UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Application of antimicrobial peptides for the control of betanodavirus in European sea bass

Aplicación de péptidos antimicrobianos para el control de betanodavirus en lubina

Dña. Laura Cervera Martínez

2024

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Autora: Dña. Laura Cervera Martínez

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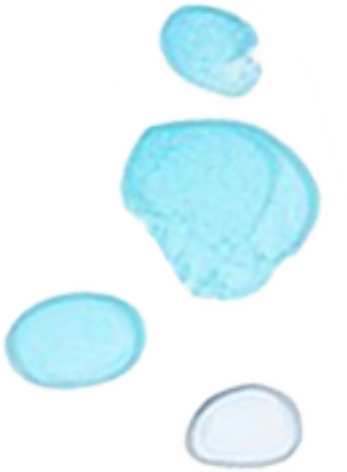
A mis padres

“A veces sentimos que lo que hacemos es tan solo una gota en el mar,
pero el mar sería menos si le faltara una gota”
Amelia Earthart (1897-1937)

“Uno nunca ve lo que ha hecho,
sino que ve lo que queda por hacer”
Marie Curie (1867-1934)



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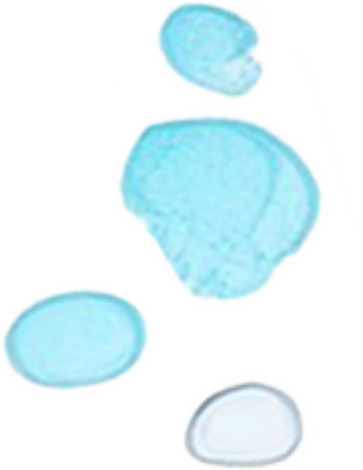

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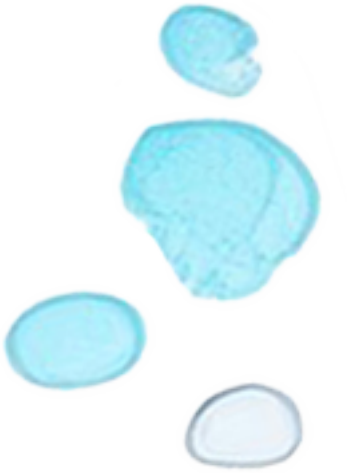

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Cervera, L., Chaves-Pozo, E., Cuesta, A. (2024). Synthetic antimicrobial peptides fail to induce leucocyte innate immune functions but elicit opposing transcriptomic profiles in European sea bass and gilthead seabream. Marine Drugs, 22(2), 86. https://doi.org/10.3390/md22020086

Cervera, L., Arizcun, M., Mercado, L., Chaves-Pozo, E., Cuesta, A. (2024). Synthetic antimicrobial Nkl and Dic peptides are immunomodulatory but only Dic peptide can be therapeutic against nodavirus infection. Fish and Shellfish Immunology, 152, 109772. https://doi.org/10.1016/j.fsi.2024.109772

Cervera, L., Arizcun, M., Mercado, L., Cuesta, A., Chaves-Pozo, E. (2024). Hepcidin peptide controls the inflammatory response induced by betanodavirus infection and improves European sea bass survival. Marine Life Science & Technology. (Submitted February 2024).

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Cervera, L., Arizcun, M., Johnstone, C., Cuesta, A., Chaves-Pozo, E. (Manuscript in preparation). The link between autophagy, inflammation and viral transcription control is key for understanding the antiviral role of hepcidin and dicentracin.

Cervera L., Álvarez-Torres D., Béjar, J., Cuesta, A., Martín, V., Jérez, S., Chaves-Pozo E. (Manuscript in preparation). Diagnosis of NNV latent infection in the gonad of greater amberjack broodstocks shows a sex-biased infection and reproductive disruption.

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García-Álvarez M.A., **Cervera L.**, Cuesta A., Chaves-Pozo E. "RGNNV and SJNNV reassortant produce mortality and replicate in gilthead seabream larvae". Poster communication in "XX International Conference of Diseases of Fish and Shellfish", 20-23 September 2021. Online, Aberdeen, United Kingdom.

Cervera L., González-Fernández C., Cano D., García-Álvarez M.A., Esteban M.A., Cuesta A. "Antimicrobial peptide (AMP)-encoding plasmids show little immunomodulatory and nodavirus-protective actions in European sea bass juveniles" Poster communication in "XX International Conference of Diseases of Fish and Shellfish", 20-23 September 2021. Online, Aberdeen, United Kingdom.

Cervera L., González-Fernández C., Arizcun M., Cuesta A., Chaves-Pozo E. "Immunological response of gilthead seabream during an outbreak of the parasite *Cryptocaryon irritans*". Poster communication in "XX International Conference of Diseases of Fish and Shellfish", 20-23 September 2021. Online, Aberdeen, United Kingdom.

García-Álvarez M.A., **Cervera L.**, González-Fernández C., Arizcun M., Campos-Sánchez J.C., Esteban M.A., Chaves-Pozo E., Cuesta A. "Potential impact in the

gilthead seabream larviculture by Nodavirus". Poster communication in "IX Iberian Congress of Ichtyology". 20-23 June 2022. Porto, Portugal.

Cervera L., Chaves-Pozo E., Cuesta A. "Los péptidos antimicrobianos Hepcidina y Dicentracina producen inmunomodulación en juveniles de lubina y confieren protección parcial frente a nodavirus". Oral communication in "VII Jornadas Doctorales de la Universidad de Murcia". 04-07 July 2022. Murcia, Spain.

Cervera L., Arizcun M., Cuesta A., Chaves-Pozo E. "European sea bass defensin beta genes are modulated upon NNV-infeccion and vaccination" Poster communication in "XVI Congreso Nacional de Virología", 6-9 September 2022. Málaga, Spain.

Cervera L., Arizcun M., Cuesta A., Chaves-Pozo E. "Antimicrobial peptides Hepcidin and Dicentracin produce immunomodulation and confer partial protection against NNV". Oral communication in "XVI Congreso Nacional de Virología", 6-9 September 2022. Málaga, Spain.

Cervera L., Arizcun M., Johnston C., Cuesta A., Chaves-Pozo E. "Hepcidin and Dicentracin increase the viral clearance via autophagy in European sea bass". Poster communication in "XVI Congreso Nacional de Virología", 6-9 September 2022. Málaga, Spain.

Cervera L., Arizcun M., Chaves-Pozo E., Cuesta A. "El tratamiento con péptidos antimicrobianos sintéticos mejora la supervivencia de la lubina a la infección por nodavirus y modula el curso de la enfermedad". Poster communication in "XVIII Congreso Nacional de Acuicultura", 21-14 November 2022. Cádiz, Spain.

Cervera L., García-Álvarez M.A., Valero Y., González-Fernández C., Arizcun M., Chaves-Pozo E., Esteban M.A., Cuesta A. "Herramientas biotecnológicas para el estudio y control de nodavirus". Poster communication in the "I Congreso sobre Avance Europeo en Crecimiento Azul", 7-8 June 2023. Murcia, Spain.

Cervera L., Chaves-Pozo E., Esteban M.A., Cuesta A. Capacidad inmunomoduladora *in vitro* por AMPs de lubina. Oral communication in the "VIII Jornadas Doctorales de la Universidad de Murcia", 03-05 July 2023. Murcia, Spain.

Cervera L., Arizcun M., Chaves-Pozo E., Cuesta A. "Antimicrobial peptide Dicentracin shows prophylactic and therapeutic actions against NNV infection in European sea bass". Poster communication in the "XXI International Conference on Diseases of Fish and Shellfish", 11-14 September 2023. Aberdeen, United Kingdom.

García-Álvarez M.A., **Cervera L.**, Valero Y., González-Fernández C., Guardiola F.A., Arizcun M., Chaves-Pozo E., Esteban M.A., Cuesta A. "Biotechnological tools for the research and control of nodavirus in aquaculture". Poster communication in "Aquaculture Europe 2023". 18-21 September 2023. Vienna, Austria

Chaves-Pozo E., **Cervera L.**, Valero Y., García-Álvarez M.A., Cuesta A. "Biotechnological tools for the study and control of nodavirus". Oral communication in "European congress of Marine Biotechnology", 21- 23 November 2023. Málaga, Spain.

Cervera L., Chaves-Pozo E., Cuesta A. "El péptido antimicrobiano hepcidina induce inmunomodulación en lubina y previene parcialmente la mortalidad por nodavirus". Oral communication in "XII Foro Iberoamericano de los Recursos Marinos y la Acuicultura", 27 November - 1 December, 2023. Online.

Cervera L., Chaves-Pozo E., Cuesta A. "Capacidad inmunomoduladora *in vitro* del péptido antimicrobiano NK-lisina de lubina". Poster communication in "XII Foro Iberoamericano de los Recursos Marinos y la Acuicultura", 27 November - 1 December, 2023. Online.

Valero Y., **Cervera L.,** Chaves-Pozo E., Cuesta A. "Péptidos antimicrobianos derivados de peces para la prevención y el tratamiento de nodavirus". Oral communication in "VI Workshop de Péptidos terapéuticos para bioaplicaciones", 11-12 January 2024. Santiago, Chile. Online.

Arizcun, M., García-Beltrán, J.M., **Cervera, L.**, Valero, Y., Mercado, L., Cuesta, A., Chaves-Pozo, E. Inheritance of maternally trained immunity in teleost fish. Oral communication in the "Vaccine Congress 2024", 24-25 June 2024. Prague, Czech Republic.

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ABBREVIATIONS INDEX

A	Alanine
AA	Amino acid
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
APC	Antigen presenting cells
B1	Nodavirus B1 protein
B2	Nodavirus B2 protein
BCR	B cell receptor
BFNNV	Barfin flounder nervous necrosis virus
BLASTp	Protein basic local alignment search tool
bw	Body weight
CD	Cluster of differentiation
CD4	Cluster of differentiation 4
cd4	Cluster of differentiation 4 coding gene
CD8	Cluster of differentiation 8
cd8a	Cluster of differentiation 8 alpha coding gene
cDNA	Copy desoxyribonucleic acid
CMC	Cell-mediated cytotoxicity
COMU-IEO	*Centro Oceanográfico de Murcia, Instituto Español de Oceanografía*
COX2	Cyclooxigenase 2
CP	NNV coat protein
CPE	Cytopathic effects
cp	NNV coat protein coding gene
csf1r	Colony-stimulation factor receptor 1 coding gene
Ct	Cycle threshold
CTLs	T cytotoxic cells
CXCR3/*cxcr3*	C-X-C motif chemokine receptor 3
CXCL9/*cxcl9*	CXC chemokine 9
DAB	3,3’ - diaminobenzidine tetrahydrochloride
DB1	Defensin beta 1
defb1	Defensin beta 1 coding gene
Dic	Dicentracin

DNA	Desoxyribonucleic acid
DNase	Deoxyribonuclease
dpi	Days post infection
E-11	E-11 cell line
ef1a	Elongation factor 1 alpha coding gene
ELISA	Enzyme-linked immunoabsorbent assay
EU	European Union
fasl	Fas ligand coding gene
FBS	Foetal bovine serum
FC	Fold change
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G3BP	Galectin-3 binding protein
GF-1	Grouper fin-1 cell line
GALT	Gut-associated lymphoid tissue
GIALT	Gills-associated lymphoid tissue
gzmb	Granzyme B coding gene
h	Hour
Hamp	Hepcidin antimicrobial peptide
hamp1	Hepcidin antimicrobial peptide 1
hamp2	Hepcidin antimicrobial peptide 2
HBSS	Hank's balanced salt solution
HK	Head kidney
HKLs	Head kidney leukocytes
IEO	*Instituto Español de Oceanografía*
IFN	Interferon
Ig	Immunoglobulin
IgD	Immunoglobulin D
ighm	Immunoglobulin M heavy chain coding gene
IgG-HRP	Immunoglobulin G conjugated with horseradish peroxidase
IgM	Immunoglobulin M
IgT/Z	Immunoglobulin T/Z
ILs	Interleukins

il1b	Interleukin-1 beta coding gene
il6	Interleukin 6 coding gene
il8	Interleukin 8 coding gene
il10	Interleukin 10 coding gene
IL-21	Interleukin 21
im	Intramuscularly
ISG	Interferon stimulated genes
ISG15	Interferon-stimulated gene 15
IPNV	Infectious pancreatic necrosis virus
irf3	Interferon regulatory factor 3 coding gene
K	Lysin
L	Leucine
l13a	L13 alpha coding gene
L-15	Leibovitz's culture medium
lyz	Lysozyme coding gene
MALT	Mucosa-associated lymphoid tissue
mcsf1r	Macrophage colony stimulatory factor 1 receptor coding gene
min	Minutes
MHC	Major histocompatibility complex
mhc2	Major histocompatibility complex 2 coding gene
MM	Melanomacrophages
mm	Milimeters
mpo	Mieloperoxidase coding gene
mx	Interferon-induced GTP-binding protein Mx coding gene
Myd88	Myeloid differentiation factor 88
NCCs	Non-specific cytotoxic cells
NCCRP1	Non-specific cytotoxic cells receptor protein 1
NK	Natural killer
Nkl	NK-lysin
NLRs	NOD-like receptors
nm	Nanometers
NNV	Nervous necrosis virus
nRT-PCR	Nested real time polymerase chain reaction

OD	Optical density
Oligo-DT	Oligo deoxy-thymine nucleotides
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline supplemented with Tween-20
pDB1	Plasmid encoding defensin beta 1
pDIC	Plasmid encoding dicentracin
pcDNA	Empty plasmid
pHAMP	Plasmid encoding hepcidin antimicrobial peptide 2
PI	Propidium iodide
pis	Piscidin coding gene
PMA	Phorbol 12-myristate 13-acetate
pNKL	Plasmid encoding NK-lysin
prf	Perforin
PRRs	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RAS	Recirculating aquaculture systems
RdRp	RNA-dependent RNA-polymerase or NNV protein A
rdrp	NNV protein A coding gene
RGNNV	Red-spotted grouper nervous necrosis virus
RNA	Ribonucleic acid
rpm	Revolution per minute
RPS	Relative percent survival
rps18	Ribosomal protein S18
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
SALT	Skin-associated lymphoid tissue
SEM	Standard error of the mean
SJNNV	Striped Jack nervous necrosis virus
SPSS	Statistical package for the social sciences
SSC	Side scatter
SVCV	Spring viremia carp virus
Th	T helper cells

$TCID_{50}$	Median tissue culture infectious dose
TCR	T cell receptor
tcrb	T cell receptor beta chain coding gene
tgfb	Transforming growth factor beta coding gene
TLRs	Toll-like receptors
TLR9	Toll-like receptor 9
TMB	3,3′,5,5′-tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
TNV	Turbot betanodavirus
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TPNNV	Tiger puffer nervous necrosis virus
VER	Viral encephalopathy and retinopathy
Vh	*Vibrio harveyi*
VHSV	Viral haemorragic septicaemia virus

LIST OF SPECIES

Scientific name	Common name
Acipenser baeri	Siberian sturgeon
Acipenser gueldenstaedtii	Russian surgeon
Boleophthalmus pectinorostris	Blue spotted mudskipper
Cynoglossus semilaevis	Tongue sole
Danio rerio	Zebrafish
Dicentrarchus labrax	European sea bass
Epinephelus coioides	Orange-spotted grouper
Gadus morhua	Altantic cod
Hemibarbus labeo	Barbel steed
Hipoglossus hipoglossus	Atlantic halibut
Hyporthodus septemfascicitus	Convict grouper
Ictalurus punctatus	Channel catfish
Lates calcarifer	Barramundi/ Asian sea bass
Megalobrama amblycephala	Wuchang bream
Misgurrus anguillicadatus	Loach
Morone saxatilis	Striped bass
Oncorhynchus mykiss	Rainbow trout
Oplegnathus fasciatus	Striped beakfish
Oreochromis niloticus	Nile tilapia
Oryzias latipes	Medaka
Paralichthys olivaceus	Olive flounder
Plecoglossus altivelis	Ayu
Pseudopleuronectes americanus	Winter flounder
Salmo trutta caspius	Caspian trout
Salmo salar	Atlantic salmon
Salvelinus alpinus	Arctic char
Salvelinus fontinalis	Brook trout
Schophtalmus maximus	Turbot
Sebastes schlegelii	Black rockfish
Siniperca chuatsi	Mandarin fish
Solea senegalensis	Sole
Sparus aurata	Gilthead seabream
Trachinotus cuatus	Pompano

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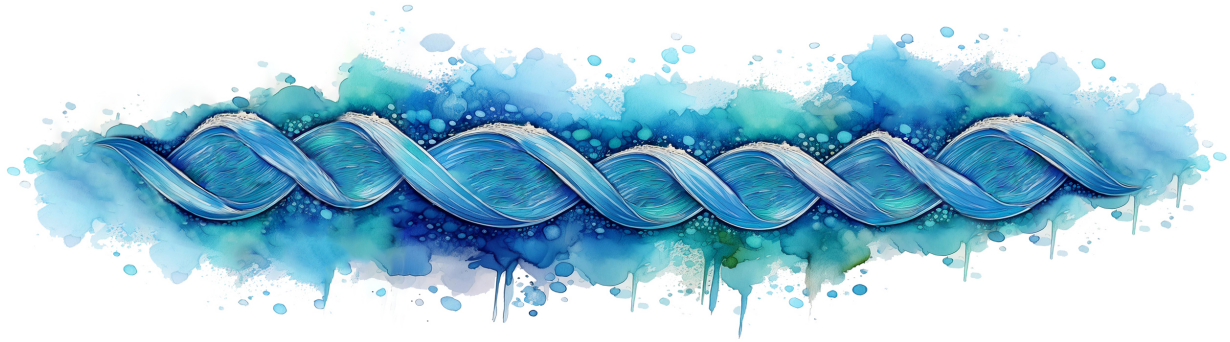
SUMMARY



Summary

The growing demand of sea-derived food leads to the development of aquaculture as a prosperous sector. Gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) are the most produced and traded species in Spain. The appearance of viral outbreaks, such as the one provoked by nervous necrosis virus (NNV), threatens this industry. So, in recent years, most studies have focused on the analysis of the immune response against NNV and the development of strategies to control this infection. Thus, the aim of this Doctoral Thesis is to evaluate the capability of European sea bass-derived antimicrobial peptides (AMPs), endogenous proteins involved in the anti-NNV response, to be used as preventive or palliative treatments against NNV.

As a first step of our research, we evaluated the structure of the European sea bass-derived peptides *in silico* and their immunomodulatory roles *in vitro*. NK-lysin (Nkl) and dicentracin (Dic) peptides showed a predominant alpha helix structure while hepcidin (Hamp) presents beta sheet structure stabilized by disulfide bonds. These secondary structures and the high sequence homology between European sea bass and gilthead seabream peptides suggest antiviral properties, conserved functions and application in both species indistinctly. To test this, their immunomodulatory roles in head-kidney leukocytes (HKLs) from European sea bass and gilthead seabream were assessed *in vitro.* Despite the synthetic AMPs failed to modulate respiratory burst and phagocytosis activities in both species, the modulation of the gene expression profile of immune-related genes were completely different in sea bass and seabream. In gilthead seabream, a tendency to immunosuppression was evidenced by the down-regulation of genes related to inflammation, cell-mediated cytotoxicity (CMC), interferon (IFN), AMPs and cellular markers. These data discard the potential of European sea bass-derived peptides as biotechnological tool to control viral infections in gilthead seabream. On the contrary, in European sea bass, the promotion of the anti-inflammatory response by up-regulating genes such as *interleukin(il)10* or *tgfb* (transforming growth factor beta) along with the up-regulation of CMC markers and AMPs encoding genes were observed. Besides, all AMPs up-regulated *ighm* (immunoglobulin M heavy chain) gene expression pointing to their role in bridging innate and adaptive responses. However, only Nkl peptide was able to up-regulate IFN stimulated genes such as *mx* (Interferon-induced GTP-binding protein Mx) and *irf3* (IFN regulating factor 3), crucial to fight against viruses. Therefore, Nkl, Hamp and Dic show promising properties to be used as preventive and palliative treatments against NNV in European sea bass.

Next experiments included in this Doctoral Thesis consisted in *in vivo* assays in which AMPs, via expressing plasmids or synthetic peptides, were administered to ascertain their immunomodulatory role and the protection conferred against NNV challenge. First, the suitability of plasmids encoding AMPs as NNV-preventive agents administered intramuscularly was evaluated. All tested plasmids resulted in the up-regulation of proinflammatory interleukins and cell-adhesion molecules, such as *il1b*, *il8*, *cxcr3* (C-X-C receptor 3) and *cxcl9* (C-X-C ligand 9), and the neutrophils marker, *mpo* (myeloperoxidase), in muscle. Considering this immunomodulation, a NNV challenge was performed 3 days after plasmid injection. Unfortunately, AMP-encoding plasmids were unable to increase the survival rates and pHAMP2 even worsen them. The proinflammatory status triggered by plasmids might be the cause of the high mortality rates due to the relation between exacerbated inflammation and brain lesions. For these reasons, the employment of plasmids encoding AMPs as anti-NNV agents were discarded and their potential as palliative was not evaluated.

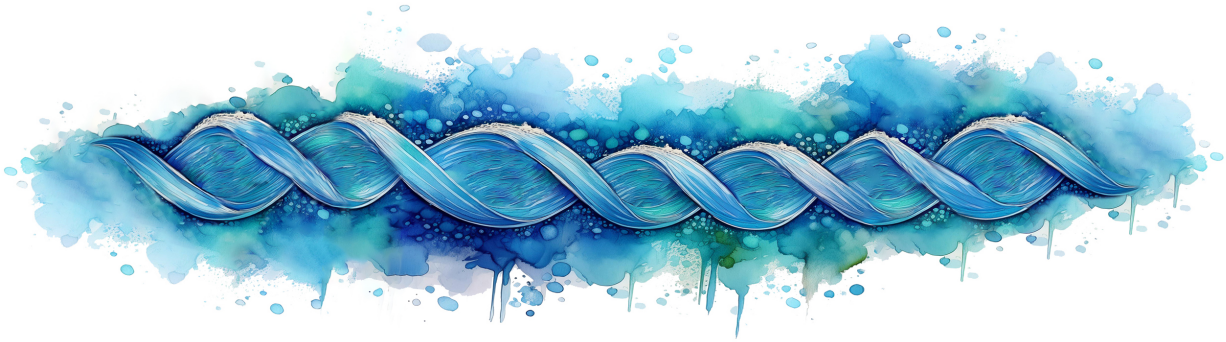
Subsequently, Hamp and Dic synthetic peptides were evaluated as preventive tools against NNV since Nkl had been previously tested with successful results. The administration of Hamp and Dic induced the opposite effects on the inflammatory response than plasmids. Indeed, Hamp and Dic resulted in the down-regulation of proinflammatory markers such as *il1b*, *il6* and *cox2* (cyclooxygenase 2) and although no modifications were observed for cell-adhesion molecules, the markers for neutrophils, macrophages and B cells *mpo*, *mcsf1r* (macrophage colony stimulatory factor 1 receptor) and *ighm*, respectively, were up-regulated. This boosted immune status might be responsible for the increased survival rates, reaching a relative percentage of survival (RPS) of 26.6% for Hamp and 33.3% for Dic. The analysis of viral markers revealed that there was no difference in viral load or titers among treated and non-treated groups pointing to non-lytic effects of Hamp and Dic directly on NNV. However, when evaluating the immune parameters in the target site of NNV, the brain, we observed an up-regulation of *il10*, *cxcr3*, *cxcl9* and gene markers for neutrophils, macrophages, and T cells along with the blockage of *il6*. This anti-inflammatory status might permit the influx of leukocytes via Cxcr3/Cxcl9 complex that allow the recruitment of key cells for viral infection resolution. So, the great amelioration of survival rates triggered by Hamp and Dic administered prior to infection as well as the immunostimulation observed point to AMPs as candidates to prevent NNV induced disease in fish farms.

Summary

Due to the successful immunostimulatory and preventive properties, Hamp and Dic synthetic peptides were tested as palliative agents. Similar to the observations for preventive experiments, Hamp and Dic post-treatments resulted in 30% RPS, while Nkl only delayed the appearance of mortalities but did not ameliorate this rate. Again, the treatments did not alter viral markers confirming our previous hypothesis of non-lytic effects on NNV. Regarding the immune response triggered in brain by Hamp and Dic post-treatments, our data revealed the up-regulation of *il10* favoring the anti-inflammation but keeping the expression of some proinflammatory cytokines at the same level than the control group. These findings along with the up-regulation of chemokines favored macrophage recruitment, a cell type which can restore cytokine production and revert brain damage induced by inflammation. By its side, Nkl did not stimulate macrophage infiltration, but enhance CMC response by up-regulating *nccrp1* (nonspecific cytotoxic cell receptor protein 1) and *nkl* expression. Although CMC has been related to viral clearance, our data point to the inefficiency of this molecular pathway to combat NNV infection and the key role of macrophages in NNV infection resolution. Strikingly, our data revealed the up-regulation of the marker of B cells and increased levels of IgM in brain upon Hamp and Dic treatments point to their role in the promotion of adaptive responses and suggesting their potential as vaccine adjuvants.

In conclusion, European sea bass AMPs are not efficient tools to modulate gilthead seabream immune response *in vitro*, contrary to what happened in European sea bass. The administration of these AMPs as expression plasmid *in vivo* fails to prevent NNV infection in European sea bass due to the potent inflammatory response triggered. Otherwise, Hamp and Dic synthetic peptides induced great RPS when administered as preventive or palliative treatments against NNV through their effect as immunomodulatory agents. Indeed, Hamp and Dic reverted the inflammatory response triggered by NNV via up-regulating anti-inflammatory cytokines such as IL-10 and promoting the infiltration of key cell types in antiviral response such as neutrophils, macrophages or T and B cells. Unfortunately, and despite the good results of Nkl as NNV-preventive agent, this peptide failed to confer protection against NNV when administered as palliative treatment. The main reason could be the incapability of Nkl to recruit macrophages into the brain, even when it increases the CMC response.

INTRODUCTION



Aquaculture: status and challenges

The food demand of the growing population due to the impossibility of satisfying it by traditional means of production, such as fisheries, has pointed to aquaculture as an excellent sector to fight against malnutrition and diet-related diseases (Fiorella *et al*., 2021). In this context, aquaculture is the fastest growing food production sector and is becoming one of the most prosperous markets in the economic sector worldwide (FAO, 2022). In the European Union (EU), aquaculture sector reached €3.9 billion in sales value and employed around 57,000 people in mainly four EU countries, in which Spain is included (STECF, 2021). Focusing on Spain, the most cultured teleost species are gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) and the most prosperous in terms of trading (APROMAR, 2023). Thus, in this Doctoral Thesis, we will focus on these two species.

Nevertheless, and despite the promising prospects of this sector, aquaculture is facing great difficulties due to the management of pathogens, sustainability issues or climate change conditions (Naylor *et al*., 2021). The natural outbreaks of pathogens in fish farms worldwide are threatening the economic viability of the sector due to the lack of proper available treatments (Buchmann, 2022). Among these outbreaks, viral infections have emerged as a great emerging problem for fish producers. The high culture density makes that viruses, which normally coevolve with their host and are harmless under natural conditions, become pathogenic developing severe diseases (Kibenge, 2016). Therefore, many efforts must be done in the line of developing new molecules with interest in preventing or treating fish diseases in aquaculture.

Immune response in teleost fish

Immune response is formed by innate and adaptive responses, both constituted by a complex network of specialized cell types and molecules. Innate immunity constitutes a rapid initial response of the host to eliminate invading pathogens and promotes disease resistance (Abbas *et al*., 2012). Adaptive response is a more complex, sophisticated, and specialized response against antigens (Abbas *et al*., 2012) that orchestrates the activation of specific cells with immunological memory, acquired in the first contact with an antigen, allowing a rapid secondary response (Stosik *et al*., 2021).

Innate immunity

In fish, gills, skin, and gut constitute mucosal barriers and serve as physical first line of defense against microbes. A complex network of immune cells is associated to these mucosal surfaces forming mucosa-associated lymphoid tissues (MALT) such as gills-, skin- or gut-associated lymphoid tissue (GIALT, SALT or GALT, respectively) (Salinas, 2015). In addition, these epithelia are covered by a mucus layer rich in bioactive compounds (Dezfuli *et al*., 2023).

The main leukocyte types involved in innate immunity are monocytes/macrophages, granulocytes, melanomacrophages, natural killer (NK)-like and non-specific cytotoxic cells (NCCs). Monocytes/macrophages and acidophilic granulocytes/neutrophils are myeloid cells produced in the head-kidney (HK), the main lymphopoietic and hematopoietic organ with important implications in the induction and elaboration of the immune response (Press and Evensen, 1999). These cell types take part in phagocytosis and can be recruited to the inflamed tissues due to the damage caused by a pathogen (Ellis, 2001; Jørgensen, 2014; Somamoto *et al*., 2013). Melanomacrophages (MMs) are pigmented cells produced and distributed in the spleen and HK. MMs are potent phagocytic cells capable to destroy endogenous and exogenous bodies, such as erythrocytes or pathogens, respectively (Gómez-Manrique *et al*., 2019; Steinel and Bolnick, 2017). Teleost fish possess cells equivalent to mammalian NK cells: NCCs and NK-like cells, which perform the cell-mediated cytotoxicity (CMC). Both cell types are widely located in the lymphomyeloid tissues able to kill tumor and virus-infected cells without requiring any induction period and with no major histocompatibility (MHC) restriction (Jørgensen, 2014; Mali *et al*., 2017). While fish NK-like cells are large granular lymphocytic cells, morphologically similar to mammalian NK cells, NCCs are formed by a heterogeneous population consisting of lymphocytes, monocyte/macrophages and/or granulocytes. Although they are functionally equivalent, NCCs express in their surface the non-specific cytotoxic cells receptor protein 1 (NCCRP-1) (Evans *et al*., 2001, 1992; Jaso-Friedmann *et al*., 2001) whilst NK-like cells do not. Both cell types use perforin and granzymes, as granule-dependent, and the Fas/FasL system, as granule independent, as the main cytolytic mechanisms. Perforin is a pore-forming molecule, which acts synergistically with granzymes favoring their entry into target cells leading to the target cell death (Praveen *et al*., 2006, 2004).

Introduction

Once a pathogen reaches an organism, pathogen associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Sahoo, 2020). This recognition lead to the leukocyte activation and promote the orchestration of other immune humoral components such as complement, acute phase proteins, antimicrobial peptides (AMPs) or cytokines among others (Zou and Secombes, 2016). Considering that fish show a great variety of humoral processes, we will focus on those with antiviral implications since this response is the main interest of the present Doctoral Thesis.

AMPs are short aminoacidic sequences which play a critical role in the innate immunity by mediating and regulating key immune processes as well as directly killing pathogens (Katzenback, 2015). AMPs will be described in more detail in next sections.

Cytokines are a diverse group of soluble extracellular proteins involved in the regulation of a wide variety of immune processes including immune cell mobilization, antigen presentation or the initiation and development of the adaptive response, although they are considered as innate effectors (Cao *et al*., 2023). Among cytokines, interferon, interleukins, and chemokines are the most relevant for this work. Interferon (IFN) are cytokines secreted in the defense against viruses in vertebrates, induce cellular antiviral state (Robertsen, 2006) and can be classified into two categories: type I and type II. Type I IFN are homologous to the IFNα/β of mammals and induced by the presence of viruses in all nucleated cells while type II is formed by the IFNγ mainly produced by T cells (Boudinot *et al*., 2016). IFN can activate a signal transduction cascade inducing interferon stimulated genes (ISG), such as *mx*, *isg15* or *pkr,* which act as antiviral effectors (Collet and Secombes, 2002). The induction of ISGs can promote the activation of other immune routes such as the activation of adaptive leukocytes such as CD8+ T cells (Zou and Secombes, 2011). Other important cytokines are interleukins (ILs), involved in the regulation and coordination of immunity. ILs are mainly involved in inflammatory processes, but with opposing functions. IL-1 or IL-6 are pro-inflammatory cytokines that also regulate other immune processes such as the non-specific amplification of T and B cell responses, the induction of IFN production or the stimulation of antibody production (Boraschi, 2022; Chen *et al*., 2012; Kaneda *et al*., 2012). Otherwise, ILs from the IL-10 family mainly exert anti-inflammatory functions and compensate the roles of the pro-inflammatory ones leading also to the promotion of the adaptive responses (Cao *et al*.,

2023; Secombes *et al*., 2011; Zou and Secombes, 2016). Chemokines are chemoattractant molecules that coordinate the migration of immune cells to damaged or infected tissues guaranteeing efficient innate and adaptive responses (Bird and Tafalla, 2015). The chemokine Cxcl9 is known to be induced by IFN activation and can bind to the chemokine's receptor Cxcr3 promoting T cell recruitment (Groom and Luster, 2013; Valdés *et al*., 2022). Also, IL-8 is a chemokine involved in leukocyte migration that is also categorized as a pro-inflammatory cytokine (Zhao *et al*., 2022).

Adaptive immunity

The adaptive or specific immune response is started when the innate cells recognize and present the antigens to the lymphocytes to induce their maturation, selection and generation of memory. For this, antigen presenting cells (APCs), T cytotoxic (CTLs), T helper (Th) and B cells are the main cells involved in the adaptive immunity.

APCs are a diverse group of specialized immune cells which process and present antigens in their surface through the MHC-II complex, which can interact with T cell receptor (TCR), activating T cells (Bassity and Clark, 2012). T cells proliferate and mature in the thymus. There are two main types of T cells: CTLs and Th cells showing, in their surface, the TCR and the coreceptor cluster of differentiation (CD) 3, but differ in the expression of CD8 or CD4, respectively. Thus, CTLs are the main effector cells of the CMC and interact with the MHC-I, present in all cell types (Yamaguchi *et al*., 2019), releasing the content of their cytoplasmatic granules, granzymes and perforins among other effectors, that allow the elimination of abnormal and/or virus-infected cells (Mutoloki *et al*., 2014). On the other hand, T helper (Th) cells interact with the MHC-II and induce the secretion of cytokines which facilitate the pathogen elimination (Mutoloki *et al*., 2014). The roles of Th cells are diverse and orchestrate multiple immune processes including the defense against intracellular or extracellular pathogens or the regulation of the immune response to prevent exacerbated reactions.

B lymphocytes express in their surface the B cell receptor (BCR), which can recognize antigens activating the proliferation and differentiation of other immune cell types but also regulated by Th cells (Harwood and Batista, 2010). B cells secrete immunoglobulins (Ig), which are the main actors of the humoral adaptive responses. Three antibody types have been described in teleost fish: IgM, IgD and IgT/IgZ (Mutoloki *et al*., 2014). The most common isoform is IgM, present in serum, mucus and B cells

surface and mediates both innate and adaptive responses (Salinas *et al*., 2011). IgT/IgZ is the main immunoglobulin present in fish mucosal tissues and it is considered as the potential equivalent of the IgA of mammals (Ye *et al*., 2013). Otherwise, IgD is also present in the B cells surface or secreted, but their role remains an enigma though its participation in the homeostasis of fish microbiota has been suggested (Salinas *et al*., 2017). B cells role in phagocytosis has also been described in fish and bridge the innate and the adaptive immunity (Wu *et al*., 2020), as well as their key participation in antiviral response in teleosts (Díaz-Rosales *et al*., 2018).

Antimicrobial peptides

In general, AMPs are gene-encoded peptides that share some common structural features such as their short amino acid (aa) sequences (10-50 aa), low molecular weight (less than 10 KDa), mostly positive charged, amphiphilic and amphipathic (Valero *et al*., 2013). AMPs can form alpha-helix, beta-sheets, or a mixture of both secondary structures, that confer bioactivity to AMPs by potentiating their stability and favoring membrane binding and disruption (Kumar *et al*., 2018). Alpha helix peptides are enriched in Leu, Ala, Gly and Lys residues (Personne *et al*., 2023), while in beta-sheet structure the presence of disulfide bonds formed by cysteines is a key motif in their antimicrobial activity forming cycled peptides (Freitas *et al*., 2020). All the mentioned structural features contribute to achieve AMP functions by favoring their interaction with membranes (Ahmed and Hammami, 2019). In fact, AMPs can be grouped into different families attending to their structure. The most representative families in teleost are piscidins (marine exclusive), defensins, hepcidins, cathelicidins, histone-derived peptides and saponins (Masso-Silva and Diamond, 2014).

Mode of action

AMPs show a wide range mode of actions leading to reduce the possibilities of the appearance of bacterial resistances (Mwangi *et al*., 2019). Thus, AMPs can exert their antimicrobial properties in two separate ways: i) direct lytic effects against a wide range of pathogens, and ii) modulating the immune response of the host (Katzenback, 2015) as shown in Figure 1.

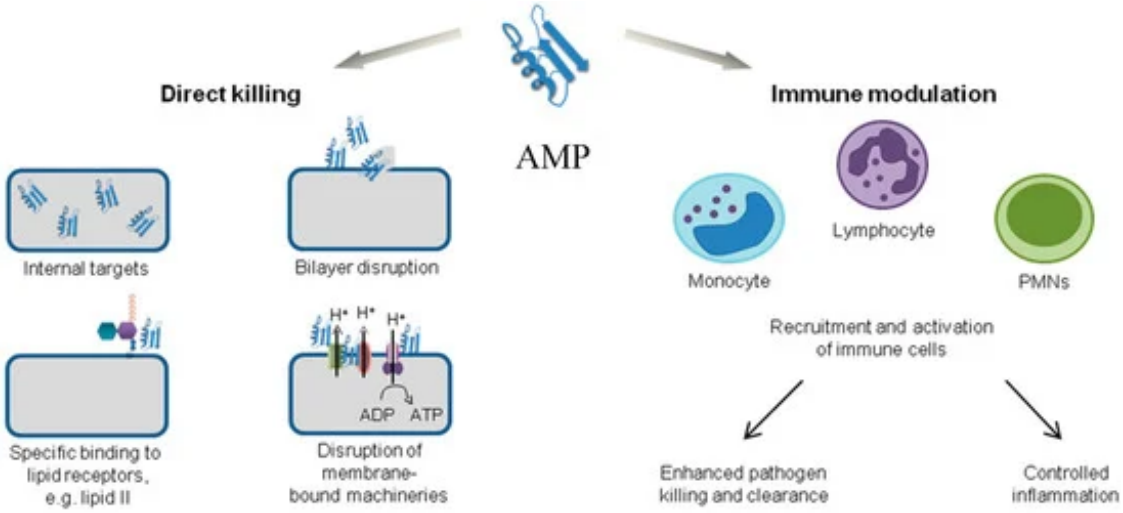


Figure 1. Different mode of actions of antimicrobial peptides (Kumar *et al*., 2018).

Two different ways of direct lytic effects have been proposed: membrane targeting and non-membrane targeting mechanisms. Many AMPs can interact with the components of the membrane without interacting with specific receptors. AMPs are normally positively charged and have strong electrostatic interaction with the phospholipids in bacterial membranes, which are negatively charged, and can disrupt them, but not in animal cells in which most lipids are neutral (Kumar *et al*., 2018). The electrostatic and hydrophobic interactions lead to two direct modes of action explained by the transmembrane pore and non-pore models (Kumar *et al*., 2018). The pore formation model is tightly related to amphipathic AMPs since this structure promotes the interaction between hydrophobic regions with lipids (Brogden, 2005; Kumar *et al*., 2018). Other AMPs can act without forming membrane pores by establishing unfavorable interaction with the membranes, which lose their integrity, and finally disintegrate through the formation of micelles. The non-membrane targeting mechanisms of action could be by means of: (i) targeting bacterial cell walls or (ii) intracellular targets. Briefly, AMPs can inhibit cell wall synthesis or interact with precursor molecules that are required for cell wall synthesis, exerting, therefore, antibacterial activity. Nevertheless, there are other AMPs that interact with cytoplasmic components blocking critical cellular processes such as the inhibition of protein/nucleic acid synthesis or the disruption of protein functions among others (Avila, 2016; Kumar *et al*., 2018; Scocchi *et al*., 2016).

The antiviral mechanisms of AMPs are still unclear, and poorly studied, but the ability of AMPs to agglutinate viral particles into clumps, the inhibition of the viral adsorption capacity hampering them to enter into the cells or the inhibition of the viral

replication cycle have been proposed (Avila, 2016; Chia *et al*., 2010) as represented in Figure 2.

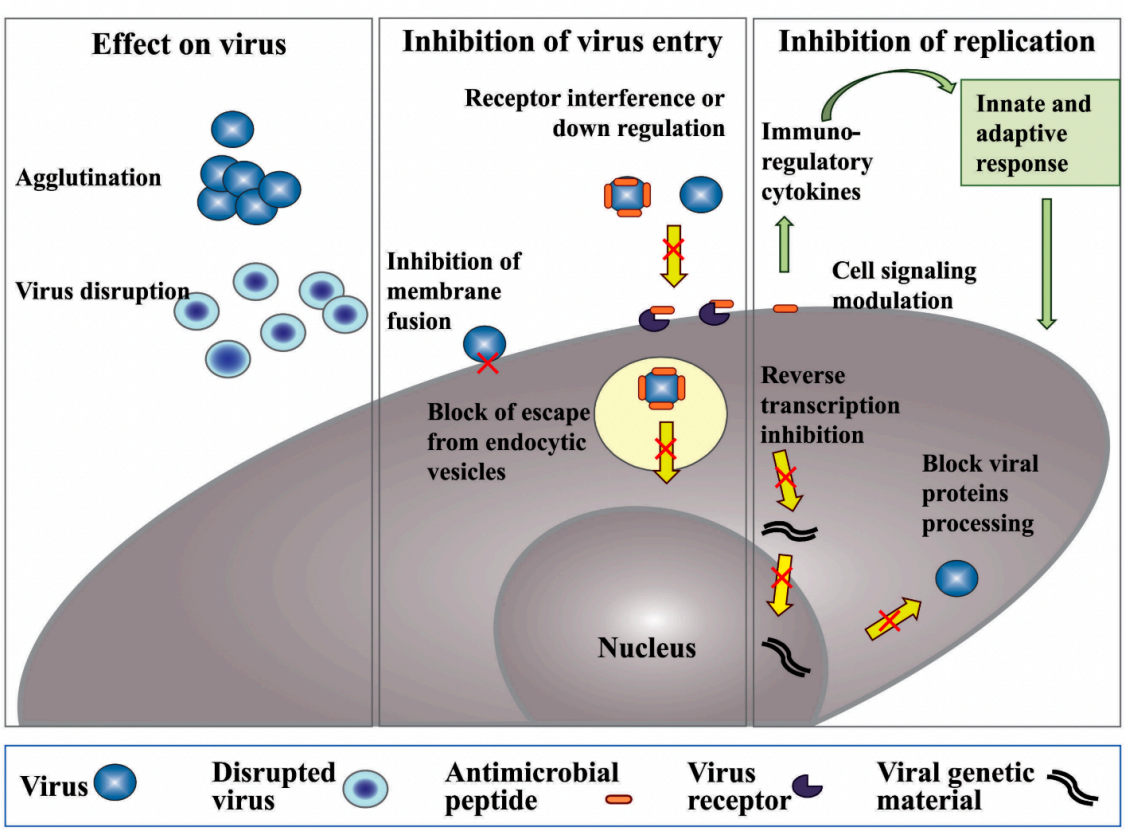


Figure 2. Antiviral mechanism of action of AMPs (Avila, 2016).

Beside their direct lytic effect on pathogens, AMPs are also able to recruit and activate immune cells and control inflammation (Fig. 3) resulting in an enhanced response to eliminate pathogens (Katzenback, 2015). Their modulatory effects on the immune response are the consequences of the promiscuous interaction between AMPs with various cellular receptors, which transduce regulatory elements of the immune response (Luo and Song, 2021). Thus, AMPs have the ability to attract leukocytes and promote their expression and secretion of cytokines and chemokines (Haney et al., 2016). In the early stages of infection, AMPs may induce a transitory inflammatory status allowing a local leukocyte infiltration and activation that favors the elimination of the microbe (Masso-Silva and Diamond, 2014). Nevertheless, AMPs are also able to revert this condition when the prolonged exposition would lead to tissue damage by inducing anti-inflammatory molecules and blocking pro-inflammatory ones (Méndez-Samperio, 2013).

Recently, AMPs have also been described to serve as a bridge between innate and adaptive responses (Ma *et al*., 2020). The stimulation of antibody production, the induction of T helper and B cells and the secretion of cytokines by these cell types have been described to be regulated by AMPs (Avila, 2016; Xia *et al*., 2018).

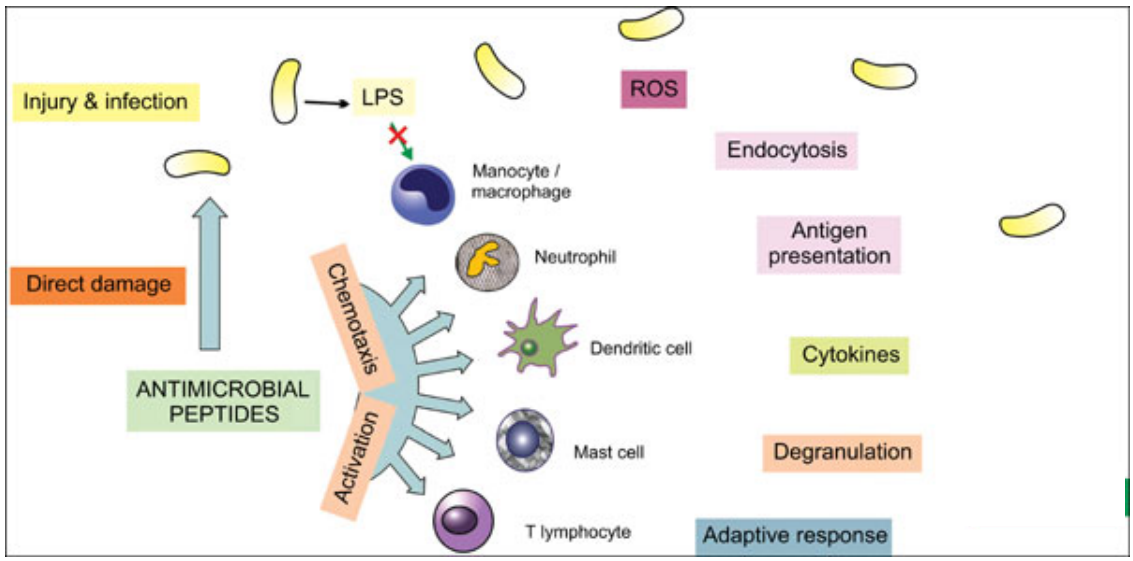


Figure 3. Immune modulation triggered by AMPs upon *stimuli* such as an injury or infection (Modified from Avila 2016).

Main AMPs in teleosts

According to the "Antimicrobial Peptide Database" (https://aps.unmc.edu/), up to date, there are around 146 peptides from fish with known antimicrobial activity. The most representative studies in which the role of the main AMPs families and members have been explored are summarized in Table 1. In this section, we are going to highlight 4 of the most characterized fish AMPs that serve as models in this Doctoral Thesis.

Fish NK-lysin (Nkl) was first characterized as an AMP in channel catfish (Wang *et al*., 2006). Nkl is synthetized by CTLs and stored into granules until their release upon *stimuli* (Valero *et al*., 2020b). Nkl expression is induced upon bacterial, viral and parasitic challenges pointing to its role in combating pathogens (Cervera *et al*., 2022; Ding *et al*., 2019; Pereiro *et al*., 2017; Valero *et al*., 2020b; Zhou *et al*., 2016). Otherwise, Nkl can act as a modulator of the immune response (Ding *et al*., 2019; Hao *et al*., 2022) along with its direct lytic effect on bacteria and viruses such as betanodavirus (NNV) (León *et al*., 2020a; Valero *et al*., 2019)

Table 1. Representative fish AMP families and main properties.

Family	Structure	Main members	Species	Effects	References
Cathelicidins	β-sheet and random coil structure	Cathelicidin-1 Cathelicidin-2	*Gadus morhua, Salvelinus alpinus, Salvelinus fontinalis, Salmo salar, Plecoglossus altivelis, Oncorhynchus mykiss*	Lytic activity against bacteria and fungi. Involved in the regulation of inflammation.	(Acosta et al., 2019; Broekman et al., 2011; Lu et al., 2011; Maier et al., 2008; Zhang et al., 2015)
Piscidins	α-helix, 44 amino acid mature peptide	Dicentracin Epinecidin-1 Pleurocidin Piscidin-1 Piscidin-2	*Dicentrarchus labrax, Epinephelus coioides, Sparus aurata, Pseudopleuronectes americanus, G. morhua, Morone saxatilis, Oreochromis niloticus, Oplegnathus fasciatus*	Lytic activity against bacteria, viruses, parasites, and fungi. Induce the expression of TLR, IFN and several ILs. Regulate apoptosis.	(Bae et al., 2014; Douglas et al., 2003; Lin et al., 2012; Peng et al., 2012; Salerno et al., 2007; Serna-Duque et al., 2022)
Hepcidins	β-sheet, 8 Cys forming 1-4 disulphide bridges, 20-26	Hepcidin-1 Hepcidin-2	*D. labrax, S. aurata, E. coiodes, O. niloticus, Scophtalmus maximus, Siniperca chuatsi, O. fasciatus, S. salar, O. mykiss*	Lytic activity against bacteria, viruses, and parasites. Selectively promote the transcription of proinflammatory cytokines.	(Álvarez et al., 2022, 2016, 2014; Cho et al., 2009; Cuesta et al., 2008; Neves et al., 2017; Pan et al., 2011; Rajanbabu and Chen,

	amino acid mature peptide			Induce the expression of TLR. Mediate iron homeostasis.	2011; Rodrigues et al., 2006; Shen et al., 2019; Yang et al., 2013)
Saposin	α-helix, saponin-B domain with 6 Cys forming 3 sulphide bonds, 74-78 amino acid mature peptide	NK-lysin-1	*D. labrax, S. aurata, O. niloticus, I. punctatus, Danio rerio, S. maximus, S. salar, Hyporthodus septemfasciatus, Cynoglossus semilaevis, O. mykiss, Boleophthalmus pectinirostris*	Lytic activity against bacteria, viruses, and parasites. Effector of the CMC response. Contained in NCCs and CTLs as granules. Control the expression ILs, chemokines, IFN. Promote autophagy.	(Cervera et al., 2022; Ding et al., 2019; Y. Huang et al., 2018; Ma et al., 2021; Pereiro et al., 2015; Valero et al., 2019, 2020b; Wang et al., 2023, 2006; Zhang et al., 2013; Zhang et al., 2014)
		NK-lysin-2			
		NK-lysin-3			
		NK-lysin 4			
		NK-lysin-like a			
		NK-lysin-like b			
Defensins	α-helix and β-sheet, 6 conserved Cys, 39-45 amino acid mature peptide	Defensin-beta 1	*D. labrax, Trachinotus ovatus, Plecoglossus altivelis, Paralichthys olivaceus, S. salar, S. aurata*	Lytic activity against bacteria, parasites, fungi, and viruses. Control the levels of inflammation. Chemoattract leucocytes and promote phagocytosis. Regulation of reproductive and endocrine processes.	(Barroso et al., 2021; Cuesta et al., 2011; Das et al., 2022; Hao et al., 2021; Harte et al., 2020; Zhou et al., 2020, 2019)
		Defensin-beta 2			
		Defensin-beta 3			
		Defensin-beta 4			
		Defensin-beta 5			

Histone-derived peptides	Low molecular weight peptides generated from proteolytic cleavage of histones	Histone 1 Histone 2A Histone 2B	*O. mykiss, S. salar, Acipenser gueldenstaedtii, D. labrax, S. aurata*	Lytic activity against bacteria, parasites, fungi, and viruses.	(Fernandes et al., 2004; Luders et al., 2005; Noga et al., 2002; Valero et al., 2016a)

Hepcidin (Hamp) was first described in mammals but also in a large number of vertebrates including fish. The immunomodulatory effects of Hamp have been well-described in several fish species, promoting mainly anti-inflammatory responses (Masso-Silva and Diamond, 2014). Hamp antiviral roles in teleost have been described (Gui *et al*., 2016; Zhang *et al*., 2014) and Hamp peptides have been tested as anti-NNV agent in species such as medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*) or grouper (*Epinephelus coioides*) (Chia *et al*., 2010; Hsieh *et al*., 2010; Wang *et al*., 2010a, 2010b). Antibacterial and antiparasitic activities are part of the bioactivity of Hamp (Cervera *et al*., 2022; Huang *et al*., 2007). Besides, Hamp also has a role in iron metabolism in vertebrates (Neves *et al*., 2017).

Dicentracin (Dic) was first characterized in European sea bass and belongs to the exclusive marine family of the piscidins (Salerno *et al*., 2007). Although Dic display direct lytic effect on NNV *in vitro* (León *et al*., 2020b), very little is known about the immune implications of this peptide. Dic encoding gene modulation against NNV and *Vibrio anguillarum* infection has been reported (Meloni *et al*., 2015; Valero *et al*., 2015b), being altered in the HK, brain, spleen and muscle upon NNV infection (Valero *et al*. 2020a).

Defensin beta (BD) was discovered in various fish such as zebrafish (*Danio rerio*), pufferfish (*Takifugu rubripes*) and tetraodon (*Tetraodon nigroviris*) showing high homology with other vertebrate defensins (Zou *et al*., 2007). Antiviral and antibacterial roles have been described for this group of peptides in fish (Jin *et al*., 2010). With regards to their immunomodulatory roles, defensin beta has been related to the recruitment of leukocytes, the control of inflammation as well as bridging innate and adaptive responses (Das *et al*., 2022; Ruangsri *et al*., 2013).

Nodavirus

Etiological agent, disease, transmission, and diagnosis

Nervous necrosis virus (NNV), or Betanonodavirus, is the etiological agent causing viral encephalopathy and retinopathy (VER) disease, which affects to cultured fish species with devastating consequences for their production (Low *et al*., 2017). The first reports of VER disease existence go back to the 90s when fish farms were severely impacted by massive mortalities (Bloch *et al*., 1991; Breuil *et al*., 1991; Mori *et al*., 1992). Up to date, NNV is known to infect more than 170 fish species including marine or freshwater species but not all of them show the same clinical signs nor mortality rates (Bandín and Souto, 2020). The two main traded species in Spain, gilthead seabream and European sea bass, are a good example of this issue since the former is resistant to the disease produced by the main viral genotypes while the latter is highly susceptible (Chaves-Pozo *et al*., 2012). The central nervous system is the main target tissue of the virus; brain, spinal cord or retina showed vacuolation and the formation of necrosis areas, which explain the observed symptomatology (Azad *et al*., 2005; Munday *et al*., 2002). These lesions induce movement abnormalities such as spiral swimming, difficulties to control the swim bladder, paler skin coloration or anorexia (Munday *et al*., 2002). The virus has also been detected in other tissues such as blood or immune organs in experimentally infected and symptomatic fish (Poisa-Beiro *et al*., 2008). Interestingly, in the fish that overcome the infection, NNV can colonize the gonad causing an asymptomatic latent infection which is difficult to diagnose (Valero *et al*., 2018b). Hence, NNV can be transmitted vertically or horizontally from apparently healthy specimens via gametes or fluids, respectively (Azad *et al*., 2006; Breuil *et al*., 2002; Grotmol *et al*., 1999; Valero *et al*., 2018b, 2015a).

NNV diagnosis is normally performed by RT-PCR or nested RT-PCR (nRT-PCR) in the target site of infection, the brain, but also histological methods, isolation of viral particles in cell lines or enzyme-linked immunosorbent assay (ELISA) are employed as postmortem ways to detect NNV (Breuil and Romestand, 1999; Dalla Valle *et al*., 2005; Olveira *et al*., 2008). The antemortem diagnosis methods have also been explored such as the use of RT-PCR or nRT-PCR in blood samples (Olveira *et al*., 2008).

NNV structure

NNV is a small non-enveloped, spherical in shape with icosahedral symmetry and with a genome composed of two-single-stranded, positive-sense RNA molecules: RNA1 and RNA2 (Bandín and Souto, 2020). RNA1 is the largest segment, composed by 3100 nucleotides (nt) and codifies for the RNA-dependent RNA-polymerase (RdRp) or protein A, which plays a key role in the viral genome replication (Munday *et al*., 2002), but, recently, other functions have been attributed to this protein such as the capability of triggering the activation of type I IFN route (Huang *et al*., 2018). RNA2 is a smaller segment (1410-1433 nt) and codifies for the structural capsid protein (CP), which is implicated in viral-host pathogenesis such as host specificity, cell cycle regulation or autophagy (Sommerset and Nerland, 2004; Zhang *et al*., 2022). Additionally, there is also a subgenomic RNA, called RNA3, composed by 371-378 nt that codes for two non-structural viral proteins: protein B1 and B2 (Iwamoto *et al*., 2005; Nagai and Nishizawa, 1999), which regulate the host cell survival or induces mitochondria-mediated cell death, respectively (Chen *et al*., 2009; Su *et al*., 2009). The replication cycle of NNV is shown in Figure 4.

Four traditional NNV genotypes according to differences in the variable region, T4, of the RNA1 have been described: Red-spotted grouper nervous necrosis virus (RGNNV), Striped Jack nervous necrosis virus (SJNNV), Barfin flounder nervous necrosis virus (BFNNV) and Tiger puffer nervous necrosis virus (TPNNV) though another additional genotype has been proposed and called Turbot betanodavirus strain (TNV) (Costa and Thompson, 2016). In addition, two natural reassortants between RGNNV and SJNNV strains have been isolated: RGNNV/SJNNNV and SJNNV/RGNNV and pointed to as pathogenic but with modified host specificity and resistance when compared to the parentals (Biasini *et al*., 2022).

Host immune response against NNV

The study of the immune response triggered by NNV in the host has drawn attention due to the necessity of understanding the host-virus interaction to design methods for controlling the outbreaks. Among the humoral innate immune responses, most of the studies focused on the type I IFN pathway upon NNV infection. Thus, some members of this route have been evaluated at transcriptional levels in Atlantic cod (*Gadus morhua*), barramundi (*Lates calcarifer*), gilthead seabream, European sea bass, zebrafish (*Danio*

rerio), orange-spotted grouper (*Epinephelus coiodes*), among other species, in brain, retina and/or cell lines resulting up-regulated in all cases and demonstrating its role in the response against NNV (Álvarez-Torres *et al*., 2018; Huang *et al*., 2015; Krasnov *et al*., 2013; Lu *et al*., 2008; Valero *et al*., 2015c; Wu *et al*., 2010). Interestingly, the role of AMPs during the NNV infection has been highlighted. Thus, NNV infection in European sea bass resulted in the up-regulation in the expression of some AMP genes, but not in the resistant gilthead seabream (Valero *et al*., 2015b, 2016a, 2020a, 2020b). Strikingly, these data were not parallel to the protein levels and suggest certain post-transductional regulation (Valero *et al*., 2020a).

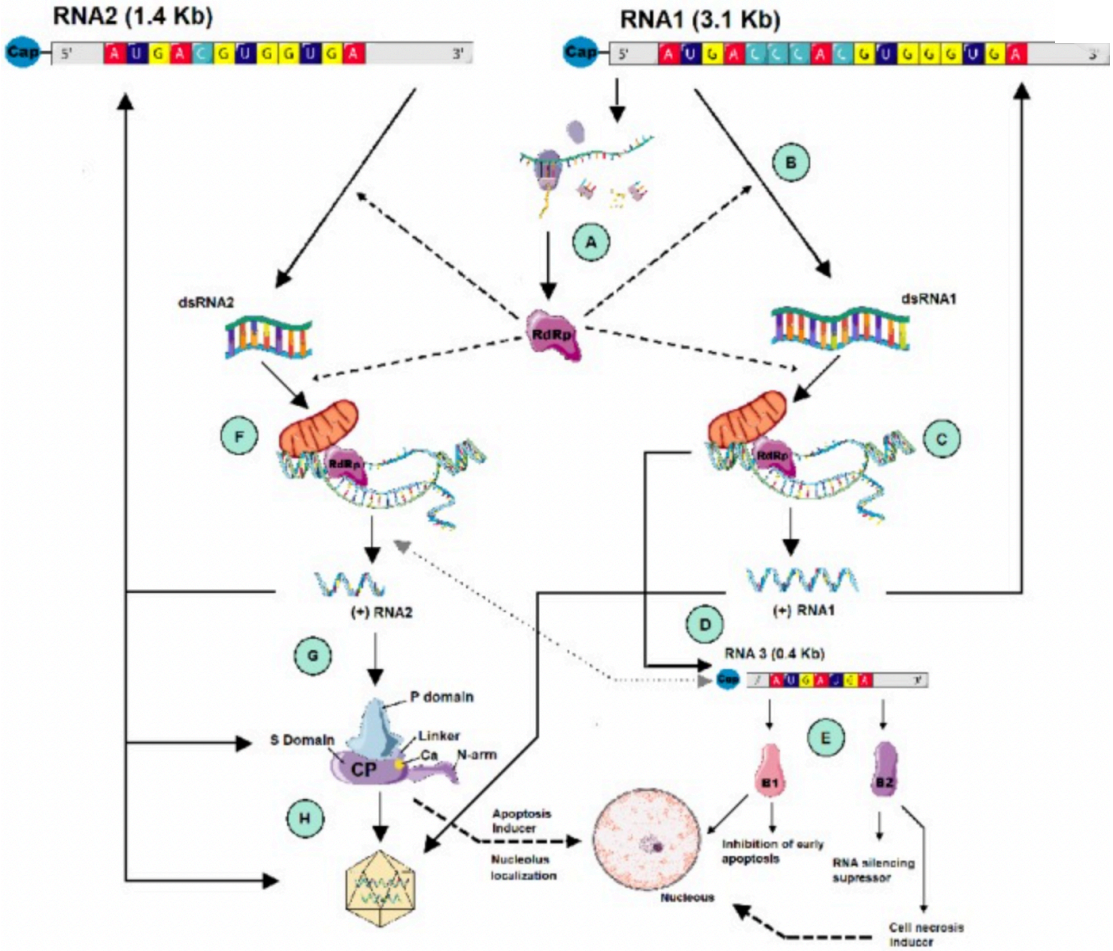


Figure 4. Schematic view of the replication cycle of NNV (Bandín and Souto, 2020).

Despite the fact that IFN activation might be not enough to combat the infection in susceptible species such as European sea bass considering the high mortalities registered (Chaves-Pozo *et al*., 2019a; Valero *et al*., 2015c), cellular immune processes have also

been studied. The mobilization of neutrophils and macrophages towards the brain favor the NNV infection resolution in zebrafish larvae or red-spotted grouper, respectively (Lama *et al*., 2022). However, this infiltration is a double-edge sword since they induce an acute cytokine storm (Wang *et al*., 2021) leading to an exacerbated inflammatory response, which in turn is associated with high host susceptibility due to the neuronal damage (Poisa-Beiro *et al*., 2008). The implication of the CMC response has been also documented by the increased NCC and CD8+ CTL activity, and the general up-regulation of NCC and CTL markers as well as related genes, including granzyme A, granzyme B, perforin, and the antimicrobial peptide Nkl, in NNV target tissues, brain and retina, as well as in HK or HK leukocytes (HKLs) pointing to its role at both local and systemic levels (Chaves-Pozo *et al*., 2019b, 2017, 2012; García-Álvarez *et al*., 2024b, 2024a; Øvergård *et al*., 2013; Patel *et al*., 2008; Valero *et al*., 2020b, 2018a). Interestingly, these studies have evidenced an important CMC response during NNV infection in resistant fish and minor in susceptible ones like the European sea bass suggesting a factor of resistance or viral evasion.

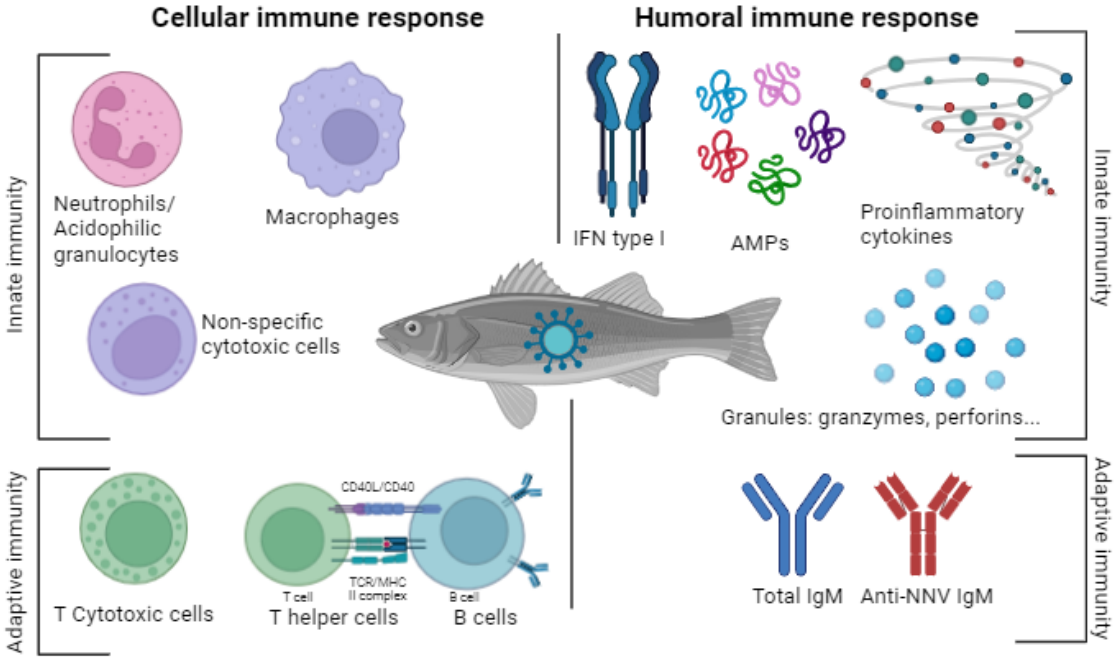


Figure 5. Schematic representation of the immune response triggered by NNV.

Otherwise, the up-regulation of adaptive markers such as *ighm* or *cd4* have been reported as well as the increase of antibody secretion (Buonocore *et al*., 2017; López-Muñoz *et al*., 2012; Moreno *et al*., 2018; Scapigliati *et al*., 2010; Valero *et al*., 2018a). Moreover, high titers of antibodies have been related to the capability of neutralizing

NNV antigens and favoring viral clearance (López-Jimena *et al*., 2012; Scapigliati *et al*., 2010; Skliris and Richards, 1999). This information is schematically represented in Figure 5.

Treatments against NNV

Although NNV is one of the most contagious and devastating viruses in fish farms, the availability of preventive or palliative methods is very limited. Vaccines are not only the prophylactic method more explored by researchers worldwide to control natural NNV outbreaks, but also has been proposed to be an excellent tool to prevent vertical transmission (Kai *et al*., 2010). Most of the proposed vaccines are administered by intramuscular injection but oral administration is gaining relevance, nowadays. Three types of vaccines have been proven against NNV infection up to date. The first type of vaccines are those composed by recombinant NNV proteins which strongly elicit the humoral immune responses when administered intramuscularly or orally and induce the highest survival rates, more than 60% (González-Silvera *et al*., 2019; Hegde *et al*., 2005; Kim *et al*., 2014; Øvergård *et al*., 2013; Sommerset *et al*., 2005; Thiéry *et al*., 2006; Thwaite *et al*., 2020; Vimal *et al*., 2016, 2014; Yuasa *et al*., 2002). The second type is based on inactivated virus, administered intramuscularly or intraperitoneally, and elicit a less potent immune response and exhibit lesser protection (Coeurdacier *et al*., 2003; Liu *et al*., 2006; Pakingking *et al*., 2009; Valero *et al*., 2018c). The last type of vaccine used for teleost fish against NNV are DNA vaccines which show good rates of protection, around 50% of survival when administered by intramuscular injection or orally (Ramya *et al*., 2014; Sommerset *et al*., 2003; Valero *et al*., 2016b). Nowadays, there are two commercial vaccines against NNV in Spain according to Cimavet (www.cimavet.aemps.es). Alpha Ject Micro 1 Noda Emulsion is commercialized by Pharmaq As. This vaccine is composed by inactivated NNV (strain ALV1107) and is intraperitoneally injectable. Ichtiovac VNN Emulsion is commercialized by Hipra S.A. This vaccine is composed by inactivated NNV (strain 1103) and is intraperitoneally injectable.

Even if anti-viral treatments are very few employed for the management of NNV in aquaculture facilities, efforts are being done to find molecules with potential to be used in farms. The hugest experiment done up to date screened more than 1,000 drugs in a cell line to evaluate their potential as anti-NNV compounds (Huang and Han, 2014).

Moreover, the known antivirals ribavirin and amantadine have also been tested as anti-NNV candidates (Huang *et al*., 2016; Morick and Saragovi, 2017; Zhu *et al*., 2022). Aptamers and affinity peptides have also been proposed as anti-NNV molecules (Bandín and Souto, 2020). Otherwise, in recent years, AMPs have arisen as potential and effective molecules to become anti-NNV agents due to their lytic effect against viruses and their role in the stimulation of the immune response (Masso-Silva and Diamond, 2014). The use of AMPs as pharmacological compounds with application in the aquaculture area will be discussed in the following section.

AMPs applicability in aquaculture

As previously mentioned, the development of new molecules with therapeutic prospects for aquaculture is crucial to control pathogen outbreaks. AMPs possess both a wide range of antimicrobial properties as well as immunomodulatory roles becoming great candidates to fight against viral, bacterial, parasitic or fungal infections (Freitas *et al*., 2020). The widespread distribution of AMPs among all life kingdoms, from plant to humans, points to the existence of an evolutionary ancient precursor and their persistence over the evolution is an evidence of the low generation of microbial resistance (Katzenback, 2015). In this context, AMPs have arisen as potential candidates for developing new pharmacological treatments for aquaculture (García-Beltrán *et al*., 2023; Katzenback, 2015). AMPs potential as antiviral agents in aquaculture has also been pointed out to solve the scarcity of proper drugs to control this kind of diseases (Falco *et al*., 2009). In this line, some researches have put their focus on the potential use of AMPs, administered as synthetic or recombinant peptides or via plasmids (Chia *et al*., 2010; Hsieh *et al*., 2010; Pan *et al*., 2011; Valero *et al*., 2021; Wang *et al*., 2010a, 2010b; Zhang *et al*., 2014; Zhang *et al*., 2013). The *in vivo* experiments in which the applicability of AMPs in fish farms are evaluated are summarized in Table 2. First, *in vitro* analysis have revealed the direct lytic effect of tilapia Hamp against NNV (GF-1) (Chia *et al*., 2010). Tilapia Hamp has also been tested *in vivo* in zebrafish via plasmids resulting in the stimulation of innate immune related genes such as interleukins and conferring protection against the bacteria *Vibrio vulnificus* (Hsieh *et al*., 2010; Pan *et al*., 2011). Moreover, tilapia Hamp synthetic peptide has been administered to grouper and medaka resulting in the increase of survival against NNV administered prior, at the same time or after the infection (Wang *et al*., 2010b, 2010a). Otherwise, plasmids encoding Nkl as well as Nkl synthetic peptide administered to tongue sole (*Cynoglossus semilaevis*) resulted in the

Table 2. *In vivo* studies about the potential applicability of AMPs in aquaculture.

AMPs	Specie tested	Way of administration	Effect	References
Tilapia hepcidin 2-3	Zebrafish	Plamids	↑ protection against *Vibrio vulnificus* ↓ inflammation-related genes	(Hsieh *et al.*, 2010)
Tilapia hepcidin 1-5	Medaka	Synthetic peptide	↑ protection against NNV	(Wang *et al.*, 2010a)
Tilapia hepcidin 1-5	Grouper	Synthetic peptide	↑ protection against NNV when coadministered No preventive nor therapeutic against NNV ↓ *mx* gene expression	(Wang *et al.*, 2010b)
Turbot hepcidin 1 and hepcidin 2	Turbot	Recombinant peptides	↓ CFU/g *Edwardsiella tarda* ↓ Copies/g megalocytovirus	(Zhang *et al.*, 2014)
European sea bass hepcidin	European sea bass	Synthetic peptides	↑ protection against *Vibrio anguillarum*	(Álvarez *et al.*, 2016)
Grouper epinecidin-1	Medaka	Synthetic peptide	↑ protection against NNV	(Wang *et al.*, 2010a)
Grouper epinecidin-1	Grouper	Synthetic peptide	↑ protection against NNV when coadministered ↑ protection against NNV when administered before or after infection	(Wang *et al.*, 2010b)

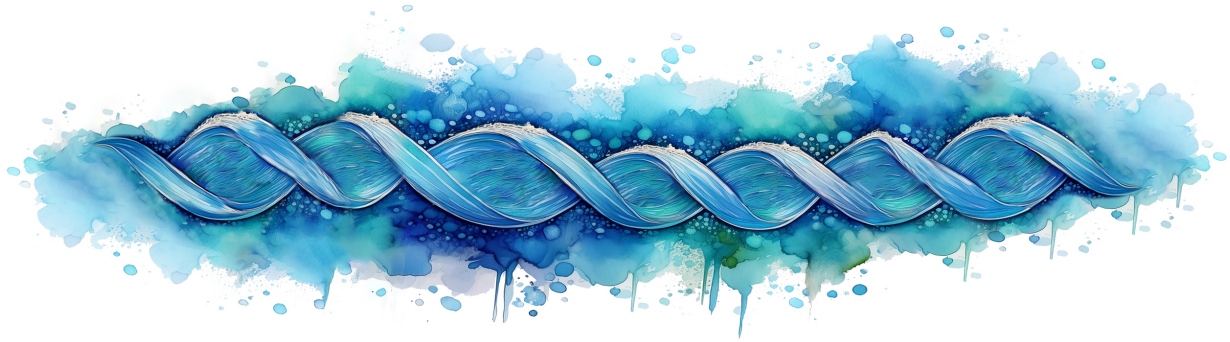
			↓ *mx* gene expression	
Tongue sole NK-lysin	Tongue sole	Plasmids	↓ CFU/g *Vibrio anguillarum* ↓ Copies/g megalocytovirus ↑ Proinflammatory cytokines ↑ Innate cell markers	(Zhang *et al*., 2013)
Tongue sole NK-lysin	Tongue sole	Synthetic peptides	↓ CFU/g *Vibrio anguillarum* ↓ Copies/g megalocytovirus ↑ Proinflammatory cytokines and chemokines	(Zhang *et al*., 2014)
European sea bass NK-lysin	European sea bass	Synthetic peptides	↑ protection against NNV ↑ macrophages, ILs and chemokines transcription	(Valero *et al*., 2021)
Grouper defensin-beta	Pearl gentian grouper	Vaccine adjuvant	↑ protection against NNV ↑ anti-RGNNV CP specific antibodies ↑ immune and inflammatory levels	(Zheng *et al*., 2023)

stimulation of interleukins or chemokines exerting direct activity against Gram-positive and Gram-negative bacteria (Zhang *et al*., 2014; Zhang *et al*., 2013). In European sea bass, synthetic Nkl peptide enhanced the NNV-survival by modulating the expression levels of leukocytes such as B and T cells (Valero *et al*., 2021). More recently, AMPs are starting to be considered as candidates to be adjuvants to vaccines because of their

property as activators of the adaptive response (Acosta *et al*., 2014; Huang *et al*., 2011; Liu *et al*., 2020).

The promising results about the anti-NNV roles of AMPs in teleost fish open the door to a key research field to develop effective and safe drug to control viral outbreaks in fish farms. The evaluation of different ways of administration as well as their potential preventive or palliative effects are mandatory to assess which AMPs can be used in aquaculture.

OBJECTIVES

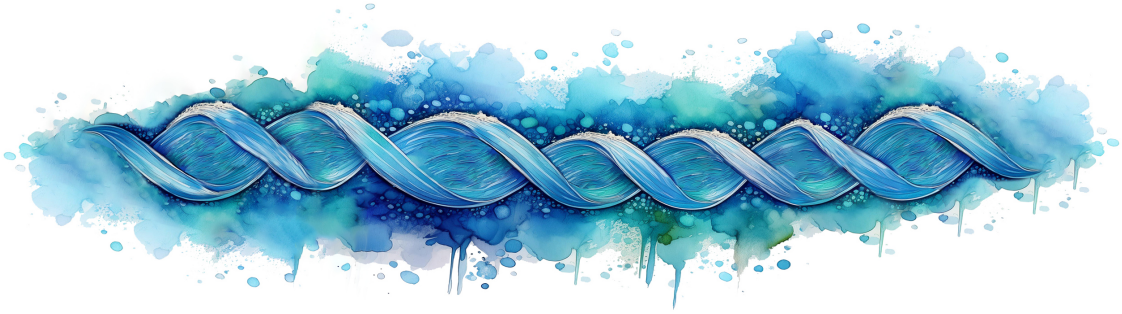


The general objective of this Doctoral Thesis is to evaluate the implication of AMPs in the immune response of teleost fish with special emphasis in their application as antiviral agents in European sea bass against NNV infection.

To achieve our general objective, we proposed the following specific objectives:

1. Evaluate the *in vitro* immunomodulatory roles of synthetic European sea bass-derived AMPs on head-kidney leukocytes (HKLs) from European sea bass and gilthead seabream.
2. Administrate AMP-encoding plasmids to European sea bass juveniles to determine their potential as immunostimulatory and/or NNV preventive agents.
3. Apply the synthetic AMPs hepcidin and dicentracin to European sea bass as preventive agents against NNV.
4. Evaluate the therapeutic application of synthetic NK-lysin, hepcidin and dicentracin peptides to European sea bass as anti-NNV treatment.

CHAPTER 1



marine drugs

MDPI

Article

Synthetic Antimicrobial Peptides Fail to Induce Leucocyte Innate Immune Functions but Elicit Opposing Transcriptomic Profiles in European Sea Bass and Gilthead Seabream

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Abstract: Antimicrobial peptides (AMPs) are promising molecules in diverse fields, including aquaculture. AMPs possess lytic effects on a wide range of pathogens, resulting in a potential replacement for traditional antimicrobials in aquaculture. In addition, they also have modulatory effects on host immune responses. Thus, the objective of this work was to evaluate the immunomodulatory capability of three known synthetic AMPs derived from European sea bass, NK-lysin (Nkl), hepcidin (Hamp), and dicentracin (Dic), in head-kidney cell suspensions from European sea bass and gilthead seabream. The tested peptides were neither cytotoxic for European sea bass nor gilthead seabream cells and failed to modulate the respiratory burst and phagocytosis activities. However, they modified the pattern of transcription of immune-related genes differently in both species. Peptides were able to promote the expression of marker genes for anti-inflammatory (*il10*), antiviral (*mx*, *irf3*), cell-mediated cytotoxicity (*nccrp1*, *gzmb*), and antibody responses (*ighm*) in European sea bass, with the Nkl peptide being the most effective. Contrary to this, the effects of those peptides on gilthead seabream mainly resulted in the suppression of immune responses. To conclude, European sea bass-derived peptides can be postulated as potential tools for immunostimulation in European sea bass fish farms, but more efforts are required for their universal use in other species.

Keywords: antimicrobial peptides (AMPs); NK-lysin; hepcidin; dicentracin; immunity; European sea bass; gilthead seabream

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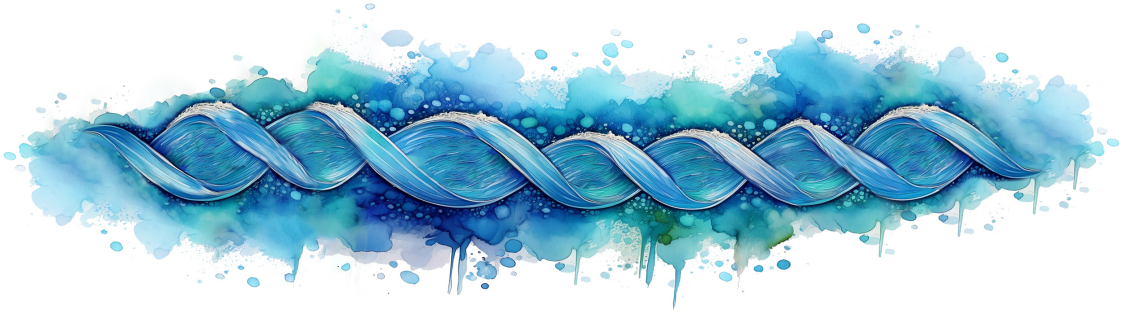
1. Introduction

The discovery of antimicrobial peptides (AMPs) constitutes a promising field of research thanks to their potential applications in medicine, pharmaceutics, cosmetics, or animal production. Their complexity and variety of modes of action make AMPs excellent candidates as antimicrobial drugs claimed to solve the increasing problems with antimicrobial resistance [1], as they show low toxicity, biocompatibility, and a lack of resistance generation [2]. AMPs are structurally a very diverse group, but with some common features such as their short aminoacidic sequences (<50 aa), low molecular weight, positive charges, cationic and amphipathic residues, and a predominant structure in the alpha-helix [3]. The structure of AMPs determines their specificity and biological activity [2]. Thus, based on their structure, AMPs can be grouped into different families, such as defensins, hepcidins (Hamp), saponins, in which NK-lysin (Nkl) is included, or piscidins, which are only present in marine organisms and include dicentracin (Dic), among others [4]. Regarding their functional properties, a dual mode of action has been described: (1) AMPs possess direct lytic effects against a wide range of pathogens such as viruses, fungi, bacteria, or parasites [5]; and (2) AMPs can modulate host immune responses by promoting cell recruitment, modulating inflammatory responses, and bridging innate and adaptive responses [3].

CHAPTER 2



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Full length article

Immunity elicited by AMP-encoding plasmids fails to increase the protection of European sea bass against nodavirus

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ABSTRACT

Antimicrobial peptides (AMPs) are a potent arm of the innate immune system that can directly kill pathogens and induce immunomodulation. In the marine aquaculture, European sea bass (*Dicentrarchus labrax* L.) is one of the most prosperous species but is highly susceptible to nodavirus (NNV), which produces high rates of mortality in larvae and juvenile stages. Thus, we aimed to evaluate whether AMPs exert immunomodulatory and/or NNV-preventive actions in sea bass. To do this, plasmids encoding the sea bass AMPs dicentracin (pDIC), beta-defensin (pDB1), hepcidin (pHAMP2) or NK-lysin (pNKL) were generated and intramuscularly injected into sea bass juveniles to evaluate their immunomodulatory and anti-NNV roles. Sea bass muscle transcribes the AMPs and produces an increase in their circulating levels, along with an increase of the antibacterial activity. Immune-related gene analysis revealed a great activation of the inflammatory response and the recruitment of neutrophilic granulocytes at the site of injection. However, AMP-encoding plasmids, namely pHAMP2, negatively affected to NNV disease by increasing fish mortality. In conclusion, plasmids encoding AMPs show immunostimulatory effects on European sea bass but do not improve the resistance to NNV.

1. Introduction

Antimicrobial peptides (AMPs) are among the most important arms of the innate immune system to combat infections. AMPs are widespread in the nature, representing an evolutionary ancient mechanism of host defense [1]. AMPs show a dual mode of action: they can directly kill a wide range of pathogens, including viruses, and might act as immunostimulatory molecules. Structurally, AMPs are small peptides, mostly cationic and amphipathic, which properties allow their interaction with anionic molecules from pathogens [2,3]. Thus, AMPs are able to interact and inhibit both surface and intracellular proteins, as well as DNA or RNA, leading to pathogen killing by membrane disruption or inhibition of viral assembly [2,4]. In addition, their immunomodulatory actions include leucocyte recruitment [5], inflammation [6] or adaptative responses [7]. Therefore, AMPs have been postulated as good preventive agents against fish pathogens due to their effectiveness in the modulation of the host response [8] and the lack to produce antimicrobial resistance (AMR), when compared to traditional antibiotics.

AMPs have been widely identified in teleost fish and grouped into different families including piscidins, β-defensins, hepcidins or cathelicidins, among others. Although the direct lytic activity of AMPs against bacteria has been broadly evaluated, however, very few studies have drawn the attention on their immunostimulatory and antiviral activities [9–11]. NK-lysin (Nkl), which is mainly produced by cytotoxic T (CTLs) and natural killer (NK) cells [10] playing a role in the cell-mediated cytotoxicity (CMC), shows important AMP functions [12] and is the most studied AMP in fish. Nkl synthetic peptides exhibit antiviral properties by inhibiting the fusion of viruses to the membrane of cells, as well as inhibiting the union of viral particles to the cell host [13]. The *in vivo* administration of Nkl synthetic peptides to European sea bass (*Dicentrarchus labrax*) confers partial prevention against the nervous necrosis virus (NNV) or nodavirus infection [9]. Meanwhile, *in*

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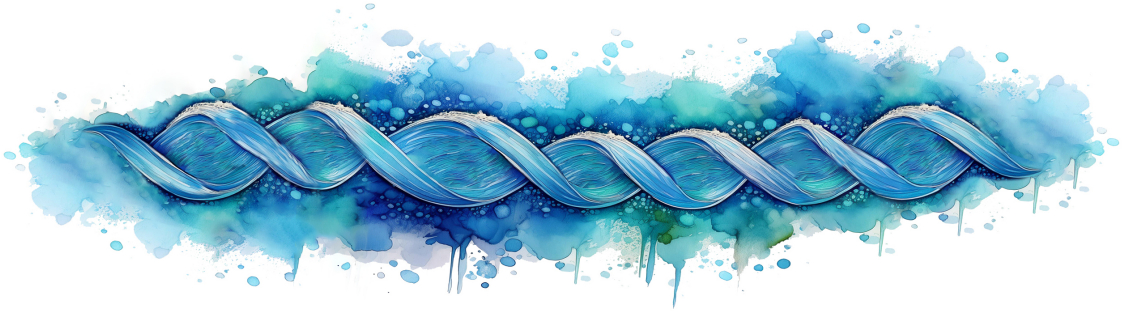
CHAPTER 3



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Hepcidin and dicentracin peptides show preventive antiviral applications against NNV infection in European sea bass through immunomodulatory roles

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European sea bass
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ABSTRACT

Aquaculture is an extremely prosperous market threatened by pathogen outbreaks, including viruses as nodavirus (NNV), which infect fish species with special interest in trading such as European sea bass. Antimicrobial peptides (AMPs) might constitute potential antiviral agents, which had been previously evaluated in fish with positive prospects, based on their properties as immunomodulators or directly killing pathogens. In this line, we aimed to evaluate this dual role by administering two European sea bass synthetic AMPs (Hamp or Dic) prior to NNV infection. Both treatments conferred partial protection against NNV though viral replication and load were not affected. Both AMPs elicited, prior to infection, AMP response and leukocyte mobilization whilst down-regulated pro-inflammatory markers. Upon infection, Hamp and Dic peptides abrogated the inflammatory response provoked by NNV as well as avoid NNV-induced disturbance of the leucocyte distribution in the brain, mainly neutrophils, macrophages and $CD8^+$ T cells. This study points that preventive applications of synthetic Hamp and Dic peptides exert their antiviral actions through the immunomodulatory role and not by a direct action of the antimicrobial on NNV. This work opens the door to the use of AMPs as potential prophylactic tools against NNV as well as immunostimulant in fish farms.

1. Introduction

Human consumption of aquatic organisms is continuously growing due to their excellent nutritional profiles, including essential fatty acids and minerals. Traditional fisheries are not able to satisfy the food demand of the population; hence, aquaculture has arisen as one of the most prosperous economic sectors with promising prospects worldwide (FAO, 2020). Nevertheless, aquaculture is facing difficulties due to pathogen outbreaks, which are favored by the high density and the chronic stress culture conditions (Kibenge, 2019). Among pathogens, viruses are severe threatens for fish hatcheries and pre-ongrowing facilities. In the case of fish culture, at industrial levels, the available antiviral treatments are extremely limited with very few effective commercial vaccines and no antiviral agents. By contrast, there are some effective commercial vaccines and antibiotics to control bacterial infections. Therefore, viruses are one of the main biological problems in the modern aquaculture and practical solutions to combat them are of great priority.

Nervous necrosis virus (NNV; family *Nodaviridae*, genus *Betanodavirus*), or Betanodavirus, is the causative agent of the viral encephalopathy and retinopathy due to its neurotropic tropism, being brain and retina the main target tissues for NNV replication. NNV affects >177 fish species, some of them with special interest for the aquaculture industry such as European sea bass (*Dicentrarchus labrax*), Asian sea bass (*Lates calcacifer*) or sole (*Solea senegalensis*), among others (Bandín and Souto, 2020; Munday et al., 2002). Structurally, NNV are non-enveloped icosahedral RNA virus composed by two molecules of single-stranded and positive sense RNA: RNA1 and RNA2, which codify for the RNA-dependent RNA polymerase and the capsid protein, respectively (Low et al., 2017). In addition, there is a subgenomic RNA3, which encodes proteins B1 and B2, with anti-necrotic death and RNA silencing-suppression functions, respectively (Chen et al., 2009; Su et al., 2009). European sea bass is a very susceptible species to NNV infection, with

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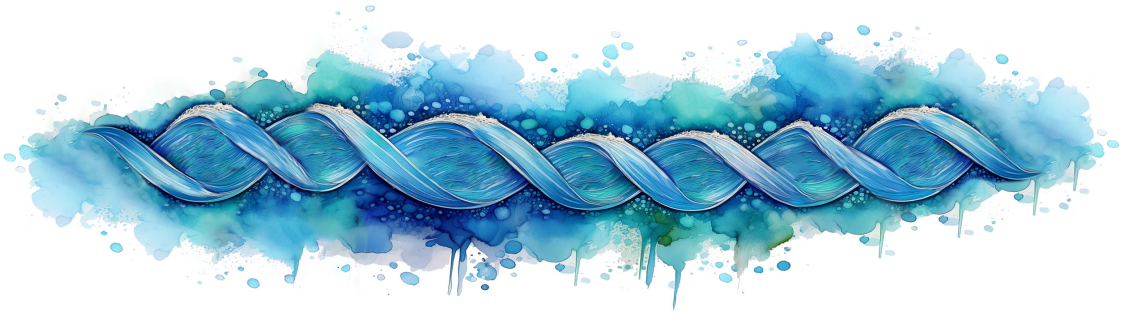
CHAPTER 4



Synthetic antimicrobial Nkl, Hamp and Dic peptides are immunomodulatory but only Hamp and Dic peptide can be therapeutic against betanodavirus infection

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Abstract

Aquaculture is a prosperous economic sector threatened by viral infections. Among the viruses threatening fish culture, nervous necrosis virus (NNV) is extremely important in the Mediterranean Sea affecting to highly traded species as European sea bass in economic aspects. In this context, application of antimicrobial peptides (AMPs) has arisen as a potential biotechnological tool. The aim of this work was to evaluate the therapeutic application of two European sea bass-derived AMPs, NK-lysin (Nkl), hepcidin (Hamp) and dicentracin (Dic), against NNV infections. Synthetic Hamp and Dic peptides were able to significantly reduce NNV-mortalities while Nkl failed to do so. However, neither Dic, Hamp nor Nkl peptides were able to alter the transcriptional levels of NNV and the number of infected cells. Interestingly, all tested peptides showed immunomodulatory roles. For instance, our data revealed an interplay among different AMPs, at both gene and protein levels. Otherwise, Nkl, Hamp and Dic peptides provoked an anti-inflammatory balance upon NNV infection, as well as the recruitment of macrophages and B cells to the target site of the infection, the brain, which might prevent neuronal damage. Besides, these AMPs seem to orchestrate the adaptive response by increasing the total levels of immunoglobulin M (IgM) in brain, in case of Hamp and Dic. In conclusion, Hamp and Dic can be proposed as a therapeutic candidate to combat NNV and as immunomodulatory agents to be used in aquaculture.

Keywords

Dicentrarchus labrax; Betanodavirus (NNV); Antimicrobial peptides (AMPs); Hepcidin; Dicentracin; NK-lysin; Immunostimulation

Introduction

Aquaculture is considered the world fastest growing sector constituting a sustainable way of producing food and reducing the pressure exerted to marine resources has made this sector one of the most prosperous worldwide (FAO, 2022). However, the culture conditions promote the appearance of viral outbreaks (Kibenge, 2019), including nervous necrosis virus (NNV), belonging to Betanodavirus genus. NNV is the causing agent of the viral encephalopathy and retinopathy disease (VER), which affects to more than 180 species including those of commercial interest such as European sea bass (*Dicentrarchus labrax*), orange spotted grouper (*Epinephelus coioides*) or Atlantic halibut (*Hipoglossus hipoglossus*) among others, reaching the 100% of mortalities in larvae and juvenile stages (Bandín and Souto, 2020). NNV is a small non-enveloped virus composed by two single-stranded positive-sense RNA molecules: RNA1 and RNA2. RNA1 encodes for the RNA-dependent RNA polymerase (RdRp) or protein A whilst RNA2 codifies for capsid protein (CP) (Mori *et al*., 1992; Nagai and Nishizawa, 1999). NNV also possess a subgenomic RNA molecule called RNA3 which codes for two non-structural proteins: B1 and B2, which are involved in the viral pathogenesis (Low *et al*., 2017). Molecular mechanisms involved in its pathogenesis and effective diagnosis methods have been widely studied (Bandín and Souto, 2020). However, few treatments against VER disease have been developed and most of them are related to preventive measures against this infection by the development of vaccines (Buonocore *et al*., 2019). Up to the date, very scarce therapeutic antiviral treatments have been explored for aquaculture, but their profit constitutes a novel and promising research area (Baek *et al*., 2023; Huang *et al*., 2016; Li *et al*., 2023; Zhu *et al*., 2022).

Molecules such as antimicrobial peptides (AMPs) or antivirals have been proposed as possible treatments for NNV infection (Chia *et al*., 2010; Costa and Thompson, 2016; Valero *et al*., 2021; Zhang *et al*., 2014). In this study, we focused on three fish well-characterized AMPs: NK-lysin (Nkl), hepcidin (Hamp) and dicentracin (Dic). The antiviral role of these peptides has been assessed by our lab in *in vitro* assays (León *et al*., 2020a). In addition, AMP transcription and protein levels are increased upon NNV infection in European sea bass (Valero *et al*., 2020a) pointing to their involvement in combating this disease. Otherwise, the selected AMPs are potent immunomodulatory molecules in teleosts, regulating immune pathways such as inflammation or cell

recruitment (Katzenback, 2015). Among the immunomodulatory roles of AMPs their anti-inflammatory and proinflammatory properties are controversial and under debate. Concretely, Hamp administered to zebrafish (*Dario rerio*) up-regulates the anti-inflammatory interleukin (*il)10* while in European sea bass the pro-inflammatory cytokines *il6*, *il1b* and tumor necrosis factor alpha (*tnfa*) resulted up-regulated upon *in vivo* and *in vitro* administration (Álvarez *et al*. 2022; Cervera *et al*. 2023; Neves *et al*. 2015). Otherwise, Nkl can induce the inflammatory response mediating the up-regulation of proinflammatory *il1b*, *myd88* or *il8* in Atlantic salmon (*Salmo salar*), tongue sole (*Sole solea*) and European sea bass, *in vitro* and/or *in vivo* (Acosta *et al*., 2019; Cervera *et al*., 2024b; Valero *et al*., 2021; Zhang *et al*., 2013). The scarce knowledge of Dic is limited to a single species, European sea bass, where Dic is known to stimulate the inflammatory response via *il1b* up-regulation when administered as plasmid but down-regulated proinflammatory interleukins *il1b* and *il6* after peptide injection (Cervera *et al*., 2024a, 2024b, 2023). Additionally, Nkl and Hamp regulates the expression of several chemokines or interleukins related to cell infiltration (Hsieh *et al*., 2010; Pan *et al*., 2011b; Valero *et al*., 2021). In fact, several studies have showed that Nkl, Hamp and Dic can up-regulate T, B cells and/or macrophages cell markers pointing to these AMPs to serve as a bridge to the innate and adaptive responses (Cervera *et al*., 2024a, 2024b, 2023; Ting *et al*., 2019; Valero *et al*., 2021).

These functional features make AMPs potential candidates to combat NNV infections in aquaculture. Preventive approaches have been made in this. In brief, the administration of Hamp synthetic peptide at the time of infection or before the infection had a positive impact on the survival of medaka (*Oryzias latipes*) or orange-spotted grouper (Wang *et al*., 2010b, 2010a) as well as Nkl, Hamp and Dic administration to European sea bass prior the infection also resulted in increased survival rates (Cervera *et al*., 2024a; Valero *et al*., 2021). Nevertheless, the administration of Nkl, Hamp or Dic-expressing plasmids did increase the mortalities in European sea bass (Cervera *et al*., 2023).

Considering the promising results obtained for these peptides in NNV-preventive assays performed in our research group and considering their immunomodulatory roles, this work aimed to evaluate the potential use of the synthetic Nkl, Hamp or Dic peptides as a therapeutic tool to combat NNV in fish farms. Thus, synthetic peptides were

administered after NNV infection to European sea bass to evaluate the disease development and immunity. Data will be discussed to shed light in the anti-NNV applications as well as its ability to modulate host immune responses.

Material and methods

Animals

Apparently healthy juveniles of European sea bass (*Dicentrarchus labrax* L.; 5.94 ± 0.27 g body weight) were bred at COMU-IEO, CSIC facilities. Animals were kept in 200 L tanks with an independent recirculation system of natural seawater (38‰ salinity), suitable aeration and filtration systems, temperature of 25 ± 1ºC and 12h light:12h dark photoperiod. Fish were fed *ad libitum* with a commercial pellet diet (Skretting). Specimens were allowed to acclimatize during 1 week before starting the experiment. Handling of the specimens was always performed under the Guidelines of the European Union Council (2010/63/UE), the Bioethical Committees of the IEO (REGA code ES300261040017) and the University of Murcia and the approval of the Ministry of Water, Agriculture and Environment of the Autonomous Community Region of Murcia (Permit Numbers A13210701 and A13202602).

Peptides and NNV production

European sea bass derived Nkl (acc. nº A0A218MG56; NH_2-KLLAVCDQIGLLKSLCRKFVKKH-COOH), hepcidin (Hamp 2, variant 1; acc. nº KJ890397.1; NH_2-HSSPGGCRFCCNCCPNMSGCGVCCTF-COOH) and Dic (acc. nº P59906; NH_2-DAFFHHIFRGIVHVGKSIHKLVTGGKAQQD-COOH) peptides were chemically synthesized by GeneScript (Purity ≥90%). The bioactivity of these peptides had been demonstrated by analyzing direct antibacterial and/or antiviral activities of the synthetic peptides (León *et al*., 2020a; Neves *et al*., 2015). These AMPs were purchased as lyophilizates, so AMPs were first resuspended in ultrapure water at 1 mg/mL and then, stored at -20ºC. NNV (strain It/411/96; genotype RGNNV) was propagated in the E-11 cell line as described elsewhere (Iwamoto *et al*., 2001). NNV stocks were titrated (Reed and Müench, 1938) and the viral dilution infecting 50% of the cell cultures ($TCID_{50}$) calculated.

Experimental design

To evaluate the potential therapeutic application of Nkl, Hamp and Dic peptides against NNV infection, fish were randomly divided into five experimental groups (55 fish/group): mock, infected with NNV (NNV), or infected with NNV and treated with Nkl (NNV+Nkl), with Hamp (NNV+Hamp) or with Dic (NNV+Dic). For NNV infection, fish from NNV-infected groups were intramuscularly (im) injected with 50 µL of NNV ($TCID_{50}$/mL = 2.8×10^6), while the fish from the mock group were injected with 50 µL of PBS. One-day post-infection (dpi), fish conforming the NNV+Nkl, NNV+Hamp and NNV+Dic groups were im injected with 50 µL ~1 µg Nkl, Hamp or Dic per g of fish, respectively, whilst those from the mock and NNV groups were injected with 50 µL of PBS. The injection site was consistently situated between the dorsal fin and the lateral line on the right side of the dorsal muscle throughout all procedures. Each fish was individually captured, anesthetized with 40 µL/L of clove oil in a 10 L tank of marine water, and subsequently injected using an insulin syringe approximately in the same area and with uniform handling duration for all specimens.

Mortality and clinical signs of infection were daily recorded. The clinical signs were ranked from 1 to 4 according to their severity (1: changes in the color of skin, slower rhythm of swimming and reaction to external stimuli; 2: alteration in the swimming balance and/or erratic swimming spams; 3: continuous erratic swimming; 4: complete incapacity to keep balance or swim). The experiment finished when three consecutive days without deaths occurred.

Sampling

Fish were sampled (n=6/group) after 3 dpi (2 days from Nkl, Hamp or Dic treatment; dpt). Briefly, specimens were anesthetized with 40 µL/L of clove oil, completely exsanguinated and rapidly decapitated. Blood samples were collected from the caudal vein with an insulin syringe and centrifuged at 10,000xg for 10 min at 4ºC to obtained serum samples that were stored at -80ºC. Fragments from brain, head-kidney (HK) and muscle were immediately frozen and stored at -80ºC until used for ELISA analysis or frozen and stored at -80ºC in DNA shield for gene expression analysis. For histological analysis, brain samples were collected (n=3/group) and processed for light microscopy analysis as previously described (Chaves-Pozo *et al*., 2021).

NNV detection by immunohistochemistry (IHC)

Brain samples were fixed in Bouin's solution for 16 h at 4ºC. Then, they were dehydrated using increasing solutions of ethanol in water (70%, 96%, and twice 100%) baths for 60 min each, cleared with two baths in xylene substitute (Sigma-Aldrich; 30 min in each bath), and embedded in paraffin (Paraplast Plus; Sherwood Medical) for 16 h. Brain sections of 5 µm thickness were subjected to an indirect IHC method using a commercial anti-RGNNV antiserum (Abcam: 26812), which specifically binds to the capsid protein of NNV, using a previously described protocol with slight modifications (Chaves-Pozo *et al*., 2021). The slides were examined with an Eclipse E600 light microscope (Nikon). The images were obtained with an Olympus SC30 digital camera (Olympus soft imaging solutions). For each fish (n=3 fish/group), three different sections and four optical areas/section were used to determine the area of anti-NNV immunostaining. Images at 20x magnification were processed with ImageJ software.

ELISA analysis

The total proteins present in serum, and in brain and HK homogenates (1g of tissue/mL of PBS) were measured using Bradford reagent (Bradford, 1976). Then, an indirect ELISA technique was used for detecting the NNV capsid protein in brain, the total IgM in serum and brain homogenate and the European sea bass AMPs, Nkl, Hamp and Dic, in serum, brain and HK homogenates as described (Valero *et al*., 2020a).The primary antibodies were used at their optimal dilutions: the anti-RGNNV for NNV detection at 1:5,000, polyclonal anti-Nkl, anti-Hamp and anti-Dic antisera for AMPs detection at 1:200 or the anti-European sea bass IgM (Aquatic Diagnosis) at 1:100 (Valero *et al*., 2020a). Positive controls were always included in the reactions. Negative controls lacking serum or primary antisera were also used. Data were expressed as the OD per µg of protein.

Antibacterial activity

The antibacterial activity of serum, and tissues homogenates (HK or brain) was determined by evaluating their effects on the bacterial growth of *Vibrio harveyi* (Vh) (strain Lg 16/ 100) curves (Cervera *et al*., 2022; Sunyer and Tort, 1995). Aliquots of 10 µL of sample from serum or the same volume from a dilution of tissues homogenates (1 mg/mL of protein) were used. A negative control (0% bactericidal activity, 0% bacterial

growth) was prepared replacing the sample and the bacteria solution by Tryptic Soy Broth (TSB), while a positive control (0% bactericidal activity, 100% bacterial growth) was prepared replacing the sample by TSB but maintaining the bacterial solution.

Gene expression analysis

Total RNA was isolated using TRIzol Reagent (Invitrogen) following the manufacturer's instructions and treated with amplification grade DNase I (1 unit/µg RNA, Invitrogen). SuperScript IV Reverse Transcriptase (Invitrogen) was used to synthesize the first strand cDNA with random hexamers from 1 µg of total RNA as previously described. qPCR was performed in individual cDNA samples with a Quant Studio 5 instrument (Applied Biosystems) using SYBR Green PCRCore Reagents (Applied Biosystems) as described (Cervera *et al*., 2023). For each mRNA, gene expression was corrected by the geometric mean of three housekeeping genes (elongation factor 1 alpha (*ef1a*), ribosomal protein 18 (*rps18*) and ribosomal protein L13 alpha (*l13a*)) and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the housekeeping genes Ct values from the target Ct in each sample (Pfaffl, 2001). Genes analyzed were grouped in the following categories: AMPs, inflammatory-related cytokines, cell recruitment, leukocyte-type markers, antiviral response, and NNV markers. Gene names and primer sequences used are shown in Table 1. Negative controls with no template were always included in the reactions.

Statistical analysis

Survival was represented by the Kaplan-Meier method and statistical differences were studied using a Log-ranked (Mantel-Cox) test. All data are presented as mean ± standard error of the mean (SEM). The normality of the distributions and the homoscedasticity of the variances were tested through the Shapiro–Wilk and Levene tests, respectively. ANOVA or Student-t tests were used accordingly. When data did not meet parametric assumptions, a U-Mann–Whitney or a Kruskal-Wallis test was used. SPSS 24 and GraphPad Prism 8 software were used, and the minimum level of significance fixed at 0.05 ($p \leq 0.05$).

Table 1. Primers used in this study.

	Protein name	Gene name	Acc. number	Sequence (5'→3')
Housekeeping	Elongation factor 1 alpha	*ef1a*	AJ866727	F: CGTTGGCTTCAACATCAAGA R: GAAGTTGTCTGCTCCCTTGG
	Ribosomal protein S18	*rps18*	AY831388	F: TTCCTTTGATCGCTCTTAACG R:TCTGATAAATGCACGCATCC
	Ribosomal protein L13 alpha	*l13a*	DT044539	F: GCGAAGGCATCAACATCTCC R:AGACGCACAATCTTGAGAGCAG
Antiviral response	Interferon-induced GTP-binding protein Mx	*mx*	AM228977	F: GTATGAGGAGAAGGTGCGTCC R: CTCTTCCCCGAGCTTTGGTC
Leukocyte-type markers	Myeloperoxidase	*mpo*	DLAgn_0011834	F: GAAGAGTGGGGCCTTTGTTT R: CTGGGCCTCAGTGAAGACTC
	Macrophage colony stimulator factor 1 receptor	*mcsf1r*	KM225787	F: TTTCGGAAAGGTTGTTGAGG R: TCTCATCTGAATGGGCACTG
	Immunoglobulin M heavy chain	*ighm*	FN908858	F: AGGACAGGACTGCTGCTGTT R: CACCTGCTGTCTGCTGTTGT
	Non-specific cytotoxic cell receptor protein 1	*nccrp1*	FM022070	F: TGGGGTGAGATACGTCCACT R: TGGTTTTGGTTGCTCTGACA
Inflammation-related cytokines	Interleukin-6	*il6*	AM490062	F: ACTTCCAAAACATGCCCTGA R: CCGCTGGTCAGTCTAAGGAG
	Interleukin-8	*il8*	AM490063	F: GTCTGAGAAGCCTGGGAGTG R: GCAATGGGAGTTAGCAGGAA
	Interleukin-10	*il10*	DQ821114.1	F: ACTCCTCGGTCTCTTCTCCT R: TCCACAAAACGACAGCACTG
Cell recruitment-related molecules	Interleukin-8	*il8*	AM490063	F: GTCTGAGAAGCCTGGGAGTG R: GCAATGGGAGTTAGCAGGAA
	C-X-Cmotif chemokine receptor 3	*cxcr3*	ENSDLAT000050	F: ATCCTGTACGCCTTTGTGGG R: GTCGGCAGACTCAGACCAAA
	CXC chemokine 9	*cxcl9*	DLAgn_0001298	F: TCTGTCAGCTCGCCTTTCTG R: TTCGTACTTGGACACGCACA
Antimicrobial peptides	NK-lysin	*nkl*	KY801205	F: GAAGAAACACCTCGGGGAAT R: GCAGGTCCAACATCTCCTTC
	Hepcidin 1	*hamp1*	KJ890396	F: AAGGCATTCAGCATTGCAGTTG R: CCGCAACTGGAGTGTCATTG
	Hepcidin2	*hamp2*	DQ131605	F:CCAGTCACTGAGGTGCAAGA R:GCTGTGACGCTTGTGTCTGT
	Dicentracin	*dic*	AY303949	F: GGCAAGTCCATCCACAAACT R:ATATTGCTCCGCTTGCTGAT
	Lysozyme	*lyz*	KJ433681.1	F: ATTTCCTGGCTGGAACACAG R:GAGCTCTGGCAACAACATCA
	Defensin beta 1	*defb1*	DLAgn_00041270	F: CCTTTCCTTGGTCTTGCCCA R: ACACACAGCACAAGAAGCCT
NNV markers	NNV coat protein	*cp*	D38636	F: CAACTGACAACGATCACACCTTC R: CAATCGAACACTCCAGCGACA
	Protein A	*rdrp*	AF319555	F: GTGTCCGGAGAGGTTAAGGATG R: CTTGAATTGATCAACGGTGAACA

Results

Hamp and Dic peptides increase European sea bass survival against NNV

The administration of Hamp and Dic peptides to NNV-infected European sea bass significantly increased the survival when compared to those only infected, leading in both cases to a relative percentage of survival (RPS) of 30%. (Fig. 1A). However, Nkl administration failed to do so. In addition, Dic treatment prevented the most severe signs of infection while Hamp prevented all clinical signs of infection (Fig. 1B).

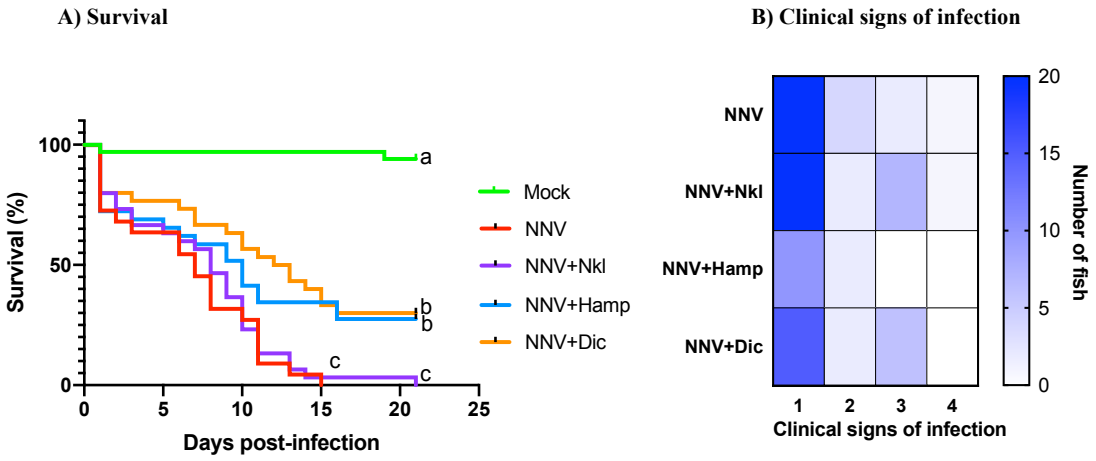
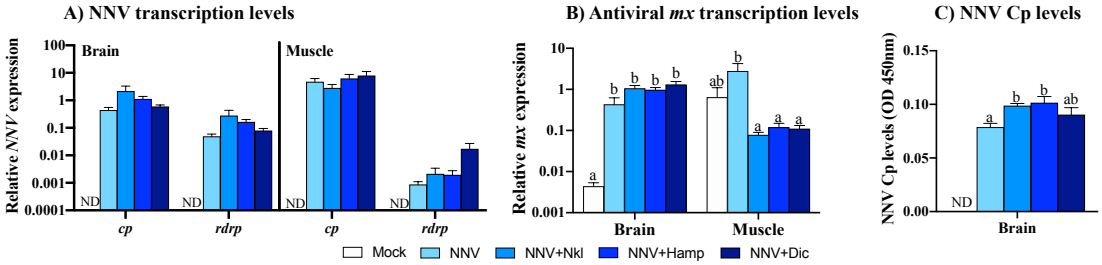
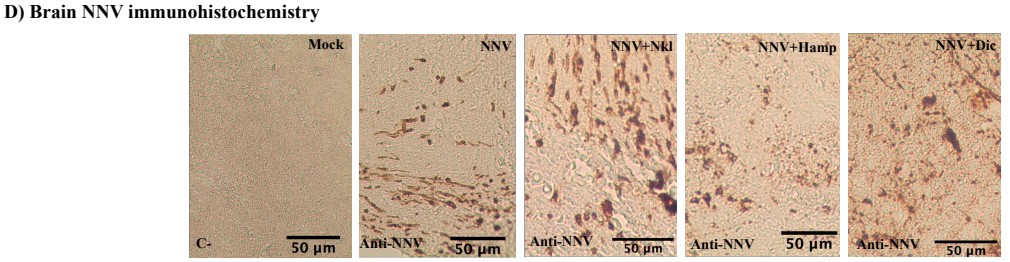


Fig. 1. Therapeutic administration of synthetic hepcidin (Hamp) and dicentracin (Dic) peptide partly protects European sea bass juveniles against nodavirus (NNV). European sea bass juveniles were intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish=5.6×10^{6}) and 1 day later injected with PBS (NNV group), Nkl (NNV+Nkl), Hamp (NNV+Hamp) or Dic (NNV+Dic) (~1 µg peptide per g of fish). A mock group was injected twice with only PBS. (a) Kaplan-Meier survival curves showing the proportion of European sea bass survivors upon NNV infection. Different letters indicate differences between groups according to a long-rank test ($p\leq0.05$). (b) Diagram representing the cumulated number of fish showing clinical signs of NNV disease attending to their severity: 1) changes of the color of the skin, slower rhythm of swimming and/or slower reaction to external stimuli as feeding; 2) alterations in the swimming balance and/or erratic swimming spasms; 3) continuous erratic swimming; and 4) complete incapacity to keep balance, swim and/or move without external stimuli. PBS, phosphate buffer; NNV, nodavirus; Nkl, NK-lysin; Hamp, hepcidin; Dic, dicentracin.

Nevertheless, the presence of the virus in fish tissues was not affected by the AMP treatments (Fig. 2). Thus, though the transcription levels of NNV were similar amongst NNV-infected groups (Fig. 2A) the NNV Cp protein levels in the brain were slightly increased by Nkl and Hamp treatments, but not by Dic (Fig. 2C). Despite that, the percentage area immunostained with anti-NNV antibody in the brain was similar in all infected groups (NNV, NNV+Nkl, NNV+Hamp and NNV+Dic; Fig. 2D). Concerning the antiviral response, the transcriptional level of the antiviral gene *mx*, in brain, was significantly up-regulated in the NNV, NNV+Nkl, NNV+Hamp and NNV+Dic groups in comparison to the mock fish (Fig. 2B) with similar levels in the NNV+Nkl, NNV+Hamp and NNV+Dic fish groups (Fig. 2B). However, this transcription of *mx*, in muscle, was significantly reduced by AMP treatments when compared to the NNV group in the muscle (Fig. 2B).





Immunostained area (%)	0%	7.67% ± 0.94 %	7.59 ± 0.75 %	6.98 ± 0.72 %	7.2 ± 0.75 %

Fig. 2. NNV transcription and load were not altered by Nkl, Hamp or Dic treatments, while NNV-Cp levels are increased by Nkl and Hamp treatments. European sea bass juveniles were intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish=5.6×10^6) and 1 day later treated with Nkl, Hamp or Dic (~1 µg peptide per g of fish; NNV+Nkl and NNV+Dic groups) or vehicle (PBS; NNV group). A mock group was injected twice with only PBS. Muscle and brain samples (n = 6) were obtained 2 days after AMP treatment. (A) Transcription levels of NNV *rdrp* and *cp* genes. (B) Transcription levels of the antiviral marker, *mx*. (C) Brain levels of NNV capsid protein (Cp) determined by ELISA. Data from ELISA represent the mean optical density (OD) at 450 nm ± SEM (n = 6). Data from transcriptional levels represent the mean relative gene expression ± SEM (n=6) obtained by real-time PCR. (D) Sections of brain immunostained with anti-RGNNV serum from mock, NNV, NNV+NKl, NNV+Hamp or NNV+Dic (g) infected fish. Brown cell corresponded to NNV-infected cells. The total immunostained area was expressed as percentage (%) of the total area from 12 optical area randomly distributed in the brain of 3 specimens. Different letters indicate significant statistical differences among the experimental groups according to ANOVA followed by Tukey post-hoc ($p\leq0.05$). ND, no detection.

Synthetic Nkl and Dic peptides modulate the NNV-induced humoral immunity

The humoral innate immune response was evaluated in NNV-infected European sea bass (Fig. 3). Regarding the total IgM level, they were increased in serum from NNV+Nkl, NNV+Hamp and NNV+Dic groups in comparison to mock and NNV groups, whereas, in the brain, only Hamp and Dic peptide were able to increase these levels (Fig. 3A). The antibacterial activity was greatly up-regulated in the NNV group in all tested tissues, but in the groups treated with all the three peptides, this activity recorded similar

levels as in the control group (Fig. 3B). On the other hand, the levels of the AMPs analyzed were not significantly affected by the NNV infection (Fig. 3C-E). Interestingly, Nkl, Hamp and Dic levels were significantly increased in the brain from the treated groups, except to Hamp in the Hamp-treated group, respect to those only infected or mock-infected (Fig. 3C-E). However, Nkl or Dic treatments increased the Nkl levels respect to the NNV alone in the HK or in serum and HK tissues, respectively (Fig. 3C). Dic levels were increased in serum but decreased in HK in the NNV+Dic group (Fig. 3E).

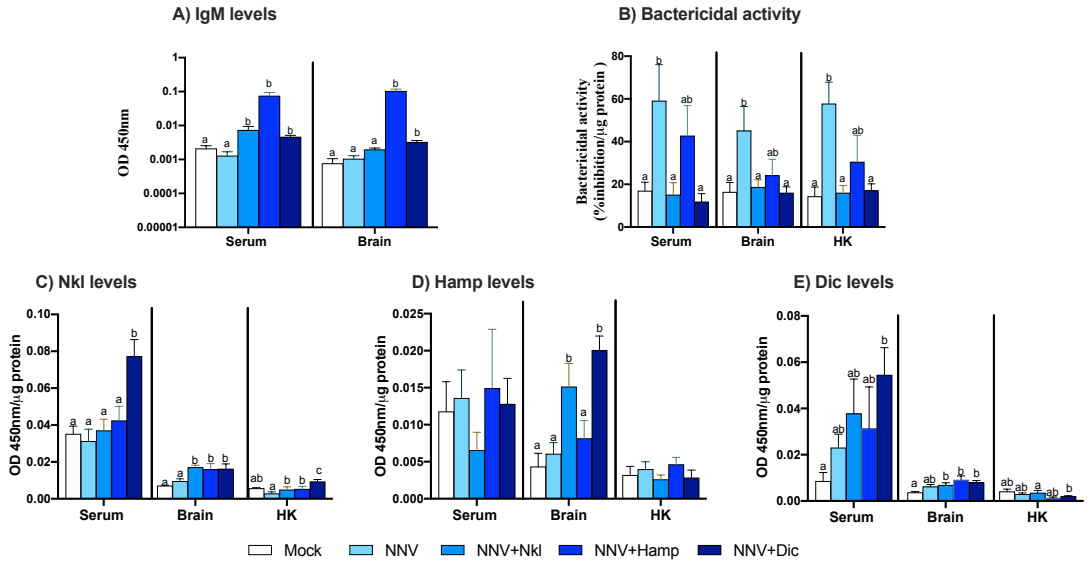


Fig. 3. Nkl and Dic peptides modulate the immune parameters triggered by NNV challenge. European sea bass juveniles were intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish=5.6×10^6) and 1 day later treated with Nkl, Hamp or Dic (~1 µg peptide per g of fish; NNV+Nkl or NNV+Dic groups) or vehicle (PBS; NNV group). A mock group was injected twice with only PBS. Serum, brain, and head-kidney (HK) samples (n = 6) were obtained 2 days after peptide treatment. (A) Total IgM levels in serum and brain. (B) Antibacterial activity against *Vibrio haveryi* in all tissues analyzed. (C-E). Protein levels of Nkl (C), Hamp (D) and Dic (E) in all tissues analyzed. In all cases data represent the mean ± SEM (n = 6) whilst different letters indicate significant statistical differences among groups according to ANOVA followed by Tukey post-hoc ($p \leq 0.05$).

AMPs regulated the transcriptional profile of immune-related genes against NNV infection

Firstly, we evaluated the gene expression induced by NNV infection (Fig. 4). In the brain, we found that NNV infection up-regulated the *nkl*, *hamp1*, *hamp2*, *defb1*, *il6*, *il10*, *cxcl9*, *mcsf1r* and *ighm* transcripts when compared to the mock group. In the HK,

however, only *dic*, *nccrp1* and *mpo* were up-regulated while *il8* and *cxcr3* were down-regulated. No significant differences were observed in the muscle of NNV- and mock-infected European sea bass specimens.

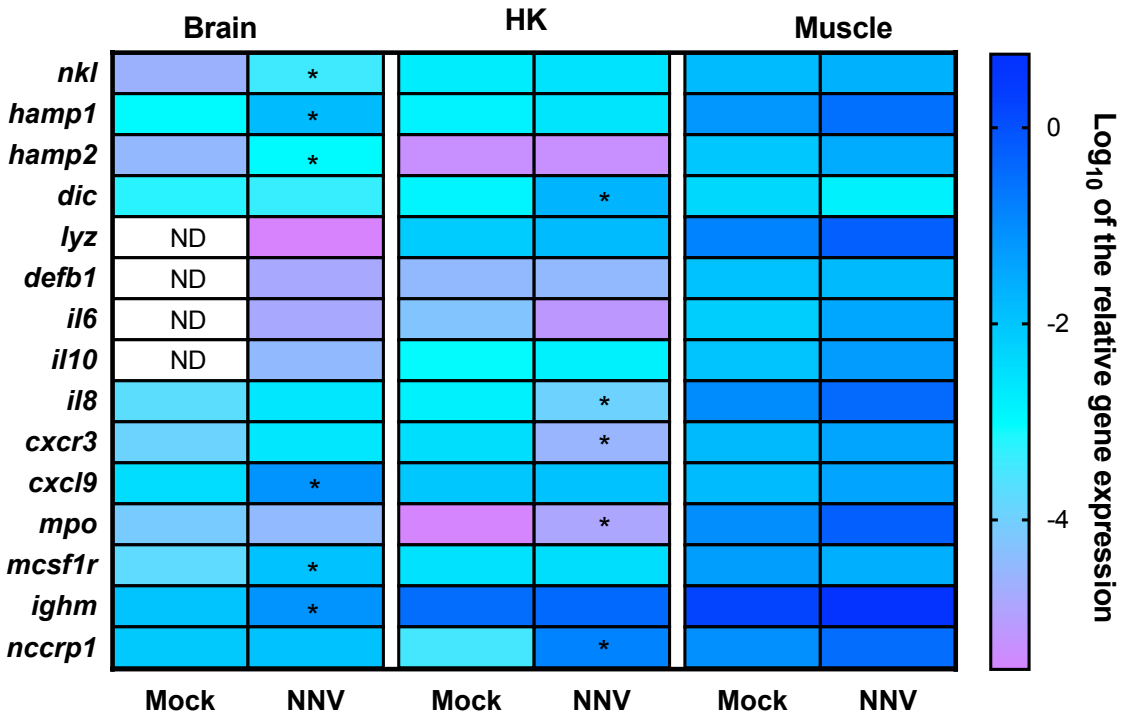


Fig. 4. The pattern of expression of several immune-related genes are modulated upon NNV challenge in European sea bass. European sea bass juveniles were intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish=5.6×10^{6}) and 1 day later treated with vehicle (PBS; NNV group). A mock group was injected twice with only PBS. Brain, head-kidney (HK) and muscle samples (n = 6) were obtained 2 days after Nkl or Dic treatment. Heatmap showing the relative gene expression levels of immune-related genes in the mock and NNV groups. Data represent the mean ± SEM (n = 6). Asterisks indicate significant statistical differences against mock and NNV group ($p \leq 0.05$). Gene abbreviations are described in the Table 1.

Then, we evaluated the differences between NNV-infected fish due to the post-treatment with Nkl, Hamp or Dic synthetic peptides (Fig. 5). In the brain, Nkl peptide up-regulated the expression of *nkl* gene, the anti-inflammatory interleukin *il10* and the B-cell and NCC markers, *ighm* and *nccrp1*, respectively with respect to the NNV-infected group alone (Fig. 5). Hamp treatment up-regulated *nkl*, *il10*, *il8*, *cxcr3*, *cxcl9* and *ighm* whilst *lyz* was down-regulated. By its side, Dic peptide up-regulated the expression levels of the AMPs *nkl*, *hamp2*, *lyz*, the interleukins *il6, il10* and *il8*, the chemokine *cxcr3* and the cell markers for macrophages and B-cells *mcsf1r* and *ighm*, respectively. In the HK, Nkl treatment increased the transcriptional levels of *nkl*, *hamp1*, *hamp2*, *lyz*, *defb1*, *il8*, *cxcr3*

and *nccrp1* while *dic* and *il6* were down-regulated (Fig. 5). Hamp peptide up-regulated *il10*, *cxcr3* and *mpo* whilst down-regulated *dic* and *nccrp1* (Fig. 5). By contrast, Dic treatment increased the *cxcr3* and decreased the *dic*, *lyz* and *cxcl9* expression levels (Fig. 5). In the muscle, both Nkl and Dic down-regulated the transcriptional levels of *nkl*, *hamp1*, *lyz*, *il6*, *il10*, *il8*, *cxcr3*, *mpo*, *ighm* and *nccrp1* (Fig. 5). Besides, Nkl also down-regulated *cxcl9* expression while Dic up-regulated *defb1* (Fig. 5). Otherwise, Hamp promoted a generalized down-regulation of immune markers (*nkl*, *lyz*, *il6*, *cxcr3*, *mpo*, *ighm* and *nccrp1* genes) whilst only *il10* resulted up-regulated (Fig. 5).

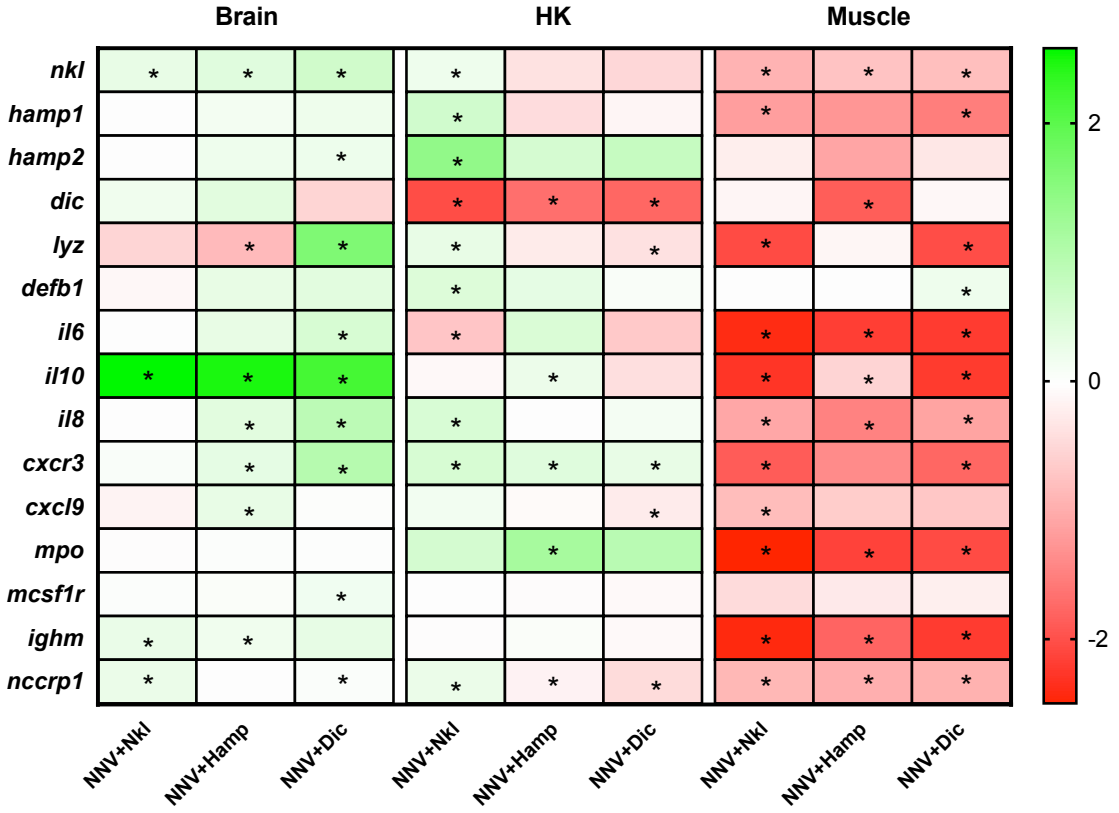


Fig. 5. Nkl and Dic treatment after NNV challenge in European sea bass modulate several immune-related genes in brain. European sea bass juveniles were intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish=5.6×10^6) and 1 day later treated with Nkl, Hamp or Dic (~1 µg peptide per g of fish; NNV+Nkl, NNV+Hamp and NNV+Dic groups) or vehicle (PBS; NNV group). A mock group was injected twice with only PBS. Brain, head-kidney (HK) and muscle samples (n = 6) were obtained 2 days after Nkl or Dic treatment. Heatmap showing the fold change of the gene expression in NNV+Nkl, NNV+Hamp and NNV+Dic groups respect to the NNV group. Data represent the mean ± SEM (n = 6). Asterisks indicate significant statistical differences against mock or NNV groups ($p \leq 0.05$). Gene abbreviations are described in the Table 1.

Discussion

Aquaculture sector is facing a great problematic related to viral infections. Viral infections have devastating consequences in fish farms such as diminished fish quality and production, leading to severe economic losses. Many research and field efforts have focused on the prevention of fish viral diseases by the generation of vaccines, leading to the commercialization of few vaccines for farmers and much more restricted to laboratory trials (Ahmed *et al*., 2019). However, and as far as we are concerned, nothing is registered and marketed to combat the virus after the infection, highlighting the necessity of research for therapeutic treatments. NNV prevalence in the Mediterranean area is very high and constitutes one of the main challenges for European sea bass farms (Muniesa *et al*., 2020). Regarding this, increasing studies are trying to solve this problematic by developing new therapeutic molecules (Chia *et al*., 2010; Morick and Saragovi, 2017; Sushila *et al*., 2018). To fulfil this goal, AMPs have arisen as most promising molecules based on their dual role as pathogen lytic molecules and immunomodulators and on the lack of resistance associated to them (Shabir *et al*., 2018). In this context, we focused on AMPs as a potential therapeutic solution due to: i) their dual properties as direct lytic effectors and immunostimulants, ii) the lack of antimicrobial resistance generation and goodness for the environment, and iii) the fact that some AMPs are excellent tools to prevent viral diseases, including VER. Thus, since Nkl, Hamp and Dic peptides are known to partly prevent the mortalities caused by the NNV infection to European sea bass juveniles (Cervera *et al*., 2024a; Valero *et al*., 2021) we aimed herein to evaluate their potential therapeutic application.

We firstly observed that the mortalities provoked by the NNV infection in European sea bass were decreased upon Hamp and Dic peptide therapeutic treatments. This result is similar to the therapeutic application of piscidin to grouper and epinecidin to medaka or when administering Hamp or Epinecidin to medaka or Nkl to European sea bass before NNV-infection (Valero *et al*., 2021; Wang *et al*., 2010b, 2010a). However, the therapeutic administration of Hamp and Epinecidin failed to ameliorate the survival rates in grouper specimens infected with NNV (Wang *et al*., 2010b). All these data suggesting differential effects of AMPs among species. Besides, Hamp and Dic blocked the appearance of the most severe clinical signs of the disease (level 4: complete incapacity to keep balance or swim), which might be related to the protection conferred by these treatments.

Nevertheless, Nkl peptide failed to do so even when the appearance of mortalities was partly delayed. Nevertheless, none of the tested treatments altered the viral load at transcriptional levels as also occurred when Nkl, Hamp and Dic were used as NNV-preventive treatments in European sea bass, but disagreeing with Nkl effect on megalocytic virus transcription (Cervera *et al*., 2024a; Valero *et al*., 2021; Zhang *et al*., 2014). Supporting this, NNV immunostaining in the brain revealed that the infected areas in the brain were not modified upon treatments even though the levels of viral capsid protein, which is involved in pathogenesis and viral invasion (Zhang *et al*., 2022), were increased in Hamp and Nkl treated-groups. The lack of direct effects on NNV might be a consequence of the rapid degradation of the synthetic peptides upon administration since 24 h post-injection they are not detected at the injection site (Cervera *et al*., 2024a). So, our data evidenced that the direct anti-NNV properties of AMPs, which have been probed for tilapia Hamp and European sea bass Nkl, Hamp and Dic *in vitro* (Chia *et al*., 2010; León *et al*., 2020b), are not extrapolated to *in vivo* studies as previous studies in our lab (Cervera *et al*., 2024a, 2023) and this work demonstrate. Indeed, the antiviral roles observed for these AMPs might be caused by the immunomodulation elicited.

To confirm this hypothesis, we next evaluated the immune response triggered by the tested peptides. Firstly, we evaluated the serum antibacterial activity. Unfortunately, the NNV-induced antibacterial activity was reduced by all treatments in serum, brain, and HK pointing to lower levels of bacterial lytic compounds, including AMPs. However, we reported in the target site of NNV replication, the brain, increased levels of Nkl, Hamp and Dic peptides along with *nkl*, *hamp1*, *lyz* and *defb1* transcripts after AMPs administration, corroborating the crosstalk among AMPs previously observed in European sea bass (Cervera *et al*., 2024a, 2024b, 2023). Regarding on the immunity elicited by the AMPs, we started analyzing the expression of a type-I interferon induced genes, the *mx*, as the key marker of the antiviral immune response. Thus, all the tested peptides decreased the antiviral *mx* transcription in the muscle but unaltered it in the brain with respect to the levels observed in the NNV infected group, indicating that there must be other immune processes controlling the course of infection. Following with the innate response, we observed the *nkl* expression up-regulation in all treated groups. As Nkl is involved in the cell-mediated cytotoxic (CMC) response and mainly produced by cytotoxic cells (Hao *et al*., 2022; Pereiro *et al*., 2017; Valero *et al*., 2020b), we further studied the modulation of the cytotoxic immune response by means of the transcription

levels of the *nccrp1*, a marker for the innate non-specific cytotoxic cells (NCC) (Nakanishi *et al*., 2011). Our data showed a systemic great up-regulation of this gene upon Nkl treatment, but not upon Hamp nor Dic treatments. Nevertheless, even if this innate CMC response has also been proposed as a key antiviral pathway in European sea bass (Chaves-Pozo *et al*., 2019, 2017, 2012), our data suggest that this response might not be the most effective against NNV in this species due to the fact that *nccrp1* up-regulation driven by Nkl did not resulted in amelioration of the survival rates. In addition, the increase of sea bass survival upon NNV infection elicited by Hamp and Dic administration was accompanied by the down-regulation of *nccrp1* expression in both HK and muscle. Taking all these data together, the innate CMC or type-I IFN routes triggered by NNV might not be enough to reduce NNV-induced mortalities, so there must be other immune pathways mobilized upon AMPs treatment that explained the survival rates observed upon Hamp and Dic treatments.

It is well known that NNV infection elicits an exacerbated inflammation in brain (Chaves-Pozo *et al*., 2019; Moreno *et al*., 2020), being the main cause of mortalities. AMPs, including Nkl, Hamp and Dic, are known to play a role in the control of the inflammatory response in teleost fish (Katzenback, 2015). In fact, the administration of preventive treatments with anti-inflammatory capability resulted in a decrease of mortalities in NNV infected fish (Cervera *et al*., 2024a; Valero *et al*., 2021). All treatments showed anti-inflammatory properties because of the high up-regulation of brain *il10* and down-regulation of HK *il6*, which might be able to calm down the exacerbated inflammation triggered by the virus, similar to what occurred during AMPs treatments in other fish trials (Álvarez *et al*., 2022; Cervera *et al*., 2024b, 2024a, 2023; Pan *et al*., 2011a; Valero *et al*., 2021). An effective therapeutic treatment might also allow at the same time the acute inflammation, which promotes the leukocyte recruitment to the damaged tissue, the brain (Soliman and Barreda, 2023). In this sense, we observed up-regulation of *cxcr3* and *il8,* molecules involved in cell adhesion, leukocyte recruitment and acute inflammation (Bird and Tafalla, 2015), in both brain and HK in NNV+Hamp and NNV+Dic groups, but not in NNV+Nkl group. These data are in agreement with the maintenance of some pro-inflammatory levels in immune tissues that allow the cell recruitment to the infection site and also the antigen presenting process in those tissues (Chaves-Pozo *et al*., 2005). *Cxcr3*, expressed by many cell types including monocytes, dendritic cells, neutrophils, T or NK-like cells, endothelial cells, neurons, and astrocytes

(Valdés *et al*., 2022), was increased in the brain together with its interferon-gamma inducible ligand, *cxcl9*, and *il8*, and the macrophages markers *mcsf1r* in NNV+Hamp group and NNV+Dic group. In addition, macrophages have been described as capable to revert the exacerbated inflammatory-induced status by modulating cytokines release (Wang *et al*., 2021), So, macrophages infiltration into the brain might help to combat the ongoing infection and resolving the acute inflammation as previously described under naïve conditions for teleost (Rieger *et al*., 2015; Soliman and Barreda, 2023). Thus, the Hamp and Dic-induced macrophage recruitment to brain might be one of the main causes of the survival promotion and can suppose a great difference with Nkl treatment.

On the other hand, AMPs are known to promote the adaptive response as well as linkers of the innate and adaptive immunity (van der Does *et al*., 2019). Total IgM levels were increased upon all treatments in serum. Although the transcriptional levels of *ighm* were up-regulated upon Nkl and Hamp administration, the total IgM levels, in brain, was increased in Hamp and Dic-treated groups. These data point to a higher production of total antibodies which might promote the selection of specific antibodies. In fact the co-stimulation of *ighm* and some genes related to cell-adhesion such as *cxcr3* let us to hypothesize that B cells might be mobilized to the NNV-replication tissue upon Hamp and Dic treatments. Some studies have already demonstrated the role of fish AMPs as promoters of the adaptive response due to their adjuvant properties as described for pleurocidin (Liu *et al*., 2020), oreochromicin (Acosta *et al*., 2014), epinecidin-1 (Huang *et al*., 2011), or β-defensin (Zheng *et al*., 2023). Therefore, it is tempting to speculate that Hamp and Dic administration would show adjuvant properties and promising applications in teleost fish.

Very interestingly, the same immune-related genes that were stimulated in both, HK, and brain tissues, were down-regulated in the injection site, the muscle. Concretely, even when NNV did not altered the immune response parameters at the site of injection, after the administration of the treatments we observed a great and generalized down-regulation of all studied genes, suggesting that AMPs play an immunosuppressive role at the site of injection, promoting the recruitment of immune cells to the target site of the virus, the brain, and suggesting that the tested treatments show certain specificity. The mechanisms underlying this issue deserves more efforts to be understood.

To conclude, therapeutic administration of Hamp and Dic synthetic peptides to European sea bass infected with NNV reduces mortalities and the severity of the clinical signs. These therapeutic actions might be due thanks to the anti-inflammatory status induced, which could control the exacerbated inflammation and promote the recruitment of macrophages and/or B cells to the brain and the production of AMPs, leading to prevent the cell damage induced by NNV in the brain. Although Nkl peptide shows immunomodulatory roles, it failed to promote survival probably since the changes induced are lower than those reached by the other peptides. Therefore, Hamp and Dic peptides can be postulated as promising therapeutic tools to treat NNV outbreaks in European sea bass farms.

References

Acosta, J., Carpio, Y., Valdés, I., Velázquez, J., Zamora, Y., Morales, R., Morales, A., Rodríguez, E., Estrada, M.P., 2014. Co-administration of tilapia alpha-helical antimicrobial peptides with subunit antigens boost immunogenicity in mice and tilapia (*Oreochromis niloticus*). Vaccine 32, 223–229. https://doi.org/10.1016/j.vaccine.2013.11.009

Acosta, J., Roa, F., González-Chavarría, I., Astuya, A., Maura, R., Montesino, R., Muñoz, C., Camacho, F., Saavedra, P., Valenzuela, A., Sánchez, O., Toledo, J.R., 2019. *In vitro* immunomodulatory activities of peptides derived from *Salmo salar* NK-lysin and cathelicidin in fish cells. Fish Shellfish Immunol. 88, 587–594. https://doi.org/10.1016/j.fsi.2019.03.034

Ahmed, F., Soliman, F.M., Adly, M.A., Soliman, H.A.M., El-Matbouli, M., Saleh, M., 2019. Recent progress in biomedical applications of chitosan and its nanocomposites in aquaculture: A review. Res. Vet. Sci. 126, 68–82. https://doi.org/10.1016/j.rvsc.2019.08.005

Álvarez, C.A., Santana, P.A., Salinas-Parra, N., Beltrán, D., Guzmán, F., Vega, B., Acosta, F., Mercado, L., 2022. Immune modulation ability of hepcidin from teleost fish. Animals 12, 1–14. https://doi.org/10.3390/ani12121586

Baek, E.J., Kim, M.J., Kim, K. Il, 2023. *In vitro* and *in vivo* evaluation of the antiviral activity of arctigenin, ribavirin, and ivermectin against viral hemorrhagic septicemia virus infection. Fish Shellfish Immunol. 132, 108456. https://doi.org/10.1016/j.fsi.2022.108456

Bandín, I., Souto, S., 2020. Betanodavirus and VER disease : A 30-year. Pathogens 9, 1–

46. https://doi.org/10.3390/pathogens9020106

Bird, S., Tafalla, C., 2015. Teleost chemokines and their receptors. Biology (Basel). 4, 756–784. https://doi.org/10.3390/biology4040756

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 254, 248–254.

Buonocore, F., Nuñez-Ortiz, N., Picchietti, S., Randelli, E., Stocchi, V., Guerra, L., Toffan, A., Pascoli, F., Fausto, A.M., Mazzini, M., Scapigliati, G., 2019. Vaccination and immune responses of European sea bass (*Dicentrarchus labrax* L.) against betanodavirus. Fish Shellfish Immunol. 85, 78–84. https://doi.org/10.1016/j.fsi.2017.11.039

Cervera, L., Arizcun, M., Mercado, L., Chaves-Pozo, E., Cuesta, A., 2024a. Hepcidin and dicentracin peptides show preventive antiviral applications against NNV infection in European sea bass through immunomodulatory roles. Aquaculture. https://doi.org/10.1016/j.aquaculture.2024.740592

Cervera, L., Chaves-Pozo, E., Cuesta, A., 2024b. Synthetic antimicrobial peptides fail to induce leucocyte innate immune functions but elicit opposing transcriptomic profiles in European sea bass and gilthead seabream. Mar. Drugs 22, 1–16. https://doi.org/10.3390/md22020086

Cervera, L., González-Fernández, C., Arizcun, M., Cuesta, A., Chaves-Pozo, E., 2022. Severe Natural Outbreak of Cryptocaryon irritans in Gilthead Seabream Produces Leukocyte Mobilization and Innate Immunity at the Gill Tissue. Int. J. Mol. Sci. 23. https://doi.org/10.3390/ijms23020937

Cervera, L., González-Fernández, C., Cano, D., Esteban, M.Á., Mercado, L., Chaves-Pozo, E., Cuesta, A., 2023. Immunity elicited by AMP-encoding plasmids fails to increase the protection of European sea bass against nodavirus. Fish Shellfish Immunol. 132, 1–9. https://doi.org/10.1016/j.fsi.2022.108507

Chaves-Pozo, E., Arizcun, M., Cuesta, A., 2021. Betanodavirus genotypes produce clinical signs and mortality in the shi drum (*Umbrina cirrosa*), and infective particles are isolated from the damaged brain. Aquaculture 541, 736777. https://doi.org/10.1016/j.aquaculture.2021.736777

Chaves-Pozo, E., Bandín, I., Olveira, J.G., Esteve-Codina, A., Gómez-Garrido, J., Dabad, M., Alioto, T., Ángeles Esteban, M., Cuesta, A., 2019. European sea bass brain DLB-1 cell line is susceptible to nodavirus: A transcriptomic study. Fish Shellfish

Immunol. 86, 14–24. https://doi.org/10.1016/j.fsi.2018.11.024

Chaves-Pozo, E., Dabad, M., Valero, Y., Cuesta, A., Esteban, M.Á., Gómez-Garrido, J., Meseguer, J., Alioto, T., Esteve-Codina, A., 2017. Innate cell-mediated cytotoxic activity of European sea bass leucocytes against Nodavirus-infected cells: A functional and RNA-seq study. Sci. Rep. 7, 1–15. https://doi.org/10.1038/s41598-017-15629-6

Chaves-Pozo, E., Guardiola, F.A., Meseguer, J., Esteban, M.A., Cuesta, A., 2012. Nodavirus infection induces a great innate cell-mediated cytotoxic activity in resistant, gilthead seabream, and susceptible, European sea bass, teleost fish. Fish Shellfish Immunol. 33, 1159–1166. https://doi.org/10.1016/j.fsi.2012.09.002

Chaves-Pozo, E., Muñoz, P., López-Muñoz, A., Pelegrín, P., García Ayala, A., Mulero, V., Meseguer, J., 2005. Early innate immune response and redistribution of inflammatory cells in the bony fish gilthead seabream experimentally infected with *Vibrio anguillarum*. Cell Tissue Res. 320, 61–68. https://doi.org/10.1007/s00441-004-1063-7

Chia, T.J., Wu, Y.C., Chen, J.Y., Chi, S.C., 2010. Antimicrobial peptides (AMP) with antiviral activity against fish nodavirus. Fish Shellfish Immunol. 28, 434–439. https://doi.org/10.1016/j.fsi.2009.11.020

Cordero, H., Morcillo, P., Martínez, S., Meseguer, J., Pérez-Sirvent, C., Chaves-Pozo, E., Martínez-Sanchez, M.J., Cuesta, A., Ángeles Esteban, M., 2018. Inorganic arsenic causes apoptosis cell death and immunotoxicity on European sea bass (*Dicentrarchus labrax*). Mar. Pollut. Bull. 128, 324–332. https://doi.org/10.1016/j.marpolbul.2018.01.052

Costa, J.Z., Thompson, K.D., 2016. Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. Fish Shellfish Immunol. 53, 35–49. https://doi.org/10.1016/j.fsi.2016.03.033

FAO, 2022. The state of world fisheries and aquaculture 2022, Towards Blue Transformation. FAO, Rome. https://doi.org/10.4060/cc0463en

Hao, D. fang, Wang, G. hua, Li, N. qiu, Liu, H. mei, Wang, C. biao, Liu, W. qing, Yan, X., Zhang, M., 2022. Antimicrobial and immunoregulatory activities of the derived peptide of a natural killer lysin from black rockfish (Sebastes schlegelii). Fish Shellfish Immunol. 123, 369–380. https://doi.org/10.1016/j.fsi.2022.03.020

Hsieh, J.C., Pan, C.Y., Chen, J.Y., 2010. Tilapia hepcidin (TH)2-3 as a transgene in

transgenic fish enhances resistance to *Vibrio vulnificus* infection and causes variations in immune-related genes after infection by different bacterial species. Fish Shellfish Immunol. 29, 430–439. https://doi.org/10.1016/j.fsi.2010.05.001

Huang, H.N., Pan, C.Y., Rajanbabu, V., Chan, Y.L., Wu, C.J., Chen, J.Y., 2011. Modulation of immune responses by the antimicrobial peptide, epinecidin (Epi)-1, and establishment of an Epi-1-based inactivated vaccine. Biomaterials 32, 3627–3636. https://doi.org/10.1016/j.biomaterials.2011.01.061

Huang, Y.C., Lin, T.S., Peng, C., Chan, N.L., Han, Y.S., 2016. Strong inhibition of betanodavirus replication by ribavirin targeting RNA-dependent RNA polymerase. J. Fish Dis. 39, 619–623. https://doi.org/10.1111/jfd.12398

Iwamoto, T., Mise, K., Mori, K.I., Arimoto, M., Nakai, T., Okuno, T., 2001. Establishment of an infectious RNA transcription system for Striped jack nervous necrosis virus, the type species of the betanodaviruses. J. Gen. Virol. 82, 2653–2662. https://doi.org/10.1099/0022-1317-82-11-2653

Katzenback, B.A., 2015. Antimicrobial peptides as mediators of innate immunity in teleosts. Biology (Basel). 4, 607–639. https://doi.org/10.3390/biology4040607

Kibenge, F.S., 2019. Emerging viruses in aquaculture. Curr. Opin. Virol. 34, 97–103. https://doi.org/10.1016/j.coviro.2018.12.008

León, R., Ruiz, M., Valero, Y., Cárdenas, C., Guzman, F., Vila, M., Cuesta, A., 2020a. Exploring small cationic peptides of different origin as potential antimicrobial agents in aquaculture. Fish Shellfish Immunol. 98, 720–727. https://doi.org/10.1016/j.fsi.2019.11.019

León, R., Ruiz, M., Valero, Y., Cárdenas, C., Guzman, F., Vila, M., Cuesta, A., 2020b. Exploring small cationic peptides of different origin as potential antimicrobial agents in aquaculture. Fish Shellfish Immunol. 98, 720–727. https://doi.org/10.1016/j.fsi.2019.11.019

Li, X., Chi, H., Dalmo, R.A., Tang, X., Xing, J., Sheng, X., Zhan, W., 2023. Anti-microbial activity and immunomodulation of recombinant hepcidin 2 and NK-lysin from flounder (*Paralichthys olivaceus*). Int. J. Biol. Macromol. 253. https://doi.org/10.1016/j.ijbiomac.2023.127590

Liu, S.P., Chuang, S.C., Yang, C. Da, 2020. Protective immunity against Vibrio harveyi in grouper induced by single vaccination with poly (Lactide-co-glycolide) microparticles releasing pleurocidin peptide and recombinant glyceraldehyde-3-phosphate dehydrogenase. Vaccines 8. https://doi.org/10.3390/vaccines8010033

Low, C.F., Syarul Nataqain, B., Chee, H.Y., Rozaini, M.Z.H., Najiah, M., 2017. Betanodavirus: Dissection of the viral life cycle. J. Fish Dis. 40, 1489–1496. https://doi.org/10.1111/jfd.12638

Moreno, P., Gemez-Mata, J., Garcia-Rosado, E., Bejar, J., Labella, A.M., Souto, S., Alonso, M.C., 2020. Differential immunogene expression profile of European sea bass (*Dicentrarchus labrax*, L.) in response to highly and low virulent NNV. Fish Shellfish Immunol. 106, 56–70. https://doi.org/10.1016/j.fsi.2020.06.052

Mori, K.I., Nakai, T., Muroga, K., Arimoto, M., Mushiake, K., Furusawa, I., 1992. Properties of a new virus belonging to nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. Virology 187, 368–371. https://doi.org/10.1016/0042-6822(92)90329-N

Morick, D., Saragovi, A., 2017. Inhibition of nervous necrosis virus by ribavirin in a zebrafish larvae model. Fish Shellfish Immunol. 60, 537–544. https://doi.org/10.1016/j.fsi.2016.11.015

Muniesa, A., Basurco, B., Aguilera, C., Furones, D., Reverté, C., Sanjuan-Vilaplana, A., Jansen, M.D., Brun, E., Tavornpanich, S., 2020. Mapping the knowledge of the main diseases affecting sea bass and sea bream in Mediterranean. Transbound. Emerg. Dis. 67, 1089–1100. https://doi.org/10.1111/tbed.13482

Nagai, T., Nishizawa, T., 1999. Sequence of the non-structural protein gene encoded by RNA1 of striped jack nervous necrosis virus. J. Gen. Virol. 80, 3019–3022. https://doi.org/10.1099/0022-1317-80-11-3019

Nakanishi, T., Toda, H., Shibasaki, Y., Somamoto, T., 2011. Cytotoxic T cells in teleost fish. Dev. Comp. Immunol. 35, 1317–1323. https://doi.org/10.1016/j.dci.2011.03.033

Neves, J. V., Caldas, C., Vieira, I., Ramos, M.F., Rodrigues, P.N.S., 2015. Multiple hepcidins in a teleost fish, *Dicentrarchus labrax* : Different hepcidins for different roles. J. Immunol. 195, 2696–2709. https://doi.org/10.4049/jimmunol.1501153

Pan, C.Y., Peng, K.C., Lin, C.H., Chen, J.Y., 2011a. Transgenic expression of tilapia hepcidin 1-5 and shrimp chelonianin in zebrafish and their resistance to bacterial pathogens. Fish Shellfish Immunol. 31, 275–285. https://doi.org/10.1016/j.fsi.2011.05.013

Pan, C.Y., Wu, J.L., Hui, C.F., Lin, C.H., Chen, J.Y., 2011b. Insights into the antibacterial and immunomodulatory functions of the antimicrobial peptide, epinecidin-1, against *Vibrio vulnificus* infection in zebrafish. Fish Shellfish Immunol. 31, 1019–1025.

https://doi.org/10.1016/j.fsi.2011.09.001

Pereiro, P., Romero, A., Díaz-Rosales, P., Estepa, A., Figueras, A., Novoa, B., 2017. Nucleated teleost erythrocytes play an Nk-lysin- and autophagy-dependent role in antiviral immunity. Front. Immunol. 8, 1–15. https://doi.org/10.3389/fimmu.2017.01458

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 2002–2007.

Reed, L.., Müench, H., 1938. A simple method of estimating fifty per cent endopoints. J. Hygene 27, 493–497.

Rieger, A.M., Havixbeck, J.J., Belosevic, M., Barreda, D.R., 2015. Teleost soluble CSF-1R modulates cytokine profiles at an inflammatory site, and inhibits neutrophil chemotaxis, phagocytosis, and bacterial killing. Dev. Comp. Immunol. 49, 259–266. https://doi.org/10.1016/j.dci.2014.12.003

Shabir, U., Ali, S., Magray, A.R., Ganai, B.A., Firdous, P., Hassan, T., Nazir, R., 2018. Fish antimicrobial peptides (AMP's) as essential and promising molecular therapeutic agents: A review. Microb. Pathog. 114, 50–56. https://doi.org/10.1016/j.micpath.2017.11.039

Soliman, A.M., Barreda, D.R., 2023. The acute inflammatory response of teleost fish. Dev. Comp. Immunol. 146, 104731. https://doi.org/10.1016/j.dci.2023.104731

Sunyer, J.O., Tort, L., 1995. Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. Vet. Immunol. Immunopathol. 2427, 333–345.

Sushila, N., Hameed, A.S.S., Prasad, K.P., Majeed, S.A., Tripathi, G., 2018. *In vitro* screening of selected antiviral drugs against betanodavirus. J. Virol. Methods 259, 66–73. https://doi.org/10.1016/j.jviromet.2018.06.005

Ting, C.H., Pan, C.Y., Chen, Y.C., Lin, Y.C., Chen, T.Y., Rajanbabu, V., Chen, J.Y., 2019. Impact of Tilapia hepcidin 2-3 dietary supplementation on the gut microbiota profile and immunomodulation in the grouper (*Epinephelus lanceolatus*). Sci. Rep. 9, 1–17. https://doi.org/10.1038/s41598-019-55509-9

Valdés, N., Cortés, M., Barraza, F., Reyes-López, F.E., Imarai, M., 2022. CXCL9-11 chemokines and CXCR3 receptor in teleost fish species. Fish Shellfish Immunol. Reports 3, 100068. https://doi.org/10.1016/j.fsirep.2022.100068

Valero, Y., Arizcun, M., Cortés, J., Ramírez-Cepeda, F., Guzmán, F., Mercado, L., Esteban, M.Á., Chaves-Pozo, E., Cuesta, A., 2020a. NK-lysin, dicentracin and

hepcidin antimicrobial peptides in European sea bass. Ontogenetic development and modulation in juveniles by nodavirus. Dev. Comp. Immunol. 103, 103516. https://doi.org/10.1016/j.dci.2019.103516

Valero, Y., Chaves-Pozo, E., Cuesta, A., 2020b. NK-lysin is highly conserved in European sea bass and gilthead seabream but differentially modulated during the immune response. Fish Shellfish Immunol. 99, 435–441. https://doi.org/10.1016/j.fsi.2020.02.049

Valero, Y., González-Fernández, C., Cárdenas, C., Guzmán, F., León, R., Cuesta, A., 2021. NK-lysin peptides ameliorate viral encephalopathy and retinopathy disease signs and provide partial protection against nodavirus infection in European sea bass. Antiviral Res. 192, 1–8. https://doi.org/10.1016/j.antiviral.2021.105104

van der Does, A.M. van der, Hiemstra, P.S., Mookherjee, N., 2019. Antimicrobial host defence peptides: immunomodulatory functions and translational prospects, in: antimicrobial peptides. pp. 149–171.

Wang, Y. Da, Kung, C.W., Chen, J.Y., 2010a. Antiviral activity by fish antimicrobial peptides of epinecidin-1 and hepcidin 1-5 against nervous necrosis virus in medaka. Peptides 31, 1026–1033. https://doi.org/10.1016/j.peptides.2010.02.025

Wang, Y. Da, Kung, C.W., Chi, S.C., Chen, J.Y., 2010b. Inactivation of nervous necrosis virus infecting grouper (*Epinephelus coioides*) by epinecidin-1 and hepcidin 1-5 antimicrobial peptides, and downregulation of Mx2 and Mx3 gene expressions. Fish Shellfish Immunol. 28, 113–120. https://doi.org/10.1016/j.fsi.2009.10.001

Wang, Q., Peng, C., Yang, M., Huang, F., Duan, X., Wang, S., Cheng, H., Yang, H., Zhao, H., Qin, Q., 2021. Single-cell RNA-seq landscape midbrain cell responses to red spotted grouper nervous necrosis virus infection. PLoS Pathog. 17, 1–26. https://doi.org/10.1371/journal.ppat.1009665

Zhang, M., Li, M.F., Sun, L., 2014. NKLP27: A teleost NK-lysin peptide that modulates immune response, induces degradation of bacterial DNA, and inhibits bacterial and viral infection. PLoS One 9. https://doi.org/10.1371/journal.pone.0106543

Zhang, M., Long, H., Sun, L., 2013. A NK-lysin from *Cynoglossus semilaevis* enhances antimicrobial defense against bacterial and viral pathogens. Dev. Comp. Immunol. 40, 258–265. https://doi.org/10.1016/j.dci.2013.03.005

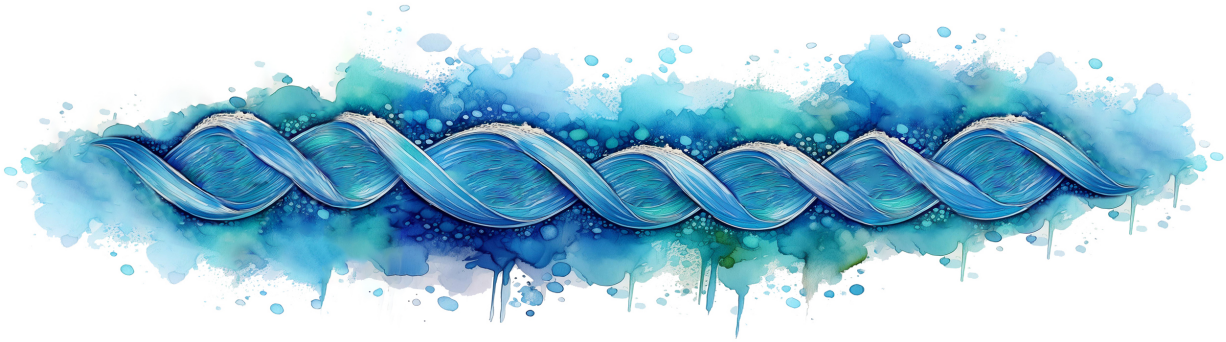
Zhang, W.W., Jia, P., Lu, X.B., Chen, X.Q., Weng, J.H., Jia, K.T., Yi, M.S., 2022. Capsid protein from red-spotted grouper nervous necrosis virus induces incomplete autophagy by inactivating the HSP90ab1-AKT-MTOR pathway. Zool. Res. 43, 98–

110. https://doi.org/10.24272/J.ISSN.2095-8137.2021.249

Zheng, J., Yang, J., Zhang, Z., Liang, X., Liu, S., Pan, Y., Wei, J., Huang, Y., Huang, X., Qin, Q., 2023. An improved oral vaccine with molecular adjuvant β-defensin protects grouper against nervous necrosis virus infection. Fish Shellfish Immunol. 136, 108709. https://doi.org/10.1016/j.fsi.2023.108709

Zhu, S., Miao, B., Zhang, Y.Z., Wang, D.S., Wang, G.X., 2022. Amantadine, a promising therapeutic agent against viral encephalopathy and retinopathy. J. Fish Dis. 45, 451–459. https://doi.org/10.1111/jfd.13574

GENERAL DISCUSSION



Aquaculture is a prosperous and expanding sector alowing to satisfy the food demand of the world population (FAO, 2022). However, this sector faces serious difficulties that compromises its economic viability. Viral infections are one of the main biological threatens in fish farms favored by the high-density culture conditions and chronic stress (Kibenge, 2019). In this sense, NNV emerge as a great viral problem in the Mediterranean area farms affecting to very traded species in the zone. Among those, European sea bass is one of the most susceptible species to NNV, while gilthead seabream had recently showed susceptibility to natural reassortant strains localized in the Mediterranean basin (Biasini *et al*., 2022; Munday *et al*., 2002). In both cases, outbreaks of VER disease are bringing devastating consequences (APROMAR, 2020; Bandín and Souto, 2020). Unfortunately, there are no effective and approved commercial antiviral treatments available for producers. Research in this field is very limited to laboratory trials about vaccines and few studies dealt with therapeutic molecules administered after NNV infection (Bandín and Souto, 2020). In this context, finding antiviral molecules with potential application in aquaculture is a great priority. After NNV infection, in European sea bass, AMPs are increased at both gene and protein levels pointing to their importance in the infection resolution (Valero *et al*., 2020a, 2015b). In this line, previous studies have proposed AMPs as potential antiviral agents in aquaculture due to their lack of cytotoxicity, the little likely to generate antimicrobial resistance, their direct antiviral effects *in vitro* and immunomodulatory roles (Masso-Silva and Diamond, 2014). Taking all this into account, this Doctoral Thesis aims to give response to this urgent problematic by evaluating the suitability of different AMPs as preventive or palliative treatments upon NNV.

Among the large number of AMPs studies in fish, there are only four well-characterized AMPs in the immune response of European sea bass and gilthead seabream against NNV: Nkl, Hamp, Dic and DB1. (Barroso *et al*., 2021; Cuesta *et al*., 2011, 2008; Neves *et al*., 2015; Salerno *et al*., 2007; Valero *et al*., 2020b, 2015b). The *in silico* analysis of the secondary structure of these four AMPs pointed out the high complexity of DB1 that makes very difficult its chemical synthesis, hampering its production, application and evaluation of its functionality. For this reason, DB1 was only used via plasmid in this Thesis. Among the other three AMPs, Nkl, Hamp and Dic, Nkl and Dic showed a predominant alpha-helix structure stabilized by Alanine (A), Leucin (L) and Lysin (K)

residues which confer antiviral properties (Personne *et al*., 2023). Otherwise, Hamp is mainly composed by strand conformations forming the disulfide bridges characteristic of this peptide and responsible for their antimicrobial activities (Freitas *et al*., 2020). The great homology and conservation between AMPs of European sea bass and gilthead seabream point to similar functions. Thus, European sea bass Nkl, Hamp and Dic synthetic peptides were chemically synthetized and *in vitro* tested in European sea bass and gilthead seabream HKLs to assess their potential use in both species as a common treatment for natural disease outbreaks. Firstly, the non-cytotoxic effect on both species HKLs was assessed confirming that all peptides can be used without cytotoxic consequences confirming a key property of AMPs to be used as pharmacological drugs (Boparai and Sharma, 2019). Since their bactericidal and/or antiviral direct killing functions were already demonstrated (León *et al*., 2020; Neves *et al*., 2015) we next evaluated the immunomodulation triggered by these peptides by analyzing some cellular immune activities and gene expression profiles.

To ascertain the role of Nkl, Hamp and Dic in the modulation of phagocytosis and respiratory burst *in vitro*, several concentrations that have demonstrated to be active in other fish species were used (Álvarez *et al*., 2022; Chen *et al*., 2019, 2021; Hao *et al*., 2022; Wang *et al*., 2023). By contrast, in our work, these activities remained unaltered by all the peptides and concentrations tested. However, the gene expression studies revealed a completely opposite transcriptional pattern between both species in five functional categories: inflammation, type-I IFN, CMC, AMPs, and leukocyte markers; suggesting species-dependence for immunomodulation. In gilthead seabream, most evaluated genes were down-regulated leading to an immunosuppressed status of both innate and adaptive responses. So, for this reason, Nkl, Hamp and Dic peptides are not good candidates to prevent or treat viral infections in gilthead seabream and this species was discarded for next studies in the present Doctoral Thesis. As regards European sea bass, we found several stimulations in key aspects of the anti-NNV response. Our *in vitro* results show anti-inflammatory roles for all our peptides by the up-regulation of molecules such as *il10* or *tfgb* as pointing in some studies (Huang *et al*., 2019; Pan *et al*., 2011; Valero *et al*., 2021), but contrary to others which described pro-inflammatory roles for AMPs (Álvarez *et al*., 2022; Masso-Silva and Diamond, 2014; Neves *et al*., 2011; M. Zhang *et al*., 2014). Inflammation control is crucial to design new pharmacological treatments avoiding tissue damage (Valero *et al*., 2020c). Moreover, an exacerbated inflammation has been related

to larger mortalities upon NNV infection (Poisa-Beiro *et al*., 2008). Tightly related to AMPs role as antiviral agents, our data showed Nkl capability to up-regulate ISGs confirming their antiviral potential previously described (Ding *et al*., 2019; León *et al*., 2020b; Zhang *et al*., 2014). Otherwise, we found that the tested AMPs induced the transcription of CMC effectors pointing to a synergic effect of both routes promoting infection resolution as other studies point to (Chaves-Pozo *et al*., 2019b, 2017, 2012; Valero *et al*., 2020a, 2020b, 2018a, 2015b). Regarding cellular markers, we found the up-regulation of *ighm*, a B cells marker, establishing a link between the innate and adaptive immune responses (Ma *et al*., 2020). All this data show that AMPs play a role in regulating the immune response of European sea bass and point to them as excellent candidates to be used as *in vivo* preventive or palliative treatments in this species.

We next analyzed the immune processes regulated by *in vivo* administration of AMPs at two different administration forms. For this, four plasmids were constructed encoding Nkl (pNKL), Hamp (pHAMP2), Dic (pDIC) and DB1 (pDB1) and three peptides chemically synthesis (Nkl, Hamp and Dic) and all of them were intramuscularly administrated as preventive or palliative treatment. Regarding plasmids, an effective transcription at the site of injection concomitantly with an increase of seral AMPs levels and bactericide activity allowed the study of the effect of the encoding AMPs on immune regulation and as anti-viral treatments. In the case of the Nkl synthetic peptide, its effects on NNV prevention were previously evaluated in our lab (Valero *et al*., 2021), so our study with this synthetic peptide was restricted to its use as palliative treatment.

Inflammation control is crucial to design new pharmacological treatments avoiding tissue damage (Valero *et al*., 2020c). The role of AMPs in the control of inflammation is contradictory as anti-inflammatory or pro-inflammatory roles have been described in several fish species (Acosta *et al*., 2019; Guo *et al*., 2012; Hsieh *et al*., 2010; Valero *et al*., 2021). Our data showed that plasmids encoding AMPs led to a greatly enhanced inflammatory response in muscle through the up regulation of genes involved in cell recruitment and induction of inflammation as also described in other fish species (Acosta *et al*., 2019; Jiang *et al*., 2018; Lin *et al*., 2016; Pan *et al*., 2017; Valero *et al*., 2021; Zhang *et al*., 2013). On the other hand, Hamp and Dic synthetic peptides were able to down-regulate or kept steady the inflammatory levels in muscle or HK, demonstrating its ability to control this response as other studies also pointed to (Drayton *et al*., 2021; Kang

et al., 2017; Luo and Song, 2021; Wang *et al*., 2020). Interestingly, the up-regulation of the chemotaxis complex Cxcr3/Cxcl9 is triggered by inflammation in humans (Guo *et al*., 2018) and was also observed in European sea bass treated with AMP encoding plasmids, but not upon peptide treatments. Despite the up-regulation of chemotactic molecules, plasmids only promoted neutrophil infiltration and in the case of pHAMP2 also B cells. However, synthetic AMPs induced the recruitment of macrophages and neutrophils and promote T and B cells formation.

Considering that the immune status of European sea bass was differently boosted upon both ways of administration, NNV challenge was performed to ascertain which one conferred more protection. Regarding plasmid administration, the improved immune status triggered by them did not serve to ameliorate survival rates and even worsen it after pHAMP2 treatment contradicting previous studies in which the antiviral effect of AMPs was proposed (León *et al*., 2020b; Valero *et al*., 2021). This failure as preventive agents might be a consequence of the exacerbated inflammatory response induced by the plasmids. In addition, the worsen mortality rates induced by pHAMP2 could also be related to the positive feedback between *hamp2* and *hamp1* observed. As Hamp is responsible for iron homeostasis, the unbalance on these peptides production could cause anemia episodes altering the immune capability of erythrocytes (Neves *et al*., 2016, 2015; Stosik *et al*., 2018). So, although plasmids encoding AMPs elicited immunity, they might not be considered as proper antiviral tools against NNV, and their potential as palliative treatments was not evaluated. Contrary to these results, Hamp and Dic synthetic peptides enhanced the survival rates when administered prior or after NNV infection as also described for Hamp and epinecidin in grouper and medaka (Wang *et al*., 2010b, 2010a). However, Nkl peptide administrated after NNV infection partly delayed the appearance of mortalities but failed to confer protection disagreeing with its role as a preventive agent previously described in the same species (Valero *et al*., 2021).

Focusing on the potential mechanisms behind the protection, we observed that Hamp and Dic peptides failed to alter the viral load at transcriptional levels and the size of brain NNV-immunostained areas when comparing to non-treated and infected fish. In fact, the *mx* antiviral marker levels were not stimulated in Hamp or Dic treated fish as also described in other species (Chia *et al*., 2010; Wang *et al*., 2010b, 2010a). All these findings together with the fact that synthetic peptides are not detected at the site of

injection after 24 h when administrated prior to the infection, discard direct lytic effects of these peptides on NNV *in vivo* contrary to what has been proposed using *in vitro* approaches (Chia *et al*., 2010; León *et al*., 2020b). Discarding a direct lytic effect, the increased survival observed in the AMPs treated fish might only be explained due to the regulation of the immune response.

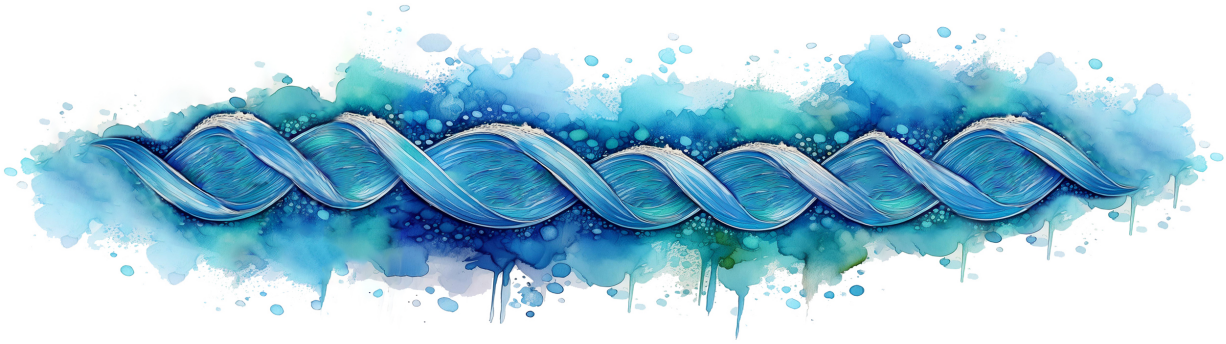
In that sense, we observed that Hamp and Dic synthetic peptides used as pre- or post-treatments control the exacerbated inflammation triggered by NNV in brain in contrast to what plasmid administration did. Our data showed a great up-regulation of the anti-inflammatory interleukin *il10* along with the maintenance of the expression levels of other inflammatory molecules as other authors also described (Álvarez *et al*., 2022; Pan *et al*., 2011; Valero *et al*., 2021). So, although a predominant anti-inflammatory status might be triggered by peptide administration, we observed the up-regulation of other genes related to acute inflammation and cell adhesion and attraction such as *il8*, *cxcl9* and *cxcr3,* allowing cell infiltration into the target tissue as previously proposed for other AMPs (Drayton *et al*., 2021; Hao *et al*., 2021; Li *et al*., 2015). Indeed, upon Hamp and Dic pre- and post-treatment up-regulation of the macrophage marker in brain might explain the great differences in mortalities between Hamp- or Dic-treated groups and Nkl-treated group where macrophages are not recruited. Thus, macrophages are capable to revert the exacerbated inflammation induced by NNV infection through the modulation of cytokines release, restoring normal levels (Rieger *et al*., 2015; Soliman and Barreda, 2023; Wang *et al*., 2021). Regarding the clearance of the virus through CMC activation (Chaves-Pozo *et al*., 2019a, 2017, 2012), only Nkl stimulate this pathway while Hamp and Dic did not. These data suggest that CMC activation might not be enough to combat NNV infection and that other immune processes might be activated to overcome the disease. In this scenario, the ability of AMPs to bridge the innate and adaptative immunity is reinforced. Thus, our data showed that B cells marker was only up-regulated upon pHAMP2, Hamp or Dic administration, although Nkl, Hamp or Dic increased the total IgM levels in serum. These data, taken together, demonstrated that only Hamp and Dic induced a higher production of total antibodies which might promote the selection of specific antibodies together with an effective mobilization of B cells into the brain probably by means of the Cxcr3 cell-adhesion protein, which gene expression in brain is also up-regulated. So, all these data confirmed that AMPs can act as linkers between innate and adaptive responses in European sea bass as other AMPs previously described

(Magrone *et al*., 2018). In fact, and according to our data, several studies have already demonstrated the uses of AMPs as adjuvant for vaccines (Acosta *et al*., 2014; Huang *et al*., 2011; Liu *et al*., 2020; Zheng *et al*., 2023). Therefore, and considering that only Hamp and Dic recruit B cells to the site of infection, we could highlight another promising application of these two AMPs as vaccine adjuvants in European sea bass.

Finally, our data demonstrated the existence of AMPs crosstalk in European sea bass, as also suggested in other fish species (Pan *et al*., 2011). The promotion of AMPs production by other AMPs that favor viral disease overcome (Hamp and Dic) or not (pHamp2 and Nkl), is something that deserves further studies to develop effective antiviral treatments for fish aquaculture applications.

In conclusion, this Doctoral Thesis shows that the improvement of survival evidences the great prospects of the application of synthetic AMPs as pharmacological tools to be used in European sea bass farms against NNV. Moreover, the immunomodulatory data obtained in the present Doctoral Thesis support previous studies in this topic and highlight the necessity to ascertain the precise AMP treatments to fine tunning the immunity for a better fish resistance to diseases and welfare. In this framework, control of the inflammatory response and induction of the adaptive response by AMPs seem to be key aspects to focus on for their application as NNV treatments or vaccine formulations in the culture of European sea bass, properties that could be expanded to other fish species and aquaculture.

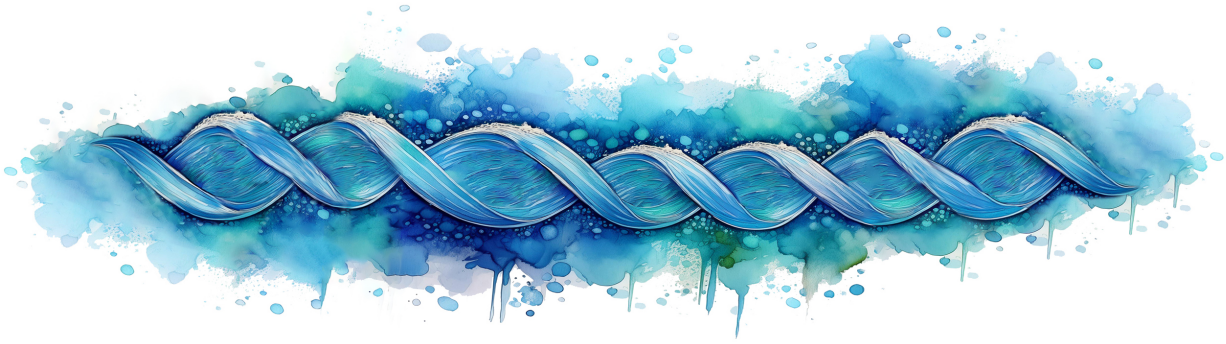
CONCLUSIONS



The conclusions of this Doctoral Thesis are:

1. European sea bass Nkl, Hamp and Dic synthetic peptides are not cytotoxic for European sea bass or gilthead seabream HKLs but are unable to modulate phagocytosis and respiratory burst activities *in vitro*.
2. Synthetic Nkl, Hamp and Dic peptides induce genes related to anti-inflammatory, IFN, CMC and antibody responses in European sea bass HKLs while in gilthead seabream HKLs resulted in immunosuppression.
3. The tested peptides might be employed as biotechnological tools for immunostimulation and viral control in European sea bass farms but not for gilthead seabream.
4. Plasmids encoding Nkl, Hamp, Dic or DB1 are transcribed in the European sea bass muscle promoting serum circulating levels of the correspondent AMP as well as enhancing antibacterial activity.
5. The plasmids encoding AMPs induced local inflammation and leukocyte infiltration in European sea bass but failed to induce NNV protection and pHAMP2 even worsen the survival rates.
6. Injection of Hamp and Dic synthetic peptides blocked the production of proinflammatory molecules at systemic and local level in European sea bass while induced macrophage and neutrophil trafficking.
7. Hamp and Dic synthetic peptides administered prior and after NNV infection ameliorated the clinical signs and enhanced survival rates in European sea bass juveniles, while Nkl administered after NNV did not.
8. Administration of Hamp and Dic peptides failed to alter the viral replication levels indicating that the mode of action of the tested AMPs is by their immunomodulatory actions and not by direct antiviral properties.
9. Preventive and therapeutic role of Hamp and Dic peptides promoted leukocyte infiltration in the brain restoring the exacerbated inflammation provoked by the NNV infection.
10. Hamp and Dic synthetic peptides can be postulated as excellent tools to deal with NNV outbreaks in European sea bass farms as both preventive and palliative treatments.

RESUMEN



Introducción

Actualmente, los métodos de pesca tradicional no pueden dar suministro a la elevada demanda de pescado de una población cada vez más numerosa y preocupada por su alimentación. En este contexto, la acuicultura se erige como un próspero sector económico en todo el mundo para solventar esta problemática (APROMAR, 2023; FAO, 2022; STECF, 2021). En España, las dos especies más producidas y comercializadas son la lubina (*Dicentrarchus labrax*) y la dorada (*Sparus aurata*). Sin embargo, la aparición de brotes infecciosos, incluidos los virales, en las granjas acuícolas compromete su viabilidad económica y genera una problemática que necesita ser solventada (Buchmann, 2022; Kibenge, 2016).

La respuesta inmunitaria de peces está compuesta por una compleja red de células y moléculas que desarrollan respuestas de tipo innato y adaptativo. La respuesta inmunitaria innata constituye una primera línea de defensa que se activa rápidamente para eliminar patógenos aumentando la resistencia a la enfermedad (Abbas *et al*., 2012). La piel, las branquias y el intestino constituyen una primera barrera mucosa frente a patógenos constituida principalmente por las células epiteliales, además de células inmunitarias, que conforman el tejido linfoide asociados a mucosas (MALT), y de una capa superficial de moco rica en compuestos bioactivos (Dezfuli *et al*., 2023; Salinas, 2015). Sin embargo, cuando estas barreras son insuficientes para prevenir la invasión del patógeno, los patrones moleculares asociados a patógenos (PAMPs) son reconocidos, desencadenando una temprana activación leucocitaria (Sahoo, 2020). Entre las principales células implicadas en este tipo de respuesta se encuentran los monocitos/macrófagos, los granulocitos, los melanomacrófagos, las células *natural killer* y las células citotóxicas no específicas (Jørgensen, 2014). En líneas generales, estas células participan en procesos como la fagocitosis y la citotoxicidad mediada por células (CMC), sin requerir ningún tiempo de inducción previo y sin restricción por el complejo mayor de histocompatibilidad (MHC) (Jørgensen, 2014; Mali *et al*., 2017). Además, orquestan una respuesta humoral que incluye el sistema del complemento, proteínas de fase aguda, péptidos antimicrobianos (AMPs) y citoquinas. Las citoquinas son un grupo variado de proteínas solubles extracelulares que regulan diversos procesos inmunitarios como la movilización leucocitaria y la presentación de antígenos (Cao *et al*., 2023). Entre las citoquinas, se puede destacar el interferón (IFN), que induce el estado antiviral celular,

las interleuquinas (ILs), implicadas en procesos inflamatorios, o las quimioquinas, encargadas de coordinar la migración de células inmunitarias hacia los tejidos dañados (Zou and Secombes, 2016). Una vez que las células de la respuesta innata reconocen los antígenos se induce una respuesta inmune más sofisticada y específica conocida como la respuesta inmunitaria adaptativa. La presentación de antígenos a los linfocitos induce su maduración, selección y generación de memoria. Las células presentadoras de antígeno (APC), los linfocitos T citotóxicos (CTLs), T colaboradores (Th) y linfocitos B son las principales células implicadas en la respuesta adaptativa (Bassity and Clark, 2012; Mutoloki *et al*., 2014; Yamaguchi *et al*., 2019). Los linfocitos B, por su parte, secretan anticuerpos que son los principales actores de las respuestas adaptativas humorales. En peces teleósteos se han descrito tres tipos de anticuerpos: IgM, IgD y IgT/IgZ (Mutoloki *et al*., 2014). La forma más común es IgM que se encuentra en suero, moco y la superficie de los linfocitos B y median tanto respuestas innatas como adaptativas (Salinas *et al*., 2011).

Los AMPs son péptidos o proteicas pequeñas que comparten algunas características estructurales comunes como su bajo peso molecular, carga neta positiva, carácter anfílico y anfipático (Valero *et al*., 2013). Principalmente, los AMPs pueden formar estructuras en alfa hélice o lámina beta que favorecen su bioactividad, estabilidad y la interacción con membranas (Kumar *et al*., 2018). Existen varias familias de AMPs, en peces las más destacables son las piscidinas, defensinas, hepcidinas, catelicidinas, péptidos derivados de histonas y saponinas (Masso-Silva and Diamond, 2014). Los AMPs presentan un modo de acción dual con efectos líticos directos contra los patógenos o, bien, modulando la respuesta inmunitaria del huésped. Por un lado, la carga positiva de los AMPs les permite establecer interacciones electrostáticas con las membranas bacterianas formando poros o disgregando la membranas en micelas (Brogden, 2005; Kumar *et al*., 2018) aunque también pueden inhibir la síntesis de moléculas implicadas en la formación de la pared celular bacteriana o bloquear procesos celulares críticos (Kumar *et al*., 2018). Sin embargo, hasta el momento poco se sabe sobre su mecanismo antiviral. Se ha propuesto la capacidad de los AMPs para aglutinar partículas virales o para inhibir la capacidad de adsorción o el ciclo de replicación viral (Avila, 2016; Chia *et al*., 2010). Por otro lado, los AMPs también pueden ayudar a eliminar patógenos a través de la inmunomodulación en el huésped, originada por la interacción promiscua de los AMPs con varios receptores celulares que transducen elementos de la respuesta inmunitaria (Katzenback, 2015; Luo

and Song, 2021). Los AMPs son capaces de modular la expresión y secreción de citoquinas y quimioquinas por los leucocitos, promover la infiltración leucocitaria y la activación de linfocitos (Méndez-Samperio, 2013). Además, se ha descrito que a pesar de que los AMPs son fundamentalmente efectores de la respuesta innata, también pueden servir de puente a la respuesta adaptativa, fomentando la secreción de anticuerpos (Avila, 2016; Xia *et al*., 2018).

Entre los aproximadamente 146 AMPs descritos en peces, en esta Tesis Doctoral nos centramos en 4 de los mejor caracterizados y que sirven como modelo: NK-lisina (Nkl), hepcidina (Hamp), dicentracina (Dic) y beta-defensina (DB1). En líneas generales, estos péptidos poseen actividad antibacteriana, antiviral, antifúngica y antiparasitaria, así como moduladora de la respuesta inmune, principalmente mediando la respuesta inflamatoria (Barroso *et al*., 2021; Cuesta *et al*., 2008, 2011; Neves *et al*., 2015; Salerno *et al*., 2007; Valero *et al*., 2015b, 2020b).

El virus de la necrosis nerviosa (NNV, familia Betanodaviridae) es el agente causante de la encefalopatía y retinopatía viral (VER) que afecta a más de 170 especies de peces con consecuencias económicas devastadoras (Low *et al*., 2017). Existen especies susceptibles a la infección como la lubina, que presenta mortalidades del 100% en estadios de larvas y juveniles, o resistentes a ella como es el caso de la dorada. Aunque la aparición de recombinantes virales naturales, principalmente en el Mediterráneo, está afectando a dicha susceptibilidad. NNV afecta a órganos del sistema nervioso central, principalmente al cerebro y la retina, en los que aparecen vacuolas y zonas de necrosis que justifican las mortalidades (Bandín and Souto, 2020; Chaves-Pozo *et al*., 2012). En cuanto a su estructura, NNV es un virus pequeño no envuelto, esférico con simetría icosaédrica y con un genoma compuesto por dos moléculas de ARN de cadena simple y de sentido positivo: RNA1 que codifica la polimerasa de ARN dependiente de ARN (RdRp) implicada en la replicación viral y RNA2 que codifica la proteína estructural de la cápside (CP) implicada en la patogénesis (Sommerset and Nerland, 2004; Zhang *et al*., 2022). También se ha descrito un RNA3 obtenido mediante un procesamiento alternativo del RNA1 que codifica dos proteínas no estructurales: proteínas B1 y B2 implicadas en apoptosis (Chen *et al*., 2009; Su *et al*., 2009). En función de las diferencias de la región variable T4 del segmento de RNA1 se han descrito 4 genotipos diferentes, siendo el más

común en el área mediterránea RGNNV (Biasini *et al*., 2022; Costa and Thompson, 2016).

El conocimiento de la respuesta inmunitaria frente a NNV es fundamental para poder desarrollar tratamientos que controlen esta infección. En cuanto la respuesta humoral, la inducción de la ruta del IFN ha sido ampliamente estudiada observándose un aumento significativo en la misma en diversas especies (Álvarez-Torres *et al*., 2018; Huang *et al*., 2015; Krasnov *et al*., 2013; Lu *et al*., 2008; Valero *et al*., 2015c; Wu *et al*., 2010). De igual forma, se induce la expresión de los genes de AMPs, así como sus niveles de proteína (Valero *et al*., 2015b, 2016a, 2020a, 2020b). A nivel celular, se ha descrito una movilización de neutrófilos y macrófagos hacia el cerebro que puede inducir una tormenta de citoquinas produciendo niveles de inflamación exacerbados que aumentan el daño neuronal (Lama *et al*., 2022). Además, también se ha documentado la infiltración en los tejidos diana del virus de NCCs y CTLs $CD8^+$, además de una alta expresión de otras moléculas que participan en la CMC como las granzimas A y B o las perforinas (Chaves-Pozo *et al*., 2012, 2017, 2019b; García-Álvarez *et al*., 2024b, 2024a; Øvergård *et al*., 2013; Patel *et al*., 2008; Valero *et al*., 2018a, 2020b). La respuesta adaptativa también puede ser clave en la respuesta frente a NNV ya que se ha descrito una sobreexpresión de marcadores de linfocitos B y Th a la vez que aumentan los niveles de anticuerpos que pueden neutralizar los antígenos de NNV (Buonocore *et al*., 2017; López-Muñoz *et al*., 2012; Moreno *et al*., 2018; Scapigliati *et al*., 2010; Valero *et al*., 2018a).

A pesar de la gran importancia de NNV a nivel económico, hasta el momento hay muy pocos tratamientos disponibles para frenar su avance. Por ahora, el método profiláctico mejor estudiado son las vacunas. Se han descrito tres tipos de vacunas: de proteínas recombinantes, virus inactivados o vacunas de ADN; todas ellas pueden ser administradas de forma oral, intramuscular o intraperitoneal, aunque la mayoría de las vacunas estudiadas son del tipo recombinante y de administración intramuscular (González-Silvera *et al*., 2019; Hegde *et al*., 2005; Kim *et al*., 2014; Øvergård *et al*., 2013; Sommerset *et al*., 2005; Thiéry *et al*., 2006; Thwaite *et al*., 2020; Vimal *et al*., 2016, 2014; Yuasa *et al*., 2002). Se han propuesto otras moléculas con potencial anti-NNV entre las que destacan los AMPs. Se han realizado estudios en los que se confirma *in vivo* el potencial de los AMPs como agentes profilácticos o terapéuticos frente a virus o bacterias, además de su potencial como modulador de la respuesta inmunitaria (Masso-Silva and

Diamond, 2014). Por ello, la presente Tesis Doctoral parte de estos estudios y pretende solventar la problemática actual de brotes virales en granjas de peces aplicando los AMPs como potenciales herramientas para el control de NNV.

Objetivos

El objetivo general de esta Tesis Doctoral es evaluar la implicación de los AMPs en la respuesta inmunitaria de peces teleósteos con especial énfasis en su aplicación como agentes antivirales en lubina contra la infección por NNV.

Para alcanzar el objetivo general, se plantean los siguientes objetivos específicos:

1. Evaluar la capacidad inmunomoduladora *in vitro* de AMPs de lubina en leucocitos de riñón cefálico (HKLs) de lubina y dorada.
2. Administrar plásmidos codificantes de AMPs a juveniles de lubina para determinar su potencial inmunoestimulador y/o como agentes preventivos frente a NNV.
3. Aplicar los AMPs sintéticos hepcidina y dicentracina a lubina como agentes preventivos frente a NNV.
4. Evaluar la aplicación terapéutica de péptidos NK-lisina, hepcidina y dicentracina sintética a lubinas como tratamiento anti-NNV.

Principales resultados y discusión

La aparición de brotes naturales de infecciones, como NNV, que amenazan la viabilidad económica de la acuicultura hacen necesario el desarrollo de nuevas moléculas que puedan servir como fármacos. Por ello, esta Tesis Doctoral se centra en estudiar la aplicación de AMPs como agentes profilácticos y terapéuticos frente a NNV.

El primer paso del estudio fue la caracterización estructural *in silico* de los AMPs de lubina. Este análisis reveló que Nkl y Dic presentan una estructura en alfa hélice mientras que Hamp posee estructura en lámina beta con enlaces disulfuro. Ambas estructuras se sabe que confieren propiedades antimicrobianas y, en concreto, antivirales (Freitas *et al*., 2020; Personne *et al*., 2023). Además, se encontró una alta homología entre los AMPs de lubina y dorada sugiriendo funciones conservadas en ambas especies, así como su potencial para ser usados indistintamente en ambas especies. Los AMPs no

fueron citotóxicos para los HKLs de dorada y lubina por lo que se prosiguió a realizar los ensayos en los que se evaluó el potencial inmunomodulador *in vitro* de los péptidos. Desafortunadamente, los péptidos utilizados no fueron capaces de modular el estallido respiratorio ni la fagocitosis. Sin embargo, alteraron el patrón de expresión génica en ambas especies, aunque de forma completamente opuesta. En dorada, se apreció una marcada tendencia a la inmunosupresión reflejada por la inhibición de genes relacionados con la inflamación, el interferón, la CMC, AMPs y marcadores celulares. Por lo tanto, los AMPs de lubina no parecen ser efectivos para su aplicación en esta especie como inmunoestimulantes. De manera contraria a estos resultados, en lubina, varios genes del sistema inmunitario se vieron modulados. Nkl, Hamp y Dic inducen un estado antiinflamatorio mediante el aumento de la expresión de genes como *il10* o *tgfb* como ya apuntaban algunos estudios (Huang *et al*., 2019; Pan *et al*., 2011; Valero *et al*., 2021) pero contradiciendo otros en los que se describía su acción proinflamatoria (Álvarez *et al*., 2022; Masso-Silva and Diamond, 2014; Neves *et al*., 2011; Zhang *et al*., 2014). Todos los AMPs probados aumentaron los niveles de *ighm*, considerado un marcador de linfocitos B, conectando así la respuesta innata con la adaptativa, función que se está describiendo recientemente para los AMPs (Ma *et al*., 2020). También, se incrementaron los niveles de expresión de diversos genes de AMPs, así como de marcadores de la respuesta de CMC datos que sugieren una acción sinérgica entre ambas rutas, como también lo hacen otros estudios (Chaves-Pozo *et al*., 2012, 2017, 2019b; Valero *et al*., 2015b, 2018a, 2020a, 2020b). Nkl fue el único péptido que incrementó la transcripción de genes relacionados con la ruta del interferón. Este resultado confirmó el potencial de Nkl como agente antiviral como ya lo sugerían algunos estudios (Ding *et al*., 2019; León *et al*., 2020b; Zhang *et al*., 2014). La modulación de la respuesta inmune descrita unida a su incapacidad de los AMPs de generar citotoxicidad hace de ellos una excelente opción para su aplicación *in vivo* en lubina.

El siguiente paso de la investigación fue probar el poder de los AMPs seleccionados como agentes preventivos o paliativos frente a la infección por NNV administrándolo en forma de plásmidos o péptidos sintéticos. Primero se estudió la respuesta inmunitaria que desencadena la administración de 4 plásmidos codificantes de diferentes AMPs: pNKL (Nkl), pHAMP2 (Hamp), pDIC (Dic) y pDB1 (DB1). Dichos plásmidos fueron transcritos correctamente y aumentaron los niveles circulantes de dichos AMPs y la capacidad bactericida. Se observó, además, un gran aumento de los marcadores proinflamatorios y

de adhesión celular como *il1b*, *il8*, *cxcr3* y *cxcl9* en el lugar de la inyección, el músculo, concordando con los estudios que describen propiedades proinflamatorias para los AMPs (Acosta *et al*., 2019; Jiang *et al*., 2018; Lin *et al*., 2016; Pan *et al*., 2017; Valero *et al*., 2021; Zhang *et al*., 2013). Tras comprobar que los plásmidos eran capaces de estimular la respuesta inmunitaria, se realizó una infección con NNV para evaluar su capacidad preventiva. Desafortunadamente, ninguno de los plásmidos testados mejoró la supervivencia frente a NNV y, de hecho, el tratamiento con pHAMP2 aumentó la mortalidad. El aumento de los marcadores inflamatorios parece ser la causa del incremento de las mortalidades debido a que los procesos inflamatorios exacerbados se han relacionado con aumento de lesiones tisulares y daños cerebrales durante la infección por NNV (Chiang *et al*., 2017; Montes *et al*., 2010; Poisa-Beiro *et al*., 2008). En el caso concreto de pHAMP2, se observó una retroalimentación positiva entre el gen *hamp2*, con funciones antimicrobianas, y *hamp1*, relacionado con el metabolismo del hierro. Esto puede derivar en episodios de anemia que disminuyan la capacidad inmunitaria de los eritrocitos y comprometan la eliminación viral (Neves *et al*., 2016, 2015; Stosik *et al*., 2018). Por lo tanto, el tipo de estimulación de la respuesta inmunitaria generada hace que los plásmidos codificantes de AMPs no sean un buen agente antiviral y su evaluación como método paliativo fue descartada.

Los estudios continuaron con la evaluación de la capacidad inmunomoduladora de los péptidos sintéticos Hamp y Dic, ya que la evaluación de Nkl había sido previamente realizada (Valero *et al*., 2021). Al contrario de lo observado tras la administración de plásmidos, se redujeron los niveles de marcadores proinflamatorios *il1b*, *il6* y *cox2*, pero no se modificó la expresión de ningún gen relacionado con la adhesión celular. A pesar de esto, sí que se observó un incremento de los niveles transcripcionales de los marcadores de neutrófilos, macrófagos y linfocitos B, *mpo*, *mcsf1r* e *ighm* en el sitio de inyección del tratamiento, el músculo. Esta modulación de la respuesta inmunitaria podría suponer para el hospedador una ventaja a la hora de luchar contra infecciones virales, por lo que se valoró su potencial preventivo. Estos resultados fueron muy satisfactorios ya que se mejoraron los niveles de supervivencia, siendo el índice relativo de supervivencia (RPS) con respecto a los peces no tratados de un 26,6% en el caso de Hamp y de un 33,3% para Dic. Estos datos están en concordancia con los obtenidos en otros estudios previos para Hamp y epinecidina (Wang *et al*., 2010b, 2010a). Sin embargo, no se modificaron los niveles de transcripción de NNV ni los títulos virales sugiriendo que el mecanismo de

acción de los AMPs testados no es lítico directo contra el virus sino mediante la modulación de la respuesta inmunitaria del huésped. Para determinar qué mecanismos inmunes están siendo estimulados, se analizaron genes clave de distintas rutas inmunitarias tras la infección y el tratamiento. En el tejido diana del virus, el cerebro, se observó la inducción de un estado antiinflamatorio gracias al aumento de la expresión de *il10* y el bloqueo de la de *il6* que podría explicar el aumento de supervivencia observado. A la vez, se incrementa la expresión del complejo Cxcr3/Cxcl9, que se induce por interferón y promueve la infiltración de linfocitos T y otros tipos celulares (Guo *et al*., 2018). De hecho, nuestros datos mostraron un aumento del reclutamiento de CTLs, neutrófilos y macrófagos, tipos celulares clave en la resolución de infecciones virales (Lama *et al*., 2022; Nakanishi *et al*., 2011; Poisa-Beiro *et al*., 2008). En suma, estos datos demuestran el potencial de aplicación de Hamp y Dic para conferir protección parcial a la infección por NNV.

Por último, se evaluó la capacidad terapéutica de Nkl, Hamp y Dic frente a NNV. De manera similar a lo observado cuando se administraban antes de la infección, se registró un RPS de 30% para Hamp y Dic similar a lo observado en otras especies como mero o medaka (Wang *et al*., 2010b, 2010a). Sin embargo, Nkl sólo fue capaz de retrasar la aparición de las mortalidades, pero no de frenarlas. Al igual que en el caso de su aplicación preventiva, la administración de Hamp y Dic después de la infección no redujo la carga viral ni la cantidad de proteína de la cápside de NNV producida, descartando, de nuevo, un efecto directo sobre el virus. En relación con el potencial inmunoestimulador de Hamp y Dic, nuestros resultados mostraron un aumento muy significativo de los niveles de *il10* en cerebro tras la administración de todos los tratamientos, promoviendo un ambiente antiinflamatorio, pero manteniendo los niveles de expresión de citoquinas proinflamatorias inducidas por la infección. Esto, unido al incremento de la transcripción de quimioquinas implicadas en el reclutamiento celular, favorece la llegada de leucocitos al sitio de infección. Entre ellos, se observó un aumento de la transcripción del marcador de macrófagos, un tipo celular capaz de restaurar los niveles normales de citoquinas y revertir el daño tisular inducido por la inflamación (Rieger *et al*., 2015; Soliman and Barreda, 2023; Wang *et al*., 2021). No obstante, en el caso de Nkl, no se estimuló este marcador, pero sí marcadores relacionados con la respuesta citotóxica, *nccrp1* y *nkl*. A pesar de que estos genes habían sido previamente relacionados con la resolución de infecciones virales (Chaves-Pozo *et al*., 2019a, 2017, 2012), nuestros datos evidencian

que la estimulación de esta ruta no es suficiente para combatir la infección por NNV en lubina.

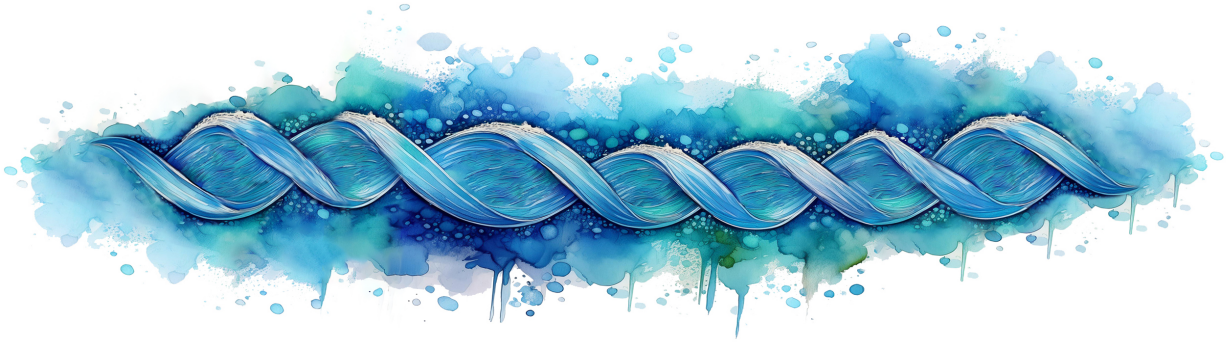
Conclusiones

Las conclusiones de esta Tesis Doctoral son las siguientes:

1. Los péptidos sintéticos Nkl, Hamp y Dic de lubina no son citotóxicos para los leucocitos de riñón cefálico de lubina ni de dorada, pero tampoco son capaces de modular el estallido respiratorio ni la fagocitosis in vitro.
2. Los péptidos Nkl, Hamp y Dic inducen la expresión de genes relacionados con procesos antinflamatorios y respuesta del interferón, citotoxicidad mediada por leucocitos y producción de anticuerpos en los leucocitos de lubina, mientras que producen inmunosupresión en los de dorada.
3. Los péptidos sintéticos evaluados podrían ser empleados como herramientas biotecnologías para la inmunoestimulación y el control antiviral en el cultivo de la lubina, pero no para la dorada.
4. Los plásmidos codificantes de Nkl, Hamp, Dic y DB1 se transcriben en el músculo de la lubina aumentando los niveles del AMP correspondiente en suero y potenciando la actividad bactericida.
5. Los plásmidos codificantes de AMPs testados inducen inflamación local e infiltración leucocitaria en el músculo de lubinas, pero no son capaces de aumentar la resistencia frente a NNV, teniendo el pHAMPs incluso efectos negativos en esta.
6. La inyección de los péptidos sintéticos Hamp y Dic bloqueó la producción de moléculas proinflamatorias a nivel local y sistémico en lubina mientras que indujo una fuerte movilización de macrófagos y neutrófilos.
7. Los péptidos sintéticos Hamp y Dic administrados antes o después de la infección con NNV mejoraron la supervivencia y los síntomas clínicos de la enfermedad, mientras que Nkl administrado después de la infección no.
8. La administración de los péptidos Hamp y Dic es incapaz de alterar los niveles virales en lubina, lo que sugiere que su mecanismo de acción es mediante su capacidad inmunomoduladora, y no por sus propiedades microbicidas directas.

9. La acción tanto profiláctica como terapéutica de los péptidos Hamp y Dic se debe a la inducción de la infiltración leucocitaria en el cerebro que revierte la inflamación exacerbada provocada por la infección por NNV.
10. Los péptidos sintéticos Hamp y Dic pueden ser considerados como buenas herramientas para tratar los brotes de NNV en granjas de lubina, ya sea como tratamiento preventivo o paliativo.

REFERENCES



Abbas, A.K., Lichtman, A.H., Pillai, S., 2012. Inmunología celular y molecular, 7º Edition. ed. Elsevier B.V., Philadelphia.

Acosta, J., Carpio, Y., Valdés, I., Velázquez, J., Zamora, Y., Morales, R., Morales, A., Rodríguez, E., Estrada, M.P., 2014. Co-administration of tilapia alpha-helical antimicrobial peptides with subunit antigens boost immunogenicity in mice and tilapia (*Oreochromis niloticus*). Vaccine 32, 223–229. https://doi.org/10.1016/j.vaccine.2013.11.009

Acosta, J., Roa, F., González-Chavarría, I., Astuya, A., Maura, R., Montesino, R., Muñoz, C., Camacho, F., Saavedra, P., Valenzuela, A., Sánchez, O., Toledo, J.R., 2019. *In vitro* immunomodulatory activities of peptides derived from *Salmo salar* NK-lysin and cathelicidin in fish cells. Fish Shellfish Immunol. 88, 587–594. https://doi.org/10.1016/j.fsi.2019.03.034

Ahmed, T.A.E., Hammami, R., 2019. Recent insights into structure–function relationships of antimicrobial peptides. J. Food Biochem. 43, 1–8. https://doi.org/10.1111/jfbc.12546

Álvarez-Torres, D., Gómez-Abellán, V., Arizcun, M., García-Rosado, E., Béjar, J., Sepulcre, M.P., 2018. Identification of an interferon-stimulated gene, isg15, involved in host immune defense against viral infections in gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 73, 220–227. https://doi.org/10.1016/j.fsi.2017.12.027

Álvarez, C.A., Acosta, F., Montero, D., Guzmán, F., Torres, E., Vega, B., Mercado, L., 2016. Synthetic hepcidin from fish: Uptake and protection against *Vibrio anguillarum* in sea bass (*Dicentrarchus labrax*). Fish Shellfish Immunol. 55, 662–670. https://doi.org/10.1016/j.fsi.2016.06.035

Álvarez, C.A., Guzmán, F., Cárdenas, C., Marshall, S.H., Mercado, L., 2014. Antimicrobial activity of trout hepcidin. Fish Shellfish Immunol. 41, 93–101. https://doi.org/10.1016/j.fsi.2014.04.013

Álvarez, C.A., Santana, P.A., Salinas-Parra, N., Beltrán, D., Guzmán, F., Vega, B., Acosta, F., Mercado, L., 2022. Immune modulation ability of Hepcidin from teleost

fish. Animals 12, 1–14. https://doi.org/10.3390/ani12121586

APROMAR, 2023. La Acuicultura en Espana. La Asoc. Empres. Acuic. España 107.

APROMAR, 2020. Informe La Acuicultura en España, in: Informe Realizado Por La Asociación Empresarial de Acuicultura de España (APROMAR). p. 95.

Avila, E.E., 2016. Functions of antimicrobial peptides in vertebrates. Curr. Protein Pept. Sci. 18, 1098–1119. https://doi.org/10.2174/1389203717666160813162629

Azad, I.S., Jithendran, K.P., Shekhar, M.S., Thirunavukkarasu, A.R., de la Pena, L.D., 2006. Immunolocalisation of nervous necrosis virus indicates vertical transmission in hatchery produced Asian sea bass (*Lates calcarifer* Bloch)-A case study. Aquaculture 255, 39–47. https://doi.org/10.1016/j.aquaculture.2005.04.076

Azad, I.S., Shekhar, M.S., Thirunavukkarasu, A.R., Poornima, M., Kailasam, M., Rajan, J.J.S., Ali, S.A., Abraham, M., Ravichandran, P., 2005. Nodavirus infection causes mortalities in hatchery produced larvae of *Lates calcarifer*: first report from India. Dis. Aquat. Organ. 63, 113–118.

Bae, J., Hee, S., Don, S., Park, M., Jee, B., An, C., Kim, Y., Kim, J., Park, C., 2014. Expression analysis and biological activity of moronecidin from rock. Fish Shellfish Immunol. 40, 345–353. https://doi.org/10.1016/j.fsi.2014.07.023

Bandín, I., Souto, S., 2020. Betanodavirus and VER disease : A 30-year. Pathogens 9, 1–46. https://doi.org/10.3390/pathogens9020106

Barroso, C., Carvalho, P., Gonçalves, J.F.M., Rodrigues, P.N.S., Neves, J. V., 2021. Antimicrobial peptides: Identification of two beta-defensins in a teleost fish, the European sea bass (*Dicentrarchus labrax*). Pharmaceuticals 14, 1–16. https://doi.org/10.3390/ph14060566

Bassity, E., Clark, T.G., 2012. Functional identification of dendritic cells in the teleost model, rainbow trout (*Oncorhynchus mykiss*). PLoS One 7. https://doi.org/10.1371/journal.pone.0033196

Biasini, L., Berto, P., Abbadi, M., Buratin, A., Toson, M., Marsella, A., Toffan, A., Pascoli, F., 2022. Pathogenicity of different betanodavirus RGNNV/SJNNV

reassortant strains in European sea bass. Pathogens 11, 1–15. https://doi.org/10.3390/pathogens11040458

Bird, S., Tafalla, C., 2015. Teleost chemokines and their receptors. Biology (Basel). 4, 756–784. https://doi.org/10.3390/biology4040756

Bloch, B., Gravningen, K., Larsen, J., 1991. Encephalomyelitis among turbot associated with a picornavirus-like agent. Dis. Aquat. Organ. 10, 65–70. https://doi.org/10.3354/dao010065

Boparai, J.K., Sharma, P.K., 2019. Mini review on antimicrobial peptides, sources, mechanism and recent applications. Protein Pept. Lett. 27, 4–16. https://doi.org/10.2174/0929866526666190822165812

Boraschi, D., 2022. What is IL-1 for? The functions of interleukin-1 across evolution. Front. Immunol. 13, 1–12. https://doi.org/10.3389/fimmu.2022.872155

Boudinot, P., Langevin, C., Secombes, C.J., Levraud, J.P., 2016. The peculiar characteristics of fish type I interferons. Viruses 8, 1–17. https://doi.org/10.3390/v8110298

Breuil, G., Bonami, J.R., Pepin, J.F., Pichot, Y., 1991. Viral infection (picorna-like virus) associated with mass mortalities in hatchery-reared sea-bass (*Dicentrarchus labrax*) larvae and juveniles. Aquaculture 97, 109–116. https://doi.org/10.1016/0044-8486(91)90258-9

Breuil, G., Pépin, J.F.P., Boscher, S., Thiéry, R., 2002. Experimental vertical transmission of nodavirus from broodfish to eggs and larvae of the sea bass, *Dicentrarchus labrax* (L.). J. Fish Dis. 25, 697–702. https://doi.org/10.1046/j.1365-2761.2002.00406.x

Breuil, G., Romestand, B., 1999. A rapid ELISA method for detecting specific antibody level against nodavirus in the serum of the sea bass, *Dicentrarchus labrax* (L.): Application to the screening of spawners in a sea bass hatchery. J. Fish Dis. 22, 45–52. https://doi.org/10.1046/j.1365-2761.1999.00136.x

Broekman, D.C., Zenz, A., Gudmundsdottir, B.K., Lohner, K., Maier, V.H., Gudmundsson, G.H., 2011. Functional characterization of codCath, the mature

cathelicidin antimicrobial peptide from Atlantic cod (*Gadus morhua*). Peptides 32, 2044–2051. https://doi.org/10.1016/j.peptides.2011.09.012

Brogden, K.A., 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3, 238–250. https://doi.org/10.1038/nrmicro1098

Buchmann, K., 2022. Control of parasitic diseases in aquaculture. Parasitology 149, 1985–1997. https://doi.org/10.1017/S0031182022001093

Buonocore, F., Stocchi, V., Nunez-Ortiz, N., Randelli, E., Gerdol, M., Pallavicini, A., Facchiano, A., Bernini, C., Guerra, L., Scapigliati, G., Picchietti, S., 2017. Immunoglobulin T from sea bass (*Dicentrarchus labrax* L.): Molecular characterization, tissue localization and expression after nodavirus infection. BMC Mol. Biol. 18, 1–14. https://doi.org/10.1186/s12867-017-0085-0

Cao, J., Xu, H., Yu, Y., Xu, Z., 2023. Regulatory roles of cytokines in T and B lymphocytes-mediated immunity in teleost fish. Dev. Comp. Immunol. 144, 104621. https://doi.org/10.1016/j.dci.2022.104621

Cervera, L., González-Fernández, C., Arizcun, M., Cuesta, A., Chaves-Pozo, E., 2022. Severe natural outbreak of *Cryptocaryon irritans* in gilthead seabream produces leukocyte mobilization and innate immunity at the gill tissue. Int. J. Mol. Sci. 23. https://doi.org/10.3390/ijms23020937

Chaves-Pozo, E., Bandín, I., Olveira, J.G., Esteve-Codina, A., Gómez-Garrido, J., Dabad, M., Alioto, T., Ángeles Esteban, M., Cuesta, A., 2019a. European sea bass brain DLB-1 cell line is susceptible to nodavirus: A transcriptomic study. Fish Shellfish Immunol. 86, 14–24. https://doi.org/10.1016/j.fsi.2018.11.024

Chaves-Pozo, E., Dabad, M., Valero, Y., Cuesta, A., Esteban, M.Á., Gómez-Garrido, J., Meseguer, J., Alioto, T., Esteve-Codina, A., 2017. Innate cell-mediated cytotoxic activity of European sea bass leucocytes against Nodavirus-infected cells: A functional and RNA-seq study. Sci. Rep. 7, 1–15. https://doi.org/10.1038/s41598-017-15629-6

Chaves-Pozo, E., Guardiola, F.A., Meseguer, J., Esteban, M.A., Cuesta, A., 2012. Nodavirus infection induces a great innate cell-mediated cytotoxic activity in

resistant, gilthead seabream, and susceptible, European sea bass, teleost fish. Fish Shellfish Immunol. 33, 1159–1166. https://doi.org/10.1016/j.fsi.2012.09.002

Chaves-Pozo, E., Valero, Y., Lozano, M.T., Rodríguez-Cerezo, P., Miao, L., Campo, V., Esteban, M.A., Cuesta, A., 2019b. Fish granzyme a shows a greater role than granzyme b in fish innate cell-mediated cytotoxicity. Front. Immunol. 10, 1–14. https://doi.org/10.3389/fimmu.2019.02579

Chen, H.H., Lin, H.T., Foung, Y.F., Han-You Lin, J., 2012. The bioactivity of teleost IL-6: IL-6 protein in orange-spotted grouper (*Epinephelus coioides*) induces Th2 cell differentiation pathway and antibody production. Dev. Comp. Immunol. 38, 285–294. https://doi.org/10.1016/j.dci.2012.06.013

Chen, J., Lv, Y.P., Dai, Q.M., Hu, Z.H., Liu, Z.M., Li, J.H., 2019. Host defense peptide LEAP-2 contributes to monocyte/macrophage polarization in barbel steed (*Hemibarbus labeo*). Fish Shellfish Immunol. 87, 184–192. https://doi.org/10.1016/j.fsi.2019.01.015

Chen, L.J., Su, Y.C., Hong, J.R., 2009. Betanodavirus non-structural protein B1: A novel anti-necrotic death factor that modulates cell death in early replication cycle in fish cells. Virology 385, 444–454. https://doi.org/10.1016/j.virol.2008.11.048

Chen, R.Y., Chen, J., Liu, Z.M., Lin, Z.H., Guo, Z.P., 2021. Barbel steed (*Hemibarbus labeo*) NK-lysin protects against *Aeromonas hydrophila* infection via immunomodulatory activity. Dev. Comp. Immunol. 122, 104114. https://doi.org/10.1016/j.dci.2021.104114

Chia, T.J., Wu, Y.C., Chen, J.Y., Chi, S.C., 2010. Antimicrobial peptides (AMP) with antiviral activity against fish nodavirus. Fish Shellfish Immunol. 28, 434–439. https://doi.org/10.1016/j.fsi.2009.11.020

Chiang, Y.H., Wu, Y.C., Chi, S.C., 2017. Interleukin-1β secreted from betanodavirus-infected microglia caused the death of neurons in giant grouper brains. Dev. Comp. Immunol. 70, 19–26. https://doi.org/10.1016/j.dci.2017.01.002

Cho, Y.S., Lee, S.Y., Kim, K.H., Kim, S.K., Kim, D.S., Nam, Y.K., 2009. Gene structure and differential modulation of multiple rockbream (*Oplegnathus fasciatus*) hepcidin

isoforms resulting from different biological stimulations. Dev. Comp. Immunol. 33, 46–58. https://doi.org/10.1016/j.dci.2008.07.009

Coeurdacier, J.L., Laporte, F., Pepin, J.F., 2003. Preliminary approach to find synthetic peptides from nodavirus capsid potentially protective against sea bass viral encephalopathy and retinopathy. Fish Shellfish Immunol. 14, 435–447. https://doi.org/10.1006/fsim.2002.0449

Collet, B., Secombes, C.J., 2002. Type I-interferon signalling in fish. Fish Shellfish Immunol. 12, 389–397. https://doi.org/10.1006/fsim.2001.0405

Costa, J.Z., Thompson, K.D., 2016. Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. Fish Shellfish Immunol. 53, 35–49. https://doi.org/10.1016/j.fsi.2016.03.033

Cuesta, A., Meseguer, J., Esteban, M.Á., 2011. Molecular and functional characterization of the gilthead seabream β-defensin demonstrate its chemotactic and antimicrobial activity. Mol. Immunol. 48, 1432–1438. https://doi.org/10.1016/j.molimm.2011.03.022

Cuesta, A., Meseguer, J., Esteban, M.Á., 2008. The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream. Mol. Immunol. 45, 2333–2342. https://doi.org/10.1016/j.molimm.2007.11.007

Dalla Valle, L., Toffolo, V., Lamprecht, M., Maltese, C., Bovo, G., Belvedere, P., Colombo, L., 2005. Development of a sensitive and quantitative diagnostic assay for fish nervous necrosis virus based on two-target real-time PCR. Vet. Microbiol. 110, 167–179. https://doi.org/10.1016/j.vetmic.2005.07.014

Das, S., Pradhan, C., Pillai, D., 2022. β-Defensin: An adroit saviour in teleosts. Fish Shellfish Immunol. 123, 417–430. https://doi.org/10.1016/j.fsi.2022.03.017

Dezfuli, B.S., Lorenzoni, M., Carosi, A., Giari, L., Bosi, G., 2023. Teleost innate immunity, an intricate game between immune cells and parasites of fish organs: who wins, who loses. Front. Immunol. 14, 1–12.

https://doi.org/10.3389/fimmu.2023.1250835

Díaz-Rosales, P., Muñoz-Atienza, E., Tafalla, C., 2018. Role of teleost B cells in viral immunity. Fish Shellfish Immunol. 86, 135–142. https://doi.org/10.1016/j.fsi.2018.11.039

Ding, F.F., Li, C.H., Chen, J., 2019. Molecular characterization of the NK-lysin in a teleost fish, *Boleophthalmus pectinirostris*: Antimicrobial activity and immunomodulatory activity on monocytes/macrophages. Fish Shellfish Immunol. 92, 256–264. https://doi.org/10.1016/j.fsi.2019.06.021

Douglas, S.E., Patrzykat, A., Pytyck, J., Gallant, J.W., 2003. Identification, structure and differential expression of novel pleurocidins clustered on the genome of the winter flounder , *Pseudopleuronectes americanus* (Walbaum). Eur. J. Biochem. 270, 3720–3730. https://doi.org/10.1046/j.1432-1033.2003.03758.x

Drayton, M., Deisinger, J.P., Ludwig, K.C., Raheem, N., Müller, A., Schneider, T., Straus, S.K., 2021. Host defense peptides: Dual antimicrobial and immunomodulatory action. Int. J. Mol. Sci. 22, 1–16. https://doi.org/10.3390/ijms222011172

Ellis, A.E., 2001. Innate host defense mechanisms of fish against viruses and bacteria. Dev. Comp. Immunol. 25, 827–839.

Evans, D.L., Harris, D.T., Jaso-Friedmann, L., 1992. Function associated molecules on nonspecific cytotoxic cells: Role in calcium signaling, redirected lysis, and modulation of cytotoxicity. Dev. Comp. Immunol. 16, 383–394. https://doi.org/10.1016/0145-305X(92)90040-J

Evans, D.L., Leary, J.H., Jaso-Friedmann, L., 2001. Nonspecific cytotoxic cells and innate immunity: Regulation by programmed cell death. Dev. Comp. Immunol. 25, 791–805. https://doi.org/10.1016/S0145-305X(01)00036-2

Falco, A., Ortega-Villaizan, M., Chico, V., Brocal, I., Perez, L., Coll, J., Estepa, A., 2009. Antimicrobial peptides as model molecules for the development of novel antiviral agents in aquaculture. Mini-Reviews Med. Chem. 9, 1159–1164. https://doi.org/10.2174/138955709789055171

FAO, 2022. The state of world fisheries and aquaculture 2022, Towards Blue Transformation. FAO, Rome. https://doi.org/10.4060/cc0463en

Fernandes, J.M.O., Molle, G., Kemp, G.D., Smith, V.J., 2004. Isolation and characterisation of oncorhyncin II , a histone H1-derived antimicrobial peptide from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 28, 127–138. https://doi.org/10.1016/S0145-305X(03)00120-4

Fiorella, K.J., Okronipa, H., Baker, K., Heilpern, S., 2021. Contemporary aquaculture: implications for human nutrition. Curr. Opin. Biotechnol. 70, 83–90. https://doi.org/10.1016/j.copbio.2020.11.014

Freitas, E.D., Bataglioli, A., Oshodi, J., Beppu, M.M., 2020. Antimicrobial peptides and their potential application in antiviral coating agents. Colloids Surfaces B Biointerfaces.

García-Álvarez, M.A., Cervera, L., Valero, Y., González-Fernández, C., Mercado, L., Chaves-Pozo, E., Cuesta, A., 2024a. Regulation and distribution of European sea bass perforins point to their role in the adaptive cytotoxic response against NNV. Fish Shellfish Immunol. 144. https://doi.org/10.1016/j.fsi.2023.109244

García-Álvarez, M.A., Chaves-Pozo, E., Cuesta, A., 2024b. Cytotoxic activity and gene expression during in vitro adaptive cell-mediated cytotoxicity of head-kidney cells from betanodavirus-infected European sea bass. Dev. Comp. Immunol. 152. https://doi.org/10.1016/j.dci.2023.105124

García-Beltrán, J.M., Arizcun, M., Chaves-Pozo, E., 2023. Antimicrobial Peptides from Photosynthetic Marine Organisms with Potential Application in Aquaculture. Mar. Drugs 21, 1–17. https://doi.org/10.3390/md21050290

Gómez-Manrique, W., Pereira Figueiredo, M.A., Charlie-Silva, I., Antonio de Andrade Belo, M., Dib, C.C., 2019. Spleen melanomacrophage centers response of Nile tilapia during *Aeromanas hydrophila* and *Mycobacterium marinum* infections. Fish Shellfish Immunol. 95, 514–518. https://doi.org/10.1016/j.fsi.2019.10.071

González-Silvera, D., Guardiola, F.A., Espinosa, C., Chaves-Pozo, E., Esteban, M.Á., Cuesta, A., 2019. Recombinant nodavirus vaccine produced in bacteria and

administered without purification elicits humoral immunity and protects European sea bass against infection. Fish Shellfish Immunol. 88, 458–463. https://doi.org/10.1016/j.fsi.2019.03.013

Groom, J.R., Luster, A.D., 2013. CXCR3 ligands: redundant, collaborative and antagonistic functions. Immunol Cell Biol. 89. https://doi.org/10.1038/icb.2010.158.CXCR3

Grotmol, S., Bergh, Ø., Totland, G.K., 1999. Transmission of viral encephalopathy and retinopathy (VER) to yolk-sac larvae of the Atlantic halibut *Hippoglossus hippoglossus*: Occurrence of nodavirus in various organs and a possible route of infection. Dis. Aquat. Organ. 36, 95–106. https://doi.org/10.3354/dao036095

Gui, L., Zhang, P., Zhang, Q., Zhang, J., 2016. Two hepcidins from spotted scat (*Scatophagus argus*) possess antibacterial and antiviral functions in vitro. Fish Shellfish Immunol. 50, 191–199. https://doi.org/10.1016/j.fsi.2016.01.038

Guo, M., Wei, J., Huang, X., Huang, Y., Qin, Q., 2012. Antiviral effects of β-defensin derived from orange-spotted grouper (*Epinephelus coioides*). Fish Shellfish Immunol. 32, 828–838. https://doi.org/10.1016/j.fsi.2012.02.005

Guo, Y.C., Chiu, Y.H., Chen, C.P., Wang, H.S., 2018. Interleukin-1β induces CXCR3-mediated chemotaxis to promote umbilical cord mesenchymal stem cell transendothelial migration. Stem Cell Res. Ther. 9, 1–15. https://doi.org/10.1186/s13287-018-1032-9

Haney, E.F., Mansour, S.C., Hancock, R.E.W., 2016. Antimicrobial peptides: An introduction. Methods Mol. Biol. 1548, 3–22. https://doi.org/10.1007/978-1-4939-6737-7_1

Hao, D. fang, Wang, G. hua, Li, N. qiu, Liu, H. mei, Wang, C. biao, Liu, W. qing, Yan, X., Zhang, M., 2022. Antimicrobial and immunoregulatory activities of the derived peptide of a natural killer lysin from black rockfish (*Sebastes schlegelii*). Fish Shellfish Immunol. 123, 369–380. https://doi.org/10.1016/j.fsi.2022.03.020

Hao, X., Chi, H., Tang, X., Xing, J., Sheng, X., Zhan, W., 2021. The functions of β-defensin in flounder (*Paralichthys olivaceus*): Antibiosis, chemotaxis and

modulation of phagocytosis. Biology (Basel). 10, 1247. https://doi.org/10.3390/biology10121247

Harte, A., Tian, G., Xu, Q., Secombes, C.J., Wang, T., 2020. Five subfamilies of β-defensin genes are present in salmonids: Evolutionary insights and expression analysis in Atlantic salmon *Salmo salar*. Dev. Comp. Immunol. 104, 103560. https://doi.org/10.1016/j.dci.2019.103560

Harwood, N.E., Batista, F.D., 2010. Early events in B cell activation. Annu. Rev. Immunol. 28, 185–210. https://doi.org/10.1146/annurev-immunol-030409-101216

Hegde, A., Lam, T.J., Sin, Y.M., 2005. Immune response of freshwater fish, guppy, *Poicelia reticulata* and gouramy, *Trichogaster trichopterus* to recombinant coat protein of *Epinephelus tauvina* nervous necrosis virus. Aquaculture 249, 77–84. https://doi.org/10.1016/j.aquaculture.2005.04.053

Hsieh, J.C., Pan, C.Y., Chen, J.Y., 2010. Tilapia hepcidin (TH)2-3 as a transgene in transgenic fish enhances resistance to *Vibrio vulnificus* infection and causes variations in immune-related genes after infection by different bacterial species. Fish Shellfish Immunol. 29, 430–439. https://doi.org/10.1016/j.fsi.2010.05.001

Huang, H.N., Pan, C.Y., Rajanbabu, V., Chan, Y.L., Wu, C.J., Chen, J.Y., 2011. Modulation of immune responses by the antimicrobial peptide, epinecidin (Epi)-1, and establishment of an Epi-1-based inactivated vaccine. Biomaterials 32, 3627–3636. https://doi.org/10.1016/j.biomaterials.2011.01.061

Huang, P.H., Chen, J.Y., Kuo, C.M., 2007. Three different hepcidins from tilapia, *Oreochromis mossambicus*: Analysis of their expressions and biological functions. Mol. Immunol. 44, 1922–1934. https://doi.org/10.1016/j.molimm.2006.09.031

Huang, R., Zhou, Q., Shi, Y., Zhang, J., He, J., Xie, J., 2018. Protein A from orange-spotted nervous necrosis virus triggers type I interferon production in fish cell. Fish Shellfish Immunol. 79, 234–243. https://doi.org/10.1016/j.fsi.2018.05.006

Huang, X., Hu, B., Yang, X., Gong, L., Tan, J., Deng, L., 2019. The putative mature peptide of piscidin-1 modulates global transcriptional profile and proliferation of splenic lymphocytes in orange-spotted grouper (*Epinephelus coioides*). Fish

Shellfish Immunol. 86, 1035–1043. https://doi.org/10.1016/j.fsi.2018.12.045

Huang, Y., Huang, X., Cai, J., Yang, Z., Wei, S., Wei, J., Qin, Q., 2015. Identification of orange-spotted grouper (*Epinephelus coioides*) interferon regulatory factor 3 involved in antiviral immune response against fish RNA virus. Fish Shellfish Immunol. 42, 345–352. https://doi.org/10.1016/j.fsi.2014.11.025

Huang, Y., Zheng, Q., Niu, J., Tang, J., Wang, B., Abarike, E.D., Lu, Y., Cai, J., Jian, J., 2018. NK-lysin from *Oreochromis niloticus* improves antimicrobial defence against bacterial pathogens. Fish Shellfish Immunol. 72, 259–265. https://doi.org/10.1016/j.fsi.2017.11.002

Huang, Y.C., Han, Y.S., 2014. Determining anti-betanodavirus compounds through a GF-1 cell-based screening platform. Antiviral Res. 105, 47–53. https://doi.org/10.1016/j.antiviral.2014.02.018

Huang, Y.C., Lin, T.S., Peng, C., Chan, N.L., Han, Y.S., 2016. Strong inhibition of betanodavirus replication by ribavirin targeting RNA-dependent RNA polymerase. J. Fish Dis. 39, 619–623. https://doi.org/10.1111/jfd.12398

Iwamoto, T., Mise, K., Takeda, A., Okinaka, Y., Mori, K., Arimoto, M., Okuno, T., Nakai, T., 2005. Characterization of Striped jack nervous necrosis virus subgenomic RNA3 and biological activities of its encoded protein B2. J. Gen. Virol. 86, 2807–2816. https://doi.org/10.1099/vir.0.80902-0

Jaso-Friedmann, L., Leary, J.H., Evans, D.L., 2001. The non-specific cytotoxic cell receptor (NCCRP-1): Molecular organization and signaling properties. Dev. Comp. Immunol. 25, 701–711. https://doi.org/10.1016/S0145-305X(01)00031-3

Jiang, H., Hu, Y., Wei, X., Xiao, X., Jakovlić, I., Liu, X., Su, J., Yuan, G., 2018. Chemotactic effect of β-defensin 1 on macrophages in *Megalobrama amblycephala*. Fish Shellfish Immunol. 74, 35–42. https://doi.org/10.1016/j.fsi.2017.12.016

Jin, J.Y., Zhou, L., Wang, Y., Li, Z., Zhao, J.G., Zhang, Q.Y., Gui, J.F., 2010. Antibacterial and antiviral roles of a fish β-defensin expressed both in pituitary and testis. PLoS One 5. https://doi.org/10.1371/journal.pone.0012883

Jørgensen, J.B., 2014. The innate inmune response in fish, in: Gudding, R., Lillehaug, A., Evensen, Ø. (Eds.), Fish Vaccination. John Wiley & Sons, pp. 85–97.

Kai, Y.H., Su, H.M., Tai, K.T., Chi, S.C., 2010. Vaccination of grouper broodfish (*Epinephelus tukula*) reduces the risk of vertical transmission by nervous necrosis virus. Vaccine 28, 996–1001. https://doi.org/10.1016/j.vaccine.2009.10.132

Kaneda, M., Odaka, T., Suetake, H., Tahara, D., Miyadai, T., 2012. Teleost IL-6 promotes antibody production through STAT3 signaling via IL-6R and gp130. Dev. Comp. Immunol. 38, 224–231. https://doi.org/10.1016/j.dci.2012.02.002

Kang, H.K., Kim, C., Seo, C.H., Park, Y., 2017. The therapeutic applications of antimicrobial peptides (AMPs): a patent review. J. Microbiol. 55, 1–12. https://doi.org/10.1007/s12275-017-6452-1

Katzenback, B.A., 2015. Antimicrobial peptides as mediators of innate immunity in teleosts. Biology (Basel). 4, 607–639. https://doi.org/10.3390/biology4040607

Kibenge, F.S., 2019. Emerging viruses in aquaculture. Curr. Opin. Virol. 34, 97–103. https://doi.org/10.1016/j.coviro.2018.12.008

Kibenge, F.S.B., 2016. Determinants of emergence of viral diseases in aquaculture, Aquaculture Virology. Elsevier Inc. https://doi.org/10.1016/B978-0-12-801573-5.00006-1

Kim, H.J., Lee, J.Y., Kang, H.A., Lee, Y., Park, E.J., Kim, H.J., 2014. Oral immunization with whole yeast producing viral capsid antigen provokes a stronger humoral immune response than purified viral capsid antigen. Lett. Appl. Microbiol. 58, 285–291. https://doi.org/10.1111/lam.12188

Krasnov, A., Kileng, Ø., Skugor, S., Jørgensen, S.M., Afanasyev, S., Timmerhaus, G., Sommer, A.I., Jensen, I., 2013. Genomic analysis of the host response to nervous necrosis virus in Atlantic cod (*Gadus morhua*) brain. Mol. Immunol. 54, 443–452. https://doi.org/10.1016/j.molimm.2013.01.010

Kumar, P., Kizhakkedathu, J.N., Straus, S.K., 2018. Antimicrobial peptides: Diversity, mechanism of action and strategies to improve the activity and biocompatibility in

vivo. Biomolecules 8, 1–24. https://doi.org/10.3390/biom8010004

Lama, R., Pereiro, P., Figueras, A., Novoa, B., 2022. Zebrafish as a vertebrate model for studying nodavirus infections. Front. Immunol. 13, 1–17. https://doi.org/10.3389/fimmu.2022.863096

León, R., Ruiz, M., Valero, Y., Cárdenas, C., Guzmán, F., Vila, M., Cuesta, A., 2020a. Exploring small cationic peptides of different origin as potential antimicrobial agents in aquaculture. Fish Shellfish Immunol. 98, 720–727. https://doi.org/10.1016/j.fsi.2019.11.019

León, R., Ruiz, M., Valero, Y., Cárdenas, C., Guzmán, F., Vila, M., Cuesta, A., 2020b. Exploring small cationic peptides of different origin as potential antimicrobial agents in aquaculture. Fish Shellfish Immunol. 98, 720–727. https://doi.org/10.1016/j.fsi.2019.11.019

Li, C.H., Lu, X.J., Li, M.Y., Chen, J., 2015. Cathelicidin modulates the function of monocytes/macrophages via the P2X7 receptor in a teleost, *Plecoglossus altivelis*. Fish Shellfish Immunol. 47, 878–885. https://doi.org/10.1016/j.fsi.2015.10.031

Lin, A., Lin, H., Huang, T., Muthusamy, S., Lee, J., Duann, Y., Lin, C., 2012. Piscidin-1 , an antimicrobial peptide from fish (Hybrid Striped Bass *Morone saxatilis* x *M . chrysops*), induces apoptotic and necrotic activity in HT1080 cells piscidin-1, an antimicrobial peptide from fish Hybrid Striped Bass (*Morone saxatilis*). Zool. Soc. Japan 29, 327–332. https://doi.org/10.2108/zsj.29.327

Lin, W.C., Chang, H.Y., Chen, J.Y., 2016. Electrotransfer of the tilapia piscidin 3 and tilapia piscidin 4 genes into skeletal muscle enhances the antibacterial and immunomodulatory functions of *Oreochromis niloticus*. Fish Shellfish Immunol. 50, 200–209. https://doi.org/10.1016/j.fsi.2016.01.034

Liu, S.P., Chuang, S.C., Yang, C. Da, 2020. Protective immunity against Vibrio harveyi in grouper induced by single vaccination with poly (Lactide-co-glycolide) microparticles releasing pleurocidin peptide and recombinant glyceraldehyde-3-phosphate dehydrogenase. Vaccines 8. https://doi.org/10.3390/vaccines8010033

Liu, W., Hsu, C.H., Chang, C.Y., Chen, H.H., Lin, C.S., 2006. Immune response against

grouper nervous necrosis virus by vaccination of virus-like particles. Vaccine 24, 6282–6287. https://doi.org/10.1016/j.vaccine.2006.05.073

López-Jimena, B., García-Rosado, E., Thompson, K.D., Adams, A., Infante, C., Borrego, J.J., Alonso, M.C., 2012. Distribution of red-spotted grouper nervous necrosis virus (RGNNV) antigens in nervous and non-nervous organs of European seabass (*Dicentrarchus labrax*) during the course of an experimental challenge. J. Vet. Sci. 13, 355–362. https://doi.org/10.4142/jvs.2012.13.4.355

López-Muñoz, A., Sepulcre, M.P., García-Moreno, D., Béjar, J., Manchado, M., Álvarez, M.C., Meseguer, J., Mulero, V., 2012. Viral nervous necrosis virus persistently replicates in the central nervous system of asymptomatic gilthead seabream and promotes a transient inflammatory response followed by the infiltration of IgM+ B lymphocytes. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2012.02.007

Low, C.F., Syarul Nataqain, B., Chee, H.Y., Rozaini, M.Z.H., Najiah, M., 2017. Betanodavirus: Dissection of the viral life cycle. J. Fish Dis. 40, 1489–1496. https://doi.org/10.1111/jfd.12638

Lu, M.W., Chao, Y.M., Guo, T.C., Santi, N., Evensen, Ø., Kasani, S.K., Hong, J.R., Wu, J.L., 2008. The interferon response is involved in nervous necrosis virus acute and persistent infection in zebrafish infection model. Mol. Immunol. 45, 1146–1152. https://doi.org/10.1016/j.molimm.2007.07.018

Lu, X.J., Chen, J., Huang, Z.A., Shi, Y.H., 2011. Identification and characterization of a novel cathelicidin from ayu, *Plecoglossus altivelis*. Fish Shellfish Immunol. 31, 52–57. https://doi.org/10.1016/j.fsi.2011.03.005

Luders, T., Birkemo, G.A., Nissen-meyer, J., Andersen, Ø., Nes, I.F., 2005. Proline conformation-dependent antimicrobial activity of a proline-rich histone H1 N-terminal peptide fragment isolated from the skin mucus of Atlantic salmon. Antimicrob. Agents Chemother. 49, 2399–2406. https://doi.org/10.1128/AAC.49.6.2399

Luo, Y., Song, Y., 2021. Mechanism of antimicrobial peptides: Antimicrobial, anti-inflammatory and antibiofilm activities. Int. J. Mol. Sci. 22. https://doi.org/10.3390/ijms222111401

Ma, H., Han, Y., Palti, Y., Gao, G., Liu, S., Palmquist, D.E., Wiens, G.D., Shepherd, B.S., 2021. Structure and regulation of the NK-lysin (1–4) and NK-lysin like (a and b) antimicrobial genes in rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. 116, 103961. https://doi.org/10.1016/j.dci.2020.103961

Ma, J.Y., Shao, S., Wang, G., 2020. Antimicrobial peptides: Bridging innate and adaptive immunity in the pathogenesis of psoriasis. Chin. Med. J. (Engl). 133, 2966–2975. https://doi.org/10.1097/CM9.0000000000001240

Magrone, T., Russo, M.A., Jirillo, E., 2018. Antimicrobial peptides: phylogenic sources and biological activities. First of two parts. Curr. Pharm. Des. 24, 1043–1053. https://doi.org/10.2174/1381612824666180403123736

Maier, V.H., Dorn, K. V, Gudmundsdottir, B.K., Gudmundsson, G.H., 2008. Characterisation of cathelicidin gene family members in divergent fish species. Mol. Immunol. 45, 3723–3730. https://doi.org/10.1016/j.molimm.2008.06.002

Mali, P., Bhattacharya Sanyal, K., Dash, G., Mukherjee, D., 2017. Nonspecific cytotoxic cells (NCC) in fish: a review. Interacad 21, 372–378.

Masso-Silva, J.A., Diamond, G., 2014. Antimicrobial peptides from fish. Pharmaceuticals 7, 265–310. https://doi.org/10.3390/ph7030265

Meloni, M., Candusso, S., Galeotti, M., Volpatti, D., 2015. Preliminary study on expression of antimicrobial peptides in European sea bass (*Dicentrarchus labrax*) following in vivo infection with *Vibrio anguillarum*. A time course experiment. Fish Shellfish Immunol. 43, 82–90. https://doi.org/10.1016/j.fsi.2014.12.016

Méndez-Samperio, P., 2013. Recent advances in the field of antimicrobial peptides in inflammatory diseases. Adv. Biomed. Res. 2, 50. https://doi.org/10.4103/2277-9175.114192

Montes, A., Figueras, A., Novoa, B., 2010. Nodavirus encephalopathy in turbot (*Scophthalmus maximus*): Inflammation, nitric oxide production and effect of anti-inflammatory compounds. Fish Shellfish Immunol. 28, 281–288. https://doi.org/10.1016/j.fsi.2009.11.002

Moreno, P., López-Jimena, B., Randelli, E., Scapigliati, G., Buonocore, F., Garcia-Rosado, E., Borrego, J.J., Alonso, M.C., 2018. Immuno-related gene transcription and antibody response in nodavirus (RGNNV and SJNNV)-infected European sea bass (*Dicentrarchus labrax* L.). Fish Shellfish Immunol. 78, 270–278. https://doi.org/10.1016/j.fsi.2018.04.054

Mori, K.I., Nakai, T., Muroga, K., Arimoto, M., Mushiake, K., Furusawa, I., 1992. Properties of a new virus belonging to nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. Virology 187, 368–371. https://doi.org/10.1016/0042-6822(92)90329-N

Morick, D., Saragovi, A., 2017. Inhibition of nervous necrosis virus by ribavirin in a zebrafish larvae model. Fish Shellfish Immunol. 60, 537–544. https://doi.org/10.1016/j.fsi.2016.11.015

Munday, B.L., Kwang, J., Moody, N., 2002. Betanodavirus infections of teleost fish: a review. J. Fish Biol. 25, 127–142.

Mutoloki, S., Jørgensen, J.B., Evensen, Ø., 2014. The adaptative inmune response in fish, in: Gudding, R., Lillehaug, A., Evensen, Ø. (Eds.), Fish Vaccination. John Wiley & Sons, pp. 104–113.

Mwangi, J., Hao, X., Lai, R., Zhang, Z.Y., 2019. Antimicrobial peptides: new hope in the war against multidrug resistance. Zool. Res. 40, 488–505. https://doi.org/10.24272/j.issn.2095-8137.2019.062

Nagai, T., Nishizawa, T., 1999. Sequence of the non-structural protein gene encoded by RNA1 of striped jack nervous necrosis virus. J. Gen. Virol. 80, 3019–3022. https://doi.org/10.1099/0022-1317-80-11-3019

Nakanishi, T., Toda, H., Shibasaki, Y., Somamoto, T., 2011. Cytotoxic T cells in teleost fish. Dev. Comp. Immunol. 35, 1317–1323. https://doi.org/10.1016/j.dci.2011.03.033

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little, D.C., Lubchenco, J., Shumway, S.E., Troell, M., 2021. A 20-year retrospective review of global aquaculture. Nature 591, 551–563. https://doi.org/10.1038/s41586-

021-03308-6

Neves, J. V., Caldas, C., Ramos, M.F., Rodrigues, P.N.S., 2016. Hepcidin-dependent regulation of erythropoiesis during anemia in a teleost fish, *Dicentrarchus labrax*. PLoS One 11, 1–17. https://doi.org/10.1371/journal.pone.0153940

Neves, J. V., Caldas, C., Vieira, I., Ramos, M.F., Rodrigues, P.N.S., 2015. Multiple hepcidins in a teleost fish, *Dicentrarchus labrax* : Different hepcidins for different roles. J. Immunol. 195, 2696–2709. https://doi.org/10.4049/jimmunol.1501153

Neves, J. V., Caldas, C., Wilson, J.M., Rodrigues, P.N.S., 2011. Molecular mechanisms of hepcidin regulation in sea bass (*Dicentrarchus labrax*). Fish Shellfish Immunol. 31, 1154–1161. https://doi.org/10.1016/j.fsi.2011.10.006

Neves, J. V., Ramos, M.F., Moreira, A.C., Silva, T., Gomes, M.S., Rodrigues, P.N.S., 2017. Hamp1 but not Hamp2 regulates ferroportin in fish with two functionally distinct hepcidin types. Sci. Rep. 7, 1–14. https://doi.org/10.1038/s41598-017-14933-5

Noga, E.J., Fan, Z., Silphaduang, U., 2002. Host site of activity and cytological effects of histone-like proteins on the parasitic dinoflagellate *Amyloodinium ocellatum*. Dis. Aquat. Organ. 52, 207–215.

Olveira, J.G., Soares, F., Engrola, S., Dopazo, C.P., Bandín, I., 2008. Antemortem versus postmortem methods for detection of betanodavirus in Senegalese sole (*Solea senegalensis*). J. Vet. Diagnostic Investig. 20, 215–219. https://doi.org/10.1177/104063870802000212

Øvergård, A.C., Patel, S., Nøstbakken, O.J., Nerland, A.H., 2013. Atlantic halibut (*Hippoglossus hippoglossus* L.) T-cell and cytokine response after vaccination and challenge with nodavirus. Vaccine 31, 2395–2402. https://doi.org/10.1016/j.vaccine.2013.01.034

Pakingking, R., Seron, R., Dela Peña, L., Mori, K., Yamashita, H., Nakai, T., 2009. Immune responses of Asian sea bass, *Lates calcarifer* Bloch, against an inactivated betanodavirus vaccine. J. Fish Dis. 32, 457–463. https://doi.org/10.1111/j.1365-2761.2009.01040.x

Pan, C.Y., Peng, K.C., Lin, C.H., Chen, J.Y., 2011. Transgenic expression of tilapia hepcidin 1-5 and shrimp chelonianin in zebrafish and their resistance to bacterial pathogens. Fish Shellfish Immunol. 31, 275–285. https://doi.org/10.1016/j.fsi.2011.05.013

Pan, C.Y., Tsai, T.Y., Su, B.C., Hui, C.F., Chen, J.Y., 2017. Study of the antimicrobial activity of tilapia piscidin 3 (TP3) and TP4 and their effects on immune functions in hybrid tilapia (*Oreochromis* spp.). PLoS One 12, 1–23. https://doi.org/10.1371/journal.pone.0169678

Patel, S., Øvergård, A.C., Nerland, A.H., 2008. CD8α and CD8β in Atlantic halibut, *Hippoglossus hippoglossus*: Cloning, characterization and gene expression during viral and bacterial infection. Fish Shellfish Immunol. 25, 570–580. https://doi.org/10.1016/j.fsi.2008.08.007

Peng, K., Lee, S., Hour, A., Pan, C., Lee, L., Chen, J., 2012. Five different piscidins from Nile Tilapia, *Oreochromis niloticus*: analysis of their expressions and biological functions. PLoS One 7, e50263. https://doi.org/10.1371/journal.pone.0050263

Pereiro, P., Romero, A., Díaz-Rosales, P., Estepa, A., Figueras, A., Novoa, B., 2017. Nucleated teleost erythrocytes play an Nk-Lysin- and autophagy-dependent role in antiviral immunity. Front. Immunol. 8, 1–15. https://doi.org/10.3389/fimmu.2017.01458

Pereiro, P., Varela, M., Romero, A., Dios, S., Figueras, A., Novoa, B., 2015. Zebrafish Nk-lysins: First insights about their cellular and functional diversification. Dev. Comp. Immunol. 51, 148–159. https://doi.org/10.1016/j.dci.2015.03.009

Personne, H., Paschoud, T., Fulgencio, S., Baeriswyl, S., Köhler, T., Van Delden, C., Stocker, A., Javor, S., Reymond, J.L., 2023. To Fold or Not to Fold: Diastereomeric Optimization of an α-Helical Antimicrobial Peptide. J. Med. Chem. 66, 7570–7583. https://doi.org/10.1021/acs.jmedchem.3c00460

Poisa-Beiro, L., Dios, S., Montes, A., Aranguren, R., Figueras, A., Novoa, B., 2008. Nodavirus increases the expression of Mx and inflammatory cytokines in fish brain. Mol. Immunol. 45, 218–225. https://doi.org/10.1016/j.molimm.2007.04.016

Praveen, K., Evans, D.L., Jaso-Friedmann, L., 2004. Evidence for the existence of granzyme-like serine proteases in teleost cytotoxic cells. J. Mol. Evol. 58, 449–459. https://doi.org/10.1007/s00239-003-2566-7

Praveen, K., Leary, J.H., Evans, D.L., Jaso-Friedmann, L., 2006. Nonspecific cytotoxic cells of teleosts are armed with multiple granzymes and other components of the granule exocytosis pathway. Mol. Immunol. 43, 1152–1162. https://doi.org/10.1016/j.molimm.2005.07.027

Press, C.M.L., Evensen, O., 1999. The morphology of the immune system in teleost fishes. Fish Shellfish Immunol. 9, 309–318. https://doi.org/10.1006/fsim.1998.0181

Rajanbabu, V., Chen, J.Y., 2011. Antiviral function of tilapia hepcidin 1-5 and its modulation of immune-related gene expressions against infectious pancreatic necrosis virus (IPNV) in Chinook salmon embryo (CHSE)-214 cells. Fish Shellfish Immunol. 30, 39–44. https://doi.org/10.1016/j.fsi.2010.09.005

Ramya, V.L., Sharma, R., Gireesh-Babu, P., Patchala, S.R., Rather, A., Nandanpawar, P.C., Eswaran, S., 2014. Development of chitosan conjugated DNA vaccine against nodavirus in *Macrobrachium rosenbergii* (De Man, 1879). J. Fish Dis. 37, 815–824. https://doi.org/10.1111/jfd.12179

Rieger, A.M., Havixbeck, J.J., Belosevic, M., Barreda, D.R., 2015. Teleost soluble CSF-1R modulates cytokine profiles at an inflammatory site, and inhibits neutrophil chemotaxis, phagocytosis, and bacterial killing. Dev. Comp. Immunol. 49, 259–266. https://doi.org/10.1016/j.dci.2014.12.003

Robertsen, B., 2006. The interferon system of teleost fish. Fish Shellfish Immunol. 20, 172–191. https://doi.org/10.1016/j.fsi.2005.01.010

Rodrigues, P.N.S., Vázquez-Dorado, S., Neves, J. V., Wilson, J.M., 2006. Dual function of fish hepcidin : Response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*). Dev. Comp. Immunol. 30, 1156–1167. https://doi.org/10.1016/j.dci.2006.02.005

Ruangsri, J., Kitani, Y., Kiron, V., Lokesh, J., Brinchmann, M.F., Karlsen, B.O., Fernandes, J.M.O., 2013. A novel beta-Defensin antimicrobial peptide in Atlantic

Cod with stimulatory effect on phagocytic activity. PLoS One 8, 1–13. https://doi.org/10.1371/journal.pone.0062302

Sahoo, B.R., 2020. Structure of fish Toll-like receptors (TLR) and NOD-like receptors (NLR). Int. J. Biol. Macromol. 161, 1602–1617. https://doi.org/10.1016/j.ijbiomac.2020.07.293

Salerno, G., Parrinello, N., Roch, P., Cammarata, M., 2007. cDNA sequence and tissue expression of an antimicrobial peptide, dicentracin; a new component of the moronecidin family isolated from head kidney leukocytes of sea bass, *Dicentrarchus labrax*. Comp. Biochem. Physiol. - B Biochem. Mol. Biol. 146, 521–529. https://doi.org/10.1016/j.cbpb.2006.12.007

Salinas, I., 2015. The mucosal immune system of teleost fish. Biology (Basel). 4, 525–539. https://doi.org/10.3390/biology4030525

Salinas, I., Fernández-Montero, Á., Ding, Y., Sunyer, J.O., 2017. Mucosal immunoglobulins of teleost fish: a decade of advances. Physiol. Behav. 176, 139–148. https://doi.org/10.1016/j.dci.2021.104079

Salinas, I., Zhang, Y.-A., Sunyer, J.O., 2011. Mucosal immunoglobulins and B cells of teleost fish. Dev. Comp. Immunol. 35, 1346–1365. https://doi.org/10.1016/j.dci.2011.11.009

Scapigliati, G., Buonocore, F., Randelli, E., Casani, D., Meloni, S., Zarletti, G., Tiberi, M., Pietretti, D., Boschi, I., Manchado, M., Martin-Antonio, B., Jimenez-Cantizano, R., Bovo, G., Borghesan, F., Lorenzen, N., Einer-Jensen, K., Adams, S., Thompson, K., Alonso, C., Bejar, J., Cano, I., Borrego, J.J., Alvarez, M.C., 2010. Cellular and molecular immune responses of the sea bass (*Dicentrarchus labrax*) experimentally infected with betanodavirus. Fish Shellfish Immunol. 28, 303–311. https://doi.org/10.1016/j.fsi.2009.11.008

Scocchi, M., Mardirossian, M., Runti, G., Benincasa, M., 2016. Non-membrane permeabilizing modes of action of antimicrobial peptides on bacteria. Curr. Top. Med. Chem. 16, 76–88.

Secombes, C.J., Wang, T., Bird, S., 2011. The interleukins of fish. Dev. Comp. Immunol.

35, 1336–1345. https://doi.org/10.1016/j.dci.2011.05.001

Serna-Duque, J.A., Cuesta, A., Sánchez-Ferrer, Á., Esteban, M.Á., 2022. Two duplicated piscidin genes from gilthead seabream (*Sparus aurata*) with different roles *in vitro* and *in vivo*. Fish Shellfish Immunol. 127, 730–739. https://doi.org/10.1016/j.fsi.2022.07.013

Shen, Y., Zhao, Z., Zhao, J., Chen, X., Cao, M., Wu, M., 2019. Expression and functional analysis of hepcidin from mandarin fish (*Siniperca chuatsi*). Int. J. Mol. Sci. 20, 5602.

Skliris, G.P., Richards, R.H., 1999. Induction of nodavirus disease in seabass, *Dicentrarchus labrax*, using different infection models. Virus Res. 63, 85–93. https://doi.org/10.1016/S0168-1702(99)00061-1

Soliman, A.M., Barreda, D.R., 2023. The acute inflammatory response of teleost fish. Dev. Comp. Immunol. 146, 104731. https://doi.org/10.1016/j.dci.2023.104731

Somamoto, T., Nakanishi, T., Nakao, M., 2013. Identification of anti-viral cytotoxic effector cells in the ginbuna crucian carp, *Carassius auratus langsdorfii*. Dev. Comp. Immunol. 39, 370–377. https://doi.org/10.1016/j.dci.2012.11.001

Sommerset, I., Lorenzen, E., Lorenzen, N., Bleie, H., Nerland, A.H., 2003. A DNA vaccine directed against a rainbow trout rhabdovirus induces early protection against a nodavirus challenge in turbot. Vaccine 21, 4661–4667. https://doi.org/10.1016/S0264-410X(03)00526-7

Sommerset, I., Nerland, A.H., 2004. Complete sequence of RNA1 and subgenomic RNA3 of Atlantic halibut nodavirus (AHNV). Dis. Aquat. Organ. 58, 117–125. https://doi.org/10.3354/dao058117

Sommerset, I., Skern, R., Biering, E., Bleie, H., Fiksdal, I.U., Grove, S., Nerland, A.H., 2005. Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. Fish Shellfish Immunol. 18, 13–29. https://doi.org/10.1016/j.fsi.2004.03.006

STECF, 2021. A new strategic vision for sustainable aquaculture production and

consumption in the European Union – Blue farming in the European Green Deal. Publ. Off. EU.

Steinel, N.C., Bolnick, D.I., 2017. Melanomacrophage centers as a histological indicator of immune function in fish and other poikilotherms. Front. Immunol. 8, 1–8. https://doi.org/10.3389/fimmu.2017.00827

Stosik, M., Tokarz-Deptuła, B., Deptuła, W., 2021. Immunological memory in teleost fish. Fish Shellfish Immunol. 115, 95–103. https://doi.org/10.1016/j.fsi.2021.05.022

Stosik, M.P., Tokarz-deptuła, B., Deptuła, W., 2018. Specific humoral immunity in Osteichthyes. Cent. Eur. J. Immunol. 43, 335–340.

Su, Y.C., Wu, J.L., Hong, J.R., 2009. Betanodavirus non-structural protein B2: A novel necrotic death factor that induces mitochondria-mediated cell death in fish cells. Virology 385, 143–154. https://doi.org/10.1016/j.virol.2008.11.036

Thiéry, R., Cozien, J., Cabon, J., Lamour, F., Baud, M., Schneemann, A., 2006. Induction of a protective immune response against viral nervous necrosis in the European sea bass *Dicentrarchus labrax* by using Betanodavirus virus-like `particles. J. Virol. 80, 10201–10207. https://doi.org/10.1128/jvi.01098-06

Thwaite, R., Berbel, C., Aparicio, M., Torrealba, D., Pesarrodona, M., Villaverde, A., Borrego, J.J., Manchado, M., Roher, N., 2020. Nanostructured recombinant protein particles raise specific antibodies against the nodavirus NNV coat protein in sole. Fish Shellfish Immunol. 99, 578–586. https://doi.org/10.1016/j.fsi.2020.02.029

Valdés, N., Cortés, M., Barraza, F., Reyes-López, F.E., Imarai, M., 2022. CXCL9-11 chemokines and CXCR3 receptor in teleost fish species. Fish Shellfish Immunol. Reports 3, 100068. https://doi.org/10.1016/j.fsirep.2022.100068

Valero, Y., Arizcun, M., Cortés, J., Ramírez-Cepeda, F., Guzmán, F., Mercado, L., Esteban, M.Á., Chaves-Pozo, E., Cuesta, A., 2020a. NK-lysin, dicentracin and hepcidin antimicrobial peptides in European sea bass. Ontogenetic development and modulation in juveniles by nodavirus. Dev. Comp. Immunol. 103, 103516. https://doi.org/10.1016/j.dci.2019.103516

Valero, Y., Arizcun, M., Esteban, M.Á., Bandín, I., Olveira, J.G., Patel, S., Cuesta, A., Chaves-Pozo, E., 2015a. Nodavirus colonizes and replicates in the testis of gilthead seabream and European sea bass modulating its immune and reproductive functions. PLoS One 10, 1–24. https://doi.org/10.1371/journal.pone.0145131

Valero, Y., Arizcun, M., Esteban, M.Á., Cuesta, A., Chaves-Pozo, E., 2016a. Transcription of histones H1 and H2B is regulated by several immune stimuli in gilthead seabream and European sea bass. Fish Shellfish Immunol. 57, 107–115. https://doi.org/10.1016/j.fsi.2016.08.019

Valero, Y., Awad, E., Buonocore, F., Arizcun, M., Esteban, M.Á., Meseguer, J., Chaves-Pozo, E., Cuesta, A., 2016b. An oral chitosan DNA vaccine against nodavirus improves transcription of cell-mediated cytotoxicity and interferon genes in the European sea bass juveniles gut and survival upon infection. Dev. Comp. Immunol. 65, 64–72. https://doi.org/10.1016/j.dci.2016.06.021

Valero, Y., Boughlala, B., Arizcun, M., Patel, S., Fiksdal, I.U., Esteban, M.Á., Juan, J. De, Meseguer, J., Chaves-Pozo, E., Cuesta, A., 2018a. Genes related to cell-mediated cytotoxicity and interferon response are induced in the retina of European sea bass upon intravitreal infection with nodavirus. Fish Shellfish Immunol. 74, 627–636. https://doi.org/10.1016/j.fsi.2018.01.034

Valero, Y., Chaves-Pozo, E., Cuesta, A., 2020b. NK-lysin is highly conserved in European sea bass and gilthead seabream but differentially modulated during the immune response. Fish Shellfish Immunol. 99, 435–441. https://doi.org/10.1016/j.fsi.2020.02.049

Valero, Y., Chaves-Pozo, E., Meseguer, J., Esteban, M.A., Cuesta, A., 2013. Biological role of fish antimicrobial peptides. Antimicrob. Pept. Prop. Funct. Role Immune Response 31–60.

Valero, Y., Cortés, J., Mercado, L., 2019. NK-lysin from skin-secreted mucus of Atlantic salmon and its potential role in bacteriostatic activity. Fish Shellfish Immunol. 87, 410–413. https://doi.org/10.1016/j.fsi.2019.01.034

Valero, Y., Cuesta, A., Cammarata, M., Esteban, M.Á., Chaves-Pozo, E., 2018b. Immune-endocrine interactions in the fish gonad during infection: an Open door to

vertical transmission. Fishes 3, 1–22. https://doi.org/10.3390/fishes3020024

Valero, Y., García-Alcázar, A., Esteban, M.Á., Cuesta, A., Chaves-Pozo, E., 2015b. Antimicrobial response is increased in the testis of European sea bass, but not in gilthead seabream, upon nodavirus infection. Fish Shellfish Immunol. 44, 203–213. https://doi.org/10.1016/j.fsi.2015.02.015

Valero, Y., González-Fernández, C., Cárdenas, C., Guzmán, F., León, R., Cuesta, A., 2021. NK-lysin peptides ameliorate viral encephalopathy and retinopathy disease signs and provide partial protection against nodavirus infection in European sea bass. Antiviral Res. 192, 1–8. https://doi.org/10.1016/j.antiviral.2021.105104

Valero, Y., Mokrani, D., Chaves-Pozo, E., Arizcun, M., Oumouna, M., Meseguer, J., Esteban, M.Á., Cuesta, A., 2018c. Vaccination with UV-inactivated nodavirus partly protects European sea bass against infection, while inducing few changes in immunity. Dev. Comp. Immunol. 86, 171–179. https://doi.org/10.1016/j.dci.2018.05.013

Valero, Y., Morcillo, P., Meseguer, J., Buonocore, F., Esteban, M.A., Chaves-Pozo, E., Cuesta, A., 2015c. Characterization of the IFN pathway in the teleost fish gonad against vertically transmitted viral nervous necrosis virus. J. Gen. Virol. 96, 2176–2187. https://doi.org/10.1099/vir.0.000164

Valero, Y., Saraiva-Fraga, M., Costas, B., Guardiola, F.A., 2020c. Antimicrobial peptides from fish: beyond the fight against pathogens. Rev. Aquac. 12, 224–253. https://doi.org/10.1111/raq.12314

Vimal, S., Farook, M.A., Madan, N., Abdul Majeed, S., Nambi, K.S.N., Taju, G., Sundar raj, N., Venu, S., Subburaj, R., Thirunavukkarasu, A.R., Sahul Hameed, A.S., 2016. Development, distribution and expression of a DNA vaccine against nodavirus in Asian Seabass, *Lates calcarifier* (Bloch, 1790). Aquac. Res. 47, 1209–1220. https://doi.org/10.1111/are.12578

Vimal, S., Madan, N., Farook, M.A., Nambi, K.S.N., Majeed, S.A., Rajkumar, T., Venu, S., Thirunavukkarasu, A.R., Hameed, A.S.S., 2014. Production of recombinant vaccine using capsid gene of nodavirus to protect Asian sea bass, *Lates calcarifer* (Bloch, 1790). Aquaculture 418–419, 148–154.

https://doi.org/10.1016/j.aquaculture.2013.10.017

Wang, C., Yan, X., Wang, G., Liu, W., Wang, Y., Hao, D., Liu, H., Zhang, M., 2023. NKHs27, a sevenband grouper NK-Lysin peptide that possesses immunoregulatory and antimicrobial activity. Fish Shellfish Immunol. 136, 108715. https://doi.org/10.1016/j.fsi.2023.108715

Wang, Y. Da, Kung, C.W., Chen, J.Y., 2010a. Antiviral activity by fish antimicrobial peptides of epinecidin-1 and hepcidin 1-5 against nervous necrosis virus in medaka. Peptides 31, 1026–1033. https://doi.org/10.1016/j.peptides.2010.02.025

Wang, Y. Da, Kung, C.W., Chi, S.C., Chen, J.Y., 2010b. Inactivation of nervous necrosis virus infecting grouper (*Epinephelus coioides*) by epinecidin-1 and hepcidin 1-5 antimicrobial peptides, and downregulation of Mx2 and Mx3 gene expressions. Fish Shellfish Immunol. 28, 113–120. https://doi.org/10.1016/j.fsi.2009.10.001

Wang, Q., Bao, B., Wang, Y., Peatman, E., Liu, Z., 2006. Characterization of a NK-lysin antimicrobial peptide gene from channel catfish. Fish Shellfish Immunol. 20, 419–426. https://doi.org/10.1016/j.fsi.2005.05.005

Wang, Q., Peng, C., Yang, M., Huang, F., Duan, X., Wang, S., Cheng, H., Yang, H., Zhao, H., Qin, Q., 2021. Single-cell RNA-seq landscape midbrain cell responses to red spotted grouper nervous necrosis virus infection. PLoS Pathog. 17, 1–26. https://doi.org/10.1371/journal.ppat.1009665

Wang, X., Chen, J., Zhang, R., Liu, L., Ma, G., Zhu, H., 2020. Interleukin-6 in Siberian sturgeon (*Acipenser baeri*): Molecular characterization and immune functional activity. Fish Shellfish Immunol. 102, 296–306. https://doi.org/10.1016/j.fsi.2020.03.023

Wu, L., Qin, Z., Liu, H., Lin, L., Ye, J., Li, J., 2020. Recent Advances on Phagocytic B Cells in Teleost Fish. Front. Immunol. 11, 1–9. https://doi.org/10.3389/fimmu.2020.00824

Wu, Y.C., Lu, Y.F., Chi, S.C., 2010. Anti-viral mechanism of barramundi Mx against betanodavirus involves the inhibition of viral RNA synthesis through the interference of RdRp. Fish Shellfish Immunol. 28, 467–475.

https://doi.org/10.1016/j.fsi.2009.12.008

Xia, X., Cheng, L., Zhang, S., Wang, L., Hu, J., 2018. The role of natural antimicrobial peptides during infection and chronic inflammation. Int. J. Gen. Mol. Microbiol. 111, 5–26. https://doi.org/10.1007/s10482-017-0929-0

Yamaguchi, T., Takizawa, F., Furihata, M., Soto-Lampe, V., Dijkstra, J.M., Fischer, U., 2019. Teleost cytotoxic T cells. Fish Shellfish Immunol. 95, 422–439. https://doi.org/10.1016/j.fsi.2019.10.041

Yang, C., Liu, S., Sun, B., Wang, X., Wang, N., Chen, S., 2013. Iron-metabolic function and potential antibacterial role of hepcidin and its correlated genes (Ferroportin 1 and Transferrin Receptor) in Turbot (*Scophthalmus maximus*). Fish Shellfish Immunol. 34, 744–755. https://doi.org/10.1016/j.fsi.2012.11.049

Ye, J., Kaattari, I.M., Ma, C., Kaattari, S., 2013. The teleost humoral immune response. Fish Shellfish Immunol. 35, 1719–1728. https://doi.org/10.1016/j.fsi.2013.10.015

Yuasa, K., Koesharyani, I., Roza, D., Mori, K., Katata, M., Nakai, T., 2002. Immune response of humpback grouper, *Cromileptes altivelis* (Valenciennes) injected with the recombinant coat protein of betanodavirus. J. Fish Dis. 25, 53–56. https://doi.org/10.1046/j.1365-2761.2002.00325.x

Zhang, J., Yu, L. ping, Li, M. fei, Sun, L., 2014. Turbot (*Scophthalmus maximus*) hepcidin-1 and hepcidin-2 possess antimicrobial activity and promote resistance against bacterial and viral infection. Fish Shellfish Immunol. 38, 127–134. https://doi.org/10.1016/j.fsi.2014.03.011

Zhang, M., Li, M.F., Sun, L., 2014. NKLP27: A teleost NK-lysin peptide that modulates immune response, induces degradation of bacterial DNA, and inhibits bacterial and viral infection. PLoS One 9. https://doi.org/10.1371/journal.pone.0106543

Zhang, M., Long, H., Sun, L., 2013. A NK-lysin from *Cynoglossus semilaevis* enhances antimicrobial defense against bacterial and viral pathogens. Dev. Comp. Immunol. 40, 258–265. https://doi.org/10.1016/j.dci.2013.03.005

Zhang, W.W., Jia, P., Lu, X.B., Chen, X.Q., Weng, J.H., Jia, K.T., Yi, M.S., 2022. Capsid

protein from red-spotted grouper nervous necrosis virus induces incomplete autophagy by inactivating the HSP90ab1-AKT-MTOR pathway. Zool. Res. 43, 98–110. https://doi.org/10.24272/J.ISSN.2095-8137.2021.249

Zhang, X.-J., Zhang, X.-Y., Zhang, N., Guo, X., Peng, K.-S., Wu, H., Lu, L.-F., Wu, N., Chen, D.-D., Li, S., Nie, P., Zhang, Y.-A., 2015. Distinctive structural hallmarks and biological activities of the multiple cathelicidin antimicrobial peptides in a primitive teleost fish. J. Immunol. 194, 4974–4987. https://doi.org/10.4049/jimmunol.1500182

Zhao, M., Liu, Y., Gao, Y., Wang, X., Zhou, H., Zhang, A., 2022. Insights into the functional role of grass carp IL-8 in head kidney leukocytes: pro-inflammatory effects and signalling mechanisms. J. Fish Biol. 100, 192–202. https://doi.org/10.1111/jfb.14934

Zheng, J., Yang, J., Zhang, Z., Liang, X., Liu, S., Pan, Y., Wei, J., Huang, Y., Huang, X., Qin, Q., 2023. An improved oral vaccine with molecular adjuvant β-defensin protects grouper against nervous necrosis virus infection. Fish Shellfish Immunol. 136, 108709. https://doi.org/10.1016/j.fsi.2023.108709

Zhou, Q.J., Qiao, Y., Chen, J., Li, M.Y., 2020. The host defense peptide β-defensin confers protection against *Vibrio anguillarum* in ayu, *Plecoglossus altivelis*. Dev. Comp. Immunol. 103, 103511. https://doi.org/10.1016/j.dci.2019.103511

Zhou, Q.J., Wang, J., Liu, M., Qiao, Y., Hong, W.S., Su, Y.Q., Han, K.H., Ke, Q.Z., Zheng, W.Q., 2016. Identification, expression and antibacterial activities of an antimicrobial peptide NK-lysin from a marine fish *Larimichthys crocea*. Fish Shellfish Immunol. 55, 195–202. https://doi.org/10.1016/j.fsi.2016.05.035

Zhou, Y., Lei, Y., Cao, Z., Chen, X., Sun, Y., Xu, Y., Guo, W., Wang, S., Liu, C., 2019. A β-defensin gene of *Trachinotus ovatus* might be involved in the antimicrobial and antiviral immune response, Developmental and Comparative Immunology. Elsevier Ltd. https://doi.org/10.1016/j.dci.2018.11.011

Zhu, S., Miao, B., Zhang, Y.Z., Wang, D.S., Wang, G.X., 2022. Amantadine, a promising therapeutic agent against viral encephalopathy and retinopathy. J. Fish Dis. 45, 451–459. https://doi.org/10.1111/jfd.13574

Zou, J., Mercier, C., Koussounadis, A., Secombes, C., 2007. Discovery of multiple beta-defensin like homologues in teleost fish. Mol. Immunol. 44, 638–647. https://doi.org/10.1016/j.molimm.2006.01.012

Zou, J., Secombes, C.J., 2016. The function of fish cytokines. Biology (Basel). 5. https://doi.org/10.3390/biology5020023

Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Dev. Comp. Immunol. 35, 1376–1387. https://doi.org/10.1016/j.dci.2011.07.001