain) and the Instituto Español de Oceanografía (Spain) for the use of laboratory animals.

Cell lines were cultured at 25 °C in 25 cm² plastic tissue culture flasks (Nunc) and maintained at exponential growth. The established striped snakehead SSN-1 (Frerichs et al., 1996) and sea bream SAF-1 (Béjar et al., 2005) cell lines were cultured using Leibovitz's L15-medium (Life Technologies) supplemented with 10 % FBS (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 IU penicillin ml⁻¹ (Life Technologies) and 100 μg streptomycin ml⁻¹ (Life Technologies), whilst a new cell line derived from the European sea bass brain (DLB-1) obtained in our laboratory was cultured using Eagle’s minimal essential medium (Life Technologies) supplemented with 15 % FBS, glutamine and antibiotics, as above.

VNNV stocks. VNNVs [strain 411/96, genotype RGNNV (redspotted grouper nervous necrosis virus)] were propagated in the SSN-1 cell line, which was persistently infected with a snakehead retrovirus (Frerichs et al., 1996). Cells were inoculated with VNNV and incubated at 25 °C until the cytopathic effect was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates before use in the experiments (Reed & Muench, 1938).

Gene search and bioinformatic analysis. According to the literature (Sun et al., 2011; Takeuchi & Akira, 2008; Zhang et al., 2014), virally activated RLRs (MDA5, LGP2 or RIG-I) initiate a molecular pathway leading to the expression of IFN and ISGs creating the cellular antiviral state. Thus, these receptors interact with the RLR adaptor protein MAVS and then associate with TRAF3, which recruits and facilitates the interaction between, but not exclusively, TANK and TBK1, also activated by TLR3, and therefore the TLR and RLR IFN activation pathways by viral RNA are shared from this point. TBK1, in turn, phosphorylates and activates IRF3 and IRF7.

IRF3 and IRF7 are then translocated to the nucleus where they bind to the IFN-stimulated response elements (ISREs), and activate the expression of ifn and ISGs, including those coding for Mx and PKR.

Therefore, in this work, the corresponding coding sequences for zebrafish proteins were selected and launched using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) within the EST databases from gilt-head sea bream and European sea bass as well as within the European sea bass gill transcriptome (Núñez Ortiz et al., 2014). Thus, deduced protein sequences from the full or partial gene sequences were obtained and analysed for similarity with known orthologue sequences and domain conservation using the BLAST program (Altschul et al., 1990) within the ExpASy Molecular Biology server (http://us.expasy.org). Phylogenetic and molecular evolution-ary analyses were conducted using MEGA version 6 (Tamura et al., 2013) to confirm that they were expected bona fide sequences. The sequences found and studied, related to IFN pathway activation by RLRs, are described in this work (Fig. 1).

In vitro infections. Duplicate cultures of SAF-1 and DLB-1 cells were incubated for 24 h with culture medium alone (controls) or containing 50 μg polyl: C ml⁻¹ or VNNV at 10⁶ TCID₅₀ ml⁻¹. After treatment, monolayers were carefully washed with PBS and stored in TRIzol (Life Technologies) at −80 °C for later isolation of RNA.

In vivo infections with VNNV. Thirty specimens of gilt-head sea bream or European sea bass were randomly divided into two tanks. Each group received a single intramuscular injection of 100 μl SSN-1 culture medium (mock-infected) or culture medium containing VNNV at 10⁶ TCID₅₀ per fish, as this route of infection has been proven as the most effective (Aranguren et al., 2002). Fish were sampled at 1, 7 and 15 days p.i., and fragments of brain and gonad tissues were stored in TRIzol at −80 °C for later isolation of RNA.

Analysis of gene expression by real-time (qPCR). We studied the transcription of selected genes in brain and gonad from naive fish, SAF-1 and DLB-1 cell lines, as well as after in vitro treatments with polyl: C or VNNV and after in vivo infection with VNNV. Total RNA was isolated from stored TRIzol samples following the manufacturer’s instructions. Total RNA (1 μg) was treated with DNase 1 to remove genomic DNA and the first strand of cDNA synthesized by reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) with an oligo-dT₁₂-₁₈ primer (Invitrogen) followed by RNase H (Invitrogen) treatment.

qPCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the elongation factor 1α (ef1α) content in each sample and expressed as 2⁻ΔΔCt, where ΔΔCt was determined by subtracting the ef1α Ct value from the target Ct. Gene names followed the accepted nomenclature for zebrafish (https://wiki.zfin.org). The primers used were designed using the Oligo Perfect software tool (Invitrogen) and are shown in Table 2. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. Amplified products from positive samples were run in 2 % agarose gels and sequenced. After these verifications, all amplifications were performed in duplicate cDNAs and repeated once to confirm the results. Negative controls with no template were always included in the reactions.

Statistical analysis. Data in figures are represented as mean ± SEM (n=4–6 individuals in the in vivo experiment and n=2 independent in vitro experiments). Statistical differences between control and
treated groups were analysed by ANOVA \( (P \leq 0.05) \) using SPSS 20 (IBM) software.

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