UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Immune Response in Fish Gonad Upon Nodavirus Infection and Vaccines Development

Implicación de la Respuesta Inmunitaria de la Gónada de Peces durante una Infección con Nodavirus y Desarrollo de Vacunas

Dña. Yulema Valero Cuesta

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CENTRO OCEANOGRÁFICO DE MURCIA

INSTITUTO ESPAÑOL DE OCEANOGRAFÍA

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A mis padres

A mi hermana

“Ve con confianza en la dirección de tus sueños.
Vive la vida que has imaginado”
H. D. Thoreau

“Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life”

Marco Aurelio

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INDEX

ABBREVIATIONS INDEX

11KT	11-ketotestosterone
A	androstenedione
ABC-AP	avidin-biotin alkaline phosphatase
ACH_{50}	50% alternative complement haemolysis
actb	beta actin beta chain coding gene
AG	acidophilic granulocyte
AMP	antimicrobial peptide
ANOVA	analysis of variance
APC	antigen presenting cell
APROMAR	asociación empresarial de productores de cultivos marinos de españa
B2	nodavirus B2 protein
bdef	beta defensin coding gene
Bf	B factor
BFNNV	barfin flounder nervous necrosis virus
Bl	blood
BLAST	basic local alignment search tool
Br	brain
BSA	bovine serum albumin
BV	blood vessels
bw	body weight
C3	complement factor 3
CARD	caspase recruitment domain
CD	cluster of differentiation co-receptor
cDNA	complementary deoxyribonucleic acid
ChP	chitosan nanoparticles
ChP-CP-pNNV	DNA vaccine codifying the capsid protein of nodavirus encapsulated in chitosan nanoparticles
ChP-pcDNA3.1	chitosan nanoparticles containing the empty plasmid
CMC	cell-mediated cytotoxicity
COM-IEO	Centro Oceanográfico de Murcia, Instituto Español de Oceanografía
ConA	concanavalin A
CP	capsid protein
CP-pNNV	DNA vaccine codifying the capsid protein of nodavirus
CPE	cytopathic effect
CpG ODN	cytosine-phosphodiester-guanosine oligodeoxynucleotide
csfr1	colony-stimulating factor receptor 1 coding gene
Ct	cycle threshold
CTL	cytotoxic T cell
cyp11b1	steroid 11-β-hydroxylase coding gene
cyp19a1	gonadal aromatase coding gene of European sea bass
cyp19a1a	gonadal aromatase coding gene of gilthead seabream
cyp19a2	neural aromatase coding gene

DAB	3,3’ - diaminobenzidine tetrahydrochloride
DC	dendritic cell
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
DEPC	diethylpyrocarbonate
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58
dic	dicentracin coding gene
DIG-11-dUTP	digoxigenin-11-2'-deoxy-uridine-5'-triphosphate
DLB-1	*Dicentrarchus labrax* brain cell line 1
DMRT1	double sex-and mab3-related transcription factor
DNA	deoxyribonucleic acid
DNase	deoxynuclease
dpf	days post-fertilization
dph	days post-hatching
DSRM	double stranded RNA-binding domain
dsRNA	double stranded ribonucleic acid
E-11	E-11 cell line
E_2	17β-estradiol
EDTA	ethylenediaminetetraacetic acid
ef1a	elongation factor 1 alpha coding gene
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle`s Minimal Essential medium
ER	nuclear estrogen receptor
esr	nuclear estrogen receptor coding gene
EST	Expressed Sequence Tag
ExPASy	SIB bioinformatics resource portal
FAO	Food and Agriculture Organization of the United Nations
FBS	foetal bovine serum
fH1LP	flounder histone 1 like protein
FSH	follicle stimulating hormone
FSH-R	follicle stimulating hormone receptor
GCRV	grass carp reovirus
Gi	gills
Go	gonad
GPER	G-protein coupled estrogen receptor
GPR30	G-protein coupled estrogen receptor 30
GSI	gonadosomatic index
GTH	gonadotrophin
GTP	trimeric G-protein
H1	histone 1
H2A	histone 2A
H2B	histone 2B
H3	histone 3
H4	histone 4

hamp	hepcidin coding gene
HBSS	phenol red-free Hank's buffer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEWL	hen egg white lysozyme
HK	head-kidney
HKL	head-kidney leucocyte
HLP	histone-like protein
hpf	hours post-fertilization
HRP	horseradish peroxidase
hsd11b	11β-hydroxysteroid dehydrogenase coding gene
hsd17b	17β-hydroxysteroid dehydrogenase coding gene
HUGO	human genome organization
ICC	immunocytochemistry
IEO	Instituto Español de Oceanografía
ifi25	interferon inducible protein 25 coding gene
IFIN1	interferon induced with helicase C domain 1
IFN	interferon
Ig	antibody / immunoglobulin
IL	interleukin
IPNV	infectious pancreatic necrosis virus
IPS-1	interferon promoter stimulator 1
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
isPCR	*in situ* polymerase chain reaction
ISRE	interferon-stimulated response elements
iTreg	induced T-regulatory cells
Jak-Stat	janus kinase-signal transducer and activator of transcription
JFNNV	Japanese flounder nervous necrosis virus
l13a	ribosomal protein L13 alpha coding gene
L-15	Leibovitz’s culture medium
LC	Leydig cell
Lck	lymphocyte-specific protein tyrosine kinase
LGP2	laboratory of genetics and physiology 2 or DHX58 protein
LH	luteinizing hormone
LHH1M	linker histone H1M
LH-R	luteinizing hormone receptor
Li	liver
LPS	lipopolysaccharide
Lv	lipovitellin
lyz	lysozyme coding gene
M	100-bp ladder marker
mAb	monoclonal antibody
MALT	mucosa-associated lymphoid tissue

MATH_TRAF3	meprin and tumour necrosis factor receptor-associated factor 3 homology domain
MAVS	mitochondrial antiviral-signalling protein or interferon β promoter stimulator-1
MB	body mass
MDA5	melanoma differentiation-associated gene 5 or IFIN1
MG	gonad mass
MHC	major histocompatibility complex
MMP	metalloproteinase
MO	macrophage
mpx	myeloid-specific peroxidase coding gene
mRNA	messenger ribonucleic acid
Mx	myxovirus (influenza) resistance protein or interferon-induced GTP-binding protein
NCC	non-specific cytotoxic cell
NCCRP-1	non-specific cytotoxic cell receptor protein 1
ND	non detected
NET	neutrophil extracellular trap
NF-kB	inhibitor of nuclear factor kappa-B kinase subunit beta
NK	natural killer
nk-lys	NK-lysin coding gen
NLR	nucleotide-oligomerization domain (NOD)-like receptor
NNV	nodavirus or nervous necrosis virus
NO	nitric oxide
NOD	nucleotide-oligomerization domain
OAS	2,5-oligoadenylate synthetase
OD	optical density
OHT	11β-hydroxytestosterone
OIE	World Organisation for Animal Health
oligo-dT	oligo deoxy-thymine nucleotides
One Shot TOP10	chemically competent *Escherichia coli* cells
ORF	open reading frame
P	significance level
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline supplemented with Tween-20
pcDNA3.1	religated empty mammalian vector plasmid
pcDNA3.1/V5-His-TOPO	eukaryotic expression vector
PCR	polymerase chain reaction
Pd	*Photobacterium damselae*
Pe	peritubular cell
PHA	phytohemagglutinin
pI:C or poly I:C	polyinosinic-polycytidylic acid

pis	piscidin coding gene
PKR	double stranded (ds)RNA dependent protein kinase receptor
PRR	pattern recognition receptor
PSG	primary spermatogonia
Pv	phosvitin
RdRp	RNA dependant RNA polymerase
RGNNV	red-spotted grouper nervous necrosis virus
RIG-I	retinoic-acid-inducible gene I protein
RING	really interesting new gene finger domain
RLR	retinoic-acid-inducible gene I (RIG-I)-like receptor
RNA	ribonucleic acid
RNA1	nodavirus ribonucleic acid 1
RNA2	nodavirus ribonucleic acid 2
RNA3	nodavirus ribonucleic acid 3
RNase	ribonuclease
RNA-seq	RNA sequencing
RNI	reactive nitrogen intermediary species
ROI	reactive oxygen intermediary species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute culture medium
RPS	relative percent survival
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
S	Sertoli cell
SACS	sacsin molecular chaperone protein
SAF-1	*Sparus aurata* fibroblast-like cell line 1
SC	spermatocyte
SD	spermatid
SEM	standard error of the mean
SG	spermatogonia
SGA	spermatogonia A
SGB	spermatogonia B
SGSC	spermatogonia stem cell
siRNA	silencing RNA
Sk	skin
sL-15	Leibovitz's culture medium supplemented with sodium chloride
SnRV	snakehead retrovirus
Sp	spleen
SRBC	sheep red blood cells
sRPMI	Roswell Park Memorial Institute culture medium supplemented with sodium chloride
SSC	sodium citrate dehydrate with NaCl buffer
SSN-1	striped snakehead cell line

ssRNA	single stranded ribonucleic acid
STECF	scientific, technical and economic committee for fisheries
STKc_EIF2AK2_PKR	smart sticky-C eukaryotic translation initiation factor 2-alpha PKR domain
STKc_TBK1	smart sticky-C TBK1 domain
sTSB	tryptic soy broth supplemented with sodium chloride
SVCV	spring viremia of carp virus
SZ	spermatozoa
T	testosterone
T4	variable region (T4) of the RNA1 of nodavirus
TANK	TRAF family member-associated NF-kappa-B activator
TBD	tbk1/Ikki binding domain
TBK1	TANK-binding kinase 1
TBS	tris buffered saline
TCA	trichloroacetic acid
$TCID_{50}$	median tissue culture infective dose
TCR	T cell receptor
TGF	transforming growth factor
Th	T-helper
Thy	thymus
TLR	Toll-like receptor
TMB	3.3',5.5' – tetramethylbenzidine hydrochloride
TNF	tumour necrosis factor
TPNNV	tiger puffer nervous necrosis virus
TRAF3	tumour necrosis factor (TNF) receptor-associated factor 3
TRIM	tripartite motif
TRIM39	tripartite motif (TRIM)-containing protein 39
TSA	tryptic soy agar
TSB	tryptic soy broth
UV	ultraviolet
Va	*Vibrio anguillarum*
VER	viral encephalopathy and retinopathy
Vg	vitellogenin
Vh	*Vibrio harveyi*
VHSV	viral haemorrhagic septicaemia virus
vs	versus
ZF-4	zebrafish cell line 4
ZNC	zebrafish nomenclature committee

SPECIES, VIRUSES AND CELL LINES INDEX

Fish species

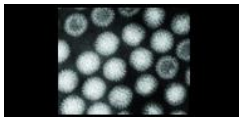
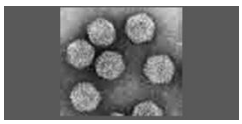
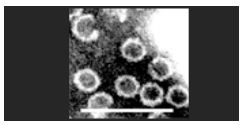
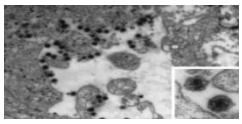
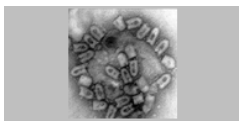
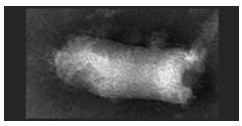
Common name	Scientific name	
Asian sea bass	*Lates calcarifer*	
Atlantic cod	*Gadus morhua*	
Atlantic halibut	*Hippoglossus hippoglossus*	
Atlantic salmon	*Salmo salar*	
Channel catfish	*Ictalurus punctatus*	
Common carp	*Cyprinus carpio*	
European flounder	*Platichthys flesus*	
European sea bass	*Dicentrarchus labrax*	
Fugu	*Takifugu rubripes*	
Gilthead seabream	*Sparus aurata*	
Goldfish	*Carassius auratus*	

Guppy	*Poecilia reticulata*	
Medaka	*Oryzias latipes*	
Olive flounder	*Paralichthys olivaceus*	
Orange-spotted grouper	*Epinephelus coioides*	
Rainbow trout	*Oncorhynchus mykiss*	
Rohu	*Labeo rohita*	
Shi drum	*Umbrina cirrosa*	
Silver seabream	*Sparus sarba*	
Striped jack	*Pseudocaranx dentex*	
Sturgeon	*Acipenser gueldestaedi*	
Tench	*Tinca tinca*	
Turbot	*Psetta maxima*	

Crustacean species

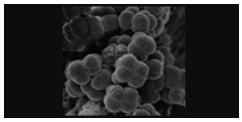
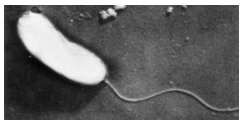
Common name	Scientific name	
Artemia	*Artemia salina*	

Virus

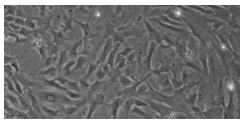
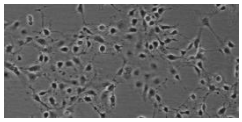
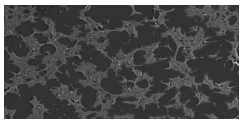
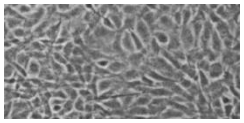
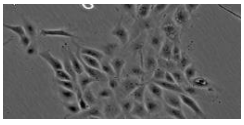
Common name	Family	
Grass carp reovirus (GCRV)	*Reoviridae*	
Infectious pancreatic necrosis virus (IPNV)	*Birnaviridae*	
Nodavirus or nervous necrosis virus (NNV)	*Nodaviridae*	
Snakehead retrovirus (SnRV)	*Retroviridae*	
Spring viremia of carp virus (SVCV)	*Rhabdoviridae*	
Viral haemorragic septicaemia virus (VHSV)	*Rhabdoviridae*	

Bacteria

Superior classification	Scientific name	
Aeromona	*Aeromonas hydrophila*	

Micrococco	*Micrococcus lysodeikticus*	
Photobacteria	*Photobacterium damselae*	
Vibrio	*Vibrio harveyi*	
Vibrio	*Vibrio anguillarum*	
Escherichia	*Escherichia coli*	

Cell lines

Name	Species of origin	
DLB-1	*Dicentrarchus labrax*	
E-11	*Channa striata*	
SAF-1	*Sparus aurata*	
SSN-1	*Channa striata*	
ZF-4	*Danio rerio*	

SUMMARY

Gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) are the most important fish species for Mediterranean aquaculture and also for Spain. Among the problems to improve their culture are the high economic losses produced by pathologies. Thus, nodavirus (NNV), which actually is spreading worldwide, produces very high mortalities in European sea bass, a very susceptible species, and uses seabream as a resistant reservoir. In addition, this virus shows vertical transmission through gametes and gonadal fluids and no clearly effective preventive methods are available yet. Thus, in this Doctoral Thesis, we aimed to evaluate the interaction between European sea bass and gilthead seabream gonad and NNV (Part 1) and how to prevent its pathology and dissemination (Part 2).

The Part 1 of this Doctoral Thesis contains four chapters (III.1.1 to III.1.4). In Chapter III.1.1., we studied whether NNV is able to colonize and replicate in the gonad of European sea bass and gilthead seabream males, and demonstrate whether the virus is able to replicate into the tissue. We firstly demonstrated that NNV infects and replicates into the testis of both species since we localized viral RNA in somatic cells of testis of both species and in germ cells of gilthead seabream by *in situ* PCR (isPCR) whether capsid protein (CP) and B2 peptide of the virus were only detected in gilthead seabream testis by immunocytochemistry. Furthermore, infective viral particles were rescued from the gonad of both species using cell culture techniques. Regarding reproductive functions, NNV infection altered sex hormones 17β-estradiol and 11-ketotestosterone synthesis and their serum levels and the sensitivity of brain and testis to these hormones, whereas there was no disruption of testicular functions according to the results related to gonadosomatic index (GSI), the gene expression level of *dmrt1* and the morphology of the testis.

In Chapters III.1.1 to III.1.4, we also determined how the immune responses are affected in gonad and brain, the main target tissue of the virus. Our results (Chapter III.1.1) showed a higher inflammatory response in gonad and brain of European sea bass, showing the up-regulation of all the pro-inflammatory genes analysed (*tnfa*, *il6* and *il1b*), compared to gilthead seabream specimens, in which only *il6* gene was slightly up-regulated in brain. These results are in concordance with the fact that European sea bass specimens reached mortality rates up to 55% after only 15 days of infection, whilst gilthead seabream was able to overcome the infection. Type I interferon (IFN) response (Chapter III.1.2) is one of the most powerful innate immune

responses against virus. 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 the IFN production are scarcely known. Thus, we have searched for the presence of these genes in both fish models used in this Doctoral Thesis and evaluated their expression upon NNV infection in brain and gonad, usually used by certain pathogens to be transmitted vertically. We succeeded in identifying the sequences coding for MDA5, TBK1, IRF3, IFN, Mx and PKR proteins in both species, and, in addition, for LGP2, MAVS, TRAF3, TANK and IRF7 in European sea bass. In order to characterize the IFN pathway, we studied their expression in two different cell lines, SAF-1 from gilthead seabream and DLB-1 from European sea bass brain, in which most of the genes were up-regulated after the *in vitro* treatment with NNV or polyinosinic-polycytidylic acid (pI:C). After the *in vivo* infection with NNV in gilthead seabream, most of studied genes were up-regulated in brain and unaltered in gonad, probably favouring the dissemination through gonad fluids or gametes. Strikingly, in European sea bass, all the genes analysed were up-regulated in the gonad but only *mda5*, *lgp2*, *irf3*, *mx* and *pkr* did in brain. In both species, the *tbk1* gene was not stimulated by any treatment; neither *in vivo* nor *in vitro*, suggesting that it could not be relevant in this route. These findings support the idea that the innate immune response in European sea bass brain is unable to clear the virus and points to the importance of the gonad immunity to control the dissemination of NNV, an aspect that is worthy to investigate in aquatic animals.

Antimicrobial peptides (AMPs) have a crucial role in fish innate immune response, being considered an essential component of the first line of defence against pathogens. Moreover, the importance of AMPs in fish gonad is based on the needed to develop an efficient immune response without damage the germ cells to avoid the used of the tissue by some pathogens as a vehicle or a reservoir to be transmitted to the progeny, as occurs with NNV, but no study has looked into the gonad of infected fish. Regarding this, we have characterized the antimicrobial response triggered by NNV in the testis of European sea bass and in the gilthead seabream, both *in vivo* and *in vitro*, and compared with that present in the serum and brain (Chapter III.1.3.). First, our data show a great antiviral response in the brain of gilthead seabream and in the gonad of European sea bass since *mx* gene expression was greatly up-regulated. In addition, for the first time, our results demonstrate that the antimicrobial activities (complement,

lysozyme and total bactericidal) and the expression of several AMP genes (*c3*, *lyz*, *hamp*, *dic*, *pis* or *bdef*) in the gonad and brain of both species are very different, but generally activated in the European sea bass, probably related with the differences of susceptibility upon NNV infection. Furthermore, our *in vitro* results suggest that some AMPs (*lyz*, *hamp* and *dic*) are locally regulated playing a local immune response in the gonad, while others (*c3*) are more dependent of the systemic immune system.

Histones (H1 to H4) are the primary proteins which mediate the folding of DNA into chromatin, however, and in addition to this function, histones have also been related to antimicrobial activity in vertebrates, in fact, mammalian H1 is mobilized as part as the anti-viral immune response. In fish, histones with AMP activity have been isolated and characterized mainly from skin and gonads. Taking all this into account, we studied the histones immune related role in European sea bass and gilthead seabream (Chapter III.1.4.). Firstly, we identified the complete H1 and H2B coding sequences in both fish species and studied their pattern of expression under naïve conditions and upon NNV *in vivo* infection. Our results showed the highest expression of *h1* gene in peripheral blood of both species and in thymus of European sea bass whilst in the case of *h2b* gene expression, the tissues where the *h2b* expression reached highest levels were European sea bass thymus and gilthead seabream blood and gonads. Upon *in vivo* infection with NNV, we found that *h1* gene expression was stimulated in the gonad or brain of European sea bass or gilthead seabream, respectively, but never in head-kidney. However, *h2b* gene expression was exclusively up-regulated in the head-kidney of both species. These genes seemed to be systemically regulated since they were not altered in the male gonad of both species after an *in vitro* infection with NNV. All these data obtained prompted us to study their role on the immune response of head-kidney leucocytes upon viral (NNV), bacterial (*Vibrio anguillarum* or *Photobacterium damselae*) or chemical stimulation. Although further studies are needed, our data suggest that H1 has a role in the immune response against NNV in brain, while H2B seems to be more important in head-kidney. Moreover, the potential role of histones as anti-viral agents is suggested and further characterization is in progress.

The Part 2 of this Thesis contains two chapters (III.2.1 and III.2.2) and is focused on the development of preventive tools to improve the survival of European sea bass larval and juveniles upon NNV infection. NNV is extremely harmful during larval development of European sea bass specimens, and at these early stages fish are very

difficult to be vaccinated due to their small size or their immature immune system. Among preventive tools to avoid fish viral infections, vaccines need to be designed and created to improve aquaculture sector production, being the DNA vaccines the most promising. Moreover, oral administration must be investigated for mass vaccination of early stages due to their no stress-associated problems. Taking into account the difficulty of injecting a vaccine to a very small immunocompetent juvenile fish, we have created an oral chitosan-encapsulated DNA vaccine specifically against NNV (ChP-CP-pNNV), and administered orally to early juveniles of European sea bass during two days (Chapter III.2.1). After 90 days of vaccination, we studied the total and the specific anti-NNV IgM levels in serum, and also the gene expression of different sequences coding for proteins involved in cell-mediated cytotoxicity (CMC: *tcrb* and *cd8a*), the antigen presentation (*mhc1a* and *mhc2b*), antibodies (*igmh* and *igth*) and IFN (*ifn*, *mx* and *ifng*) responses after 7, 30 and 90 post-vaccination in the posterior gut. Our study shows that the oral ChP-CP-pNNV vaccine failed to show circulating specific antibodies after 90 days of vaccination and differences on immunoglobulin (*igmh* or *igth*) gene expression levels in the posterior gut of vaccinated and non-vaccinated fish from 30 days after vaccination onwards. However, the vaccine up-regulated the expression of genes related to CMC (*tcrb* and *cd8a*) and IFN response (*ifn*, *mx* and *ifng*). In addition, three months after vaccination the resistance to an experimental NNV infection was significantly improved by retarding the onset of the fish death and the accumulative mortality, with a relative percent survival (RPS) of 45% in ChP-CP-pNNV vaccinated sea bass. In conclusion, we created a chitosan-encapsulated DNA vaccine against NNV that is partly protective to European sea bass juveniles and this protection seems to be mediated by CMC and IFN pathways, although further studies are needed to improve the anti-NNV vaccine and to understand their mechanisms of action.

The continuous transport of eggs and larvae between fish farms worldwide favours the spread of viral infections, especially, in the case of NNV. Although the first larval stages lack a mature immune system, the exposition to environmental pathogens is not a threshold thanks to the maternal transfer of immune factors from broodfish to their progeny. Thus vaccination of broodstock seems to be a feasible method to enhance the survival of the progeny eliciting the transfer of passive immunity. As the studies performed until now have mainly focussed on maternal transfer of specific immune

factors and the innate immune response is also essential to understand fish immunity, we have studied the role of innate immune effectors on the maternal transfer of immunity (Chapter III.2.2). In this work, we used the previously constructed and partially protective DNA vaccine against NNV (CP-pNN) to inject mature fluent females of European sea bass. As DNA vaccines elicit few specific antibodies in vaccinated specimens, we focussed on different antimicrobial activities (peroxidase, protease, anti-protease, lysozyme and total bactericidal) in the serum of vaccinated or sham-vaccinated females and in eggs or larval homogenates of their progeny, from eggs to 69 days post-fertilization. We have also analysed the pattern of expression of granulocytes and macrophages gene markers (*mpx* and *csf1a*, respectively) and several AMP genes (*hamp*, *dic*, *c3*, *lyz* and *nk-lys*). Our results showed that the vaccine elicits the maternal transference of some bactericidal activity effectors since eggs from vaccinated group showed increased activities at 0 days post-fertilization. Indeed, the maternal transfer described in our work seemed to be restricted to protein factors as transcript levels of mRNA of several leucocyte markers or AMPs were undetected in eggs. Interestingly, the immunisation of the broodstock females promoted a higher and earlier innate immune response in their progeny than in controls.

In conclusion, our study demonstrate for the first time the localization of NNV into the testis cells in fish and how the immune-reproductive interactions are regulated by NNV, which is hiding in the testis and likely using it to spread to the progeny. Furthermore, we have designed a DNA vaccine that shows promising properties as preventive tools against NNV disease when administered to juveniles or broodstocks females.

I. INTRODUCTION

According to the Food and Agriculture Organization (FAO) of the United Nations, in 2012, the extractive fishing captures only covered 58.7% of the fish population demand, in a framework in which the human demand of seafood to cover human needs of animal proteins is increasing (FAO, 2014). A situation in which aquaculture seems to be the most feasible option to increase human supply of fish. Thus, the estimated global seafood (excluding plants) supply increased from 155.8 million tonnes in 2011 to 158 million tonnes in 2012, being this increase driven by the aquaculture sector, which compensated a 2.6% decrease in capture fisheries production (FAO, 2014; STECF, 2014). Similarly, in the European Union, the capture fisheries have been decreasing from 1992 to 2012, while aquaculture has become relatively more important to supply the seafood market. In 2012 the aquaculture sector provided the 21% of the seafood supplied in Europe (STECF, 2014). The aquaculture production in Europe is mainly concentrated in a few countries, being the Spanish production up to 24% of the total European production in volume (STECF, 2014). Taking into account only the Spanish production of marine fish, the gilthead seabream (*Sparus aurata* L.) and the European sea bass (*Dicentrarchus labrax* L.) are the most relevant species in terms of production (tonnes) and commercial value (APROMAR, 2015).

In intensive farming facilities, the confinement of large amounts of specimens in a reduced space and the regular handling subject the animals to stress conditions, weakening their immune system, increasing their susceptibility to pathogens and favouring, in turn, the emergence of diseases. In addition, the actual movement of eggs, larvae and juveniles between facilities worldwide favour the spread of pathogens to wider areas, resulting in many economic losses (Fukuda, *et al.*, 1996; Grau, *et al.*, 2003; Villamil, *et al.*, 2003). All these issues together, make mandatory to know how to prevent pathogen spread all around the world. For that reason, the study of the immune responses at different tissues and life stages, the different transmission strategies of pathogens between specimens, together with the development of different vaccines and preventive strategies have become the major objectives in present aquaculture research.

1. Immune system of teleost fish

The term immunity is used to designate the immune reaction against foreign agents, including microorganisms (viruses, bacteria, fungi, protozoa and multicellular

parasites) and macromolecules (proteins and polysaccharides) without the consequences of disease of such pathological reaction (Abbas, *et al.*, 2012). The immune system is formed by cells and molecules responsible for this immunity, which collective and coordinated action against foreign agents composes the immune response (Abbas, *et al.*, 2012; Male and Roitt, 2001).

In fish, in sharp contrast to mammals, tissues related to the immune system comprise a haematopoietic area where blood cells of the myeloid lineage (erythrocytes, monocyte-macrophages, granulocytes and thrombocytes) are produced and a lymphoid area where lymphocytes are generated (Manning, 1998). Regarding the head-kidney (HK), the cephalic portion of the kidney, is the main hematopoietic organ in fish, although it also possesses lymphopoietic *foci* (Lin, *et al.*, 2005). Thus, the HK displays lymphoid functions as primary lymphoid organ (where lymphocyte maturation occurs) or even as secondary lymphoid organ (where mature lymphocytes establish contact with antigens) since some cells containing bacterial antigen have been observed in this tissue upon bacterial infection in some species (Chaves-Pozo, *et al.*, 2005a). Moreover, data related to dendritic cells (DCs) and DCs markers support the role of HK as secondary lymphoid tissue in fish (Zoccola, *et al.*, 2015). However, the thymus, located near the gills is the primary source of mature T lymphocytes, being the spleen (the main secondary organ in fish), located in the peritoneal cavity, composed by T and B lymphocytes (Zapata, *et al.*, 1996). In addition, there is a disperse and diffuse mucosa-associated lymphoid tissue (MALT) in the gut, gill and skin (Salinas, 2015) that is also part of the secondary lymphoid tissue.

In vertebrates, including fish, the immune response is divided in two types: innate (also known as natural or non-specific) and adaptive (also called acquired or specific) immune responses, whereas invertebrates only own innate immune responses (Abbas, *et al.*, 2012). Thus, the innate immune response is the collection of defence mechanisms that protect an organism against infection without any prior exposure to the pathogen, acting as the first line of defence against foreign agents until the specific response is activated. The adaptive immune response is characterized by its memory and specificity. Both types of responses are greatly interconnected, involving a wide variety of components. The immune response generally starts with the innate response that regulates and triggers the activation of lymphocytes determining the self-nature of the adaptive response and co-operating in the maintenance of the homeostasis (Fearon

and Locksley, 1996; Fearon, 1997). Fish constitute the first group of vertebrates possessing a complete immune system and owing cellular and humoral responses with specificity and memory (Van Muiswinkel, 1995). Although the fish immune system possesses relevant differences compared to mammals, fish are considered not only very interesting models for evolution of immunity but also excellent scientific biological models for addressing studies on biomedical research.

1.1. Innate immunity

In fish, the innate response is considered a pivotal component in the fight against pathogens due to their poikilothermic characteristic, their limited repertoire of antibodies and the slow proliferation and maturation of their lymphocytes (Whyte, 2007). The innate immune response is composed by three types of components: physical barriers, humoral factors and cellular elements (Abbas, *et al.*, 2012). Different pathogen-associated molecular patterns (PAMPs) are recognized thanks to a wide variety of pattern recognition receptors (PRRs), which activation trigger different molecular cascades (Fig. 1). These PRRs are classified in three types: Toll-like receptors (TLRs), retinoic-acid-inducible gene I (RIG-1)-like receptors (RLRs) and nucleotide-oligomerization domain (NOD)-like receptors (NLRs) (Shayakhmetov, 2010).

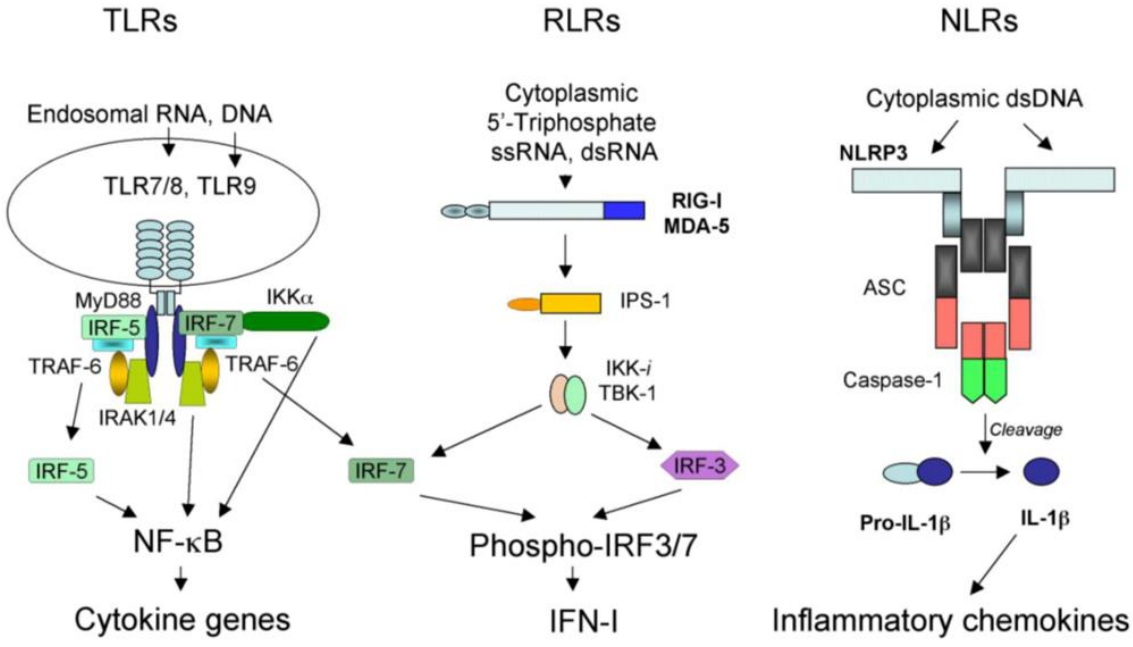


Figure 1: Pattern recognition receptors (PRRs) and molecular cascades triggered after their activation in fish (Shayakhmetov, 2010).

1.1.1. Physical barriers

Skin, gills and gastric tract act as the first barrier to infection (Magnadottir, 2010). The mucus, secreted at these barriers, contains many humoral factors such as lectins, pentraxins, lysozyme, complement components, antibacterial proteins and antibodies (immunoglobulins, Igs), that play a relevant role in the inhibition of pathogen invasion. Moreover, several immune cell types are present at these barriers such as lymphocytes, macrophages and eosinophilic granular cells (Magnadottir, 2006).

1.1.2. Humoral factors

1.1.2.1. Antimicrobial peptides

The antimicrobial peptides (AMPs) are considered to play a crucial role in the innate immune system and have been extensively studied in different invertebrates and vertebrate species, including fish (Mulero, *et al.*, 2008; Smail and Munro, 2008). These proteins are quite small (from 12 to 80 amino acids) with low molecular weight (from 1 to 5 kDa). In most of the cases they are cationic and amphipathic, though a minority of them are anionic peptides (Brogden, *et al.*, 2003). Their biochemical qualities allow them to directly interact with certain microbial components leading to a rapid death (Hancock and Rozek, 2002; Patrzykat and Douglas, 2003). Although the molecular mechanisms of action of these peptides are poorly understood, they show a broad-spectrum of antimicrobial activities against bacteria, virus, fungi, protozoa, and even tumour cells (Chia, *et al.*, 2010; Cuesta, *et al.*, 2008; Mihajlovic and Lazaridis, 2010). Furthermore, these peptides have other important functions such as regulators of inflammatory processes, orchestrating the adaptive immune response or recruiting effector cells (Chertov, *et al.*, 1996; Cuesta, *et al.*, 2008; Oppenheim, *et al.*, 2003).

1.1.2.2. Interferons (IFNs)

IFNs are secreted cytokines (low molecular weight proteins frequently with no more that 8-25 kDa) which induce an antiviral status in cells and are the most powerful mechanisms in the defence against viral infections (Robertsen, 2006). In fish, there are two different IFN families (type I and type II) attending to their gene sequences, protein structure and functional characteristics. Type I IFNs are similar to mammalian IFNα and IFNβ and are induced by viruses in all nucleated cells, whereas type II IFNs are counterparts to mammalian IFNγ, which is produced by activated natural killer (NK)

cells and T lymphocytes (Robertsen, 2006), being also involved in the adaptive immune response. In addition, type II FNs protect the surrounding cells of an infected one from viral infection by binding to different receptors and activating the Janus kinase-signal transducer and activator of transcription (Jak-Stat) pathway. This results in the induction of several hundred of genes named as IFN-stimulated genes (ISGs), that include molecules with direct anti-viral activity such as the protein myxovirus (influenza) resistance protein (Mx). Other molecules also related to virus response and/or IFN also have direct antiviral function such as the protein kinase double stranded (ds) RNA activated (PKR) or the 2,5-oligoadenylate synthetase (OAS) (de Veer, *et al.*, 2001; Robertsen, 2006). Although some viral sensors have been studied in fish (Aoki, *et al.*, 2013; Zou, *et al.*, 2009), the IFN pathway is not completely characterized and most of the studies used the *mx* gene expression as an indicator of viral infection and activation of the INF response.

1.1.2.3. Complement system

The complement system is composed by different proteins present on serum in soluble form or associated to cells as membrane receptors and is well developed in fish. The activation of the complement system at the site of infection leads to a complex cascade of proteases, consecutively activated by proteolytic cleavage, that mediate inflammatory responses and the killing and clearance of pathogens through the formation of membrane attack complexes and the opsonisation of the antigenic particles (Boshra, *et al.*, 2006; Nonaka and Smith, 2000). The complement system is activated through three different activation routes merging at the point of the central pivotal component, the C3 protein. The three activation pathways are: (i) the classical pathway triggered by antibody binding to the antigen, (ii) the alternative pathway activated directly by foreign microorganisms and (iii) the lectin pathway, which is triggered through the formation of a protein complex consisting of mannose/mannan-binding lectin binding to a sugar present at the surface of foreign particles and represent the most ancient (Holland and Lambris, 2002).

1.1.2.4. Proteases and protease inhibitors

Proteases are responsible for the complete hydrolysis of peptide bonds in both starting amino and ending carboxyl groups. Taking into account the functionality of the active site of the protease they can be classified in (i) serine-proteases, (ii) aspartic-

proteases, (iii) cysteine-proteases and (iv) metalloproteases (Rao, *et al.*, 1998). Apart from their important role in the cellular physiology, they are involved in pathological processes such as inflammation or tumour growth, and all of them are found in fish (Rao, *et al.*, 1998; Sriket, 2014). On the other hand, different protease inhibitors are present in serum and other body fluids of fish (Bowden, *et al.*, 1997). Despite the fact that their main function is the maintenance of body homeostasis, these molecules are also implicated in acute phase reactions and defense against pathogens that secrete proteolytic enzymes (Magnadottir, 2010).

1.1.2.5. Lysozyme

Among the lytic enzymes with relevance in immunity lysozyme is the most important. It is widely distributed throughout the body and able to hydrolyse the peptidoglycan present on Gram-positive and Gram-negative bacteria cell walls (Saurabh and Sahoo, 2008). Besides its antibacterial function, it also promotes phagocytosis by directly activating polymorphonuclear leucocytes and macrophages or indirectly acting as an opsonin or activating the complement system (Magnadottir, 2006; Saurabh and Sahoo, 2008).

1.1.2.6. Natural antibodies

Although antibodies are an adaptive humoral factor, natural antibodies may be included into the innate immune response and are found in the serum of most vertebrates (Boes, 2000; Haury, *et al.*, 1997). Natural antibodies seem to be elicited without any apparent specific and prior antigen stimulation and provide immediate and broad protection against bacterial and viral pathogens (Boes, 2000). The relevance of natural antibodies in fish might be even greater than in higher vertebrates (Magor and Magor, 2001). Furthermore, natural antibodies have been shown to take part in the immune defence of rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) against both viral and bacterial diseases (González, *et al.*, 1989; Sinyakov, *et al.*, 2002).

1.1.2.7. Lectins

Agglutinins and precipitins are highly specific sugar-binding proteins implicated in the recognition processes of pathogens that trigger the opsonization, phagocytosis and activation of the complement system (Du Clos and Mold, 2004). All these proteins

are considered as major factors in the acute phase response and important pathogen recognition proteins (Bayne and Gerwick, 2001).

1.1.3. Cells

1.1.3.1. Non-specific cytotoxic cells

Cells responsible for non-specific cytotoxicity in mammals are the NK cells, which are capable of recognizing and killing viral infected and tumour cells without any prior induction, but can also respond to other microbial infections (Albertsson, *et al.*, 2003; Bancroft, 1993; Biron and Brossay, 2001; Herberman, *et al.*, 1975). In fish, non-specific cytotoxic cells (NCCs) are functionally equivalent to the NK cells of mammals, but formed by a variety of different population of cells with typical morphological characteristic of monocyte-macrophages, granulocytes, platelets, NK cells and/or lymphocytes which have the ability to kill tumour cells, xenogeneic cells, virus-infected cells and parasites (Cuesta, *et al.*, 1999; Ellis, 2001; Fischer, *et al.*, 2006; Manning, 1998; Meseguer, *et al.*, 1996; Secombes, 1996).

1.1.3.2. Phagocytic cells

Cells responsible for phagocytosis in fish are monocyte-macrophages and granulocytes, also known as professional phagocytic cells. Phagocytosis is one of the most important processes in poikilothermic animals (Blazer, 1991; Froystad, *et al.*, 1998; Neumann, *et al.*, 2001). The phagocytic process starts with the contact between the microorganisms and the phagocytic cells and finishes with three different possible mechanisms responsible for the death of the phagocytosed microorganism: (i) lysosomal enzyme digestion of pathogen into the phagolysosome, (ii) the production of reactive oxygen intermediates (ROIs) with a rapid and sudden increase in the rate of oxygen consumption which is known as respiratory burst and is independent of mitochondrial respiration, and (iii) the production of nitric oxide (NO) and other reactive nitrogen intermediates (RNIs), all of them with bactericidal activity (Sharp and Secombes, 1993; Skarmeta, *et al.*, 1995).

1.2. Adaptive immunity

Teleost fish is the first evolutionary group which possess an adaptive immune response (Magnadottir, 2010). This response is mediated through diverse mechanisms

involving humoral factors (antibodies), cellular elements (B and T lymphocytes) and specific proteins such as the major histocompatibility complex (MHC) proteins and T cell receptors (TCRs) (Magnadottir, 2010).

1.2.1. Humoral factors (antibodies)

Antibodies are Igs produced by B lymphocytes and secreted into plasma or bound to the membrane as membrane receptors. In teleost fish, there are three major types of Igs: IgM, IgD and IgT/IgZ, being this last one specific of this group of vertebrates (Hordvik, 2015). The predominant fish Ig is the IgM, a tetramer which contains eight antigenic combining sites and is able to bind to a particular antigen or pathogen being a strong complement activator (Acton, *et al.*, 1971). Furthermore, the IgM heavy chain of fugu (*Takifugu rubripes*) acts as an N-acetyl-glucosamine binding protein having a potent inhibitory effect on the growth of many types of bacteria (Tsutsui, *et al.*, 2013). By its side, IgD is known to be expressed in all immune-related tissues at transcriptional level while its function still remains uncertain (Ramírez-Gómez, *et al.*, 2013). On the other hand, IgT, although is expressed in several tissues, is predominantly found in the mucosal surfaces such as gut and skin, suggesting that it is an equivalent of mammalian IgA (Xu, *et al.*, 2013; Zhang, *et al.*, 2010). As occurs with IgM, the IgT production and secretion to mucus is triggered by pathogens but its transport mechanisms to mucosal surfaces are still unknown.

1.2.2. Cells

1.2.2.1. Antigen presenting cells

Antigen presenting cells (APCs) highly express MHC II and co-stimulatory factors such as CD80 and CD86 (CD, cluster of differentiation co-receptor) (Mutoloki, *et al.*, 2014). In fish, the cells identified as APCs are: macrophages, DCs and B lymphocytes, although granulocytes also seem to be involved in this process (Cuesta, *et al.*, 2006; Lewis, *et al.*, 2014). When APCs phagocyte a pathogen, the cell presents small peptide fragments of the pathogen associated with MHC II proteins on its surface (Secombes, 1996; Vallejo, *et al.*, 1992). Additionally, other innate cells including monocyte-macrophages, also activated by PAMPs, secrete cytokines leading to a subsequent activation of the APCs (Mutoloki, *et al.*, 2014). These activated APCs

migrate to lymphoid organs and present the antigens to naïve T lymphocytes, starting the adaptive immune response (Mutoloki, *et al.*, 2014).

1.2.2.2. T lymphocytes

T lymphocytes constitute a minor population in blood, but they are distributed throughout the body particularly in lymphoid tissues such as the thymus, kidney and spleen and also in mucosal tissues such as the intestine, gill and skin (Mutoloki, *et al.*, 2014; Nakanishi, *et al.*, 2015). These cells are subdivided into cytotoxic T cells (CTLs) and T-helper (Th) cells. CTLs directly kill infected and not normal cells and express CD8 co-stimulatory factor, TCR and co-receptors involved in the MHC I interaction with cells (Fig. 2). Th cells modulate other immune cell responses through cytokine production and are crucial in the cell- and humoral-mediated immune responses (Magnadottir, 2010). Th cells express CD4 molecules on their surface, responsible for the specificity in their interaction with MHC II proteins (Mutoloki, *et al.*, 2014). In mammals, when CD4 expressing Th cells recognize a foreign antigen peptide presented by APCs (Fig. 3), a massive proliferation occurs and the cell can be differentiated in at least four different Th cell types: Th1, Th2, Th17 and induced T-regulatory (iTreg) cells accordingly to specific arrays of cytokines that drive their differentiation and the unique set of cytokines they produce (Mutoloki, *et al.*, 2014).

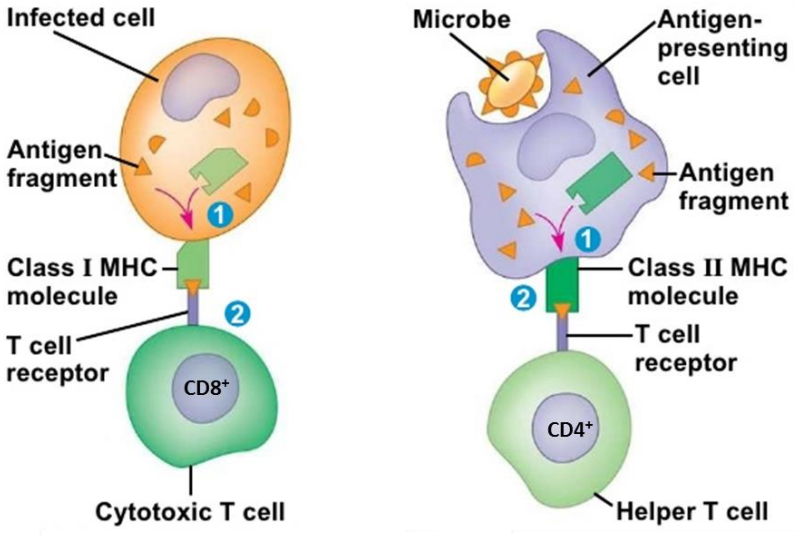


Figure 2: Schematic representation of antigen presentation and T lymphocytes activation (modified from Campbell and Reece, 2008).

In fish, many molecules representing different helper T cell subsets and their transcription factors have been demonstrated either at genetic or functional level (Mutoloki, *et al.*, 2014). Thus, interleukin (IL)-2, IL-4/13, IL-7, IL-12, IL-15, IL-18 and IL-21, and their cognate receptors, have been described in fish (Huising, *et al.*, 2004; Mutoloki, *et al.*, 2014; Nascimento, *et al.*, 2007; Wang, *et al.*, 2007; Wang and Secombes, 2013; Yoshiura, *et al.*, 2003; Zou, *et al.*, 2004). Type I and type II IFNsare a hallmark of Th1 cell differentiation in fish (Wang, *et al.*, 2007). Interestingly and regarding Th2 type cytokines, a molecule that seems to be homologue to IL-4 of mammals has been found recently in fish; however, it has no obvious similarity to known vertebrate *il4* genes (Li, *et al.*, 2007).

1.2.2.3. B Lymphocytes

B lymphocytes produce and secrete Igs and different B cells subsets can be identified in fish depending on the type of Igs that they produce, although the amount of B lymphocyte subsets is species-specific (Mutoloki, *et al.*, 2014). Thus, three subsets of B lymphocytes have been identified in channel catfish (*Ictalurus punctatus*): IgM^+/IgD^-, IgM^+/IgD^+ and IgM^-/IgD^+. However, in other species such as rainbow trout, only two subsets have been reported: $IgM^+/IgD^+/IgT^-$ and $IgM^-/IgD^-/IgT^+$. Interestingly, the most relevant differences with mammalian lymphocytes, is that fish B lymphocytes have phagocytic activity (Li, *et al.*, 2006; Randelli, *et al.*, 2008).

In general, fish B lymphocytes can be activated directly by multivalent antigens without T lymphocytes cooperation or upon T-dependent responses in which case, the B lymphocytes that present microbial antigens to Th cells are, in turn, activated (Abbas, *et al.*, 2012).

1.2.3. Memory of the immune system

One feature of the adaptive immune response is the generation of memory cells that develop a more rapid response that the primary one (first exposure to an antigen) (Uribe, *et al.*, 2011). Although different aspects of T cells responses are still poorly understood in fish, T cells clonal expansion and repertoire diversity has been characterized in rainbow trout by immunoscope spectratyping and many markers usually used to differentiate between naïve, memory and effectors T cells have been described in fish (Boudinot, *et al.*, 2001; Laing and Hansen, 2011). Regarding the

secondary antibody production in fish, the data obtained suggest that the secondary response is caused by the expansion of a pool of memory B cells obtained from the selection of B cells with high affinity receptors to the antigen (Kaattari, 1992; Morrison and Nowak, 2002). In fact, the secondary antibody production is often more extensive and rapid than the primary, but this immune memory is not as developed as in mammals (Kaattari and Piganelli, 1996; Manning and Nakanishi, 1996; Manning, 1998).

1.3. Immunocompetence acquisition and maternal immunity

As happens in other vertebrates, immunocompetence appears in fish during their ontogenic development, but in contrast to mammals, fish are in close contact with their environment long time before to gain complete immunocompetence, which greatly varies and depends on the age and the species (Mulero, *et al.*, 2007a). In the case of European sea bass, T lymphocytes emerge in early stages during larval development, between 5-12 days post hatching (dph) whether B lymphocytes with cytoplasmic Igs appear around 52 dph. However, definitive levels of T and B lymphocytes are reached between 137-145 dph, suggesting immunologically maturity in this species at this age (Dos Santos, *et al.*, 2000). The thymus and the HK become lymphoid tissues at around 30 dph (Breuil, *et al.*, 1997). In gilthead seabream, non-determined lymphocytes were detected by microscopy in thymus at 29 dph, while in HK or spleen they were found at 54 dph (Mulero, *et al.*, 2007a). Interestingly, acidophilic-granulocytes were observed in the posterior intestine and in blood vessels earlier than in the haematopoietic kidney (21 *vs* 27 dph) (Mulero, *et al.*, 2007b).

Maternal immunity refers to the immunity transferred from mother to progeny through the placenta, colostrum, milk or eggs. This transfer seems to be crucial to protect the offspring when their own immune response is still undeveloped and has been described in all vertebrates including fish (Hanif, *et al.*, 2004; Hasselquist and Nilsson, 2009; Zhang, *et al.*, 2013). Thus, in teleost fish, a maternally-derived IgM and some innate immune factors such as several complement proteins, lectins, protease inhibitors, lysozyme or cathelicidin have been reported to be transferred to oocytes together with vitellogenin (Vg), and are present during the first developmental stages of larvae until complete development of the immune system (Fig. 3) (Swain and Nayak, 2009; Zhang, *et al.*, 2013). In addition to immune transferred proteins, antibacterial and antiviral functions of Vg and Vg-derived yolk proteins (phosvitin and lipovitellin) have also been

described in fish (Zhang, *et al.*, 2013). Apart of the protein transmission to oocytes, transfer of mRNAs is also possible due to the fact that a significant level of IgM, C3, transferrin, transforming growth factor (TGF9-β1, IL-1β and lysozyme gene transcripts has been detected in teleost oocytes (Mingming, *et al.*, 2014; Picchietti, *et al.*, 2006). Interestingly, immunization of broodstock specimens results in a significant increase in IgM levels and anti-protease, lysozyme and complement activities, in their progeny compared to non-vaccinated broodstock's progeny (Hanif, *et al.*, 2004; Swain and Nayak, 2009; Wang, *et al.*, 2009). In fact, in some studies the parental immunitation triggered a significant reduction in mortality of the larvae upon a bacterial challenge (Swain, *et al.*, 2006). However, little is known about the molecular mechanisms that allow maternal transfer of immunity in fish and most of the information deal with IgM transfer.

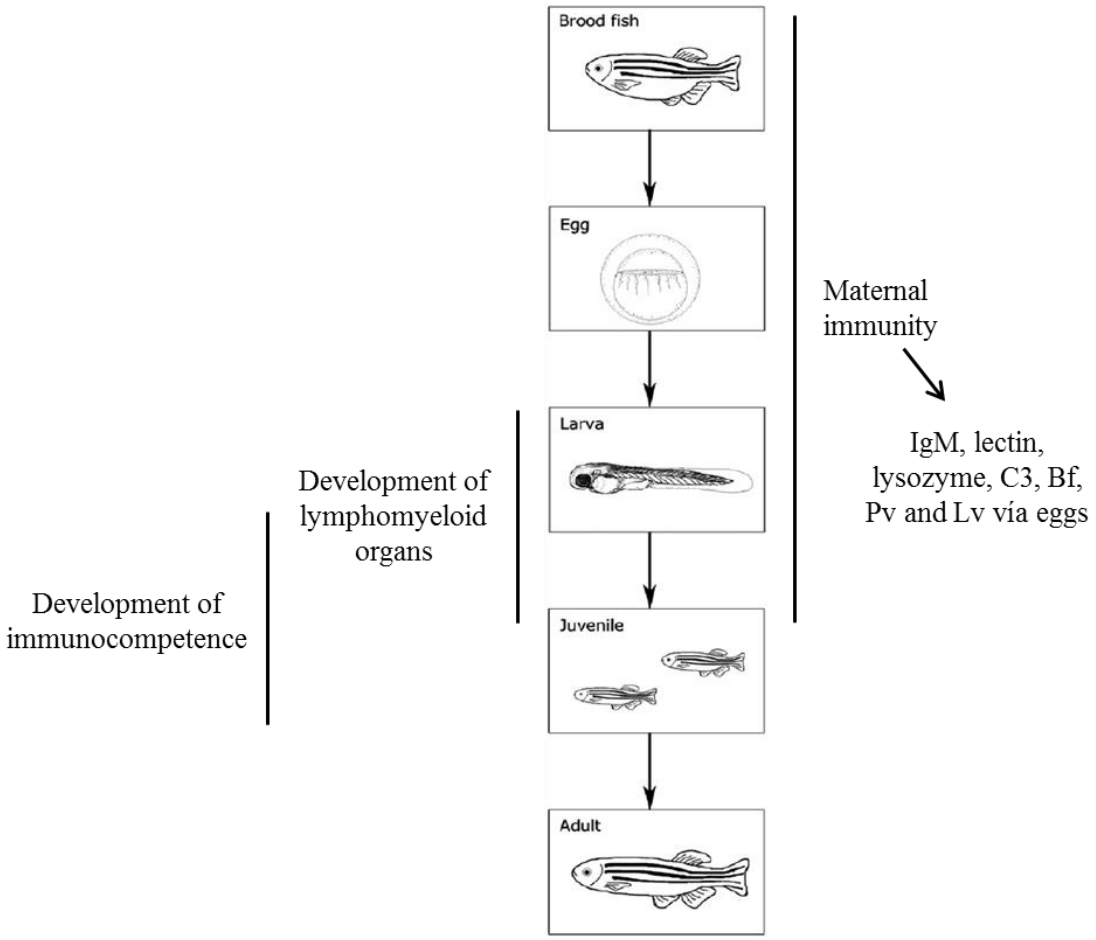


Figure 3: Schematic diagram representing the transfer and persistence of maternal immunity and the acquisition of the immunocompetence at different development stages of fish (modified from Swain and Nayak, 2009 and Zhang, *et al.*, 2013). IgM: immunoglobulin M, C3: complement factor 3, Bf: B factor, Pv: phosvitin, Lv: lipovitellin.

2. Reproductive system

The great diversity of fish species implies a great variety in reproductive strategies and reproductive cycles. Thus, based on their reproductive strategy fish are classified as gonochoristic species; the species in which each specimen develop a single functional sex as happens with the European sea bass; or as hermaphrodite species, the species in which the same specimen develops two functional sexes throughout its life, as the gilthead seabream. The gonochoristic species can be: (i) primary or differentiated when their development proceeds to directly form an ovary or testis; (ii) undifferentiated if all individuals initially develop an immature ovary to subsequently half of them develop a functional testis; or (iii) secondary, when appears intersexual gonads prior to the unisex and functional one. By their side, the hermaphrodite species can be: (i) sequential protogynous or protandrous depending on the functional sex developed earlier, female or male, respectively; or (ii) ambisexuals, when both sexes are functional at the same time (Devlin and Nagahama, 2002). Despite this high reproductive diversity, the underlying structures which constitute the teleost gonads are very similar to other vertebrates, with the exception of its ontogenetic non-double origin since fish gonad lacks of marrow tissue (Nagahama, 1983). In this Doctoral Thesis we will mainly focus on several aspects of the immune-reproductive interactions of the male gonad of European sea bass and gilthead seabream although the last experiment deals with female specimens and the transfer of immunity so that both organs are described below.

2.1. Ovary

Fish ovaries consist in a hollow sac-like organ composed by numerous ovigenous lamella extended into the central ovarian lumen (Kagawa, 2013). Attending to their relation with the peritoneal tissue, they can divide into two different ovarian structures: (i) closed ovaries (cystovarian) which are fully enclosed by peritoneal tissue and after maturation, oocytes are released into the central ovarian lumen as happens in European sea bass and gilthead seabream; and (ii) open ovaries (gymnovarian) which are only partially covered by peritoneal tissue, and the oocytes are released into the abdominal cavity, as occurs in Atlantic salmon (*Salmo salar*) and rainbow trout (Coward, *et al.*, 2002; García-López, *et al.*, 2011; Kagawa, 2013; Liarte, *et al.*, 2007). Teleost fish ovary is formed by two cell types; those forming the ovarian structures

(somatic cells) and the germinal line cells consisting in the oogonia lineage, which will derive in the haploid reproductive cells (gametes) during the oogenesis process (Coward, *et al.*, 2002; Kagawa, 2013). Up to date, all teleost studied possess the same basic pattern of oogenesis that can be divided into four different phases (Coward, *et al.*, 2002; Kagawa, 2013):

(i) Proliferation phase. Characterized by oogonia proliferation by mitosis forming oogonia nests. Once the oogonia differentiate into primary oocytes they become surrounded by a layer of follicle cells and meiosis starts until the meiotic arrest. As oocyte is growing, the follicular cells proliferate forming a continuous inner layer (granulosa cell layer) as the same time as the surrounding stromal connective tissue become organized to form the outer follicular envelope (thecal cell layer).

(ii) Primary growth phase. It occurs when cell and nucleolus are increasing their size and organelles and molecules that will be used later are synthesised. Oocytes are defined as pre-perinucleolar and perinucleolar oocytes due to multiple nucleoli become located around the periphery of the nucleus.

(iii) Secondary growth phase. It is characterized by vitellogenesis, a process in which the oocyte accumulates nutritional reserves needed for the development of the embryo. Thus, liver-derived Vg is up-taken and processed into yolk protein. Depending on the location of the yolk granules and their staining characteristic oocytes at this stage are divided into three different stages: yolk vesicle (named cortical alveoli), oil droplet and yolk globule stages. At this phase the oocyte also accumulates RNA (known as maternal RNA) and other maternal proteins different from Vg (Swain and Nayak, 2009). The follicle layers complete their differentiation and the oocytes become surrounded by cellular and non-cellular envelopes.

(iv) Maturation and ovulation phase. Endocytosis is reduced or stopped and the meiosis is resumed. At this phase the oocytes possess a large nucleus (called germinal vesicle), often localised in a peripheral position, surrounded by a relatively thick acellular envelope exhibiting a porous fibrillary structure, the zona radiata, and by several cell layers. This phase ends in the release of the mature eggs from the surrounding follicular cells.

Once the eggs are released at spawning, they must be fertilized by a male gamete to form new individuals. Breeding teleost fish, such as European sea bass and gilthead seabream, exhibit different periods of oogonial proliferation immediately before, during or after the main spawning. This determines that their ovaries show several oocyte types at several phases and stages of development at the gametogenesis stage including nest of oogonia always available for being recruited. This type of ovary is usually defined as asynchronous ovary (Coward, *et al.*, 2002; Lubzens, *et al.*, 2010).

2.2. Testis

Fish testes are formed by the interstitial tissue that surrounds the seminiferous tubules which house the germinal epithelium. The interstitial tissue is composed by Leydig cells (the main steroidogenic site), immune cells such as macrophages, acidophilic granulocytes or lymphocytes, fibroblasts, blood vessels, collagen fibres and connective tissue cells, the latter being continuous with the tunica albuginea (the testis organ wall) (Cabas, *et al.*, 2011; Chaves-Pozo, *et al.*, 2003; Grier, 1981; Loir, *et al.*, 1995; Nagahama, 1983; Schulz, *et al.*, 2010). The seminiferous tubules are delineated by a basement membrane and peritubular myoid cells. This germinal epithelium, located within the seminiferous tubules, is formed by two different types of cells: (i) germ cells; and (ii) Sertoli cells, which support germ cells structurally, nutritionally and with regulatory factors (Rocha and Rocha, 2006; Schulz, *et al.*, 2010). The fish germinal epithelium, in contrast to mammalian, form a cystic structure in which all germ cells develop synchronously surrounded by a cohort of Sertoli cells, which also proliferate (Billard, *et al.*, 1982; Callard, 1991; Chaves-Pozo, *et al.*, 2005b; Grier, 1981; Miura, 1999; Nagahama, 1983).

The two species studied in this Doctoral Thesis, European sea bass and gilthead seabream, are seasonally breeder species that develop different reproductive strategies. Thus, the European sea bass is a gonochoristic species with the singularity of its temperature-dependent sex differentiation due to polygenic sex determination (Piferrer, *et al.*, 2005; Vandeputte, *et al.*, 2007). However, the gilthead seabream is a protandrous hermaphrodite specie that develops as male during the first two reproductive cycles being the male gonad formed by a functional testicular area and an immature ovarian area (Chaves-Pozo, *et al.*, 2005b). At the end of the second reproductive cycle, the testis undergoes a degenerative process in order to sex change, which is characterized by high

rates of apoptosis and necrosis, encompassed by a massive infiltration of acidophilic granulocytes and the decrease of DMRT1 (double sex-and mab3- related transcription factor 1) encoding gene expression levels (Liarte, *et al.*, 2007). Moreover, *dmrt1* gene expression reaches its highest level during the spermatogenesis stages of the first two reproductive cycles, suggesting its involvement in testis maintenance (Liarte, *et al.*, 2007), as previously described in other vertebrates (Don, *et al.*, 2011).

The male reproductive cycle of seasonally breeding species such as European sea bass and gilthead seabream, can be divided into four stages: spermatogenesis, spawning, post-spawning and resting, which are described below attending to the morphology of the testis and cell renewal data (Fig. 4):

(i) Spermatogenesis. During spermatogenesis (male gametogenesis), different cell types: the spermatogonia stem cells, the Sertoli cells, and the developing germ cells (primary spermatogonia, A and B spermatogonia, spermatocytes and spermatids) form the germinal epithelium of the testis (Chaves-Pozo, *et al.*, 2005b; Valero, *et al.*, 2015a). The beginning of spermatogenesis triggers quick mitotic division enhancing the number of spermatogonia and Sertoli cells per cyst (Koulish, *et al.*, 2002; Schulz, *et al.*, 2012). The number of mitotic divisions at this stage is very variable between, and genetically determined within, species (Nobrega, *et al.*, 2009). Afterwards, spermatogonia differentiate into primary spermatocytes, starting the first meiotic division and originating secondary spermatocytes, which quickly differentiate into spermatids. Later on, spermatozoa maturation occurs, acquiring mobility and fertilization capacity (Schulz, *et al.*, 2010).

(ii) Spawning. In most of the teleost species studied, when spawning starts, testes are conformed by various spermatogonia and spermatocyte cysts and certain quantity of spermatozoa, which gradually increases through this stage. When testes are fully developed, the tunica albuginea becomes thinner and the gonadosomatic index (GSI) reaches maximum levels, as happens in the European sea bass (Valero, *et al.*, 2015a), whilst in the gilthead seabream this occurs in late spermatogenesis (Chaves-Pozo, *et al.*, 2005b). At the same time, spermatozoa are released to the lumen, appearing the cysts as open structures and the lumen full of spermatozoa. At the end of

the spawning stage, a sharp decrease in testis size occurs as a consequence of the release of spermatozoa, being the majority of the tubules empty (Chaves-Pozo, *et al.*, 2005b; Valero, *et al.*, 2015a).

(iii) Post-spawning. At this stage, testis become smaller, the interstitial tissue reaches its maximum thickness and the germinal epithelium of testes is composed by spermatogonia, some spermatocytes, few spermatids and residual spermatozoa (Chaves-Pozo, *et al.*, 2005b; Valero, *et al.*, 2015a).

(iv) Resting. The testicular size and volume reaches its minimum size and the tubules showed no lumen or a very small one, and are mainly formed by cysts of spermatogonia (Chaves-Pozo, *et al.*, 2005b; Valero, *et al.*, 2015a).

Regarding proliferation, the highly proliferative activity observed during spermatogenesis contrasted with the scarce proliferative activity of early gametogenetic cells observed during spawning (Chaves-Pozo, *et al.*, 2005b; Valero, *et al.*, 2015a). The proliferative activity is resumed at post-spawning or resting stages depending on the species, although necrotic areas and apoptotic germ cells can also be observed in some species (Chaves-Pozo, *et al.*, 2005b; Liarte, *et al.*, 2007; Valero, *et al.*, 2015a). In contrast to what happened in gilthead seabream, apoptosis is involved in the elimination of spermatozoa in European sea bass (Valero, *et al.*, 2015a). In addition, in the gilthead seabream the immature ovarian area proliferates at each resting stage of the male reproductive cycles (Chaves-Pozo, *et al.*, 2005b; Liarte, *et al.*, 2007).

2.3. Endocrine regulation of the male reproductive process

In teleost fish, spermatogenesis is regulated by the interplay of systemic and intragonadal factors and the importance of each type of regulation vary depending on the developmental stage of the gonad (Schulz, *et al.*, 2010). Thus, gonadotrophins (GTHs) are synthesized and secreted by the pituitary and are structurally homologous to the follicle stimulating hormone (FSH) and luteinizing hormone (LH) of mammals (Miura, 1999; Schulz, *et al.*, 2001). In several fish species, the receptors for these two hormones (FSH-R and LH-R, respectively) have been detected mainly in the gonads, but also in other tissues such as HK, liver, heart or gills (Hirai, *et al.*, 2002; Shinoda, *et al.*, 2010; Wong and Van Eenennaam, 2004; Zanuy, *et al.*, 2009). In fish testis, Leydig cells seem to express and have both LH-R and FSH-R, whilst the Sertoli cells only

show the FSH-R (García-López, *et al.*, 2009, 2012; Miwa, *et al.*, 1994; Yan, *et al.*, 1992). In contrast to mammals, the FSH-R of fish is also activated by LH, although

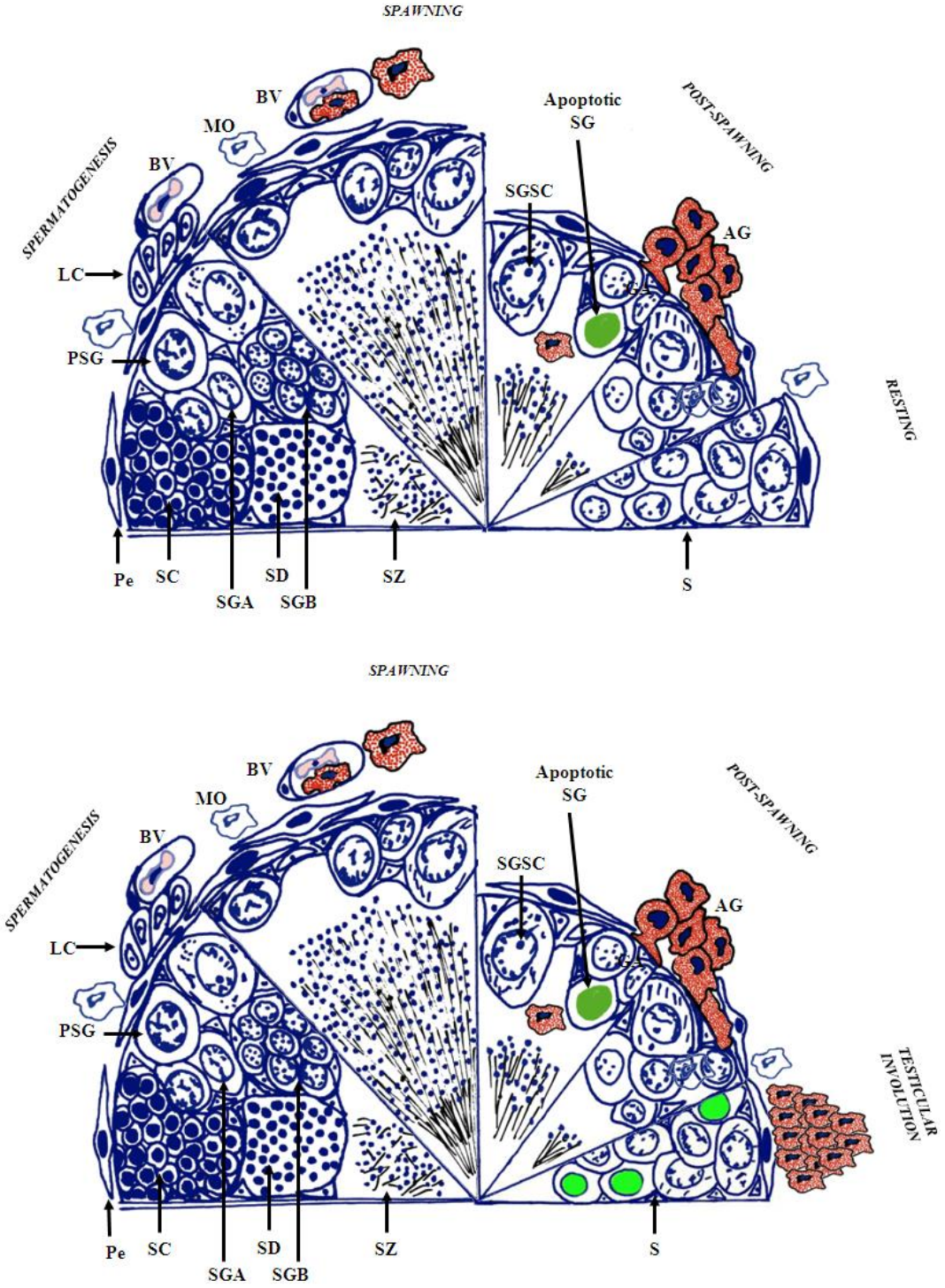


Figure 4: Schematic illustration of the morphological changes that undergo by the testicular area during the first (a) and second (b) reproductive cycles of gilthead. BV, blood vessels; LC, Leydig cell; MO, macrophage; Pe, peritubular cell; PSG, primary spermatogonia; S, Sertoli cell; SC, spermatocyte; SD, spermatid; SG, spermatogonia; SGA, spermatogonia A; SGB, spermatogonia B; SGSC, spermatogonia stem cell; SZ, spermatozoa (modified from García-Ayala, *et al.*, 2010).

it requires high LH concentrations in plasma and may be restricted to periods in which LH plasma levels peak (Schulz, *et al.*, 2010). Although, the biological activities of fish gonadotropins seem less clearly separated than in mammals, piscine GTHs are potent steroidogenic hormones that regulates sex steroid hormone production in the gonad (Schulz, *et al.*, 2010). The production of sex steroids (progestagens, androgens, and estrogens) are mainly produced in the gonads (Stocco, 2001), which is controlled by rapid changes in the activities of steroidogenic enzymes through the regulation of the amounts of the specific mRNAs encoding for them (Miller, 1989).

Testosterone (T) and 11-ketotestosterone (11KT) are considered the main sex hormones in male fish (Borg, 1994). The transformation of T into 11KT is mediated by two-step process: the hydroxylation of T to 11β-hydroxytestosterone (OHT) by the enzyme 11β-hydroxylase (coding gene: *cyp11b1*), followed by its conversion to 11KT via 11β-hydroxysteroid dehydrogenase (coding gene: *hsd11b*) (Feswick, *et al.*, 2014; Liu, *et al.*, 2000; Lokman, *et al.*, 2002). On the other hand, T is also transformed into 17β-estradiol (E_2) in the gonad, by aromatase activity (coding gene: *cyp19a1a*) (Miller, 1989). E_2 is the main female hormone in fish; nevertheless, this estrogen is also essential for male reproduction (Amer, *et al.*, 2001; Diotel, *et al.*, 2011; Hess, 2003; Miura, *et al.*, 1999, 2002). Interestingly, other tissues, as brain, can transform some steroids into others such as T and androstenedione (A) into E_2 (Diotel, *et al.*, 2011). The enzyme responsible for T and A transformation to E_2 in the brain is the neural aromatase (coding gene: *cyp19a2*) (Balthazart and Ball, 1998). These final steps of the steroidogenic process are schematically represented in Fig. 5.

In fish, three distinct estrogen receptors (ERs) have been cloned and characterized: ERa, ERb1, and ERb2, corresponding to *esr1*, *esr2b*, and *esr2a* coding genes, respectively (Diotel, *et al.*, 2011; Liu, *et al.*, 2010). The expression of ERs in fish testis and brain suggest that E_2 is involved in the physiology of these tissues (Andreassen, *et al.*, 2003; Bardet, *et al.*, 2002; Diotel, *et al.*, 2011; Legler, *et al.*, 2000). Moreover, recent data document the expression of G-protein coupled estrogen receptor, GPR30 or GPER, in the brain, testis and leucocyte of adult fish (Cabas, *et al.*, 2013; Liu, *et al.*, 2009).

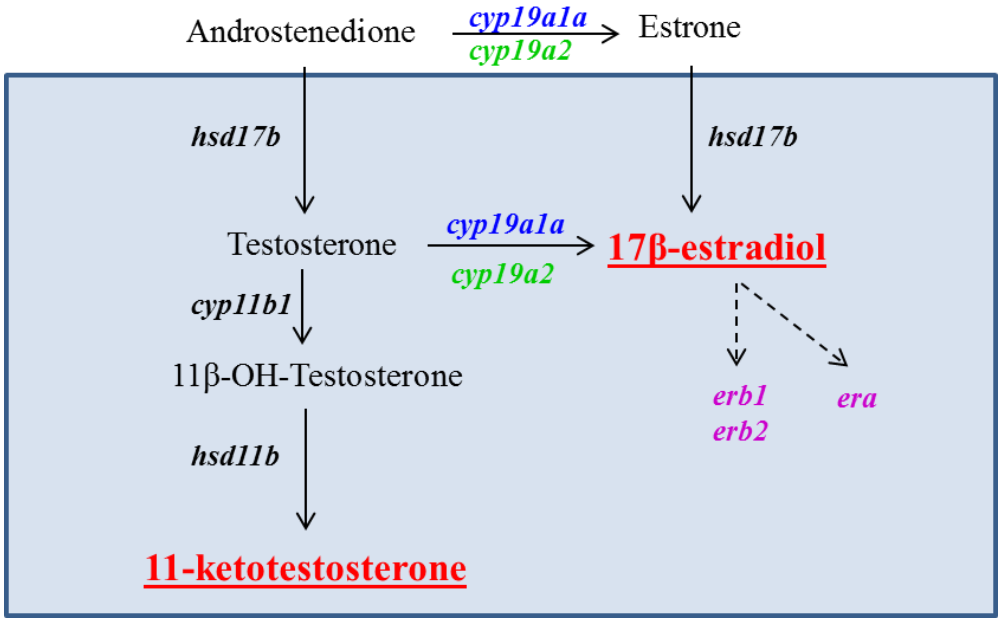


Figure 5: Schematic representation of the key steps involved in the last steps of fish androgens and estrogen production. The coding genes for steroidogenic enzymes involved in the aromatization of androgens present in the gonad are written in blue and those present in the brain in green. The genes coding for the three estrogen nuclear receptors characterized in fish are written in pink (modified from Villeneuve, *et al.*, 2007).

3. Immune-reproductive interaction

In fish, most existing information on reproductive-immune interactions deal with the modulation of immune responses by circulating hormones, including cortisol, growth hormone, prolactin and reproductive hormones and some proopiomelanocortin-derived peptides (Engelsma, *et al.*, 2002; Harris and Bird, 2000). Although, the effects of circulating hormones on the immune response depend on the species, in general, they regulate the whole system to adapt it to its environment (Lutton and Callard, 2006).

Some fish species show altered sex steroid hormone levels upon infection. In fact, during an infective period of vibriosis, silver seabream (*Sparus sarba*) showed gradually increasing T serum levels, whereas E_2 serum levels significantly decreased at early stages of infection and remained low until death. Moreover, this process coincided with increasing phagocytic activity of macrophages (Deane, *et al.*, 2001). In fish, as in mammals, sex hormones modulate leucocyte functions. In fact, the biological effect of E_2 on fish is considered mainly anti- inflammatory, although it seems to depend on the species. Thus, intra-peritoneal injections of E_2 in common carp (*Cyprinus carpio*) inhibit phagocytosis and the production of ROIs and RNIs by HK macrophages in a

dose-dependent manner (Watanuki, *et al.*, 2002). However, in goldfish macrophages, E_2 only inhibited the percentage of phagocytic cells (Wang and Belosevic, 1995). Expression studies have revealed that E_2 suppresses immune system-related transcripts in liver of European flounder (*Platichthys flesus*) and rainbow trout (Tilton, *et al.*, 2006; Williams, *et al.*, 2007). However, in gilthead seabream HK macrophages, E_2 up-regulates some genes coding for key immune molecules, including inflammatory and anti-inflammatory molecules, innate immune receptors, molecules related to leucocyte infiltration, matrix metalloproteinases (MMP) and the Mx. Moreover, the soluble factors produced by those E_2-stimulated macrophages modify the immune functions of HK leucocytes (HKLs) (Liarte, *et al.*, 2011a,b,c). In European sea bass larvae, E_2 up- or down-regulated the gene expression patterns of pro-inflammatory cytokines (*il1β*, *tnfa* and *il6*) and the production of IL-1β at protein levels depending on the age of the specimens during the critical period of HK development (Seemann, *et al.*, 2013). On the other hand, the biological effect of androgens, T and 11KT, on immune cells seems to be completely different. Thus, T does not suppress phagocytic activity in common carp or tench (*Tinca tinca*), inhibits B cell activation in salmonids and enhances several innate immune parameters in gilthead seabream (Cuesta, *et al.*, 2007; Law, *et al.*, 2001; Saha, *et al.*, 2003; Vainikka, *et al.*, 2005). Moreover, in gilthead seabream, T modulates the gene expression of cytokines, chemokines and immune related receptors and induces the expression of *il1b* gene (Águila, *et al.*, 2013; Castillo-Briceño, *et al.*, 2013).

On the other hand, it is worthy to notice that different types of leucocytes are present in teleost gonad and, as occurs in mammals, they seem to orchestrate important reproductive physiological processes, including gametogenesis and steroidogenesis. Thus, a massive infiltration of leucocytes, mainly acidophilic granulocytes, is orchestrated by gonadal factors including sex steroid hormones and occurs during post-spawning and testicular involution in the gilthead seabream testis (Chaves-Pozo, *et al.*, 2012a). Interestingly, when the acidophilic granulocytes infiltrate the testis, they show heavily impaired ROIs production and phagocytic activity (hardly 1% of the testicular acidophilic granulocytes are able to phagocytose) while the production of IL-1β is sharply induced (Chaves-Pozo, *et al.*, 2012a). These functional changes are known to be mediated by the action of estrogens through nuclear estrogen receptors and GPER expressed on leucocytes (Chaves-Pozo, *et al.*, 2012a). In the European sea bass, the expression of pro-inflammatory genes such as *il1b* and *il6* are low during

spermatogenesis stage, whilst transcript levels of *igmh* and *tcrb* as well as different anti-viral molecules and some AMPs increased at spermatogenesis and/or spawning stages (Valero, *et al.*, 2015a). Moreover, this special regulation of testicular leucocytes avoid the development of an immune response against germ cells, due to the fact that meiotic and haploid germ cells show non-self-antigens as they appear at the time of puberty, long after the complete development of the immune system and the establishment of self-tolerance in early stages of individual development. Moreover, the quantitative different inflammatory responses displayed in fish testis against infection allow the establishment of chronic and asymptomatic infections in this tissue (Chaves-Pozo, *et al.*, 2010a; Romalde, *et al.*, 1999; Sitja-Bobadilla and Álvarez-Pellitero, 1993).

4. Nodavirus

4.1. Aetiological agent and disease

During last decades, a neural infectious pathology called viral encephalopathy and retinopathy (VER) caused by nervous necrosis virus (NNV; *Nodaviridae* family, *Betanodavirus* genus) has been described to affect several species of marine fish, especially in farms (Athanassopulou, *et al.*, 2004; Bovo, *et al.*, 2011; Haddad-Boubaker, *et al.*, 2013; Johansen, *et al.*, 2004; John, *et al.*, 2014; Keawcharoen, *et al.*, 2015; Olveira, *et al.*, 2013). This infective disease has been increasingly spread across large geographical areas worldwide (Bovo, *et al.*, 2011; Munday, *et al.*, 2002). This expansion represents a serious economic threat to the aquaculture industry all around the world (Munday, *et al.*, 2002). Furthermore, this pathology has also been described in several freshwater species such as guppy (*Poecilia reticulata*) or sturgeon (*Acipenser gueldestaedi*) among others (Athanassopulou, *et al.*, 2004; Bovo, *et al.*, 2011; Hedge, *et al.*, 2003). Very recently, NNV has also been detected in apparently healthy marine invertebrates in Korea, suggesting that these animals could be a reservoir source which could lead to spread viral infections and, subsequently, mortalities in fish that cohabitate with these species (Gómez, *et al.*, 2008).

NNV is a bipartite, naked, icosahedral virus of 25-30 nm, composed by 2 positive single-stranded RNA fragments (Fig. 6), RNA1 and RNA2, which are capped but not polyadenylated (Sommerset and Nerland, 2004). The capsid is composed by

multiple units of a single protein of 42 kDa, the capsid protein (CP), encoded by the RNA2 and implicated in host specificity (Delsert, *et al.*, 1997; Sommerset and Nerland, 2004; Tan, *et al.*, 2001). The RNA1 encodes a non-structural protein of approximately 110 kDa, designated RNA-dependent RNA polymerase (RdRp) that is vital for replicating of the viral genome (Munday, *et al.*, 2002). Additionally, there is a sub-genomic RNA transcript, the RNA3, which is synthesized during an infection from the 3' terminus of the RNA1 and encodes the protein B2 (Iwamoto, *et al.*, 2005). The B2 protein is important for high-level accumulation of the viral RNA1 in the cells and could efficiently antagonize host siRNA (Fenner, *et al.*, 2006; Iwamoto, *et al.*, 2005). Interestingly, B2 protein is only expressed in newly infected but not in chronically infected fish cells (Mezeth, *et al.*, 2009).

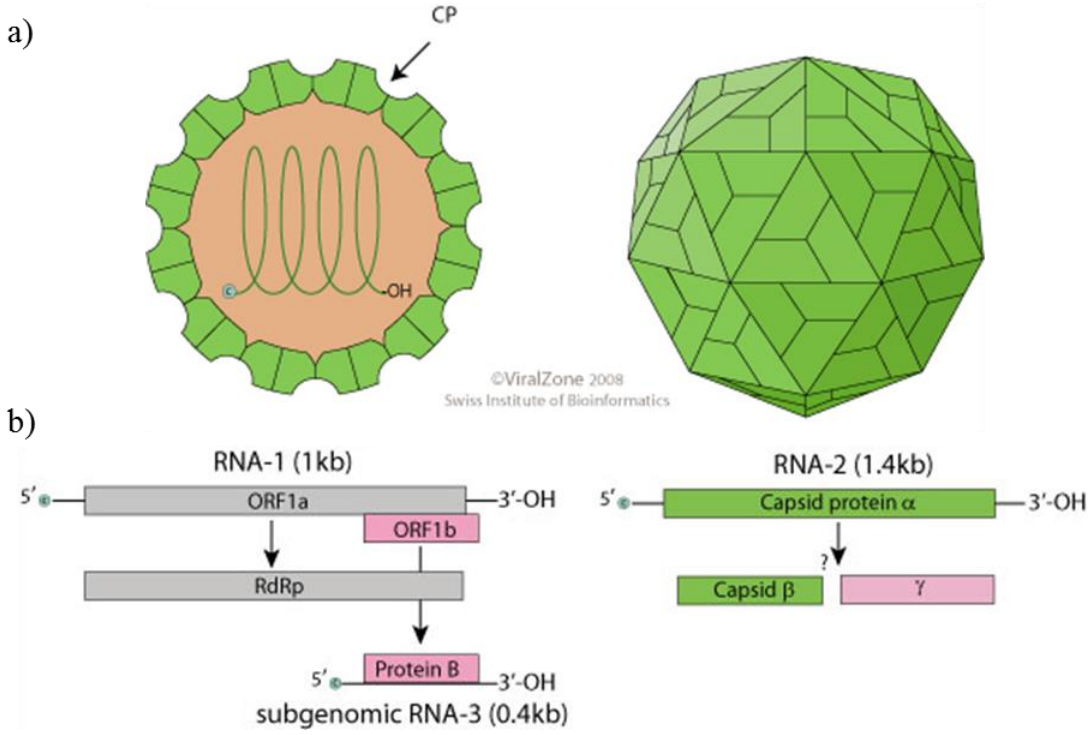


Figure 6: Schematic representation of NNV virion structure (a) and genome (b). The capsid protein (CP) is composed by multiple peptides forming an icosahedral naked structure (a). The genome is composed by two RNA strands in positive sense coding for the RNA-dependent RNA polymerase (RdRp), B2 and CP proteins (b). (http://viralzone.expasy.org/all_by_species/47.html).

Piscine NNVs are usually classified in different genotypes based on the phylogenetic analyses of the variable region (T4) of the RNA1 (Nishizawa, *et al.*, 1995; 1996). Thus, four genotypes have been described: tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV), Japanese flounder nervous

necrosis virus (JFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa, *et al.*, 1995). The NNV strains isolated from infected fish in different Mediterranean areas, including Spain, belongs to the RGNNV and SJNNV genotypes (Skliris, *et al.*, 2001; Thiéry, *et al.*, 2004).

NNV infects and alters the brain, spinal cord and retina where the virus actively replicates causing extensive tissue degradation (Munday, *et al.*, 2002). Thus, infected fish frequently exhibit symptoms of neurological damage such as erratic swimming behaviour (Comps, *et al.*, 1994; Mori, *et al.*, 1992). Usually, the mortality rates caused by NNV seem to be age-dependent, being the most affected stages, larvae and juveniles. In fact, when larval stages are affected, high mortalities, generally up to 100%, are observed whilst in affected juveniles and older fish lower losses are reported (OIE, 2013). However, outbreaks with high mortalities have also been described in market-size and adult fish (Hellberg, *et al.*, 2010; Kara, *et al.*, 2014; Le Breton, *et al.*, 1997). Eventually, the infected adult fish, that overcome the disease, become asymptomatic carrier fish (Gjessing, *et al.*, 2009). Among Mediterranean fish species, one of the most susceptible ones is the European sea bass, especially at larvae and juvenile stages (Breuil, *et al.*, 1991; Frerichs, *et al.*, 1996). In contrast, the gilthead seabream is infected but do not suffer the disease, acting as asymptomatic host for the main genotypes of NNV and being able to spread the virus (Castric, *et al.*, 2001). However, some NNV reassortant strains, recently documented, produced high mortalities in gilthead seabream farming populations (Olveira, *et al.*, 2009).

4.2. Immune response against nodavirus

During last decades, the study of the immune response against NNV has been raising relevance due to the great impact of the viral incidence worldwide. Most of the studies have been focused on the innate immune response. As IFN response is one of most powerful antiviral mechanisms in fish, the study of the pattern of expression of several IFN-related genes has been described in different species upon NNV infection. Thus, NNV infection triggers the up-regulation of the *ifi25* (interferon inducible protein 25), *lgp2* (*laboratory of genetics and physiology 2*), *isg15* (*interferon-stimulated gene 15*), type I *ifn* and *mx* coding genes in spleen, spleen cells or kidney and also in brain and eye, target tissues of NNV (Huang, *et al.*, 2013; Krasnov, *et al.*, 2013; Overgård, *et al.*, 2012; Park, *et al.*, 2009; Rise, *et al.*, 2010). However, in zebrafish IFN response was

inactive upon acute infection with NNV, whilst this response was activated during persistent infections (Lu, *et al.*, 2008). Regarding European sea bass and gilthead seabream, their immune response upon NNV infection has been widely studied due to their different susceptibilities to the virus and their great relevance in aquaculture in Mediterranean areas. As expected, *ifn*-related genes are up-regulated upon NNV in both species in the brain and the HK, although generally, this response is triggered in higher intensity in gilthead seabream brain (Bravo, *et al.*, 2013; Chaves-Pozo, *et al.*, 2012b; Dios, *et al.*, 2007; Novel, *et al.*, 2013; Poisa-Beiro, *et al.*, 2008, 2009; Scapigliati, *et al.*, 2010). However, two *mx* isoforms (*mxa* and *mxb*) have been described to express differently in HK and brain of European sea bass (Novel, *et al.*, 2013). Apart from IFN, NNV stimulates other innate immune responses. Several studies have demonstrated that this virus up-regulates the expression of genes related with the innate immunity in the brain and/or HK such as genes coding for different ILs, TLRs, chemokines, or lectins in European sea bass or gilthead seabream (Cuesta, *et al.*, 2010; López-Muñoz, *et al.*, 2012; Poisa-Beiro, *et al.*, 2008, 2009; Scapigliati, *et al.*, 2010). Curiously, previous studies in this laboratory indicated that, upon *in vivo* infection with NNV, NCCs cytotoxicity of HKLs is increased in both species as well as the expression of the gene coding for non-specific cytotoxic cell receptor protein 1 (NCCRP-1), whereas the respiratory burst is only increased in gilthead seabream and the phagocytic activity is unchanged (Chaves-Pozo, *et al.*, 2012b). Otherwise, in turbot (*Psetta maxima*), the expression of serum lectin isoform 4, the serum-inducible protein kinase, saxitoxin binding protein 1 genes and others related to the complement pathway are up-regulated in the kidney upon NNV infection (Park, *et al.*, 2009). The expression of several genes coding for cytokines, TLRs, sacsin molecular chaperone protein (SACs), viperin or chemokines are also stimulated in brain of Atlantic cod (*Gadus morhua*) upon infection (Rise, *et al.*, 2010). Very recently, a tripartite motif (TRIM)-containing protein (TRIM39) human homolog involved in several innate immune responses, has been shown to inhibit NNV gene transcription when it is over-expressed in spleen cells from orange-spotted grouper (*Epinephelus coioides*) (Wang, *et al.*, 2016). Moreover, a beta-defensin cloned from liver of orange-spotted grouper exert antiviral activity against NNV through the activation of type I IFN and IFN-stimulated response elements (ISRE) *in vitro* (Guo, *et al.*, 2012). Regarding antimicrobial functions, upon NNV infection, antimicrobial activities such as the complement and bactericidal activity are mainly increased in the serum of European sea bass and gilthead seabream species in different

crowding conditions whilst the lysozyme is not affected or decreased (Mauri, *et al.*, 2011). Interestingly, upon NNV infection, the gene expression of gene coding for C3 is up-regulated in liver of both species upon low crowding conditions whereas only does in gilthead seabream upon high crowding conditions (Mauri, *et al.*, 2011). Moreover, the Apolipoprotein A-1, which has bactericidal activity (Valero, *et al.*, 2013), is increased in the liver of European sea bass upon NNV infection, as the gene expression of transferrin and ferritin coding genes, which could have a role in macrophages activation in fish (Sarropoulou, *et al.*, 2009).

Regarding the adaptive immune response, NNV triggers the production of specific antibodies against different viral proteins in several fish species as European sea bass and gilthead seabream at least from 2 weeks post-infection in sea bass (Grove, *et al.*, 2006; López-Jimena, *et al.*, 2012; Scapigliati, *et al.*, 2010; Skliris and Richards, 1999; Woo, *et al.*, 2009). Furthermore, these specific antibodies display neutralizing activity against NNV (Grove, *et al.*, 2003, 2006; Hegde, *et al.*, 2005; Tanaka, *et al.*, 2001; Woo, *et al.*, 2009). In Atlantic halibut (*Hippoglossus hippoglossus*), IgM+ cells and NNV infected cells co-localize in viral target tissues such as retina or brain, but not in muscle, kidney or spleen (Grove, *et al.*, 2006). Furthermore, in gilthead seabream, infiltration of IgM+B cells has been suggested in brain since this type of cells has been detected in brain of infected fish (López-Muñoz, *et al.*, 2012). In addition, several T cell related genes are up-regulated in infected tissues of Atlantic halibut. Thus, the genes coding for the proteins TCRb, CD4-2, CD4, CD8α, IFNg and Lck were up-regulated in brain but only those coding for TCRb, CD4, IFNg and Lck did in eye, appearing those increments earlier in eye (Overgård, *et al.*, 2012). Moreover, T cell activation is also supported by *in vitro* infections in which *ifng* coding gene was up-regulated (Overgård, *et al.*, 2012) and by a transcriptomic analysis of NNV-infected brain from Atlantic cod which showed the up-regulation of B and T lymphocytes related genes (Krasnov, *et al.*, 2013). In European sea bass, both B and T cells have been detected in peripheral blood leucocytes during an infection with NNV, showing the leucocytes from blood, HK and gills proliferative activity upon inactivated viral particles *in vitro* (Scapigliati, *et al.*, 2010). Moreover, *cd83* transcription is suggested to be modulated by NNV since it is down-regulated in HKLs upon *in vivo* infection with NNV (Buonocore, *et al.*, 2012).

4.3. Transmission of nodavirus

Several studies about the transmission mechanisms of NNV point to both horizontal and vertical transmission routes (Breuil, *et al.*, 1991; Grotmol, *et al.*, 1999; Korsnes, *et al.*, 2012; Kuo, *et al.*, 2012; Sinyakov, *et al.*, 2011). The horizontal transmission should be considered the most common route since water is the most important abiotic vector (OIE, 2013). However, vertical transmission is also an important issue. In fact, the virus has been detected in broodstocks of different fish species by means of polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) techniques (Hodneland, *et al.*, 2011; Munday, *et al.*, 2002). Furthermore, in orange-spotted grouper, NNV was detected into the embryos in which the virus continues replicating during the early stages of development, demonstrating that it is able to be vertically transmitted (Kuo, *et al.*, 2012). In addition, several studies have detected NNV in sperm and ovarian biopsies of European sea bass, shi drum (*Umbrina cirrosa*) and striped jack (*Pseudocaraux dentex*) brood-fish by PCR techniques (Dalla Valle, *et al.*, 2000; Mushiake, *et al.*, 1994). However, although all this data support the idea of a *sensu stricto* route of vertical transmission of NNV (into the germ cells), the methodology was not able to clearly demonstrate if the virus is into the gametes or surrounding them. Furthermore, up to date, strategies focused on the control of vertical transmission include the segregation or elimination of NNV carrier-spawners (Mushiake, *et al.*, 1994; Watanabe, *et al.*, 2000), and/or sterilization of the eggs by ozone or other chemicals (Arimoto, *et al.*, 1996; Grotmol and Totland, 2000), nevertheless on some occasions failure of the PCR method to detect the virus in selected spawners have been reported (Nishizawa, *et al.*, 1996).

4.4. Prevention of nodavirus infection

In all forms of fish culture, infectious disease agents are easily transmitted between individuals and, independently of the technology used for farming, good environmental conditions are important to maintain a healthy fish population. For farms located in open aquatic environment, the exposure to pathogens is an unavoidable fact. In order to prevent the appearance of infectious outbreaks, several types of vaccines have been developed as inactivated pathogens, recombinant or DNA vaccines (Sommerset, *et al.*, 2005a). The DNA vaccines are increasingly considered as a potential method to solve the lack of efficient and feasible treatments against virus

(Kurath, 2008). In contrast to other vaccines, DNA vaccines are relatively inexpensive and easy to produce, being very stable and also avoiding the risk of undercover infections as could happen with attenuated or inactivated vaccines. Furthermore, multivalent vaccines may easily be prepared by blending different vaccines (Heppell and Davis, 2000). However, they have the disadvantage of the administration route (by injection), which is invasive and economically less feasible than other methods such as oral administration (Heppell and Davis, 2000). Up to date, recent approaches have demonstrated that oral management of encapsulated DNA vaccines improves the survival to different pathogen infections (de las Heras, *et al.*, 2010; Lin, *et al.*, 2007; Rajesh Kumar, *et al.*, 2008). .

Regarding NNV vaccines, different types such as heated- or formalin-killed virus or recombinant viral capsid protein have been tested (Dos Santos, *et al.*, 2009; Hall, 2003; Thiéry, *et al.*, 2006; Yamashita, *et al.*, 2004). Despite the studies reporting that inactivated NNV or recombinant protein vaccines triggered an immune response or improve the survival of affected species (Kai and Chi, 2008; Kim, *et al.*, 2000; Nishizawa, *et al.*, 2012; Sommerset, *et al.*, 2005b), there were no available commercial vaccines providing fish farms a solution to the devastating mortality rates provoked by NNV until 2015. A vaccine composed by formalin inactivated cultured NNV specific for European sea bass specimens and intraperitoneally administrated has recently been patented (PharmaQ). In addition, a first study showed that the intramuscular injection of a NNV DNA vaccine failed to protect Atlantic halibut (Sommerset, *et al.*, 2005b) whilst more recently the oral administration of an encapsulated DNA vaccine to Asian sea bass (*Lates calcarifer*) resulted partly protective against NNV (Vimal, *et al.*, 2014). However, most vaccines administered by injection cannot be used in specimens with less than 1 g body weight and the most susceptible life stages to NNV are larvae and juveniles. Larvae cannot be vaccinated at the early stages of their development due to a non-completed immunocompetence and later on due to their small sizes. Taking all this into account, the development of different strategies to stablish an effective maternal vaccination that protect the progeny upon a specific pathogens together with an adequate oral vaccine of easy administration in recently weaned larvae seem to be appropriated.

II. OBJECTIVES

The present Doctoral Thesis proposes the following objectives:

1. Evaluate the ability of NNV to colonize and replicate in the testis of European sea bass and gilthead seabream.

2. Study the potential effects of NNV infection on reproductive parameters.

3. Evaluate the innate immune response, with especial focus on the interferon pathway and antimicrobial peptides, orchestrated on the testis of European sea bass and gilthead seabream upon NNV infection and compare them to the same responses triggered in the brain.

4. Generate a DNA vaccine against NNV and study the immune response and protection elicited in European sea bass juveniles upon oral vaccination.

5. Evaluate whether the DNA vaccine administration to European sea bass females improves the maternal transfer of innate immune factors to their progeny.

III. EXPERIMENTAL CHAPTERS

III.1. NNV LOCALIZATION AND IMMUNE RESPONSE ON EUROPEAN SEA BASS AND GILTHEAD SEABREAM TESTIS

III.1.1. NODAVIRUS COLONIZES AND REPLICATES IN THE GONAD OF GILTHEAD SEABREAM AND EUROPEAN SEA BASS MALES MODULATING ITS IMMUNE AND REPRODUCTIVE FUNCTIONS

1. Introduction

Viruses and viral diseases have become one of the unsolved problems in modern aquaculture (since there are no effective preventive measures available to control them) resulting in major economic loses. Amongst the most threatening viruses is nervous necrosis virus (NNV; *Nodaviridae* family, *Betanodavirus* genus), causative agent of the viral encephalopathy and retinopathy (VER), a disease that alters the brain and retina structure and function (Munday, *et al.*, 2002) and provokes mortality rates up to 100% in more than 50 fish species (Gómez, *et al.*, 2004; Munday, *et al.*, 2002). NNV is a small, naked icosahedral virus, composed of 2 positive single stranded RNA fragments, RNA1 and RNA2, which are capped but not polyadenylated (Sommerset and Nerland, 2004). The capsid is composed of multiple units of a single protein, the capsid protein (CP) (Delsert, *et al.*, 1997) coded by the RNA2 (Sommerset and Nerland, 2004; Tan, *et al.*, 2001) and involved in host specificity. It has been recently described that each units of the CP shows three major domains: the N-terminal arm, the shell domain (S-domain) and the protrusion domain (P-domain) formed by three-fold trimeric protrusions with hypervariable surface regions that contribute to host binding and specificity (Chen, N.C., *et al.*, 2015). The RNA1 encodes the viral RNA-dependent RNA polymerase (RdRp). In addition, a sub-genomic RNA transcript, called RNA3, is originated from the 3' terminus of the RNA1. The RNA3 of betanodavirus has been considered to have a single open reading frame encoding for protein B2 (Iwamoto, *et al.*, 2005). B2 is important for high-level accumulation of viral RNA1 in the cell and could efficiently antagonize host siRNA silencing (Fenner, *et al.*, 2006; Iwamoto, *et al.*, 2005; Nagai and Nishizawa, 1999). Interestingly, B2 is only detected when the virus is actively replicating, but not in persistent infections (Mezeth, *et al.*, 2009).

Moreover, in the Mediterranean aquaculture, European sea bass (*Dicentrarchus labrax*) is one of the most susceptible species, especially at larvae and juvenile stages, resulting in 100% mortalities at these stages (Breuil, *et al.*, 1991; Frerichs, *et al.*, 1996). On the other hand, gilthead seabream (*Sparus aurata*) is less susceptible species than European sea bass and a carrier of the infection for most of the NNV strains (Castric, *et al.*, 2001). However, this species suffered mortalities when infected with some NNV reassortant strains (Olveira, *et al.*, 2009).

Several studies about the transmission mechanisms of the virus point to both horizontal and vertical transmission routes (Breuil, *et al.*, 2002; Grotmol, *et al.*, 1999; Korsnes, *et al.*, 2012; Kuo, *et al.*, 2012; Sinyakov, *et al.*, 2011). Thus, although adult specimens do not suffer the disease, NNV has been detected in broodstocks of different fish species by PCR and ELISA techniques (Hodneland, *et al.*, 2011; Munday, *et al.*, 2002). The infection of the gonad by pathogens is the initial step to promote horizontal transmission through gonadal fluids and/or vertical transmission through infected gametes (Inoue and Ushida, 2003; Sinyakov, *et al.*, 2011). In all vertebrates, the gonad is considered an immunologically privileged site, as also the brain and retina, due to the fact that in those tissues, the immune response proceeds in a different manner in order to avoid cell damage (Chaves-Pozo, *et al.*, 2005c; Hedger, 2002). These physiological characteristics of the gonad are used by a certain number of viral pathogens to evade the immune system, replicate and be transmitted to other specimens. In fish, the immune functions inside the reproductive organs and its implication on pathogen dissemination through the gonad have recently been documented. In rainbow trout (*Oncorhynchus mykiss*), viral haemorrhagic septicaemia virus (VHSV), a Rhabdovirus, and infectious pancreatic necrosis virus (IPNV), an Aquabirnavirus, can be transmitted through the ovary in two different ways. In fact, some studies have detected infective particles of VHSV in the ovary and ovarian fluids, allowing horizontal and/ or vertical transmission through fluids (Kocan, *et al.*, 2001), while infective particles of IPNV has only been detected in homogenates of oocytes, being transmitted in a *sensu stricto* vertical way (Smail and Munro, 2008). Interestingly, once they reach the ovary, their replication cycles are completely different. While VHSV is able to actively transcribe and translate its genes and increase the virus load in the tissue, the mRNA levels of IPNV remain undetectable, but infective particles of the virus can be isolated from the ovary by cell culture (Chaves-Pozo, *et al.*, 2010a). In addition, trout leucocytes present into the gonads showed altered immune response (including leucocyte markers, antigen presentation, interferon response, chemokines or cytokines) allowing VHSV to cause chronic infections and IPNV to keep latent into the tissue (Chaves-Pozo, *et al.*, 2010a). Strikingly, there are no studies focused on NNV and its ability to colonize the gonad, even when NNV has demonstrated vertical transmission (Breuil, *et al.*, 2002). In the other hand, sex steroid hormones, regulated by the brain-pituitary-gonadal axis (Migaud, *et al.*, 2012; Weltzien, *et al.*, 2004) modulate the immune response in vertebrates; including fish (Chaves-Pozo, *et al.*, 2008a; Muñoz-Cruz, *et al.*, 2011). As a

consequence, some pathogens modify the sex steroid hormone levels of the host when they spread an infection (Deane, *et al.*, 2001; Gómez, *et al.*, 2000).

Taking all this into consideration, in this work we have studied whether NNV colonized the testis in European sea bass and gilthead seabream, a very susceptible and asymptomatic host fish species, respectively. Moreover, we will analyse how the immune response and the production of reproductive hormones, 17β-estradiol (E_2) and 11-ketotestosterone (11KT) in the testis are modified upon NNV infection. In addition, we determined whether the sensitivity of brain and testis to these reproductive hormones are modified by NNV infection. Furthermore and with the aim to elucidate which of the alterations observed in the testis might be due to NNV localization in this tissue with no interference of other systemic factors or tissue alterations, an *in vitro* challenge of the testis of both species with pI:C or NNV was performed for 24 hours.

2. Materials and Methods

2.1. Animals

Healthy specimens of European sea bass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.) were bred and kept at the *Centro Oceanográfico de Murcia, Instituto Español de Oceanografía* (COM-IEO, Mazarrón, Murcia). The European sea bass larvae were bred at warm water temperature (around 20°C) obtaining a high proportion of males in the population (Blázquez, *et al.*, 2009). The fish from juveniles to adults were kept in 14 m^3 tanks with the natural water temperature, a flow-through circuit, a suitable aeration and filtration system and a natural photoperiod. Fish were fed daily with 1% biomass of a commercial pellet diet (Skretting). The environmental parameters and food intake were recorded daily. Specimens of European sea bass (n = 50) or gilthead seabream (n = 50) of the same age with a mean body weight (bw) of 125 ± 25 and 305 ± 77 g respectively, were transported to the University of Murcia (Spain) aquaria in order to perform *in vivo* infections (see below). Fish were randomly divided into two tanks, kept in 450–500 L running seawater (28‰ salinity) aquaria at 25°C and with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the experiments. Some other specimens with a bw of 509 ± 38 g and 530 ± 148 g, respectively, were used for *in vitro* experiments (see below). Before sampling, all specimens were anesthetized with 40 µl/l of clove oil, completely bled and immediately decapitated and weighed. The experiments described comply with the Guidelines of the

European Union Council (2010/63/UE). The protocol was approved by the Committee on the Ethics of Animal Experiments of the *Instituto Español de Oceanografía* (IEO) (Permit Number: 2010/02) and of the University of Murcia (Permit Number: A13150104).

2.2. Nodavirus stock

NNV (strain 411/96, genotype RGNNV) was propagated in the SSN-1 cell line (Frerichs, *et al.*, 1996). The SSN-1 cells were grown at 25°C in Leibovitz's L15-medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 50 µg/ml gentamicin (Gibco) using Falcon Primaria cell culture flasks (Becton Dickinson). Inoculated cells were incubated at 25°C until the cytopathic effect (CPE) was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stock was titrated in 96-well plates and expressed as the viral dilution infecting 50% of the cell cultures ($TCID_{50}$), following the methodology previously described (Reed and Müench, 1938), before use in the experiments.

2.3. Testis culture and *in vitro* treatments

Specimens of European sea bass (n = 6) or gilthead seabream (n = 6) males were completely bled and the testis removed without taking the afferent and efferent blood vessels that are located in the mesentery (dorsally) connecting the testis to the body wall. The testis were weighed and chopped in 1 mm^2 fragments to culture them in flat-bottomed 96-well microtiter plates (Nunc) with sL-15 culture medium [Leibovitz's L15-medium supplemented with 2 mM glutamine, 100 u.i./ml penicillin, 100 µg/ml streptomycin, 2 µg/ml fungizone (Life Technologies), 2% FBS serum and 0.35% of NaCl] alone (control) or with NNV (10^7 $TCID_{50}$/ml) or polyinosinic-polycytidylic acid (pI:C; a synthetic analogous of double-stranded RNA) (62.5 µg/ml; Sigma) for 24 hours at 25°C. After incubation, fragments of tissue were washed with 0.01 M phosphate buffered saline (PBS) and processed for gene analysis as described below.

2.4. *In vivo* infection

The infection was performed either by injecting with a single intramuscular injection of 100 µl of SSN-1 culture medium (mock-infected) or with culture medium containing 10^6 $TCID_{50}$/fish of NNV since this route of infection has been proven to be

the most effective (Aranguren, *et al.*, 2002). Mortality was also recorded through the experiment. Fish (n = 5 fish/group and time) were sampled 1, 7 or 15 days after the viral infection and serum, testis and brain were removed. Testis was sampled without taking the afferent and efferent blood vessels that are located in the mesentery (dorsally) connecting the testis to the body wall. Blood samples were obtained from the caudal peduncle and, after clotting; serum samples were collected by centrifugation at 10,000 g for 1 min at 4°C, and immediately frozen and stored at -80°C until use. The testis were weighed and fragments of testis and brain tissues were either immediately frozen in TRIzol Reagent (Life Technologies) and stored at -80°C for later RNA isolation or fixed in 4% paraformaldehyde in PBS for 24 h at 4°C for light microscopy examination as described below.

2.5. Light microscopy and immunocytochemistry

Testis fragments from the *in vivo* experiment (n = 4–5 fish/group and time) fixed in 4% paraformaldehyde in PBS for 24 h at 4°C were embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 5 μm. After dewaxing and rehydration, some sections were stained with haematoxylin–eosin in order to determine the changes in the morphology of the organs through the infection. Other sections were subjected to a direct immunocytochemical method using two antibodies specific to: (i) the NNV capsid protein (anti-CP, Ø233 antibody) or (ii) the NNV B2 protein (anti-B2, Ø6073 antibody) at the optimal dilution of 1:500 as previously described (Mezeth, *et al.*, 2009). In brief, the sections were incubated at 60°C for 30 min, dewaxed in xylene, rehydrated in a series of ethanol baths and washed in running water. Prior to staining the tissue sections were autoclaved for 15 min in 0.01 mM citric acid (pH 6.0) for antigen retrieval. To prevent non-specific antibody binding, sections were blocked by using 5% bovine serum albumin (BSA; Sigma) in Tris buffered saline (TBS; Merck, 0,05 M, pH 7.6) for 20 min. The primary anti-B2 or anti-CP sera were diluted in TBS containing 2.5% BSA and incubated for 30 min at 37°C, and washed for 5 min with TBS. The Vectostain1 universal ABC-AP kit (Vector Laboratories), which provides both the secondary antibody (biotinylated anti-mouse/rabbit immunoglobulin) and avidin-biotin alkaline phosphatase (ABC-AP) was used. After TBS wash, the sections were incubated for 5 min with DAKO Fuchsin Substrate- and Cromogen system (Dako), followed by washing in running tap water before counterstaining with Shandon's haematoxylin and mounting in aqueous mounting medium (Aquatex, BDH laboratory). The specificity of

the reaction was determined by using sections of tissue from control fish and by omitting the primary antibody on section of tissue from infected fish.

2.6. Localization of gene expression by *in situ* PCR

Testis sections were used to perform *in situ* PCR (isPCR) analysis using a modified protocol previously described (Nuovo, 1995). Sections were dewaxed in xylene for 5 min, dried in 100% ethanol, and air-dried for 5 min. Protease digestion was performed with 2 mg/ml of proteinase K (Invitrogen) for 5 min at room temperature and washed in DEPC–treated water for 1 min. After that, sections were dried with 100% ethanol and air-dried for 1 minute. The sections were treated with DNAse I (300 u/ml; Biotools) for 20 min at room temperature to remove any genomic DNA traces that might interfere with the PCR reactions, washed in DEPC-treated water for 1 min, dried with 100% ethanol, and air-dried for 1 minute. Retrotranscription and amplification reactions were performed using the primers for the gene coding for CP (Table 1) and with MyTaq One-Step RT-PCR kit (Bioline) following the manufacturer's instructions. All sections were incubated with a total volume of 50 µl of 2% BSA, 50 mM $MgCl_2$, 1 mM DIG-11-dUTP alkali-stable buffer 5x, 10 u/µl Ribosafe RNAse inhibitor and 5 µl of Reverse Transcriptase for 20 min at 45°C and 1 min at 95°C. Amplification was carried out by running, 25 cycles for 10 s at 95°C, 10 s at 60ºC and 30 s at 72°C; and a final step of 10 min at 72°C. Afterwards, all sections were washed with 2% BSA in 1x SSC [15 mM sodium citrate dehydrate and 0.15 M NaCl] during 10 min at 52°C. The DIG-11-dUTP incorporated in the PCR products was detected by means of a direct immunocytochemistry method using a specific antiserum anti-digoxigenin-HPR (Roche) in 0.1 M Tri-HCl with 0.1 M NaCl at the optimal dilution of 1:100 for 60 min at room temperature. The peroxidase activity was revealed by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.01 M Tris-HCl with 0.1 M NaCl and 0.05% H_2O_2 at room temperature for 5 min. Tissue from non-infected fish was used as negative controls. In order to determine the ability of the amplified products to diffuse into the aqueous phase, liquid phase was collected after performing the *in situ* RT-PCR reactions, run in 1% agarose gel for electrophoresis (Bioline) with 0.5 µg/ml of ethidium bromide (Sigma) and visualized under UV light.

Table 1: Information about the studied genes, primer sequences and applications used in this work.

	Protein	RNA molecule or gene abbreviation	Accession number		Sequence (5'-3')	Tm	Use	Tissue
NNV	Capsid protein	*cp*	D38636	F	AAATTGCACACCACCTGTGA	60ºC	TaqMan real-time PCR	Gonad
				R	ACCCAGAATGGAATGTCAGC			
				Probe	6FAM-ACTGCACGTGTGGTCCAGTA-MGB			
				F	CGTGTCAGTCATGTGTCGCT	60ºC	SYBR real-time PCR/ *in situ* PCR	Gonad / E-11
				R	CGAGTCAACACGGGTGAAGA			
	RNA-dependent RNA polymerase	*rdrp*	AF319555	F	GAGGGTGCGATTGCTATTGT	60ºC	TaqMan real-time PCR	Gonad
				R	ACTGGCACCCAATTAAGCAC			
				Probe	6FAM-CGCTTGAAGGCCTATACACG-MGB			
Gilthead seabream	Tumour necrosis factor alpha	*tnfa*	AJ413189	F	TCGTTCAGAGTCTCCTGCAG	60ºC	SYBR real-time PCR	Gonad Brain
				R	TCGCGCTACTCAGAGTCCATG			
	Interleukin 6	*il6*	AM749958	F	AGGCAGGAGTTTGAAGCTGA	60ºC	SYBR real-time PCR	Gonad Brain
				R	ATGCTGAAGTTGGTGGAAGG			
	Interleukin 1 beta	*il1b*	AJ277166	F	GGGCTGAACAACAGCACTCTC	60ºC	SYBR real-time PCR	Gonad Brain
				R	TTAACACTCTCCACCCTCCA			
	T cell receptor beta chain	*tcrb*	AM261210	F	AAGTGCATTGCCAGCTTCTT	60ºC	SYBR real-time PCR	Gonad Brain
				R	TTGGCGGTCTGACTTCTCTT			
	Immunoglobulin M heavy chain	*igmh*	AM493677	F	CAGCCTCGAGAAGTGGAAAC	60ºC	SYBR real-time PCR	Gonad Brain
				R	GAGGTTGACCAGGTTGGTGT			
	Double sex-and mab3-related transcription factor 1	*dmrt1*	AM493678	F	GATGGACAATCCCTGACACC	60ºC	SYBR real-time PCR	Gonad
				R	GGGTAGCGTGAAGGTTGGTA			
	Gonadal aromatase	*cyp19a1a*	AF399824	F	CACCATGGATCTGATCTCTGCCTGT	60ºC	SYBR real-time PCR	Gonad
				R	GAGCGTTTGCCAGCTGCCTC			
	Steroid 11-β-hydroxylase	*cyp11b1*	FP332145	F	GCTATCTTTGGACCCCATCA	60ºC	SYBR real-time PCR	Gonad
				R	CTTGACTGTGCCTTTCAGCA			
	Estrogen receptor α	*era*	AF136979	F	GCTTGCCGTCTTAGGAAGTG	60ºC	SYBR real-time PCR	Gonad Brain
				R	TGCTGCTGATGTGTTTCCTC			
	Estrogen receptor β1	*erb1*	AF136980	F	CAGCTCCAGAAGGTGGACTC	58ºC	PCR	Gonad Brain
				R	GGATTGGCATAGCTGAAAT			
	Estrogen receptor β2	*erb2*	AJ580050	F	TGATGATGTCACTCACCAACC	58ºC	PCR	Gonad Brain
				R	TTCAGCTCACGAAACCGA			

	Elongation factor 1 alpha	*ef1a*	AF184170	F	CTGTCAAGGAAATCCGTCGT	60ºC	SYBR real-time PCR	Gonad Brain
				R	TGACCTGAGCGTTGAAGTTG			
	β-Actin	*actb*	AJ493428	F	ATCGTGGGGCGCCCCAGGCACC	55ºC	PCR	Gonad Brain
				R	CTCCTTAATGTCACGCACGATTTC			
European sea bass	Tumour necrosis factor alpha	*tnfa*	DQ200910	F	CGAGGGCAAGACTTTCTTTG	60ºC	SYBR real-time PCR	Gonad Brain
				R	GCACTGCCTGTTCAGCTACA			
	Interleukin 6	*il6*	AM490062	F	ACTTCCAAAACATGCCCTGA	60ºC	SYBR real-time PCR	Gonad Brain
				R	CCGCTGGTCAGTCTAAGGAG			
	Interleukin 1 beta	*il1b*	AJ269472	F	CAGGACTCCGGTTTGAACAT	60ºC	SYBR real-time PCR	Gonad Brain
				R	GTCCATTCAAAAGGGGACAA			
	T cell receptor beta chain	*tcrb*	FN687461	F	GACGGACGAAGCTGCCCA	60ºC	SYBR real-time PCR	Gonad Brain
				R	TGGCAGCCTGTGTGATCTTCA			
	Immunoglobulin M heavy chain	*igmh*	FN908858	F	AGGACAGGACTGCTGCTGTT	60ºC	SYBR real-time PCR	Gonad Brain
				R	CACCTGCTGTCTGCTGTTGT			
	Gonadal aromatase	*cyp19a1*	AJ298290 AJ311177	F	CTGGAGCCACACAGACAAGA	60ºC	SYBR real-time PCR	Gonad
				R	AACTGAGGCCCTGCTGAGTA			
	Neural aromatase	*cyp19a2*	AY138522	F	CATGTTCTGAGGAGCGTTCA	60ºC	SYBR real-time PCR	Brain
				R	AAGGGAGTCCACATGTCCTG			
	Steroid 11-β-hydroxylase	*cyp11b1*	AF449173	F	CCCATCTACAGGGAGCATGT	60ºC	SYBR real-time PCR	Gonad
				R	GGAAGACTCCTTTGCTGTGC			
	Estrogen receptor β1	*erb1*	AJ489523	F	GGGTGAGAGAGCTCAAGCTC	60ºC	SYBR real-time PCR	Gonad Brain
				R	AAGCTAAGGCCGGTTTTGGC			
	Estrogen receptor β2	*erb2*	AJ489524	F	AGTGGGCATGATGAAGTGCG	60ºC	SYBR real-time PCR	Gonad Brain
				R	TGCACGTGGTTCACCTGAGG			
	Elongation factor 1 alpha	*ef1a*	FM019753	F	CGTTGGCTTCAACATCAAGA	60ºC	SYBR real-time PCR	Gonad Brain
				R	GAAGTTGTCTGCTCCCTTGG			

2.7. Isolation of NNV infective particles from the testis

After 15 days of infection, testis fragments from mock- or NNV-infected fish were homogenized in 1 ml of 0.01 M PBS. E-11 cells (Iwamoto, *et al.*, 2005), derived from the SSN-1, cultured in L-15 culture medium supplemented with antibiotics and 2% FBS were inoculated with the testis samples and incubated at 25°C. Infected monolayers were examined daily for the presence of CPE. Those cultures showing CPE

were further processed to isolate the total RNA and confirm the identity of NNV by real-time PCR as described below.

2.8. cDNA synthesis

Total RNA was extracted from gonad and brain fragments from both, the *in vivo* (n = 4-5 fish/group and time) and the *in vitro* (n = 6 fish/group) experiments, with TRIzol Reagent (Life Technologies) following the manufacturer's instructions, and quantified with a spectrophotometer (Cecil Instruments Ltd). In the cell culture experiments total RNA was extracted using RNeasy Mini kit (Qiagen) following the manufacturer´s instructions.

Isolated RNA was DNase I treated (amplification grade, 1 unit/µg RNA, Life Technologies) and the SuperScript III RNase H−Reverse Transcriptase (Life Technologies) was used to synthesize first strand cDNA with 1 µl of random primers (0.25 µg/µl; Life Technologies) from 1µg of total RNA, at 50ºC for 60 min.

2.9. Confirmation of NNV gene expression

With the aim of determining the levels of transcription of RNA-dependent RNA polymerase (*rdrp*) and capsid protein (*cp*) genes of NNV in the testis and brain of *in vivo* infected gilthead seabream and European sea bass specimens, real-time PCR with TaqMan probe was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using TaqMan® Gene Expression Master Mix (Applied Biosystems). The primers and TaqMan fluorogenic probes are detailed in Table 1. Reaction mixtures were incubated for 2 min at 50°C and subsequently 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 to finish the reaction. To confirm the results, conventional PCR using the standard F2 and R3 primers for NNV T4 region was also applied to brain of mock-infected and NNV infected sea bass specimens as described elsewhere (Nishizawa, *et al.*, 1994).

In order to identify the presence of NNV in the E-11 cell line inoculated with testis homogenates from control and *in vivo* infected fish at day 15 post-infection, real-time PCR reactions were carried out in a final volume of 50 µl, containing 200 nM of each primer coding for the *cp* gene of NNV (Table 1), as previously described (Olveira, *et al.*, 2013), and 2 µl of cDNA template in iQ™ SYBRGreen Supermix (Bio-Rad). Following an initial 15 min denaturation/activation step at 95°C, the mixture was

subjected to 45 cycles of amplification (denaturation for 15 s at 95°C, annealing and extension for 15 s at 60°C) in a CFX96™ Real-time PCR detection system (BioRad).

2.10. Evaluation of immune- and reproductive-related genes expression

The gilthead seabream and European sea bass genes coding for: (i) pro-inflammatory cytokines such as the tumour necrosis factor alpha (*tnfa*) and the interleukin 6 (*il6*) and 1 beta (*il1b*); (ii) specific cellular immune response markers as the beta chain of the T cell receptor (*tcrb*) and the heavy chain of the immunoglobulin M (*igmh*); (iii) the sex specific gene double sex-and mab3-related transcription factor 1 (*dmrt1*); (iv) steroidogenic enzymes such as gonadal aromatase (*cyp19a1a* of gilthead seabream or *cyp19a1* of European sea bass), neural aromatase (*cyp19a2*) and steroid 11-β-hydroxylase (*cyp11b1*); and (v) the estrogen receptors as the estrogen receptor α (*era*) of gilthead seabream, and the European sea bass estrogen receptor β1 (*erb1*) and β2 (*erb2*) were analysed in the testis and brain by real-time PCR using an ABI PRISM 7500 instrument and SYBR Green PCR Core Reagents (Applied Biosystems) as previously described (Chaves-Pozo, *et al.*, 2010a). The specific primers are shown in Table 1. For each sample, gene expression was normalised by its elongation factor 1 alpha coding gene (*ef1a*) content presented as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primer for specificity. Negative controls with no template were always included in the reactions.

The *erb1* and *erb2* genes of gilthead seabream were analysed by semi-quantitative PCR performed with a Flexcycler (Analitikjena). Reaction mixtures were incubated for 2 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at the specific annealing temperature for each gene (see Table 1), 1 min at 72°C, and finally 10 min at 72°C. For visualizing and comparing the groups, the PCR products were run on a 2% agarose gel. As internal control, the expression of β-actin coding gene (*actb*) was used.

2.11. Serum sex hormone levels

Serum levels of 17β-estradiol (E_2) and 11-ketotestosterone (11KT) were quantified by ELISA following the method previously described in European sea bass (Rodríguez, *et al.*, 2000) and adapted to gilthead seabream (Chaves-Pozo, *et al.*, 2008a).

Steroids were extracted from 10 or 20 µl individual serum (n = 5 fish/group and time) from sea bass or seabream, respectively, in 1.3 ml of methanol (Panreac). Then, methanol was evaporated at 37°C and the steroids were resuspended in 400 µl of reaction buffer [0.1 M phosphate buffer with 1 mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1.5 mM NaN_3 (Sigma) and 0.1% BSA]. 50 µl of extracted sample (1.25 or 2.5 µl of serum per reaction, respectively) were used for each ELISA reaction. The standard, mouse anti-rabbit IgG monoclonal antibody (mAb), and specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical while the microtiter plates (MaxiSorp) were purchased from Nunc. A standard curve from 6.13×10^{-4} to 5 ng/ml (0.03–250 pg/well), a blank and a non-specific binding control (negative control) was established in all the assays. Standards and extracted serum samples were run in duplicate. The lower limit of detection for European sea bass assays was 24.41 pg/ml and for gilthead seabream assays was 12.21 pg/ml. The intra-assay coefficients of variation (calculated from sample duplicates) were 9.3 ± 4.3% for E_2 and 9.1 ± 3.8% for 11KT assays for serum. Details on cross-reactivity for specific antibodies were provided by the supplier (0.01% of anti-11KT reacts with testosterone (T); and 0.1% of anti-E_2 reacts with T; no cross-reaction between 11KT and E_2 was described).

2.12. Calculations and statistical analysis

The gonads were weighed and the gonadosomatic index (GSI) was calculated as an index of the reproductive stage [100×(MG/MB) (%)], where MG is gonad mass (in grams), and MB is body mass (in grams).

All slides were examined with a Nikon eclipse E600 light microscope. The images were obtained with an Olympus SC30 digital camera (Olympus soft imaging solutions GMBH) and Spot 3.3 software (Diagnostic instruments).

The genetic nomenclature used in this manuscript follows the guidelines of Zebrafish Nomenclature Committee (ZNC) for fish genes and proteins and the HUGO Gene Nomenclature committee for mammalian genes and proteins.

The quantification of gilthead seabream *erb1* and *erb2* gene expression was determined by means of 2% agarose gel densitometrically scanned using an image analysis using the Gel Logic 100 Imaging System (Kodac) and ImageJ 1.44p software

(National Institute of Health). The data are showed as the mean value ± standard error to the mean (SEM) of the gene expression relative to *actb* gene expression.

All data were analysed by t-Student test to determine statistical differences between infected and control groups, or one-way ANOVA to denote statistical differences at different points in the infected group ($P < 0.05$). A non-parametric Kruskal–Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 15.0 application. All data are presented as mean ± SEM. Minimum level of significance was fixed in 0.1 (*$P < 0.1$; **$P < 0.05$; ***$P < 0.01$). Letters denote statistical differences between different time points ($P < 0.05$).

3. Results

The experimental infection showed no mortality or disease signs in gilthead seabream specimens, but reached 55% mortality in European sea bass (Fig. 1A).

3.1. NNV colonizes and replicates in the gonad of gilthead seabream and European sea bass males

All the techniques performed to detect NNV were firstly applied to control fish confirming that these fish were free of NNV (Fig. 1, 2 and S1A).

In the testis and brain of gilthead seabream and European sea bass specimens experimentally infected *in vivo* with NNV were determined the transcription levels of the mRNA coding for the two main proteins of the virus, the RdRp and the CP proteins, using a specific probe for each, by TaqMan real-time PCR. Surprisingly, no transcripts of these genes were found in the testis of neither gilthead seabream nor European sea bass, neither in the gilthead seabream brain (data not shown). However, both were detected in the brain of European sea bass after 15 days of infection (Fig. 1B).

The *cp* gene in the testis of gilthead seabream and European sea bass analysed by isPCR determined transcripts of this gene in the testicular cells of both species at 15 days post infection (Fig. 1C–1F and Table 2). Thus, the *cp* mRNA was found in somatic cells (black arrow head in Fig. 1D) and germ cells (G in Fig. 1D) in the testis of gilthead seabream (Fig. 1C), while in European sea bass testis, the mRNA of the *cp* was localized only in somatic cells (black arrow head in Fig. 1F), but not in germ cells (G in

Fig. 1F). After analysing by electrophoresis the liquid phase of each isPCR reaction, we found no amplicons, confirming that there is no diffusion of PCR products between cells (Fig. S1B).

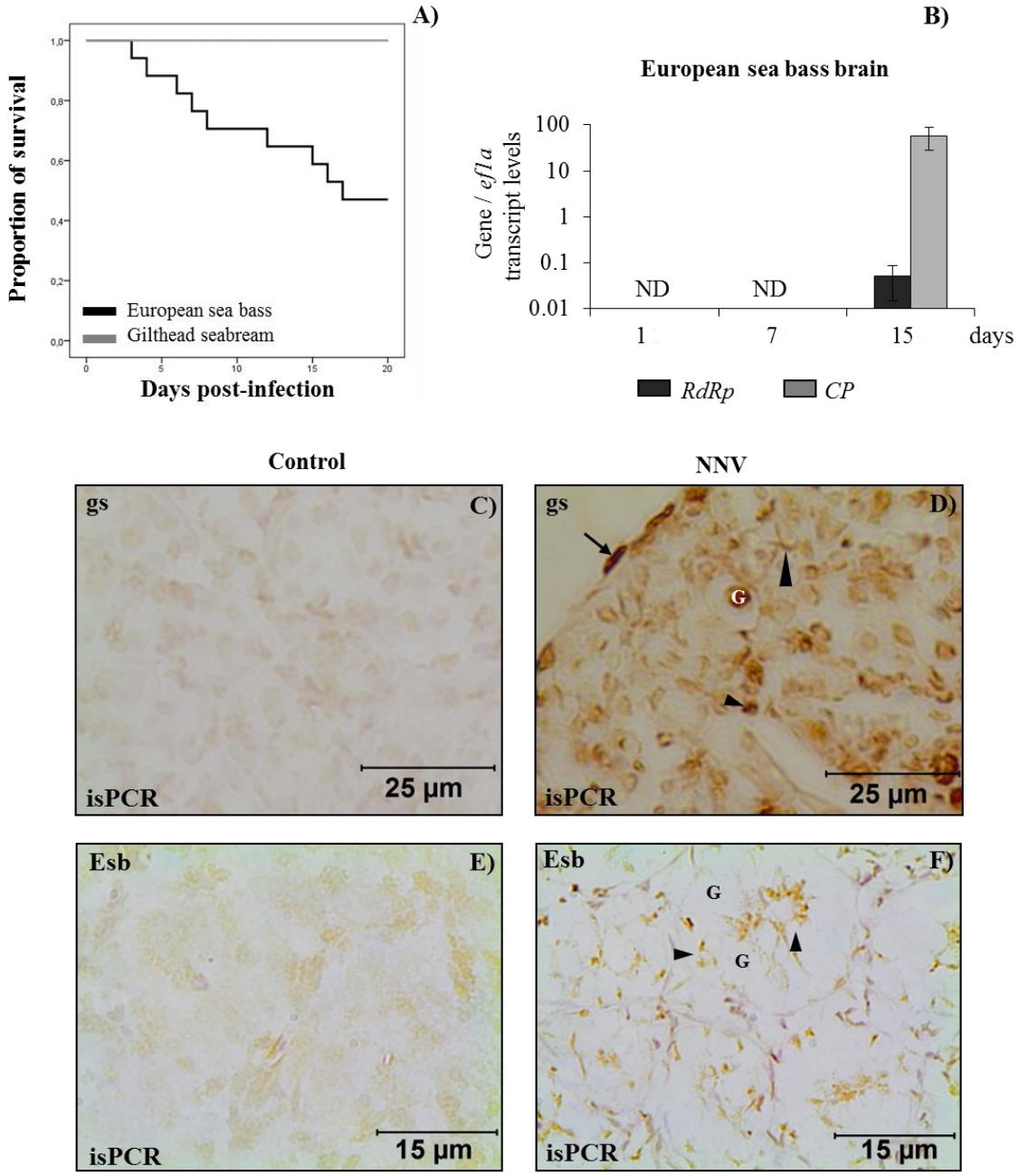


Figure 1: Nodavirus causes mortality and viral RNA is detected in the brain and testis. (A) Kaplan-Meier survival curves showing the proportion of European sea bass (black line) and gilthead seabream (grey line) survivors after intramuscular injection with 10^6 $TCID_{50}$ nodavirus/fish. (B) Transcript levels of the NNV genes, coding for polymerase (RdRp) and capsid (CP) proteins, analysed by real-time-PCR with TaqMan fluorogenic probes, in European sea bass brain after 1, 7 and 15 days of *in vivo* infection with NNV. Viral genes expression was never detected in mock-infected samples and is not shown. (C-F) Detection of RNA coding for the CP protein of NNV by *in situ* PCR (isPCR) in the testis of gilthead seabream (gs; C,D) or European sea bass (Esb; E,F) after 15 days of *in vivo* infection with NNV. Control group (mock infected; C,E) and NNV group (10^6 $TCID_{50}$/fish; D,F). Germ cells (G) and somatic cells (arrow head) are labelled in gilthead seabream (D), while in the European sea bass, somatic cells, mainly the Sertoli cells located between germ cells were stained (F). Scale bars = 25 µm (C,D) or 15 µm (E,F).

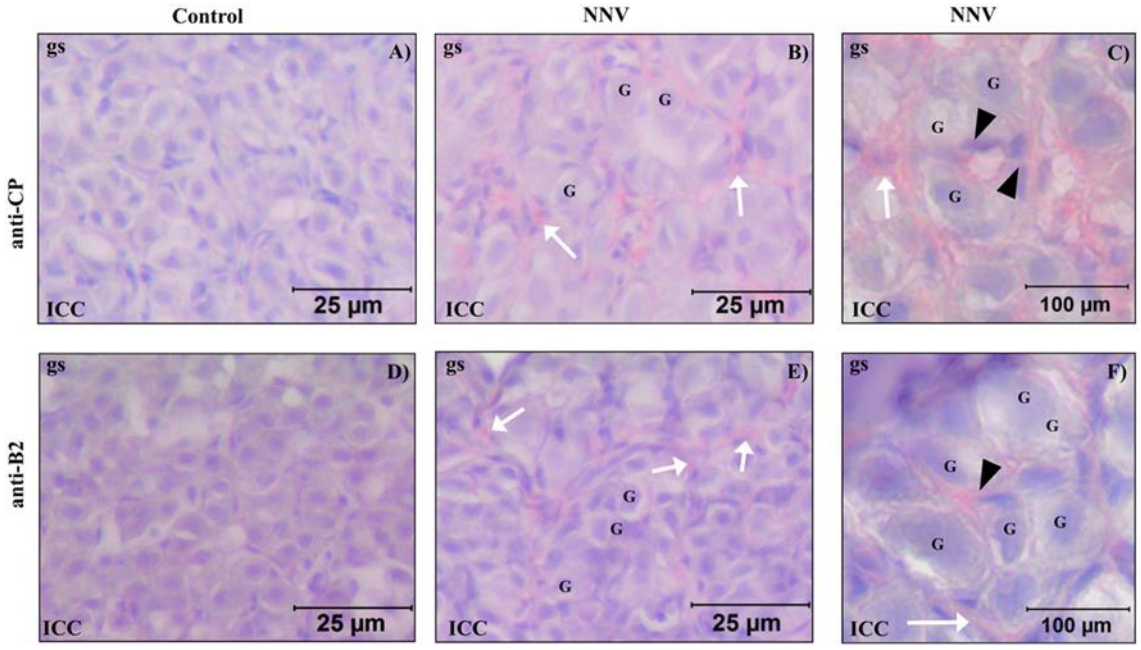


Figure 2: The NNV proteins CP and B2 are detected in the testis of infected gilthead seabream specimens. (A-F) Immunocytochemistry (ICC) in paraffin embedded sections of gilthead seabream testis of mock infected (Control; A,D) or NNV infected (10^6 $TCID_{50}$/fish) specimens (NNV; B,C,E,F) at day 15 using the anti-capsid (Ø233 antibody; 1:500; A,B,C) or the anti-B2 (Ø6073 antibody; 1:500; D,E,F) sera. Scale bars = 25 µm (A,B,D,E) and 100 µm (C,F). Infected cells are shown in dark red stain. The interstitial cells (white arrows) are located surrounding the tubules and the Sertoli cells (arrow heads) located inside the tubules and between the unstained germ cells (G).

Conventional light microscopy showed that at 15 days post infection, the testis of both species had normal morphology characteristic of the resting stage of the reproductive cycle, in which the tubules of the testis are only formed by Sertoli cells that enclosed germ cells (Chaves-Pozo, *et al.*, 2005b; Liarte, *et al.*, 2007; Valero, *et al.*, 2015a). Interestingly, using specific antibodies against CP (Fig. 2A, 2B and 2C) and B2 (Fig. 2D, 2E and 2F), both NNV proteins were localized in somatic cells of the testis. Taking into account the morphology of fish testis (for review on fish testicular morphology see (Schulz, *et al.*, 2010)), we observed stained Sertoli cells, located inside the tubules and between non stained germ cells (black arrow heads in Fig. 2C and 2F), and stained interstitial cells, located around the tubules (white arrows in Fig. 2B, 2C, 2E and 2F) of the testis of gilthead seabream after 15 days of NNV infection (Fig. 2B, 2C, 2E and 2F and Table 2). However, none of these proteins were found in European sea bass testis (data not shown and Table 2).

In order to confirm the presence of infective viral particles of NNV in the testis, the permissive E-11 cell line monolayers were incubated with testis homogenates from infected fish of both species. Although none of the inoculated monolayers developed

extensive CPE after 10 days of inoculation, after a blind passage, partial CPE was observed in three cell cultures inoculated with three samples, two from gilthead seabream and one from European sea bass infected testis. The CPE was characterized by partial disintegration of the monolayer and rounded granular cells with vacuoles (Fig. 3 and Table 2). The identity of isolates was confirmed by real-time PCR (data not shown and Table 2). These data determine that NNV reached the testis and probably maintained very low expression levels of its proteins.

Table 2: The number of NNV positive fish/total number of fish analysed. The analyses were performed in the gonad of fish with three different techniques upon 15 days of infection except for the data in bold that correspond to samples obtained upon 28 days of infection.

Detection technique	Gilthead seabream	European sea bass
isPCR	1/5	3/5
	4/5	
Immunocytochemistry	**3/5**	0/5
Virus recovery	2/5	1/5

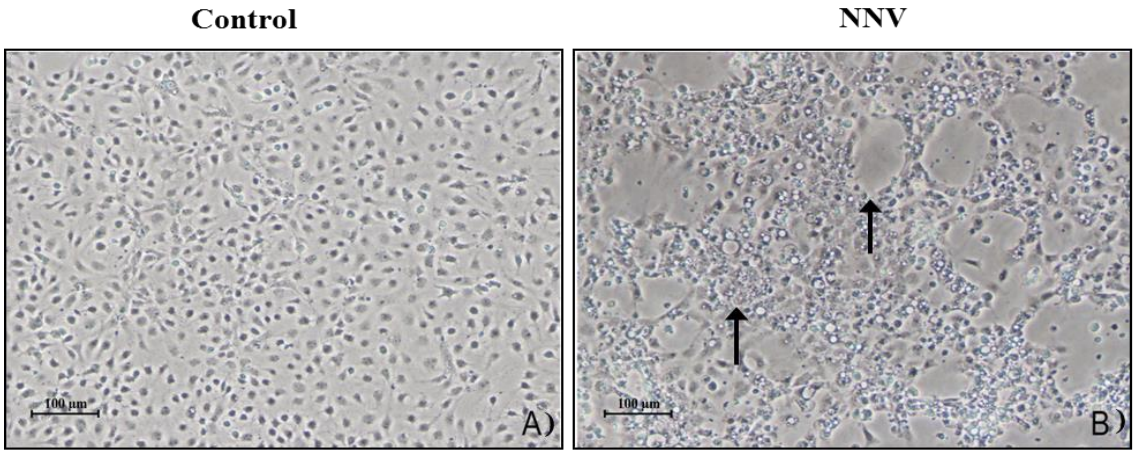


Figure 3: Recovery of infective particles of NNV from testis of gilthead seabream and European sea bass. (A-B) Cytopathic effect (CPE, arrow) in a monolayer of E-11 cells inoculated with testis homogenates from control (A) or infected (B) specimens after 15 days of *in vivo* infection with NNV. Scale bars = 100 µm (A,B).

3.2. NNV triggers immune response in the testis and brain of European sea bass but not in gilthead seabream

Once we knew that the NNV colonized and/or replicated in the testis and brain of gilthead seabream and European sea bass, we studied the pattern of expression of pro-inflammatory cytokines *tnfa*, *il6* and *il1b* and T and B lymphocyte markers (*tcrb*

and *igmh*, respectively) genes in both tissues and species upon *in vivo* infection and in the testis upon *in vitro* infection (Fig. 4) and found that, in gilthead seabream (Fig. 4A–4E), all the cytokine genes were unchanged in the testis (Fig. 4A–4E), whilst in the brain, the *tnfa* and *il1b* gene expression was down-regulated after 7 days of infection (Fig. 4A and 4C) and the *il6* gene expression was increased after 15 days of infection (Fig. 4B). Regarding the lymphocyte markers, the *tcrb* gene was down-regulated from day 7 onwards in the testis and up-regulated at day 15 in the brain (Fig. 4D). Similarly, the *igmh* gene expression was down-regulated at day 7 in the testis and upregulated at day 1 and 15 in the brain (Fig. 4E). When the testis was *in vitro* challenged with NNV or pI:C the expression levels of all these genes were down-regulated (*il6*, *il1b*, *igmh*) or unchanged (*tnfa*, *tcrb*) (Fig. 4F).

In contrast, in the European sea bass upon *in vivo* infection (Fig. 4A–4E), all the cytokine genes analysed, were up-regulated at least at one time point in the testis (Fig. 4A–4E). In the brain, however, the *tnfa* gene expression was down-regulated at day 1 and up-regulated from day 7 onwards (Fig. 4A) and the *il6* gene expression was completely blocked at days 1 and 7 and up-regulated at day 15 (Fig. 4B). Finally, the *il1b* gene expression was up-regulated after 1 and 15 days of infection (Fig. 4C). Regarding the lymphocyte marker genes, the *tcrb* transcription levels were only up-regulated at day 15 in the brain and kept unmodified in the testis (Fig. 4D), while the *igmh* transcription levels were up-regulated at day 7 in the testis and down and upregulated at day 1 and 15 in the brain, respectively (Fig. 4E). None of these genes were modified in the testis upon an *in vitro* challenge with NNV or pI:C (Fig. 4F).

3.3. NNV alters steroidogenesis and sex steroid hormones in the testis of gilthead seabream and European sea bass

As an index of the reproductive stage, we analysed the GSI and the E_2 and 11KT serum levels (Fig. 5) and found that in the gilthead seabream, the GSI was increased after 15 days of NNV infection (Fig. 5A), while in the European sea bass no changes were observed (Fig. 5B). Regarding the hormonal levels in serum (Fig. 5C–5F), in gilthead seabream, NNV induced a high increment in E_2 serum levels after 1 and 7 days of infection (Fig. 5C), whereas in European sea bass E_2 serum levels were not modified compared to controls (Fig. 5D). On the other hand, the 11KT levels in gilthead seabream serum were strongly decreased at day 1 and 7 and increased at day 15 upon

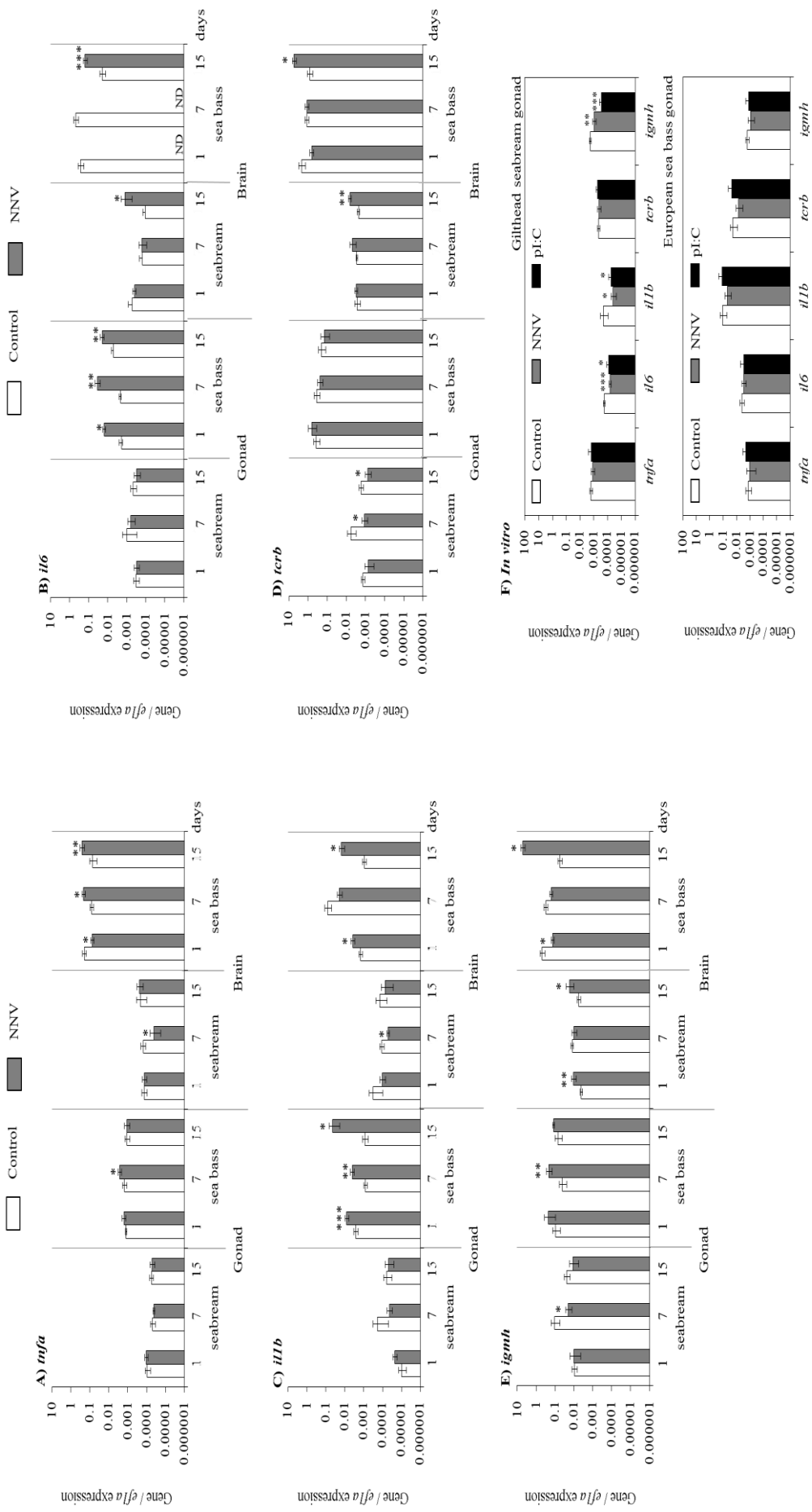


Figure 4: NNV triggers immune responses in the testis and brain of European sea bass but not in gilthead seabream. (A-E) Transcription of *tnfa* (A), *il6* (B), *il1b* (C), *tcrb* (D) and *igmh* (E) genes in the testis and brain of gilthead seabream and European sea bass, after 1, 7 and 15 days of *in vivo*.infection with NNV (F) Transcription of *tnfa*, *il6*, *il1b*, *tcrb* and *igmh* genes after 24 hours of *in vitro* infection with NNV. Data represent the mean ± SEM (n = 5/ group and time). Significance level (P) was fixed at 0.1 (P<0.1*; P<0.05**; P<0.01***). ND, not detected.

infection (Fig. 5E), while in European sea bass, 11KT serum level was only decreased at 1 day of infection (Fig. 5F). Interestingly as the infection progressed an increment in the serum level of E_2 and 11KT were observed in gilthead seabream (Fig. 5C and 5E) but not in European sea bass, where the E_2 serum levels decrease throughout the infection period (Fig. 5F).

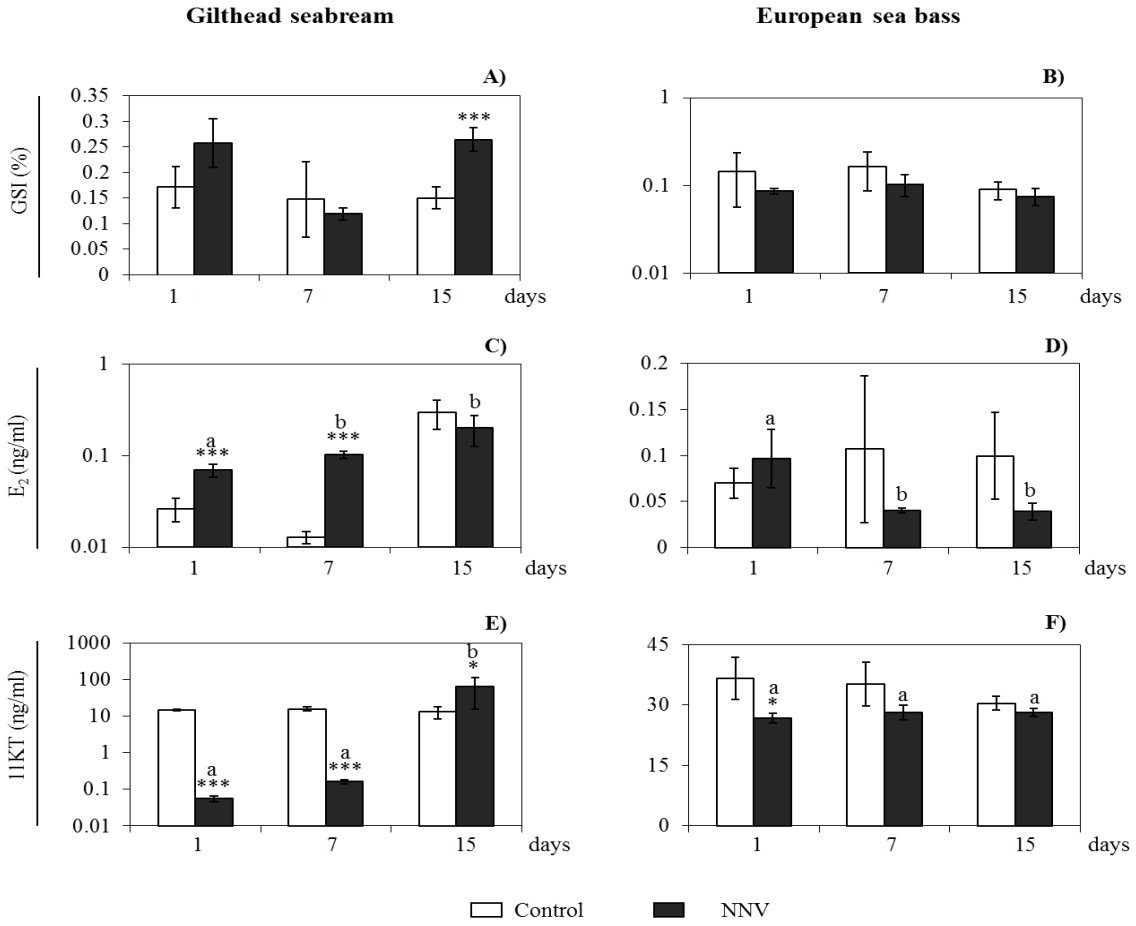


Figure 5: NNV alters sex steroid hormones in gilthead seabream and sea bass. (A-B) Gonadosomatic index (GSI) in gilthead seabream (A) and European sea bass (B) after 1, 7 and 15 days of *in vivo* infection with NNV. (C-F) Sex steroid serum levels of 17β-estradiol (E_2; C,D), and 11-ketotestosterone (11KT; E, F) in gilthead seabream (C,E) and European sea bass (D,F) after 1, 7 and 15 days of *in vivo* infection with NNV. Control group (mock infected) and NNV group (10^6 $TCID_{50}$/fish). Data represent the mean ± SEM (n = 5/group and time). Significance level (P) was fixed at 0.1 (P<0.1*; P<0.01***). Letters denote statistically significant differences within the infected group over time (P<0.05).

In order to determine whether the NNV infection or the changes in the sex-steroid hormone levels detected in gilthead seabream serum affects the functionality of the testis, we next analysed the expression of some reproduction-related genes in the testis. Firstly, *dmrt1* gene expression, a marker of male function in the gilthead seabream testis (Liarte *et al.*, 2007), was slightly down-regulated at day 15 upon

infection (Fig. 6A). Secondly, the expression of genes coding for aromatase (*cyp19a1a*) and 11β-hydroxylase (*cyp11b1*), the enzymes involved in E_2 and 11KT production, respectively; and several E_2 nuclear receptors (*era*, *erb1* and *erb2*) were differently regulated. The *cyp19a1a* expression was up-regulated at 1 day post infection (Fig. 6B), whilst the *cyp11b1* expression was unchanged (Fig. 6C). On the other hand, all estrogen nuclear receptor genes were down-regulated at different time points (Fig. 6D, 6E and 6F). As the E_2 regulated the reproductive behaviour of fish through signalling by its receptor in the brain, the target tissue of the NNV, we also analysed the expression of *era* in this tissue (Fig. 6G), and found that *era* transcript levels were increased at day 1 and decreased at day 7 (Fig. 6G), whereas *erb1* and *erb2* gene expressions were undetected in the brain of both controls and infected fish samples (data not shown). Interestingly, when the expression pattern of these genes was analysed in the testis upon *in vitro* challenge with NNV or pI:C, we found that NNV up-regulated the *cyp11b1* and down-regulated the *erb1* gene expression, while pI:C up-regulated the *cyp11b1* and down-regulated the *cyp19a1a*, *erb1* and *erb2* gene expression (Fig. 6H,I).

Regarding the European sea bass, we found that the steroidogenic enzymes and hormonal receptor genes analysed were also altered upon *in vivo* infection with NNV in testis and brain. Thus, the expression of *cyp11b1* was up-regulated at day 7 and *erb1* and *erb2* genes at day 1 and 7, respectively, while the three genes were down-regulated at day 15 of infection (Fig. 7A, 7B and 7C). The *cyp19a1* gene expression was undetectable in both control and infected specimens (data not shown). However, in the brain, NNV modified the expression pattern of *cyp19a2*, the neural aromatase, which was down-regulated at day 1 and up-regulated at day 7 post infection (Fig. 7D). Otherwise, *erb1* and *erb2* transcription was decreased after 1 and 7 days of infection (Fig. 7E and 7F). In addition, NNV infection completely blocked the expression of the *erb1* gene after 15 days of infection, when *erb2* gene expression was up-regulated (Fig. 7E and 7F).

Finally, in the testis of European sea bass challenged *in vitro* with NNV and pI:C (Fig. 7G), the *cyp19a1* gene expression was undetectable even in the control fish (data not shown), whereas the expression of *cyp11b1* gene was down-regulated upon pI:C exposure. Interestingly, the transcription of the genes coding for both estrogen receptors, *erb1* and *erb2*, was upregulated upon NNV challenge.

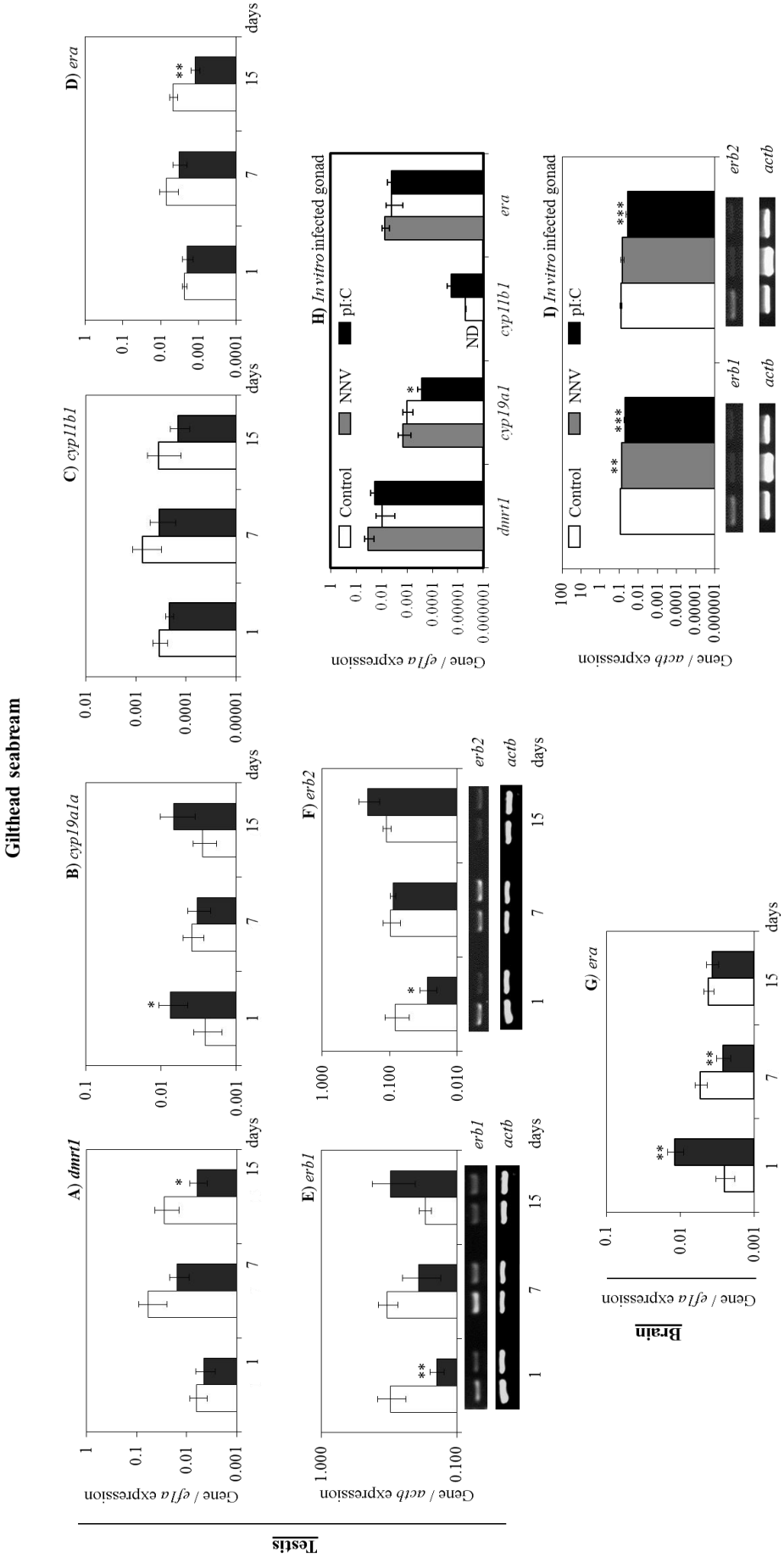


Figure 6: NNV alters the expression of some reproductive and steroidogenic relevant genes in gilthead seabream testis. (A-F) Transcription of *dmrt1* (A), *cyp19a1a* (B), *cyp11b1* (C), *era* (D), *erb1* (E) and *erb2* (F) in the testis of gilthead seabream after 1, 7 and 15 days of *in vivo* infection with NNV. (G) Transcription of *era* in the brain of gilthead seabream after 1, 7 and 15 days of *in vivo* infection with NNV. (H-I) Transcription levels of *dmrt1*, *cyp19a1a*, *cyp11b1*, *era*, *erb1* and *erb2* after 24 hours of *in vitro* infection with NNV. Data represent the mean ± SEM (n = 5/group and time). Significance level (P) was fixed at 0.1 (P<0.1*; P<0.05**; P<0.01***).

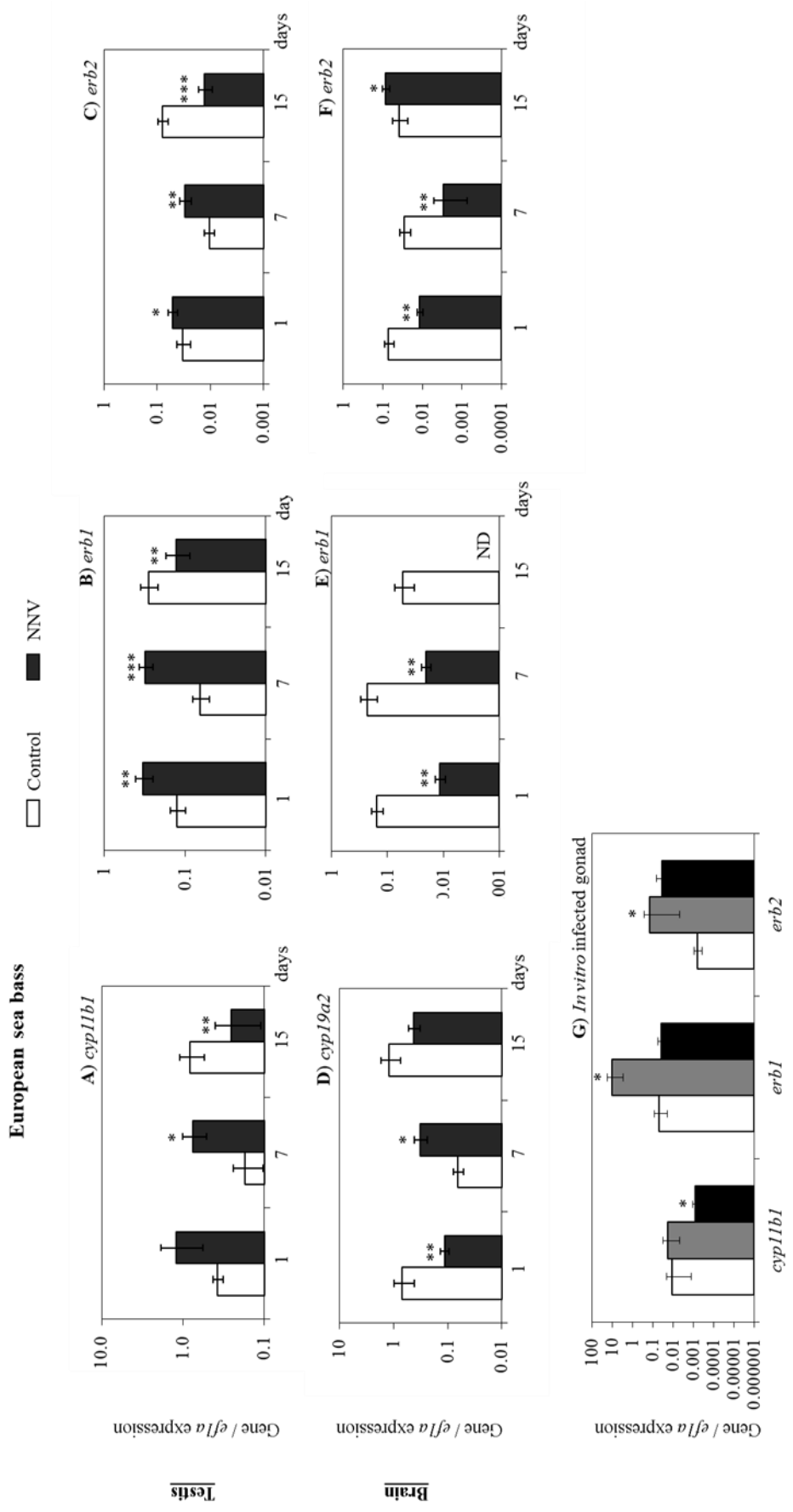


Figure 7: NNV alters the expression of some reproductive and steroidogenic relevant genes in European sea bass testis. (A-C) Transcription of *cyp11b1* (A), *erb1* (B) and *erb2* (C) in the testis of European sea bass after 1, 7 and 15 days of *in vivo* infection with NNV. (D-F) Transcription of *cyp19a2* (D), *erb1* (E) and *erb2* (F) in the brain of European sea bass after 1, 7 and 15 days of *in vivo* infection with NNV. (G) Transcription of *cyp11b1*, *erb1* and *erb2* after 24 hours of *in vitro* infection with NNV. Data represent the mean ± SEM (n = 5/group and time). Significance level (P) was fixed at 0.1 (P<0.1*; P<0.05**; P<0.01***). ND, not detected.

4. Discussion

NNV is a single stranded RNA virus which causes VER disease and provokes high mortality rates in several Mediterranean fish species (Munday, *et al.*, 2002). European sea bass is one of the most susceptible species to this disease and undergoes high mortalities at larvae and juvenile stages (Breuil *et al.*, 1991; Frerichs *et al.*, 1996). However, other species such as gilthead seabream have been considered to be an asymptomatic carrier, although recent outbreaks of VER disease resulting in high mortalities have threatened this species too (Maltese and Bovo, 2007; Olveira, *et al.*, 2009). NNV is a known vertically transmitted pathogen (Kuo, *et al.*, 2012; Munday, *et al.*, 2002). The knowledge about the ability of NNV to colonize and evade the immune response in the gonad represents the initial step to understand how pathogens are vertically transmitted to the progeny and also potentially improve and develop new strategies to prevent NNV infections. In this study, and for the first time, we demonstrate that NNV colonizes and replicates into the testis of gilthead seabream and European sea bass males although its level of gene expression is very low and not easily detected by conventional or even real-time PCR methodologies. However, we localized the expression of the *cp* gene using isPCR as previously described on grouper embryos (Kuo, *et al.*, 2012). Thus, we localize the NNV *cp* RNA on Sertoli cells in the testis of both species, and on tunica albuginea's and germ cells in the gilthead seabream testis. These results suggest that NNV could be spread into the germ cells of gilthead seabream and within the gonadal fluid of both species. However, as Sertoli cells are intimately associated to germ cells in fish (Schulz, *et al.*, 2010), the shed of the virus into European sea bass germ cells cannot be discarded. Regarding viral proteins, we also immuno-detected the CP and B2 proteins in the Sertoli and/or interstitial cells of gilthead seabream testis. Interestingly, B2 protein production is only detected when the virus is performing an active infection instead of a persistent one (Mezeth, *et al.*, 2009). In contrast to gilthead seabream, no proteins were detected in the testis of European sea bass; however we cannot discard very low rates of viral protein production in this specie. In fact, viral infected particles seem to be present in the testis of both species as we succeeded to recover the virus after one blind passage in a permissive cell line inoculated with testis homogenates from infected specimens of both species. Therefore, the lack of immunoreactivity with the anti-NNV sera could be due to the very low amount of NNV infective particles together with very low levels of gene transcription.

Our data also exclude the possibility of blood contamination since the testis was rid of blood in most of the blood vessels, as observed in the histology samples, and the isPCR and immunocytochemistry techniques never showed viral mRNA or protein staining in blood vessels or cells (data not shown).

Other viruses have also demonstrated to colonize the gonad, such as VHSV and IPNV in the rainbow trout and showed different replicating capacity and in turn they elicited a different immune response (Chaves-Pozo, *et al.*, 2010a). Interestingly, upon *in vivo* infection, IPNV did not trigger an effective immune response, which was triggered upon *in vitro* infection, so some extragonadal factors might block the immune response in the gonad improving the transmission of the virus (Chaves-Pozo, *et al.*, 2010a). NNV, however, triggered in the gilthead seabream testis a slight down-regulation of *tcrb* and *igmh* genes upon *in vivo* infection and of *il6* and *il1b* genes upon *in vitro* infection, whilst in the testis of European sea bass we observed up-regulations of the pro-inflammatory cytokine and *igmh* gene expressions upon *in vivo* infection and no changes upon *in vitro* infection. In fact, the same pattern in the testis expression of others immune-related genes, including antimicrobial peptides (AMPs) and interferon (IFN) response (Valero, *et al.*, 2015b,c), of both species was observed. Thus, the AMPs and IFN transcription is unaltered or inhibited in gilthead seabream testis, while in the European sea bass testis most of those genes are up-regulated upon *in vivo* and *in vitro* infections (Valero, *et al.*, 2015b,c). Interestingly, in the gilthead seabream brain and head-kidney (HK) upon *in vivo* infection with NNV, the phagocytosis, the cell-mediated cytotoxic activity and the *mx* transcription started very early upon the infection and stayed at high up to 15 days, while in the European sea bass brain these activities and the *mx* transcription was up-regulated at day 1 upon infection and quickly decreased to control levels (Chaves-Pozo, *et al.*, 2012b). Furthermore, our data showed that the pro-inflammatory cytokines are much more up-regulated in the European sea bass brain upon infection than in gilthead seabream. All these data, taken together, suggest that while gilthead seabream overcome the NNV infection and remove the virus from the brain probably due to a successful anti-viral immune response with little inflammatory consequences, the European sea bass fail to do so and the inflammatory response is up-regulated and probably produce high cell damage (Chaves-Pozo, *et al.*, 2012b; this study). Moreover, in the testis of gilthead seabream the immune response is tightly controlled and the virus succeeds to be transmitted as suggested by greater detection of

NNV at gene, protein and infective particle levels. However, in the European sea bass, as the infection proceeds, the reproductive process become less important and the immune response try to keep the specimen alive, even when the tissue will be damaged, so the inflammatory response increases into the testis, together with the AMP and IFN responses, and could be the reason to the very low and limited detection of NNV. Interestingly, other studies described that NNV increased the number of cytotoxic T lymphocytes in the blood and up-regulates the expression of CD8α gene in groupers (Chang, *et al.*, 2011). Similarly to what happened with the anti-viral immune response and the pro-inflammatory cytokines expression, the *tcrb* and *igmh* gene expression is up-regulated in the brain of gilthead seabream and down-regulated in the testis, while in the European sea bass only the *tcrb* is up-regulated in the brain upon 15 days of infection. Regarding the *igmh* transcription in European sea bass tissues, we observed a decrease at day 1 and an increase at day 15 in the brain, while in the testis this gene expression was up-regulated at day 7.

Several pathogen infections change the sex steroid hormone levels of the infected specimens (Deane, *et al.*, 2001; Gómez, *et al.*, 2000). Curiously, low levels of E_2 and high levels of testosterone (T) have been related with the progression of vibriosis symptoms (Deane, *et al.*, 2001). Our data support this hypothesis as the E_2 serum levels decreased as the infection proceeds only in the European sea bass, which showed mortality during the infection and displayed an earlier but less effective immune response in the brain (Chaves-Pozo, *et al.*, 2012b). In contrast, in the gilthead seabream, the E_2 serum levels increased as the infection progressed. Moreover, in the last years, it has been described in the gilthead seabream that estrogens regulated the inflammatory immune response through endothelial cells and macrophage activation and increased leucocytes recruitment in the testis (Cabas, *et al.*, 2011; Chaves-Pozo, *et al.*, 2007; Liarte, *et al.*, 2011b,c). Furthermore, androgens such as testosterone also induced the recruitment of acidophilic granulocytes and IgM-positive cells in the testis of gilthead seabream and modulated *in vitro* the activity of gilthead seabream phagocytes and their sensitivity to pathogens (Águila, *et al.*, 2013; Sánchez-Hernández, *et al.*, 2013). On the other hand, increases on E_2 and T serum levels in the gilthead seabream, increased the complement and peroxidase activities at different time points although unmodified or decreased other humoral immune responses such as anti-protease activity and IgM serum levels (Cuesta, *et al.*, 2007). Our data showed that NNV infection induced a

strong increase on E_2 serum levels at the beginning of the infection, whilst the serum levels of 11KT were decreased at these time points and increased at day 15. Taking into account that a certain increase in E_2 serum levels stimulates the immune response in the gilthead seabream, this data also supports the ability of this species to fight against the NNV infection and overcome the disease.

In mammals, E_2 regulates the inflammatory response as two-edges-sword, triggering stimulation or inhibition of this response depending on several parameters such as the immune stimulus, the tissue involved, the concentration of E_2, the expression of estrogen receptors and so on (Straub, 2007). Similarly, in fish, controversial data about the ability of sex steroid hormones to modulate the immune response has been published upon exogenous administration of several sex steroid hormones in several fish species (Chaves-Pozo, *et al.*, 2012a; Cuesta, *et al.*, 2007; Hou, *et al.*, 1999). We therefore studied the expression of some genes coding for some steroidogenic enzymes (*cyp11b1* and *cyp19a1a* and *cyp19a2*) and estrogen receptors (*era*, *erb1* and *erb2*) in the testis, the main organ that synthesizes biologically active steroids *de novo* (Stocco, 2001), and brain, as the brain is the main target tissue of the virus, of both species upon *in vivo* infection and in the testis upon *in vitro* infection. Our data showed that in the gilthead seabream testis, the expression of the *cyp19a1a* gene that coded for the aromatase enzyme that transforms T to E_2, was increased, coinciding with an increase on the E_2 serum level. The expression of this gene was undetected in the European sea bass testis, and therefore the E_2 serum levels decreased. However, the sensitivity of the gilthead seabream testis to E_2 is decreased as the expression of the *era* at day 15 and of *erb1* and *erb2* at day 1 decreased after infection. On the other hand, we analysed the sensitivity of E_2 and the local E_2 production in the brain as locally produced E_2 in the brain regulated important biological functions including reproduction and neuroprotection (García-Segura, *et al.*, 2001). Thus, we found that the expression of *era* in the brain increased at day 1 and decreased at day 7 upon infection whilst neither *erb1* nor *erb2* were detected. This is not surprising as it has been suggested that the expression of *er* genes is not detected in some areas of the brain probably because its expression is very low (Diotel, *et al.*, 2011). In contrast to gilthead seabream brain, in European sea bass brain, the expression of the gene coding for the neural aromatase (*cyp19a2*), *erb1* and *erb2* were differently regulated. Regarding androgens, NNV decreases the 11KT serum levels of gilthead seabream without

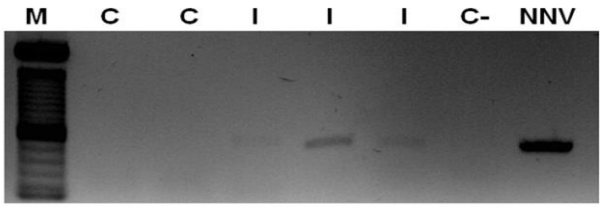
affecting, in the testis, the expression of the *cyp11b1* gene; that coded for the key enzyme in the transformation of T to 11KT in the testis. Although the testis is the main steroidogenic tissue, other tissues can synthesize 11KT or transform T to 11KT (Arukwe, 2008) producing the increase of serum 11KT observed upon NNV infection. Neither 11KT nor *cyp11b1* transcription has been detected in the brain of teleost (Diotel, *et al.*, 2011). Regarding testicular functionality, the changes observed on sex steroid hormones levels and steroidogenic enzymes and estrogen receptor gene transcriptions seems not to be disrupted for the gilthead seabream testis as the GSI was increased and the *dmrt1* gene expression was slightly decreased upon 15 days of infection. However, the *dmrt1* transcription levels were not as low as needed to produce testicular disruption in gilthead seabream males (Liarte, *et al.*, 2007). Interestingly, the changes observed on the expression of all these genes in the testis of both species upon *in vitro* infection with NNV are different to those observed *in vivo* in most of the cases. This suggests that the changes in other tissues due to NNV infection; modifies the gonadal response upon infection.

5. Conclusions

In conclusion, we have proved for the first time the ability of NNV to colonise the male testis of gilthead seabream and European sea bass and produce infective particles by means of isPCR, immunocytochemistry and cell culture. However, the response to the virus in both species is very different. In addition, NNV triggers the inflammatory immune response in the testis of European sea bass whilst seems to be overlooked in the gilthead seabream testis. This also applies to other immune responses, as AMPs or IFN production, previously studied, which could account for the higher presence of NNV in the seabream testis. Furthermore, we had also determined whether NNV is able to modulate the reproductive system to improve its transmission and could demonstrated that NNV alters E_2 and 11KT production and the sensitivity of brain and testis to these hormones. Whether this occurs due to changes in the fish physiological abilities to modulate the immune response or by NNV to improve its ability to replicate and be transmitted is still undetermined and further studies will be needed to understand these mechanisms. Apparently, according to the GSI and *dmrt1* expression levels, there is no disruption of testicular functions upon infection, which could favour the shedding and dissemination of the NNV to the water and/or the surrounding animals.

6. Supplementary data

A)



B)

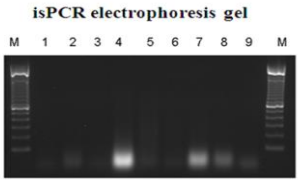


Figure S1: Fig.(A) Transcription of the NNV capsid (*cp*) gene in European sea bass brain from control (C) and infected (I) fish at 15 days of *in vivo* infection with NNV. M, 100-bp ladder; lanes 1–2, control fish; lines 3–5, infected fish; line 6, negative control of the PCR; and line 7, purified NNV genome. (Fig. B) The isPCR products do not diffuse to the aqueous phase. The liquid phase of each isPCR was run in a 2% agarose gel and showed no amplicons. M, 100-bp ladder; lanes 1–9 correspond to different samples and the bands to primers.

III.1.2. CHARACTERIZATION OF THE INTERFERON PATHWAY IN THE TELEOST FISH GONAD AGAINST THE VERTICALLY TRANSMITTED VIRAL NERVOUS NECROSIS VIRUS

1. Introduction

The innate immune response against virus infections uses different mechanisms such as the interferon (IFN), the complement system or the cytotoxic cells (Ellis, 2001) being the IFN response 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 and Williams, 2008). In fish, apart from the type II, the genome sequencing projects have detected different *ifn* genes ranging from 1 in fugu (*Takifugu rubripes*) or medaka (*Oryzias latipes*) to 11 genes in Atlantic salmon (*Salmo salar*) belonging to the types I and III (Sun, *et al.*, 2009; Zou and Secombes, 2011). Evolutionary and phylogenetical studies have demonstrated the problems in the fish *ifn* gene nomenclature. In fact, they share characteristics with the mammalian type I and III IFNs, and act as co-orthologues, being suggested to be renamed as IFNφ (Hamming, *et al.*, 2011; Levraud, *et al.*, 2007). Fish IFNs can be divided into two groups: 2 cysteine-containing group I and 4 cysteine-containing group II (Zou, *et al.*, 2007). In addition, group I *ifn* can be subdivided into subgroup-a and subgroup-d and the group II into subgroup-c and subgroup-b. Group I *ifn* genes are found in all the fish species whilst the group II is only found in the most primitive fish such as salmonids and cyprinids (Sun, *et al.*, 2009; Zhang and Gui, 2012; Zou, *et al.*, 2007). Therefore, several names have been proposed for fish IFNs: type I IFNs, virus-induced IFNs, IFNλ, IFNφ or even simply IFNs (Langevin, *et al.*, 2013). Although, it is 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 the IFN nomenclature, all 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 Jak-Stat (Janus kinase-signal transducer and activator of transcription) pathway. Their activation create in the cells an antiviral state through the induction of many IFN-stimulated genes (ISGs), including genes such as the antiviral molecule myxovirus (influenza) resistance protein (Mx), with a 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 although 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 among 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 (NOD)-like receptors (NLRs). In the case of fish viruses, TLR3 and TLR22 are induced by dsRNA viruses (Matsuo, *et al.*, 2008), whilst TLR7 and TLR8 are by ssRNA viruses (Crozat and Beutler, 2004), which in both cases induces a type I IFN-mediated response. To date, the involvement of the 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), MDA5 (Melanoma Differentiation-Associated gene 5 or IFIN1) and LGP2 (Laboratory of Genetics and Physiology 2 or DHX58). These sensors are up-regulated by viral haemorrhagic septicaemia virus (VHSV), spring viremia of carp virus (SVCV), grass carp reovirus (GCRV), nervous necrosis virus (NNV) or infectious pancreatic necrosis virus (IPNV), as well as by polyinosinic acid (pI:C; a synthetic analogue of viral dsRNA), leading to an increase in the IFN-mediated antiviral response (Chen, H.Y., *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 role in the antiviral response and the identification and characterization of their mediators in the molecular pathway leading to the IFN activation.

In all vertebrates, the gonad is considered an immunologically-privileged site, as also occurs with the brain and retina, where the immune response proceeds in a different manner in order to avoid cell damage (Chaves-Pozo, *et al.*, 2005c; Hedger, 2002), and therefore, it is used by some pathogens to be hidden and scape to the immunological control. NNV, or nodavirus, a bipartite and positive single-stranded RNA virus, is a known vertical and horizontal transmitted pathogen (Arimoto, *et al.*, 1992; Kuo, *et al.*, 2012) able to infect more than 50 marine fish species, some of them especially sensitive, as the European sea bass (*Dicentrarchus labrax*), and others only susceptible to some strains, as occurs with the gilthead seabream (*Sparus aurata*) (Castric, *et al.*, 2001; Frerichs, *et al.*, 1996). Interestingly, though the main target tissues of NNV are the brain and the retina (Castric, *et al.*, 2001; Frerichs, *et al.*, 1996), both immune-privileged tissues, as 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 it into, and isolated from, the gonad (Valero, *et al.*, 2015d). Previous studies have documented that NNV infection induces the immune response with especial emphasis in the type I IFN response. Thus, expression of *ifn* and/or *mx* genes was

greatly up-regulated in the brain or immune-relevant tissues of gilthead seabream, orange-spotted grouper (*Epinephelus coioides*) or Atlantic halibut (*Hippoglossus hippoglossus*) but lightly in the European sea bass (Chaves-Pozo, *et al.*, 2012b; Chen, Y.M., *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 up-regulated in the brain of gilthead seabream (Dios, *et al.*, 2007) and Atlantic cod (*Gadus morhua*) (Rise, *et al.*, 2010) by NNV infection. Unfortunately, any study has investigated the IFN response into the gonad of NNV-infected fish, taking into consideration that this virus uses the gonad to hide and be transmitted.

Taking in mind the previous information, we aimed in this study to deepen in the characterization of the type I IFN pathway of European sea bass and gilthead seabream, and its involvement upon infection with NNV, as well as in their respective cell lines, focusing on the gonad, and compared to that found in the brain, the target tissue for NNV.

2. Materials and methods

2.1. Animals and cell lines

Adult specimens of the marine teleost gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*; 125 ± 25 and 305 ± 77 g body weight, respectively) were bred at the *Centro Oceanográfico de Murcia*, *Instituto Español de Oceanografía* (COM-IEO) with natural conditions of photoperiod, temperature, salinity and aeration and translated to the University of Murcia aquaria. Fish were kept in 450-500 L running seawater (28‰ salinity) aquaria at 24 ± 2ºC and 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 25ºC 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 seabream SAF-1 (Béjar, *et al.*, 2005) cell lines were cultured using Leibovitz's L15-medium (Life Technologies) supplemented with 10% fetal bovine

serum (FBS; Life Technologies), 2 mM L-glutamine (Life Technologies), 100 i.u. ml^{-1} penicillin (Life Technologies) and 100 µg ml^{-1} streptomycin (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 (EMEM; Life Technologies) supplemented with 15% FBS, glutamine and antibiotics as above.

2.2. NNV stocks

NNV (strain 411/96, genotype RGNNV) were propagated in the SSN-1 cell line which is persistently infected with a snakehead retrovirus (SnRV) (Frerichs, *et al.*, 1996). Cells were inoculated with NNV and incubated at 25ºC until the cytopathic effect was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates before used in the experiments (Reed and Müench, 1938).

2.3. Gene search and bioinformatic analysis

According to the literature (Sun, F., *et al.*, 2011; Takeuchi and Akira, 2008; Zhang, *et al.*, 2014), virally activated RLRs (MAD5, LGP2 or RIG-I) initiate a molecular pathway leading to the expression of *ifn* and IFN-induced genes creating the cellular antiviral state. Thus, these receptors interact with the RLR adaptor protein, MAVS (or the IFN-β promoter stimulator-1 IPS-1), then it associates with tumour necrosis factor (TNF) receptor-associated factor 3 (TRAF3), which recruits and facilitates the interaction between, but not exclusively, TRAF family member-associated NF-kB activator (TANK) and TANK-binding kinase 1 (TBK1), also activated by TLR3, and therefore the TLR and RLR IFN-activation pathways by viral RNA are shared from this point. TBK1, in turns, phosphorylates and activates IFN regulatory factors (IRF)-3 and -7. These IRF3 and 7 are then translocated to the nucleus where bind to the IFN-stimulated response elements (ISRE) and activate the expression of *ifn* and IFN-stimulated genes, including the Mx and PKR (dsRNA-dependent protein kinase receptor) coding genes.

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 expressed sequence tags (ESTs) databases from gilthead seabream 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 evolutionary analyses were conducted using MEGA version 6 (Tamura, *et al.*, 2013) to confirm that they are expected *bona fide* sequences. The sequences found and studied, related to the IFN pathway activation by RLRs, are described in this work (Fig. 1).

2.4. *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 ml^{-1} polyinosinic acid (pI:C) or 10^6 $TCID_{50}$ ml^{-1} NNV. After treatment, monolayers were carefully washed with PBS and stored in TRIzol Reagent (Life Technologies) at -80ºC for latter isolation of RNA.

2.5. *In vivo* infections with NNV

Thirty specimens of gilthead seabream or European sea bass were randomly divided into two tanks. Each group received a single intramuscular injection of 100 µl of SSN-1 culture medium (mock-infected) or culture medium containing 10^6 $TCID_{50}$ $fish^{-1}$ since this route of infection has been proven as the most effective (Aranguren, *et al.*, 2002). Fish were sampled 1, 7 and 15 days after the viral infection and fragments of brain and gonad tissues were stored in TRIzol Reagent at -80ºC for latter isolation of RNA.

2.6. Analysis of gene expression by real-time PCR

We studied the transcription of selected genes in brain and gonad from naïve fish, SAF-1 and DLB-1 cell lines, as well as after *in vitro* treatments with pI:C or NNV and after *in vivo* infection with NNV. Total RNA was isolated from TRIzol Reagent frozen samples following the manufacturer's instructions. One µg of total RNA was treated with DNAse I to remove genomic DNA and the first strand of cDNA synthesized by reverse transcription using the SuperScriptTM III Reverse Transcriptase (Invitrogen) with an oligo-dT12-18 primer (Invitrogen) followed by RNAse H (Invitrogen) treatment.

Real-time PCR 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 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1 alpha coding gene (*ef1a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. Gene names follow 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 1. 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.

2.7. Statistical analysis

Data in figures are represented as mean ± standard error of the 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 one-way analysis of variance (ANOVA; $p \leq 0.05$) using the SPSS 20 software.

3. Results

3.1. Identification of genes involved in the IFN pathway

We have identified most of the known genes involved in the RLR-activation pathway of the IFN (Fig. 1). In gilthead seabream 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 EST databases, we found partial or full-length sequences of seabream *mda5*, *tbk1*, *irf3* and *pkr* genes 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-seq (Núñez-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 seabream and sea bass, both belonging to

the modern teleost. The predicted length, homology and E-values obtained from the gene sequences were compared with their zebrafish orthologues (Table 2) resulting in *bona fide* sequences, which were further confirmed by the analysis of the predicted protein domains and its conservation (Supplementary data; Table S1). These domains include: helicase domain in MDA5 and LGP2, CARD domain in MAVS, RING domain and MATH_TRAF3 in TRAF3, TBD domain in TANK, STKc_TBK1 domain in sea bass TBK1, IRF-3 domain in both IRF3 and 7, STKc_EIF2AK2_PKR domain in seabream PKR and DSRM domain in sea bass PKR. All these domains were also found and conserved in the respective zebrafish and human orthologues.

Table 1: Primers used for analysis of gene expression by real-time PCR.

Gene name	Gene abbrev.	Fish specie	Acc. numbers	Sequence (5'-3')
Melanoma differentiation-associated 5 protein	*mda5*	Seabream	HS988207	CATCGAGATCATCGAGGACA
				CCAGATGTCGCTCTTGAAGG
		Sea bass	AM986362	AATTCGGCAATGGTGAAGTC
				TCATTGGTCACAAGGCCATA
Laboratory of genetics and physiology 2 protein	*lgp2*	Sea bass	AM984225	TGATGGCAGTCAGTGGAGAG
				TGAGAGCTCAACGTGTTTGG
Mitochondrial antiviral-signaling protein	*mavs*	Sea bass	KP861888	GCACAAGCTCAAAGCATCAA
				TCACTGGAGGGGGTGTTTAC
TNF receptor-associated factor 3	*traf3*	Sea bass	KP861887	CGATTAGCCGACATGGATCT
				TGCTTCCTGTTTCCGTCTCT
TRAF family member-associated nuclear factor-kappa-B activator	*tank*	Sea bass	KP861886	GCGGACAGCGAATATGACTT
				GCAATGTGGAGGGGACACTA
TANK-binding kinase 1	*tbk1*	Seabream	HS988213	AGGAACAGCTGCCTCAGAAG
				CAGCTTCTTCATCCCCAGAG
		Sea bass	FM013306	ACAAGGTCCTGGTGATGGAG
				CGTCCTCAGGAAGTCCGTAA
Interferon regulatory factor 3	*irf3*	Seabream	AM956899	TCAGAATGCCCCAAGAGATT
				AGAGTCTCCGCCTTCAGATG
		Sea bass	CBN81356	AGAGGTGAGTGGCAATGGTC
				GAGCAGTTTGAAGCCTTTGG
Interferon regulatory factor 7	*irf7*	Sea bass	KP861885	ATTCACCAACCGCATCCTTA
				GCCTCCAGGCATAGATACCA
dsRNA-dependent protein kinase receptor	*pkr*	Seabream	HS988732	TCCTTTGGAACCTCCCTACC
				TCGAGGGGGAAATGTTGTAA
		Sea bass	FM008342	AGGGTCAGAGCATCAAGGAA
				GACACCTTGCTGTCCCAGTC
Type I Interferon	*ifn*	Seabream	FM882244	ATGGGAGGAGAACACAGTGG
				GGCTGGACAGTCTCTGGAAG
		Sea bass	AM765847	GGCTCTACTGGATACGATGGC
				CTCCCATGATGCAGAGCTGTG
Myxovirus (influenza) resistance proteins	*mx*	Seabream	FJ490556, FJ490555, FJ652200	AAGAGGAGGACGAGGAGGAG
				TTCAGGTGCAGCATCAACTC
		Sea bass	AM228977, HQ237501, AY424961	GAAGAAGGGCTACATGATCGTC
				CCGTCATTGTAGAGAGTGTGGA
Elongation factor 1 alpha	*ef1a*	Seabream	AF184170	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG
		Sea bass	AJ866727	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG

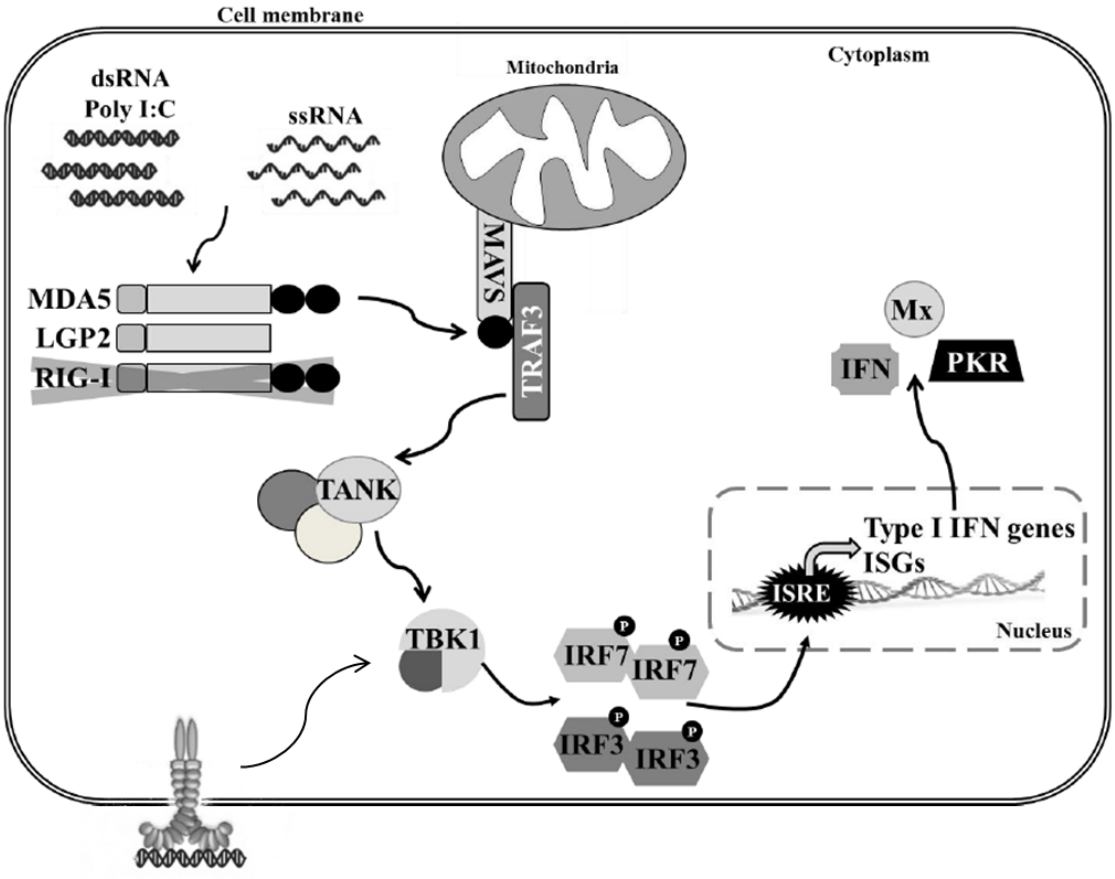


Figure 1: RLR-activation of the IFN response in gilthead seabream and European sea bass. RLR [retinoic-acid-inducible gene I (RIG-I)-like receptors), MDA5 (Melanoma Differentiation-Associated 5], LGP2 (Laboratory of Genetics and Physiology 2 protein), MAVS (Mitochondrial antiviral-signalling protein), TRAF3, [tumour necrosis factor (TNF) receptor-associated factor 3], TANK (TRAF family member-associated NF-kB activator), TBK1 (TANK-binding kinase 1), IRF3 or 7 [interferon (IFN) regulatory factor 3 or 7], Mx [myxovirus (influenza) resistance proteins], PKR (dsRNA-dependent protein kinase receptor), ISRE (IFN-stimulated response elements), ISG (IFN-stimulated genes). This figure contains the molecules found and analysed in this study and is inspired in the literature (Aoki, *et al.*, 2013; Hansen, *et al.*, 2011; Takeuchi and Akira, 2008; Verrier, *et al.*, 2011; Zhang, *et al.*, 2014).

3.2. 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 naïve gilthead seabream and European sea bass specimens and cell lines (Fig. 2). In gilthead seabream, all genes were similarly expressed in the brain and gonad whilst their transcription levels in the SAF-1 cells were much lower for *pkr*, *ifn* and *mx*. In European sea bass, all the genes were constitutively expressed with little variations between the tissues and usually lower in the DLB-1 cell line, derived from sea bass brain.

Table 2. Identification of the selected genes in the expressed sequence tags (ESTs) databases and European sea bass gill transcriptome and their relation with the zebrafish orthologues.

Predicted protein	Fish species	Gene accession number	Protein length	% protein homology[a]	E-value[b]
MDA5	Seabream	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/IPS-1	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	Seabream	HS988213	301	77	5e-154
	Sea bass	FM013306	220	95	3e-33
	Zebrafish	NP_001038213	727*		
IRF3	Seabream	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	Seabream	HS988732	306	52	3e-88
	Sea bass	FM008342	304	41	1e-41
	Zebrafish	CAM07151	682*		

Percentage of homology ([a]) and E-value ([b]) of the predicted proteins respect to the zebrafish ortholog. Asterisk denotes the sequences with predicted full length.

3.3. Most of the genes were up-regulated *in vitro* by pI:C and NNV infection

In the gilthead seabream SAF-1 cell line, *mda* and *irf3*, but not *tbk1* transcription levels were similarly induced by pI:C or NNV, except in the case of *ifn* transcription levels, which were unaffected by pI:C and greatly up-regulated by NNV infection (Fig. 3). However, whilst the *mx* gene expression was greatly induced, the *pkr* transcription was down-regulated by both *stimuli*. In a similar way, both pI:C and NNV induced most

of the genes related to the IFN-production pathway in the sea bass DLB-1 cell line though pI:C usually provoked a greater induction (Fig. 3). Interestingly, NNV failed to induce the RNA sensors *mda5* and *lgp2* transcription, although the downstream genes were significantly up-regulated. Moreover, in sea bass DLB-1 cell line, *tbk1* expression resulted unaltered with both, pI:C and NNV, whilst *pkr* was increased only with pI:C treatment.

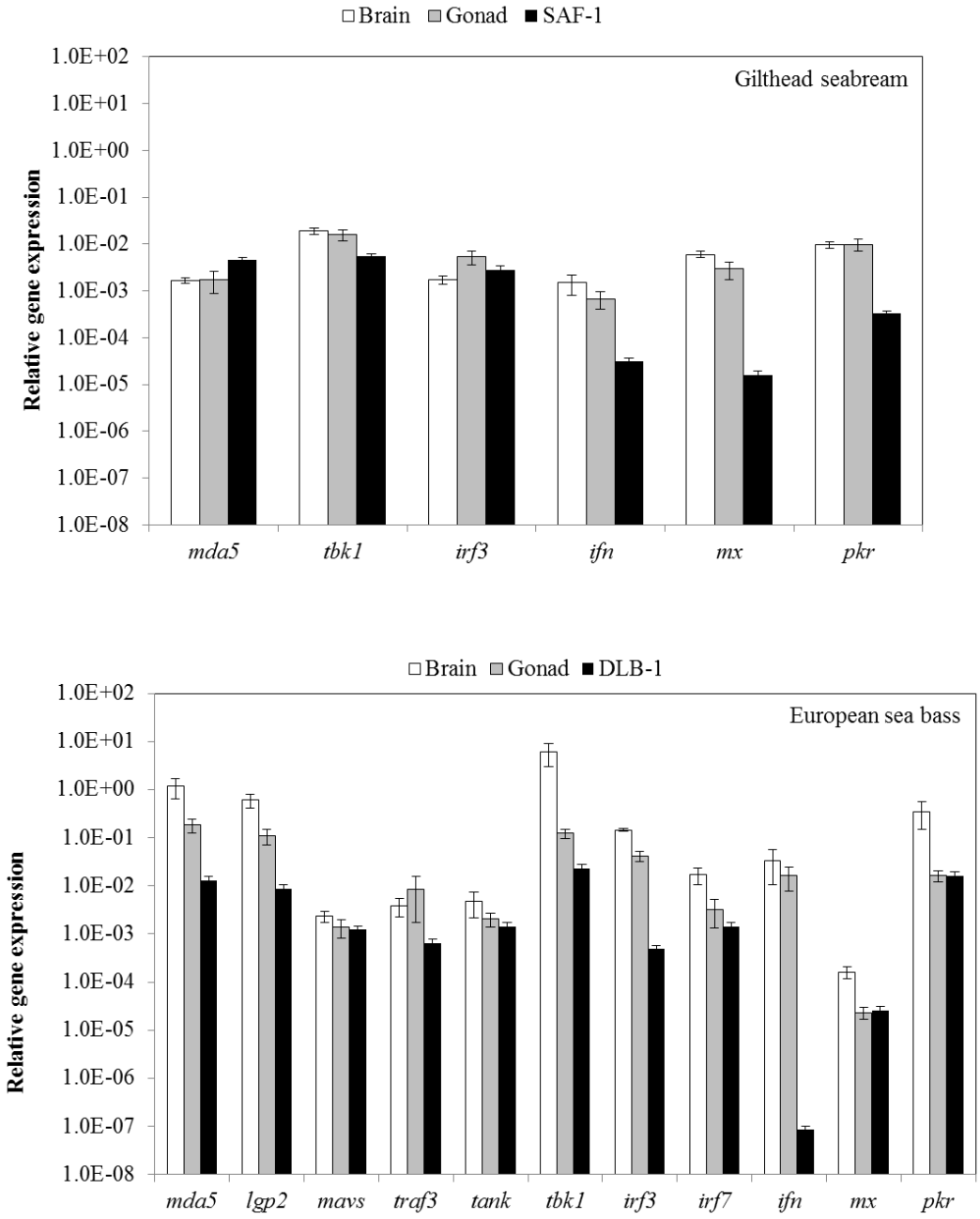


Figure 2: Expression of genes related to the IFN-induced response pathway in naïve gilthead seabream and European sea bass. The constitutive mRNA level of genes was studied by real-time PCR from naïve brain, gonad or cell lines. Data represent mean relative expression to the expression of endogenous control *ef1a* gene ± SEM of six specimen tissues or two cell cultures.

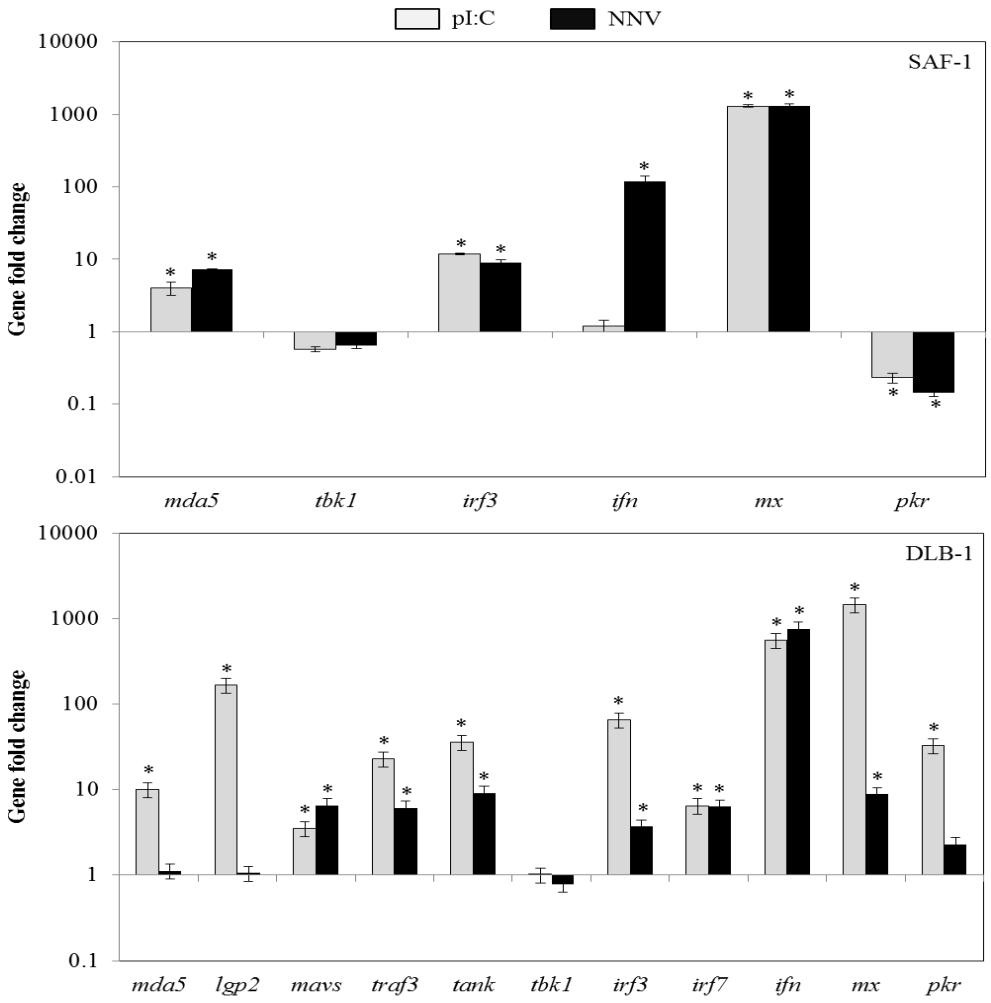


Figure 3: pI:C and NNV treatments up-regulate most of the IFN-production pathway genes (abbreviated as in Fig. 1) in SAF-1 and DLB-1 cell lines derived from gilthead seabream and European sea bass, respectively. Results are expressed as the mean ± SEM (two independent experiments) of mRNA fold increase respect to control samples. Significant differences (ANOVA, $P < 0.05$) with the controls are denoted by an asterisk.

3.4. Sensors of the viral dsRNA are up-regulated in the gonad of NNV-infected European sea bass

We evaluated the expression of the two identified RLRs, *mda5* and *lgp2*, which are the sensors for dsRNA, after NNV infection (Fig. 4). In seabream, *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 NNV infection in both tissues. Thus, in the brain, they were down-regulated after 1 and 7 days of infection to be up-regulated later on. In contrast, these genes were up-regulated in the gonad after 1 and 7 days of infection and unchanged afterwards.

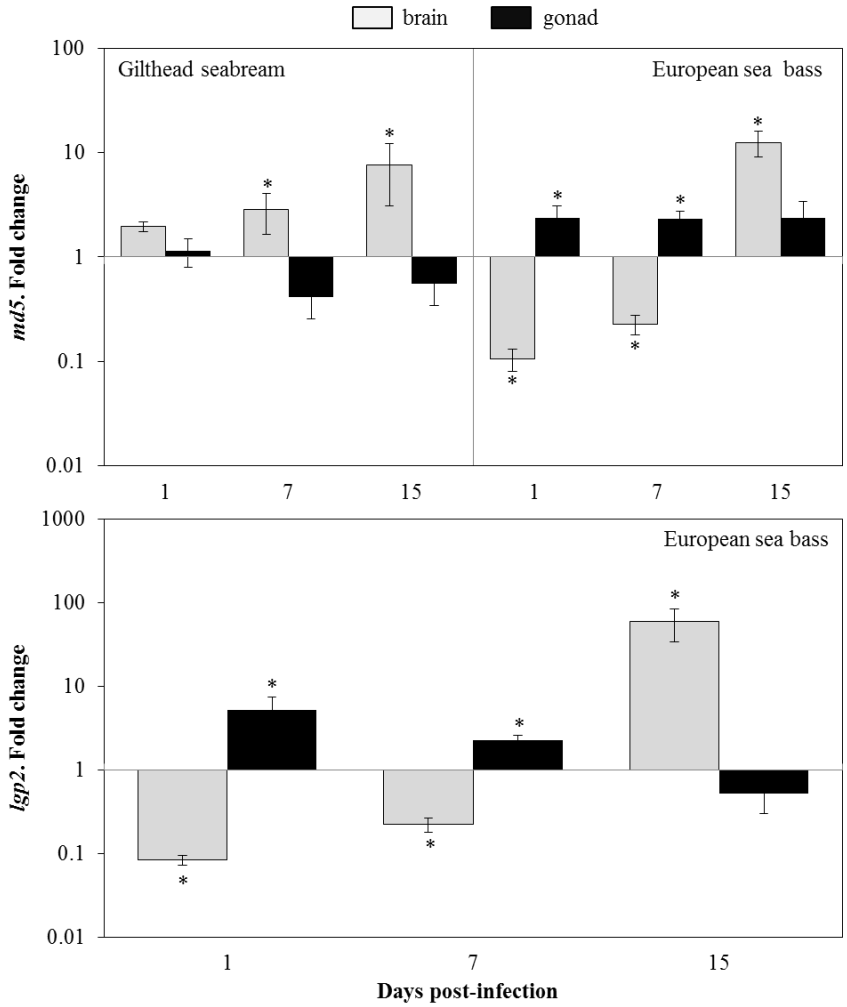


Figure 4: *In vivo* NNV infection modifies the expression of the sensors *mda5* and *lgp2* in the brain and/or gonad. Gene expression was studied by real-time PCR after 1, 7 and 15 days of infection (10^6 $TCID_{50}$ per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n = 4–6) of mRNA fold increase respect to control samples. Significant differences (ANOVA, $P < 0.05$) with the controls at each sampling time are denoted by an asterisk.

3.5. Adaptor and intermediaries are triggered by NNV infection in the gonad of European sea bass

In gilthead seabream, we only identified the *tbk1* and *irf3* intermediaries (Fig. 5). Transcription of *tbk1* was unaltered by NNV infection in any tissue whilst *irf3* gene expression was induced after 7 and 15 days of NNV infection in the brain and only after 1 day in the gonad. In European sea bass, the RLR adaptor, *mavs*, and most of the IFN-production pathway intermediary genes were identified. As occurred with the receptors, all the studied genes were down-regulated in the brain of sea bass infected with NNV except the *irf3* gene that was induced after 15 days of infection (Fig. 4). By contrast, in

the gonad, all of them (*mavs*, *traf3*, *tank*, *tbk1*, *irf3* and *irf7*) were up-regulated at different time points, mainly after 1 and 7 days of infection.

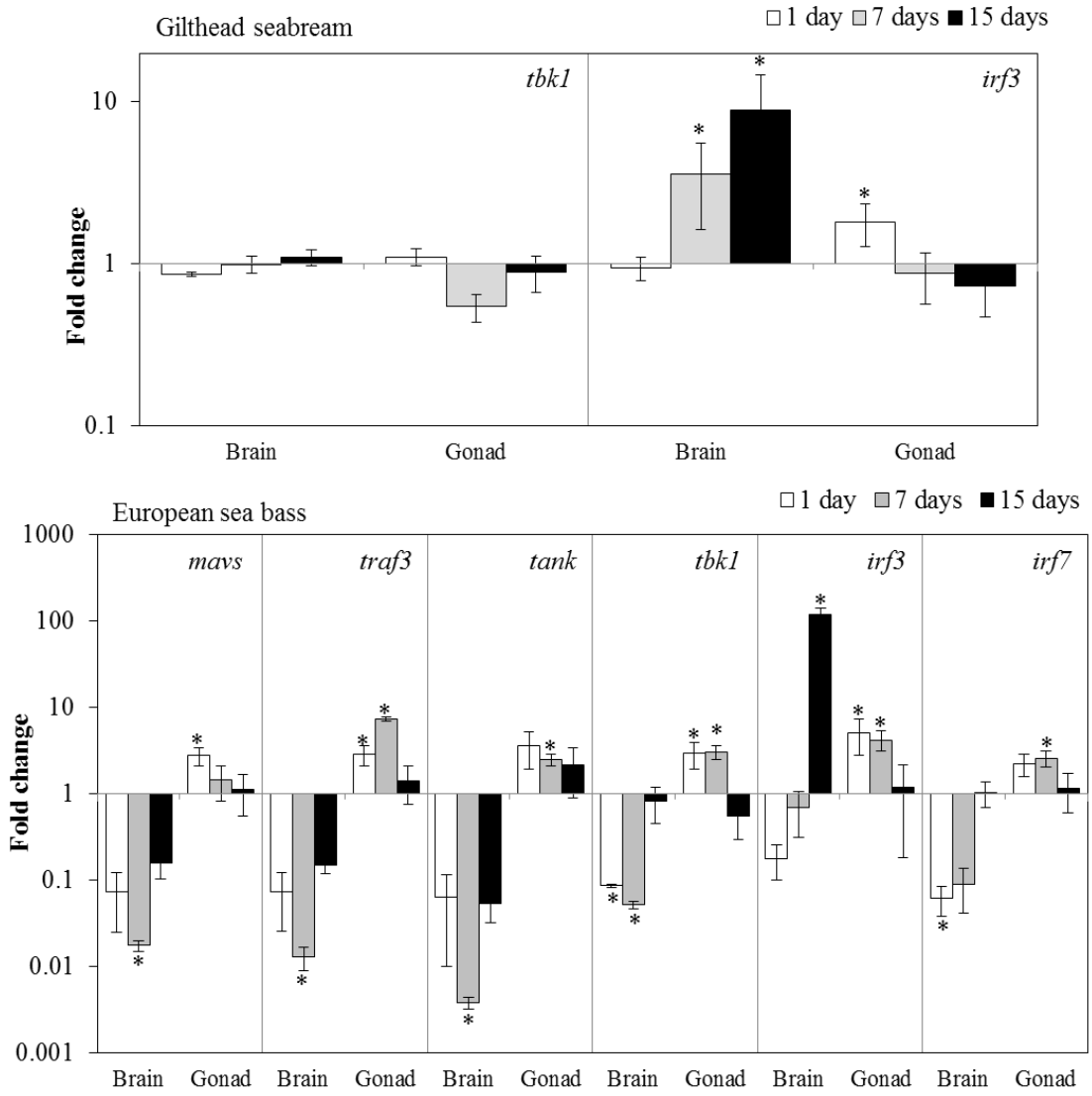


Figure 5: In vivo NNV infection modifies the expression of *tbk1* and *irf3* genes in gilthead seabream and *mavs*, *traf3*, *tank*, *tbk1*, *irf3* and *7* genes in European sea bass. Gene expression was studied by real-time PCR after 1, 7 and 15 days of infection (10^6 $TCID_{50}$ per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n = 4–6) of mRNA fold increase respect to control samples. Significant differences (ANOVA, $P < 0.05$) with the controls at each sampling time are denoted by an asterisk.

3.6. NNV greatly induced *ifn*, *mx* and *pkr* gene expression in the European sea bass gonad

Finally, the *ifn* gene was unaltered upon NNV infection in the gilthead seabream brain and reduced its expression in the European sea bass brain (Fig. 6). On the other

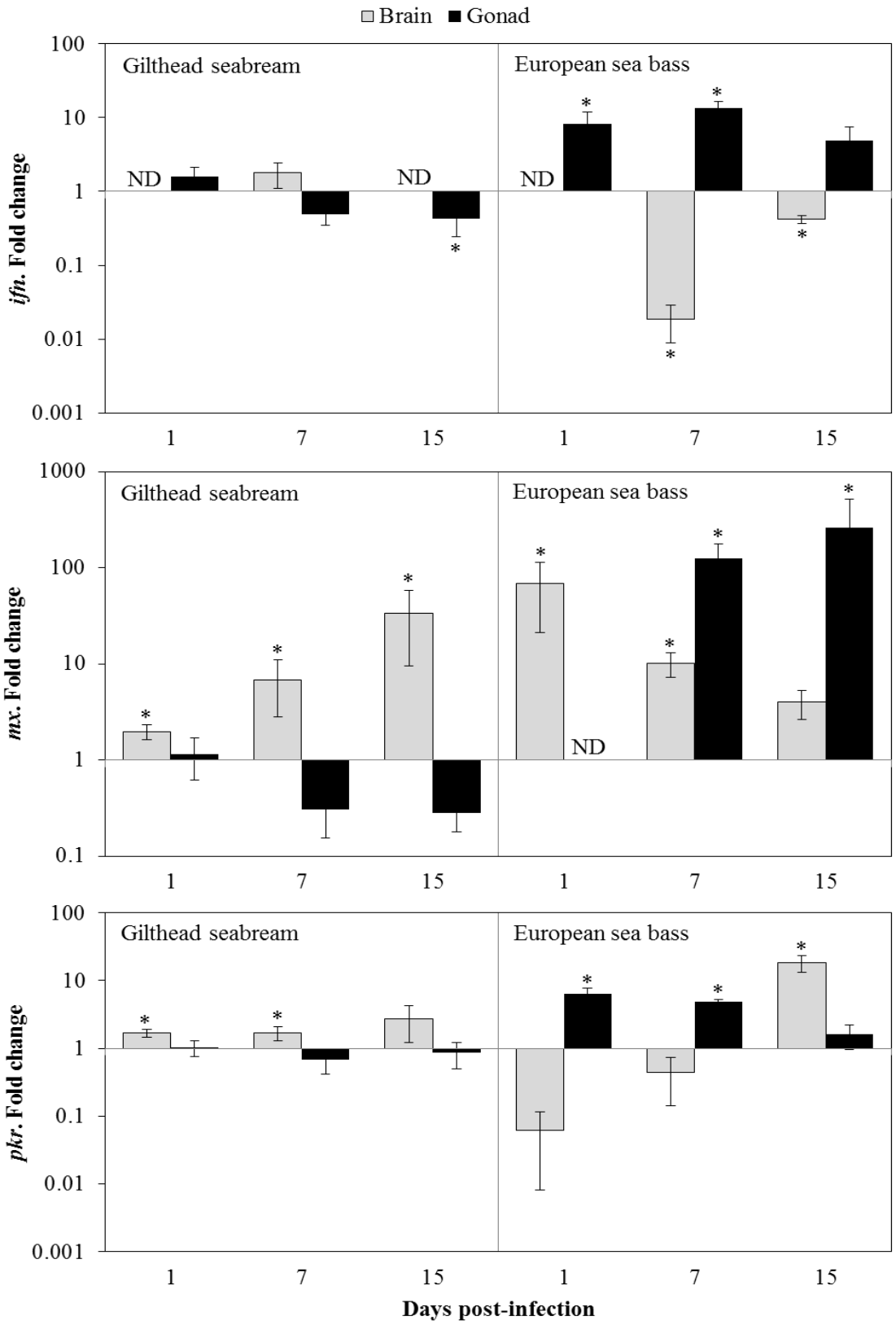


Figure 6: *ifn*, *mx* and *pkr* gene expressions are regulated upon NNV infection in gilthead seabream and European sea bass specimens. Gene expression was studied by real-time PCR after 1, 7 and 15 days of infection (10^6 $TCID_{50}$ per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n = 4–6) of mRNA fold increase respect to control samples. Significant differences (ANOVA, $P<0.05$) with the controls at each sampling time are denoted by an asterisk. ND, not detected.

in the gonad, the *ifn* transcription was decreased in seabream after 15 days of infection but induced in sea bass at days 1 and 7. After IFN production, we evaluated the transcription of two IFN-stimulated genes, which are responsible of the antiviral response, in our case *mx* and *pkr*. Thus, in seabream, both genes were up-regulated upon NNV infection in the brain, increasing their levels along the infection, but unaltered in the gonad (Fig. 6). By contrast, sea bass brain mRNA levels of *mx* were greatly increased after 1 day of infection and decreased thereafter at day 7 whilst the *pkr* was only induced after 15 days of infection (Fig. 6). In the gonad, however, *mx* was greatly induced after 7 and 15 days of infection but undetected at day 1. Nevertheless, *pkr* transcription was always induced being the highest levels reached at day 1 and decreasing thereafter.

4. Discussion

Gilthead seabream and European sea bass are the most important fish species in the Mediterranean aquaculture. So far, single *ifn* genes, belonging to the type I IFN, have been documented and partially characterized together to the IFN-induced *mx* gene (Casani, *et al.*, 2009; Fernández-Trujillo, *et al.*, 2011; Scapigliati, *et al.*, 2010). Focusing on NNV, it has been recognized that NNV infections induce a great 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 gilthead seabream whilst low activity is observed in those susceptible species such as European sea bass (Chaves-Pozo, *et al.*, 2012b; Chen, Y.M., *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 the type I IFN activation in fish induced by virus, and in particular by NNV (Dios, *et al.*, 2007; Rise, *et al.*, 2010). Moreover, none of these studies have looked at the gonad immune response on these species, an issue that it is highlighted taking into account that this tissue is used to vertically transmit NNV to the progeny (Arimoto, *et al.*, 1992; Kuo, *et al.*, 2012). Concretely, though we have failed to detect any viral gene expression by conventional and real-time PCR, we have already shown that NNV is able to replicate into the gonad of gilthead seabream and European sea bass by *in situ* PCR, immunohistochemistry and viral recovery using cell culture (Valero, *et al.*, 2015d). In addition, and most strikingly, the activity of antimicrobial peptides, and its transcription, was greatly up-regulated in the gonad of NNV-infected sea bass specimens, but failed to do so in the sea bass brain

and in the gonad of seabream specimens (Valero, *et al.*, 2015b). These data point to the importance of the gonad immunity in NNV establishment and dissemination, and prompted us to carry out this study.

We have searched ESTs databases of gilthead seabream and European sea bass as well as European sea bass gill transcriptome to search for RLR genes and mediators leading to IFN production. Firstly, we found some RNA sensors like *mda5* sequences in both fish species and *lgp2* in only sea bass, but failed to detect any *rig1* mRNA. In a similar way, *mda5* and *lgp2* genes have been identified in all teleost fish studied so far though the presence of *rig1* gene is limited to the ancient and never identified in the modern fish (class *Acanthopterygii*) (Aoki, *et al.*, 2013), in which our fish species are included. Our data showed that the expression levels of *mda5* was up-regulated in the SAF-1 cell line, which supports NNV replication (Bandín, *et al.*, 2006), in a similar way to the zebrafish ZF-4 cell line, which also supports NNV replication, in which *rig1*, *mda5* and *lgp2* transcription was up-regulated by NNV infection (Chen, H.Y., *et al.*, 2015). However, neither *mda5* or *lgp2* genes were altered in the newly obtained sea bass DLB-1 cells in contrast to what happens with pI:C stimulation. This could indicate that NNV is not able to replicate into sea bass DLB-1 cells, although this needs to be further confirmed. Moreover, up-regulation of the transcription of *mda5* and *lgp2* after NNV 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 seabream brain and in sea bass gonad indicating that these tissues would exert a high antiviral response. Similar up-regulations have been already documented in the brain of sea bass of Atlantic halibut exposed to NNV (Dios, *et al.*, 2007; Rise, *et al.*, 2010) and support our data. Moreover, these sensors are also up-regulated by several fish RNA virus or pI:C in several tissues of fish such as spleen, HK, 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 lead to the induction of the *ifn* expression and conferred an antiviral state (Biacchesi, *et al.*, 2009; Sun, F., *et al.*, 2011). Very recently, in addition, *rig1* knock-down in ZF-4 cells has demonstrated the importance of the group II of type I IFN pathway in NNV infections (Chen, H.Y., *et al.*, 2015). However, *lgp2* overexpression can produce both inducing and inhibitory effects on the *ifn* expression as

evidenced in fish and mammals (Komuro and Horvath, 2006; Ohtani, *et al.*, 2012; Sun, F., *et al.*, 2011), probably due to the lack of the CARD, which is only present in RIG-I and MDA5 proteins.

We also investigated the presence and regulation of genes between the RLRs and IFN. Thus, we looked for and found in the gilthead seabream ESTs databases sequences two intermediates 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, and also *pkr* in both species. Though most of them are only partial sequences the analysis of the predicted proteins resulted in *bona fide* orthologues to the expected proteins. Their expression in naïve conditions and upon NNV infection in brain and gonad correlated with the expression of *ifn* and two IFN-stimulated genes: *mx* and *pkr*. Regarding these genes, our results showed that NNV 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 down-regulated in the brain of sea bass specimens infected with NNV but up-regulated in the gonad. This fact would suggest a high IFN or antiviral response in the sea bass gonad and very low 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 up-regulation of most of these genes after virus infection in several tissues or their antiviral function after cell lines over-expression (Biacchesi, *et al.*, 2009; Chen, H.Y., *et al.*, 2015; Feng, *et al.*, 2011; Rise, *et al.*, 2008, 2010; Skjesol, *et al.*, 2011; Su, *et al.*, 2010; Sun, F., *et al.*, 2011; Xiang, *et al.*, 2011; Yang, *et al.*, 2011) and support the fact that the sequences identified in our study are mediating in the IFN activation cascade. In the case of *tbk1*, which is also activated by the TLR response, it is only up-regulated in sea bass specimens infected with NNV. However, fish *tbk1* has been shown to be activated by virus, pI:C, peptidoglycan and/or lipopolysaccharide indicating that this molecule can be activated by both viral and bacterial pathogens (Chi, *et al.*, 2007; Feng, *et al.*, 2011, 2014; Zhang, *et al.*, 2014). Moreover, some data point to 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). Now, our data showed that in the case of gilthead seabream which is able to clear the NNV infection (Chaves-Pozo, *et al.*,

2012b), *tbk1* expression is not up-regulated suggesting that this molecule is not essential to gilthead seabream anti-viral immune response.

Finally, this cascade leads to the activation of the IFN response. Our data showed that *ifn* transcription in gilthead seabream was not achieved though the down-stream activation of IFN-stimulated genes such as *mx* and *pkr* that were mainly observed in the brain of NNV-infected specimens. This could be explained by the different induction times, since *ifn* expression is usually very fast and last for short period, or to the presence of different *ifn* forms and splicing variants, which is unknown so far and deserves further work. By contrast, in the European sea bass, inhibition of the brain expression of *ifn* gene, as most of those genes involved in the induction cascade, was concomitant with an increase in the transcription of *mx* and *pkr*. All this data pointed to the existence of other activation pathways in fish as previously suggested (Feng, *et al.*, 2014) and demonstrated in ZF-4 cells in which the involvement of the TLR activation pathway is evidenced after NNV infection (Chen, H.Y., *et al.*, 2015). In addition, *pkr* is designed as an IFN-stimulated gene 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 NNV showed an up-regulated *pkr* expression (Chen, H.Y., *et al.*, 2015). Interestingly, in the gonad of NNV-infected sea bass specimens, *ifn*, *mx* and *pkr* genes were also up-regulated as 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.*, 2007; Feng, *et al.*, 2011, 2014), but never included the fish gonad. This is important since it is known that gonad immunity is tissue-specifically regulated in fish (Chaves-Pozo, *et al.*, 2010b) and used by pathogens for its dissemination (Arimoto, *et al.*, 1992; Kuo, *et al.*, 2012). The up-regulation of the antiviral response in the gonad of European sea bass specimens surviving to the NNV infection could be a mechanism in which fight the pathogen is more important than maintain the functionality of the gonad for reproductive purposes. However, in the gilthead seabream, specimens which overcome the infection, the tight regulation of the gonadal 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 pattern in the brain and gonad of NNV-infected

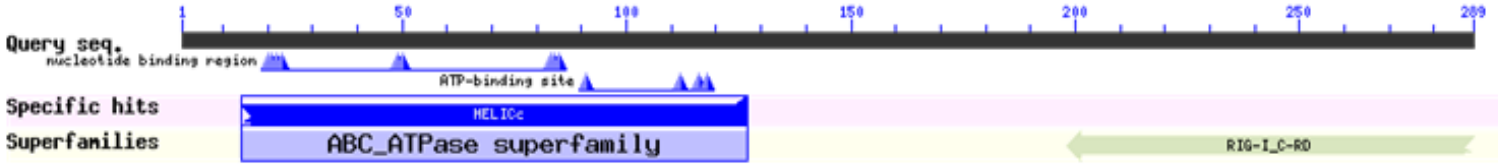
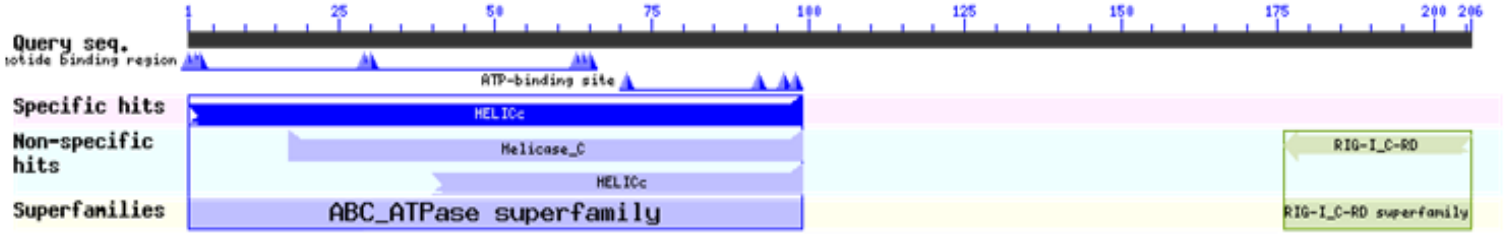
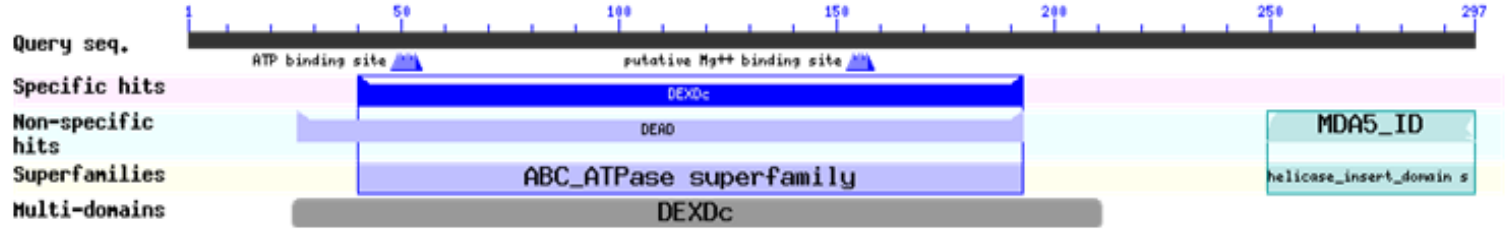
sea bass are similar (Valero, *et al.*, 2015b). However, the antiviral immune response in the reproductive organs deserved further investigation since in immature rainbow trout (*Oncorhynchus mykiss*) females, VHSV infection provoked an up-regulation of the type I IFN genes (*ifn1*, *ifn*2, *ifn*3/4, *mx1*, *mx2* and *mx3*) in the ovary (Chaves-Pozo, *et al.*, 2010b). In addition, recombinant IFN1 and IFN2 were able to induce the expression of *mx* genes and confer antiviral activity against VHSV *in vitro*, being the *mx3* which showed the highest up-regulation (Chaves-Pozo, *et al.*, 2010b). This points to the importance of the gonad IFN response to control the dissemination of viral pathogens in fish, an aspect that has been clearly unconsidered in the past.

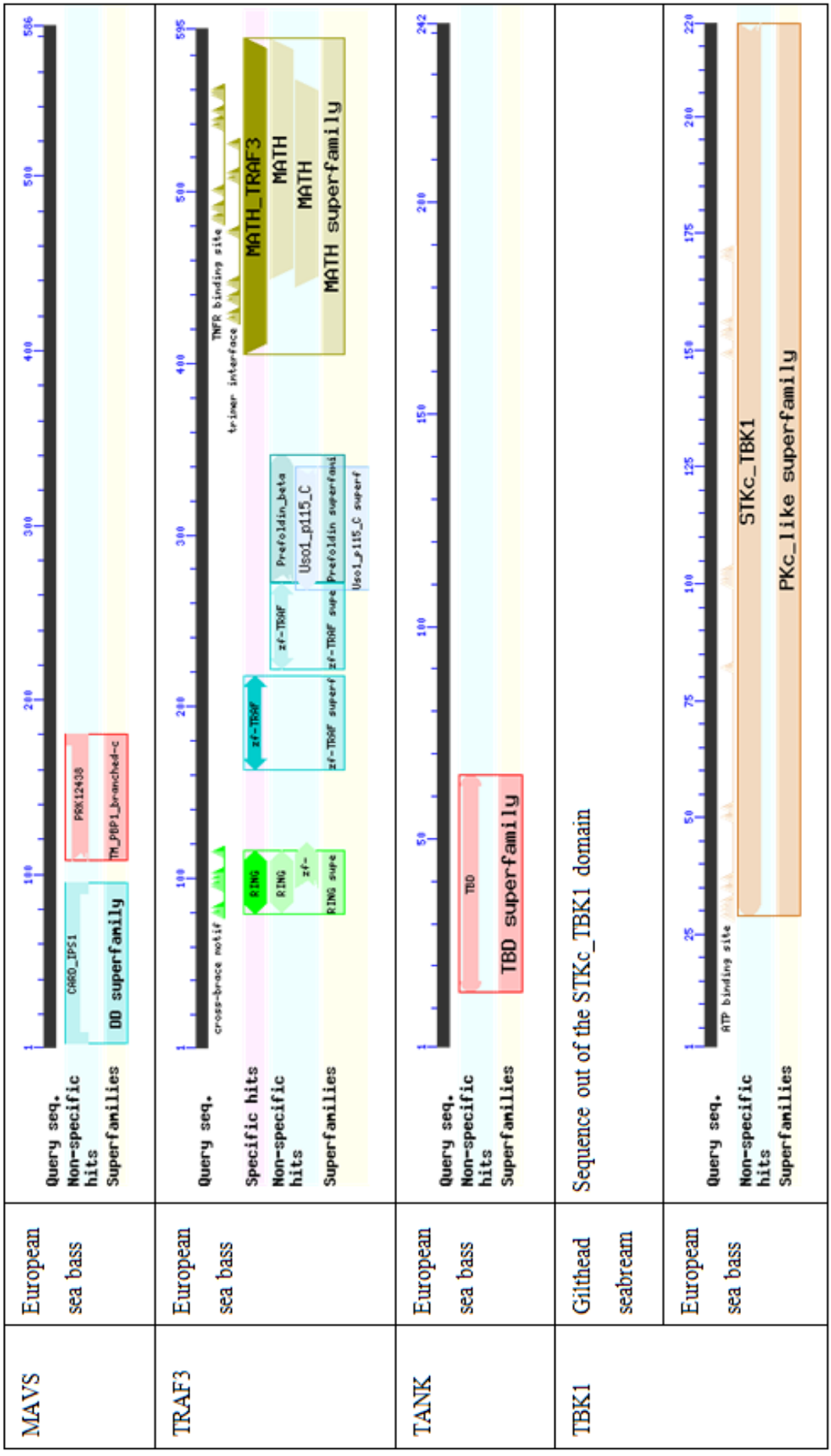
5. Conclusions

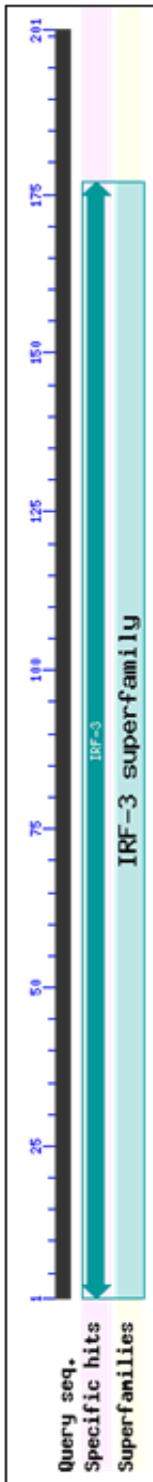
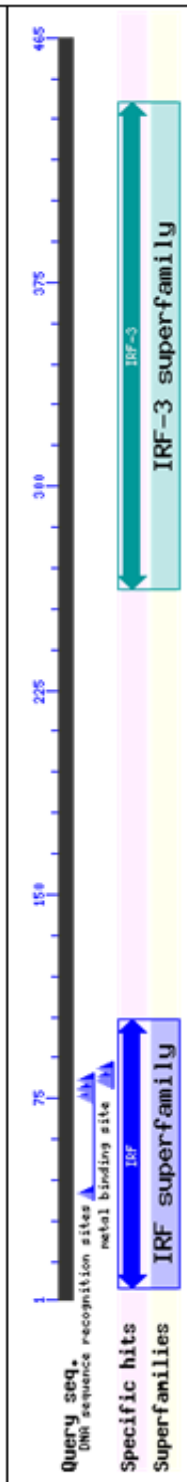
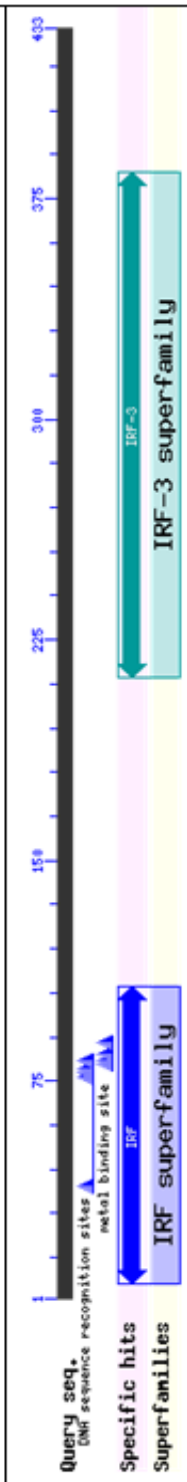
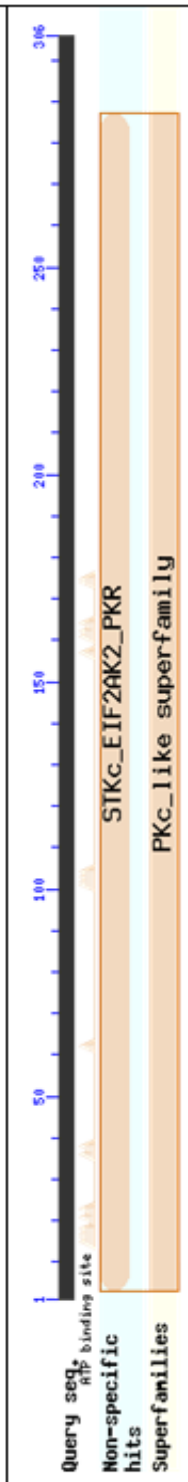
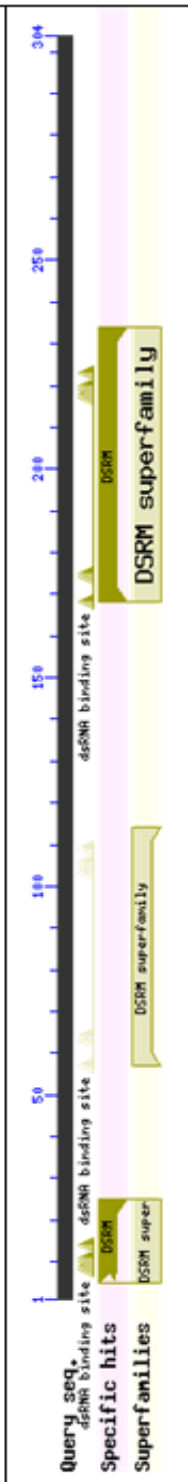
In conclusion, this study represents one of the most complete characterizations of the genes leading to the IFN response after viral infection by RLRs in fish. Thus, we have identified several molecules of gilthead seabream and European sea bass involved in the activation cascade of the interferon including viral RNA receptors (*mda5* and *lgp2*), the RLR adaptor (*mavs*) and intermediaries (*traf3*, *tank*, *tbk1*, *irf3* and *irf7*) for the first time. We also reported their simultaneous regulation upon NNV infection. Thus, in seabream, we found that *mda5*, *irf3*, *mx* and *pkr* genes were up-regulated in the brain but not in the gonad. However, in the susceptible European sea bass, the expression of most of the genes were down-regulated in the brain but significantly up-regulated in the gonad what resulted in an enhanced transcription of *ifn*, *mx* and *pkr* genes in this tissue. This is the first time since a study covered a wide view of 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.

6. Supplementary data

Table S1. Identification of the predicted protein domains by the GenBank databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Predicted protein	Fish species	Domains and motifs identified in the predicted protein sequences
MDA5	Gilthead seabream	
	European sea bass	
LGP2	European sea bass	

MAVS	European sea bass	
TRAF3	European sea bass	
TANK	European sea bass	
TBK1	Gilthead seabream	Sequence out of the STKc_TBK1 domain
	European sea bass	

IRF3	Gilthead seabream	
	European sea bass	
IRF7	European sea bass	
PKR	Gilthead seabream	
	European sea bass	

III.1.3. ANTIMICROBIAL RESPONSE IS INCREASED IN THE TESTIS OF EUROPEAN SEA BASS, BUT NOT IN GILTHEAD SEABREAM, UPON NNV INFECTION

1. Introduction

The infection of the gonad by pathogens is the initial step to promote horizontal transmission through gonadal fluids and/or vertical transmission through infected gametes (Inoue and Ushida, 2003; Sinyakov, *et al.*, 2011). In all vertebrates, the gonad is considered an immunologically-privileged site, as also occurs with the brain and retina. In those tissues, the immune response proceeds in a different manner in order to avoid cell damage (Chaves-Pozo, *et al.*, 2005c; Hedger, 2002), which is used by some pathogens to be hidden and scape to the immunological control. In fish, the implications of this different regulation of the immune functions inside the reproductive organs and its implication on pathogen dissemination through the gonad have very recently been documented (Chaves-Pozo, *et al.*, 2010a,b; Deane, *et al.*, 2001). However, this immune response in the gonad from infected fish deserves deeper characterization as a mean to control this route of pathogens dissemination. In that sense, antimicrobial peptides (AMPs) are increasingly recognized as a critical first line of defence against many pathogens and have been extensively studied in invertebrates and vertebrate species, including fish (Brogden, *et al.*, 2003). Their specific characteristics of low molecular weight, polarity or amino acid composition confer them a broad-spectrum of antimicrobial activities against bacteria, virus, fungi, protozoa, and even tumour cells (Chia, *et al.*, 2010; Cuesta, *et al.*, 2011; Mihajlovic and Lazaridis, 2010). The AMPs expressed in the mammalian gonad are considered to assume an important part of a highly effective immune response against pathogens since the production of pro-inflammatory factors is strictly restrictive in this tissue in order to avoid germ cell damage (Gerdprasert, *et al.*, 2002; MacKenzie, *et al.*, 2006) as also described in gilthead seabream gonad (Chaves-Pozo, *et al.*, 2005c). In teleost fish, more than 60 AMPs have been described and determined its expression in several tissues, including gonad (Valero, *et al.*, 2013). Unfortunately, to our knowledge, nothing is known about their regulation and immunological role in the fish gonad despite the immune peculiarities of this organ and the important roles attributed to AMPs.

Nodavirus (NNV), a bipartite and positive single-stranded RNA virus, is a known vertical and horizontal transmitted pathogen (Arimoto, *et al.*, 1992; Breuil, *et al.*, 2001, 2002; Frerichs, *et al.*, 1996; Korsnes, *et al.*, 2012; Kuo, *et al.*, 2012) able to infect more than 50 marine fish species, some of them especially sensitive, as the European sea bass (*Dicentrarchus labrax*), and others only susceptible to some strains of NNV, as

occurs with gilthead seabream (*Sparus aurata*) (Castric, *et al.*, 2001; Frerichs, *et al.*, 1996). Interestingly, though the main target tissues of NNV are the brain and the retina (Castric, *et al.*, 2001; Frerichs, *et al.*, 1996), both immune-privileged tissues, as 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 it into the gonad (Valero, *et al.*, 2015d). Previous studies have evaluated the role of several immune responses in the head-kidney (HK) or brain after NNV infections such as the gene expression of interferon, pro-inflammatory cytokines, chemokines or leucocyte markers as well as leucocyte functions such as proliferation, respiratory burst or cell-mediated cytotoxic activity but never the role of AMPs (Chaves-Pozo, *et al.*, 2012b; Cuesta, *et al.*, 2010; López-Muñoz, *et al.*, 2012; Poisa-Beiro, *et al.*, 2008; Scapigliati, *et al.*, 2010). Regarding the AMPs, it is unknown if they are triggered upon nodavirus infection but it has been well demonstrated that some isolated AMPs showed anti-NNV activity *in vitro* (Chia, *et al.*, 2010). However, no other study has evaluated the role of AMPs into the gonad from fish infected with nodavirus, nor any other immune response.

Therefore, with the knowledge that NNV uses the fish gonad to be transmitted and that it is detected and isolated form European sea bass and gilthead seabream gonads (Valero, *et al.*, 2015d), we aimed in the present study to assess the potential role of AMPs in the innate immune response triggered by NNV in the gonad. Thus, we have evaluated the antimicrobial activities (complement, lysozyme and bactericidal activities) in serum and gonad extracts, as well as the expression profiles of several AMP coding genes (*c3*, *lyz*, *hamp*, *dic*, *pis* or *bdef*) in brain and gonad, upon *in vivo* infection, in two fish species with different susceptibility to NNV, the European sea bass and the gilthead seabream. The local immune response triggered in the gonad by NNV without the systemic influence by means of an *in vitro* challenge of the gonad with NNV and pI:C have also been determined. Moreover, the capability of NNV to infect brain and gonad causing different effect on tissue functionality prompted us to elucidate the possible correlations between the AMPs gene expression levels found in the brain and the gonad.

2. Material and methods

2.1 Animals

Healthy specimens of European sea bass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.) were bred and kept at the *Centro Oceanográfico de*

Murcia, Instituto Español de Oceanografía (COM-IEO, Mazarrón, Murcia). The fish were kept in 14 m^3 tanks with the water temperature ranging from 14.6 to 17.8ºC, a flow-through circuit, a suitable aeration and filtration system, natural photoperiod and fed daily with 1% of biomass of a commercial pellet diet (Skretting). Before sampling, all specimens were anesthetized with 40 µl/l of clove oil, bled and immediately decapitated and weighed. All animal studies were carried out in accordance with the European Union regulations for animal experimentation and the Bioethical Committee of the *Instituto Español de Oceanografía* and of the University of Murcia.

2.2. Nodavirus stocks

Nodavirus (NNV) (strain 411/96, genotype RGNNV) were propagated in the SSN-1 cell line (Frerichs, *et al.*, 1996). The SSN-1 cells were grown at 25ºC in Leibovitz's L15-medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 i.u./ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco) and 50 mg/ml gentamicin (Gibco) using Falcon Primaria cell culture flasks (Becton Dickinson). Cells were inoculated with NNV and incubated at 25ºC until the cytopathic effect was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates as previously described (Reed and Müench, 1938).

2.3. *In vivo* infection

Specimens of European sea bass [n = 50; 125 ± 25 g body weight (bw)] and gilthead seabream (n = 50; 305 ± 77 g bw) were translated to the University of Murcia aquaria. Fish were randomly divided into two tanks, kept in 450-500 L running seawater (28‰ salinity) aquaria at 22-26ºC and with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the experiments. Each group received a single intramuscular injection of 100 ml of SSN-1 culture medium (mock-infected) or culture medium containing 10^6 $TCID_{50}$/fish of NNV since this route of infection has been proven as the most effective (Aranguren, *et al.*, 2002). Fish (n = 6 fish/group and sampling time) were sampled 1, 7, or 15 days after viral infection and blood serum, gonad and brain were removed. The blood was obtained from the caudal peduncle and the serum samples were obtained by centrifugation at 10,000 g during 1 min at 4ºC, and immediately frozen and stored at -80ºC until used. Fragments of gonad and brain were immediately frozen in TRIzol® Reagent (Invitrogen) and stored at -80ºC for later RNA

isolation. Fragments of gonads were also immediately frozen in liquid nitrogen and stored at -80ºC for later analysis of antimicrobial activities.

2.4. *In vitro* treatments

Specimens of naïve European sea bass males (n = 6) or gilthead seabream males (n = 6) were bled and the gonad removed, weighed and chopped into 1 mm^2 fragments to culture them in flat-bottomed 96-well microtiter plates (Nunc) with sL-15 [Leibovitz's L15-medium supplemented with 2 mM glutamine, 100 u.i./ml penicillin, 100 mg/ml streptomycin, 2 mg/ml fungizone (Invitrogen), 2% FBS and 0.35% of NaCl] culture medium (control) or containing NNV (10^7 $TCID_{50}$/ml) or pI:C (62.5 mg/ml; Sigma) during 24 h at 25ºC. Afterwards, fragments of tissue were washed with 0.01 M PBS and stored in TRIzol® Reagent at -80ºC for later isolation of RNA.

2.5. Analysis of gene expression by real-time PCR

Total RNA was isolated from TRIzol® Reagent frozen samples following the manufacturer's instructions. One mg of total RNA was treated with DNAse I (1 unit/mg RNA, Promega) to remove genomic DNA. The first strand of cDNA was synthesized by reverse transcription using the Superscript III (Invitrogen) with an oligodT12-18 primer (Promega) followed by RNAse H (Invitrogen) treatment, at 50ºC for 60 min. The expression of the genes codifying for the interferon-induced GTP-binding protein Mx (*mx*), complement component 3 (*c3 1-2*), lysozyme (*lyz*), hepcidin (*hamp*), dicentracin (*dic*), piscidin (*pis*) or beta-defensin (*bdef*) was analysed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) as previously described (Chaves-Pozo, *et al.*, 2012b). Reaction mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1 alpha coding gene (*ef1a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. The specific primers used were designed using the Oligo Perfect software tool (Invitrogen) and are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for specificity. Negative controls with no template were always included in the reactions.

Table 1: Gene accession numbers and primer sequences used for gene expression analysis.

Specie	Name	Abbreviation	Accession number	Sequence (5'-3')
European sea bass	Interferon-induced GTP-binding protein Mx	*mx*	AM228977, HQ237501, AY424961	GAAGAAGGGCTACATGATCGTC CCGTCATTGTAGAGAGTGTGGA
	complement component 3-1 and 3-2	*c3 1-2*	HM563079, HM563078	ACCAAAGAACTGGCAACCAC CTAGCAGTCGGTCAGGGAAC
	lysozyme	*lyz*	FN667957	ATTTCCTGGCTGGAACACAG GAGCTCTGGCAACAACATCA
	hepcidin	*hamp*	DQ131605	CCAGTCACTGAGGTGCAAGA GCTGTGACGCTTGTGTCTGT
	dicentracin	*dic*	AY303940	GGCAAGTCCATCCACAAACT ATATTGCTCCGCTTGCTGAT
	elongation factor 1 alpha	*ef1a*	FM019753	CGTTGGCTTCAACATCAAGA GAAGTTGTCTGCTCCCTTGG
Gilthead seabream	Interferon-induced GTP-binding protein Mx	*mx*	FJ490556, FJ490555, FJ652200	AAGAGGAGGACGAGGAGGAG CATCCCAGATCCTGGTCAGT
	complement component 3	*c3*	CX734936	ATAGACAAAGCGGTGGCCTA GTGGGACCTCTCTGTGGAAA
	lysozyme	*lyz*	AM749959	CCAGGGCTGGAAATCAACTA CCAACATCAACACCTGCAAC
	hepcidin	*hamp*	CB184616	GCCATCGTGCTCACCTTTAT CTGTTGCCATACCCCATCTT
	beta-defensin	*bdef*	FM158209	CCCCAGTCTGAGTGGAGTGT AATGAGACACGCAGCACAAG
	piscidin	*pis*	FM158699	CCTTGTGTTGTCCATGGTTG ACTGCTCCAGCTGCAAGTCT
	elongation factor 1 alpha	*ef1a*	AF184170	CTGTCAAGGAAATCCGTCGT TGACCTGAGCGTTGAAGTTG

2.6. Antimicrobial activities

Antimicrobial activities were determined in serum and homogenated gonad samples. Fragments of gonad were weighed and mechanically homogenized in 1 ml of 0.01 M PBS (9 mM sodium phosphate dibasic, 2 mM, sodium phosphate monobasic and 0.15M NaCl), and centrifuged at 10,000 g during 10 min at 4ºC to avoid cell debris. The supernatants of homogenated gonads, as well as the serum, were used for natural haemolytic complement, lysozyme and bactericidal activity assays.

2.6.1. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets (Ortuño, *et al.*, 1998). Equal volumes of

SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} (Panreac) and EGTA (Sigma) were mixed with serially diluted serum or gonad homogenates (5.2 ± 0.2 or 4.9 ± 0.01 mg of protein/ml of sea bass or gilthead seabream, respectively) to give final serum concentrations ranging from 10% to 0.078% or gonad homogenates ranging from 0.5 to 0.004 mg of protein/ml. After incubation for 90 min at 22ºC, the samples were centrifuged at 400 g during 5 min at 4ºC to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader (Thermo Fisher Multickan GO). The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 ml of distilled water or HBSS to 100 ml samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen that was obtained by plotting Y/(1-Y) against the volume of serum or gonad homogenates added (ml) on a log-log scaled graph. The volume of serum or gonad homogenates producing 50% haemolysis (ACH_{50}) was determined and the results were represented as ACH_{50} units/ml of serum or ACH_{50} units/g of gonad. Results were expressed as fold change of the infected group compared with the control group.

2.6.2. Lysozyme activity

The lysozyme activity of serum or gonad homogenates was measured according to a turbidimetric method modified from a protocol previously described (Parry, *et al.*, 1965). Briefly, 100 ml of serum or gonad homogenates diluted 1:2 with 0.01 M PBS at pH 6.2, were placed in flat-bottomed 96-well plates in triplicate. To each well, 100 ml of 0.3 mg/ml freeze-dried *Micrococcus lysodeikticus* (Sigma) in phosphate citrate buffer (0.13 M disodium phosphate, 0.11 M citrate and 0.015 M NaCl, pH 6.2) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured immediately every 30 s during 15 min at 22ºC in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. The units of lysozyme present in serum and gonads homogenates were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) and the results were calculated as units/ml of serum or units/mg of gonad. Results were expressed as fold change of the infected group compared with the control group.

2.6.3. Bactericidal activity

The pathogenic marine bacteria *Vibrio harveyi* (*Vh*) (strain Lg 16/ 100) was grown in agar plates at 25ºC in tryptic soy agar (TSA, Sigma). Then, fresh single colonies of 1-2 mm were diluted in 5 ml of tryptic soy broth (TSB; Laboratorios Conda), cultured for 16 h at 25ºC on an orbital incubator at 200-250 revolutions per minute (rpm) and adjusted to 10^8 bacteria/ml TSB. The absorbance of bacteria cell cultures were measured at 600 nm and used to know the concentration based on growth curves.

The antibacterial activity of serum or gonad homogenates was determined by evaluating their effects on the bacterial growth of *Vh* curves using a method modified previously (Sunyer and Tort, 1995). Aliquots of 100 ml of the bacterial dilutions of *Vh* (1/10) were placed in flat-bottomed 96-well plates and cultured with 100 ml of European sea bass or gilthead seabream serum or gonad homogenates dilutions (1/10). The absorbance of the samples was measured at 620 nm every 30 min intervals during 36 h at 25ºC. Samples without bacteria were used as blanks (negative control). Samples without serum or gonad homogenates were used as positive controls (100% growth or 0% antibacterial activity). Bactericidal activity was calculated as % of bacterial growth inhibition per ml of serum or mg of gonad. Results were expressed as fold change of the infected group compared with the control group.

2.7. Statistical analysis

The data were analysed by a t-Student to determine differences between control and infected groups at each time point and specie (*P < 0.1; **P < 0.05; ***P < 0.01). In addition, Pearson correlation tests were applied to test correlations among antibacterial activities and gene expression levels in the gonad or among the gene expression levels in gonad or brain after *in vivo* infections with NNV using Statgraphics 15.0 (StatPoint, Inc).

The genetic nomenclature used in this manuscript follows the guidelines of Zebrafish Nomenclature Committee (ZNC) for fish genes and proteins and the HUGO Gene Nomenclature committee for mammalian genes and proteins.

3. Results

3.1. NNV infection induces the antimicrobial response in serum and gonad

The natural haemolytic complement, lysozyme and bactericidal activity in serum and gonad homogenates of European sea bass and gilthead seabream upon an *in vivo* infection with NNV were analysed (Fig. 1).

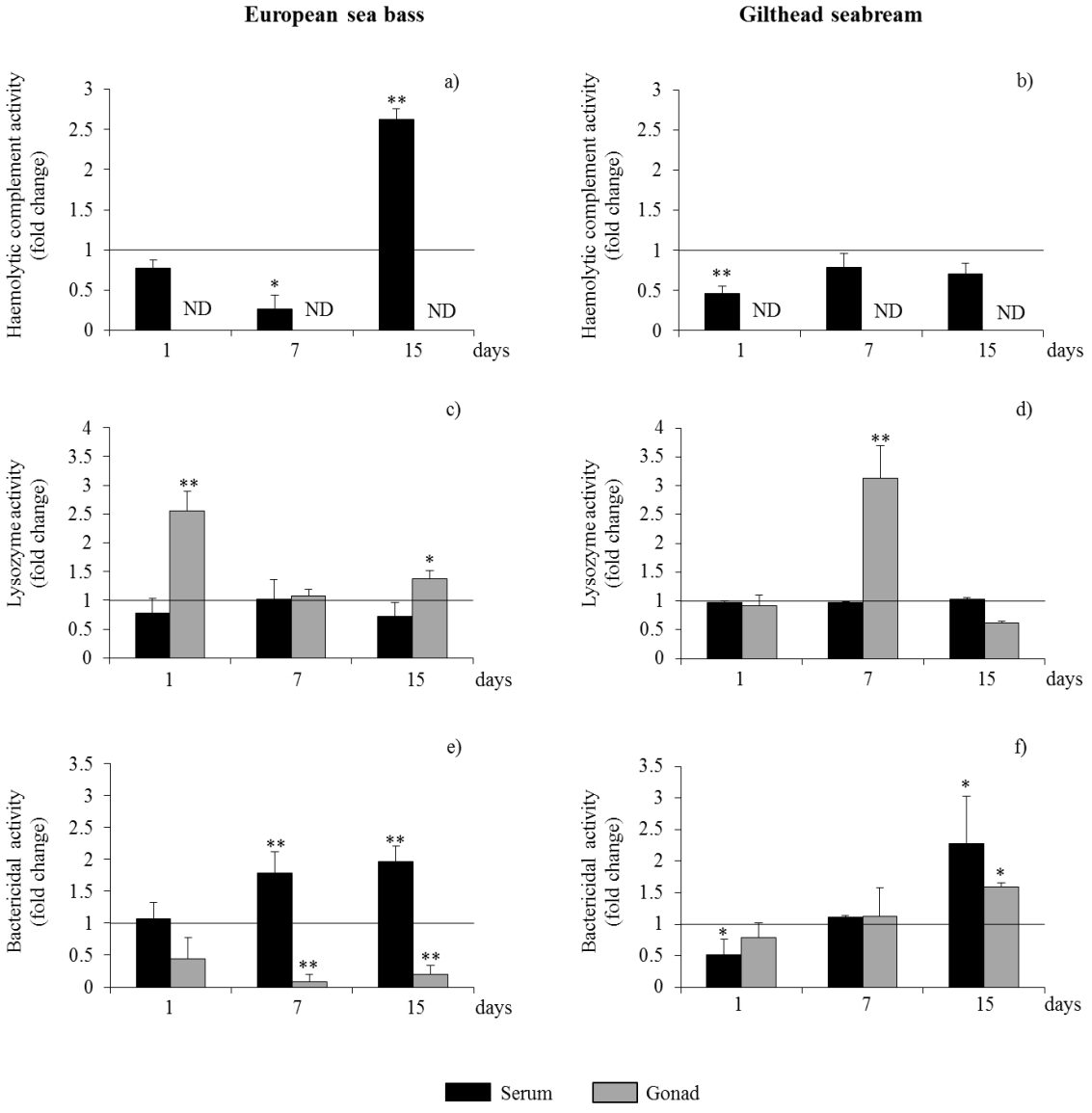


Figure 1: European sea bass and gilthead seabream antimicrobial activities in serum (black bars) and gonad homogenates (grey bars) upon *in vivo* NNV infection. Haemolytic complement activity (a, b), lysozyme activity (c, d) and bactericidal activity (e, f). Data represent the mean ± standard error of the activity of NNV-infected group respect to the control group (n = 6/group and time). Significance level (P) was fixed at 0.1 (P < 0.1*; P < 0.05**; P < 0.01***). ND, not detected.

Haemolytic activity in the gonad of any specie was non-detectable for any group and at any assayed time. In serum, the haemolytic activity was inhibited at the beginning of the infection in both species (7 or 1 day post-infection in European sea bass or gilthead seabream, respectively), while only in the European sea bass the serum haemolytic activity increased and reached 2.6-fold after 15 days of infection (Fig. 1a,b). In contrast, lysozyme activity was unchanged in serum, but greatly increased in the gonad homogenates of both species upon NNV infection (Fig. 1c,d). Thus, this activity increased 2.5- and 1.4-fold after 1 and 15 days of infection, respectively, in European sea bass gonad (Fig. 1c), and 3.1- fold increase after 7 days of infection in gilthead seabream gonad (Fig. 1d). Regarding the bactericidal activity, some differences were observed between species and tissues analysed (Fig. 1e,f). Thus, in the European sea bass, the bactericidal activity increased in serum, while decreased in the gonad after 7 and 15 days of infection (Fig. 1e). However, in the gilthead seabream, the bactericidal activity of serum decreased after 1 day and increased after 15 days of infection coinciding with an increase of the bactericidal activity in the gonad (Fig. 1f).

3.2. NNV *in vivo* infection up-regulates the AMPs gene expression in sea bass gonad

Firstly, we evaluated the expression of *mx* gene upon *in vivo* challenge with NNV as indicator of the antiviral response (Figs. 2a,b and 3a,b). The results showed a lower *mx* gene induction in the brain of European sea bass (Fig. 2b) than in the gilthead seabream (Fig. 3b), as previously documented (Chaves-Pozo, *et al.*, 2012b; Fernández-Trujillo, *et al.*, 2008; Poisa-Beiro, *et al.*, 2008). Surprisingly, the transcription of *mx* gene in the gonad was up-regulated at all time points in European sea bass (Fig. 2a), while kept unaltered in gilthead seabream (Fig. 3a). Afterwards, we have studied the expression profiles in the brain and in the gonad of some known AMP genes upon *in vivo* challenge with NNV and found a different pattern of expression between the both analysed species (Figs. 2 and 3). Thus, in the European sea bass (Fig. 2), the transcription of all AMPs analysed increased in at least one tissue, gonad or brain, while in the gilthead seabream (Fig. 3), those genes were down-regulated or kept steady.

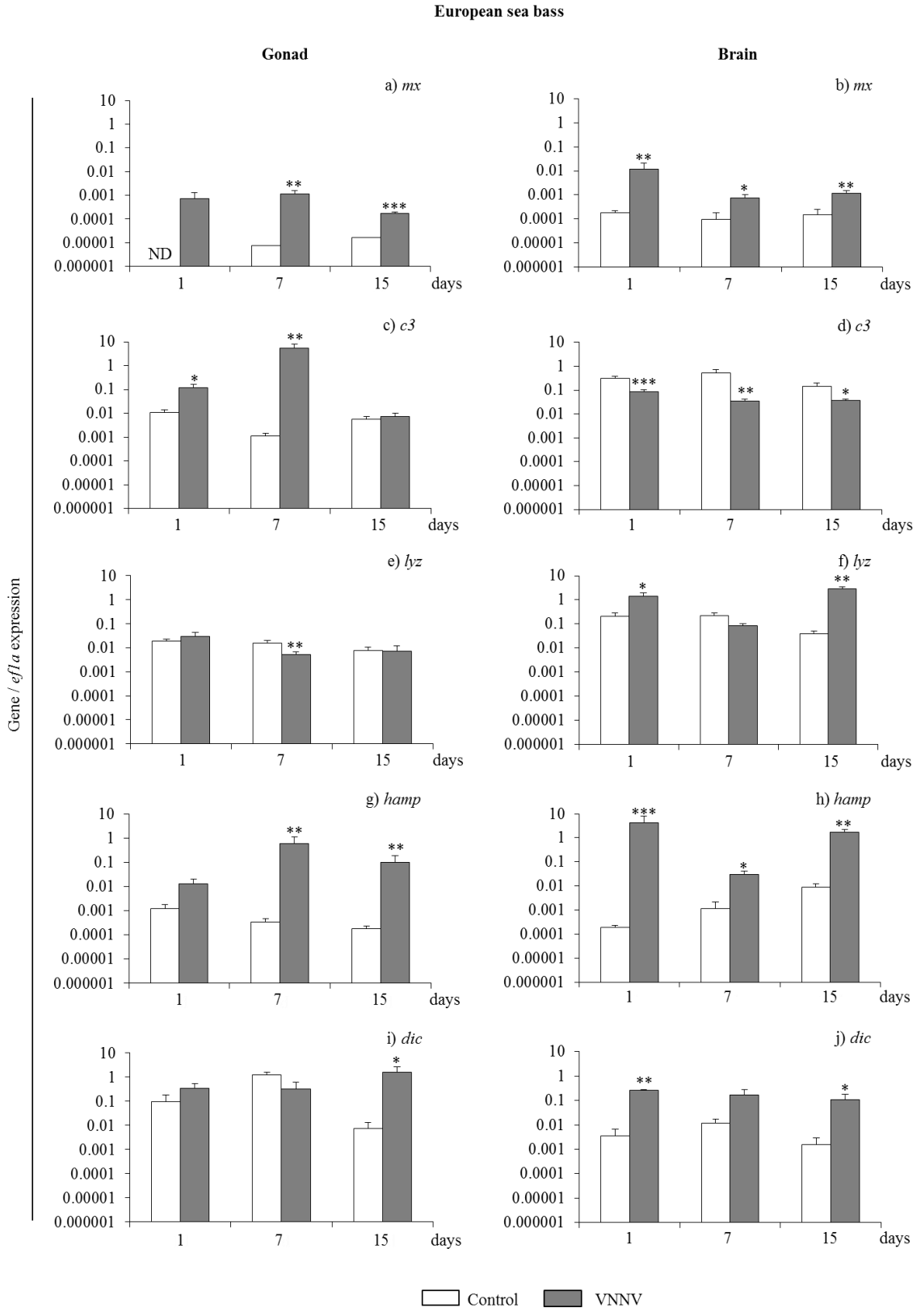


Figure 2: Expression of *mx* (a,b) and antimicrobial peptide genes *c3* (c,d), *lyz* (e,f), *hamp* (g,h) and *dic* (i,j) in the gonad (a,c,e,g,i) or brain (b,d,f,h,j) from control (white bars) or NNV-infected (grey bars) European sea bass specimens. Data represent the mean ± standard error (n = 6/group and time). Significance level (P) was fixed at 0.1 (P < 0.1*; P < 0.05**; P < 0.01***).

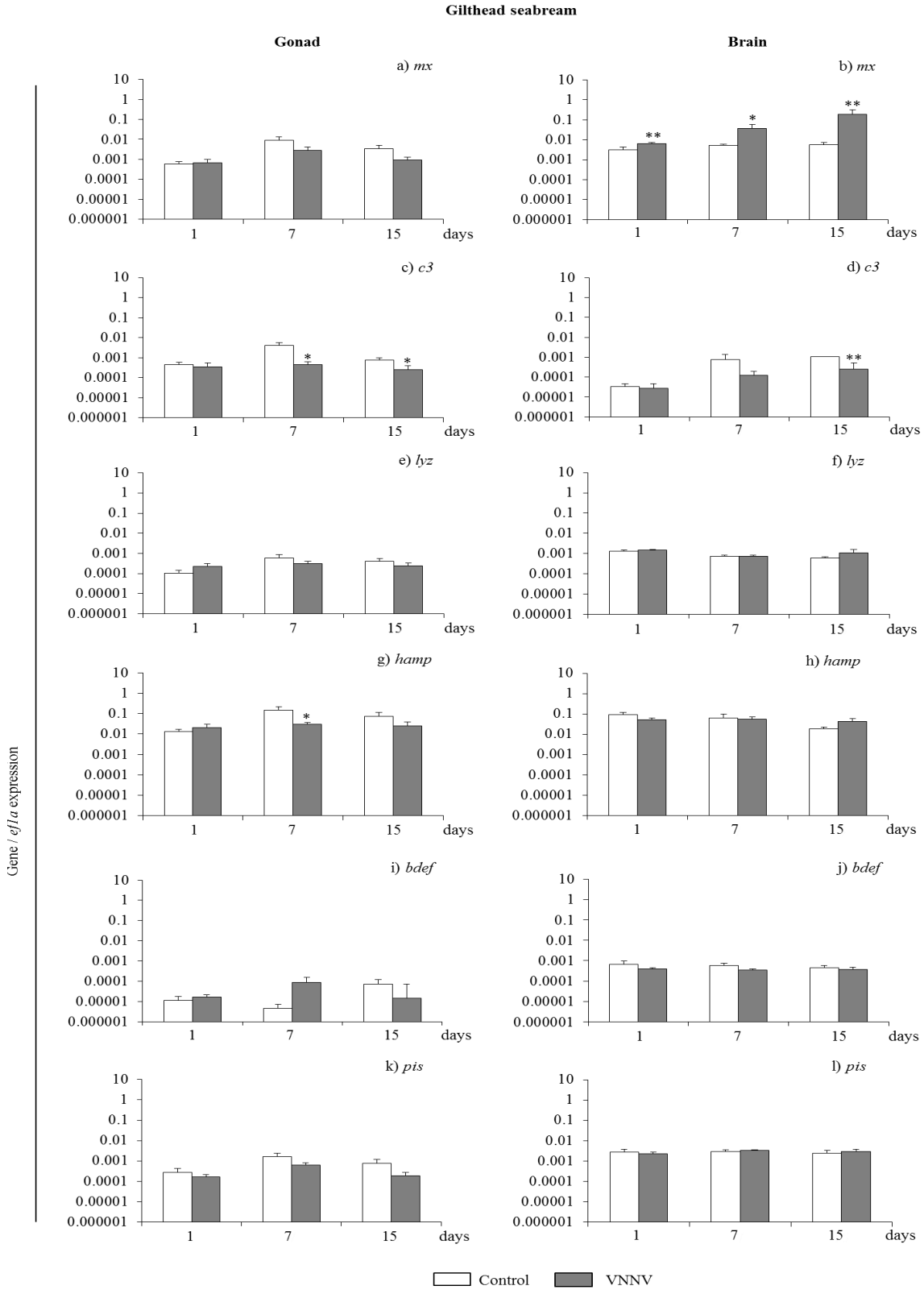


Figure 3: Expression of *mx* (a,b) and antimicrobial peptide genes *c3* (c,d), *lyz* (e, f), *hamp* (g,h), *bdef* (i,j) and *pis* (k,l) in the gonad (a,c,e,g,i,k) or brain (b,d,f,h,j,l) from control (grey white bars) or NNV-infected (grey bars) gilthead seabream specimens. Data represent the mean ± standard error (n = 6/group and time). Significance level (P) was fixed at 0.1 (P < 0.1*; P < 0.05**; P < 0.01***).

Thus, in the European sea bass gonad (Fig. 2c,e,g,i), NNV infection increased the level of expression of *c3*, *hamp* and *dic* at days 1 and 7, 7 and 15 and 15 of infection, respectively, and decreased the levels of expression of *lyz* at day 7 of infection. However, in the brain (Fig. 2d,f,h,j), the NNV infection decreased the expression level of *c3* gene throughout the trial and increased the expression levels of *lyz* and *dic* at days 1 and 15 of infection, and of *hamp* at all assayed times. In the gilthead seabream (Fig. 3), nevertheless, the NNV infection decreased the expression levels of *c3* and *hamp* in the gonad (Fig. 3c,g) at days 7 and 15 and 7, respectively, and the expression levels of *c3* in the brain (Fig. 3d) at day 15, while no differences were observed in the expression levels of *lyz*, *bdef* and *pis* in any of the tissues and sampled times analysed (Fig. 3e,f,i,j,k,l).

3.3. Gonad AMPs gene expression and some antimicrobial activities are negatively correlated

There are previous available evidences about the interrelation of complement, lysozyme and bactericidal activities (Bugla-Plskonska, *et al.*, 2008; Wardlaw, 1962), for this reason the correlation between these measured activities and the gene expression level of AMPs in the gonad upon *in vivo* infection with NNV (Tables S1, S2) have also been studied.

We found that, in both species, the expression of AMP genes negatively correlated with some antimicrobial activities. Thus, the gene expression of *lyz* negatively correlated with lysozyme activity in the gonad of European sea bass (Table S1), while in gilthead seabream gonad only the expression levels of *c3* gene negatively correlated with bactericidal activity (Table S2).

3.4. AMPs gene expression negatively correlated between gonad and brain upon NNV infection

When the correlation between the different gene expression in gonad and brain was studied, some differences between species were observed (Tables 2 and 3). In the European sea bass brain, the expression of *mx* gene, up-regulated upon viral-infection, negatively correlated with *c3* or *hamp* gene expression (Table 2), while no correlations were observed in the gilthead seabream brain (Table 3).

Table 2: Correlation observed between *mx* and AMP gene expressions in brain and gonad of European sea bass after *in vivo* infection with NNV. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P < 0.05. Written in bolds are the parameters correlated.

European sea bass		Gonad					Brain			
		mx	*c3*	*lyz*	*hamp*	*dic*	*mx*	*c3*	*lyz*	*hamp*
Gonad	*c3*	0.62 0.08								
	lyz	-0.30 0.39	0.54 0.14							
	hamp	0.40 0.28	**0.76** **0.02**	0.34 0.37						
	dic	-0.36 0.38	0.11 0.82	0.47 0.29	0.17 0.72					
Brain	*mx*	0.56 0.11	0.39 0.16	-0.19 0.49	**0.65** **0.02**	0.17 0.56				
	c3	-0.32 0.43	-0.09 0.78	0.47 0.34	-0.07 0.90	0.44 0.56	**-0.62** **0.03**			
	lyz	0.05 0.89	**-0.72** **0.03**	-0.61 0.08	-0.32 0.40	0.36 0.43	0.01 0.98	-0.16 0.76		
	hamp	0.42 0.24	-0.59 0.10	-0.16 0.68	-0.22 0.57	0.35 0.44	**-0.74** **0.01**	0.29 0.58	**0.81** **0.01**	
	dic	0.30 0.55	0.59 0.21	0.55 0.26	0.09 0.86	0.28 0.65	-0.20 0.55	0.21 0.79	-0.54 0.27	-0.38 0.45

On the other hand, in the gilthead seabream gonad, positive correlations were observed between *mx* and either *c3*, *lyz*, *hamp* or *pis* gene expressions (Table 3), while no correlations were observed in the European sea bass gonad (Table 2). Moreover, in European sea bass gonad, the expression pattern of *c3* positively correlated with *hamp* gene expression (Table 2), while in the gilthead seabream gonad, strong correlations were observed between *c3* and either *lyz*, *hamp* or *pis* gene expression and between *hamp* and either *lyz* or *pis* gene expression (Table 3). Regarding the brain, the transcription levels of *hamp* and *lyz* as well as *hamp* and *pis* positively correlated in European sea bass and gilthead seabream, respectively (Tables 2 and 3).

Interestingly, in the European sea bass positive correlation was observed between the *mx* gene expression in brain and the *hamp* gene expression in gonad (Table 2). However, negative correlations were found between some AMPs gene expression in brain and gonad in both species. Thus, in European sea bass (Table 2), the transcription

levels of *lyz* gene in brain was negatively correlated either with *c3* expression levels in gonad, while in the gilthead seabream (Table 3), the transcription levels of *bdef* in the brain were negatively correlated either with *c3* or *lyz* gene expression in the gonad.

Table 3: Correlation observed between *mx* and AMP gene expressions in brain and gonad of gilthead seabream after *in vivo* infection with NNV. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P < 0.05. Written in bolds are the parameters correlated.

Gilthead seabream		Gonad						Brain				
		mx	*c3*	*lyz*	*hamp*	*bdef*	*pis*	*mx*	*c3*	*lyz*	*hamp*	*bdef*
Gonad	*c3*	**0.68**										
		0.00										
	lyz	**0.79**	**0.74**									
		0.00	**0.00**									
	hamp	**0.79**	**0.71**	**0.77**								
		0.00	**0.00**	**0.00**								
	bdef	0.30	0.01	-0.34	0.28							
		0.16	0.98	0.31	0.37							
	pis	**0.76**	**0.67**	0.48	**0.57**	0.26						
		0.00	**0.01**	0.09	**0.03**	0.41						
Brain	*mx*	0.06	-0.22	-0.01	-0.03	-0.04	-0.13					
		0.76	0.29	0.97	0.89	0.85	0.51					
	c3	0.57	0.43	0.09	0.25	-0.03	-0.43	-0.18				
		0.16	0.34	0.85	0.54	0.95	0.29	0.66				
	lyz	-0.34	0.05	-0.06	-0.42	-0.54	0.12	-0.06	-0.61			
		0.09	0.86	0.85	0.12	0.07	0.67	0.75	0.14			
	hamp	-0.21	0.07	-0.07	-0.10	-0.05	0.32	-0.19	-0.61	0.50		
		0.28	0.82	0.83	0.74	0.87	0.26	0.34	0.14	0.06		
	bdef	-0.15	**-0.60**	**-0.65**	-0.35	0.41	-0.50	-0.11	-0.04	-0.24	-0.34	
		0.46	**0.02**	**0.02**	0.19	0.18	0.07	0.56	0.93	0.40	0.22	
	pis	0.02	0.32	0.03	0.20	0.05	0.44	0.15	-0.54	0.12	**0.77**	-0.28
		0.93	0.26	0.92	0.47	0.88	0.12	0.45	0.19	0.67	**0.00**	0.31

3.5. *In vitro* exposure to NNV differently alters the expression of AMP genes

Firstly, we found that both European sea bass and gilthead seabream gonad failed to mount an antiviral immune response after incubation with pI:C or NNV during 24 h (Fig. 4a,b), suggesting that these conditions might be suboptimal for this response. Regarding the AMPs, in the European sea bass (Fig. 4a), the expression of *lyz* gene was down-regulated upon NNV infection, while *hamp* and *dic* gene expression was up-

regulated upon NNV and NNV or pI:C challenges, respectively. In the gilthead seabream (Fig. 4b), however, the *c3* gene expression was up-regulated upon pI:C challenge, while *pis* and *bdef* gene expression were down-regulated upon NNV. Moreover, pI:C completely blocked the transcription of *bdef* gene (Fig. 4b). Noteworthy, there was not transcription of *c3* gene in control gilthead seabream gonad unless stimulated with pI:C (Fig. 4b).

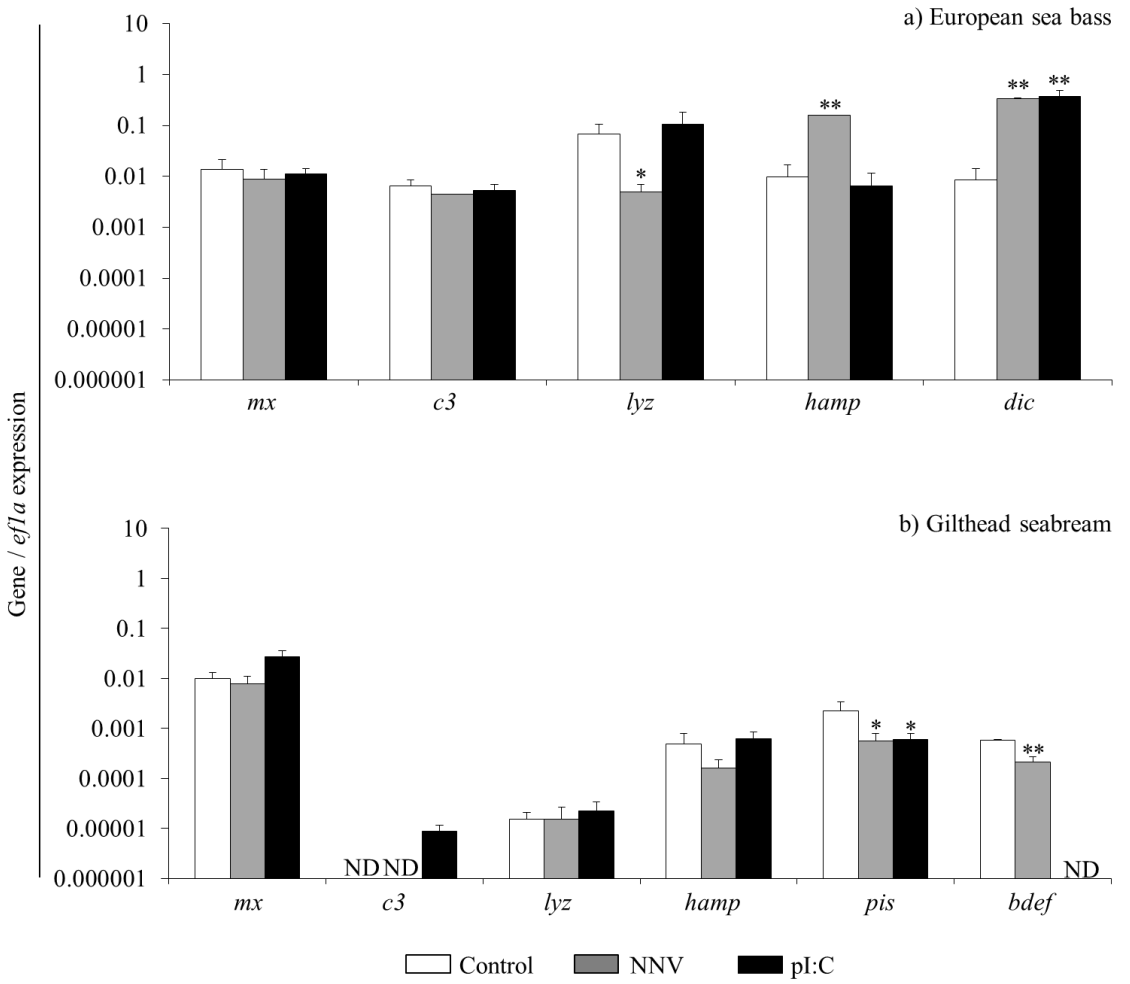


Figure 4: Expression of *mx* and antimicrobial peptide genes in the gonad of European sea bass (a) or gilthead seabream (b) upon *in vitro* treatment with medium (control group, white bars), NNV (grey bars) or pI:C (black bars) during 24 h. Data represent the mean ± standard error (n = 6/group). Significance level (P) was fixed at 0.1 (P<0.1*; P<0.05**; P<0.01***)

4. Discussion

The role of AMPs as a part of innate immune response, as well as its regulation, has been studied in many vertebrates (Hancock and Diamond, 2000). Interestingly, immune privilege is a term applied to eye, brain and reproductive organs where immune

responses either do not act, or act in a different manner from other parts of the animal body. In this framework, the AMPs expressed in the reproductive system of vertebrates probably assume an important role in the innate immune response against pathogens (Biswas and Yenugu, 2010; Chia, *et al.*, 2010; Collin, *et al.*, 2008; Jin, *et al.*, 2010). In fish, AMPs have been mainly studied in the immune organs (Valero, *et al.*, 2013), however, it is worth to study their role in the reproductive organs since the regulation of the immune response in those organs is different and it is also known that they allow to several virus colonize the gonad, persist and be transmitted (Chaves-Pozo, *et al.*, 2005c, 2010a). Between those pathogens, viruses and in particular NNV can spread both horizontally and vertically from mother to offspring, producing persistent infections and giving raise to asymptomatic carriers in European sea bass and gilthead seabream specimens (Gómez-Casado, *et al.*, 2011; López-Muñoz, *et al.*, 2012; Munday, *et al.*, 2002). In fact, in addition to the nodavirus detection by PCR and ELISA techniques in asymptomatic brood fish and their embryos (Breuil, *et al.*, 2001; Frerichs, *et al.*, 1996; Kuo, *et al.*, 2012), we have already detected and isolated infective NNV particles from the gonads of infected European sea bass and gilthead seabream specimens (Valero, *et al.*, 2015d). Interestingly, in the brain, one of the target tissues of NNV, the antiviral activity, determined as *mx* gene expression, was higher in gilthead seabream than in European sea bass, a fact that has been related with the resistance and susceptibility of these fish species to NNV disease. Conversely, the European sea bass gonad from NNV-infected fish showed an important up-regulation of the *mx* gene, which failed to do so in the gilthead seabream, indicating a strong interferon and antiviral response in this specie, which has never been observed. Therefore, we are trying to characterize the gonadal immune response under NNV infection, focussing in this study on the AMP response.

C3, a major component of the complement system, is considered as an AMP because of their direct implication in the defence against pathogens (Boshra, *et al.*, 2006; Saurabh and Sahoo, 2008). Thus, when the haemolytic activity of the complement in European sea bass and gilthead seabream serum were studied, small alterations were detected from its basal levels upon infection in contrast to the low increase in other trial (Mauri, *et al.*, 2011). However, non-detectable activity was observed in the gonads of any specie neither in control nor in infected specimens. Interestingly, a detectable transcription level of *c3* gene was observed in the gonad of control specimens of both

species, which was greatly up-regulated in the European sea bass whilst down-regulated in gilthead seabream. Interestingly, upon *in vitro* challenge of the gonad, no variation on *c3* gene expression was observed in the European sea bass neither by pI:C or NNV. Curiously, in the gonad of gilthead seabream challenged *in vitro*, non-detectable transcription of *c3* gene was observed, except after pI:C stimulation, as also reported in a previous *in vivo* study (Chaves-Pozo, *et al.*, 2008b). However, in the *in vivo* experiment, we reported basal and regulated *c3* gene expression in the gonad. These differences could be due to different acute stress conditions, which could affect a gene expression during weeks (Noga, *et al.*, 2011). Overall, these data suggest that C3 convertase is produced and this production is regulated upon infection in the testis of both species of teleost, however its activity could be inactivated by specific inhibitors produced locally, as occurs in humans (Vanderpuye, *et al.*, 1992). All these data together suggest that *c3* gene expression might be regulated and influenced by multiple *stimuli* that could affect the local immune response of both gonad and brain.

Regarding lysozyme activity, previous data showed that this activity was decreased in European sea bass and gilthead seabream specimens exposed to NNV (Mauri, *et al.*, 2011). We found that in the gonad, but not in serum, this activity was changed upon infection in both species. However, the lysozyme activity recorded in the gonad of European sea bass increased earlier (1 day upon NNV infection) and lasted longer (15 days upon infection) than in gilthead seabream gonad (7 days upon NNV infection). Interestingly, in the gonad, we found that lysozyme activity negatively correlated with the expression levels of *lyz* gene of European sea bass, suggesting that there are regulatory mechanisms of the protein activity involved in the up-regulation of lysozyme activity upon 1 day of infection and that this increased activity triggers a down-regulation of *lyz* gene transcription later on. In mammals, *lyz* gene is selectively expressed in the testis and its expression levels differ during its different developmental stages (Huang, *et al.*, 2011; Mandal, *et al.*, 2003; Narmadha, *et al.*, 2011; Sun, R., *et al.*, 2011) as also occurs during the reproductive cycle of European sea bass (Valero, *et al.*, 2015a). Furthermore, in both species, the *lyz* expression data obtained from the *in vivo* infection are in concordance with those observed in the gonad after 24 h of *in vitro* challenge, suggesting that the *lyz* expression changes are triggered by a local immune response.

In this study, the bactericidal activity against *Vh* upon NNV infection was clearly different in European sea bass and gilthead seabream. Thus, in European sea bass, this activity decreased in gonad and increased in serum from 7 days onwards; whether in gilthead seabream, after a slight down-regulation in serum at day 1, this activity was increased at day 15 post-infection in both gonad and serum. Evaluation of the direct lytic activity against pathogens is the most practical determination awaited for farmers whilst researchers also try to identify and characterize the molecules involved in this activity. Thus, determination of the bactericidal activity of the gonad might be more important than single AMP activities. In that sense, we have analysed the expression of several AMP coding genes known in both species, hepcidin and dicentracin in the European sea bass and hepcidin, piscidin and beta-defensin in the gilthead seabream; all of them cationic antimicrobial peptides (Valero, *et al.*, 2013). Some of these peptides are suggested to be involved in the defence against virus, as happens with C3 and piscidin (Dezfuli, *et al.*, 2012; Overturf and LaPatra, 2006). Furthermore, some of them have demonstrated antiviral function, as occurred with lysozyme, hepcidin and beta-defensin (Chia, *et al.*, 2010; Siwicki, *et al.*, 1998; Valero, *et al.*, 2013). Our data showed that in the European sea bass gonad, where the bactericidal activity is inhibited upon infection, the expression of most of the genes is up-regulated (*c3*, *hamp*, *dic*) with the exception of *lyz* mRNA. However, in the gilthead seabream gonad, where the activity is stimulated upon infection, the expression of most of the genes is down-regulated (*c3* and *hamp*) or unchanged (*lyz*, *bdef* and *pis*). Interestingly, the bactericidal activity was inversely correlated with *c3* gene expression in gilthead seabream gonad, while no correlations were found between the bactericidal activity and the expression of any of the AMP genes analysed in the European sea bass gonad. These data point to the complexity in the regulation of the processes analysed and to the need of further and deeper studies at molecular and functional levels.

When compared those AMPs gene expression in the gonad upon an *in vivo* infection with an *in vitro* challenge of the gonad with NNV or pI:C, we found that *lyz*, *hamp* and *dic* gene expression (but not *c3* gene expression) was similarly modified in both experimental situations in the European sea bass gonad, suggesting this that the expression profile changes observed are triggered by immune local responses. However, our *in vitro* and *in vivo* data are difficult to compare since the *in vitro* stimulation of the gonad failed to change the antiviral *mx* gene expression in sharp contrast to what

occurred in the *in vivo* infection. This fact would suggest that the antiviral and antimicrobial responses, at least those studied in this work, have different regulations and mediators. Furthermore, we also found that even when brain and gonad are immune privileged organs, those tissues behave differently upon infection with NNV at the gene expression level. Thus, in the European sea bass, the changes observed in the expression of *c3* and *lyz* genes were different in gonad and brain, while the *hamp* and *dic* expression levels were similarly modified upon infection in both tissues. Regarding gilthead seabream, all the genes analysed were similarly modified upon infection in both tissues, except *hamp* gene, which transcription was down-regulated in the gonad and unchanged in the brain. Interestingly, transcription of *mx* and some AMP genes were positively correlated in the gilthead seabream brain, adding more data to the idea that the high immune response in this tissue is the responsible for the viral clearance. Taking into account the brain-pituitary-gonadal axis, where both brain and gonad are closely linked by positive or negative feedback mechanisms (Weltzien, *et al.*, 2004) and that NNV is capable to infect both, brain and gonad, and alter some reproductive functions as the steroid serum levels (Valero, *et al.*, 2015d), we have analysed the relation between gonad and brain responses at the gene expression levels. Our analysis showed that in the European sea bass, *lyz* gene expression in brain negatively correlated with *c3* and *hamp* gene expressions in the gonad. Similarly, in the gilthead seabream, the *bdef* gene expression in brain negatively correlated with *c3* and *lyz* gene expressions in gonad. This data showed that the AMPs response, at the gene expression level, is inversely regulated in both tissues. This could partially explain the ability of NNV to be transmitted through the gonad without severely affecting the reproductive functions of the specimens.

5. Conclusions

To conclude, the results obtained in this study demonstrate that the immune response based on AMPs in the European sea bass or gilthead seabream gonads are clearly different upon NNV infection, at both expression and activity levels. These differences could be due to different susceptibility of the species to the infection and could determine the transmission rates of NNV in each species. Moreover, the differences observed between the *in vivo* and *in vitro* experiment suggest that some AMPs are locally regulated by a local immune response in the gonad while others might be more dependant of the systemic immune responses. In addition, our results determine

clear differences in the immune responses triggered by NNV in brain and gonad, explaining this, the differences observed in the affection of the functionality of both tissues upon NNV infection.

6. Supplementary data

Table S1: Correlation observed between AMPs gene expression and antimicrobial activities in gonad of European sea bass after in vivo infection with NNV. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference $P \leq 0.05$. Written in bolds are the parameters correlated.

European sea bass		Gene expression				Antimicrobial activities
		c3	*lyz*	*hamp*	*dic*	lysozyme
Antimicrobial activities	lysozyme	-0.68	**-0.72**	-0.29	0.17	
		0.07	**0.04**	0.48	0.75	
	bactericidal	-0.31	0.34	-0.21	-0.06	0.19
		0.46	0.41	0.62	0.91	0.65

Table S2: Correlation observed between AMPs gene expression and antimicrobial activities in gonad of gilthead seabream after *in vivo* infection with NNV. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference $P \leq 0.05$. Written in bolds are the parameters correlated.

Gilthead seabream		Gene expression					Antimicrobial activities
		c3	*lyz*	*hamp*	*bdef*	*pis*	lysozyme
Antimicrobial activities	lysozyme	-0.50	-0.08	-0.17	-0.25	-0.52	
		0.08	0.80	0.55	0.47	0.07	
	bactericidal	**-0.80**	-0.48	-0.31	-0.23	-0.54	0.33
		0.00	0.11	0.28	0.50	0.06	0.25

III.1.4. TRANSCRIPTION OF FISH HISTONES H1 AND H2B SUGGESTS A POTENTIAL ROLE IN IMMUNITY AS ANTIMICROBIAL PEPTIDES

1. Introduction

Histones are usually classified as core (H2A, H2B, H3 and H4) and linker histones (H1) due to their localization forming the basic units of the chromatin, the nucleosome. Thus, the nucleosome is formed by 146 base pairs of DNA wrapped around a protein octamer of two molecules of core histones. The linker H1 binds the DNA at the union sites whether it enters or exits the core nucleosome (Wolffe, 1998). Histones, mainly core ones, are greatly conserved in eukaryotic cells along evolution and therefore their functions might be also conserved. In addition to this function, they are also involved in other cellular functions and their implication in the epigenetic control of gene expression is nowadays in fashion. However, they have been also linked to immunity being their role as antimicrobial peptides (AMPs) the most described, which were first characterized in mammals long time ago (MacMillan and Hibbitt, 1969). Thus, histones and histone-derived fragments act as physiological barriers of cells exerting a variety of antimicrobial actions and functions, including bacterial cell membrane permeabilization, penetration into the membrane followed by binding to bacterial DNA and/or RNA, binding to bacterial lipopolysaccharide (LPS) in the membrane, neutralizing the toxicity of bacterial LPS, and entrapping pathogens as a component of neutrophil extracellular traps (NETs) (Kawasaki and Iwamuro, 2008).

In fish, the connections between histones and immunity have been established. First characterized was a channel catfish (*Ictalurus punctatus*) AMP isolated from the skin, the H2B (Robinette, *et al.*, 1998). Since then, proteins highly homologous to histones or fragments derived by cleavage processes from histones (eg. Parasin I, hipposin) have been defined as histone-like proteins (HLPs) and identified in some fish species (Birkemo, *et al.*, 2003; Fernandes, *et al.*, 2003, 2004; Narváez, *et al.*, 2010; Park, *et al.*, 1998). Most studies in fish have focused on the antimicrobial function of HLP-1 and HLP-2 proteins homologous to H2B and H1, respectively; and usually isolated from skin or gills (Bergsson, *et al.*, 2005; Fernandes, *et al.*, 2002; Noga, *et al.*, 2002; Richards, *et al.*, 2001; Robinette, *et al.*, 1998; Terova, *et al.*, 2011). However, other AMPs have been widely distributed among several tissues including immune-privileged tissues such as brain or gonads (Valero, *et al.*, 2013). Recently, a H1-like protein has been isolated from acidified testis extracts (fH1LP) of olive flounder (*Paralichthys olivaceus*) and shown to be constitutively expressed in ovary and testis and to have antibacterial (Gram+ and Gram-) and antifungal activity (Nam, *et al.*,

2012). In addition, several variants of H1 protein have been described to be mobilized upon viral infection in mammals (Conn, *et al.*, 2008; Hoeksema, *et al.*, 2015). In European sea bass (*Dicentrarchus labrax*), H2B and H1 coding genes were cloned and their expression levels have been reported to be altered under stress conditions (Terova, *et al.*, 2011), and also after *Vibrio anguillarum* (*Va*) infection (Meloni, *et al.*, 2015).

Nodavirus (NNV) is a naked bipartite single stranded RNA virus which severely affects European sea bass larvae and juveniles provoking high mortality rates (Breuil, *et al.*, 1991; Frerichs, *et al.*, 1996). Nevertheless, other species such as the gilthead seabream (*Sparus aurata*) are infected without showing disease symptoms, acting as a natural reservoir for most of the virus strains (Castric, *et al.*, 2001). NNV has demonstrated vertical transmission (Breuil, *et al.*, 2002) and is able to colonize and replicate in very low levels into the European sea bass and gilthead seabream testis in order to not being detected by the immune response (Valero, *et al.*, 2015d), altering the antimicrobial activities and pattern of expression of several AMPs (Valero, *et al.*, 2015b).

In this study, we identify the complete sequences of H1 and H2B coding genes in European sea bass and gilthead seabream and study their pattern of expression in immune, reproductive and metabolic important tissues in naïve specimens and under NNV infection. The results obtained, prompted us to analyse the modulation of both genes upon *in vitro* viral or bacterial infection or chemical stimulation of the immune response in gonad and HK leucocytes (HKLs) in order to determine whether this two proteins might have a role in the antimicrobial immune response of fish.

2. Material and methods

2.1. Animals

Healthy specimens of European sea bass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.) were bred and kept at the *Centro Oceanográfico de Murcia* of *Instituto Español de Oceanografía* (COM-IEO, Mazarrón, Murcia) in 14 m^3 tanks with the water temperature ranging from 14.6 to 17.8°C, flow-through circuit, suitable aeration, filtration systems and natural photoperiod. The environmental parameters, mortality and food intake, were recorded daily. Juvenile specimens of both species with a mean body weight (bw) of 325 ± 37.5 g were used for the analysis of

constitutive gene expression in naïve conditions (see below). Adult specimens of both species with a bw of 774 ± 93 g were used for *in vitro* treatments of the gonads (see below). Juvenile specimens of European sea bass (n = 50) or gilthead seabream (n = 50) with a mean bw of 200 ± 15 g, were transported to the University of Murcia (Spain) aquaria in order to perform *in vivo* infections (see below). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE). The protocol was approved by the Committee on the Ethics of Animal Experiments of the *Instituto Español de Oceanografía* (IEO) (Permit Number: 2010/02) and of the University of Murcia (Permit Number: A13150104).

2.2. Fish sampling

All specimens were anesthetized with 40 µl/l of clove oil before sampling, then weighed, completely bled and immediately decapitated. Blood was obtained from the caudal peduncle and the serum samples obtained by centrifugation (10,000 g, 1 min, 4°C), were immediately frozen in liquid nitrogen and stored at -80°C until use.

In order to analyse the constitutive expression in naïve conditions, brain, gill, liver, skin, gonad, gut, HK, spleen and thymus fragments from 6 independent fish were removed and immediately frozen in TRIzol® Reagent (Life Technologies) at -80ºC until used for RNA isolation. HKL suspensions were obtained as previously described (Esteban, *et al.*, 2013). In brief, fragments of HK tissue were transferred to 7 ml of sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35% NaCl, 100 IU/ml penicillin (Life Technologies), 100 mg/ml streptomycin (Life Technologies) and 5% foetal bovine serum (FBS; Life Technologies)] under sterile conditions. Cell suspensions were obtained by forcing fragments of the organ through a 100 µm nylon mesh, washed twice by centrifugation [400 g, 10 min, room temperature (RT)], counted and adjusted to 10^7 cells/ml in sRPMI. In all cases, leucocyte viability was determined by the trypan blue exclusion test and resulted higher than 98%.

2.3. Viruses and bacteria

NNV (strain 411/96, genotype RGNNV) was propagated in the SSN-1 cell line (Frerichs, *et al.*, 1996). The SSN-1 cells were grown in Leibovitz's L-15 medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine (Life Technologies), 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin (Gibco) at 25°C

using Falcon Primaria cell culture flasks (Becton Dickinson). Inoculated cells were incubated at 25ºC until the cytopathic effect (CPE) was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stock was titrated in 96-well plates and expressed as the viral dilution infecting 50% of the cell cultures ($TCID_{50}$), following a methodology previously described (Reed and Müench, 1938)

Pathogenic bacteria *Vibrio anguillarum* (*Va*) R-82 and *Photobacterium damselae* subsp. piscicida (*Pd*) were grown in sTSB [tryptic soy broth (Laboratorios Conda) supplemented with 1.5% NaCl] at 22°C for 24 h. Absorbance at 600 nm was measured and used to know the concentration based on growth curves. Both bacterial cell cultures were washed in sterile 0.01 M phosphate-buffered saline (PBS, pH 7.4) by centrifugation (6,000 g, 15 min, 4°C) and adjusted to 10^{10} bacteria/ml. For heat-killing, cultures were washed with PBS, incubated at 60ºC for 30 min, washed and adjusted to 10^{10} bacteria/ml with 0.01 M PBS.

2.4. *In vivo* infection

Once at the University of Murcia (Spain) facilities, juvenile specimens (n= 50) of both species were randomly divided into two tanks, kept in 450–500 L running seawater (28‰ salinity) aquaria at 25°C and with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the infection. The infection was performed by intramuscular injection of 100 µl containing 10^6 $TCID_{50}$/fish of NNV in SSN-1 culture medium, a mock-infected group was injected with 100 µl of SSN-1 culture medium since this route of infection has been proven to be the most effective (Aranguren, *et al.*, 2002). Fish (n = 5 fish/group and time) were sampled 1, 7 or 15 days upon infection and testis and brain were removed and immediately frozen in TRIzol® Reagent and stored at -80ºC for later RNA isolation as described below.

2.5. *In vitro* treatments

Fragments of European sea bass ovaries (n = 6) or testis (n = 6) or gilthead seabream gonads (n = 6) were removed, weighted and chopped into 1 mm^2 to culture them in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 µg/ml fungizone (Life Technologies) and 2% FBS. Six fragments of each tissue from independent fish specimens were incubated in flat-bottomed 96-well microtiter plates (Nunc) with 200 µl of: culture medium alone

(control), NNV (10^7 $TCID_{50}$/ml), *Va* (4 x 10^7 bacteria/ml) or polyinosinic:polycytidic acid (pI:C; 62,5 µg/ml; Sigma) at 25ºC during 24 h. Afterwards, the fragments of tissue were washed in 0.01 M PBS and stored in TRIzol® Reagent at -80ºC for later isolation of RNA as described below.

HKLs from healthy fish (n = 5) were isolated and maintained in Leibovitz's L-15-medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 20 mM HEPES (Gibco). Aliquots of 10^7 HKLs/ml were incubated in flat-bottomed 48-well microtiter plates (Nunc) at 22ºC during 24 h with: culture medium alone (control), 10^6 $TCID_{50}$ NNV/ml, 10^8 live bacteria/ml (*Va* or *Pd*), 50 µg/ml synthetic unmethylated cytosine-phosphodiester-guanosine oligodeoxynucleotide 1668 (CpG ODN 1668; sequence 5'-TCCATGACGTTCCTGATGCT-3'; Eurogentec), 25 µg/ml pI:C, 5 µg/ml lipopolysaccharide (LPS; Sigma), 10 µg/ml phytohemagglutinin (PHA; Sigma) or 5 µg/ml concanavalin A (ConA; Sigma). Afterwards, leucocytes were washed with 0.01M PBS and stored in TRIzol® Reagent at -80ºC for later isolation of RNA as mentioned below.

2.6. Gene sequences search and bioinformatics analysis

Complete sequences of European sea bass *h1* and *h2b* genes were obtained from the European sea bass genome (http://seabass.mpipz.mpg.de/) and analysed for similarity with known orthologue sequences using the BLAST program (Altschul, *et al.*, 1990) within the ExPASy Molecular Biology server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This program was also used to compare European sea bass sequences with the gilthead seabream expressed sequence tags (ESTs) databases. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura, *et al.*, 2013) to confirm that they are *bona fide* gilthead seabream sequences.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the optimal tree with the sum of branch length = 4.84862174 was obtained. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The

evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 16 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 231 positions in the final dataset.

2.7. Analysis of gene expression by real-time PCR

Total RNA was isolated from TRIzol® Reagent frozen samples following the manufacturer's instructions. One µg of total RNA was treated with DNAse I (Promega) to remove genomic DNA and the first strand of cDNA synthesized by reverse transcription using the Superscript III (Life Technologies) with an oligo-dT12-18 primer (Life Technologies) followed by RNAse H (Life Technologies) treatment. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated at 95ºC for 10 min, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC , and finally 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1 alpha coding gene (*ef1a*) expression in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. The primers used were designed using the Oligo Perfect software tool (Thermo Fisher Scientific) and are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primer for specificity. 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.

2.8. Statistical analysis

The constitutive expression data of the different genes in each tissue were analysed by one-way ANOVA to denote statistical differences between tissues. The data related to *in vivo* infections or *in vitro* treatments were analysed by a t-Student test to determine statistical differences between infected/treated or control groups. A non-parametric Kruskal–Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 20 software. All data are presented as mean ± standard error of the mean (SEM). Minimum level of significance was fixed in 0.1.

Table 1: Primers used for analysis of gene expression by real-time PCR.

Species	Gene name	Gene Abbrev.	Accession number	Primer sequence
European sea bass	Histone 1	*h1*	DLAgn00119260	AAGAAGACGGGTCCCTCAGT
				CTTGACCTTCTTCGCTTTGG
	Histone 2b	*h2b*	DLAgn00179560	GGAGAGCTACGCCATCTACG
				GCTCAAAGATGTCGCTCACA
	Elongation factor 1 alpha	*ef1a*	AJ866727	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG
Gilthead seabream	Histone 1	*h1*	FM151953	CGTGGTGAAGAACAGAGCAA
				TTGACCCTTTTCGTCTTTGG
	Histone 2b	*h2b*	AM953480	AGACGGTCAAAGCACCAAAG
				AGTTCATGATGCCCATAGCC
	Elongation factor 1 alpha	*ef1a*	AF184170	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG

3. Results

3.1 Identification of European sea bass and gilthead seabream *h1* and *h2b* gene sequences

Complete cDNA sequences coding for European sea bass proteins H1 and H2B were available at the European sea bass genome database (http://seabass.mpipz.mpg.de/). We found one uncharacterized clone containing the entire open reading frame (ORF) coding for each gilthead seabream proteins H1 and H2B at the ESTs databases available at the NCBI GenBank database [GenBank accession number *h1*: FM151953 (unpublished); *h2b*: AM953780 (Louro, *et al.*, 2010)]. The predicted length, homology and E-values obtained from the gene sequences were compared with their humans orthologues (Table 2) resulting in *bona fide* sequences.

Phylogenetic tree showed two distinct clades for H1 and H2B proteins (Fig. 1). The clustering provides evidences of high bootstrap support in the lineage of European sea bass and gilthead seabream. Moreover, the teleost H1 proteins form an exclusive clade opposed to human H1 sequences. Human protein H3 was used as outgroup.

Table 2: Identity (in %; [a]) and E-value ([b]) of the predicted proteins respect to the human orthologues. Asterisk denotes the sequences with predicted full length.

Predicted protein	Fish species	Gene accession number	Protein length	Identity[a]	E-value[b]
H1	Sea bass	DLAgn_0011926	188*	71	1e-25
	Seabream	FM151953	192*	67	9e-23
	Zebrafish	XP708065	199*	63	1e-31
	Human	NP005313	226*		
H2B	Sea bass	DLAgn_00179560	121*	92	6e-67
	Seabream	AM953480	134*	95	5e-67
	Zebrafish	NP 001013481	124*	98	2e-72
	Human	AAA63192	101		

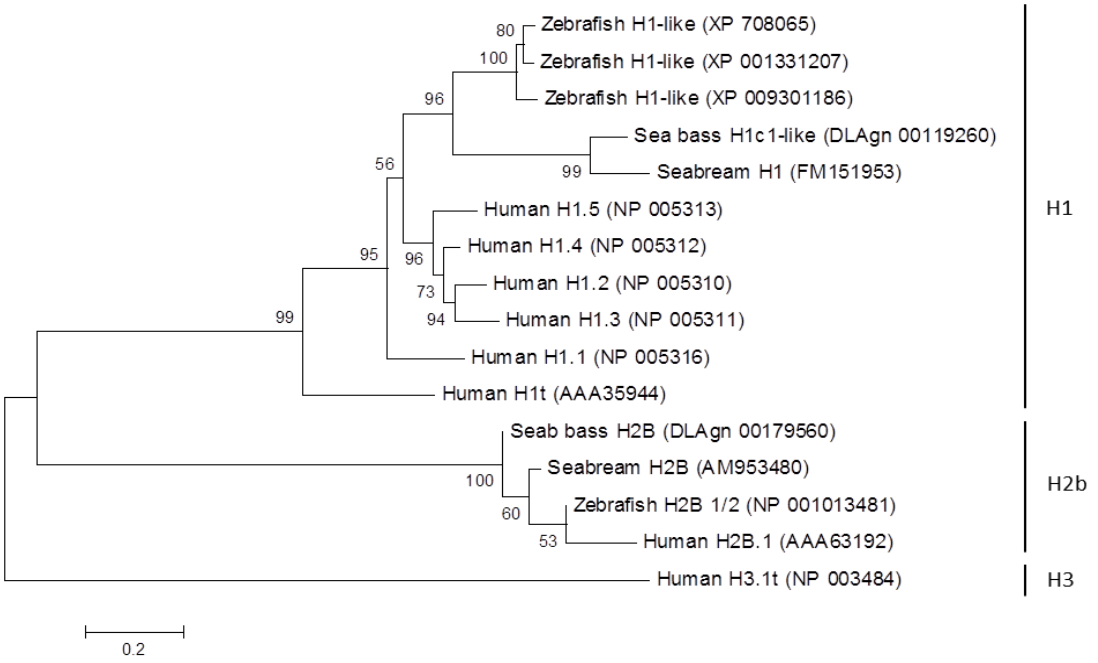


Figure 1: Phylogenetic analysis of the H1 and H2B proteins of European sea bass and gilthead seabream with related sequences of zebrafish and human histone proteins. The phylogenetic tree was drawn following the Neighbour-Joining method for the analysis of evolutionary relationship. Genbank accession numbers are shown in parentheses. Histones with "t" are thymus isolated histones.

3.2. Expression of *h1* and *h2b* under naïve conditions

We found *h1* mRNA transcripts in brain, gills, liver, skin, gonad, gut, HK, spleen, thymus and blood tissues from both species (Fig. 2), although some differences between species were observed. Thus, in European sea bass (Fig. 2a), the tissues with the highest expression of *h1* gene were in thymus and blood followed by brain and liver.

Gills and gonad were the tissues with the lowest *h1* expression levels (10,000 fold lower than thymus). However, in the gilthead seabream (Fig. 2b), the blood showed the highest *h1* gene expression levels, followed by HK and spleen whereas liver, gut and gonad showed the lowest expression (100,000-fold lower than blood).

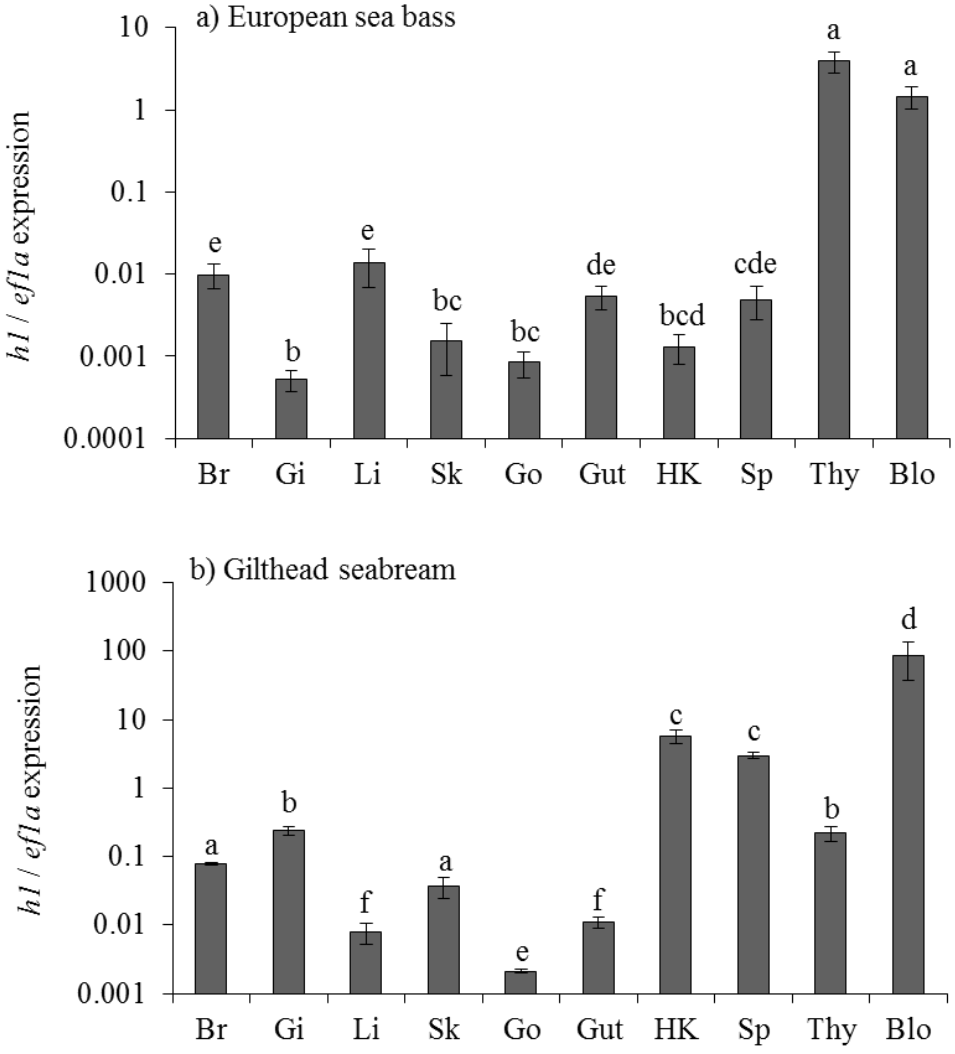


Figure 2: Levels of expression of *h1* gene in European sea bass (a) and gilthead seabream (b) tissues: brain (Br), gills (Gi), liver (Li), skin (Sk), gonad (Go), gut, head-kidney (HK), spleen (Sp), thymus (Thy) and blood (Blo). Data represent mean relative expression to the expression of endogenous control *ef1a* gene ± SEM (n = 6). Letters denote statistical differences between tissues (ANOVA; $P < 0.05$).

Regarding the expression of H2B coding gene (Fig. 3), no constitutive expression were observed in brain, skin and spleen of European sea bass (Fig. 3a), whilst the highest transcription levels were found in thymus. In contrast, in gilthead seabream (Fig. 3b), all tissues constitutively expressed this gene. The highest level of expression was found in gonad and blood while thymus has a medium level of

expression and the lowest expression was observed in liver (10,000-fold lower than in blood).

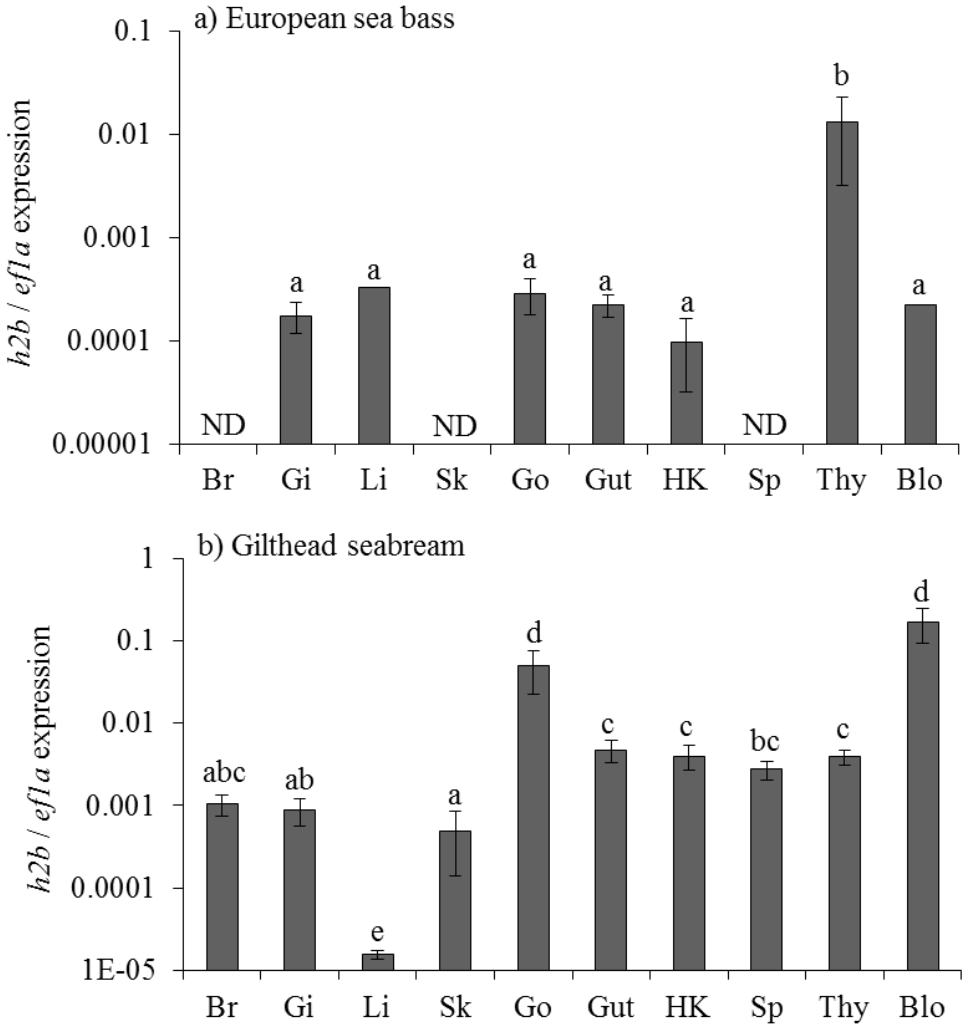


Figure 3: Levels of expression of *h2b* gene in European sea bass (a) and gilthead seabream (b) tissues: brain (Br), gills (Gi), liver (Li), skin (Sk), gonad (Go), gut, head-kidney (HK), spleen (Sp), thymus (Thy) and blood (Blo). Data represent mean relative expression to the expression of endogenous control *ef1a* gene ± SEM (n = 6). Letters denote statistical differences between tissues (ANOVA; $P < 0.05$). ND, non detected.

3.3. The expression of *h2b* but not of *h1* was increased in HK upon NNV infection in both species

The expression levels of *h1* were down-regulated in brain but up-regulated in testis of European sea bass after 7 days of NNV infection (Fig. 4a). In contrast, in gilthead seabream, the transcription levels of *h1* were down- and up-regulated in brain after 7 and 15 days post-infection, respectively, and down-regulated in testis at day 15 post-infection (Fig. 4b).

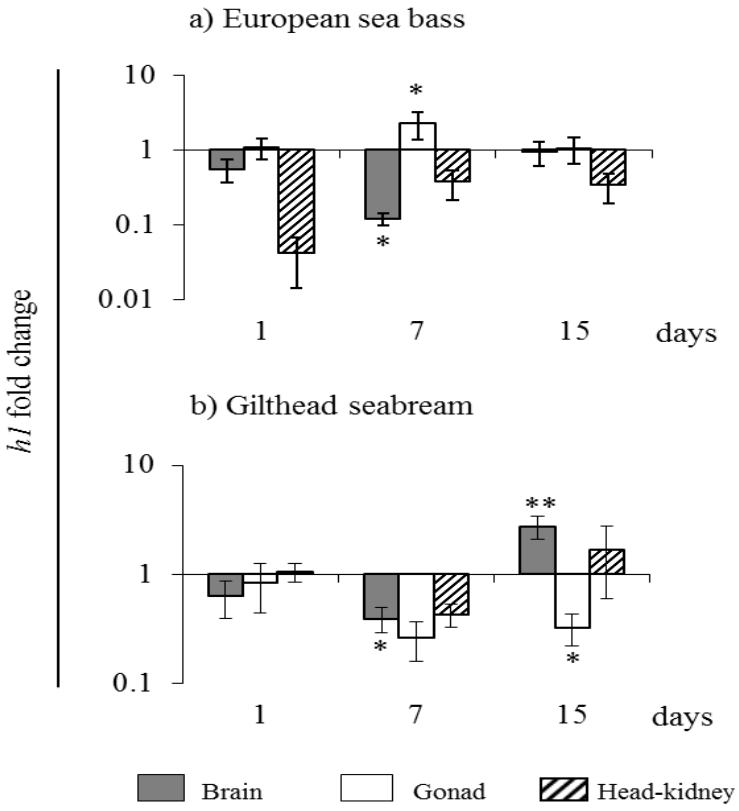


Figure 4: Expression levels of *h1* gene in European sea bass (a) and gilthead seabream (b) brain, testis and HK after 1, 7 and 15 days of *in vivo* NNV infection (10^6 $TCID_{50}$ per fish) studied by real-time PCR. Data are expressed as the mean ± SEM (n = 5) of mRNA fold increase respect to control samples. Asterisks denote significant differences with the controls at each sampling time (t Student test; *P<0.1, **P < 0.05).

In European sea bass (Fig. 5a), the *h2b* gene expression was down-regulated in brain at day 1 and in testis at day 15 post-infection whilst it was down-regulated in gilthead seabream gonad after 7 days (Fig. 5b). Interestingly, in both species the *h2b* transcription was up-regulated in HK at different time post-infection (7 days in European seabass or 15 days in gilthead seabream).

3.4. The expression of *h1* in European sea bass ovary and of *h2b* in the gilthead seabream testis were inhibited after some *in vitro* treatments

When we analysed the pattern of expression of *h1* in the gonad of European sea bass and gilthead seabream after 24 hours of *in vitro* treatment, we found that only European sea bass ovaries showed down-regulated *h1* gene expression levels after NNV infection, whilst in European sea bass and gilthead seabream testis was unchanged (Fig. 6a). However, the pattern of expression of *h2b* gene in European sea bass gonads was unaltered by any treatment while was down-regulated after the challenge with *Va* and pI:C in gilthead seabream testis (Fig. 6b).

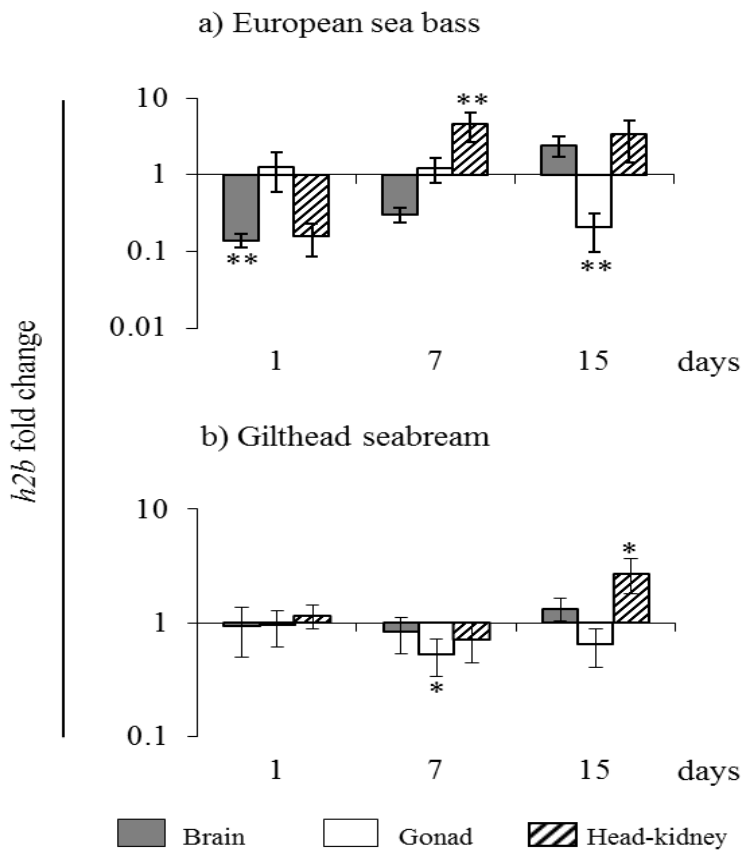


Figure 5: Expression levels of *h2b* gene in European sea bass (a) and gilthead seabream (b) brain, testis and HK after 1, 7 and 15 days of *in vivo* NNV infection (10^6 $TCID_{50}$ per fish) studied by real-time PCR. Data are expressed as the mean ± SEM (n = 5) of mRNA fold increase respect to control samples. Asterisk denote significant differences with controls at each sampling time (t Student test; *P<0.1, **P < 0.05).

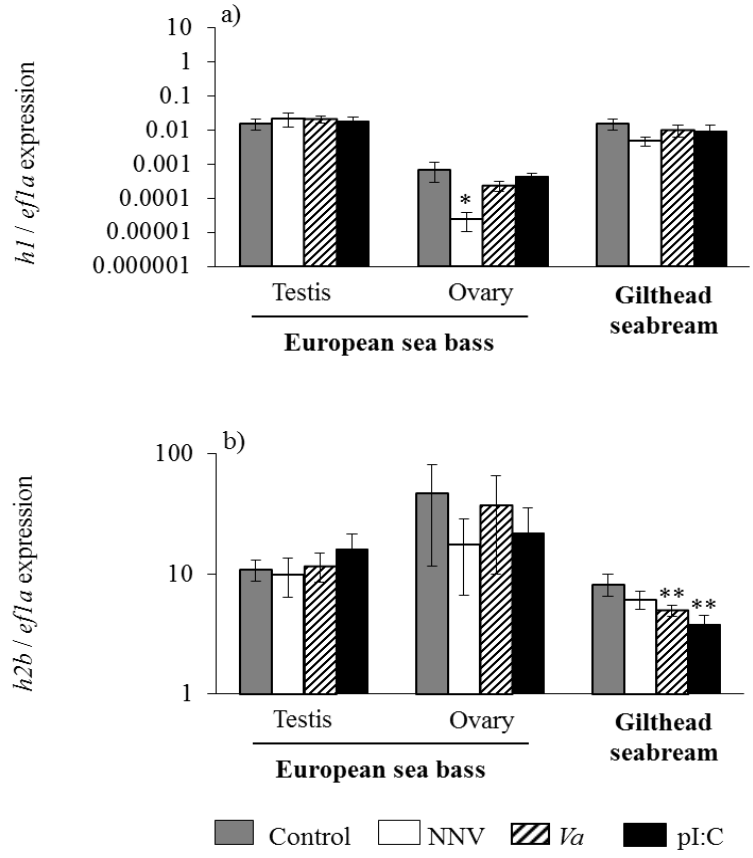


Figure 6: Expression levels of *h1* (a) and *h2b* (b) genes in European sea bass testis and ovaries and gilthead seabream gonad after 24 h of *in vitro* challenge with NNV (10^7 $TCID_{50}$/ml), *Va* (4 × 10^7 bacteria/ml) and poly I:C (pI:C 62,5 µg/ml), studied by real-time PCR. Data are expressed as the mean ± SEM (n = 6) of mRNA transcripts relative to *ef1a* gene expression. Asterisks denote significant differences with controls (t Student test; *P<0.1, **P < 0.05).

3.5. The expression of *h2b* gene was exclusively up-regulated in gilthead seabream HKLs

Finally, we studied the pattern of expression of *h1* (Fig. 7) and *h2b* (Fig. 8) genes in HKLs after 24 hours of treatment with known *stimuli* and our data showed that *h1* gene expression was down-regulated after NNV, *Va* or *Pd* treatment in European sea bass HKLs (Fig. 7a), and after NNV, *Va*, LPS, PHA or ConA treatment in gilthead seabream HKLs (Fig. 7b).

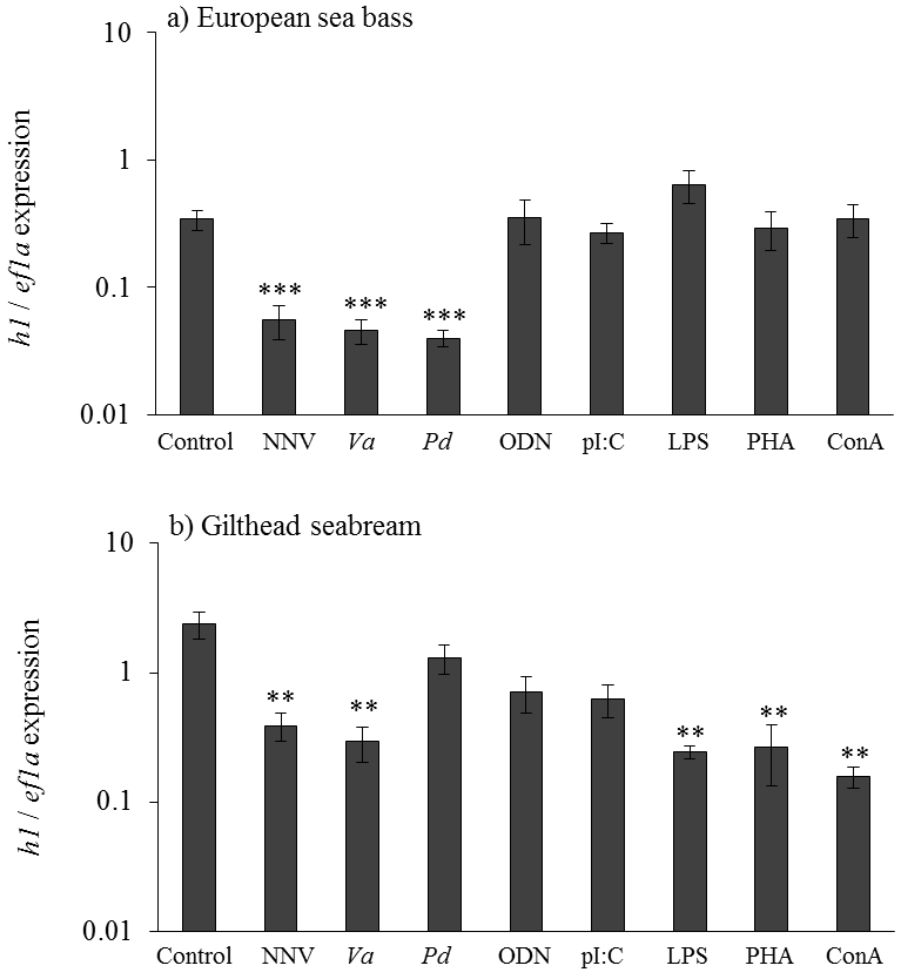


Figure 7: The expression of *h1* gene in HKLs of European sea bass (a) and gilthead seabream (b) after 24 h of *in vitro* challenge with culture medium alone (control), 10^6 $TCID_{50}$ NNV/ml, 10^8 live bacteria/ml (*Va* or *Pd*), 50 µg/ml CpG ODN 1668, 25 µg/ml pI:C, 5 µg/ml LPS, 10 µg/ml PHA or 5 µg/ml ConA studied by real-time PCR. Data are expressed as the mean ± SEM (n = 5) of mRNA transcripts relative to *ef1a* gene expression. Asterisks denote significant differences with controls (t Student test; **P<0.05, ***P < 0.01).

Similarly, the *h2b* gene expression in HKLs of European sea bass was down-regulated upon NNV, *Va*, *Pd*, pI:C or ConA treatments (Fig. 8a). In contrast to this and

what happened with *h1* gene expression, in gilthead seabream HKLs the *h2b* gene expression was up-regulated after all *stimuli* assayed except with PHA (Fig. 8b).

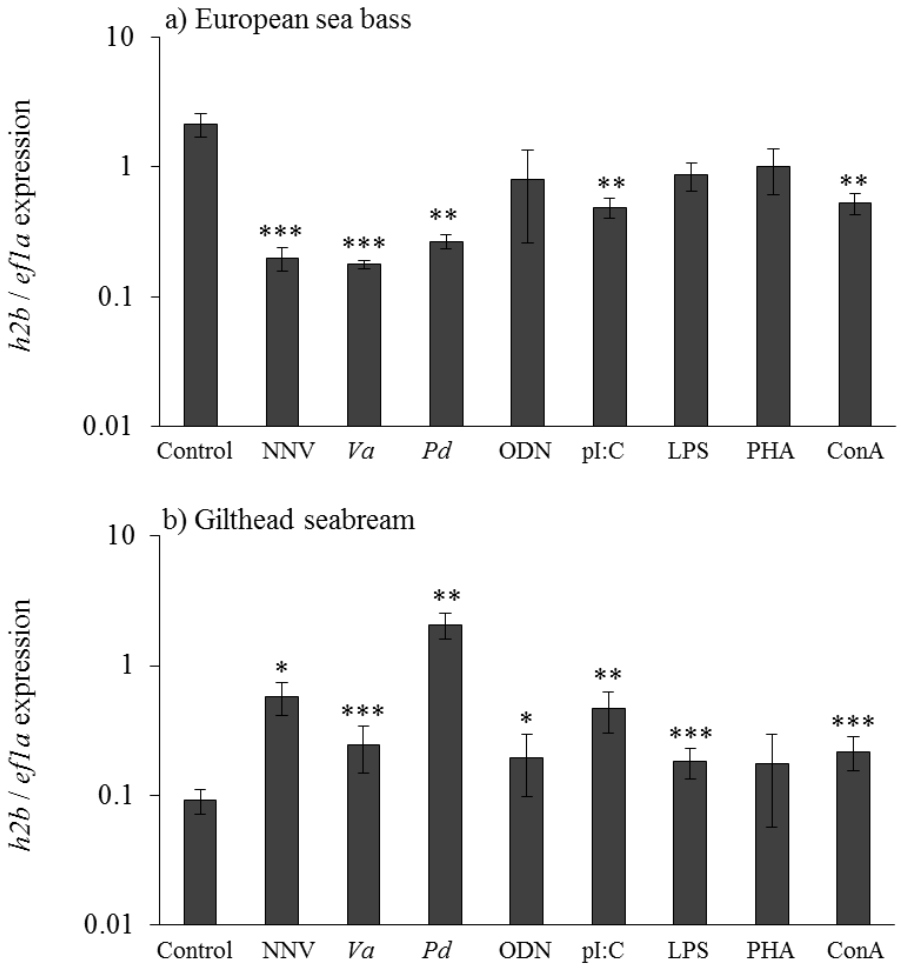


Figure 8: The expression of *h2b* gene in HKLs of European sea bass (a) or gilthead seabream (b) after 24 h of *in vitro* challenge with culture medium alone (control), 10^6 $TCID_{50}$ NNV/ml, 10^8 live bacteria/ml (*Va* or *Pd*), 50 µg/ml CpG ODN 1668, 25 µg/ml pI:C, 5 µg/ml LPS, 10 µg/ml PHA or 5 µg/ml ConA studied by real-time PCR. Data are expressed as the mean ± SEM (n = 5) of mRNA transcripts relative to *ef1a* gene expression. Asterisks denote significant differences with the controls (t Student test; *P<0.1, **P<0.05, ***P < 0.01).

4. Discussion

Histones, as chromatin structure proteins, were thought to be confined to the nucleus. However, different studies have detected various histones and their fragments in the cytoplasm of several cell types including leucocytes from mammals, bird, frogs, fish and shrimps, showing those proteins a broad spectrum of antimicrobial activities (Parseghian and Luhrs, 2006). Both, core (H2A, H2B) and the linker (H1) histones showed antimicrobial activity in several fish species (Fernandes, *et al.*, 2002, 2004;

Parseghian and Luhrs, 2006; Richards, *et al.*, 2001; Robinette, *et al.*, 1998; Valero, *et al.*, 2013). Concretely, in the European sea bass, partial cDNA sequences coding for H1 and H2B proteins were isolated and their pattern of expression analysed under stress conditions, resulted on a similar pattern of expression in gills and epidermis than haemoglobin-like protein, a known antimicrobial peptide (Terova, *et al.*, 2011; Ullal, *et al.*, 2008). In the present work we used the complete sequences coding for these proteins for searching the gilthead seabream orthologue sequences. Thus, we found two sequences annotated but not characterized in the GenBank database. After the phylogenetic analysis, European sea bass and gilthead seabream *h1* and *h2b* sequences showed a high percentage of similarity with their zebrafish and human orthologues, suggesting that their function could be conserved. Since histones with antimicrobial activity were firstly identified in fish skin, most studies in fish have avoided the study of the constitutive expression of histones in other tissues apart from skin or liver (Narváez, *et al.*, 2010; Richards, *et al.*, 2001; Robinette, *et al.*, 1998), but other AMPs have been localized in a wide range of tissues including immune-privileged tissues as brain or gonads (Valero, *et al.*, 2013, 2015b). Our data showed that *h1* was constitutively expressed in all tissues analysed in both, European sea bass and gilthead seabream, as also occurred in the olive flounder (Nam, *et al.*, 2012). Interestingly, in olive flounder the highest expression of *h1* gene was found in gonad (Nam, *et al.*, 2012), however, our data showed the highest *h1* gene expression in immune tissues of both species. As far as we are concerned, our study is the first analysing the pattern of expression of *h2b* gene covering most of the tissues in fish. Therefore, we found that in European sea bass, *h2b* gene transcription was not detected in brain, skin or spleen but, was highly expressed in thymus. In contrast, in gilthead seabream *h2b* gene was highly expressed in peripheral blood and gonad.

Several variants of H1 protein have been described to be mobilized upon viral infection (Conn, *et al.*, 2008; Hoeksema, *et al.*, 2015). In that sense we have analysed the pattern of expression of *h1* and *h2b* upon NNV infection, a virus which target tissues are the retina and brain (Munday, *et al.*, 2002) and colonizes the gonad to be vertically transmitted (Valero, *et al.*, 2015d). Our data showed that upon *in vivo* infections with NNV, *h1* is up-regulated in the gonad of European sea bass and in the brain of gilthead seabream, while *h2b* is up-regulated in the HK of both species. Interestingly, in Rohu (*Labeo rohita*), LHH1M protein, that corresponds with the linker

histone H1, is up-regulated in the brain of specimens resistant to Gram negative bacteria *Aeromonas hydrophila* (Das, *et al.*, 2014) as occurred with gilthead seabream, which is an asymptomatic carrier species of the NNV strain used to perform the experiment (Castric, *et al.*, 2001). On the other hand, European sea bass is very susceptible to NNV (Breuil, *et al.*, 1991) and our data showed that *h1* expression was down-regulated in the virus target tissue, the brain.

Taking into account the high expression of *h2b* gene in the gonad of gilthead seabream, the immune-privileged status of the gonad (Chaves-Pozo, *et al.*, 2005c; Hedger, 2002) and the ability of NNV to colonize the testis (Valero, *et al.*, 2015d), we have analysed the expression of *h1* and *h2b* genes in mature European sea bass male and females and gilthead seabream male gonads upon *in vitro* treatment with alive NNV or *Va* or pI:C, and found slight down-regulations of *h1* expression in European sea bass ovary upon NNV infection and of *h2b* expression in gilthead seabream testis upon *Va* and pI:C treatment. These data suggest that the transcriptional changes observed on both genes upon *in vivo* infections were orchestrated by the systemic immune response and not due to a local infection of the virus or bacteria. However, it has been recently demonstrated the presence of NNV in the testis of both species upon an infection (Valero, *et al.*, 2015d). Probably, the existence of other specific AMPs in the gonad together with the high proliferative rates that this tissue showed during gametogenesis, avoids the use of H1 and H2B as antimicrobial proteins, whilst in other tissue such as HKLs or brain, this function is enhanced and needed.

Histones are well known to be shed out of the cells in mammalian neutrophils extracellular traps (NETs) and recently these NETs have been described to be produced by some leucocytes of mainly cyprinid fish species (Brogden, *et al.*, 2014; Chi and Sun, 2016; Pijanowski, *et al.*, 2013, 2015). In that sense and taking into account the high expression of *h1* and of *h1* and *h2b* observed in European sea bass and gilthead seabream blood, respectively, we next analysed the transcription levels of these genes in HKLs stimulated with different immune *stimuli*. Thus, we observed that *h1* gene expression was down-regulated in European sea bass upon challenge with live virus and bacteria, while in gilthead seabream this down-regulation was also observed upon LPS, PHA and ConA treatments. In human monocytes, and upon LPS stimulation, H1 is able to bind LPS (Hampton, *et al.*, 1988). In contrast to what happened to *h1* expression, the transcription of *h2b* gene was up-regulated in gilthead seabream HKLs and down-

regulated in European sea bass HKLs upon NNV and other immune *stimuli*. Although further studies are needed, this study clearly suggests that the ability to use histones as AMPs, either in traps or not, might be a clear difference in the susceptibility to infections of each fish species.

5. Conclusions

In conclusion, this is the first study analysing the pattern of expression of H1 and H2B coding sequences in a broad spectrum of tissues of European sea bass and gilthead seabream fish species. Moreover, our data suggest that H1 might have a role in the immune response against NNV in brain of both species, due to the fact that *h1* expression pattern is similar to that found for other AMPs and the IFN pathway molecules and correlated well with the different susceptibility to infection of both species (Valero, *et al.*, 2015b,c). On the other hand, H2B seems to be more important in the HK and HKLs immune response. Although further studies are needed, the expression pattern of *h2b* gene in HK and HKLs upon infection supports that the ability to use histones as AMPs might point out the difference in the susceptibility to infection between species. Nevertheless, we consider that further functional studies are needed to understand histones implication in antimicrobial responses upon NNV infection and several studies are in progress in our laboratory.

III.2. EXPERIMENTAL PREVENTIVE MECHANISMS UPON NNV INFECTION

III.2.1. AN ORAL CHITOSAN DNA VACCINE AGAINST NODAVIRUS IMPROVES THE SURVIVAL OF EUROPEAN SEA BASS JUVENILES UPON INFECTION PROBABLY BY TRIGGERING THE CELL-MEDIATED TOXICITY AND IFN RESPONSES

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1. Introduction

Although DNA vaccines are increasingly considered as a potential method to solve the lack of available treatments to viral diseases in aquaculture (Evensen and Leong, 2013; Kurath, 2008), their administration by injection makes them a limited and inappropriate preventive measure because of its invasiveness and economically unfeasible delivery. Therefore, it remains necessary to develop simple and cost effective systems to deliver DNA vaccines for mass administration in fish farms. At this point, recent approaches have demonstrated that oral management of encapsulated vaccines improves the survival to different pathogen infections (Rajesh Kumar, *et al.*, 2008; Vimal, *et al.*, 2014). In fish vaccination studies, several types of encapsulation substances including alginate (Ballesteros, *et al.*, 2015; de las Heras, *et al.*, 2010; Maurice, *et al.*, 2004; Tian, *et al.*, 2008a), chitosan (Li, *et al.*, 2013; Rajesh Kumar, *et al.*, 2008; Tian, *et al.*, 2008a) and poly(DL-lactide-co-glycolide) (Tian, *et al.*, 2008b; Tian and Yu, 2011) have been effectively used to encapsulate bacterial or viral antigens and the data obtained demonstrate an effective immune response and resistance to pathogen challenge. Thus, encapsulated oral vaccines are easy to use for mass intake, with minimal fish stress, targetable, storable stable, and easy to produce in large quantities. In addition to the capacity of these substances to entrap and protect the antigens in the fish digestive tract, they also act by themselves as immunostimulants, offering and additional improvement of the immune response. Thus, sodium alginate or chitosan administration is able to increase the fish immune response and disease resistance (Abu-Elala, *et al.*, 2015; Cheng and Yu, 2013; Fujiki, *et al.*, 1994; Lin, *et al.*, 2011). Moreover, they are environmentally friendly since they are biodegradable and non-toxic, making the encapsulation with these polymers a possible and ideal route for general vaccination, and also for DNA vaccines.

Among fish diseases, viruses are one of the major growing problems in intensive aquaculture as no solutions are available at preventive and therapeutic levels. One of the most threatening viruses is nodavirus (*Nodaviridae* family, *Betanodavirus* genus), also named nervous necrosis virus (NNV), that causes the viral encephalopathy and retinopathy (VER) disease altering the brain and retina structure and function (Munday, *et al.*, 1992). NNV provokes mortality rates up to 100% in more than 50 marine species (Munday, *et al.*, 1992; OIE, 2013). Among them, the European sea bass (*Dicentrarchus labrax*), a very relevant species in Mediterranean aquaculture, is one of the most

susceptible ones, being larvae and juvenile stages in which causes higher mortalities (Breuil, *et al.*, 1991; Frerichs, *et al.*, 1996). Although some aspects of the fish immune response against NNV are known, very few studies have addressed the generation of an effective vaccine. So far, different studies have demonstrated the increase of the immune response and/or NNV resistance after administration of live/inactivated NNV or recombinant proteins (Kai and Chi, 2008; Kai, *et al.*, 2014; Nishizawa, *et al.*, 2012; Oh, *et al.*, 2013; Sommerset, *et al.*, 2005b). In addition, a first study showed that intramuscular injection of a NNV DNA vaccine failed to protect Atlantic halibut (*Hippoglossus hippoglossus*) (Sommerset, *et al.*, 2005b) whilst more recently the oral administration of an encapsulated DNA vaccine to Asian sea bass (*Lates calcarifer*) resulted partly protective against NNV (Vimal, *et al.*, 2014). However, further studies are needed to improve their efficiency and applicability in fish farms to control the infections and dissemination of this important virus.

In this study, we orally vaccinated healthy specimens of European sea bass juveniles with a specific DNA vaccine against NNV, encapsulated into chitosan nanoparticles, aiming to study whether the vaccine stimulates the immune response in the gut of the fish and provokes a decrease of the mortality rate after challenge. Our results point to the activation of a cell-mediated cytotoxicity (CMC) status and the use of IFN pathways in the gut of vaccinated fish that resulted in improving the survival against an *in vivo* NNV challenge after 3 months of vaccination.

2. Material and methods

2.1. Animals

Juveniles of the marine teleost European sea bass (*Dicentrarchus labrax*) (125 days post-hatching, dph; 6.02 ± 0.70 g body weight) were bred and reared in the *Centro Oceanográfico de Mazarrón, Instituto Español de Oceanografía* (COM-IEO). 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) (Permit Number: A13150104) and the *Instituto Español de Oceanografía* (Spain) (Permit Number: 2010/02) for the use of laboratory animals.

2.2. Nodavirus stocks

Nodavirus (strain 411/96, genotype RGNNV) was propagated in the SSN-1 cell line which is persistently infected with a snakehead retrovirus (SnRV) (Frerichs, *et al.*, 1996). The SSN-1 cells were grown at 25°C in Leibovitz's L15-medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 IU ml^{-1} penicillin (Gibco), 100 µg ml^{-1} streptomycin (Gibco) and 2.5 µg ml^{-1} fungizone (Gibco) using Falcon Primaria cell culture flasks (Becton Dickinson). Cells were inoculated with NNV and incubated at 25ºC until the cytopathic effect was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates according to a protocol previously described (Reed and Müench, 1938) before used in the experiments.

2.2. Plasmid constructs

For the construction of the NNV DNA vaccine (CP-pNNV), the entire open reading frame of the RNA2 gene (genotype RGNNV, strain 411/96) was amplified by a polymerase chain reaction (PCR) from a cDNA sample obtained from the NNV culture (Table 1), containing both the start and stop codons.

The PCR product was cloned into the expression vector pcDNA3.1/V5-His-TOPO according to manufacturer´s instructions (Invitrogen) and used to transform One Shot TOP10 *Escherichia coli* cells (Invitrogen). A clone containing the CP-pNNV was identified by PCR screening, and the proper orientation was verified by sequencing. A religated empty pcDNA3.1/V5-His-TOPO plasmid (pcDNA3.1) was used as a negative control.

2.3. Preparation of vaccine

We synthesized the nanoparticles by complexing high-molecular-weight (about 390,000 Da) chitosan (Sigma) with DNA plasmid. Chitosan was dissolved in 1% acetic acid with gentle heating and adjusted to pH 5.7. The solution was then sterile-filtered through a 0.45 µm filter and adjusted to pH 5.5. Several concentrations of chitosan (w/v; 0.02, 0.04, 0.06 and 0.10%) were prepared and mixed with equal volumes of plasmid (200 µg/ml in 25 mM of sodium sulphate solution) at 55°C and rapidly mixed and vortexed at maximum speed for 45 seconds. The resulting polyplexes were kept at room temperature for 30 min for stabilization. The DNA loading efficiency in the

chitosan particles was measured by spinning a sample at 13,000 g for 15 min and measuring the resulting DNA in the supernatant at 260 nm. In addition, the vaccine formulation (CP-pNNV) was studied by transmission electron microscopy to assess the heterogeneity and size of the chitosan-DNA nanoparticles. Briefly, 5 µl of the vaccine formulation were placed onto formvar-carbon-coated 400 mesh copper grids, fixed with 2% glutaraldehyde for 5 min, washed and negatively stained with 2% phosphotungstic acid, pH 7, for 1 min. Samples were then examined under a Tecnai 12 transmission electron microscope (Phillips).

Table 1: Sequence of the oligonucleotides used. Underlined are the start and stop codons.

Gene	Symbol	Acc. number		Sequence (5'3')	Use
Nodavirus capsid protein	NNV RNA2	D38636	F	ACAATGGTACGCAAAGGTGATAAG	PCR cloning
			R	TTAGTTTTCCGAGTCAACACGG	
			F	CGTGTCAGTCATGTGTCGCT	Conventional PCR
			R	CGAGTCAACACGGGTGAAGA	
Interferon	*ifn*	AM765847	F	GGCTCTACTGGATACGATGGC	Real-time PCR
			R	CTCCCATGATGCAGAGCTGTG	
Interferon gamma	*ifng*	KJ818329	F	TCAAGATGCTGAGGCAACAC	
			R	AGTGCTTTGCTCTGGACGAC	
Interferon-induced GTP-binding protein Mx	*mx*	AM228977, HQ237501, AY424961	F	GTCTGGAGATCGCCTCT	
			R	TCTCCGTGGATCCTGATGGAGA	
MHC class I alpha	*mhc1a*	AM943118	F	GGACAGACCTTCCCTCAGTG	
			R	TCCAGATGAGTGTGGCTTTG	
MHC class II beta	*mhc2b*	AM113466	F	CAGAGACGGACAGGAAG	
			R	CAAGATCAGACCCAGGA	
Immunoglobulin mu heavy chain	*igmh*	FN908858	F	AGGACAGGACTGCTGCTGTT	
			R	CACCTGCTGTCTGCTGTTGT	
Immunoglobulin tau heavy chain	*ight*	FM010886	R	TCACTTGGCAAATTGATGGA	
			F	AGAACAGCGCACTTTGTTGA	
CD8 alpha	*cd8a*	AJ846849	F	CTGTCCTCCGCTCATACTGG	
			R	TTGTAATGATGGGGGCATCT	
T cell receptor beta chain	*tcrb*	FN687461	F	GACGGACGAAGCTGCCCA	
			R	TGGCAGCCTGTGTGATCTTCA	
Elongation factor 1 alpha	*ef1a*	FM019753	F	CGTTGGCTTCAACATCAAGA	
			R	GAAGTTGTCTGCTCCCTTGG	

2.4. Fish vaccination

In all cases, the optimal chitosan formulations were prepared with 0.04% of chitosan (w/v). Diets were obtained by spreading the chitosan solutions onto the commercial diet and allowing the pellet to dry. European sea bass juveniles (125 dph of age) were randomly distributed and fed for two days with the following formulations into the commercial diet: diet alone (control), chitosan particles (ChP), chitosan

particles containing the empty plasmid (ChP-pcDNA3.1) or chitosan particles containing CP-pNNV (ChP-CP-pNNV). Fish received the ChP-pcDNA3.1 and ChP-CP-pNNV diets containing approximately 10 µg per fish of plasmid. Fish (n = 6 fish/group and time) were sampled at 7, 30 and 90 days after oral vaccination. The posterior region of gut was removed, immediately frozen in TRIzol® Reagent (Life Technologies) and stored at -80ºC for later RNA isolation to evaluate the transcription of immune-related genes since this part of the gut is the richest in B and T lymphocytes (Ballesteros, *et al.*, 2013; Picchietti, *et al.*, 2011). The blood was obtained from the caudal peduncle of specimens after 90 days of vaccination and the serum samples were obtained by centrifugation at 10,000 g during 1 min at 4ºC, and immediately frozen and stored at -80ºC until used to evaluate the immunoglobulin M (IgM) levels.

2.5. Serum IgM levels

Total serum IgM levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) (Cuesta, *et al.*, 2004). Thus, 20 µl per well of 1/100 serum diluted in PBS pH 7.4 containing 0.05% Tween 20 (PBS-T; Sigma) were placed in flat-bottomed 96-well plates (Nunc) in triplicate and the proteins were coated by overnight incubation at 4ºC with 200 µl of carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBS-T, the plates were blocked for 2 h at room temperature with blocking buffer: PBS-T containing 3% bovine serum albumin (BSA, Sigma), followed by three rinses with PBS-T. The plates were then incubated for 1 h at room temperature with 100 µl per well of mouse anti-sea bass IgM monoclonal antibody (Aquatic Diagnostics Ltd.) at the optimal dilution of 1:100 in blocking buffer, washed and incubated with the secondary antibody anti-mouse IgG-HRP (Sigma) at the optimal dilution of 1:1000 in blocking buffer. After exhaustive rinsing with PBS-T the plates were developed using 100 µl of a 0.42 mM TMB solution, daily prepared in distilled water, containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µl of 2 M H_2SO_4. The plates were read at 450 nm. Negative controls consisted of samples without serum or without primary antibody, whose OD values were subtracted for each sample value.

Serum specific IgM levels against NNV were analyzed using a slightly modified ELISA (Scapigliati, *et al.*, 2010). Briefly, 100 µl of purified NNV preparation diluted 1:5 with 50 mM carbonate-bicarbonate buffer pH 9.6 were employed to coat flat-

bottomed 96-well plates overnight at 4ºC. After three rinses with PBS-T, the plates were blocked for 2 h at room temperature with blocking buffer, followed by four rinses with PBS-T. Then, 100 µl of serial dilutions (1:50 to 1:200) of sera (n = 6 fish/group and time) diluted in PBS-T were incubated for 2 h at room temperature, followed by five rinses with PBS-T. The plates were then incubated with mouse anti-sea bass IgM monoclonal antibody and secondary anti-mouse IgG-HRP as above. The absorbance was read at 450 nm. Negative controls consisted on samples without serum or without coating and positive controls that consisted on sera from *in vivo* infected sea bass with NNV after 15 days were also used in the ELISA (Chaves-Pozo, *et al.*, 2012b). The OD values of negative controls were subtracted for each sample value.

2.6. Gene expression by real-time PCR

Total RNA was isolated from TRIzol Reagent frozen samples following the manufacturer's instructions. One µg of total RNA from each individual fish was treated with DNAse I to remove genomic DNA and the first strand of cDNA synthesized by reverse transcription using the ThermoScript^{TM} RNAse H^{-} Reverse Transcriptase (Invitrogen) with random hexamers (Invitrogen) followed by RNAse H (Invitrogen) treatment.

The vaccine expression was analysed by conventional PCR performed with a thermocycler (Mastercycler, Eppendorf). Reaction mixtures were incubated for 2 min at 94ºC, followed by 35 cycles of 45 s at 94ºC, 45 s at the specific annealing temperature of 58ºC, 1 min at 72ºC, and finally 10 min at 72ºC. Primers used are shown in Table 1. For visualizing and comparing the groups, the PCR products were run on a 1% agarose gel.

Real-time PCR 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 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1 alpha coding gene (*ef1a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. Primers used are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for

specificity. 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.

2.7. Nodavirus challenge

Three months after oral vaccination, 24 fish per group were transported to the University of Murcia aquaria. Fish were randomly divided into two tanks per group, kept in 30 L running seawater (28‰ salinity) aquaria at 24 – 26ºC, with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the experiments. Fish were fed daily with a commercial pellet diet (Skretting). Each group received a single intramuscular injection of 100 µl culture medium containing 10^6 $TCID_{50}$/fish of NNV (n = 24 fish/group) since this route of infection has been proven as the most effective (Aranguren, *et al.*, 2002). Mortality was recorded daily through the challenge and relative percent survival (RPS) was determined:

RPS =1 – [(% mortality in vaccinated fish) / (% mortality in control fish)] × 100.

2.8. Statistical analysis

Gene expression data were analysed by one-way ANOVA to denote statistical differences between groups at different sampling times ($P \leq 0.05$). A non-parametric Kruskal–Wallis assay, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Gene expression data are presented as mean ± standard error of the mean (SEM; n = 6 fish/group and time). Letters denote statistical differences between groups at the same time point (lowercase letters = 7 days, capital letters = 30 days, underlined lowercase letters = 90 days). Cumulate survival was represented for all treatments as mean ± SEM (n = 2 replicates) and analysed by a two-way ANOVA followed by Tukey's post-hoc analysis. All statistical analyses were conducted using SPSS 20 application.

3. Results

It is worthy to note that the expression of the plasmid coding for NNV detection by PCR analysis in the posterior gut from European sea bass vaccinated specimens resulted undetected for all the animals (data not shown).

3.1. Characteristics of the chitosan-DNA nanoparticles

The loading efficiency of chitosan nanoparticles with all chitosan concentrations and plasmid DNA amounts assayed; ranged from 84.71 to 97.47% of total plasmid DNA (Table 2). The optimal loading efficiency was when 0.04% chitosan concentration and 200 µg/ml of plasmid DNA were used (Table 2), beginning in turn the one selected for formulating the vaccine. Transmission electron microscopy showed that freshly prepared particles are approximately 0.05-0.2 µm in size and fairly spherical (Fig. 1).

Table 2: Loading efficiency of plasmid DNA (% of total plasmid DNA used to produce the particles) in the chitosan particles produced with different chitosan concentrations.

Plasmid DNA (µg/ml)	**Chitosan concentration (%)**			
	0.02	0.04	0.06	0.10
85	84.71	87.93	91.03	97.24
200	62.30	97.47	92.18	88.62

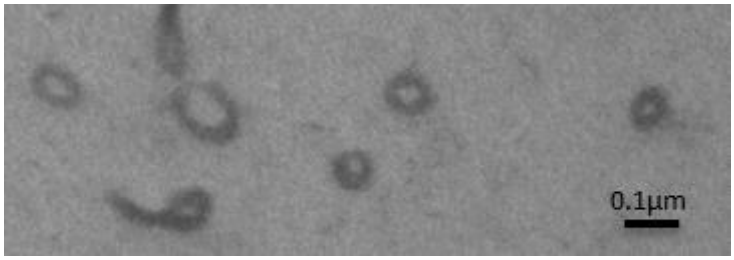

Figure 1: Appearance and size distribution of chitosan-DNA nanoparticles by transmission electron microscopy.

3.2. Encapsulated DNA vaccine failed to induce the production of specific IgM antibodies

Serum IgM levels were analysed at 90 days post-vaccination (Fig. 2) and no significant changes in the total serum IgM levels were detected between groups (Fig. 2A). Moreover, anti-NNV specific IgM levels were undetected in any experimental group and only detected in intramuscularly *in vivo* infected specimens at day 15 of infection (Fig. 2B). In addition, we evaluated the *igmh* and *igth* transcription levels at 7, 30 and 90 days post-vaccination. We found significant differences in ChP-pcDNA3.1 and ChP-CP-pNNV groups comparing to control and ChP groups only after 7 days post-vaccination in the transcription of *igmh* (Fig. 2C). In the case of *igth* transcription, sea

bass specimens receiving the ChP-CP-pNNV vaccine significantly increased this gene expression compared to control group but not to ChP-pcDNA3.1 group after 30 and 90 days of vaccination. (Fig. 2D).

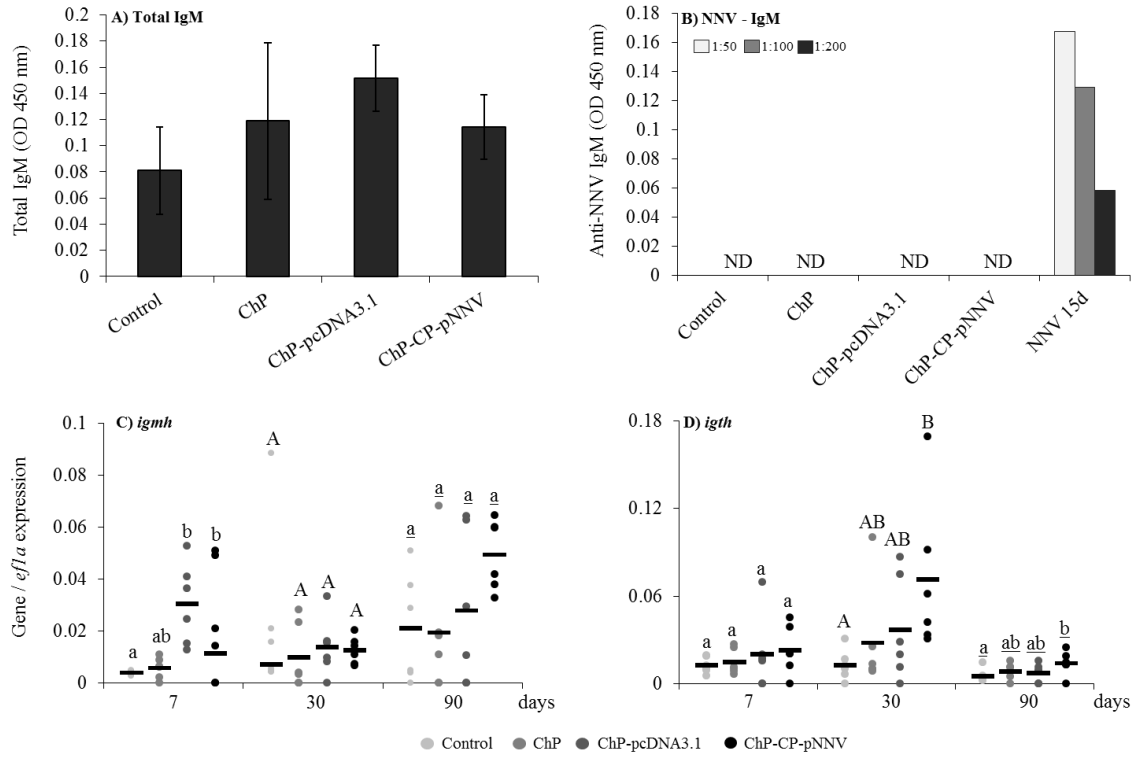

Figure 2: Total (A) or specific anti-NNV (B) IgM in the serum of European sea bass specimens after 90 days of vaccination. Transcription of *igmh* (C) and *igth* (D) genes in the posterior region of the gut of European sea bass. Data represent the mean ± standard error of the mean (SEM; n = 6/group and time). Significance level (P) was fixed at 0.05. Letters denote statistically differences between groups at the same time point (lowercase letters = 7 days, capital letters = 30 days, underlined lowercase letters = 90 days). ND, not detected.

3.3. Oral DNA vaccine did not induced antigen presentation

We evaluated the antigen presentation by the expression of MHC class I and II genes, *mhc1a* and *mhc2b*, respectively (Fig. 3). Interestingly, the ChP-CP-pNNV oral vaccine failed to up-regulate their transcription in posterior gut except the *mhc1a* gene expression that was up-regulated at 7 days post-vaccination comparing to control and ChP groups (Fig. 3A).

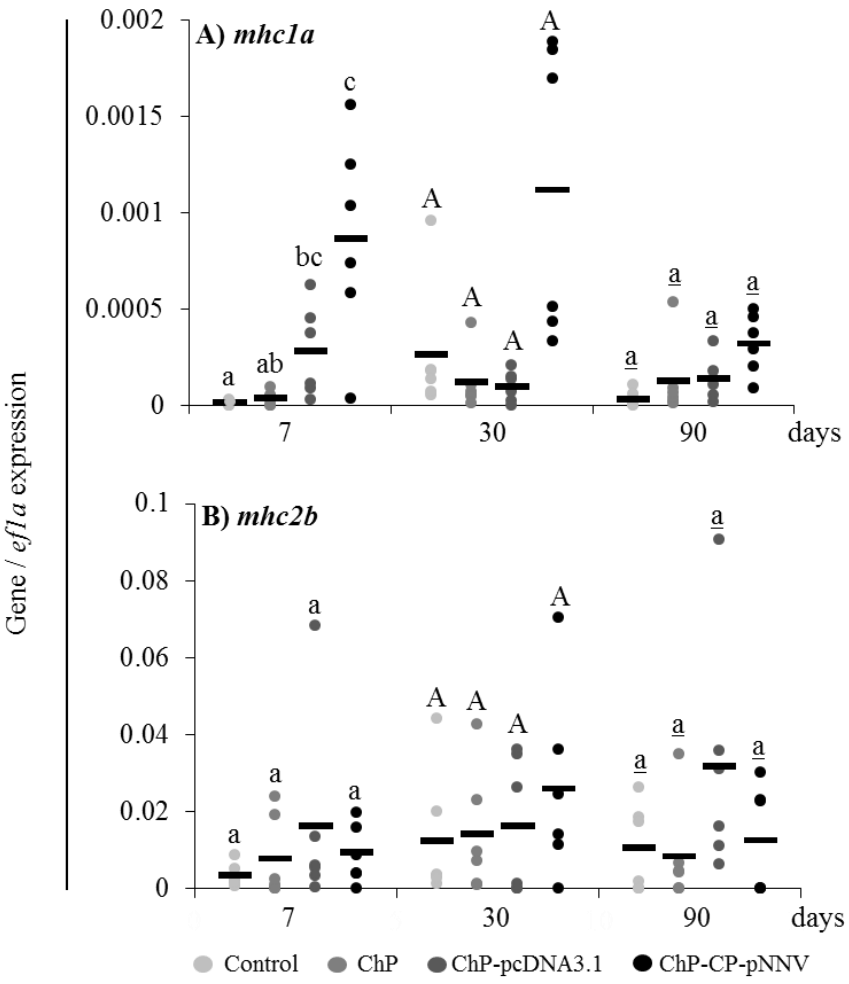

Figure 3: Expression level of genes related to antigen presentation, *mhc1a* (A) and *mhc2b* (B), in the posterior region of the gut of European sea bass after 7, 30 and 90 days of vaccination. Data represent the mean ± SEM (n = 6/group and time).

Significance level (P) was fixed at 0.05.

Letters denote statistically differences between groups at the same time point (lowercase letters = 7 days, capital letters = 30 days, underlined lowercase letters = 90 days).

3.4. The oral vaccine stimulated the expression of CMC related genes

The vaccine enhanced the expression in the posterior gut of some CMC genes as *tcrb* and *cd8a* (Fig. 4). Interestingly, *tcrb* gene expression was up-regulated in ChP-CP-pNNV group after 7 and 90 or 30 days of vaccination when compared to control or ChP groups, respectively; and with the ChP-pcDNA3.1 group at 7 and 90 time points (Fig. 4A). Otherwise, *cd8a* gene was up-regulated after 30 and 90 days post-vaccination in ChP-CP-pNNV group comparing to control (Fig. 4B). Regarding the ChP-pcDNA3.1 group, it showed stimulation of the *tcrb* gene expression after 7 days of vaccination and *tcrb* and *cd8a* gene expression after 7 and 90 days of vaccination comparing to control group (Fig. 4).

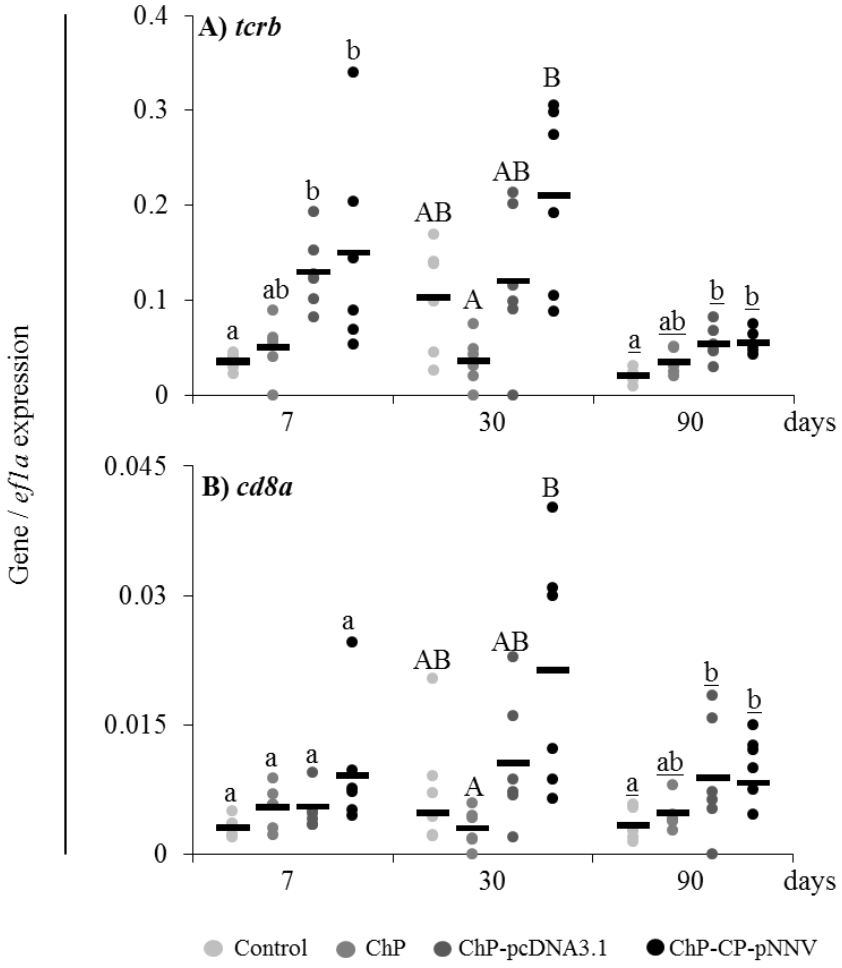

Figure 4: Expression level of genes related to CMC response, *tcrb* (A) and *cd8a* (B), in the posterior region of the gut of European sea bass after 7, 30 and 90 days of vaccination. Data represent the mean ± SEM (n = 6/group and time). Significance level (P) was fixed at 0.05. Letters denote statistically differences between groups at the same time point (lowercase letters = 7 days, capital letters = 30 days, underlined lowercase letters = 90 days).

3.5. Type I and II IFN were induced by the oral DNA vaccine

Finally, we analysed the expression of genes related to type I (*ifn* and *mx*) and II (*ifng*) IFN response (Fig. 5). Thus, in ChP-pcDNA3.1 group, the transcription of *ifn* (Fig. 5A) and *mx* (Fig. 5B) was significantly up-regulated in CP-pNNV group at day 30 and 7 and 30, respectively. The expression level of *ifng* gene was significantly up-regulated in the ChP-CP-pNNV group after 30 and 90 days of vaccination. Interestingly, ChP-pcDNA3.1 group showed enhanced expression levels when compared with ChP group (Fig. 5C).

3.6. European sea bass resistance against NNV infection was improved by the oral encapsulated DNA vaccine

Infection challenge with NNV was performed in two replicates after three months of oral vaccination (Fig. 6). Two or three days after challenge, the fish control and ChP groups developed the first symptoms of VER disease, as erratic swimming and

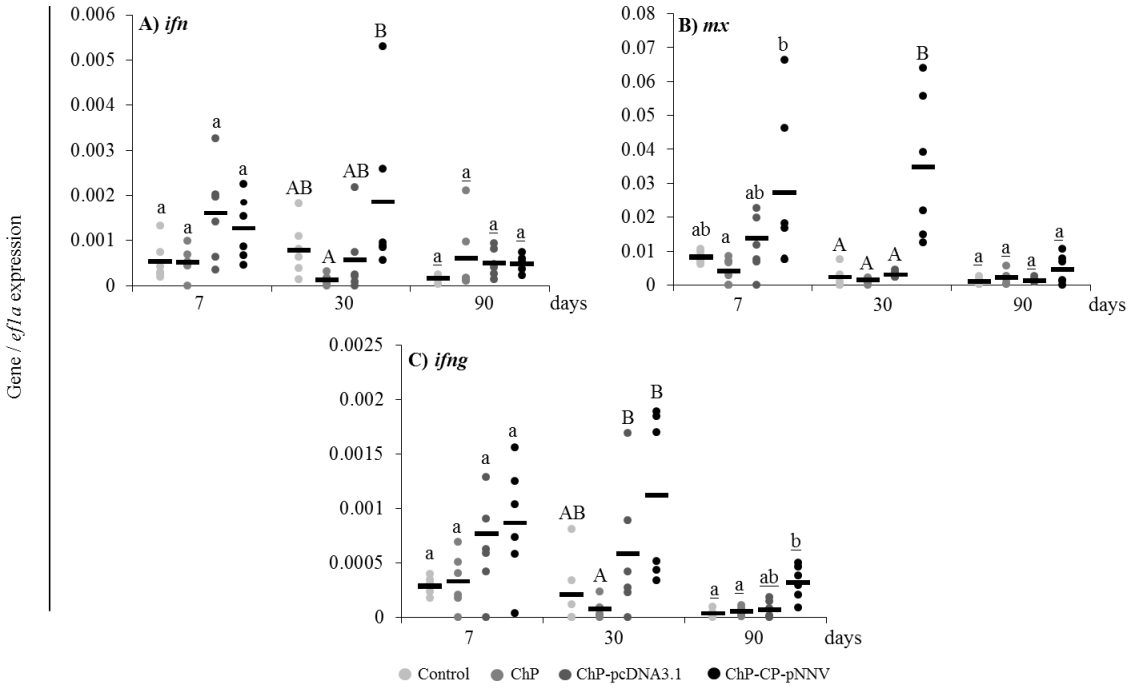

Figure 5: Expression levels of the type I, *ifn* (A), *mx* (B), and II, *ifng* (C), interferon response genes in the posterior region of the gut of European sea bass after 7, 30 and 90 days of vaccination. Data represent the mean ± SEM (n = 6/group and time). Significance level (P) was fixed at 0.05. Letters denote statistically differences between groups at the same time point (lowercase letters = 7 days, capital letters = 30 days, underlined lowercase letters = 90 days).

changes in skin colour (data not shown), starting to dye after 3 or 4 days of challenge in control or ChP groups, respectively (Fig. 6). Mortality reached 100% at day 19 in control group and at day 21 in ChP group. Interestingly, and although the fish from the ChP-pcDNA3.1 group showed a delay on the onset of mortalities that started at day 17 post-challenge, the mortality recorded in this group was of 100% at day 22 (Fig. 6). In contrast, and although the fish from ChP-CP-pNNV group started to dye at day 21 of infection, they did not show very clear signs of disease (data not shown) and showed a RPS of 45% at the end of the challenge.

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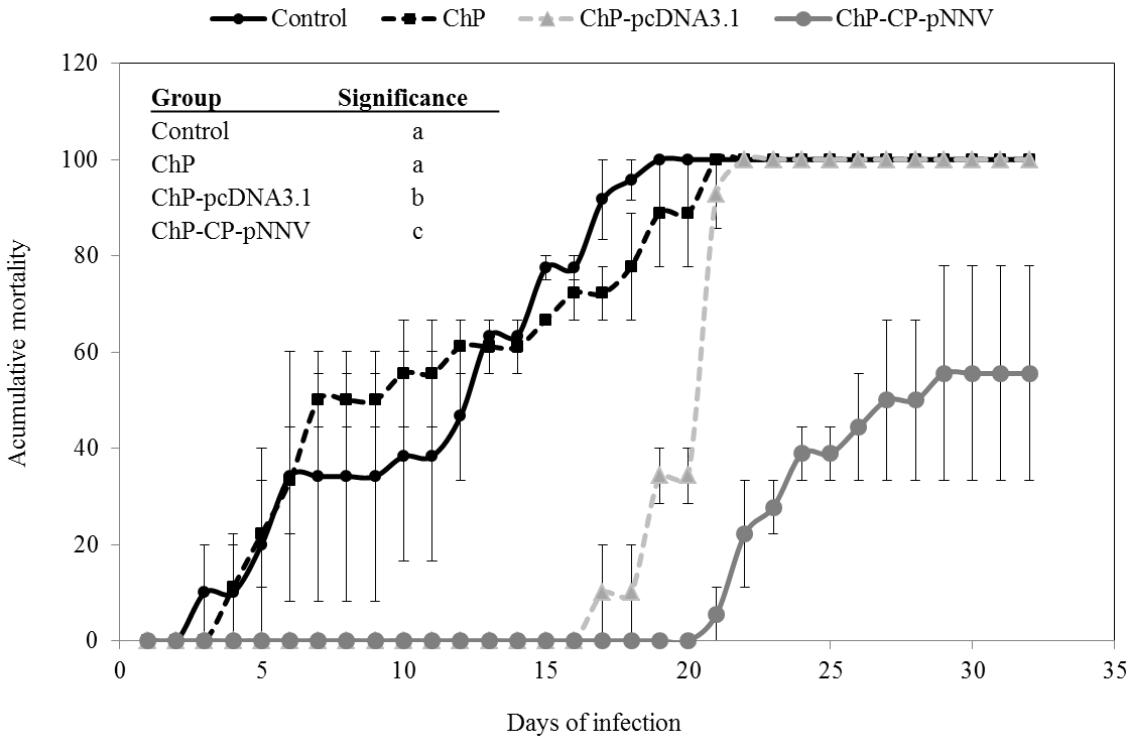

Figure 6: Accumulative mortality in orally vaccinated European sea bass juveniles after intramuscular injection with 10^6 $TCID_{50}$ NNV per fish 3 months after vaccination. Curves show the mean mortality ± SEM (n = 2 replicates). Statistical analysis was performed by two-way ANOVA followed by Tukey's post-hoc analysis. Inset: Different letters indicate significant differences ($P < 0.05$) among groups.

Two-way ANOVA found significant differences among groups ($P = 6.61\times10^{-57}$), time ($P = 5.3\times10^{-62}$) and between the two factors ($P = 2.3\times10^{-16}$). Furthermore, Tukey's analysis found significant differences among the groups (Fig. 6 inset) being Control and ChP groups similar but different to ChP-pcDNA3.1 and ChP-CP-pNNV groups as well as the ChP-pcDNA3.1 and ChP-CP-pNNV groups were significantly different.

4. Discussion

Chitosan nanoparticles seem to be ideal for DNA encapsulation and vaccine administration by oral intake because of its natural characteristics. Thus, chitosan nanoparticles are non-toxic for animals or even humans (Rao and Sharma, 1997). Moreover, their positively charged surface, complex stability at physiological pH conditions allow them to protect the encapsulated DNA plasmid from nuclease degradation in the gut (MacLaughlin, *et al.*, 1998; Mao, *et al.*, 1996). In addition, sodium alginate or chitosan also act by themselves as immunostimulants, offering and additional improvement of the immune response (Abu-Elala, *et al.*, 2015; Cheng and Yu, 2013; Fujiki, *et al.*, 1994; Lin, *et al.*, 2011). However, we did not observe any immune stimulation due to chitosan dietary intake, but we found an up-regulation of the *igmh*, *mhc1a*, *tcrb*, *cd8a* and *ifn* gene expression levels in the ChP-pcDNA3.1 group concomitantly with a delay of the start of mortality of challenged fish, indicating these data, some protective effect of the empty plasmid as previously described in other studies dealing with DNA vaccination (Kim, *et al.*, 2000; Liang, *et al.*, 2010).

We failed to detect any NNV capsid gene transcription in the posterior gut of vaccinated fish after 7 days onwards. This issue could mean that the DNA vaccine shows a very short and transitory expression by the host while at the same time confers partial protection. Our post-vaccination results also showed no alterations in total serum IgM levels in any group after 90 days of vaccination and we failed to detect specific IgM antibodies against NNV in any vaccinated group while we did in sera from sea bass specimens infected with NNV. Interestingly, the expression of *mhc2b* and *igmh* genes was unchanged in the posterior gut of vaccinated sea bass at all time points except at 7 days post-vaccination, where we found an up-regulation of *igmh* gene expression in ChP-pcDNA3.1 and ChP-CP-pNNV groups similarly to what happened with the *igth* transcription after 30 and 90 days post-vaccination. Moreover, many of the studies dealing with DNA vaccines in fish, regardless the administration route used, demonstrate that this type of vaccine elicit acquired immunity by producing specific antibodies against virus at different time point that range from 1 month to 6 month post-vaccination (de las Heras, *et al.*, 2010; MacLaughlin, *et al.*, 1998; Vimal, *et al.*, 2014; Zheng, *et al.*, 2010). However, in other cases and times assayed, specific antibodies were not detected despite good protection observed (Kurath, 2008; Lorenzen, *et al.*, 1998; McLauchlan, *et al.*, 2003). Our data are in concordance with these last data

previously described due to the fact that even when we did not detect specific antibodies against NNV, we observed a clear delay in the beginning of mortalities and a RPS of 45% at the end of the trial in ChP-CP-pNNV vaccinated fish. All this data point to specific cellular antiviral mechanisms involved in fish protection upon vaccination with DNA vaccines. In that sense, and taking into account that the IFN pathway and the CMC are considered as the major immune mechanisms to fight viral infections in fish (Ellis, 2001; Robertsen, 2006), we studied those mechanism at gene expression levels. Our data showed that the orally administrated vaccine stimulated the expression of the *tcrb* and *cd8a* genes, pointing to the activation of a stimulated CMC status in the gut. This is the first study in which a prominent role of the CMC immune response triggered by an encapsulated DNA vaccine orally administrated has been reported in fish, although it had also been demonstrated in mammals and other vertebrates (Jazayeri, *et al.*, 2012; Nixon, *et al.*, 1996). Interestingly, one study also reported an increase in the CMC antiviral activity after intramuscular DNA vaccination in fish (Utke, *et al.*, 2008). Moreover, in previous *in vivo* infections with NNV, we observed that innate CMC activity, as well as some gene transcription as NCCRP-1, related to this activity, were greatly activated, whereas other immune responses played by phagocytes (monocyte-macrophages and granulocytes) seem to be less relevant upon NNV infection in the European sea bass (Chaves-Pozo, *et al.*, 2012b). Furthermore, it is demonstrated that infected orange-spotted grouper (*Epinephelus coioides*) increased the *cd8a* gene expression as well as the number of CD8+ lymphocytes and the specific cytotoxic activity against NNV-infected cells, in a MHC I-restricted manner, but this specific CMC activity does not appear in all fish species (Chang, *et al.*, 2011; Patel, *et al.*, 2008; Scapigliati, *et al.*, 2010).

As expected, the protection was also orchestrated by an early phase of non-specific IFN mechanisms due to the up-regulating expression of *ifn*, *mx* genes at 30 or 7 and 30 days, respectively and of *ifng* gene at 30 and 90 days post-vaccination. In fact, this *ifng* up-regulation also points to the activation of the CMC since cytotoxic cells are the main producers of IFNγ. These results are in concordance with those found in juvenile specimens of European sea bass upon NNV infection *in vivo*, where different genes involved in the IFN pathway were greatly stimulated in brain (target tissue of the virus) and gonad (Valero, *et al.*, 2015c), suggesting the same unspecific immune response after vaccination. Similarly to our data, in another DNA vaccination study

performed in rainbow trout an early enhancement of IFN mechanisms were reported (Utke, *et al.*, 2008).

Independently of the mechanism of action of the oral DNA vaccine used in this study, our data showed that after 90 days post-vaccination, the ChP-CP-pNNV vaccinated fish showed a clear and complete protection against NNV until day 21, when mortality started. Taking into account that mortality on control and ChP groups started around day 4 and reached 100% before day 21 post-challenge and that the 45% of the ChP-CP-pNNV vaccinated fish kept alife at the end of the challenge, we can concluded that the encapsulated DNA vaccine used in this study protect the fish upon NNV infection.

5. Conclusions

In conclusion, our results showed that the designed oral DNA vaccine against NNV encapsulated in chitosan nanoparticles, elicits CMC and IFN immune responses in the posterior gut, but failed to trigger the production of specific antibodies after 90 days of vaccination. However, at this time, the infection with a lethal dose of NNV resulted in a relative percent of survival of 45 % of the vaccinated fish. Although this protection is slightly lower than the one observed in other studies related, most of them evaluate the protection at few weeks or a month post-vaccination but not at longer time as our study (3 months). Therefore, further studies are needed to understand the immune response in the gut after oral DNA vaccination and to improve the oral DNA vaccines against NNV in order to ameliorate or abolish the incidence of VER disease and subsequent mortalities.

III.2.2. MATERNAL TRANSFER OF INNATE IMMUNITY UPON DNA VACCINATION AGAINST NODAVIRUS IN TELEOST FISH

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1. Introduction

Fish in early stages of development (larval development) are in close contact to all environmental pathogens and are vulnerable since their immune system is not fully developed yet, being essential the maternal transfer of innate and acquired factors (Zhang, *et al.*, 2013). Fish immunocompetence is acquired during first stages of larval development, however; the moment when it is completely carried out is highly varied and depends on the age and the specie (Mulero, *et al.*, 2007a). In the case of European sea bass (*Dicentrarchus labrax*), T lymphocytes emerge in early stages during larval development, between 5-12 days post hatching (dph) whether B lymphocytes with cytoplasmic immunoglobulins (Igs) appear around 52 dph. However, adult levels of T and B lymphocytes are reached between 137-145 dph, suggesting immunologically maturity in this species at this age (Dos Santos, *et al.*, 2000). As European sea bass larvae complete immunocompetence is reached at around 50 dph, the maternal transfer of immune factors (innate and adaptive related ones) is crucial for larvae survival (Magnadottir, *et al.*, 2005; Mulero, *et al.*, 2007a). Thus, in teleost fish, maternally-derived substances such IgM and some innate immune factors such as several complement proteins, lectins, protease inhibitors, lysozyme or cathelicidin have been reported to be transferred to oocytes together with vitellogenin (Vg) (Swain and Nayak, 2009; Zhang, *et al.*, 2013). Apart from the protein transmission to oocytes, transfer of mRNAs is also possible due to the fact that a significant level of *igm*, complement component C3 (*c3*), *transferrin*, transforming growth factor beta 1 (*tgfb1*), interleukin 1 beta (*il1b*) and lysozyme (*lyz*) gene transcripts have been detected in the eggs of some species of teleost, decreasing later on during development until the onset of the own larvae gene expression (Mingming, *et al.*, 2014). In the European sea bass, different studies have described lysozyme activity in different developmental stages being detected for the first time in newly fertilised eggs (Cecchini, *et al.*, 2000), as well as other molecules such as cathepsins, which may also have bactericidal role in other species, and are detectable from morula stages (12-16 cells) at about 3.2 hours post fertilization (hpf) (Carnevali, *et al.*, 2001; Cho, *et al.*, 2002; Cucchi, *et al.*, 2011). In addition, IgM maternal transfer has also been demonstrated in European sea bass specimens (Breuil, *et al.*, 1997; Picchietti, *et al.*, 2004).

Nodavirus (NNV) is one of the most devastating virus spread worldwide (Haddad-Boubaker, *et al.*, 2013; Munday, *et al.*, 2002). NNV is a non-enveloped

bipartite and positive single-stranded RNA virus which caused up to 100% mortalities on larvae and juveniles of European sea bass (Breuil, *et al.*, 2002; OIE, 2013), stages which are difficult to be vaccinated due to their immune system immaturity or their small size. Recently, broodstock vaccination has become an important strategy to fight against larvae infections. Thus, bacterial or viral inactivated vaccines reduce the mortality of the progeny from vaccinated-broodstock concomitantly with the enhancement of IgM and non-specific humoral immune parameters levels on eggs (Hanif, *et al.*, 2004, 2005; Kai, *et al.*, 2010). Despite the fact that the innate immune response is one of most powerful tools in the control of pathogens and that antimicrobial peptides (AMPs) have a relevant role regarding host defences against viruses (Uribe, *et al.*, 2011; Valero, *et al.*, 2013), few studies focused on this type of immunity after NNV vaccination (Chen, S.P., *et al.*, 2014; Kai, *et al.*, 2014; Overgård, *et al.*, 2013). Taking into account that DNA vaccines elicit low levels of specific IgMs, but high effectiveness in protection (reviewed by Hølvold, *et al.*, 2014), in this study we describe the maternal transfer of several antimicrobial activities and some AMP transcripts upon DNA vaccination of the females of a European sea bass broodstock compared with a sham-vaccinated one.

2. Material and Methods

2.1. Animals

Mature specimens of European sea bass (*Dicentrarchus labrax*), 3 years old, with a mean body weight (bw) of 1,518 ± 85 g were bred and kept at the facilities of the *Centro Oceanográfico de Murcia, Instituto Español de Oceanografía* (COM-IEO) in Mazarrón. All specimens studied were handled in accordance with the Guidelines of the European Union Council (2010/63/UE), the Bioethical Committee of the IEO and the Bioethical Committee of the University of Murcia (reference REGA ES300305440012).

2.2. Vaccine constructs

For the construction of the NNV DNA vaccine (CP-pNNV), the entire open reading frame of the RNA2 gene (strain 411/96, genotype RGNNV) was amplified by a polymerase chain reaction (PCR) from a cDNA sample obtained after random primer retrotranscription of the NNV RNA2 obtained from a NNV culture on SSN-1 cell line, containing both the start and stop codons. The PCR product was cloned into the

expression vector pcDNA3.1/V5-His-TOPO according to manufacturer´s instructions (Invitrogen) and used to transform One Shot TOP10 *Escherichia coli* cells (Invitrogen). A clone containing the CP-pNNV was identified by PCR screening, and the proper orientation was verified by sequencing. A religated empty pcDNA3.1/V5-His-TOPO plasmid (pcDNA3.1) was used as a negative control.

2.3. Experimental design and sampling procedure

Forty adult fish were divided into two groups formed by 5 females and 15 males each. All fish were anesthetised in clove oil (20 ppm) and fluently males and females were identified after a gentle abdominal massage. A subcutaneous magnetic tag code was implanted in each female which was also intramuscularly injected with 50 µl of 1 µg/µl of CP-pNNV in 0.01 M phosphate buffer saline (PBS, pH 7,2) or with 1 µg/µl of pcDNA3.1 in 0.01 M PBS as mock-vaccinated fish (Control). The specimens were vaccinated twice: at day 0 (priming) and at day 15 (booster). Blood samples were taken at day 16 (24 h after booster) from the caudal peduncle of female specimens and the serum samples were obtained by centrifugation at 10,000 g during 1 min at 4ºC, and immediately frozen and stored at -80ºC until used. The experimental design of European sea bass female vaccination with CP-pNNV is shown in Fig. 1.

Afterwards, specimens were kept in a 15 m^3 round tank with natural sea water (38‰ salinity), temperature (around 13ºC) and photoperiod (Latitude 37.5667, Longitude -1.2; January-February). The first spawn was collected from each group and incubated in 13.5 m^3 round tanks with natural sea water (38‰ salinity) filtered through mechanical and biological substrates. The temperature was 13ºC at the beginning and increased naturally to reach 19ºC at the end of the experiment. The fry were kept in dark until 160º day. Afterwards, the larvae were kept with 16 h light:8 h dark photoperiod and a light intensity of 500 lux at the water surface. Larvae were fed with *Artemia nauplii* (Inve Animal Health) until 18 days post-hatching and with enriched Instar II *Artemia* until 45 days post-hatching and, afterwards, with commercial dry pellet diet (Skretting).

Three pools of eggs (500 mg each) were collected at 0 and 2 days post-fertilization (dpf), while three pools of larvae samples (300 mg each) were collected at 5, 7, 9, 12, 15, 19, 23, 26, 40 and 69 dpf (Fig. 1) and stored in TRIzol® Reagent (Invitrogen) at -80ºC for later gene expression analysis. Three pools of eggs (100 mg

each) and larvae (300 mg each) were homogenized in 1 ml of 0.01 M PBS. Eggs were sonicated on ice in 30 s cycles until complete homogenization and larvae were mechanically homogenized. Then, homogenates were centrifuged at 10,000 g for 10 min at 4ºC. Supernatants were collected and stored at -80ºC for later functional activities analysis.

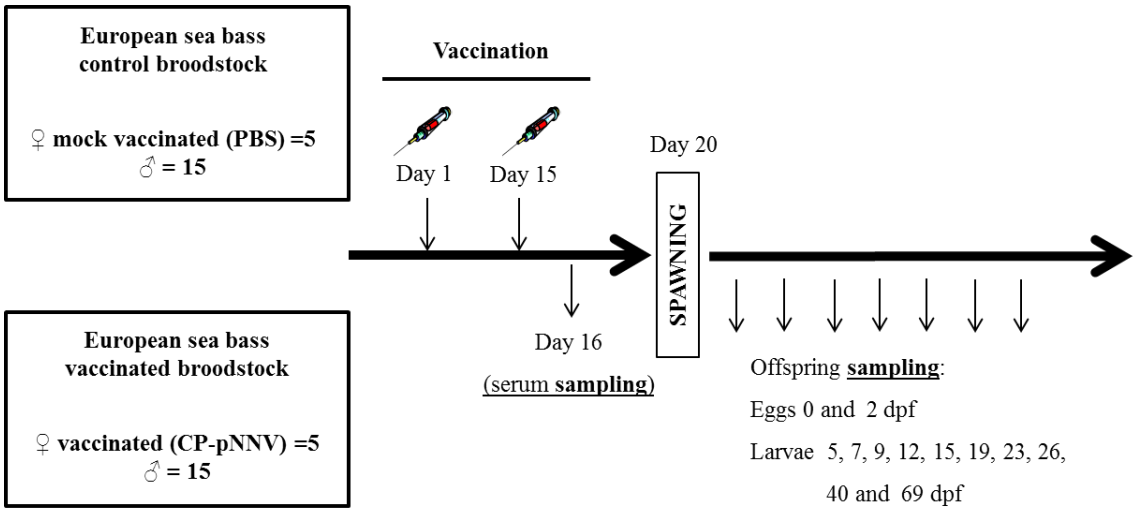

Figure 1: Flow diagram representing the experimental design of vaccination of broodstock females of European sea bass with a DNA vaccine against NNV (CP-pNNV), showing the time of vaccination and sampling of serum, eggs and larvae. Days post-fertilization (dpf).

2.4. Functional analysis

2.4.1. Peroxidase activity

The peroxidase activity in female serum, eggs or larvae homogenates was measured according to a protocol previously described (Quade and Roth, 1997). Briefly, 5 µl of serum, eggs or larvae homogenates was diluted with 45 µl of Hank's buffer (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates (Nunc). As substrate, 100 µl of 10 mM TMB (3.3',5.5'- tetramethylbenzidine hydrochloride) solution containing 0.015 % H_2O_2 was added. The colour-change reaction was stopped after 15 min by adding 50 µl of 2 M sulphuric acid and the optical density (OD) was read at 450 nm using a plate reader (MultiskanGo, Thermo Fisher Scientific). Wells with HBSS but without sample were used as blanks. Samples were run in triplicates. One unit was

defined as the amount of activity producing an absorbance change of 1 and the activity was expressed as U/ml of serum or U/mg of tissue.

2.4.2. Protease activity

Protease activity in serum, eggs or larvae homogenates was determined as the percentage of hydrolysis of azocasein using a modified protocol previously described (Charney and Tomarelli, 1947). Briefly, 10 µl of serum, eggs or larvae homogenates were incubated with 100 µl of 0.01 M PBS and 125 µl of 2% azocasein (Sigma) dissolved in 0.01 M PBS for 24 h at room temperature (RT). The reaction was stopped by adding 10% trichloroacetic acid (TCA) incubating for 30 min at RT. The mixture was centrifuged at 6,000 g for 5 min being 100 µl of the supernatants transferred to a flat-bottomed 96-well plate in duplicate. One hundred µl of 1 N NaOH were then added and the OD read at 450 nm using a plate reader. For a positive control, 10 µl of 2 mg/ml proteinase K (AppliChem) in 0.01 M PBS replaced the sample (100% of activity), and for a negative control, 0.01 M PBS replaced the sample (0% of activity). Samples were run in duplicates. The percentage of protease activity for each sample was calculated as % of the activity of the positive control. Results were expressed as % in serum or %/mg of tissue.

2.4.3. Anti-protease activity

Anti-protease activity was determined by the ability of serum, eggs or larvae homogenates to inhibit proteinase K activity using a modified protocol previously described (Ellis, 1990). Briefly, 10 µl of each sample were incubated during 10 min at RT with the same volume of 2 mg/ml proteinase K solution in 0.01 M PBS. After adding 100 µl of 0.01 M PBS and 125 µl of 2% azocasein dissolved in 0.01 M PBS (Sigma), samples were incubated during 2 h at RT. Afterwards, 250 µl of 10% TCA were added and an additional incubation during 30 min at RT was done. The mixture was then centrifuged at 6,000 g for 5 min and 100 µl of the supernatants transferred to a flat-bottomed 96-well plate. One hundred µl of 1 N NaOH were added and the OD read at 450 nm using a plate reader. For a positive control (100% of anti-protease activity), 0.01 M PBS replaced samples and proteinase K; and for a negative control (0% of anti-protease activity) 0.01 M PBS replaced only the sample. Samples were run in duplicates. The percentage of inhibition of proteinase K activity for each sample was

calculated as [100-(% of sample activity)]. Results were expressed as % in serum or %/mg of tissue.

2.4.4. Lysozyme activity

The lysozyme activity of serum, eggs or larvae homogenates was measured using a modified turbidimetric method previously described (Parry, *et al.*, 1965). Briefly, 100 µl of serum, eggs or larvae homogenates diluted 1:2 with 0.01 M PBS at pH 6.2, were placed in flat-bottomed 96-well plates in duplicate. To each well, 100 µl of 0.3 mg/ml freeze-dried *Micrococcus lysodeikticus* (Sigma) in phosphate citrate buffer [0.13 M disodium phosphate, 0.11 M citrate and 0.015 M NaCl, pH 6.2] was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured immediately every 30 s during 15 min at 22ºC using a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. A standard curve from 20 µg/ml to 0.3 µ/ml of hen egg white lysozyme (HEWL, Sigma) and a blank control (negative control) were established. Standards and samples were run in duplicates and all the measures were corrected with the blank. The results were expressed as U/ml of serum or U/mg of tissue.

2.4.5. Bactericidal activity

The pathogenic marine bacteria *Vibrio harveyi* (*Vh*) (strain Lg 16/100) was grown in agar plates at 25ºC in tryptic soy agar (TSA, Sigma). Then, fresh single colonies of 1-2 mm were diluted in 5 ml of tryptic soy broth (TSB; *Laboratorios Conda*), cultured for 16 h at 25°C on an orbital incubator at 200-250 revolutions per minute (rpm) and adjusted to 10^8 bacteria/ml of TSB.

The antibacterial activity of serum, eggs or larvae homogenates was determined by evaluating their effects on the bacterial growth of *Vh* curves using a method previously described (Sunyer and Tort, 1995). Aliquots of 100 µl of the bacterial dilution of *Vh* (1/10) were placed in flat-bottomed 96-well plates and cultured with 100 µl of European sea bass serum, eggs or larvae homogenates. The absorbance of the samples was measured at 620 nm every 30 min intervals during 36 h at 25ºC in a plate reader. Samples replacing bacteria by culture medium were used as blanks (negative control). Samples replacing serum or tissue homogenates by culture medium were used as positive controls (100% growth or 0% antibacterial activity). Samples and controls

were run in duplicates. Bactericidal activity was expressed as [100-(% of bacterial growth)]. Results were corrected with absorbance measured in each sample at initial time point and expressed as % of serum or %/mg of tissue.

2.5. Gene expression analysis

Total RNA was isolated from TRIzol Reagent® (Invitrogen) frozen samples following the manufacturer's instructions. One μg of total RNA was treated with DNAse I (1 unit/μg RNA, Promega) to remove genomic DNA. The first strand of cDNA was synthesized by reverse transcription using the Superscript III (Invitrogen) with an oligod$T_{12\text{-}18}$ primer followed by RNAse H (Invitrogen) treatment, for 60 min at 50ºC. The expression of the genes codifying for (i) leucocyte markers of granulocytes: myeloid-specific peroxidase (*mpx*); and macrophages: colony stimulating factor receptor 1 (*csfr1*); and (ii) AMPs: hepcidin (*hamp*), dicentracin (*dic*), complement factor 3-1 and -2 (*c3*), lysozyme (*lyz*) and NK-lysin (*nk-lysin*) were analysed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) as previously described (Chaves-Pozo, *et al.*, 2012b). Reaction mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15 s at 95ºC. For each mRNA sample, gene expression was corrected by the geometric average of the expression of two endogenous genes: elongation factor 1 alpha coding gene (*ef1a*) and *ribosomal protein L13 alpha* (*l13a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the endogenous Ct geometric average value from the target Ct. The specific primers used were designed using the Oligo Perfect software tool (Invitrogen) and are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for specificity. Negative controls with no template were always included in the reactions.

2.6. Statistical analysis

BestKeeper algorithm index and repeated par-wise regression analysis was determined using the BestKeeper® software according to the methodology previously described (Pfaffl, 2001). The data were analysed by a t-Student test to determine differences between control and vaccinated groups at each time point ($P \leq 0.05$). Data are represented as the mean ± standard error of the mean (SEM).

Table 1: Gene accession numbers and primer sequences used for gene expression analysis.

Molecule	Gene	Accession number		Sequence (5'→3')
Myeloid-specific peroxidase	*mpx*	DLAgn_00118340	F	GAAGAGTGGGGCCTTTGTTT
			R	CTGGGCCTCAGTGAAGACTC
Colony-stimulating factor receptor 1	*csfr1*	DLAgn_00109630	F	TTTCGGAAAGGTTGTTGAGG
			R	TCTCATCTGAATGGGCACTG
Hepcidin	*hamp*	DQ131605	F	CCAGTCACTGAGGTGCAAGA
			R	GCTGTGACGCTTGTGTCTGT
Dicentracin	*dic*	AY303940	F	GGCAAGTCCATCCACAAACT
			R	ATATTGCTCCGCTTGCTGAT
Lysozyme	*lyz*	FN667957	F	ATTTCCTGGCTGGAACACAG
			R	GAGCTCTGGCAACAACATCA
Complement factor 3-1 and 3-2	*c3*	HM563079 HM563078	F	ACCAAAGAACTGGCAACCAC
			R	CTAGCAGTCGGTCAGGGAAC
NK-lysin	*nk-lys*	DLAgn_00022970	F	GAAGAAACACCTCGGGGAAT
			R	GCAGGTCCAACATCTCCTTC
Elongation factor 1 alpha	*ef1a*	FM019753	F	CGTTGGCTTCAACATCAAGA
			R	GAAGTTGTCTGCTCCCTTGG
Ribosomal protein L13 alpha	*l13a*	DT044539	F	GCGAAGGCATCAACATCTCC
			R	AGACGCACAATCTTGAGAGCAG

3. Results

3.1. Peroxidase, protease and anti-protease activities increased at some time points of development in larvae from vaccinated females

Our data showed that peroxidase, protease and anti-protease activities were not modified in the serum of vaccinated females compared to controls (Fig. 2). However, in fry from vaccinated females the peroxidase activity was inhibited at 0, 9, 12, 15 and 26 dpf and enhanced after 40 dpf (Fig. 2b). Differently, the protease activity was stimulated in eggs (2 dpf) and larvae at 5, 7, 9, 23, 40 and 69 dpf from vaccinated females (Fig. 2d). Regarding the anti-protease activity observed in the vaccinated fry, although it was inhibited in eggs of 0 dpf, larvae of 7, 9, 23 and 40 dpf showed increased levels of this activity (Fig. 2f).

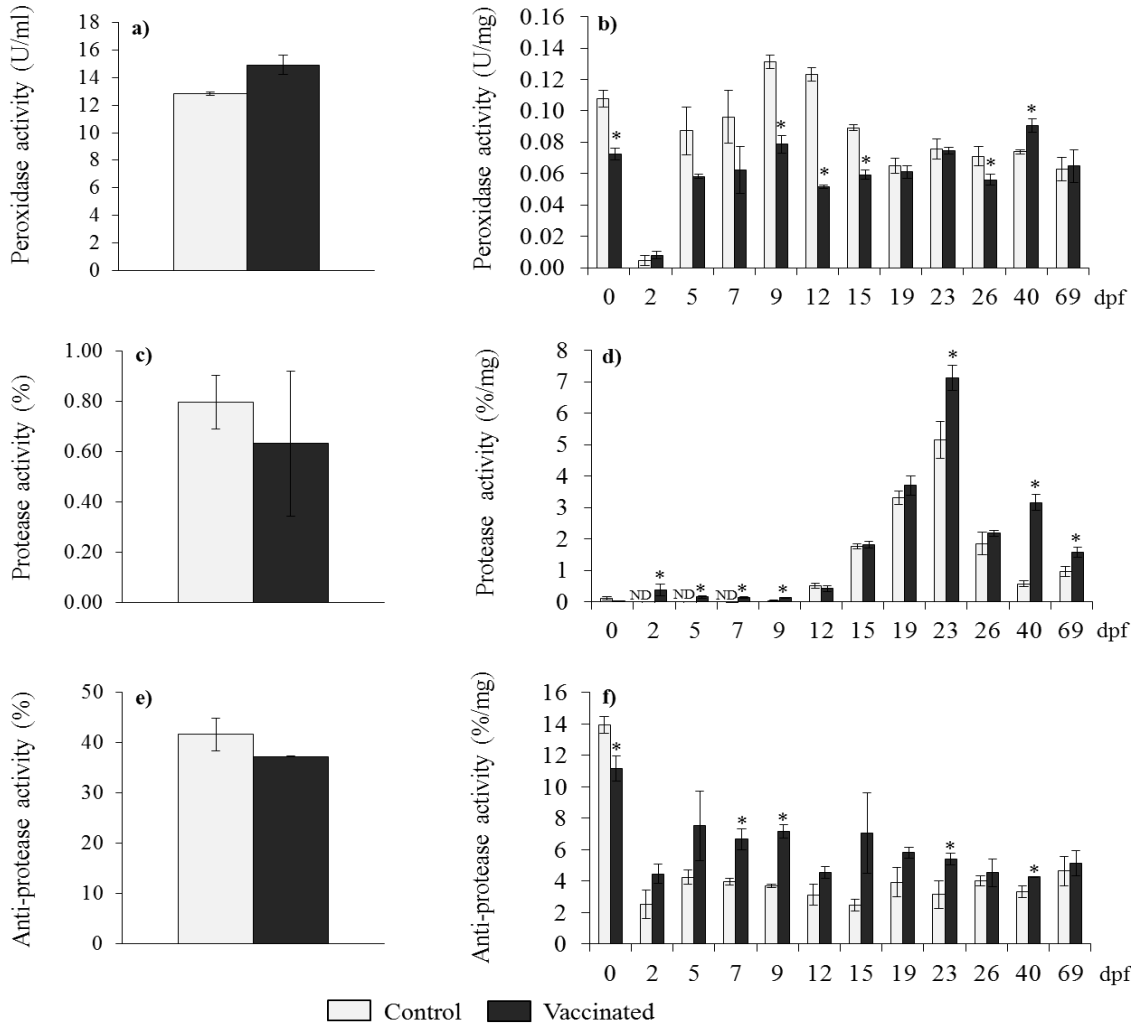

Figure 2: Peroxidase (a,b), protease (c, d) and anti-protease (e, f) activities in European sea bass serum (a, c, e) or eggs and larvae homogenates (b, d, f) at different days post-fertilization (dpf). Data represent the mean ± SEM. Asterisks denote statistical differences with controls ($P \leq 0.05$).

3.2. Lysozyme and total bactericidal activity were enhanced in larvae from vaccinated females whether only lysozyme did in serum

Lysozyme activity was greatly increased in the serum of vaccinated females (Fig. 3a) while was decreased in eggs of 0 dpf from vaccinated females compared to controls (Fig 3b). We found increments of lysozyme activity at different time points of development of vaccinated female fry (eggs of 2 dpf and larvae of 19, 23, 26 and 69 dpf, Fig. 3b). Interestingly, the total bactericidal activity was decreased in vaccinated female serum (Fig. 3c) but increased in their progeny at both eggs and larvae at different sampling times (0, 5, 9, 12, 15, 19, 26 and 40 dpf; Fig. 3d).

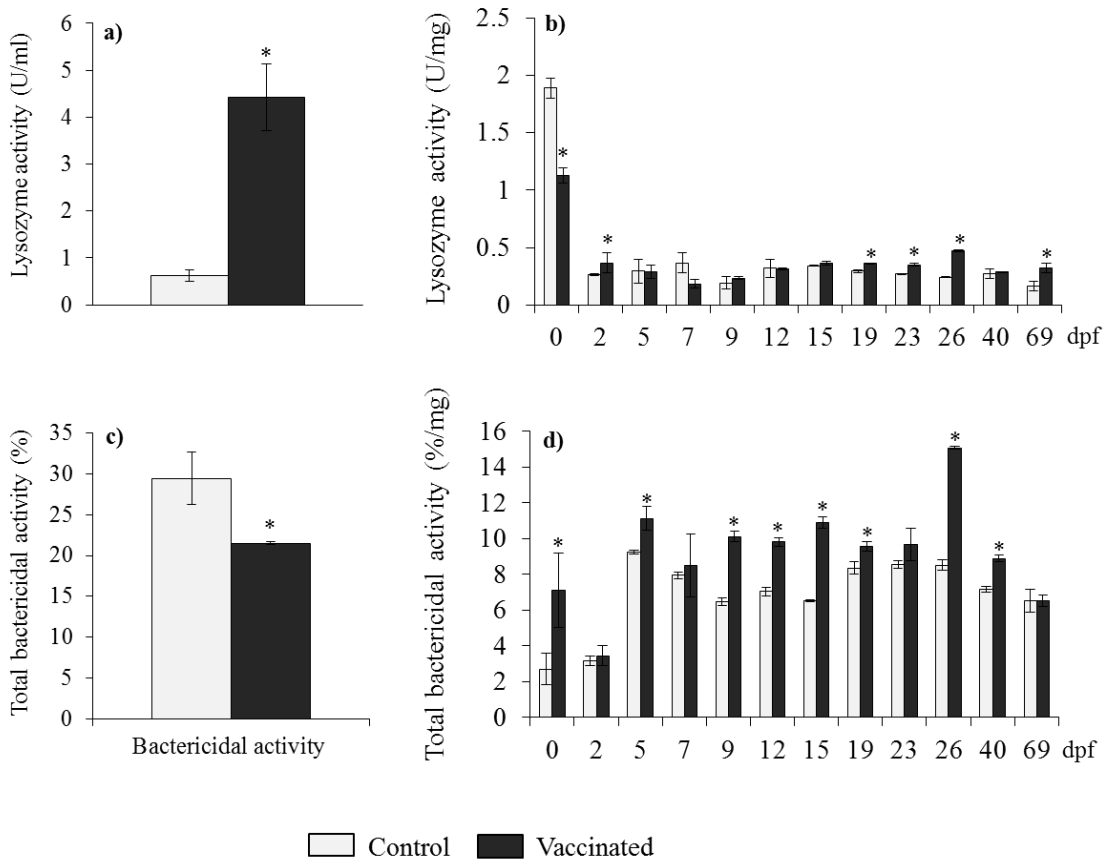

Figure 3: Lysozyme (a,b) and total bactericidal (c, d) activities in European sea bass serum (a, c) or eggs and larvae homogenates (b, d) at different days post-fertilization (dpf). Data represent the mean ± SEM. Asterisks denote statistical differences with controls (P≤0.05).

3.3. Expression levels of reference genes during the ontogeny of control and vaccinated female progenies

The reference genes (*ef1a* and *l13a*) were chosen based on the stability of their Ct values and the stability of their pattern of expression as previously described (Mitter, *et al.*, 2009). In our hands, both genes showed a high BestKeeper algorithm index along the ontogenic development of the specimens in each experimental group (control and vaccinated). However, statistically significant differences in Ct values of both genes were found between control and vaccinated specimens at some time points of development (Fig. 4). In order to minimize those differences we calculated the geometric mean of both reference genes and found that this parameter showed a better correlation index with the BestKeeper algorithm (Table 2).

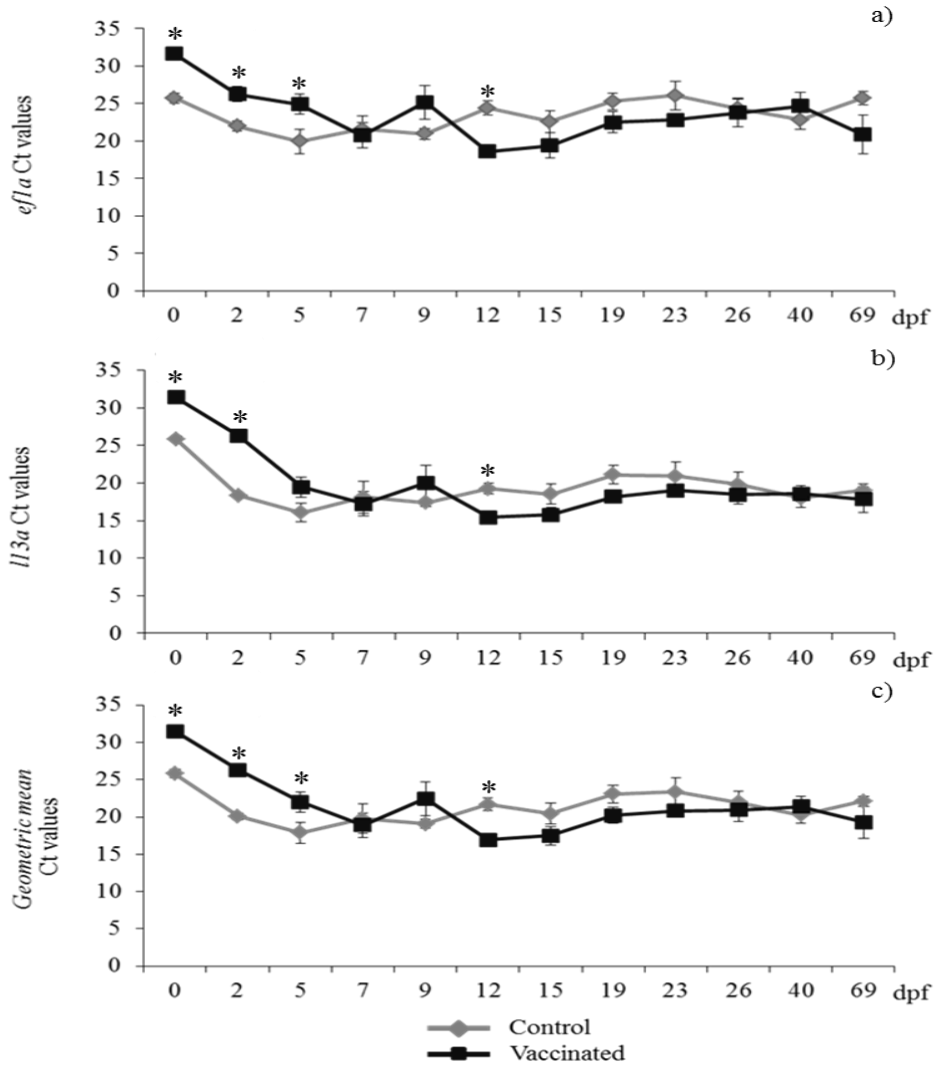

Figure 4: Ct values of endogenous genes: (a) *ef1a* and (b) *l13a*, and the (c) geometric mean used for the relative expression estimation of eggs and larvae from control and vaccinated European sea bass broodstocks. Data represent the mean ± SEM. Asterisks denote statistical differences with controls (P≤0.05).

Table 2: Repeated Par-wise regression analysis between BestKeeper index and each housekeeping gene Ct value. Represented in bold the highest correlation coefficient.

	Correlation coefficient	**p-value**
ef1a	0.956	0.001
l13a	0.976	0.001
Geometric mean	**1.000**	**0.000**

3.4. Granulocytes and macrophages gene marker expression was up-regulated in the progeny of vaccinated females

The gene expression of *mpx* gene (Fig. 5a), a granulocyte gene marker, was undetected in most of the sampling point in control group except at 2, 40 and 69 dpf.

However, the progeny from vaccinated females showed detectable expression levels of *mpx* at 2, 12, 15, 26, 40 and 69 dpf (Fig. 5a). Interestingly, the expression of *mpx* gene in controls increased from 40 dpf onwards, while in vaccinated fry the expression increased from 26 dpf reaching control levels at 69 dpf. Otherwise, the *csrf1* gene expression, a macrophage marker gene, was undetectable at 0, 15, 19 23 and 26 dpf in control fry (Fig. 5b). In the fry from vaccinated females, this gene was undetectable at 0, 19 and 23 dpf as control, but also at 9 and 40 dpf. On the other hand, higher expression of *csfr1* was observed in fry from vaccinated female at 2 and 7 dpf and lower expression at 40 dpf when compared to controls (Fig. 5b).

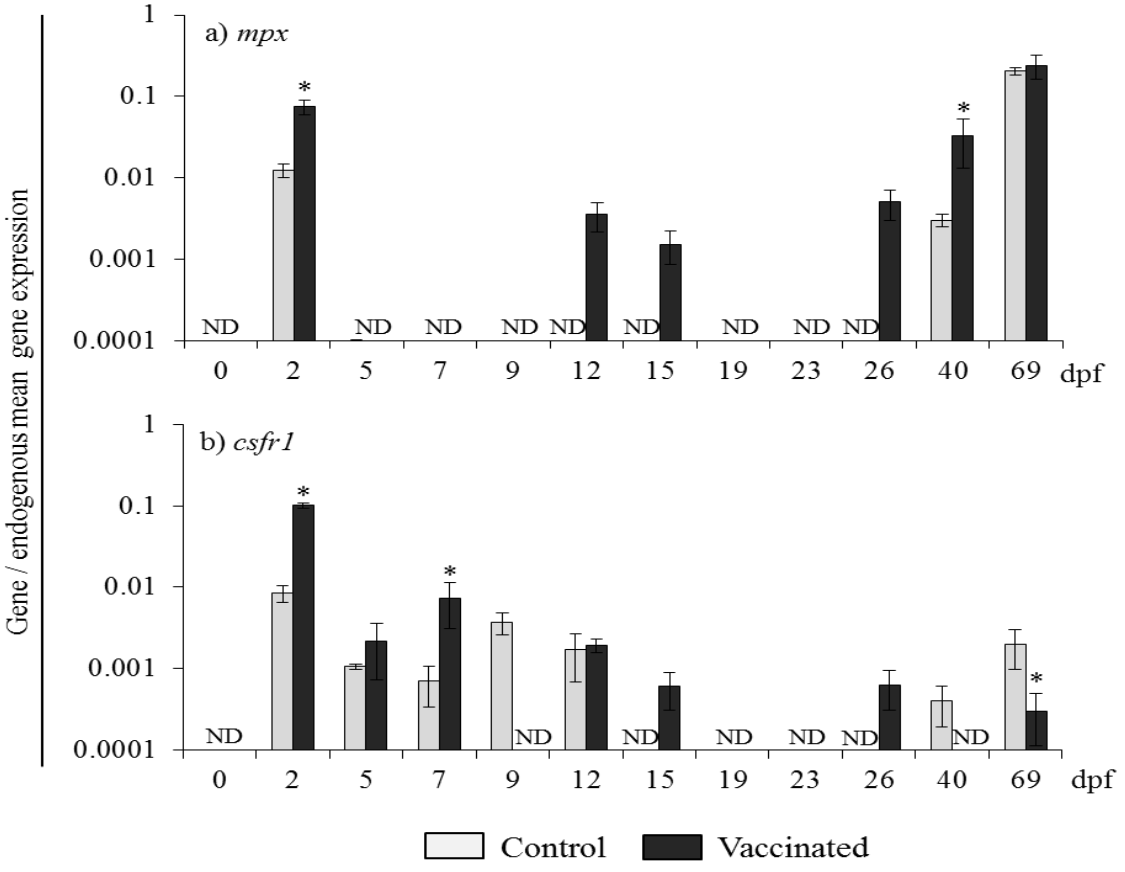

Figure 5: The expression levels of *mpx* (a) and *csf1r* (b) genes in the fry from control and vaccinated females during larval development Data represent the mean ± SEM. Asterisks denote statistical differences between vaccinated and control groups (P≤0.05). ND, non detected.

3.5. Antimicrobial peptides gene expression was up-regulated in the progeny of vaccinated females

The expression levels of *hamp*, *dic*, *lyz*, *c3* and *nk-lys* genes were higher in the fry from vaccinated females than in the fry from control females at different

developmental time points (Fig. 6). Moreover, most of the genes showed a similar pattern of expression. Thus, no transcript levels of *hamp*, *dic*, *c3*, *lyz*, and *nk-lys* were detected in eggs of 0 and 2 dpf neither in control or vaccinated group, except *c3* at 2 dpf, which was expressed in the control group (Fig. 6a,b,c,d,e). In general (Fig. 6), higher levels of expression of all genes were observed in the fry from vaccinated females between 5 and 9 pdf and between 26 and 69 dpf and some genes were also up-regulated between 12 and 19 dpf (*hamp* at 5 and 26 onwards dpf, *dic* at 5, 7, 9, and 69 dpf, *c3* at 5, 7, 12, 19, 26 and 40 dpf, *lyz* at 5, 7, 19 and 26 dpf and *nk-lys* at 5, 7, 12, 19, 26 and 40 dpf). The vaccinated group showed punctual down-regulations of *dic*, *c3* and *nk-lys* expression at 12 and 15, 69 and 15 and 23 dpf, respectively (Fig. 6b,c,e) or a complete blockage of *dic*, *lyz* and *nk-lys* expression (*dic* at 26 and 40 dpf, *c3* at 2 dpf and *lyz* and *nk-lys* at 9 dpf).

4. Discussion

The immune system of fish embryos and newly hatched larvae is not fully developed so the maternal transfer of immune factors is a crucial event in the defence against pathogens at these early stages of development (Zhang, *et al.*, 2013). In fish, the innate immune response is considered to be essential to fight against pathogens due to the temperature dependence, the limited antibodies repertoire and the slow lymphocyte proliferation rates of the specific immune response (Ellis, 1988; Whyte, 2007). Moreover, innate immune factors are known to be transmitted to the progeny such as several complement proteins, lectins, protease inhibitors, lysozyme or cathelicidin (Swain and Nayak, 2009; Zhang, *et al.*, 2013) Apart of the protein transmission to oocytes, transfer of mRNAs is also possible since high gene transcripts levels of some antimicrobial molecules has been detected in eggs of several teleost species (Løvoll, *et al.*, 2006; Mingming, *et al.*, 2014).

NNV affects more than 50 marine species, being the most susceptible the specimens at larval development (OIE, 2013). In this study, we immunized the females of European sea bass broodstock with a DNA vaccine against NNV (CP-pNNV) in order to establish whether a DNA vaccine might elicit enhanced passive immunity in their progeny. The CP-pNNV vaccine, hardly modified the innate immune activities analysed in the serum of vaccinated-females. In fact, it only increased the lysozyme activity and decreased the total bactericidal activity. A DNA vaccine against grass carp

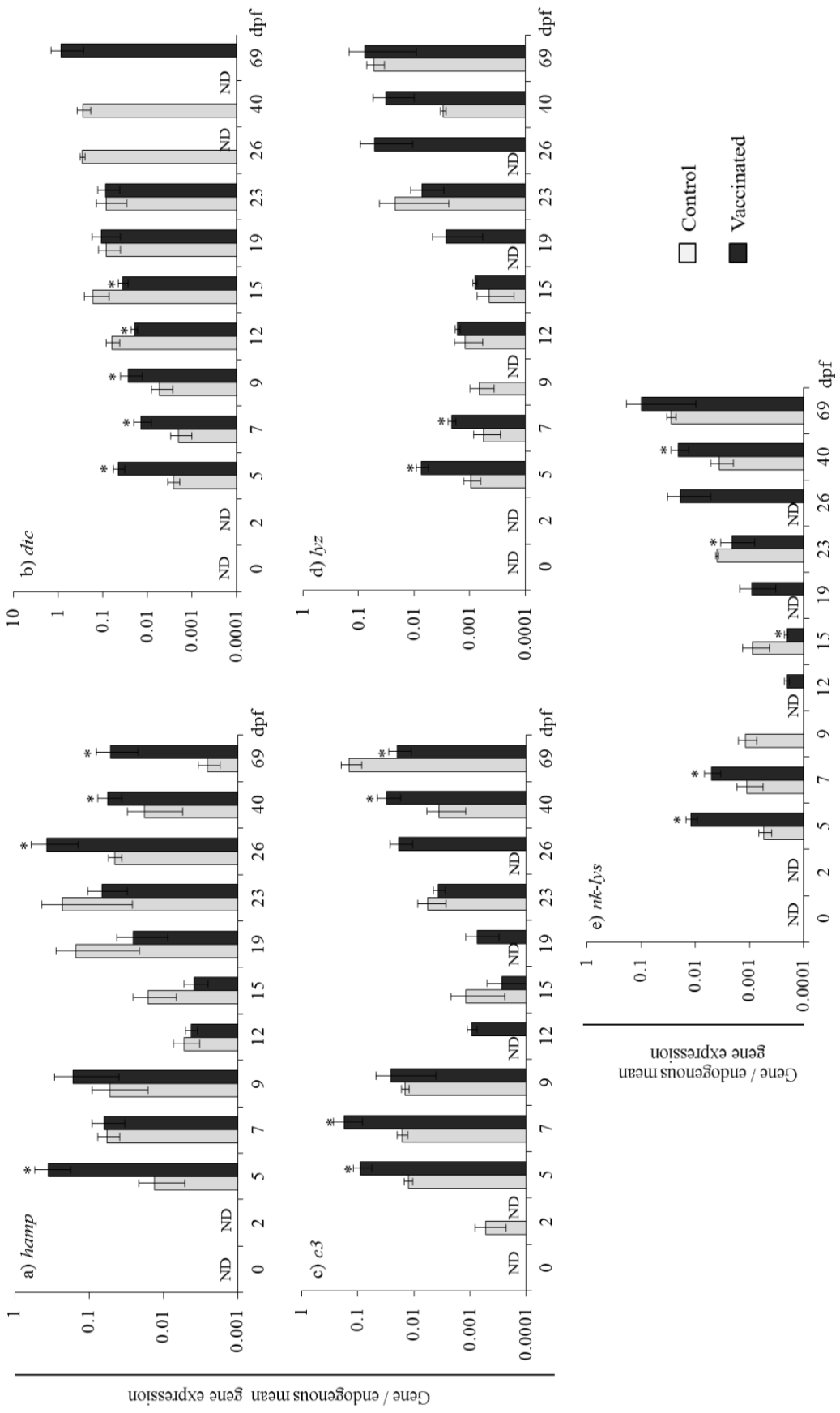

Figure 6: The expression levels of *hamp* (a), *dic* (b), *c3* (c), *lyz* (d) and *nk-lys* (e) genes in the fries from control and vaccinated females during larval development. Data represent the mean ± SEM. Asterisks denote statistical differences between control and vaccinated groups (P≤0.05). ND, non detected.

reovirus (GCRV), administrated to juvenile specimens, triggered the enhancement of lysozyme activity as on the present work (Zhu, *et al.*, 2015). However, previous studies in our laboratory demonstrated that *in vivo* infections with NNV in European sea bass juveniles elicited the increase of total bactericidal activity in serum whether lysozyme activity kept steady, contrasting with our data in vaccinated females showing different behaviours in antimicrobial responses depending on the fish size and immunization type (Valero, *et al.*, 2015b).

Regarding the progeny, all the activities except protease activity were detected from 0 dpf onwards in both, control and vaccinated, progenies. Secondly, compared to control-female's progeny, the vaccinated-female's progeny showed higher level of all these activities at certain time points of their development, even when lower levels of some of them (peroxidase, anti-protease and lysozyme activities) were recorded at earlier stages. Interestingly, the protease activity was firstly detected on vaccinated-female's progeny at 2 dpf, while in control-female's progeny this occurs from 9 dpf onwards. As these activities were down-regulated or not detected at egg stages (0 and 2 dpf), we discard that the CP-pNNV vaccine elicits an effective maternal transfer of the effectors of these activities. However, our data suggest that female vaccination with CP-pNNV vaccine elicit a better development of the innate immune response, as vaccinated-female's progeny reached higher levels of all these activities during their development. This hypothesis is also supported by the expression levels of hepcidin, dicentracin, lysozyme and NK-lysin coding genes which were not detected at egg stages, but were up-regulated at some time points later on during development. Moreover, and in order to wide this hypothesis to the main innate immune cell types, granulocytes and macrophages, we have also analysed two gene markers of this cell types, the *mpx* and *csfr1*, respectively. Despite the fact that there are no references about the ontogenic development of granulocytes and macrophages in European sea bass, in the fish species studied until now, two waves of haematopoiesis take place, as occurs in other vertebrates (Katzenback, *et al.*, 2013). Thus, the first wave is primitive haematopoiesis and occurs during embryonic development, followed by a definitive haematopoiesis wave that occurs in the post-natal or adult specimens (Katzenback, *et al.*, 2013). This issue would explain why we observed two points of *mpx* expression at 2 dpf and from 40 dpf onwards and of *csfr1* expression from 2 to 12 dpf and later from 40 dpf onwards in control-female's progeny. Interestingly, in vaccinated-female's progeny

we observed higher levels of expression of both genes at some point of both haematopoiesis waves and even at some points between them with the exception of the *csfr1* transcription that was down-regulated at 40 and 69 dpf. Moreover, the second wave of myelopoiesis seems to start earlier in vaccinated-female's progeny, as *mpx* gene expression progressively increased from 26 dpf onwards. Taken together, all these data support our hypothesis about female's vaccination as an enhancer of the development of the innate immune system in their progeny at least in the case of myelopoiesis.

In contrast to the four first activities discussed, the total bactericidal activity against *Vh* was higher in vaccinated- than in control-female's progeny in most of the time points sampled including eggs at 0 dpf, while this activity decreased in vaccinated-female's serum. These results suggest the transfer of effectors of this activity from maternal serum to eggs, leading to newly fertilized eggs with higher activity levels than those from control females. Related to this activity are the proteins coded for *hamp*, *dic*, *c3*, and *nk-lysin* genes, which expression we have analysed in both control- and vaccinated-female's progenies (Andersson, *et al.*, 1995; Bugla-Plskonska, *et al.*, 2008; Ogundele, 1998; Valero, *et al.*, 2013). In control's progeny, all these genes were not detected in eggs neither at 0 nor at 2 dpf but were expressed during larval development at some time points, although some blockages of their expression were also observed at others time points. Female's vaccination elicits higher levels of expression of those genes at certain time points of the larval development, although some down-regulations were also observed at other time points. So our data showed no clear effects of maternal vaccination on the inherited mRNAs or higher expression pattern of the AMP genes analysed. That is why our data support the idea that the up-regulated bactericidal activity upon female's vaccination might be due to maternal transfer of proteins instead of mRNAs. However, and as other molecules are also involved in total bactericidal activity (Valero, *et al.*, 2013), further studies are deserved to clearly determine the importance of transferred-proteins or -mRNAs, regarding bactericidal activity.

5. Conclusions

We can resume that broodstock immunization with a DNA vaccine against NNV initiated and promoted the innate immune response in vaccinated group earlier than in controls. Thus, the vaccine greatly increased protease, anti-protease, lysozyme and total

bactericidal activities, up-regulating granulocytes and macrophages gene markers and AMP gene expressions during early stages of development of the progeny. Furthermore, this vaccine might be eliciting the maternal transference of bactericidal activity since vaccinated females group showed decreased activities in serum and subsequently, increased activities in eggs. Indeed, maternal transfer described in our work seems to be restricted to protein factors as transcript levels of mRNAs coding for leucocyte markers or AMP genes were undetected in eggs. However, further studies are needed in order to elucidate whether vaccination against NNV could elicit increased maternal transfer which may generate an enhanced innate immune status on the progeny.

IV. GENERAL DISCUSSION

In this Doctoral Thesis, and for the first time, we demonstrate that NNV colonises, replicates and produces infective particles into the testis of fish, concretely gilthead seabream and European sea bass, although it keeps a very low level of gene expression and only in gilthead seabream viral proteins were detected. NNV was localized into somatic cells of the testis of both species, but only into gilthead seabream germ cells. Interestingly, NNV strongly increases the E_2 serum levels at the beginning of the infection and decrease the 11KT serum levels at day 15 in gilthead seabream, while in European sea bass, we observed a progressive decrease of E_2 serum levels concomitant with increasing mortalities as the infection proceeds, similarly to what happens in other studies upon bacterial infection (Deane, *et al.*, 2001). Taking into account that no mortalities were observed in gilthead seabream upon infection and that increases on E_2 and T serum levels stimulates the immune response in gilthead seabream (Cuesta, *et al.*, 2007), our results suggest that the ability to increase E_2 levels upon infection might be related to overcome the disease. On the other hand, these changes on the serum levels of reproductive hormones upon NNV infection are not enough to modify the reproductive physiology of the specimens as revealed by normal *dmrt1* transcription levels in gilthead seabream and normal morphology of the testis in both fish species. Furthermore, this normal function of the gonad guarantees the shedding and dissemination of the NNV infective particles through gonadal fluids or even into the germ cells, since the close association between Sertoli cells and germ cells (Schulz, *et al.*, 2001) did not allow us to discard the transfer of NNV through the spermatogenesis process from one cell type to another in European sea bass specimens.

As different susceptibilities to the RGNNV strain of the virus have been proven in this work between gilthead seabream and European sea bass as previously described (Breuil, *et al.*, 1991; Castric, *et al.*, 2001; Frerichs, *et al.*, 1996), differences in the immune responses elicited were also expected. In this work, we have analysed antimicrobial functions in serum as well as the expression patterns of the coding genes for several molecules of the IFN pathway, pro-inflammatory cytokines, cellular markers of T and B lymphocytes and AMPs. Our data show that all of them were up-regulated upon NNV infection in European sea bass but not in gilthead seabream testis. Moreover, these parameters of the immune response in brain (the main target tissue of the virus) were sharply increased in gilthead seabream and overlooked in European sea bass as previously observed that occurs with the cell-mediated cytotoxicity (Chaves-Pozo, *et*

al., 2012b). These differences in the immune responses in the brain have been related to the susceptibility to NNV disease and now our data in the seabream testis point to the use of this tissue to hide and be spread.

We also observed that NNV might alter the sensitivity of brain and testis to estrogens modifying the expression levels of ERs coding genes in both tissues. Interestingly, ER activity has been shown to modulate innate immune signalling pathways in dendritic cells and macrophages in humans and fish (Cabas, *et al.*, 2013; Kovats, 2015; Liarte, *et al.*, 2011a,b). Thus, ERα and physiological adult levels of E_2 promote the production of type I IFN in humans (Kovats, 2015). As our data show a great stimulation of the IFN pathway in the European sea bass testis as also occurs with the expression of *erb1* and *erb2* genes, we studied the correlation between IFN pathway and estrogen receptor genes and found positive correlations in the expression between *erb2* and most of the IFN-related genes in European sea bass testis (Table 1). However, regarding these relationships, in gilthead seabream we failed to find them.

Table 1: Correlation observed between the gene expression of *erb2* and IFN-related genes in European sea bass testis after *in vivo* infection with NNV. The first line corresponds to Pearson coefficient of correlation and the second to the P value. Written in bolds are the parameters correlated.

		lgp2	*mavs*	*traf3*	*tank*	*tbk1*	*irf3*	*irf7*	*ifn*	*mx*	*pkr*
Sea bass testis	*erb2*	**0.73**	**0.58**	**0.60**	0.46	**0.69**	**0.69**	**0.59**	**067**	**0.75**	**0.67**
		0.00	**0.02**	**0.01**	0.07	**0.00**	**0.00**	**0.01**	**0.00**	**0.01**	**0.00**

In contrast to what we observed upon the *in vivo* infection, after the *in vitro* infection only *hamp* and *dic* genes expression was up-regulated in the testis of European sea bass, whether the rest of immune genes analysed (pro-inflammatory cytokines, AMPs and lymphocyte markers) were unmodified or down-regulated. Interestingly, upon the *in vitro* infection the *erb1* and *erb2* gene expression was also up-regulated in the European sea bass testis. These results suggest that the antimicrobial responses are locally regulated by the gonad while others might be more dependent on systemic immune responses. Curiously, in a study performed during my international stay at the University of Palermo, we found a very different profile of active AMPs in the gonad of European sea bass males and females, suggesting the existence of specific AMPs in each type of gonad. However, further characterization of isolated peptides is needed to

fully characterize the molecules and determine their role upon infection (Valero, *et al.*, 2015e).

As several histones with antimicrobial activity have been described (Valero, *et al.*, 2013), and some of them are specific from testis (Nam, *et al.*, 2012), we have also studied the pattern of expression of H1 and H2B coding sequences in a broad spectrum of tissues of European sea bass and gilthead seabream and upon diverse experimental infections. Our data suggest that H1 might have a role in the immune response against NNV infection in the brain of both species, due to the fact that *h1* expression pattern is similar to other AMPs and several IFN pathway genes and correlated well with the different susceptibility to infection of both species (Valero, *et al.*, 2015b,c). On the other hand, H2B seems to be more important in the head-kidney (HK) and HK leucocytes (HKLs) immune response.

Regarding the fish-NNV interactions, the quick, intensive and diverse immune response observed in the brain of gilthead seabream with little inflammatory consequences, explain the ability of this specie to overcome the NNV infection. However, European sea bass infected specimens are not able to clear the virus from the brain, even when the infection elicited an intense inflammatory process, the activation of the IFN pathway and antimicrobial, cell-mediated cytotoxicity and adaptive responses (Chaves-Pozo, *et al.*, 2012b; present data). This intense immune response in the brain probably triggers high cell damage, leading to the mortality rates observed upon NNV infection in European sea bass specimens (Chaves-Pozo, *et al.*, 2012b; Chapter III.1.1. in this Thesis).

We next evaluated the generation and application of DNA vaccines for prevention. Thus, we developed a DNA vaccine cloning in a eukaryotic expression vector the complete open reading frame of RNA2 from NNV (CP-pNNV). The DNA vaccine was encapsulated with chitosan nanoparticles and administered to early juveniles of European sea bass. Our data demonstrate that the vaccine triggered cell-mediated cytotoxicity and IFN responses in the hindgut, similarly to what happened with the immune responses elicited after *in vivo* infections with NNV in European sea bass and gilthead seabream (Chaves-Pozo, *et al.*, 2012b; present data) and in other fish species against viral agents (Ellis, 2001; Robertsen, 2006). However, the vaccine failed to trigger the production of specific NNV antibodies after 90 days of vaccination,

contrarily to what happened in other cases (de las Heras, *et al.*, 2010; MacLaughlin, *et al.*, 1998; Vimal, *et al.*, 2014). At 90 days post-vaccination, the experimental infection with a lethal dose of NNV resulted in a quite high survival rate (45% of the vaccinated fish) highlighting that the innate immune responses are crucial in the fight against NNV. Later on, the same partly protective vaccine was administered by injection to the females of a European sea bass broodstock. Our results showed that all the antimicrobial activities analysed (peroxidase, protease, anti-protease, lysozyme and total bactericidal activity) in vaccinated and non-vaccinated female's progenies were detected from 0 dpf onwards. As these activities were not increased in egg stages (0 or 2 dpf) from vaccinated females, we discard that the CP-pNNV vaccine elicits an effective maternal transfer of the effectors of these activities, except in the case of total bactericidal activity, which increased in vaccinated-female's progeny from 0 dpf onwards. However, the vaccinated-female's progeny showed higher level of all these activities analysed at certain time points of their development, suggesting these data that female vaccination with CP-pNNV vaccine elicits a better and faster development of the innate immune response of their progeny. This hypothesis is also supported by the gene expression pattern of granulocytes and macrophages gene markers and of several genes related to the activities analysed. Furthermore, our data showed that the transcription of genes related to the activities analysed were up-regulated at several time points after vaccination as in brain and gonad upon *in vivo* infection with NNV, demonstrating that NNV elicits the antimicrobial status of the fish as the vaccine does in the progeny of vaccinated-females. Therefore, further studies are needed to completely understand the immune response in the hindgut after oral DNA vaccination and the maternal transfer of immunity upon vaccination in order to improve DNA vaccines against NNV in order to ameliorate or abolish the incidence of VER disease and subsequent mortalities.

V. CONCLUSIONS

1. Nodavirus colonises, replicates and produces infective viral particles in European sea bass and gilthead seabream testis.

2. Nodavirus modifies the serum levels of 17β-estradiol and 11-ketotestosterone and the pattern of expression of the nuclear estrogen receptor genes in both species, although it is not enough to disrupt testicular functions.

3. Nodavirus up-regulates the transcription of genes of the interferon pathway, antimicrobial peptides, cellular markers of T and B lymphocytes and pro-inflammatory cytokines in European sea bass but not in gilthead seabream testis. By contrast, in the brain, the opposite occurs. This could be related to the virus resistance or dissemination.

4. Comparison of the immune responses determined in the gonads after infection with nodavirus *in vivo* or *in vitro* indicates that this response is, to some extent, locally primed and regulated by the NNV particles.

5. The administration of a chitosan-encapsulated DNA vaccine against nodavirus to European sea bass juveniles improves their survival upon an experimental challenge. The vaccine triggers cell-mediated cytotoxicity and interferon responses in the hindgut without the production of specific antibodies in the serum.

6. The vaccination of European sea bass broodstock females with a DNA vaccine against nodavirus triggers the maternal transfer of proteins with antibacterial activity to their progeny and elicits an earlier and enhanced expression of several innate immune-related genes.

7. Our study demonstrates the importance of the interaction of the immune-reproductive responses upon nodavirus infection. Furthermore, any preventive or therapeutic strategy should take into account the role of the gonad in the clearance and transmission of nodavirus.

VI. RESUMEN EN CASTELLANO

RESUMEN

La dorada (*Sparus aurata*) y la lubina (*Dicentrarchus labrax*) son las dos especies más relevantes en la acuicultura mediterránea y española. Entre los mayores problemas de su cultivo destacan las elevadas pérdidas económicas que provocan las patologías. Entre éstas, nodavirus (NNV) produce elevadas mortalidades en lubina, especie altamente sensible al virus, mientras que el virus puede infectar a la dorada, pero no desarrolla la enfermedad, especie reservorio. Además, este virus se transmite de forma vertical a través de los gametos y los fluidos gonadales, y no existen herramientas preventivas efectivas todavía contra él. Por ello, en esta Tesis Doctoral nos propusimos evaluar la interacción entre la gónada de lubina y dorada y NNV (Parte 1), y cómo prevenir la infección y su diseminación (Parte 2).

La Parte 1 de la Tesis contiene cuatro capítulos (III.1.1 a III.1.4.). Inicialmente, (Capítulo III.1.1.) estudiamos si NNV era capaz de colonizar y replicar en la gónada de machos inmaduros de lubina y dorada y si la infección afecta a las funciones reproductivas. En primer lugar, se localizó RNA viral en células somáticas testiculares de ambas especies y también en células germinales del testículo de dorada mediante PCR *in situ* (isPCR), mientras que la proteína de la cápsida (CP) y la proteína B2 sólo se pudieron detectar en el testículo de dorada mediante inmunocitoquímica. Es más, se rescataron partículas virales infectivas de la gónada de ambas especies mediante técnicas de cultivos celulares demostrando que el virus tiene capacidad de replicación. Si nos centramos en las funciones reproductivas, la infección con NNV alteró la producción y los niveles séricos de 17β-estradiol y 11-cetotestosterona, así como la sensibilidad a estas hormonas en cerebro y testículo de ambas especies, mientras que no alteró el proceso espermatogénico si tenemos en cuenta lo valores del índice gónadosomático (GSI), los niveles de expresión del gen *dmrt1* y la morfología testicular tras la infección.

A continuación (Capítulos III.1.1. al II.1.4.), estudiamos la respuesta inmunitaria en gónada y cerebro, principal tejido diana del virus. Nuestros resultados mostraron una mayor respuesta inflamatoria en la gónada y el cerebro de lubina, ya que se estimuló la expresión de todos los genes pro-inflamatorios analizados (*tnfa*, *il6* y *il1b*), mientras que en dorada solo se estimuló en menor medida la expresión del gene *il6*. Estos resultados concuerdan con el hecho de que la lubina alcanzó una mortalidad del 55% tras tan solo

15 días de infección, mientras que la dorada fue capaz de superar la infección. La respuesta del interferón (IFN) tipo I es una de las más poderosas del sistema inmunitario innato de peces contra virus. En peces, se conocen las secuencias codificantes de INF y de muchos otros genes estimulados por IFN, mientras que los sensores de RNA viral y otras moléculas intermediarias de la ruta son muy poco conocidos. Así, nos propusimos estudiar la presencia de los genes que codifican para este tipo de proteínas en lubina y dorada, y evaluar su expresión tras una infección experimental con NNV en cerebro y gónada (Capítulo III.1.2). Así identificamos en las bases de datos las secuencias que codifican para las proteínas MDA5, TBK1, IRF3, IFN, Mx y PKR en ambas especies. Además en lubina, también encontramos las secuencias codificantes de las proteínas LGP2, MAVS, TRAF3, TANK y IRF7. Para poder caracterizar la ruta completa del IFN analizamos la expresión de estos genes en dos líneas celulares, la línea SAF-1 de dorada y la DLB-1 de cerebro de lubina, en las que la mayoría de los genes se estimularon tras la infección *in vitro* con NNV o pI:C. Tras la infección experimental con NNV *in vivo* en dorada, la mayoría de los genes de esta ruta se estimularon en el cerebro mientras que no se observaron cambios en la gónada, probablemente favoreciendo la diseminación del virus. Curiosamente en lubina todos los genes analizados se estimularon en gónada pero únicamente *mda5*, *lgp2*, *irf3*, *mx* y *pkr* lo hicieron en cerebro. En ambas especies, el gen *tbk1* no fue alterado por ningún tratamiento, *in vivo* o *in vitro*, sugiriendo esto que podría no ser un gen relevante en la ruta del IFN de tipo I. Estos resultados apoyan la hipótesis de que la respuesta inmunitaria del cerebro de lubina no es capaz de eliminar el virus tras una infección, y apuntan a la importancia de la inmunidad de la gónada para controlar la diseminación de NNV.

Los péptidos antimicrobianos (AMPs) son considerados un componente fundamental en la respuesta innata de peces. Sin embargo, no existen estudios que analicen la respuesta inmunitaria centrada en AMPs en la gónada de peces infectados a pesar del papel de la gónada en la diseminación viral. Por ello, hemos caracterizado la respuesta por AMPs desencadenada por NNV en testículo de lubina y dorada, *in vivo* e *in vitro*, y la hemos comparado con la que se desarrolla en suero y en cerebro (Capítulo III.1.3). En primer lugar, nuestros resultados muestran una elevada respuesta anti-viral en el cerebro de ambas especies ya que la expresión del gen *mx* se estimuló fuertemente tras la infección. Además, por primera vez, pudimos demostrar que las actividades antimicrobianas (hemolítica del complemento, lisozima y bactericida total) y la

expresión de determinados genes que codifican para AMPs (*c3*, *lyz*, *hamp*, *dic*, *pis* o *bdef*) en la gónada y cerebro de ambas especie es muy diferente, activándose generalmente en gónada de lubina y estando probablemente estas diferencias relacionadas con la diferente susceptibilidad a NNV. Es más, los resultados obtenidos tras el tratamiento *in vitro* con NNV indican que algunos AMPs (*lyz*, *hamp* y *dic*) se regulan de forma local en la gónada mientras que otros (*c3*) dependen de la respuesta inmunitaria sistémica.

Las histonas (H1 a H4) son las principales proteínas implicadas en la compactación del DNA en la cromatina, sin embargo, y además, las histonas ejercen funciones antimicrobianas en vertebrados. De hecho, la histona H1 de mamíferos se moviliza como parte de una respuesta antiviral. En peces, se han aislado y caracterizado histonas con actividad AMP en piel y gónada. Por ello, hemos identificado la secuencia completa de los genes que codifican para las histonas H1 y H2B en lubina y dorada y estudiado su patrón de expresión en condiciones de salud y frente a una infección *in vivo* con NNV (Capítulo III.1.4). Nuestros resultados mostraron mayores niveles de expresión del gen *h1* en sangre periférica en ambas especies, y en timo de lubina. Sin embargo, en el caso del gen *h2b*, su expresión fue mayor en timo de lubina y sangre y gónada de dorada. Tras la infección *in vivo*, pudimos observar que se estimulaba la expresión de *h1* en gónada de lubina o en cerebro de dorada, pero en ningún caso en riñón cefálico. Por el contrario, la expresión del gen *h2b* aumentaba exclusivamente en riñón cefálico de ambas especies tras la infección. Además, ambos genes parecen estar regulados de forma sistémica ya que su expresión no era alterada tras una infección *in vitro* del testículo. Todos estos datos obtenidos nos instaron a estudiar el papel de estas histonas en la respuesta inmunitaria de los leucocitos de riñón cefálico frente a virus, bacterias (*Vibrio anguillarum* o *Photobacterium damselae*) o frente a determinados estimulantes químicos. A pesar de que son necesarios más estudios, nuestros datos sugieren que la histona H1 podría tener un papel relevante en la respuesta inmunitaria del cerebro, mientras que la H2B parece estar más involucrada en la respuesta del riñón cefálico. Es más, la función potencial de las histonas como agentes anti-virales parece evidente aunque más estudios son necesarios y, en este laboratorio, se están llevando a cabo estudios funcionales adicionales para caracterizar esta actividad.

La Parte 2 de esta Tesis contiene dos capítulos (III.2.1. y III.2.2.) y se centra principalmente en el desarrollo de herramientas preventivas para mejorar la

supervivencia de lubina frente a una infección con NNV. NNV es extremadamente dañino durante estadios larvarios y juveniles. Además durante estos estadios tempranos de desarrollo los peces son difícilmente vacunables debido a su pequeño tamaño o a que su sistema inmunitario todavía no es inmunocompetente.

Entre las herramientas preventivas, más y mejores vacunas son necesarias para mejorar la producción acuícola, siendo las vacunas de DNA las que ofrecen resultados más prometedores. Es más, la administración oral es fundamental para la administración masiva de vacunas en estadios tempranos de desarrollo puesto que no ofrece problemas asociados al manejo de los ejemplares. Por ello, teniendo en cuenta la dificultad para inyectar una vacuna a individuos pequeños pero inmunocompetentes, creamos una vacuna oral de DNA encapsulada en partículas de quitosano, desarrollada específicamente contra NNV (ChP-CP-pNNV), la cual se administró oralmente a juveniles de lubina en sus primeros estadios de desarrollo durante dos días y estudiamos, a los 90 días de la vacunación, los niveles de IgM totales y específicos contra NNV en suero, así como la expresión de genes involucrados en la citotoxicidad mediada por células (CMC: *tcrb* y *cd8a*) la presentación de antígenos (*mhc1a* y *mhc2b*), distintos anticuerpos (*igmh* e *igth*) y la respuesta del IFN (*ifn*, *mx* e *ifng*) tras 7, 30 y 90 días en el intestino posterior (Capítulo III.2.1). Nuestro estudio muestra que la vacuna oral ChP-CP-pNNV no desencadenó la producción de anticuerpos específicos circulantes a los 90 días de la vacunación ni diferencias en la expresión génica de inmunoglobulinas (*igmh* e *igth*) en el intestino posterior entre los grupos experimentales a partir de los 30 días post-vacunación. Sin embargo, la vacuna provocó una estimulación de la expresión de los genes relacionados con la CMC (*tcrb* y *cd8a*) y de la respuesta del IFN (*ifn*, *mx* e *ifng*). Además, tres meses tras realizar la vacunación, el grupo vacunado (ChP-CP-pNNV) mostró un porcentaje relativo de supervivencia (RPS) del 45% tras una infección experimental. Así, podemos concluir que la vacuna (ChP-CP-pNNV) diseñada protege de forma parcial a ejemplares juveniles de lubina, y esta protección parece ser mediada por la CMC y la respuesta del IFN, aunque son necesarios más estudios para mejorar esta vacuna y entender sus mecanismos de acción.

El transporte continuo de huevos y larvas entre estaciones acuícolas por todo el mundo favorece la diseminación de infecciones virales, especialmente de NNV. Aunque los primeros estadios larvarios se caracterizan por no tener un sistema inmunitario inmunocompetente, la exposición a patógenos no es un problema debido a la

transferencia materna de factores inmunitarios, siendo los más estudiados los efectores de la respuesta inmunitaria específica, a pesar de que la respuesta innata es esencial en la inmunidad de peces. Por ello, nos propusimos estudiar si la vacuna de DNA previamente diseñada contra NNV (CP-pNNV) podría estimular la transferencia materna de efectores de la respuesta inmunitaria innata para lo que, dicha vacuna fue inyectada en hembras maduras y fluyentes de lubina (Capítulo III.2.2). En este estudio analizamos diferentes actividades antimicrobianas (peroxidasa, proteasa, anti-proteasa, lisozima y bactericida total) en el suero de las hembras vacunadas y en los huevos y larvas de su descendencia, desde los 0 a los 69 días después de la fertilización (dpf). Además, analizamos el patrón de expresión de marcadores de granulocitos y macrófagos (*mpx* y *csf1a*, respectivamente) y diversos genes codificantes de AMPs (*hamp*, *dic*, *c3*, *lyz* y *nk-lys*). Nuestros resultados mostraron que la vacuna desencadena la transferencia materna de efectores bactericidas, mostrando los huevos (0 dpf) del grupo vacunado una actividad bactericida total mayor que el control. De hecho, esta transferencia materna parece estar restringida a factores proteicos ya que no se detectó mRNA de la mayoría de los genes analizados a 0 dpf. Curiosamente, la inmunización de las hembras reproductoras favoreció un desarrollo más temprano y elevado de la respuesta inmunitaria innata en su descendencia,

En resumen, nuestro estudio demuestra por primera vez la localización de NNV en células testiculares de peces y cómo las interacciones inmuno-endocrinas son reguladas por NNV, el cual pasa inadvertido a la respuesta inmunitaria en el testículo manteniendo su capacidad de replicación y podría transmitirse a la descendencia. Es más, hemos diseñado una vacuna de DNA que muestra propiedades prometedoras como herramienta preventiva contra NNV cuando se administra a juveniles o a hembras reproductoras.

VI.1. INTRODUCCIÓN

Según la FAO, las capturas debidas a la pesca extractiva a nivel mundial solo cubren un 58,7% de la demanda de peces de toda la población mundial (FAO, 2014). En esta situación, la acuicultura parece ser la mejor opción económica para cubrir el progresivo aumento de la demanda de pescado para alimentación humana (FAO, 2014; STECF, 2014). Igualmente, en la Unión Europea, la producción obtenida mediante capturas ha ido descendiendo paulatinamente con el paso de los años, mientras que la acuicultura ha ido creciendo, llegando a proveer el 21% de la demanda europea (FAO, 2014; STECF, 2014). España ocupa uno de los primeros puestos en producción acuícola, siendo la lubina (*Dicentrarchus labrax*) y la dorada (*Sparus aurata*) las especies más relevantes en términos de producción (toneladas) y económicos (APROMAR, 2015).

La cría intensiva de peces, con el consecuente confinamiento de grandes cantidades de peces en espacios reducidos y el manejo diario, provoca situaciones de estrés que debilitan el sistema inmunitario de los peces, incrementando su susceptibilidad a patógenos. Además, el actual comercio de huevos, larvas y juveniles entre instalaciones de todo el mundo, favorecen la propagación de estos patógenos provocando grandes pérdidas económicas al sector de la acuicultura (Fukuda, *et al.*, 1996; Grau, *et al.*, 2003; Villamil, *et al.*, 2003). Todo ello nos obliga a estudiar cómo prevenir la diseminación de patógenos a nivel mundial. Por esta razón, el estudio de la respuesta inmunitaria en diferentes tejidos y estadios, las diferentes estrategias de transmisión entre especímenes junto con el desarrollo de vacunas y diferentes rutas de administración de las mismas se han convertido en uno de los objetivos primordiales en la acuicultura moderna.

Los peces tienen una posición filogenética clave representando el primer grupo animal en poseer un sistema inmunitario innato, que está constituido por barreras físicas, efectores celulares (leucocitos) y humorales, y un sistema inmunitario adaptativo, que está constituido por linfocitos T, B y los anticuerpos que éstos producen. Así, el sistema inmunitario de vertebrados presenta un patrón común aunque esto no excluye la existencia de diferencias importantes entre diferentes grupos de vertebrados. Quizás la diferencia más importante sea el desarrollo y la preponderancia de los mecanismos de defensa innatos en peces, en contraste con la respuesta adaptativa

potente y bien desarrollada de vertebrados superiores, a pesar de que ambas respuestas en peces se encuentran igual de altamente interconectadas que en el resto de vertebrados (Van Muiswinkel, 1995). En peces, al contrario que en mamíferos, los tejidos inmunitarios se dividen en una región hematopoyética donde se producen las células mieloides; y por otra parte una región linfoide, encargada de la producción de linfocitos, siendo la región cefálica del riñón el órgano hematopoyético por excelencia (Manning, 1998).

La respuesta inmunitaria innata es aquella que protege al organismo sin que éste haya sido expuesto previamente al antígeno y se compone de barreras físicas, factores humorales y elementos celulares que actúan como primera línea de defensa contra la infección hasta que la respuesta adaptativa se activa. La respuesta adaptativa se compone de factores humorales y elementos celulares además de proteínas específicas como el MHC y TCRs (Abbas, *et al.*, 2012). Una de las características más notorias de la repuesta adaptativa es la generación de memoria, que desarrolla una respuesta mucho más rápida tras la segunda exposición a un patógeno en concreto (Uribe, *et al.*, 2011). Aunque la respuesta de los linfocitos T todavía no se conoce con mucho detalle en peces, su capacidad de expansión clonal y la diversidad que presenta, su repertorio de linfocitos ha sido caracterizado en trucha arcoíris (*Oncorhynchus mykiss*) (Boudinot, *et al.*, 2001; Laing y Hansen, 2011). Sin embargo, la producción secundaria de anticuerpos ha sido más estudiada y es causada por la expansión de un subconjunto de linfocitos B con receptores de alta afinidad a un antígeno concreto (Kaattari, 1992; Morrison and Nowak, 2002), siendo esta segunda producción de anticuerpos más extensa y rápida que la primaria (Kaattari y Piganelli, 1996; Manning y Nakanishi, 1996; Manning, 1998).

Al igual que ocurre en otros vertebrados, la inmunocompetencia aparece en peces durante su desarrollo ontogénico, pero al contrario que en mamíferos, en los primeros estadios de desarrollo, los peces se encuentran en contacto directo con el ambiente durante mucho tiempo antes de que su sistema inmunitario se encuentre completamente desarrollado. En el caso de la lubina, aunque los linfocitos aparecen entre los 10 y 50 días post-eclosión (dph), se alcanzan niveles de linfocitos B semejantes a los de los individuos maduros sobre los 140 dph (Dos Santos, *et al.*, 2000). Sin embargo en la dorada se detectan linfocitos alrededor de los 30 – 55 dph, poniendo de manifiesto que las diferencias en la adquisición de la inmunocompetencia dependen en gran medida de la edad y de la especie (Mulero, *et al.*, 2007a). Con el fin de asegurar

la protección frente a las infecciones de los ejemplares a lo largo de su desarrollo, al igual que en otros grupos de vertebrados, en los que se ha desarrollado un mecanismo de transferencia materna de inmunidad a través de la placenta, el calostro, la leche o los huevos, la transferencia materna resulta en peces de crucial relevancia en la protección de los individuos todavía no completamente desarrollados (Hanif, *et al.*, 2004; Hasselquist and Nilsson, 2009; Zhang, *et al.*, 2013).

Los peces son el grupo de vertebrados con mayor diversidad de estrategias reproductivas. Así, hay especies gonocoristas que desarrollan un único sexo funcional, como la lubina; y especies hermafroditas que desarrollan ambos sexos funcionales a lo largo de su vida, como la dorada (Devlin y Nagahama, 2002). Aunque en este trabajo nos centraremos en los machos, hay que reseñar que en el caso de las hembras, el ovario consiste en una estructura hueca compuesta por una cavidad ovárica rodeada de pliegues de tejido denominados láminas ovígenas, donde se alojan los distintos tipos de células germinales rodeadas de tejido conectivo y delimitadas de la cavidad ovárica por células epiteliales (Kagawa, 2013; Liarte, *et al.*, 2007). Las células germinales proliferan y se diferencian siguiendo un patrón semejante en todas las especies de peces teleósteos. Así, este proceso de ovogénesis podemos subdividirlo en diferentes fases: proliferación de ovogonias, crecimiento primario y foliculogénesis, vitelogénesis, maduración y ovulación. En las especies de teleósteos estacionales como la dorada y lubina se suceden diferentes periodos de proliferación de ovogonias antes, durante o después del periodo principal de puesta (Coward, *et al.*, 2002). El momento crítico del crecimiento ovocítico es la vitelogénesis cuando la Vg hepática es movilizada al ovocito en maduración (Coward, *et al.*, 2002).

El testículo de los peces está formado por una región de tejido intersticial que rodea los túbulos seminíferos, los cuáles albergan el epitelio germinal. El tejido intersticial se compone, entre otros, por células de Leydig y células inmunitarias (Chaves-Pozo, *et al.*, 2003; Schulz, *et al.*, 2010). Por su parte, el epitelio germinal está formado por células germinales y de Sertoli (Rocha and Rocha, 2006; Schulz, *et al.*, 2010). Este epitelio germinal es muy diferente al encontrado en mamíferos ya que las células de Sertoli forman una estructura quística en el interior de la cual se desarrollan clones de células germinales de forma sincrónica (Billard, *et al.*, 1982; Callard, 1991; Chaves-Pozo, *et al.*, 2005b; Grier, 1981; Miura, 1999; Nagahama, 1983).

Las dos especies objeto de estudio, lubina y dorada, son especies estacionales que desarrollan estrategias reproductivas muy diferentes. Así, la lubina es una especie gonocorista que muestra la peculiaridad de poseer una diferenciación sexual dependiente de la temperatura (Piferrer, *et al.*, 2005; Vandeputte, *et al.*, 2007). Sin embargo, la dorada es una especie hermafrodita protándrica que se desarrolla como macho durante los primeros dos ciclos reproductivos y posee una gónada formada por una región testicular funcional y una pequeña región ovárica funcionalmente inmadura (Chaves-Pozo, *et al.*, 2005b). Al final del segundo ciclo reproductivo, el testículo comienza un proceso degenerativo para cambiar de sexo, caracterizado por una elevada apoptosis y necrosis acompañada por una infiltración masiva de granulocitos acidófilos y el drástico descenso de la expresión del gen que codifica para DMRT1 (Liarte, *et al.*, 2007). En ambos casos, el ciclo reproductivo de los machos se puede dividir en cuatro estadios: espermatogénesis, puesta, post-puesta y quiescencia (Chaves-Pozo, *et al.*, 2005b; Valero, *et al.*, 2015a). Durante la espermatogénesis el epitelio germinal del testículo está formado por espermatogonias, células de Sertoli y células germinales en desarrollo. A lo largo de este periodo las células germinales maduran pasando por los estadios de espermatocito, espermátida y espermatozoide, cuya maduración implica la adquisición de movilidad y capacidad de fertilización (Chaves-Pozo, *et al.*, 2005b; Schulz, *et al.*, 2010; Valero, *et al.*, 2015a). La mayoría de los peces con puesta estacional, al comienzo de la puesta, poseen un testículo formado por quistes de espermatogonias y espermatocitos inmaduros. Una vez han madurado, los espermatozoides se liberan al lumen del quiste para ser liberados al medio (Chaves-Pozo, *et al.*, 2005b; Valero, *et al.*, 2015a). Durante las etapas de post-puesta y quiescencia, la gónada sufre importantes cambios morfológicos, fruto de una remodelación tisular y a la eliminación de los espermatozoides remanentes del ciclo anterior (Chaves-Pozo, *et al.*, 2005b; Valero, *et al.*, 2015a).

En peces, la gametogénesis está regulada por factores gonadales y sistémicos que varían dependiendo del estadio en que se encuentre la gónada (Schulz, *et al.*, 2010). De esta forma, la glándula pituitaria sintetiza gonadotropinas, cuyos receptores se encuentran localizados generalmente en la gónada (Schulz, *et al.*, 2010). La producción de esteroides sexuales (progestágenos, andrógenos y estrógenos) se produce de forma generalizada en la gónada, controlada por la actividad de enzimas esteroidogénicas (Miller, 1989; Stocco, 2001). Los andrógenos mayoritarios en machos son la T y la

11KT, mientras que el estrógeno mayoritario en la hembra es el 17β-estradiol (Borg, 1994; Miller, 1989). En peces existen tres receptores de estrógenos diferentes: ERa, ERb1 y ERb2, los cuales existen en testículo, sugiriendo que el E_2 puede estar involucrado en la fisiología de este tejido en machos (Andreassen, *et al.*, 2003; Bardet, *et al.*, 2002; Diotel, *et al.*, 2011; Legler, *et al.*, 2000; Liu, *et al.*, 2010).

En peces, la mayoría de la información existente respecto a la interacción inmuno-endocrina trata sobre la regulación de la respuesta inmunitaria debida a las hormonas circulantes, incluyendo en éstas las hormonas reproductivas (Engelsma, *et al.*, 2002; Harris and Bird, 2000). Aunque los efectos de estas hormonas circulantes en la respuesta inmunitaria depende de la especie, en general, el sistema endocrino regula la respuesta inmunitaria con el fin de adaptar al individuo al medio en el que habita (Lutton and Callard, 2006). Se sabe que algunas especies de peces muestran alteraciones en los niveles de hormonas sexuales tras una infección (Deane, *et al.*, 2001). En peces, al igual que ocurre en mamíferos, las hormonas sexuales modulan las funciones leucocitarias como la fagocitosis o la producción de ROIs y RNIs, regulando también la producción de citoquinas pro-inflamatorias, quimioquinas y receptores relacionados con la respuesta inmunitaria (Águila, *et al.*, 2013; Cuesta, *et al.*, 2007; Liarte, *et al.*, 2011a,b,c; Watanuki, *et al.*, 2002). En la gónada de peces podemos encontrar diferentes tipos de leucocitos que modulan importantes procesos reproductivos incluyendo la gametogénesis y la esteroidogénesis. Por ejemplo, durante la post-puesta y la involución testicular de la dorada, se produce una infiltración masiva de leucocitos (principalmente granulocitos acidófilos) que es orquestada por factores gonadales, entre otros, hormonas sexuales (Chaves-Pozo, *et al.*, 2012a). Además, los leucocitos presentes en gónada, muestran un patrón funcional distinto a los leucocitos presentes en otros tejidos, ya que la respuesta inmunitaria en la gónada está muy restringida para evitar que puedan producir daños en las células germinales, debido a que éstas se producen durante la pubertad, mucho después de que el sistema inmunitario haya desarrollado la inmunocompetencia (Chaves-Pozo, *et al.*, 2012a; Valero, *et al.*, 2015a). Por esta razón podemos considerar la gónada de peces teleósteos como un tejido inmunoprivilegiado.

Entre los patógenos de peces más importantes se encuentran los virus, que ocasionan generalmente altas mortalidades, y frente a los que no existen tratamientos paliativos y muy pocos preventivos (vacunas). Entre los virus que afectan a especies importantes en la acuicultura marina, y concretamente a la española, nos encontramos a

NNV (familia *Nodaviridae*, género *Betanodavirus*) (Munday, *et al.*, 2002), ya que una de las especies más susceptibles a este virus es la lubina (Breuil, *et al.*, 1991; Frerichs, *et al.*, 1996). Además, NNV es capaz de infectar otras especies tales como la dorada, las cuales si bien no sufren la enfermedad, sí son capaces de transmitirlo (Castric, *et al.*, 2001). Este virus infecta fundamentalmente tejidos del sistema nervioso central (cerebro y retina) de larvas y juveniles de las especies susceptibles produciendo la encefalopatía y retinopatía viral, aunque el desarrollo de la enfermedad disminuye con el tamaño del animal generalmente. Sin embargo, se han detectado brotes con mortalidades elevadas en ejemplares adultos de talla comercial (Hellberg, *et al.*, 2010; Kara, *et al.*, 2014; Le Breton, *et al.*, 1997). NNV es un virus de RNA monocatenario con polaridad positiva que contiene dos fragmentos de RNA genómico, el RNA1 que codifica para la CP y el RNA2 que codifica para una proteína no estructural llamada RNA polimerasa dependiente de RNA que se encarga de la replicación del virus (Munday, *et al.*, 2002). Además, durante la infección el virus expresa una región sub-genómica de RNA1, denominada el RNA3, que codifica la proteína B2 que es importante para la acumulación del RNA1 en las células y que antagoniza de forma muy eficiente los siRNA del hospedador (Fenner, *et al.*, 2006; Iwamoto, *et al.*, 2005).

Durante los últimos años, el estudio de la respuesta inmunitaria frente a NNV está cobrando relevancia debido a que se ha extendido por todo el mundo, provocando grandes pérdidas económicas. Dentro de la respuesta inmunitaria innata, la respuesta del IFN es la más estudiada, ya que es la más potente en la defensa contra virus. Se han descrito algunos genes que participan en esta respuesta, caracterizando principalmente la respuesta de IFN y Mx en diversas especies (Krasnov, *et al.*, 2013; Lu, *et al.*, 2008; Overgård, *et al.*, 2012; Park, *et al.*, 2009). En dorada y lubina, la respuesta de estos dos efectores ha sido extensamente estudiada debido a la relevancia que tienen estas especies en la acuicultura Mediterránea y a sus diferentes susceptibilidades a NNV. Se ha podido observar que los genes que codifican estas proteínas se estimulan fuertemente en ambas especies tanto en el cerebro, como en riñón cefálico, aunque esta respuesta se desarrolla con mayor intensidad en el cerebro de la dorada (Bravo, *et al.*, 2013; Chaves-Pozo, *et al.*, 2012b; Dios, *et al.*, 2007; Novel, *et al.*, 2013; Poisa-Beiro, *et al.*, 2008, 2009; Scapigliati, *et al.*, 2010). Por otro lado, se han estudiado otros aspectos de la respuesta innata, demostrándose que NNV estimula en el cerebro o riñón cefálico, genes que codifican para ILs, TLRs, quimioquinas o lectinas en estas dos especies (Cuesta, *et*

al., 2010; López-Muñoz, *et al.*, 2012; Poisa-Beiro, *et al.*, 2008, 2009; Scapigliati, *et al.*, 2010). En ellas, además, este laboratorio pudo demostrar que la citotoxicidad de las NCCs en HKLs tras una infección *in vivo* con NNV aumenta al igual que lo hace el gen que codifica para la proteína NCCRP-1, aunque la explosión respiratoria sólo se incrementa en dorada (Chaves-Pozo, *et al.*, 2012b). Además, la expresión de otros genes relacionados con la respuesta inmune innata como la proteína de unión a saxitoxina 1, proteína quinasa-inducible del suero, algunos genes relacionados con la ruta del complemento, TLRs, SACs o beta-defensina entre otros, se estimulan en determinadas especies tras una infección con NNV (Guo, *et al.*, 2012; Park, *et al.*, 2009; Rise, *et al.*, 2010). En cuanto a la función antimicrobiana, NNV estimula la actividad del complemento y la bactericida en condiciones de elevado confinamiento en el caso de dorada y lubina (Mauri, *et al.*, 2011), incrementando los niveles de apolipoproteína A-1 en el hígado de lubinas infectadas con NNV y estimulando la expresión de los genes que codifican para ferritina y transferrina, que podrían estar relacionados con la activación de macrófagos en peces (Sarropoulou, *et al.*, 2009). En cuanto a la respuesta inmunitaria adaptativa, se ha observado que NNV induce la producción de anticuerpos específicos en muchas especies, incluidas dorada y lubina (Grove, *et al.*, 2006; López-Jimena, *et al.*, 2012; Scapigliati, *et al.*, 2010; Skliris y Richards, 1999; Woo, *et al.*, 2009). Es más, estos anticuerpos son capaces de neutralizar a NNV (Grove, *et al.*, 2003, 2006; Hegde, *et al.*, 2005; Tanaka, *et al.*, 2001; Woo, *et al.*, 2009). Concretamente en la dorada, un estudio sugiere la infiltración masiva de células B positivas en IgM en el cerebro de peces infectados (López-Muñoz, *et al.*, 2012). Además, algunos genes relacionados con los linfocitos T se estimulan en los tejidos infectados de rodaballo, sugiriendo la activación de estas células durante una infección con NNV (Overgård, *et al.*, 2012). En la lubina, se ha detectado un aumento de linfocitos T y B en leucocitos de sangre periférica durante una infección con NNV, además de que los leucocitos obtenidos de la sangre, riñón cefálico o branquias aumentan su proliferación tras un tratamiento *in vitro* con partículas virales inactivadas (Scapigliati, *et al.*, 2010). Sin embargo, la expresión del gen *cd83* se inhibe en HKLs tras una infección experimental por NNV (Buonocore, *et al.*, 2012).

Existen otros estudios que indican que NNV se transmite tanto horizontal como verticalmente de unos ejemplares a otros. La transmisión horizontal parece ser la más común en estaciones acuícolas, sobre todo teniendo en cuenta que el agua es el vector

principal (OIE, 2013). Sin embargo, se han detectado partículas infectivas de NNV tanto en individuos reproductores como en embriones de diferentes especies sugiriendo esto su transmisión vertical, aunque una transmisión vertical *senso stricto* no ha sido claramente demostrada (Hodneland, *et al.*, 2011; Kuo, *et al.*, 2012; Munday, *et al.*, 2002). Además, en el caso de las estaciones acuícolas situadas en ambientes acuáticos abiertos, la exposición a estos patógenos es inevitable ya que existe un constante contacto entre peces cultivados y silvestres. Para prevenir la aparición de enfermedades se han desarrollado diversos tipos de vacunas, entre las que destacan las vacunas de DNA (Kurath, 2008). Durante los últimos años, se han desarrollado diversos tipos de vacunas contra NNV formadas por partículas virales inactivadas o por proteínas virales recombinantes (Dos Santos, *et al.*, 2009; Grove, *et al.*, 2003; Thiéry, *et al.*, 2006; Yamashita, *et al.*, 2004). Además, se han desarrollado experimentalmente vacunas de DNA con resultados contradictorios. Así, la inyección intramuscular de una vacuna de DNA contra NNV no generó una protección efectiva contra el virus en ejemplares de fletán (*Hippoglossus hippoglossus*) (Sommerset, *et al.*, 2005b), mientras que otros estudios han conseguido proteger parcialmente ejemplares de barramundi (*Lates calcarifer*) tras la administración oral de este tipo de vacunas (Vimal, *et al.*, 2014). A pesar de que estas vacunas inducen la respuesta inmunitaria y mejoran la supervivencia de diferentes especies afectadas, no existía hasta 2015 una vacuna comercial para paliar las devastadoras mortalidades producidas por este virus (Kai y Chi, 2008; Kim, *et al.*, 2000; Nishizawa, *et al.*, 2012; Sommerset, *et al.*, 2005a), la cual está compuesta por partículas virales de NNV inactivadas con formalina (PharmaQ). Es destacable que la mayoría de las vacunas se administran a los individuos por inyección, por lo que no es posible aplicarla a peces de un tamaño menor a 1 g, los cuales suelen ser los más susceptibles a una infección por NNV. Es más, utilizando la vía oral para administrarlas, los especímenes en los primeros estadios larvarios no pueden ser vacunados debido a que todavía no han desarrollado completamente su sistema inmunitario y digestivo. Por todo ello se hace necesario desarrollar diferentes estrategias que permitan por un lado vacunar a ejemplares de pequeño tamaño mediante el desarrollo de vacunas orales y por otro que produzcan una inmunización efectiva de los ejemplares reproductores que permita proteger a la descendencia mediante la transferencia pasiva de la inmunidad.

VI.2. OBJETIVOS

En la presente Tesis Doctoral se proponen los siguientes objetivos:

1. Evaluar la capacidad de nodavirus para colonizar y replicar en el testículo de lubina y dorada.

2. Estudiar los efectos de la infección por nodavirus en parámetros del sistema reproductor.

3. Analizar la respuesta inmunitaria innata, especialmente la ruta del interferón y los péptidos antimicrobianos, en el testículo de lubina y dorada durante una infección con nodavirus, y compararla con la respuesta en el cerebro.

4. Generar una vacuna DNA frente a nodavirus y evaluar la respuesta inmunitaria y protección inducidas en alevines de lubina tras la vacunación por vía oral.

5. Evaluar si la vacuna de DNA administrada a lubinas hembras reproductoras mejora la transferencia materna de la inmunidad a la descendencia.

VI.3. PRINCIPALES RESULTADOS Y DISCUSIÓN

Esta Tesis se organiza en dos partes diferenciadas. En la primera, con 4 capítulos experimentales, se estudia la localización de NNV en la gónada de dorada y lubina y cómo éste afecta a la respuesta inmunitaria gonadal. En la segunda y última parte, con 2 capítulos, se ha generado una vacuna de DNA que se administra a larvas o reproductores de lubina y se estudia la respuesta inmunitaria, protección y transferencia materna de la inmunidad.

VI.3.1. LOCALIZACIÓN DE NODAVIRUS Y RESPUESTA INMUNITARIA EN TESTÍCULO DE LUBINA Y DORADA

VI.3.1.1. Nodavirus coloniza y replica en la gónada de machos de dorada y lubina modulando su respuesta inmunitaria y reproductora

En el primer capítulo de esta Tesis se ha demostrado por primera vez que NNV es capaz de colonizar y replicar en el testículo de lubina y dorada. Se ha podido localizar el RNA2 del virus en células de Sertoli del testículo de ambas especies, y en la túnica albugínea y células germinales de dorada, sugiriendo que NNV se podría propagar a través de las células germinales de dorada y en fluidos gonadales de ambas especies. No obstante, debido a la íntima asociación entre las células de Sertoli y germinales en peces (Schulz*, et al.*, 2010), no se puede descartar que el virus pueda transmitirse también mediante células germinales de lubina. Además, se detectaron las proteínas virales CP y B2 exclusivamente en células somáticas del testículo de dorada. En ambas especies, se rescataron de la gónada partículas virales infectivas mediante pases ciegos en cultivos celulares. En cuanto a la respuesta inmunitaria desencadenada en la gónada, NNV inhibió la expresión de los genes *tcrb* e *igmh* en la gónada de dorada, mientras que en el testículo de lubina se incrementó la expresión génica de varias citoquinas pro-inflamatorias (*il6*, *il1b* y *tnfa*) y de *igmh*. En el cerebro, tejido diana del virus e inmuno-privilegiado como la gónada, la infección por NNV provocó el aumento de la expresión génica de todas las citoquinas pro-inflamatorias estudiadas en lubina, mientras que en dorada sólo se estimuló la expresión de *il6*. Teniendo en cuenta que sólo los ejemplares de lubina sufrieron mortalidades, nuestros datos sugieren que la alta respuesta pro-inflamatoria en lubina probablemente produzca un alto daño celular que explica su incapacidad para reponerse de la infección (Chaves-Pozo*, et al.*, 2012b; presente Tesis). Es más, en la gónada de dorada, la escasa alteración de la expresión de genes marcadores de respuesta celular y citoquinas junto con la muy leve respuesta anti-inflamatoria favorece la replicación viral, puesto esto de manifiesto en la detección de NNV a nivel génico, protéico y de partículas virales. Sin embargo, en lubina, conforme va progresando la infección, la actividad reproductiva pasa a un segundo plano y la respuesta inflamatoria es muy intensa ya que se prima la supervivencia del individuo aun cuando el tejido testicular pueda resultar dañado.

En algunas especies de peces, tras una infección se alteran los niveles hormonales, relacionándose esto con la mortalidad (Deane, *et al.*, 2001; Gómez, *et al.*, 2000). En el presente estudio, los niveles séricos de E_2 en lubina descendieron conforme iba progresando la infección y a la vez que aumentaba la mortalidad, a pesar del desarrollo de una respuesta inmunitaria temprana pero, evidentemente, poco efectiva en el cerebro (Chaves-Pozo, *et al.*, 2012b). Sin embargo en dorada, los niveles séricos de E_2 se incrementaron al comienzo de la infección mientras que los de 11KT descendieron al comienzo pero se incrementaron al final. Teniendo en cuenta que en dorada, los estrógenos regulan procesos inflamatorios, mientras que algunos andrógenos regulan procesos de la respuesta inmunitaria celular (Águila, *et al.*, 2013; Cabas, *et al.*, 2013; Liarte, *et al.*, 2011b,c; Sánchez-Hernández, *et al.*, 2013), nuestros resultados sugieren que la alteración de los niveles de esteroides sexuales podría favorecer que esta especie sea capaz de superar la infección. Además, los resultados obtenidos muestran que en la gónada de dorada, aumentó la expresión de la aromatasa gonadal a la vez que aumentó el nivel sérico de E_2, mientras que la expresión de este gen no se detectó en el testículo de lubina en ningún grupo, antes o después de la infección. Sin embargo, la sensibilidad de la gónada de dorada al E_2 descendió durante la infección ya que la expresión del gen *era* fue inhibida. En el testículo de lubina, sin embargo, NNV produjo una estimulación de los genes que codifican para los receptores de estrógenos a tiempo corto y una inhibición generalizada a tiempo largo. Estudiamos, además, la sensibilidad del cerebro de ambas especies al E_2 ya que la producción local de esta hormona en cerebro regula importantes funciones biológicas como la reproducción y la neuroprotección (García-Segura, *et al.*, 2001). Así, pudimos observar que en el cerebro de dorada, la expresión del gen *era* descendió. Sin embargo, en lubina, la expresión de los genes que codifican para la aromatasa neural y para dos receptores de estrógenos (*erb1* y *erb2*) se regulan de forma muy diferente con respecto a dorada, pudiendo estar esto relacionado con las distintas sensibilidades al virus de ambas especies. A pesar de los cambios observados en los niveles séricos de E_2 y 11KT, y en la expresión de genes que codifican para enzimas esteroidogénicas y receptores de estrógenos, no se observó ninguna alteración de la capacidad reproductiva testicular, ya que no se observaron alteraciones morfológicas en ninguna especie; y en dorada, además, el IGS aumentó y los niveles de transcripción del gen *dmrt1* se mantuvieron dentro de los límites funcionales del tejido.

Tras el tratamiento *in vitro* de la gónada de ambas especies con NNV, la expresión génica de la mayoría de genes estudiados fue distinta a la observada durante

la infección *in vivo*, sugiriendo que las alteraciones producidas en otros tejidos tras la infección modifican la respuesta de la gónada a la misma.

En conclusión, en este estudio se ha demostrado por primera vez la habilidad de NNV de colonizar la gónada de machos de dorada y lubina, su capacidad para producir partículas virales infectivas así como para regular y modular su respuesta inmune y reproductora.

VI.3.1.2. Caracterización de la ruta del interferón en la gónada de peces teleósteos frente al virus de la necrosis nerviosa viral

El IFN es el mecanismo mejor caracterizado de la respuesta inmunitaria innata de peces frente a infecciones virales. Aun así, hasta ahora, en peces sólo se habían caracterizado de forma aislada el gen del *ifn*, perteneciente al IFN de tipo I, y el gen inducido por IFN, *mx* (Casani, *et al.*, 2009; Fernández-Trujillo, *et al.*, 2011; Scapigliati, *et al.*, 2010). NNV induce una respuesta elevada del IFN de tipo I en el cerebro, y su activación puede ser responsable de la eliminación del virus en especies resistentes como la dorada, mientras que se ha descrito una menor actividad en especies susceptibles como la lubina (Chaves-Pozo, *et al.*, 2012b; Chen, Y.M., *et al.*, 2014; López-Muñoz, *et al.*, 2012; Overgård, *et al.*, 2012; Poisa-Beiro, *et al.*, 2008; Scapigliati, *et al.*, 2010). Sin embargo, se sabe muy poco sobre los mecanismos moleculares que producen la activación del IFN de tipo I cuando es inducido por virus, particularmente NNV (Dios, *et al.*, 2007; Rise, *et al.*, 2010). Además, ninguno de los estudios encontrados se centra en la respuesta gonadal de estas dos especies, a pesar de que se trata de un virus que se transmite de forma vertical a la descendencia (Arimoto, *et al.*, 1992; Kuo, *et al.*, 2012). Por ello, nos propusimos la búsqueda en las bases de datos existentes de las secuencias de los genes que codifican receptores RLRs y los mediadores que participan en la ruta de activación del IFN y el estudio de su expresión en gónada y cerebro tras infecciones experimentales con NNV.

Inicialmente, en este laboratorio, identificamos las secuencias génicas de sensores de RNA viral como *mda5* en ambas especies y *lgp2* en lubina. Aunque en células de la línea celular SAF-1 infectadas con NNV solo se incrementó la expresión del gen *mda5* ninguno de los genes sufrió cambios de expresión en la línea de cerebro de lubina DLB-1 infectada con NNV. Ambos genes se estimularon durante la infección *in vivo* con NNV en el cerebro de ambas especies, sugiriendo que el RNA del virus es

reconocido e induce la respuesta mediada por IFN al igual que ocurre en otras especies de peces (Dios, *et al.*, 2007; Rise, *et al.*, 2010). También pudimos identificar dos secuencias codificantes de moléculas de la ruta de activación del IFN intermedias entre los RLR y el IFN en dorada (*tbk1* e *irf3*) y de la mayoría de las moléculas de esta ruta (*mavs*, *traf3*, *tank*, *tbk1*, *irf3* e *irf7*) en el caso de la lubina, y también el gen *pkr* en ambas especies. Aunque la mayoría son secuencias parciales, el análisis de predicción de proteínas las agrupó con ortólogos de las proteínas esperadas. La expresión de estos genes en cerebro y gónada tanto en condiciones naturales como tras una infección con NNV se correlacionó con la expresión del gen *ifn*. Nuestros resultados muestran que NNV incrementa la expresión de los adaptadores e intermediarios en la ruta del IFN en gónada de lubina, pero inhibe dicha expresión en cerebro, pudiendo explicar esto la elevada sensibilidad de esta especie al virus. Sin embargo, en dorada se observó el aumento de la expresión del gen *irf3* en gónada al comienzo y en cerebro a tiempos largos de infección. Estos datos coinciden con los obtenidos en otros estudios realizados en peces que muestran la estimulación de algunos genes relacionados con la ruta del IFN tras infecciones virales en diversos tejidos o líneas celulares (Biacchesi, *et al.*, 2009; Chen, H.Y., *et al.*, 2015; Feng, *et al.*, 2011; Rise, *et al.*, 2008, 2010; Skjesol, *et al.*, 2011; Su, *et al.*, 2010; Sun, F., *et al.*, 2011; Xiang, *et al.*, 2011; Yang, *et al.*, 2011), apoyando el hecho de que las secuencias identificadas en este estudio forman parte de la cascada de activación del IFN. Únicamente *tbk1*, que es activado también por los receptores Toll (TLR), sólo fue estimulado en gónada de lubinas infectadas con NNV, no así en dorada, donde no fue alterado en ningún tejido. Es más, algunos datos apuntan a la activación de este gen y la respuesta antiviral sin implicar la activación de IRF3/7, pudiendo existir otras rutas de activación en peces (Feng, *et al.*, 2014). Por todo ello, nuestros datos sugieren que *tbk1* no es relevante para la respuesta antiviral en la dorada.

Finalmente, observamos que esta cascada desencadena la activación del IFN y la expresión de los genes inducidos por el IFN. Sin embargo, en dorada la transcripción del gen del *ifn* no logra que se induzca la expresión de los genes estimulados por IFN, es decir, de los genes *mx* y *pkr*. Esto podría explicarse debido a los distintos tiempos de inducción de estas moléculas, ya que la expresión del *ifn* suele ser muy rápida y por cortos periodos de tiempo (López-Muñoz, *et al.*, 2012), o bien a la presencia de diferentes isoformas o variantes de IFN, que por el momento todavía son desconocidas y necesitarían ser estudiadas. Contrastando con los datos de dorada, en lubina se inhibió la expresión del gen del *ifn* en el cerebro, así como ocurrió con la mayoría de los genes

implicados en la cascada, al mismo tiempo que se vieron estimulados los genes *mx* y *pkr*, sugiriendo la existencia de otras vías de activación como ya se ha sugerido previamente (Feng, *et al.*, 2014). Los resultados obtenidos en la gónada de lubina son muy interesantes ya que todos los genes fueron estimulados tras la infección con NNV, sobre todo si consideramos la especial regulación de la respuesta inmunitaria en este órgano (Chaves-Pozo, *et al.*, 2010b), característica por la que algunos patógenos utilizan la gónada como vía de transmisión (Arimoto, *et al.*, 1992; Kuo, *et al.*, 2012). La estimulación de la respuesta inmunitaria en la gónada de los ejemplares de lubina infectados podría ser un mecanismo de supervivencia al virus, que supondría priorizar la lucha contra el virus frente a la función reproductiva. Sin embargo, en la dorada la respuesta en el cerebro es altamente eficaz y supone que la enfermedad no se desarrolla, así, la inhibición de la respuesta inmunitaria en gónada evita daños en las células germinales y garantiza la reproducción del ejemplar, aunque al mismo tiempo permite la transmisión del virus a través de los fluidos gonadales y los gametos. Esta hipótesis se ve apoyada por el hecho de que existen otras moléculas inmunitarias como los AMPs, cuyos patrones de expresión génica son similares, es decir, se inhiben en cerebro y se estimulan en gónada de lubinas infectadas, y a la inversa en doradas infectadas (Valero, *et al.*, 2015b).

En conclusión, este estudio representa una de las caracterizaciones más completas de los genes que codifican proteínas involucradas en la respuesta del IFN tras una infección viral desencadenada por RLR en peces. Así, en dorada, encontramos estimulaciones de la expresión de los genes *mda5*, *irf3*, *mx* y *pkr* en cerebro pero no en gónada. Sin embargo, en lubina, la expresión de la mayoría de los genes se encuentra inhibida en el cerebro pero muy estimulada en la gónada, desencadenando esto un incremento de la transcripción de los genes del *ifn*, *mx* y *pkr* en este tejido.

VI.3.1.3. La respuesta antimicrobiana es incrementada en el testículo de lubina, pero no en el de dorada, durante una infección con nodavirus

La implicación de los AMPs en la respuesta inmunitaria innata, así como su regulación, se ha investigado en vertebrados (Hancock y Diamond, 2000). En peces, los AMPs se han estudiado principalmente en los tejidos inmunitarios (Valero, *et al.*, 2013), sin embargo, merece la pena su estudio en los órganos reproductores ya que la regulación de la respuesta inmunitaria en estos órganos es singular, permitiendo a

ciertos virus colonizar la gónada y ser transmitidos a través de los fluidos o los gametos (Chaves-Pozo, *et al.*, 2005c, 2010a). Entre los virus capaces de ello, NNV produce infecciones persistentes en especies asintomáticas y se ha detectado en individuos reproductores y embriones e incluso en la gónada de dorada y lubina (Aranguren, *et al.*, 2002; Breuil, *et al.*, 2001; Frerichs, *et al.*, 1996; Kuo, *et al.*, 2012; Munday, *et al.*, 2002; Capítulo III.1.1.). Es interesante que en el cerebro, principal tejido diana de NNV, la actividad antiviral determinada por la expresión del gen *mx* es mayor en dorada que en lubina, hecho que ha sido correlacionado con la resistencia o susceptibilidad de estas especies a la infección con NNV. Sin embargo, este gen fue altamente estimulado en la gónada de lubina pero no en dorada. En este capítulo nos propusimos caracterizar la respuesta inmunitaria gonadal frente a una infección con NNV, centrándonos principalmente en la respuesta de los AMPs.

La proteína C3 es el componente central del sistema del complemento y se considera un péptido antimicrobiano debido a su implicación directa en la defensa frente a patógenos (Boshra, *et al.*, 2006). Nuestros resultados mostraron pequeñas alteraciones en la actividad hemolítica del complemento en el suero de ambas especies al contrario de lo que ocurre en otros estudios (Mauri, *et al.*, 2011). Sin embargo, en la gónada no se detectó actividad hemolítica del complemento ni en ejemplares control ni en infectados. Es interesante destacar que sí se observó una estimulación de la transcripción del gen *c3* en la gónada de lubina y una inhibición en la de dorada con respecto a los niveles de expresión de los ejemplares control. Sin embargo, tras una infección con NNV *in vitro*, la expresión de este gen en la gónada no fue modificada en ninguna de las especies. En la gónada de dorada, no se observó expresión de *c3*, excepto tras la estimulación con pI:C. Esta ausencia de expresión de *c3* en la gónada coincide con otros estudios previos *in vivo* (Chaves-Pozo, *et al.*, 2008b). Las diferencias encontradas entre los estudios *in vivo* e *in vitro* podrían ser debidas al estrés generado en el proceso de infección y muestreo posterior de la experimentación *in vivo*, que puede llegar a afectar durante semanas (Noga, *et al.*, 2011). Pero sobretodo, el hecho de existir expresión de *c3* y una regulación específica tras la infección, sugiere que la actividad convertasa de C3 tiene lugar y es regulada por la infección en el testículo de ambas especies. El no detectar actividad podría deberse a que ésta es inactivada por inhibidores específicos producidos a nivel local como ocurre en humanos (Vanderpuye, *et al.*, 1992).

La actividad lisozima, incrementó en la gónada de ambas especies tras la infección aunque no ocurrió así en suero. En la gónada de lubina se incrementó a tiempo

corto tras la infección, mientras que en la de dorada el incremento se mantuvo a más largo plazo. Además, en la gónada de lubina, la actividad lisozima se correlacionó negativamente con los niveles de expresión de *lyz*, sugiriendo esto la existencia de mecanismos regulatorios de la actividad mediante la regulación de la expresión del gen. Es más, en ambas especies, la expresión en la gónada de *lyz* tras la infección *in vivo* coincide con la observada tras el tratamiento *in vitro*, apuntando a que estos cambios están causados por una respuesta local de la gónada a la infección.

En este estudio, la actividad bactericida total contra *V. haveyi* durante una infección con NNV fue muy distinta entre lubina y dorada. En la gónada de lubina, esta actividad se inhibió pero se incrementó en el suero a partir de los 7 días de infección. Sin embargo, en dorada, tras un pequeño descenso inicial en suero, esta actividad se incrementó tras 15 días de infección tanto en gónada como en suero. Aunque la evaluación de la actividad lítica total contra patógenos es la determinación más práctica para los criadores de peces, pudiendo ser esta más relevante que el estudio independiente de cada AMP, hemos analizado la expresión génica de diversos AMPs previamente descritos en estas especies con el fin de identificar los más relevantes en la respuesta inmune gonadal. Algunas de las moléculas estudiadas parecen estar involucradas en la defensa contra virus, como sucede con C3 y piscidina (Dezfuli, *et al.*, 2012; Overturf y LaPatra, 2006). De hecho, algunos de ellos tienen demostrada actividad antiviral, como ocurre con la lisozima, la hepcidina y la beta-defensina (Chia, *et al.*, 2010; Siwicki, *et al.*, 1998; Valero, *et al.*, 2013). Nuestros datos mostraron que en la gónada de lubina, donde la actividad bactericida se encontró inhibida tras la infección, la expresión de la mayoría de los genes se estimuló, al contrario de lo que ocurrió en la gónada de dorada. A pesar de que esta actividad se correlacionó negativamente con la expresión de *c3* en la gónada de dorada, no se observaron correlaciones con el resto de AMPs en la gónada de lubina. El patrón de expresión de *lyz*, *hamp* y *dic* en la gónada de lubina tras la infección *in vivo* o tras el tratamiento *in vitro* con NNV o pI:C fue similar, sugiriendo esto que este perfil de expresión puede estar provocado por una respuesta inmunitaria local. No obstante, hay que destacar que a diferencia del estudio *in vivo*, tras el tratamiento *in vitro*, no se detectó expresión génica de *mx*, por lo que ambos experimentos son difícilmente comparables. Sin embargo, este hecho sugiere que las respuestas antiviral y antimicrobianas estudiadas en este trabajo podrían regularse mediante mecanismos distintos. Por otro lado, si comparamos la respuesta de la gónada con la del cerebro de lubina, aunque ambos son

órganos immunoprivilegiados, encontramos una regulación completamente diferente en cada uno de ellos a nivel de expresión génica, encontrando únicamente el mismo patrón de expresión en el caso de *hamp* y *dic*. En contraposición, en dorada, los genes analizados mostraron variaciones de expresión similares en ambos tejidos. Es importante destacar que tanto la expresión de algunos AMPs como la de *mx* estaban correlacionadas positivamente en el cerebro de dorada, apoyando la hipótesis de que la elevada respuesta inmunitaria en este tejido es responsable de la eliminación del virus. Es más, en esta especie, la respuesta de los AMPs a nivel de expresión génica está inversamente regulada en ambos tejidos, explicando parciamente la habilidad de NNV de ser transmitido a través de la gónada sin inducir una respuesta inmune que afecte las funciones reproductivas.

En conclusión, los resultados obtenidos en ese estudio demuestran que la respuesta inmunitaria basada en los AMPs en la gónada de lubina y dorada es claramente diferente frente a una infección con NNV, tanto a nivel de expresión génica y de actividad antimicrobiana, lo cual podría explicar la distinta susceptibilidad de ambas especies al virus. Es más, esta diversidad encontrada tras las infecciones *in vivo* e *in vitro* sugiere que algunos AMPs podrían estar regulados localmente en la gónada, mientras que otros podrían depender en mayor medida de la respuesta inmunitaria elaborada en otros tejidos. Además, nuestros resultados han determinado diferencias claras entre la respuesta inmunitaria desencadenada por NNV en cerebro y gónada, explicando la distinta funcionalidad de ambos tejidos frente a la infección

VI.3.1.4. La transcripción de genes de las histonas de peces H1 y H2B sugiere una posible función como péptidos antimicrobianos

Las histonas, como proteínas estructurales de la cromatina, se creían confinadas al núcleo. Sin embargo, algunos estudios han detectado que diversas histonas, o fragmentos de las mismas, se hallaban en el citoplasma de varios tipos celulares incluyendo leucocitos de mamíferos, aves, anfibios o peces, mostrando estas proteínas un amplio espectro de actividades antimicrobianas (Parseghian y Luhrs, 2006). Se ha demostrado que algunas histonas centrales del nucleosoma (H2A y H2B) y la histona de enlace (H1) poseen actividad antimicrobiana en diversas especies de peces (Valero, *et al.*, 2013). Concretamente, en lubina, se aislaron las secuencias parciales de las histonas H1 y H2B, y se observaron patrones de expresión en condiciones de estrés similares a

otros AMPs conocidos (Terova, *et al.*, 2011; Ullal, *et al.*, 2008). En este trabajo hemos utilizado las secuencias completas de estas proteínas de lubina para realizar la búsqueda de ortólogos en dorada. De esta forma, encontramos dos secuencias anotadas pero no caracterizadas en las bases de datos del *GenBank*. Tras un análisis filogenético, las secuencias de *h1* y *h2b* de dorada y lubina mostraron un elevado porcentaje de similitud con ortólogos de pez cebra y humanos, lo que sugiere que su función podría estar también conservada. La mayoría de las histonas se han aislado de piel de peces, estudiando su expresión constitutiva en piel o hígado principalmente (Narváez, *et al.*, 2010; Richards, *et al.*, 2001; Robinette, *et al.*, 1998). Sin embargo, otros AMPs se han localizado en un amplio conjunto de tejidos incluyendo tejidos inmunoprivilegiados (Valero, *et al.*, 2013, 2015b). Nuestros resultados mostraron que el gen *h1* se expresaba de forma constitutiva en todos los tejidos analizados, tanto de lubina como de dorada, tal y como ocurre también en lenguado (*Paralichthys olivaceus*) (Nam, *et al.*, 2012). Curiosamente, en lenguado, la mayor expresión del gen *h1* se da en la gónada (Nam, *et al.*, 2012). Sin embargo, en el presente estudio la mayor expresión se encontró en los tejidos inmunitarios de las dos especies estudiadas. Según la bibliografía existente, nuestro estudio es el primero en analizar el patrón de expresión del gen *h2b* en un amplio abanico de tejidos en dos especies de peces. Así, en lubina no detectamos niveles de transcripción del gen *h2b* en cerebro, piel o bazo, mientras que se expresaba de forma muy elevada en timo. Por el contrario, en dorada la mayor expresión de este gen se encontró en sangre periférica y gónada.

Se ha descrito que algunas variantes de la histona H1 se movilizan tras una infección viral (Conn, *et al.*, 2008; Hoeksema, *et al.*, 2015). Por ello, analizamos la expresión de *h1* y *h2b* durante una infección con NNV. Tras la infección *in vivo*, el gen *h1* se estimuló en gónada de lubina y en cerebro de dorada, mientras que *h2b* lo hizo en el riñón cefálico de ambas especies. Es interesante destacar que la histona H1 se ha visto estimulada en el cerebro de especies resistentes a infecciones con ciertas bacterias (Das, *et al.*, 2014). De forma similar, la dorada es resistente al aislado de NNV utilizado en este estudio y en ella también se estimuló este gen en cerebro. Por el contrario, el gen *h1* se inhibió en cerebro de lubina, que es una especie altamente susceptible al virus.

A continuación analizamos la expresión de *h1* y *h2b* en gónadas de machos y hembras maduros de lubina y en gónadas de machos funcionales de dorada tras un tratamiento *in vitro* con NNV, *Va* o pI:C. Nuestros resultados sugieren que los cambios transcripcionales observados fueron alterados por la respuesta generada en otros tejidos.

No obstante, puede que la existencia de otros AMPs específicos de gónada y los elevados ratios de proliferación celular de este tejido hagan prescindible la función AMPs de las histonas H1 y H2B, mientras que en otros tejidos como cerebro o HKLs, esta función sea más necesaria y activa.

En el caso de los mamíferos, se sabe que las histonas son secretadas al exterior celular junto a DNA formando las NETs. Es más, recientemente se ha descrito que estas NETs se producen también en algunos leucocitos de peces, principalmente en ciprínidos (Brogden, *et al.*, 2014; Chi y Sun, 2016; Pijanowski, *et al.*, 2013, 2015). Por ello, y teniendo en cuenta la elevada expresión de *h1* y de *h1* y *h2b* observada en sangre periférica de lubina y dorada, respectivamente, procedimos al análisis de los niveles de transcripción de estos genes en HKLs estimulados *in vitro*. Así, la expresión de *h1* se inhibió en lubina en el caso de los tratamientos con virus y bacterias, mientras que en dorada esta inhibición también se observó tras los tratamientos con LPS, PHA y ConA. Por el contrario, la transcripción de *h2b* fue estimulada en HKLs de dorada e inhibida en los de lubina tras el tratamiento con NNV y otros estímulos. Aunque se necesitan estudios adicionales, este trabajo sugiere que la utilización de histonas como AMPs, ya sea en forma de NETs o no, podría formar parte de la clara diferencia que existe en la susceptibilidad de ambas especies a una infección con NNV.

En resumen, nuestros datos sugieren que la histona H1 podría tener un papel activo en la respuesta inmunitaria del cerebro de ambas especies contra NNV debido al hecho de que la expresión de *h1* es muy similar al patrón de expresión encontrado en otros AMPs y en moléculas involucradas en la ruta de activación del IFN, y se correlaciona perfectamente con las diferentes susceptibilidades a NNV de ambas especies (Valero, *et al.*, 2015b,c). Por otro lado, la histona H2B parece tener una mayor relevancia en la respuesta inmunitaria del riñón cefálico y sus leucocitos.

VI.3.2. MECANISMOS PREVENTIVOS EXPERIMENTALES FRENTE A UNA INFECCIÓN CON NODAVIRUS

VI.3.2.1. Una vacuna de DNA administrada oralmente mejora la supervivencia de juveniles de lubina frente a nodavirus

Las partículas de quitosano parecen ser un método ideal para la encapsulación de vacunas de DNA y la administración de las mismas de forma oral debido a sus

características naturales y a que no son tóxicas ni para animales ni para humanos (Rao y Sharma, 1997). Además, actúan como inmunoestimulantes, ofreciendo una mejora adicional de la respuesta inmunitaria (Abu-Elala, *et al.*, 2015; Cheng y Yu, 2013; Fujiki, *et al.*, 1994; Lin, *et al.*, 2011). Por ello diseñamos una vacuna de DNA que fue encapsulada en nanopartículas de quitosano y administrada en la dieta a ejemplares juveniles de lubina durante dos días (grupo vacunado: ChP-CP-pNNV). Como controles, se establecieron tres grupos: uno alimentado con dieta comercial (control), otro al que se le administró en la dieta nanopartículas de quitosano (ChP) y un tercer grupo al que se le administró en la dieta el plásmido vacío encapsulado en nanopartículas de quitosano (ChP-pcDNA3.1). En estos cuatro grupos experimentales, se analizó la expresión de diversos genes inmunitarios en intestino posterior a los 7, 30 y 90 días de la vacunación y los niveles de IgM total y específicos contra NNV en suero a los 90 días. Además, algunos ejemplares de cada grupo fueron sometidos a una infección mediante inyección intramuscular con NNV a los 90 días de la vacunación.

Es de destacar que independientemente del mecanismo de acción desarrollado por la vacuna de DNA administrada oralmente, nuestros datos muestran que, a los 90 días de la vacunación, los peces vacunados se encontraban clara y completamente protegidos contra NNV los primeros 21 días tras ser infectados, momento en el cual se comenzaron a registrar mortalidades. Teniendo en cuenta que las mortalidades observadas en los grupos control y ChP comenzaron alrededor del día 4 post-infección y alcanzaron el 100% de mortalidad antes del día 21, y que el grupo vacunado alcanzó un 45% de supervivencia al final del reto, podemos concluir que la vacuna de DNA encapsulada utilizada en este estudio protege a los peces frente a una infección con NNV.

A pesar de ser inmunoestimulantes como previamente se había descrito, ofreciendo una mejora adicional de la respuesta inmunitaria (Abu-Elala, *et al.*, 2015; Cheng y Yu, 2013; Fujiki, *et al.*, 1994; Lin, *et al.*, 2011), en este trabajo no encontramos ningún tipo de estimulación de la respuesta inmunitaria provocada por la administración de nanopartículas de quitosano. Sin embargo, en el grupo ChP-pcDNA3.1, se observó una estimulación de los genes *igmh*, *mhc1a*, *tcrb*, *cd8a* e *ifn* acompañada de un retraso en el comienzo de la mortalidad de los peces de este grupo tras el reto, que comenzó en el día 17 tras la infección con NNV. Estos datos indican que el plásmido vacío confiere cierta protección al igual que se ha descrito en otros casos (Kim, *et al.*, 2000; Liang, *et*

al., 2010) aunque esta sea probablemente inespecífica y no exclusiva para NNV. Es de destacar que en este estudio no detectamos expresión de la vacuna en el intestino posterior de los peces vacunados (ChP-CP-pNNV), pudiendo significar esto una expresión de la vacuna transitoria que podría explicar la protección parcial observada. Ningún grupo experimental mostró alteraciones en los niveles séricos de IgM total a los 90 días de la vacunación, es más, no detectamos anticuerpos específicos contra NNV en el grupo ChP-CP-pNNV. Curiosamente, la expresión de los genes *mhc1a* e *igmh* se incrementó a los 7 días tras la vacunación, al igual que ocurrió con la transcripción del gen *igth* tras 30 y 90 días post-vacunación. En la mayoría de los estudios en los que tras la administración de una vacuna de DNA, independientemente de la ruta de administración, se detecta producción de anticuerpos específicos, lo hacen dentro de un rango de entre 1 y 6 meses post-vacunación (de las Heras, *et al.*, 2010; MacLaughlin, *et al.*, 1998; Vimal, *et al.*, 2014; Zheng, *et al.*, 2010). Por el contrario, en otros estudios realizados con vacunas DNA, no se detectaron anticuerpos específicos a pesar de la buena protección conferida por la vacuna (Kurath, 2008; Lorenzen, *et al.*, 1998; McLauchlan, *et al.*, 2003). Nuestros resultados se muestran en consonancia con estos últimos estudios ya que, aunque no pudimos detectar anticuerpos específicos contra NNV, en el grupo vacunado se retrasó el comienzo de la mortalidad y aumentó la supervivencia hasta un 45% al final del reto experimental con NNV. Todos estos datos apuntan a la activación de mecanismos celulares antivirales específicos implicados en la protección provocada por la vacuna de DNA. En este sentido, y teniendo en cuenta que la ruta del IFN y la CMC son los mecanismos inmunitarios más potentes en la lucha contra virus en peces (Ellis, 2001; Robertsen, 2006), nos propusimos estudiar el patrón de expresión de genes relacionados con estas respuestas tras la vacunación. Nuestros resultados mostraron una estimulación de la expresión de los genes *tcrb* y *cd8a*, sugiriendo la activación de la CMC en el intestino posterior. Este es el primer estudio en describir este tipo de respuesta activada tras una vacuna de DNA administrada de forma oral en peces, a pesar de que ya se ha demostrado en mamíferos y en otros vertebrados (Jazayeri, *et al.*, 2012; Nixon, *et al.*, 1996). Además, sólo existe un estudio que describa el incremento de la actividad antiviral mediada por CMC tras una vacunación de DNA administrada de forma intramuscular en peces (Utke, *et al.*, 2008). A pesar de la falta de estudios que demuestren este tipo de respuesta en vacunaciones, es importante destacar que, en lubinas infectadas con NNV, la actividad CMC innata fue fuertemente activada, mientras que otras respuestas inmunitarias llevadas a cabo por fagocitos parecían ser

menos relevantes (Chaves-Pozo, *et al.*, 2012b). Como era de esperar, la protección encontrada tras las vacunación también fue debida a una fase temprana de activación de la respuesta inespecífica mediada por mecanismos de la ruta del IFN ya que se observó una gran estimulación de los genes *ifn*, *mx* e *ifng* en el intestino posterior. De hecho, la estimulación de la expresión de *ifng* indica la activación de la CMC ya que las células citotóxicas son las mayores productoras de IFNγ, aunque no las únicas (Schroder, *et al.*, 2004). De hecho, estos resultados concuerdan con los encontrados en juveniles de lubinas infectados con NNV *in vivo*, donde la expresión de diversos genes relacionados con la ruta del IFN se vio incrementada fundamentalmente en gónada (Valero, *et al.*, 2015c). Estos datos sugieren que la vacunación con la vacuna de DNA, ChP-CP-pNNV, y una infección con NNV desencadenan el mismo tipo de respuesta inmunitaria inespecífica. Al igual que en este estudio, vacunaciones realizadas en trucha con vacunas de DNA también produjeron un incremento temprano de la respuesta del IFN (Utke, *et al.*, 2008).

En resumen, la vacuna de DNA encapsulada en nanopartículas de quitosano resultó parcialmente eficaz y desencadenó la CMC y la respuesta del IFN en el intestino posterior de los ejemplares vacunados oralmente y provocó un porcentaje relativo de supervivencia del 45%, aunque no indujo la producción de anticuerpos. Se requieren más estudios que nos permitan entender la respuesta inmunitaria en el intestino tras una vacunación oral con una vacuna de DNA y poder mejorar ésta contra NNV con el objetivo de minimizar o evitar la incidencia de la enfermedad y las mortalidades producidas por la misma.

VI.3.2.2. Transferencia materna de actividad bactericida tras la administración de una vacuna de DNA en lubina

El sistema inmunitario de los embriones de peces y de las larvas recién eclosionadas no se encuentra completamente desarrollado (Zhang, *et al.*, 2013). Por ello, la transferencia materna de inmunidad a nivel de transferencia de proteínas o mRNA es de gran relevancia en la defensa contra patógenos en los primeros estadios de desarrollo (Løvoll, *et al.*, 2006; Mingming, *et al.*, 2014). Además, los estadios más susceptibles a NNV son los larvarios (OIE, 2013). Por ello, hemos inmunizado intramuscularmente hembras reproductoras de lubina con una vacuna de DNA contra NNV (CP-pNNV) para intentar determinar si esta vacuna es capaz de incrementar la

inmunidad pasiva de la descendencia, centrándonos en el estudio de la respuesta inmunitaria innata. Dicha respuesta es considerada en peces esencial en la defensa debido a los condicionantes que existen para el desarrollo de la respuesta inmunitaria adaptativa como los bajos ratios de proliferación de linfocitos o la dependencia de esta respuesta con la temperatura (Ellis, 1988; Whyte, 2007). La vacuna CP-pNNV provocó una inhibición de la actividad bactericida total y un aumento en la actividad lisozima en el suero de las hembras vacunadas. No obstante, en peces de menor tamaño una infección experimental *in vivo* con NNV provoca un aumento de la actividad bactericida total mientras que no se observaban cambios en la actividad lisozima en suero (Valero, *et al.*, 2015b). Esto indica que la respuesta relacionada con la actividad antimicrobiana depende del tamaño del pez y del tipo de inmunización.

Si nos centramos en la descendencia, todas las actividades analizadas: peroxidasa, anti-proteasa, lisozima y bactericida, excepto la actividad proteasa, se detectaron en huevos desde los 0 dpf y en las larvas, tanto en el grupo control como en el vacunado. No obstante, si comparamos con el grupo control, la descendencia de hembras vacunadas mostró mayores niveles en todas las actividades analizadas a distintos tiempos de desarrollo, a pesar de que en los estadios más tempranos, algunas (peroxidasa, anti-proteasa y lisozima) mostraron niveles de actividad menores que en la progenie control. Curiosamente, la actividad proteasa se detectó antes en la descendencia del grupo vacunado, concretamente en huevos de 2 dpf, que en la del grupo control, en donde se detectó a partir del día 9. Debido a que la descendencia del grupo vacunado tiene menores niveles de actividad peroxidasa, anti-proteasa, proteasa y lisozima en huevos (0 y 2 dpf) que el grupo control, podemos descartar que la vacuna CP-pNNV desencadene una trasferencia materna efectiva de efectores de estas actividades. Sin embargo, nuestros resultados sugieren que la vacuna CP-pNNV es capaz de inducir un mejor desarrollo de la respuesta inmunitaria innata ya que la descendencia del grupo vacunado mostró mayores niveles en todas las actividades analizadas que los del grupo control. Esta hipótesis, además, se ve reforzada por los niveles de expresión de los genes que codifican para la hepcidina, dicentracina, lisozima y NK-lisina que no se detectaban en huevos, pero que se vieron muy estimulados en determinados momentos del desarrollo larvario. Además, y con el objetivo de ampliar esta hipótesis a los principales tipos celulares de la respuesta innata (granulocitos y macrófagos), analizamos el patrón de expresión de dos marcadores génicos de estos tipos celulares, los genes *mpx* y *csfr1*, respectivamente. A pesar de que no existen

referencias sobre el desarrollo ontogénico de estos tipos celulares en lubina, en las especies de peces estudiadas hasta ahora, existen dos olas de hematopoyesis, al igual que ocurre en otros vertebrados. Así, inicialmente tiene lugar una hematopoyesis primitiva durante el desarrollo embriogénico y otra definitiva en individuos recién nacidos o adultos (Katzenback, *et al.*, 2013). Esto podría explicar por qué se observaron dos puntos de expresión de *mpx*, a 2 dpf y a partir de 40 dpf, y de *csfr1* entre los 2 y 12 dpf y a partir de los 40 dpf en la descendencia del grupo control. Es interesante destacar que en la descendencia del grupo vacunado, observamos unos niveles de expresión de estos dos genes mayores en algunos puntos del desarrollo larvario exceptuando el caso del gen *csfr1* a los 40 y 69 dpf. Es más, la segunda ola de mielopoyesis parece comenzar antes en las larvas del grupo vacunado, ya que la expresión del gen *mpx* se va incrementando a partid del día 26 tras la fertilización. Todos estos datos apoyan la idea de que la vacuna actúa como un activador del desarrollo del sistema inmunitario innato, al menos en el caso de la mielopoyesis.

Al contrario de lo que ocurre con las actividades antimicrobianas discutidas anteriormente, la actividad bactericida total contra *Vibrio harveyi* fue mayor en la descendencia de las hembras vacunadas que en la del grupo control, incluyendo los huevos recién fertilizados (0 dpf). Estos resultados sugieren la transferencia de moléculas efectoras de esta actividad desde las hembras reproductoras a los ovocitos, apareciendo en los huevos recién fertilizados. Los genes *hamp*, *dic*, *c3* y *nk-lys* se encuentran íntimamente relacionados con la actividad bactericida total y cuya expresión analizamos en la descendencia de las hembras control y vacunadas (Andersson, *et al.*, 1995; Bugla-Plskonska, *et al.*, 2008; Ogundele, 1998; Valero, *et al.*, 2013). En la progenie control, no se detectó ninguno de estos genes en huevos a ningún tiempo, pero sí se expresaron durante el desarrollo larvario en diferentes tiempos, aunque se observó que se encontraban bloqueados en algunos tiempos. En la descendencia del grupo vacunado, se observaron mayores niveles de expresión de estos genes que en la del grupo control, aunque también se observaron ciertas inhibiciones en puntos concretos del desarrollo. Sin embargo, nuestros resultados no mostraron unos efectos claros de la vacunación materna en la transferencia de mRNA, por lo que el aumento de la actividad bactericida total provocado por la vacuna podría ser debido a la transferencia materna de proteínas. De todas formas, existen muchas otras moléculas involucradas en esta actividad y que no se han analizado en este trabajo (Valero, *et al.*, 2013) requiriéndose

más estudios para determinar la importancia de la transferencia de proteínas o mRNA que intervienen en esta actividad.

En definitiva, en este capítulo, podemos concluir que la inmunización de hembras reproductoras de lubina con una vacuna de DNA contra NNV hizo que la descendencia tuviese un desarrollo más rápido de la respuesta inmunitaria y que ésta fuese también mayor. No se observó una clara transferencia materna de proteínas inmunitarias, salvo en la actividad bactericida, ni de mRNA por parte de las hembras vacunadas. Sin embargo, este trabajo es un estudio preliminar, por lo que se requieren más estudios que nos permitan elucidar si la vacunación contra NNV podría desencadenar una transferencia materna efectiva que generase un mejor desarrollo del sistema inmunitario innato en la descendencia y cómo ocurre.

IV. DISCUSIÓN GENERAL

En esta Tesis, y por primera vez, demostramos que NNV coloniza, replica y produce partículas virales en el testículo de dorada y lubina, aunque mantiene niveles de expresión génica muy bajos, siendo detectables proteínas virales únicamente en el caso de la dorada. Además, pudimos localizar RNA de la cápsida del virus en células somáticas del testículo de ambas especies, y en células germinales de dorada. Es de destacar que a pesar de que los niveles séricos de E_2 y de E_2 y 11KT se vieron alterados tras la infección en lubina y dorada, respectivamente, la función reproductiva no se vio alterada como muestran los niveles de transcripción del gen *dmrt1* en dorada y la morfología del testículo de ambas especies (dato no mostrado). Es más, el funcionamiento normal del testículo garantiza la posterior diseminación de partículas infectivas a través de los fluidos gonadales o incluso de las células germinales en ambas especies, ya que la íntima asociación entre estas células y las células de Sertoli existente en el testículo (Schulz, *et al.*, 2001) no nos permite descartar que el virus pueda transmitirse durante la espermatogénesis entre ambos tipos celulares en lubina.

En dorada, la infección no generó mortalidad alguna, sin embargo en lubina la mortalidad alcanzo un 55%, como se había descrito previamente (Breuil, *et al.*, 1991; Castric, *et al.*, 2001; Frerichs, *et al.*, 1996). Con el fin de estudiar estas diferencias, analizamos los patrones de expresión de citoquinas pro-inflamatorias y marcadores celulares de linfocitos T y B, pero sobre todo de genes relacionados con la actividad

antimicrobiana y con la ruta del IFN. Nuestros datos mostraron que todos estos genes se estimulaban tras la infección experimental con NNV en testículo de lubina, pero no en el de dorada. Es más, estos parámetros se estimulan fuertemente en el cerebro (el principal órgano diana del virus) de dorada, mientras que no se alteran en el de lubina. Estas diferencias en la respuesta inmunitaria del cerebro se correlacionan con la susceptibilidad a NNV mientras que los datos obtenidos en testículo apuntan a que el virus puede estar utilizando este órgano para evadir la respuesta inmunitaria y poder trasmitirse.

Como ya se ha indicado, NNV altera los niveles hormonales en suero, pero también altera la sensibilidad del cerebro y del testículo a los estrógenos, modificando los patrones de expresión de los genes que codifican para los ERs en ambos tejidos. Curiosamente, se ha visto, tanto en humanos como en peces, que la actividad de los ERs modula las rutas de señalización de la respuesta innata en células dendríticas y macrófagos y, en concreto, promueven la producción de IFN de tipo I (Cabas, *et al.*, 2013; Kovats, 2015; Liarte, *et al.*, 2011b,c). Nuestros datos mostraron una gran estimulación de la mayoría de los genes implicados en la ruta del IFN y de los genes *erb1* y *erb2* en testículo de lubina. Tras realizar un estudio estadístico de correlaciones, observamos correlaciones positivas entre el gen *erb2* y la mayoría de los genes de la ruta de activación del IFN en el testículo de lubina tras la infección con NNV (Tabla 1).

Al contrario de lo observado durante la infección *in vivo*, en la gónada infectada *in vitro* con NNV sólo se estimula la expresión de los genes *hamp* y *dic*, mientras que el resto de los genes implicados en la respuesta inmunitaria estudiados (citoquinas pro-inflamatorias, otros AMPs y marcadores de linfocitos) quedaron inalterados o inhibidos. Es interesante resaltar que los genes *erb1* y *erb2* también se estimularon en el testículo de lubina tras ser infectado *in vitro*. Estos resultados sugieren que algunos efectores antimicrobianos podrían ser regulados a nivel local en la gónada, mientras que otras respuestas podrían depender de la respuesta inmunitaria en otros tejidos. Curiosamente, los estudios llevados a cabo durante mi estancia internacional en la Universidad de Palermo, mostraron perfiles muy diferentes de AMPs con actividad antimicrobiana en el testículo y el ovario de lubina, sugiriendo la existencia de AMPs específicos en cada tipo de gónada. Sin embargo, se requiere una caracterización más profunda a la llevada

a cabo durante mi estancia para poder caracterizar completamente estas moléculas y determinar su actividad frente a infecciones (Valero, *et al.*, 2015e).

Tabla 1: Correlación observada entre la expresión del gen *erb2* y varios genes de la ruta del IFN en testículo de lubina tras una infección experimental *in vivo* con NNV. La primera línea corresponde al coeficiente de correlación de Pearson y la segunda al valor P. En negrita se encuentran los parámetros correlacionados.

		lgp2	*mavs*	*traf3*	*tank*	*tbk1*	*irf3*	*irf7*	*ifn*	*mx*	*pkr*
Testículo de lubina	*erb2*	**0.73**	**0.58**	**0.60**	0.46	**0.69**	**0.69**	**0.59**	**067**	**0.75**	**0.67**
		0.00	**0.02**	**0.01**	0.07	**0.00**	**0.00**	**0.01**	**0.00**	**0.01**	**0.00**

Al haberse descrito previamente la actividad antimicrobiana de algunas histonas y puesto que algunas, además, son específicas de testículo en peces (Nam, *et al.*, 2012; Valero, *et al.*, 2013), a continuación estudiamos el patrón de expresión de los genes que codifican las histonas H1 y H2B en un amplio rango de tejidos de lubina y dorada y frente a diversas infecciones experimentales. Los resultados obtenidos apuntan a un papel relevante de la histona H1 en la respuesta inmunitaria frente a NNV en el cerebro de ambas especies, debido al hecho de que el patrón de expresión del gen *h1* fue muy similar al observado en otros AMPs y varios genes de la ruta del IFN, correlacionándose además con las diferentes susceptibilidades al virus por parte de ambas especies (Valero, *et al.*, 2015b,c). Por otro lado, la histona H2B parece estar más relacionada con la respuesta inmunitaria del riñón cefálico y los leucocitos que éste produce.

En resumen, la intensa, rápida y diversa respuesta inmunitaria observada en el cerebro de dorada llevada a cabo, con escasas consecuencias inflamatorias, explicaría la habilidad de esta especie para superar la infección con NNV. Sin embargo, la respuesta inmunitaria de lubina no es capaz de eliminar el virus del cerebro, aún cuando la inducción de la respuesta inflamatoria es mayor, ya que se produce una activación incompleta de la ruta del IFN, de las respuestas antimicrobianas, la CMC y la respuesta adaptativa (Chaves-Pozo, *et al.*, 2012b; datos actuales). Además, una mayor respuesta inflamatoria en el cerebro podría estar produciendo elevados daños celulares, tal y como se observa a nivel motor, explicando las elevadas mortalidades provocadas por la infección con NNV en lubina (Chaves-Pozo *et al.*, 2012b; Capítulo III.1.1.). Sin embargo, los parámetros inmunitarios analizados son más intensos en testículo de

lubina que en dorada, siendo en la lubina regulados por factores reproductores tales como el E_2 y sus receptores.

Tras analizar la respuesta inmunitaria contra NNV en dorada y lubina, nos propusimos desarrollar una vacuna de DNA (CP-pNNV) a través de la clonación del ORF completo del RNA2 de NNV en un vector de expresión de células eucarióticas. Esta vacuna fue encapsulada en nanopartículas de quitosano y administrada a juveniles de lubina. Nuestros datos demostraron que la vacuna desencadena la CMC y activa la ruta del IFN en el intestino posterior tal y como se ha descrito para otras especies de peces infectadas con otros agentes virales (Ellis, 2001; Robertsen, 2006). Sin embargo, la vacuna no produjo anticuerpos específicos contra NNV a los 90 días de la vacunación, al contrario de lo que ocurría en otros casos (de las Heras, *et al.*, 2010; MacLaughlin, *et al.*, 1998; Vimal, *et al.*, 2014). A los 90 días de haberse vacunado, los peces se infectaron experimentalmente con una dosis letal de NNV y mantuvieron una supervivencia del 45% poniendo de manifiesto una protección parcial frente a NNV. Aun así, son necesarios futuros estudios que nos permitan entender completamente la respuesta que tiene lugar en el intestino posterior tras la vacunación oral con vacunas de DNA para poder mejorar este tipo de vacunas y ser capaces, de esta forma, de disminuir la incidencia de NNV o incluso erradicarla.

Posteriormente administramos la vacuna CP-pNNV mediante inyección intramuscular a hembras reproductoras de lubina y estudiamos la respuesta inmunitaria innata en la descendencia. En este caso, nuestros resultados mostraron que es posible detectar todas las actividades antimicrobianas analizadas (peroxidasa, proteasa, anti-proteasa, lisozima y bactericida total) en la descendencia de hembras vacunadas y no vacunadas desde los estadios de huevo y a lo largo del desarrollo larvario. Descartamos que la vacuna CP-pNNV desencadene una transferencia materna efectiva de proteínas efectoras de estas actividades ya que todas estas actividades se inhibieron en la fase de huevo (0 o 2 dpf), exceptuando el caso de la actividad bactericida total, en cuyo caso se vio estimulada en huevos de 0 dpf en la descendencia de las hembras vacunadas y podría representar una efectiva trasferencia materna. Sin embargo, en este grupo pudimos observar elevados niveles de las actividades analizadas en determinados tiempos a lo largo de su desarrollo, sugiriendo que la vacunación de hembras con CP-pNNV produce un mejor desarrollo del sistema inmunitario innato en su descendencia.

Esta hipótesis se ve reforzada por el patrón de expresión de genes marcadores de granulocitos y macrófagos y algunos genes relacionados con las actividades analizadas en este trabajo. Es más, nuestros datos muestran que la transcripción de genes relacionados con las actividades antimicrobianas se estimularon en determinados tiempos tras la vacunación, al igual que ocurría en cerebro y gónada tras la infección *in vivo* con NNV, demostrando que NNV produce un estatus antimicrobiano elevado en los ejemplares infectados al igual que lo hace la vacuna en la descendencia de las hembras vacunadas.

V. CONCLUSIONES

1. Nodavirus coloniza, replica y produce partículas virales infectivas en el testículo de dorada y lubina.

2. Nodavirus modifica los niveles séricos de 17β-estradiol y 11-cetotestosterona y el patrón de expresión de los receptores nucleares de estrógenos en ambas especies, aunque no es suficiente para alterar las funciones testiculares.

3. Nodavirus estimula la transcripción de genes relacionados con la ruta del interferón y de péptidos antimicrobianos, marcadores de linfocitos T y B y citoquinas pro-inflamatorias en el testículo de lubina, pero no en el de dorada. Sin embargo, en cerebro ocurre lo contrario. Este hecho podría explicar la resistencia al virus y sus mecanismos de diseminación.

4. La comparación de la respuesta inmunitaria en la gónada tras una infección con nodavirus *in vivo* e *in vitro* indica que esta respuesta, hasta cierto punto, prevalece a nivel local y es regulada por las partículas virales.

5. La administración a juveniles de lubina de una vacuna de DNA contra nodavirus encapsulada en nanopartículas de quitosano mejora su supervivencia tras una infección experimental. La vacuna desencadena la citotoxicidad mediada por células y la

respuesta del interferón en el intestino posterior sin provocar la producción de anticuerpos específicos en suero.

6. La vacunación de hembras reproductoras de lubina con una vacuna de DNA contra nodavirus desencadena la transferencia materna de proteínas con actividad bactericida a su descendencia provocando una expresión más temprana y elevada de genes relacionados con la respuesta inmunitaria innata.

7. Nuestro estudio demuestra la importancia de la interacción inmuno-reproductora en una infección con nodavirus. Es más, cualquier estrategia preventiva o terapéutica debería tener en cuenta el papel de la gónada en la eliminación y transmisión de nodavirus.

VII. REFERENCES

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VIII. ANEXES

VIII.1. Scientific production related to the Doctoral Thesis

Chapter III.1.1.: **Valero, Y.**, Arizcun, M., Esteban, M.A., Bandín, I., Olveira, J.G., Patel, S., Cuesta, A. and Chaves-Pozo, E. (2015). "Nodavirus colonizes and replicates in the testis of gilthead seabream and European sea bass modulating its immune and reproductive functions". PLoS One (12): e0145131.

Chapter III.1.2.: **Valero, Y.**, Morcillo, P., Meseguer, J., Buonocore, F., Esteban, M.A., Chaves-Pozo, E. and Cuesta, A. (2015). "Characterization of the interferon pathway in the teleost fish gonad against the vertically transmitted viral nervous necrosis virus". J Gen Virol (8): 2176-87.

Chapter III.1.3.: **Valero, Y.**, García-Alcázar, A., Esteban, M.A., Cuesta, A. and Chaves-Pozo, E. (2015). "Antimicrobial response is increased in the testis of European sea bass, but not in gilthead seabream, upon nodavirus infection". Fish Shellfish Immunol (1): 203-13.

Chapter III.1.4.: **Valero, Y.**, Arizcun, M., Esteban, M.A., Cuesta, A. and Chaves-Pozo, E. (Submitted). "Transcription of fish histones h1 and h2b suggests a potential role in immunity as antimicrobial peptides". Fish Shellfish Immunol.

Chapter III.2.1: **Valero, Y.**, Awad, E., Buonocore, F., Arizcun, M., Esteban, M.A., Meseguer, J., Chaves-Pozo, E. and Cuesta, A. (Submitted). "An oral chitosan DNA vaccine against nodavirus improves the survival of European sea bass juveniles upon infecton probably by triggering the cell-mediated toxicity and IFN responses". Dev Comp Immunol.

Chapter III.2.2: **Valero, Y.**, Arizcun, M., Esteban, M.A., Cuesta, A. and Chaves-Pozo, E. (prepared to be submitted). "Maternal transfer of innate immunity upon DNA vaccination against nodavirus in teleost fish".

VIII.2. Additional scientific production

VIII.2.1. Scientific articles

Valero, Y., Sánchez-Hernández, M., García-Alcázar, A., García-Ayala, A., Cuesta, A. and Chaves-Pozo, E. (2015). "Characterization of the annual regulation of reproductive and immune parameters on the testis of European sea bass". Cell Tissue Res (1): 215-29.

Valero, Y., Martínez-Morcillo, F. J., Esteban, M. A., Chaves-Pozo, E. and Cuesta, A. (2015). "Fish peroxiredoxins and their role in immunity". Biology (4): 860-80.

Valero, Y., García-Alcázar, A., Esteban, M. A., Cuesta, A. and Chaves-Pozo, E. (2014). "Seasonal variations of the humoral immune parameters of European sea bass (*Dicentrarchus labrax* L.)". Fish Shellfish Immunol (2): 185-7.

VIII.2.2. Book chapter

Valero, Y., Chaves-Pozo, E., Meseguer, J., Esteban, M. A. and Cuesta, A. (2013). "Biologial Role of Fish Antimicrobial Peptides". In: Antimicrobial Peptides. Seong, M. D. and Hak, Y. I. Nova Science Publishers. 2: Pp 31-60.

VIII.2.3. Work submitted to Conferences or Congress

VIII.2.3.1. International Conferences

Valero, Y., Cuesta, A., García-Alcázar, A., Esteban, M. A., Chaves-Pozo, E. (2015). “DNA vaccination of broodstock specimens modifies the immune status of their progeny”. 17th International Conference on ‘Diseases of Fish and Shellfish’. Las Palmas de Gran Canaria. Poster.

Valero, Y., García-Alcázar, A., Esteban, M. A., Boughlala, B., de Juan, J., Meseguer, J., Chaves-Pozo, E., Cuesta, A. (2015). “Immune response against

intravitreally injected nodavirus in European sea bass specimens". 17th International Conference on 'Diseases of Fish and Shellfish'. Las Palmas de Gran Canaria. Poster.

Valero, Y., Cuesta, A., Arizcun, M., Esteban, M. A., Meseguer, J., Chaves-Pozo, E. (2015). "Nodavirus infection alterns the kisspeptins pathway in European sea bass brain". 17th International Conference on 'Diseases of Fish and Shellfish'. Las Palmas de Gran Canaria. Poster.

Valero, Y., Ewad, E., Chaves-Pozo, E., Meseguer, J., Esteban, M. A., Cuesta, A. (2015). "Oral administration of a nodavirus DNA vaccine into chitosan nanoparticles improves the survival of European sea bass juveniles upon challenge". 17th International Conference on 'Diseases of Fish and Shellfish'. Las Palmas de Gran Canaria. Poster.

Valero, Y., Arizcun, M., Trapani, M. R., Dioguardi, M., Cammarata, M., Esteban, M. A., Cuesta, A., Chaves-Pozo, E. (2015). "Identification of antimicrobial peptides in the gonad of European sea bass males and females". 13st Congress of International Society of Developmental and Comparative Immunology. Murcia. Poster.

Valero, Y., García-Alcázar, A., Esteban, M. A., Boughlala, B., de Juan, J., Meseguer, J., Chaves-Pozo, E., Cuesta, A. (2015). "Immune response against intravitreally injected VNNV in European sea bass specimens". 13st Congress of International Society of Developmental and Comparative Immunology. Murcia. Poster.

Valero, Y., Arizcun, M., Esteban, M. A., Cuesta, A., Chaves-Pozo, E. (2015). "17β-estradiol alters the susceptibility to nodavirus and the viral immune response of European sea bass". 13st Congress of International Society of Developmental and Comparative Immunology. Murcia. Poster.

López-Cánovas, A. E., **Valero, Y**., Cabas, I., Arizcun, M., Meseguer, J., Mulero, V., García-Ayala, A., Chaves-Pozo, E. (2015). "17β-ethynylestradiol alters the humoral immune response of gilthead seabream males depending on their reproductive stage". 13st Congress of International Society of Developmental and Comparative Immunology. Murcia. Poster.

Valero, Y., Chaves-Pozo, E., Esteban, M. A., Meseguer, J., Buonocore, F., Cuesta, A. (2015). "Characterization of the interferon pathway in the European sea bass after nodavirus infection". 13st Congress of International Society of Developmental and Comparative Immunology. Murcia. Oral comunication.

Chaves-Pozo, E., **Valero, Y.**, Esteba, M. A., Meseguer, J., Cuesta, A. (2015). "Fish granzymes and their role in the innate cell-mediated cytotoxicity against nodavirus-infected cells". 13st Congress of International Society of Developmental and Comparative Immunology. Murcia. Oral comunication.

López-Cánovas, A. E., Rodenas, M. C., **Valero, Y**., García-Alcázar, A., Meseguer, J., Mulero, V., Chaves-Pozo, E., García-Ayala, A. (2015). "Humoral immune responses in gilthead seabream (*Sparus aurata* L.) upon endocrine disrupter pollutant exposure". 13st Congress of International Society of Developmental and Comparative Immunology. Murcia. Poster.

Valero, Y., Cuesta, A., Arizcun, M., Esteban, M. A., Chaves-Pozo, E. (2015). "Maternal transfer of immunity upon DNA vaccination against VNNV in European sea bass larvae". 13st Congress of International Society of Developmental and Comparative Immunology. Murcia. Poster.

Valero, Y., Morcillo, P., Arizcun, M., Esteban, M. A., Meseguer, J., Chaves-Pozo, E., Cuesta, A. (2014). "Interferon-pathway in European sea bass and gilthead

seabream fish upon nodavirus infections". 9th International Symposium on Viruses of Lower Vertebrates. Málaga. Poster.

Valero, Y., Arizcun, M., Esteban, M. A., Bandín, I., Olveira, G., Patel, S., Cuesta, A., Chaves-Pozo, E. (2014). "Nodavirus replicates in the gonad of European sea bass and gilthead seabream and alters the reproductive and immune functions". 9th International Symposium on Viruses of Lower Vertebrates. Oral communication.

Valero, Y., García-Alcázar, A., Esteban, M. A., Meseguer, J., Chaves-Pozo, E., Cuesta, A. (2014). "Administration of 17-b-estradiol alters the susceptibility of European sea bass to nodavirus infection". 27th Conference of European Comparative Endocrinologists. Rennes. Poster.

Valero, Y., Chaves-Pozo, E., Abellán, E., Esteban, M. A., Meseguer, J., Cuesta, A. (2013). "Gilthead seabream and European seabass cell-mediated cytotoxicity and gonadal immunity upon nodavirus infections". 16th International Conference on Diseases of Fish and Shellfish. Tampere. Oral communication.

Valero, Y., Arizcun, M., Esteban, M. A., Meseguer, J., Chaves-Pozo, E., Cuesta, A. (2013). "Hepcidin is involved in the immune response of the gonad of gilthead seabream and European seabass against nodavirus". 16th International Conference on Diseases of Fish and Shellfish. Tampere. Poster.

Valero, Y., Arizcun, M., Cuesta, A., Esteban, M. A., Chaves-Pozo, E. (2013). "Histones *h1* and *h2b* might have a role in the innate immune response of European seabass gonad upon infection with nodavirus". First International Conference of Fish and Shellfish Immunology. Vigo. Poster.

Valero, Y., Cuesta, A., Abellán, E., Esteban, M. A., Meseguer, J., Chaves-Pozo, E. (2013). "Antimicrobial peptides in the gonad of European sea bass and gilthead seabream upon infection with nodavirus". First International Conference of Fish and Shellfish Immunology. Vigo. Poster.

Cuesta, A., García-Alcázar, A., Guardiola, F. A., **Valero, Y.**, Esteban, M. A., Meseguer, J., Chaves-Pozo, E. (2012). "Hormonal status and immune gene expression pattern in European seabass and gilthead seabream upon infection with nodavirus". 26th Conference of European Comparative Endocrinologists. Zurich. Poster.

VIII.2.3.2. National Conferences

Valero, Y., Cuesta, A., Esteban, M. A., Meseguer, J., Buonocore, F., Chaves-Pozo, E. (2015). "Estimulación de la respuesta citotóxica en cerebro y gónada de lubina tras una infección con nodavirus". XV Congreso Nacional y I Congreso Ibérico de Acuicultura. Huelva. Comunicación oral.

Valero, Y., Cuesta, A., Chaves-Pozo, E. (2015). "Vacunas DNA frente a nodavirus. Administración oral y transferencia materna para proteger los estadios larvarios más vulnerables de lubina". XV Congreso Nacional y I Congreso Ibérico de Acuicultura. Huelva. Comunicación oral.

Valero, Y., Arizcun, M., Guardiola, F. A., Cuesta, A., Esteban, M. A., Cuesta, A., Chaves-Pozo, E. (2015). "17β-estradiol alters the susceptibility to nodavirus and the viral immune response of European sea bass". 10º Congreso de la Asociación Ibérica de Endocrinología Comparada. Castellón. Poster.

Valero, Y., Cuesta, A., Arizcun, M., Guardiola, F. A., Esteban, M. A., Meseguer, J., Chaves-Pozo, E. (2015). "Kisspeptins pathway is altered in the european sea bass upon nodavirus infection". 10º Congreso de la Asociación Ibérica de Endocrinología Comparada. Castellón. Poster.

Valero, Y., Cuesta, A., Abellán, E., Esteban, M. A., Meseguer, J., Chaves-Pozo, E. (2015). "Antimicrobial peptides in the gonad of European sea bass and gilthead seabream upon infection with nodavirus". I Jornadas de Doctorado de la Universidad de Murcia. Murcia. Poster.

Valero, Y., Arizcun, M., Cuesta, A., Chaves-Pozo, E. (2013). "Estudio de la infección de la gónada de peces por nodavirus mediante técnicas de PCR *in situ*". XIV Congreso Nacional de Acuicultura. Principado de Asturias. Poster.

VIII.3. Grants and scholarships obtained

National merit-based grant "Beca predoctoral de formación de personal investigador (FPI)" in the "Instituto Español de Oceanografía (IEO)" (Spain) during 4 years in the period between 2012 and 2016.

VIII.4. Short stays at international research centres

Research stay in the Institution "Dipartamento di Science e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF)" of Palermo (Italy) during 100 days under the supervision of Dr. Matteo Cammarata.

VIII.5. Participation in competitive research projects

Caracterización de la respuesta inmune en el seno de los órganos reproductores, implicación de péptidos antimicrobianos frente a infecciones virales. Ministerio de Ciencia e Innovación. Period. 01/01/2011-31/12/2014.

Respuesta inmune celular y diseño de vacunas orales frente a nodavirus. Plan Estatal de Investigación Científica y Técnica y de Innovación 2013-2016. Ministerio de Economía y Competitividad. Period: 01/01/2014-31/12/2016.