Journal of General Virology (2015), **96**, 2176–2187 **DOI** 10.1099/vir.0.000164

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.

Received 5 March 2015
Accepted 24 April 2015

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;

One supplementary table is available with the online Supplementary Material.

Zhang & Gui, 2012; Zou *et al.*, 2007). Therefore, several names have been proposed for fish IFNs: type I IFNs, virus-induced IFNs, IFN-λ, IFN-φ 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 (Crozat & 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 IFIN1) 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; Aquareovius 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; Feng *et al.*, 2011; Rise *et al.*, 2008, 2010; Skjesol *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; Hedger, 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; Poisa-Beiro *et al.*, 2008; Scapigliati *et al.*, 2010). In addition, *mda5* and *lgp2* 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 sea bass 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 zebrafish orthologues (Table 1), resulting in bona fide sequences, which was further confirmed by the analysis of the

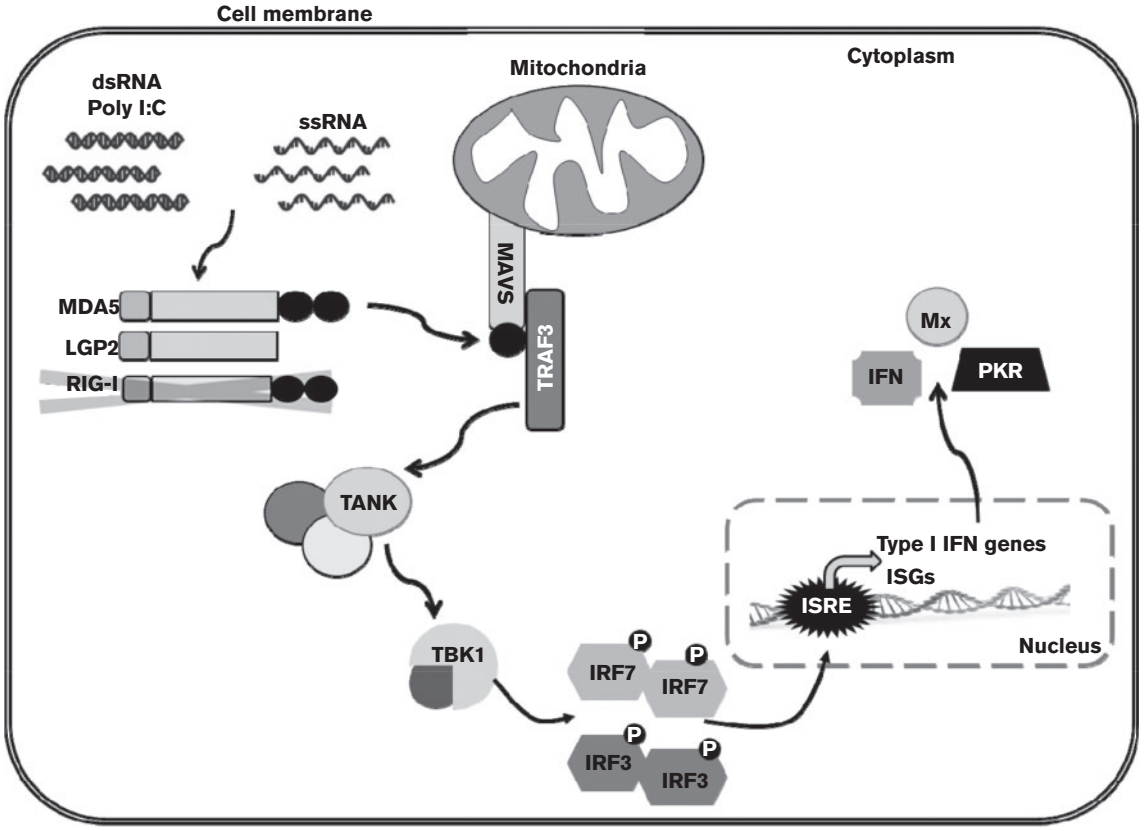

Fig. 1. RLR activation of the IFN response in gilt-head sea bream and European sea bass. RLR, retinoic acid-inducible gene I (RIG-I)-like receptor; MDA5, melanoma differentiation-associated gene 5; LGP2, Laboratory of Genetics and Physiology 2 protein; MAVS, mitochondrial antiviral signalling protein; TRAF3,, tumour necrosis factor (TNF) receptor-associated factor 3; TANK, TRAF 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).

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 (TANK), STKc_TBK1 in sea bass TANK-binding kinase 1 (TBK1), IRF-3 in both IFN regulatory factors IRF3 and IRF7, STKc_EI-F2AK2_PKR in sea bream dsRNA-dependent protein kinase receptor (PKR), and DSRM in sea bass PKR. All these domains were also found and conserved in the respective zebrafish and human orthologues.

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

Table 1. Identification of selected genes in the EST databases and European sea bass gill transcriptome, and their relation with the zebrafish orthologues (homology and *E* values of the predicted proteins with respect to the zebrafish orthologues)

Predicted protein	Fish species	GenBank accession no.	Protein length (aa)	Protein homology (%)	*E* value
MDA5	Sea bream	HS988207	289	71	1e−123
	Sea bass	AM986362	206	72	1e−91
	Zebrafish	XP_694124	997*		
LGP2	Sea bass	AM984225	297	71	2e−115
	Zebrafish	NP_001244086	679*		
MAVS	Sea bass	KP861888	586*	42	3e−18
	Zebrafish	XP_005156619	585*		
TRAF3	Sea bass	KP861887	595*	74	0.0
	Zebrafish	NP_001003513	573*		
TANK	Sea bass	KP861886	242	44	6e−42
	Zebrafish	NP_001070068	348*		
TBK1	Sea bream	HS988213	301	77	5e−154
	Sea bass	FM013306	220	95	3e−33
	Zebrafish	NP_001038213	727*		
IRF3	Sea bream	AM956899	201	44	3e−47
	Sea bass	CBN81356	465*	41	2e−87
	Zebrafish	NP_001137376	426*		
IRF7	Sea bass	KP861885	433*	51	4e−135
	Zebrafish	NP_956971	423*		
PKR	Sea bream	HS988732	306	52	3e−88
	Sea bass	FM008342	304	41	1e−41
	Zebrafish	CAM07151	682*		

*Sequences with predicted full length.

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,

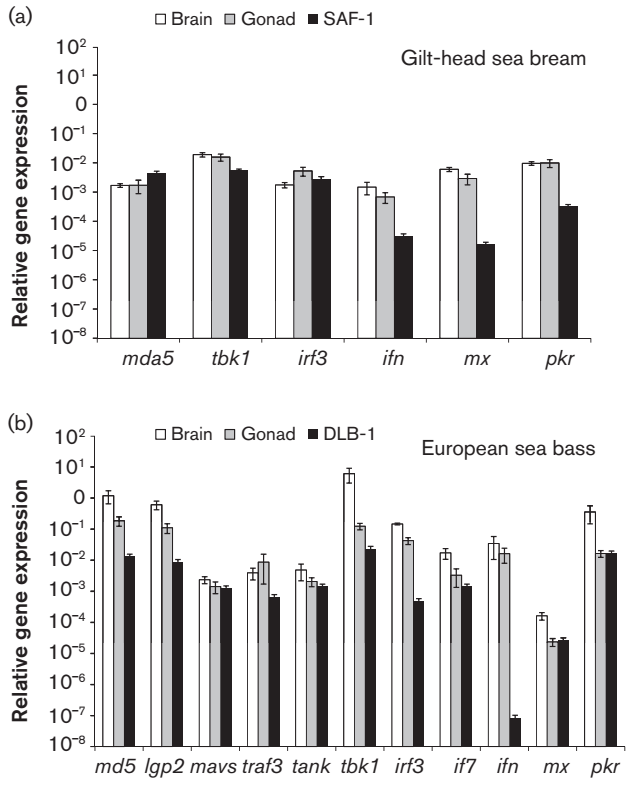

Fig. 2. Expression of genes related to the IFN-induced response pathway in naive (a) gilt-head sea bream and (b) European sea bass. The constitutive mRNA level of genes was studied by qPCR from naive brain, gonad or cell lines. Data represent mean ± SEM relative expression to the expression of endogenous control *ef1a* of six specimen tissues or two cell cultures.

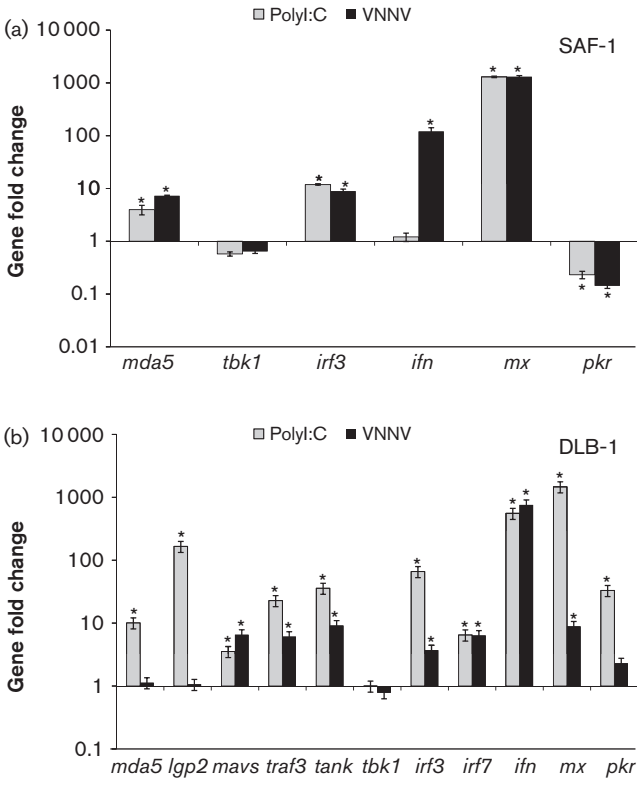

Fig. 3. Poly I: C and VNNV treatment upregulates most of the IFN production pathway genes in (a) SAF-1 and (b) DLB-1 cell lines derived from gilt-head sea bream and European sea bass, respectively. Results are expressed as the mean ± SEM (two independent experiments) mRNA fold increase with respect to control samples. Significant differences (ANOVA, $P \leq 0.05$) compared with controls are denoted by an asterisk.

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; Overgård *et al.*, 2012; Poisa-Beiro *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

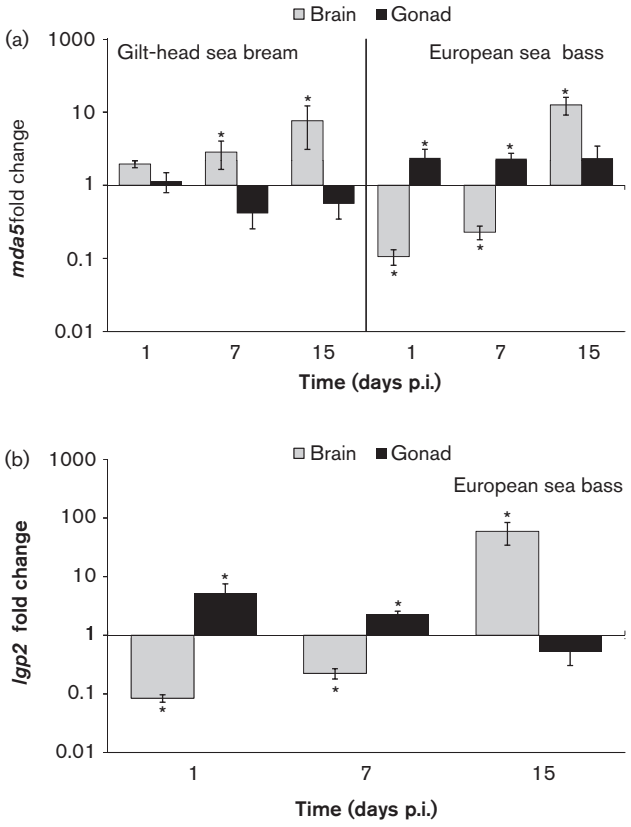

Fig. 4. *In vivo* VNNV infection modifies the expression of (a) *mda5* and (b) *lgp2* in the brain and/or gonad. Gene expression was studied by qPCR at 1, 7 and 15 days p.i. (10^6 $TCID_{50}$ per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n=4–6) mRNA fold increase with respect to control samples. Significant differences (ANOVA, $P \leq 0.05$) compared with controls at each sampling time are denoted by an asterisk.

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 gilt-head 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 *rig1* 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 *rig1* 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 (Bandín *et al.*, 2006), in a similar way to the zebrafish ZF-4 cell line, which also supports VNNV replication, in which *rig1*, *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 *rig1* and *mda5* transient overexpression leads to the induction of *ifn* expression and confers an antiviral state (Biacchesi *et al.*, 2009; Sun *et al.*, 2011). Very recently, in addition, *rig1* 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 gilt-head 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

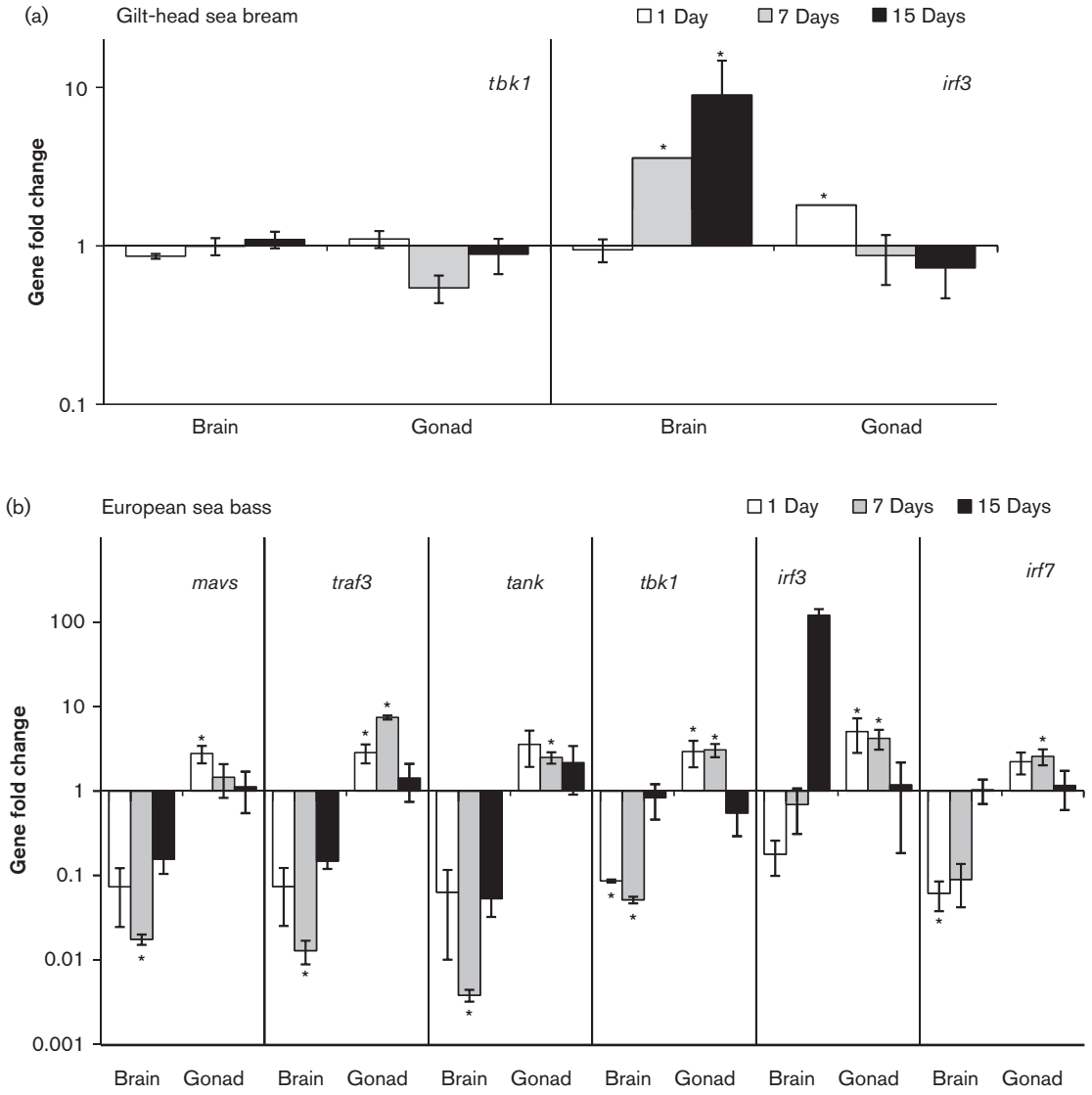

Fig. 5. *In vivo* VNNV infection modifies the expression of (a) *tbk1* and *irf3* in gilt-head sea bream and (b) *mavs*, *traf3*, *tank*, *tbk1*, *irf3* and *irf7* in European sea bass. Gene expression was studied by qPCR at 1, 7 and 15 days p.i. (10^6 $TCID_{50}$ per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n=4–6) mRNA fold increase with respect to control samples. Significant differences (ANOVA, $P \leq 0.05$) 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

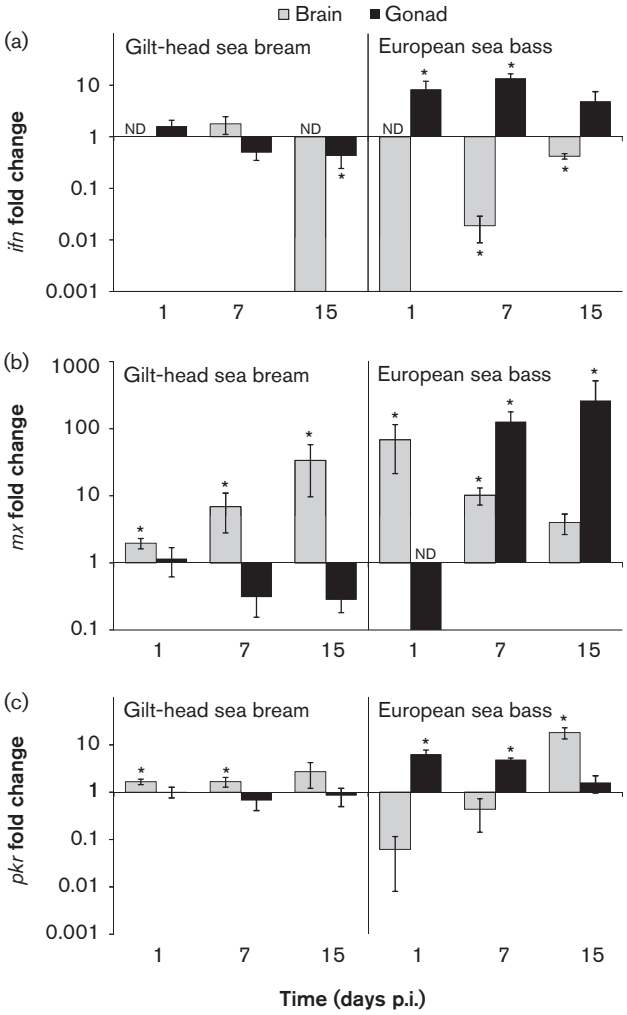

Fig. 6. (a) *ifn*, (b) *mx* and (c) *pkr* expression levels are regulated upon VNNV infection in gilt-head sea bream and European sea bass specimens. Gene expression was studied by pPCR at 1, 7 and 15 days p.i. (10^6 $TCID_{50}$ per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n=4–6) mRNA fold increase with respect to control samples. Significant differences (ANOVA, $P \leq 0.05$) compared with controls at each sampling time are denoted by an asterisk. ND, Not detected.

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 though 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 (Chi *et al.*, 2011; 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 (TRAF3, 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 ± 2 °C with a 12 h light: 12 h dark photoperiod and fed daily 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 Bioethical 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 cm^2 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 (Nuñez 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 evolutionary 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 polyI: C ml^{-1} or VNNV at 10^6 $TCID_{50}$ 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 10^6 $TCID_{50}$ 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 polyI: 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 I to remove genomic DNA and the first strand of cDNA synthesized by reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) with an oligo-$dT_{12–18}$ 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 (*ef1a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔC_t was determined by subtracting the *ef1a* C_t value from the target C_t. 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

Table 2. Primers used for analysis of gene expression by qPCR

Gene product	Gene abbreviation	Fish species	GenBank accession no.	Sequence (5′→3′)
Melanoma differentiation-associated gene 5 protein	*mda5*	Sea bream	HS988207	CATCGAGATCATCGAGGACA
				CCAGATGTCGCTCTTGAAGG
		Sea bass	AM986362	AATTCGGCAATGGTGAAGTC
				TCATTGGTCACAAGGCCATA
Laboratory of Genetics and Physiology 2 protein	*lgp2*	Sea bass	AM984225	TGATGGCAGTCAGTGGAGAG
				TGAGAGCTCAACGTGTTTGG
Mitochondrial antiviral signalling protein	*mavs*	Sea bass	KP861888	GCACAAGCTCAAAGCATCAA
				TCACTGGAGGGGGTGTTTAC
TNF receptor-associated factor 3	*traf3*	Sea bass	KP861887	CGATTAGCCGACATGGATCT
				TGCTTCCTGTTTCCGTCTCT
TRAF family member-associated NFκB activator	*tank*	Sea bass	KP861886	GCGGACAGCGAATATGACTT
				GCAATGTGGAGGGGACACTA
TANK-binding kinase 1	*tbk1*	Sea bream	HS988213	AGGAACAGCTGCCTCAGAAG
				CAGCTTCTTCATCCCCAGAG
		Sea bass	FM013306	ACAAGGTCCTGGTGATGGAG
				CGTCCTCAGGAAGTCCGTAA
IFN regulatory factor 3	*irf3*	Sea bream	AM956899	TCAGAATGCCCCAAGAGATT
				AGAGTCTCCGCCTTCAGATG
		Sea bass	CBN81356	AGAGGTGAGTGGCAATGGTC
				GAGCAGTTTGAAGCCTTTGG
IFN regulatory factor 7	*irf7*	Sea bass	KP861885	ATTCACCAACCGCATCCTTA
				GCCTCCAGGCATAGATACCA
dsRNA-dependent protein kinase receptor	*pkr*	Sea bream	HS988732	TCCTTTGGAACCTCCCTACC
				TCGAGGGGGAAATGTTGTAA
		Sea bass	FM008342	AGGGTCAGAGCATCAAGGAA
				GACACCTTGCTGTCCCAGTC
Type I IFN	*ifn*	Sea bream	FM882244	ATGGGAGGAGAACACAGTGG
				GGCTGGACAGTCTCTGGAAG
		Sea bass	AM765847	GGCTCTACTGGATACGATGGC
				CTCCCATGATGCAGAGCTGTG
Myxovirus (influenza) resistance protein	*mx*	Sea bream	FJ490556, FJ490555, FJ652200	AAGAGGAGGACGAGGAGGAG
				TTCAGGTGCAGCATCAACTC
		Sea bass	AM228977, HQ237501, AY424961	GAAGAAGGGCTACATGATCGTC
				CCGTCATTGTAGAGAGTGTGGA
Elongation factor 1α	*ef1a*	Sea bream	AF184170	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG
		Sea bass	FM019753	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG

treated groups were analysed by ANOVA ($P \leq 0.05$) using SPSS 20 (IBM) software.

ACKNOWLEDGEMENTS

E. C.-P. thanks the Ministerio de Economia y Competitividad for her Ramon y Cajal's research contract and Y. V. thanks the Instituto Espanol de Ocenaografia for her PhD grants. This work was supported by grants from the Ministerio de Economia y Competitividad and FEDER (AGL2010-20801-C02-01, AGL2010-20801-C02-02 and AGL2013-43588-P) and Fundacion Seneca (Grupo de Excelencia de la Region de Murcia 04538/GERM/06).

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