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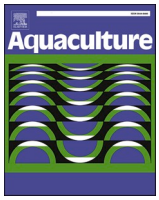

Hepcidin and dicentracin peptides show preventive antiviral applications against NNV infection in European sea bass through immunomodulatory roles

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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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mortality rates up to 100% during larvae and juvenile stages (Breuil et al., 1991), whilst adult and breeding specimens act as reservoirs with no clinical signs of disease (Valero et al., 2018). Moreover, considering that NNV can be transmitted vertically, the economic impact on hatcheries is enormously negative. Unfortunately, though a commercial vaccine against NNV exists, its application and effectiveness are extremely limited. Further research is prompted to design and generate effective control tools against NNV for intensive marine fish culture.

In this context, antimicrobial peptides (AMPs) have been postulated as good candidates to treat microbial infections, including viral pathologies. They are short peptides, endogenously produced by most organisms, that can fight microbials in a dual way either by their direct lytic effect against pathogens, or modulating the host immune response (Valero et al., 2020b). AMPs form a heterogeneous group of peptides sharing some common structural features, including short aminoacidic sequences, cationicity or amphipathicity (León et al., 2020; Valero et al., 2013), that can be grouped into different families such as hepcidins, piscidins, defensins or cathelicidins (Masso-Silva and Diamond, 2014) among others. Hepcidins (Hamp) are probably the most studied AMPs in fish (Álvarez et al., 2016). Hamp peptides, with a cysteine-rich β-sheet structure, are mainly produced in the liver with major functions on iron metabolism and immunity. Although the direct antibacterial activity of fish hepcidins has been widely explored (Cuesta et al., 2008; Neves et al., 2015; Xie et al., 2019), there are less studies dealing with its antiviral activity. Briefly, synthetic hepcidin reduced NNV mortality in Hamp-pretreated grouper (*Epinephelus coioides*) or medaka (*Oryzas latipes*) (Wang et al., 2010a, 2010b) while the administration of Hamp-expressing plasmids before NNV infection also protected European sea bass (*Dicentrarchus labrax*) from NNV infections (Cervera et al., 2023). Apart from these studies, Hamp has shown to induce an inflammatory response in several fish species (Ghodsi et al., 2020; Pan et al., 2011) or directly agglutinate and inactivate NNV infective particles (Chia et al., 2010). Taking all this into account, we can speculate that Hamp could serve as anti-NNV treatment due to both direct lytic and indirect immunomodulatory actions.

By contrast, piscidins are members from a fish-exclusive AMP family. Among them, dicentracin (Dic), described as an exclusive European sea bass piscidin (Salerno et al., 2007), has a peculiar structure, mostly formed by alpha-helix domains and lacking cysteine residues (Milne et al., 2019). Interestingly, synthetic Dic shows a great *in vitro* antibacterial and antiviral activities (León et al., 2020). Unfortunately, little is known about Dic regulation and functions *in vivo*. The first observation in that sense showed that *in vivo* NNV infection regulated mRNA and protein levels of European sea bass Dic (Valero et al., 2015a, 2020a). On the other hand, the *in vivo* administration of an expression plasmid encoding Dic triggers the European sea bass inflammatory response but fails to dismiss mortalities upon NNV infection (Cervera et al., 2023). Taking all this into account, we aimed to evaluate the potential preventive application of Hamp and Dic synthetic peptides against NNV infection on European sea bass. The potential immunomodulatory role of Hamp and Dic peptides will be discussed in relation with their importance in the promotion of survival and viral clearance.

2. Material and methods

2.1. Animals

Healthy juveniles of European sea bass (*Dicentrarchus labrax* L.; 4.64 ± 0.49 g body weight) were bred at *Centro Oceanográfico de Murcia, Instituto Español de Oceanografía* (COMU-IEO), CSIC facilities. Animals were kept in 200 L tanks with an independent recirculation system for natural sea water (38‰ salinity), suitable aeration and filtration systems, temperature of 25 ± 1 °C and 12 h light:12 h 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 Number A13210701).

2.2. Peptides and NNV production

A 26 aa peptide from the mature European sea bass hepcidin 2 variant 1 (Hamp 2.1; KJ890397.1) and the complete mature dicentracin (Dic; P59906) peptides (Table 1) were chemically synthesized by GeneScript (Purity ≥90%), dissolved in pure water at 1 mg/mL and aliquots frozen. Mature Hamp 2.1 and Dic synthetic peptides were previously tested (Álvarez et al., 2016; León et al., 2020; Neves et al., 2015, 2022).

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.

2.3. Experimental design

To evaluate the preventive application of AMPs against NNV infection, fish were intramuscularly (im) injected with AMPs or phosphate buffered saline (PBS), and one day later challenged by im injection with NNV, with the exception of the mock infected group (Control group). Thus, four experimental groups (55 fish/group) were established (Mock, PBS + NNV, Hamp+NNV and Dic + NNV). For AMP administration, fish were captured one by one, anesthetized with 40 μL/L of clove oil in marine water in a 10 L tank and im injected with 50 μL of PBS alone (Control and PBS groups) or containing ~1 μg AMP per g of fish (Hamp or Dic groups) using an insulin syringe as other authors previously used with different AMPs or fish species (Valero et al., 2021; Wang et al., 2010a, 2010b). The site of injection was located between the dorsal fin and the lateral line in the right side of the dorsal muscle. Manipulation time and injection site was roughly the same for all the specimens. For NNV infection, one-day after AMP injection, fish from PBS, Hamp and Dic groups were anesthetized and im injected with 50 μL of NNV ($TCID_{50}/mL = 2.8 \times 10^6$). Fish from the Control group were injected with 50 μL of PBS instead of virus and served as the mock-infection group. All fish were injected approximately in the same area where the peptide or PBS was previously administered.

Mortality and clinical signs of infection, 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), were daily recorded. The experiment finished when two consecutive days without deaths occurred. All dead fish, or alive at the end of the trial, were weighed.

2.4. Sampling

Fish were sampled ($n = 6$/group and time) after 1 day from AMP injection and 2 days from NNV infection (dpi). Briefly, specimens were anesthetized with 40 μL/L of clove oil, completely bled and rapidly decapitated. Blood samples were collected from the caudal vein with an

Table 1
Peptide sequences used to purchase the synthetic peptides employed in this study.

Protein name	Acc. number	Sequence
Hepcidin 2.1	KJ890397.1	HSSPGGCRFCCNCCPNMSGCGVCCTF HSSPGGCRFCCNCCPNMSGCGVCCRF
Dicentracin	P59906	DAFFHHIFRGIVHVGKSIHKLVTGGKAQQD

insulin syringe. Serum samples were obtained by centrifugation of the blood (10,000 g, 10 min, 4 °C) and stored at −80 °C. Muscle and head-kidney (HK) tissues were sampled 1 day after AMP injection while muscle, HK and brain were after 2 days of NNV infection. All these samples were stored in DNA/RNA shield (ZymoResearch) and stored at −80 °C until their use. Fish died during 15 dpi were also sampled to ascertain that the causal agent of the death was NNV.

2.5. Gene expression analysis

Total RNA from tissue samples was isolated using the Quick-RNA mini prep kit (Zymo Research) and treated with DNAse I (0.9 U/μL; Zymo Research). The Tetro Reverse Transcriptase (Bioline) was used to synthesize the first strand cDNA with random hexamers from 1 μg of total RNA, at 25 °C for 10 min, 45 °C for 30 min and 85 °C for 5 min. Real-time PCR (rtPCR) was performed with CFX96 Real-Time System (Biorad) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated at 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the *elongation factor 1 alpha* (*ef1a*) and *ribosomal protein L13 alpha* (*l13a*) expression in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the geometric mean of the *ef1a* and *l13a* Ct values from the target Ct (Pfaffl, 2001). The specific primers for the analyzed genes are shown in Table 2, and grouped in six categories: i) AMPs: *hamp1*, *hamp2*, *dic*, *nkl*, *defb1,* and *lyz*; ii) inflammation-related molecules: *il10*, *il1b, il6, il10* and *cox2*; iii) leucocyte-adhesion molecules: *il8*, *cxcr3* and *cxcl9*; iv) leucocyte-type markers: *mpo, csfr1*, *tcrb, cd8a, cd4,* and *ighm*; and, when infected, v) antiviral response: *mx*; and vi) NNV genes such as NNV capsid (*cp*) and NNV polymerase (*rprd*). Negative controls with no template were always included in the reactions.

2.6. Bactericidal activity

The pathogenic marine bacteria *Vibrio harveyi* (Vh) (strain Lg 16/100) was grown in agar plates at 25 °C in tryptic soy agar (TSA, Sigma-Aldrich). Then, fresh single colonies of 1–2 mm were diluted in 5 mL of tryptic soy broth (TSB; Laboratorios Conda), cultured for 16 h at 25 °C on an orbital incubator at 200–250 rpm and adjusted to 10^8 bacteria/mL of TSB. The absorbance of bacterial cell cultures was measured at 620 nm and used to know the concentration based on growth curves.

Table 2
Primer sequences used in this study.

	Protein name	Gene name	Accession number	Sequence (5′→3′)
House-keeping	Elongation factor 1 alpha	*ef1a*	AJ866727	F: CGTTGGCTTCAACATCAAGA
				R: GAAGTTGTCTGCTCCCTTGG
	Ribosomal protein L13 alpha	*l13a*	DT044539	F: GCGAAGGCATCAACATCTCC
				R: AGACGCACAATCTTGAGAGCAG
AMPs	Hepcidin 1	*hamp1*	KJ890396	F: AAGGCATTCAGCATTGCAGTTG
				R: CCGCAACTGGAGTGTCATTG
	Hepcidin 2	*hamp2*	DQ131605	F: CCAGTCACTGAGGTGCAAGA
				R: GCTGTGACGCTTGTGTCTGT
	Dicentracin	*dic*	AY303949	F: GGCAAGTCCATCCACAAACT
				R: ATATTGCTCCGCTTGCTGAT
	NK-Lysin	*nkl*	KY801205	F: GAAGAAACACCTCGGGGAAT
				R: GCAGGTCCAACATCTCCTTC
	Defensin beta 1	*defb1*	DLAgn_00041270	F: CCTTTCCTTGGTCTTGCCCA
				R: ACACACAGCACAAGAAGCCT
	Lysozyme	*lyz*	KJ433681.1	F: ATTTCCTGGCTGGAACACAG
				R: GAGCTCTGGCAACAACATCA
Inflammation-related molecules	Interleukin-10	*il10*	DQ821114.1	F: ACTCCTCGGTCTCTTCTCCT
				R: TCCACAAAACGACAGCACTG
	Interleukin-1 beta	*il1b*	AJ269472	F: CAGGACTCCGGTTTGAACAT
				R: GTCCATTCAAAAGGGGACAA
	Interleukin-6	*il6*	AM490062	F: ACTTCCAAAACATGCCCTGA
				R: CCGCTGGTCAGTCTAAGGAG
	Cyclooxygenase 2	*cox2*	AJ630649	F: AGCACTTCACCCACCAGTTC
				R: AAGCTTGCCATCCTTGAAGA
Leucocyte-adhesion molecules	Interleukin-8	*il8*	AM490063	F: GTCTGAGAAGCCTGGGAGTG
				R: GCAATGGGAGTTAGCAGGAA
	C-X-C motif chemokine receptor 3	*cxcr3*	ENSDLAT000050	F: ATCCTGTACGCCTTTGTGGG
				R: GTCGGCAGACTCAGACCAAA
	CXC chemokine 9	*cxcl9*	DLAgn_0001298	F: TCTGTCAGCTCGCCTTTCTG
				R: TTCGTACTTGGACACGCACA
Leucocyte markers	Myeloperoxidase	*mpo*	DLAgn_0011834	F: GAAGAGTGGGGCCTTTGTTT
				R: CTGGGCCTCAGTGAAGACTC
	Macrophage colony-stimulation factor 1 receptor	*mcsf1r*	KM225787	F: TTTCGGAAAGGTTGTTGAGG
				R: TCTCATCTGAATGGGCACTG
	T-cell receptor beta chain	*tcrb*	FN687461	F: GACGGACGAAGCTGCCCA
				R: TGGCAGCCTGTGTGATCTTCA
	Cluster of differentiation 8 alpha	*cd8a*	AJ846849	F: CTGTCCTCCGCTCATACTGG
				R: TTGTAATGATGGGGGCATCT
	Cluster of differentiation 4	*cd4*	AM849812	F: ATTCTTTGCTAAGCCAGGCG
				R: CATTGTCTTGGTCTGGCGTC
	Immunoglobulin M heavy chain	*ighm*	FN908858	F: AGGACAGGACTGCTGCTGTT
				R: CACCTGCTGTCTGCTGTTGT
Antiviral response	Interferon-induced GTP-binding protein Mx	*mx*	AM228977	F: GTATGAGGAGAAGGTGCGTCC
				R: CTCTTCCCCGAGCTTTGGTC
NNV	NNV coat protein	*cp*	D38636	F: CAACTGACAACGATCACACCTTC
				R: CAATCGAACACTCCAGCGACA
	Protein A	*rdpr*	AF319555	F: GTGTCCGGAGAGGTTAAGGATG
				R: CTTGAATTGATCAACGGTGAACA

The antibacterial activity of serum was determined by evaluating their effects on the bacterial growth of Vh curves using a method previously described (Sunyer and Tort, 1995). Aliquots of 10 μL of sample were placed in a flat-bottomed 96-wells plate, mixed with 10 μL of the bacterial culture (1/10) and incubated for 120 min at room temperature. Afterwards, 150 μL of culture medium were added to each well. Absorbance was measured at 620 nm during 38 h every 30 min at 25 °C. A negative control (0% bactericidal activity, 0% bacterial growth) was prepared replacing the sample and bacteria solution by TSB, while a positive control (0% bactericidal activity, 100% bacterial growth) was prepared replacing the sample by TSB.

2.7. AMPs quantification by ELISA

European sea bass Nkl, Dic and Hamp AMPs were detected and quantified in serum by an indirect ELISA using specific mouse polyclonal antisera previously demonstrated to be specific against European sea bass proteins (Valero et al., 2020a). Briefly, sea bass serum samples were diluted 1:1000 in coating buffer [100 mM Bicarbonate/Carbonate pH = 9,6] and incubated overnight at 4 °C in 96 Maxisorp flat-bottomed plates (Nunc). After four washes of 1 min with PBS containing 0.2% Tween-20 (PBS-T), samples were blocked with 3% BSA in PBS during 2 h. Then, samples were incubated with the corresponding AMPs' antisera (anti-sea bass Nkl or Dic and anti-rainbow trout Hamp) at their optimal dilution 1:200 for 1 h (Valero et al., 2020a). Afterwards, samples were washed five times during 1 min each, and then, incubated with the anti-mouse IgG-HRP (ThermoFisher Scientific) serum at its optimal dilution 1:2500 for 1 h. The reaction was revealed by adding 100 μL per well of 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) and stopped with 2 M sulphuric acid. Absorbance was measured at 450 nm using a microplate reader. Synthetic peptides were used instead of samples as positive controls. Negative controls lacking serum or primary antisera were also used.

2.8. Isolation of viral particles from the brain

Brain fragments from all experimental groups fish (n = 3/group) from 2 dpi were weighted and independently homogenised in 0.01 M PBS with a final concentration of 1 mg/mL. Homogenates were then tested for the NNV presence by titration on E-11 cells at 25 °C. E-11 cells were seeded in 96-well microplates (Nunc) at 4×10^4 cells well^{-1}, reaching 80% confluence the next day, when cells were incubated with the dilutions of brain homogenates for 2 h. Then, the supernatant was discarded and cells were maintained with 200 μL of medium with 2% serum. The positive control was performed by incubating with NNV (strain It/411/96, genotype RGNNV) instead of brain homogenate. Negative controls contained the same diluents without any homogenate. Cultures were daily observed under a phase contrast microscope, and cytopathic effects were monitored for 7–10 days. Finally, the $TCID_{50}$/mL was calculated for each sample (Reed and Müench, 1938).

2.9. Statistical analysis

Statistical analyses were conducted using Graphpad Prism 8.2.1 software. Statistical differences between groups were analyzed by the one-way ANOVA followed by the Tukey's test, depending on the normality and homogeneity of the variables. Minimum level of significance was fixed at 0.05 ($p \leq 0.05$) in all cases. Survival was represented by the Kaplan-Meier method and statistical differences were studied using a Log-ranked (Mantel-Cox) test. The relative percentage of survival (RPS) was calculated as RPS = 1 - [(% mortality in treated fish)/ (% mortality in control fish)] × 100.

3. Results

3.1. Hamp and Dic peptides improved the survival upon NNV infection

Fish infected with NNV suffered a mortality of 91.67% (Fig. 1A). Hamp and Dic pre-treated specimens, however, showed significantly higher percentage of survival (Fig. 1A), reaching a RPS level of 26.6% and 33.3%, respectively, compared with the mock-infected group. In contrast, none of the synthetic peptides ameliorated the clinical signs of the disease as fish from the PBS group showed less severe clinical signs before death than the fish from Hamp and Dic groups. Thus, the clinical signs before death observed in the PBS group fish only were no reaction to food stimuli or a slower rate of swimming (level 1), while the Hamp-treated fish also showed altered natation and spasms during swimming (level 2) and the Dic-treated fish showed all levels of clinical signs from mild modification of swimming rates (levels 1 and 2) to continuous erratic swimming and a complete incapacity to keep balance and swim, levels 3 and 4, respectively (Fig. 1B). As expected, the body weight, as another sign of disease, were significantly lower in all infected groups (PBS + NNV, Hamp+NNV and Dic + NNV) compared to the mock-infected group (Fig. 1 C). In order to guarantee that the cause of the death was due to the NNV infection, viral marker genes were studied in the brain of death specimens. Data showed that, in all infected groups, *cp* and *rprd* were expressed but not in the scarce Control fish (Mock group) that died due to handling (Supplementary Fig. 1).

We also tested the viral load and the antiviral response in the NNV target tissue, the brain, to find relations with the partial protection conferred by Ham and Dic peptides. Specimens pre-treated with Hamp or Dic showed increased *rprd* and *cp* NNV genes transcription (Fig. 1D) though it failed to reach significance. Moreover, we recovered infective viral particles from sampled brains from all NNV-infected fish but no from mock fish, but no statistical differences were observed among infected groups (Fig. 1 E). Interferon type-I antiviral response was also studied by the expression levels of *mx* gene in the brain. NNV elicited a strong transcription of *mx*, which was blocked by pre-treatments with Ham or Dic peptides (Fig. 1F).

3.2. Circulating AMPs are altered by Hamp and Dic upon NNV challenge

We firstly analyzed the levels of Hamp and Dic in the muscle at the moment of the NNV infection and found no differences between treated and non-treated fish (Supplementary Fig. 2). Next, we analyzed the seric AMP (Nkl, Hamp and Dic) levels before and after NNV infection. Before challenge, Hamp administration increased Dic circulating levels to a significant extent while Dic peptide failed to change the sera AMP levels studied (Fig. 2A). However, upon challenge, NNV failed to alter the AMP levels respect to the mock-infected group (Fig. 2B). However, Hamp levels were highly increased in Dic pre-treated fish (Dic + NNV), while Dic levels did in Hamp-pretreated ones (Hamp+NNV; Fig. 2 B). Interestingly, seric levels of Nkl were not statistically different in none of the tested conditions (Fig. 2A, B).

3.3. Dic peptide administration increases the bactericidal activity in serum

The bactericidal activity was increased in serum 1 day after Dic administration but not when Hamp was administered (Fig. 3). After NNV challenge, the sera bactericidal activity was increased in PBS + NNV group but not in the pre-treated groups: Hamp+NNV and Dic + NNV (Fig. 3).

3.4. Hamp and Dic peptides produce immunomodulation in muscle and head-kidney

After one day from peptide administration, the pattern of expression of several AMP coding genes was modulated in muscle and HK with a different pattern depending on the AMP injected (Fig. 4). Thus, in

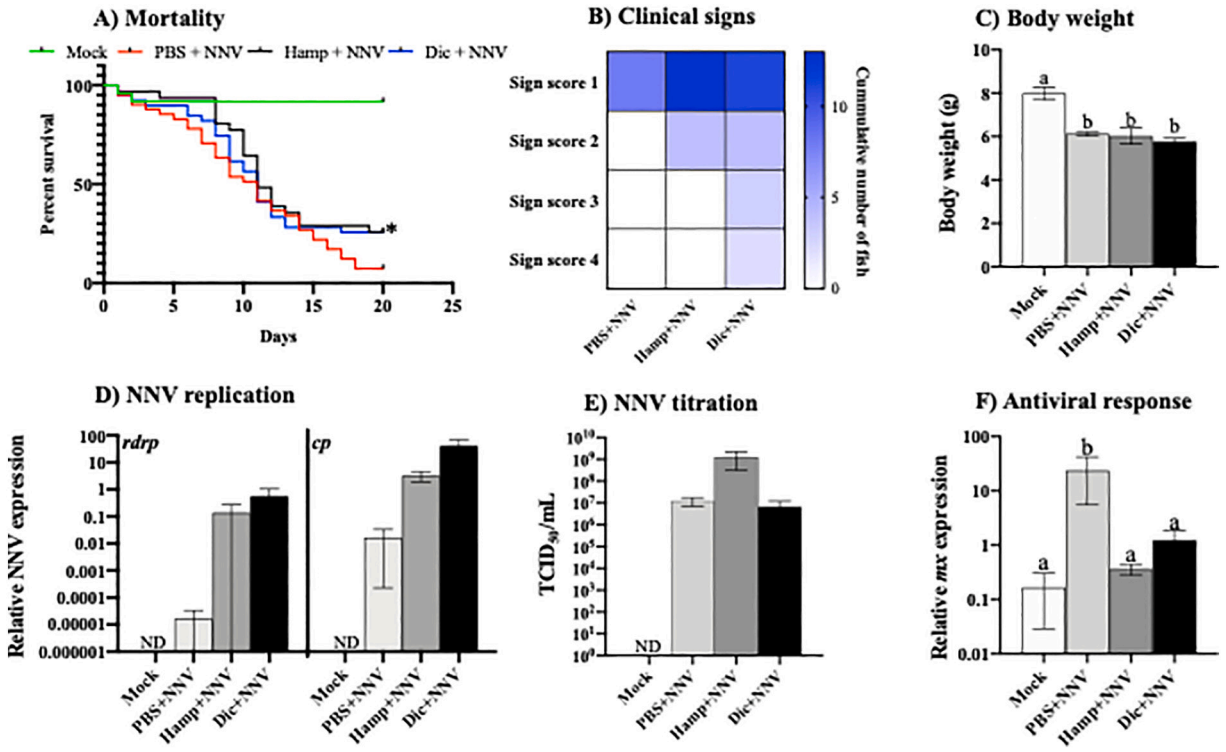

Fig. 1. Preventive administration of synthetic hepcidin (Hamp) and dicentracin (Dic) peptides improves the survival of European sea bass upon NNV infection. European sea bass juveniles were intramuscularly injected with PBS, Hamp or Dic peptides (~1 μg peptide per g of fish) and 1 day later intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish = 5.6×10^6). 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. Asterisk indicates differences between mock- and NNV-infected groups according to a long-rank test ($p \leq 0.05$). **B.** Heatmap 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. **C.** Body weight of fish specimens at death or end of the trial. Data represent the mean ± SEM ($n = 50$). **D.** Transcription levels of NNV *rdrp* and *cp* in the brain of fish from all experimental groups 2 days after NNV infection. Data represent the mean relative gene expression corrected by the *ef1a* expression in each sample ± SEM ($n = 6$) obtained by real-time PCR. **E.** Viral titration of NNV in the brain 15 days post-infection. Data represent the mean $TCID_{50}$/mL ± SEM ($n = 3$). **F.** Transcription levels of the antiviral marker *mx* in the brain of fish from all experimental groups 2 days after NNV infection. Data represent the mean relative gene expression corrected by the *ef1a* and *l13a* expression in each sample ± SEM (n = 6) obtained by real-time PCR. Different letters in **C** and **F** indicate significant statistical differences among the experimental groups according to ANOVA followed by Tukey *post-hoc* test ($p \leq 0.05$). ND, no detection.

muscle, both Hamp and Dic peptides down-regulated the expression levels of *hamp1* and *nkl,* while *dic* was up-regulated. In HK, both treatments induced the up-regulation of *hamp1* while down-regulated *nkl*. In addition, Dic treatment also led to the up-regulation of *hamp2* and *dic* and the down-regulation of *defb1* expression in HK. Regarding the expression of inflammatory-related genes, Hamp injection, only down-regulated the expression of the proinflammatory cytokine, interleukin *il6*, in HK (Fig. 4), while Dic administration resulted in decreased levels of *il6* and *cox2* transcripts in muscle and showed up-regulation and down-regulation of *il6* and *il1b* gene expressions, respectively, in HK (Fig. 4). The expression levels of specific cellular markers (Fig. 4) were mainly altered at the site of injection (muscle) of the synthetic Hamp or Dic (Fig. 4) but scarcely at the HK. Thus, the injection of Hamp increased the expression levels of *mpo, mcsf1r, tcrb and ighm* and decreased the levels of *tcrb* and *cd8a* in muscle, while in HK only the expression levels of *cd4* were decreased and those of *ighm* increased (Fig. 4). In contrast, the administration of Dic increased the expression levels of *mpo*, *mcsf1r* and *ighm* in muscle as well as the transcription of *cd8a, cd4* and *ighm* in HK (Fig. 4).

3.5. Hamp and Dic peptides are able to modulate the immunity upon NNV challenge

Firstly, to test the changes provoked by the NNV infection, we evaluated the differences in gene expression levels between Mock- and NNV-infected fish (Fig. 5A). In brain, NNV infection showed a tendency to down-regulate AMP encoding genes, such as *hamp2*, *dic*, *defb1* and *lyz*, and leucocyte recruitment, evidenced by the down-regulation of *mpo* and *cd8a*, whilst increased the inflammatory response, due to the up-regulation of *il6* and the down-regulation of *il10* (Fig. 5A). In HK, scarce immune genes such as *defb1*, *il6*, *cxcr3*, *mpo* and *cd8a* were up-regulated. In muscle, slight differences in gene expression levels were observed being *hamp2* and *il8* up-regulated and *nkl*, *defb1*, *il6*, *mpo* and *cd8a* down-regulated (Fig. 5A).

Afterwards, we analyzed the impact of the synthetic peptides injected in the response to NNV by comparing the Hamp+NNV or Dic + NNV groups with the PBS + NNV group (Fig. 5B), but not with the mock-infected one. Interestingly, both peptides produced a very similar effect on the response against NNV in the brain. Thus, treatment with Hamp resulted in the up-regulation of *nkl*, *defb1*, *il10, cxcr3*, *cxcl9*, *mpo* and *cd8a* transcription upon NNV infection though *il6* and *dic* was decreased compared to the PBS + NNV group. Similarly, Dic peptide induced, in addition, *hamp2* and *mcsf1r* in the brain while *dic* was down-regulated (Fig. 5B). Regarding the main hematopoietic organ in fish, the HK, both pretreatments down-regulated *hamp1, dic, il8, cxcr3, cxcl9, tcrb* and *cd8a* and up-regulated *defb1* and *mpo* gene expression when compared to PBS + NNV group levels (Fig. 5B). In addition, *hamp2* and *cox2* transcripts were only increased by Hamp+NNV pretreatment. At the site of peptide administration, the muscle, some relevant immune-related genes were modulated compared to NNV-infected levels. Thus,

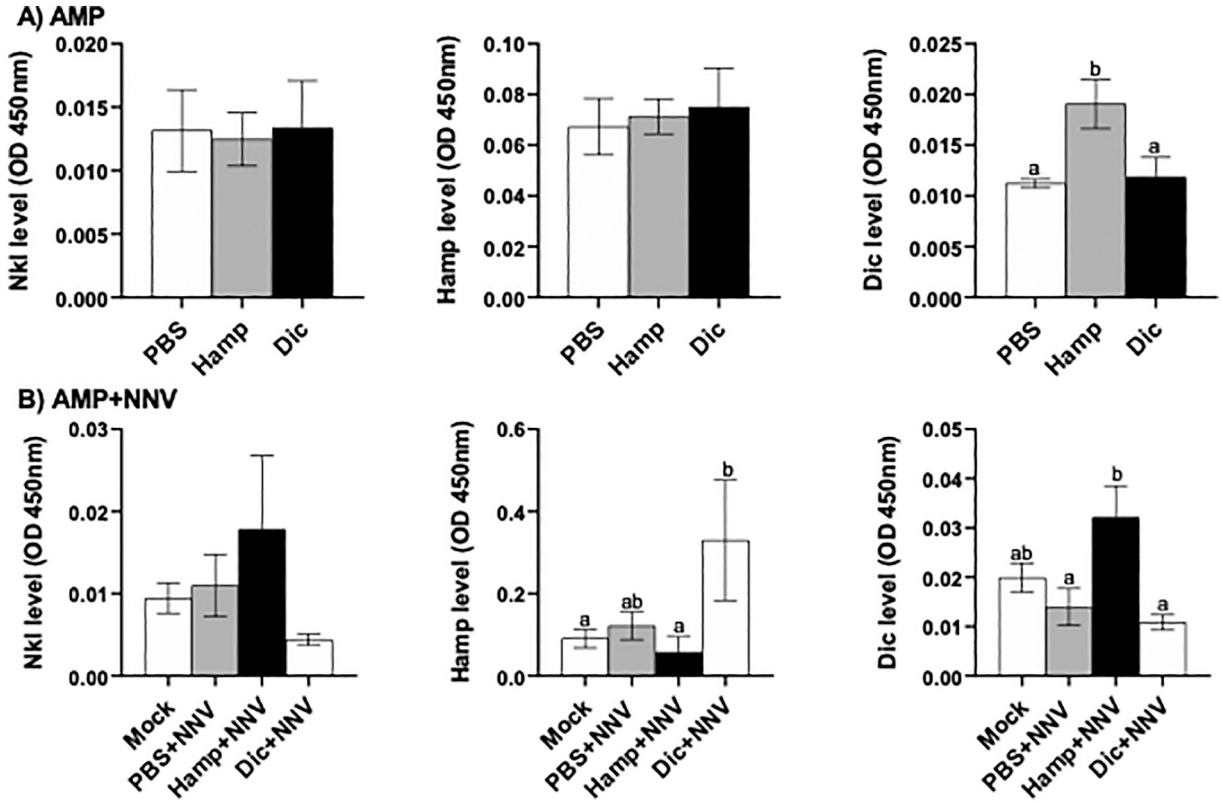

Fig. 2. Circulating levels of AMPs are altered in AMP-treated and NNV-infected fish. European sea bass juveniles were intramuscularly injected with PBS, Hamp or Dic peptides (~1 μg peptide per g of fish) and 1 day later intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish = 5.6×10^6). A mock group was injected twice with only PBS. **A.** AMP levels after 1 day of AMP-treatment and prior to the infection. **B.** AMP levels 2 days after NNV infection. Data represent the mean optical density obtained by ELISA ± SEM (n = 6). Different letters indicate significant statistical differences among groups according to ANOVA followed by Tukey *post-hoc* ($p \leq 0.05$).

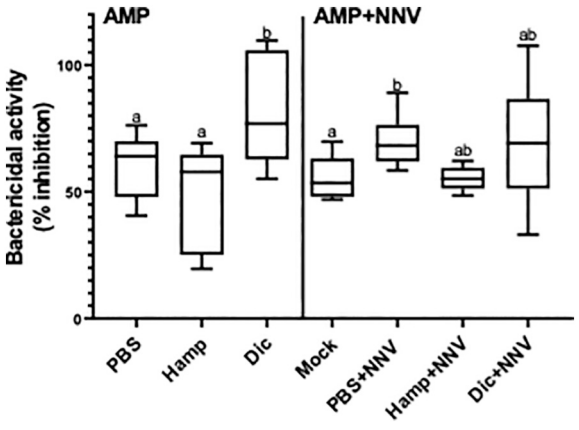

Fig. 3. Antibacterial activity is induced by AMPs but not after NNV challenge. Seric antibacterial activity against *V. harveyi* (Vh) from European sea bass juveniles intramuscularly injected with PBS, Hamp or Dic peptides (~1 μg peptide per g of fish) and 1 day later intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish = 5.6×10^6). A mock group was injected twice with only PBS. AMP levels after 1 day of AMP-treatment and prior infection (on the left) and AMP levels 2 days after NNV infection (on the right). Data represent the mean ± SEM (n = 6). Different letters indicate significant statistical differences among the experimental groups according to ANOVA followed by Tukey *post-hoc* ($p \leq 0.05$).

when fish were pretreated with Hamp or Dic *defb1, il8, cxcl9* and *mpo* gene expression was down-regulated respect to PBS + NNV fish levels whilst *dic* gene expression was up-regulated (Fig. 5B). In addition, the Hamp+NNV group showed increased *cox2* and decreased *tcrb* transcription levels compared to PBS + NNV group levels though Dic peptide up-regulated the *cd8a* transcription above the levels observed in the PBS + NNV group (Fig. 5B).

4. Discussion

The scarce availability of antiviral treatments along with the increasing aquaculture production brings great economic problems related with viral infections in this sector. Apart from the generation of vaccines, which are very scarce and limited in the field, the use of AMPs has brought our attention due to their immunostimulatory and direct antiviral potential. NNV is one of the viruses that causes a great negative impact on fish marine farms around the world (Bandín and Souto, 2020). European sea bass antimicrobial peptides Hamp and Dic are known to exert direct antiviral effects against NNV *in vitro* (Chia et al., 2010; León et al., 2020). Recently, the *in vivo* administration of an expression plasmid coding for Hamp or Dic, as a potential preventive tool in aquaculture, has been tested with poor NNV-protective effects but modulating the host immune response (Cervera et al., 2023). In contrast, the administration of Nkl synthetic peptides to European sea bass specimens conferred up to 80% RPS against NNV (Valero et al., 2021). In this context, we aimed to *in vivo* evaluate the potential effects of two chemically synthetized European sea bass AMPs, Hamp and Dic, as immune regulators and antiviral preventive treatments.

From a practical point of view, we evaluated whether Hamp or Dic peptides could prevent and/or combat NNV infections in European sea bass, whatever the mechanisms involved, to then going further analyzing the biological reasons that explain this issue. Thus, Hamp and Dic treatments resulted in higher survival rates, although more severe

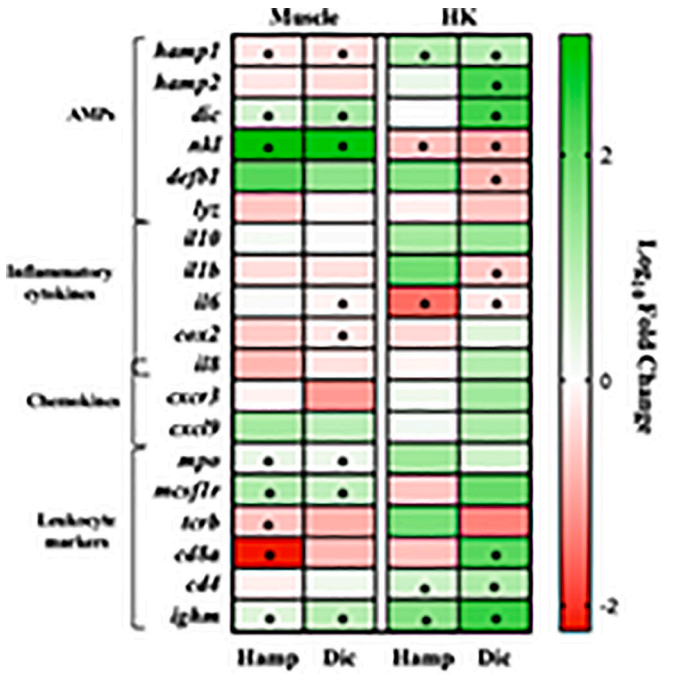

Fig. 4. Hamp and Dic peptides produce immunoregulation at gene level. Heatmap of immune-related gene transcription in the muscle and head-kidney (HK) from European sea bass juveniles intramuscularly injected with PBS, Hamp or Dic peptides (~1 μg peptide per g of fish) after 1 day of treatment. Transcription was corrected by the *ef1a* and *l13a* expression in each sample. Data are expressed as the $\log_{10}$ fold change respect to the PBS group. Asterisks indicate significant differences between each AMP respect to the PBS group ($p \leq 0.05$). Gene abbreviations are described in Table 2.

clinical signs were also observed. Similar to our data, the survival rate against NNV increased in medaka treated with Hamp or epinecidin (Wang et al., 2010a, 2010b) and in sea bass treated with Nkl peptides (Valero et al., 2021). The levels of Hamp and Dic at the site (muscle) and moment (24 h) of infection showed no differences between treated and non-treated fish, suggesting they might be degraded. In fact, Hamp and Dic peptide-treated fish showed, upon infection, similar levels of viral replication and loads, and a reduced antiviral *mx* transcription in brain. These data suggest that the antiviral activity of the AMPs *in vivo* is not mediated by a direct lysis/agglutination of NNV particles at the site of infection, as evidenced *in vitro* (Chia et al., 2010; León et al., 2020), since viruses reach the brain where they replicate and produce infective particles.

The blockage on the NNV-induced *mx* antiviral marker levels triggered by Hamp and Dic pre-treatments could explain the increased, although not significant, viral replication and load, probably resulting in higher neuronal cell death that explain the increment in the severity of the disease signs before death. Supporting our data, *in vitro* and *in vivo* epinecidin treatments also blocked the up-regulation of *mx* upon NNV infections in several fish species (Chia et al., 2010; Wang et al., 2010a, 2010b). So, taking into account that Hamp- and Dic-treated fish are partially protected against NNV but show similar viral levels and reduced interferon type-I response, carried out by all nucleated cells, the protection might be explained through immunomodulatory actions of the AMPs instead of their direct lytic capacity.

Next, and in order to ascertain the role in immunity of the AMPs studied, we evaluated some immune functional parameters as well as key immune gene expression levels before and after the NNV challenge. Before NNV challenge, both AMP treatments resulted in a strong modulation of the expression levels of several AMP genes, such as *hamp*, *nkl*, *dic* or *lyz*, mainly in the HK but also at the site of injection. These data support previous evidences about the existence of a crosstalk between different AMPs (Cervera et al., 2023). In fact, Hamp administration led to increased Dic sera levels while Dic administration led to increased sera bactericidal activity levels. According to the available data, the bactericidal activity is carried out by mostly unknown humoral factors in which AMPs might be included (Guardiola et al., 2014). There are evidences that AMPs act as anti-inflammatory mediators in the immune response (Luo and Song, 2021). In concordance, our data showed a scarce cytokine and chemokine response in muscle and HK. Thus, after Dic injection, *cox2* and *il6* expressions were down-regulated in muscle and *il1b* and *il6* were down-regulated in the HK as occurred when recombinant IL-6 was administered to Siberian sturgeon (*Acipenser baeri*) (Wang et al., 2020). In addition, a local recruitment of neutrophils and macrophages was observed in muscle upon AMP treatments, as previously observed upon AMPs-encoding plasmid injection (Cervera et al., 2023). Similarly, other AMPs such as NK-Lysin can induce macrophage-related genes in mudskipper (*Bolephthalmus pectinirostris*), Barbel steed (*Hemibarbus labeo*) or black rockfish (*Sebastes schlegelii*) (Chen et al., 2021; Ding et al., 2019; Hao et al., 2022), pointing to macrophage recruitment. The recruitment of leukocytes might in turn favor the regulation of the immune responses as fish neutrophils and macrophages are involved in the fine-tune of the innate response, guaranteeing host integrity, and promoting the adaptative response (Havixbeck and Barreda, 2015; Hodgkinson et al., 2015). In that sense our data also show that AMPs injection triggered the formation of T and B cells in HK, which are key cells in the immune regulation and the adaptative immune response against viruses (Castro et al., 2013; Chang et al., 2011; Nakanishi et al., 2015; Øvergård et al., 2013). Thus, Hamp and Dic treatments might be acting as linkers between innate and adaptive response as previously described for AMPs (Magrone et al., 2018). All these data suggest that, at the moment of infection, the immune system of the specimens was boosted permitting a better and faster response to combat the infection.

With the knowledge that synthetic Hamp and Dic elicited a partial protection upon NNV challenge, we evaluated and compared the changes in the anti-NNV immune response. We first observed the increase in the sera levels of Dic and Hamp in Hamp+NNV and Dic + NNV groups, respectively. At this point, we must consider that NNV is a neurotropic virus; however, AMPs act systemically modulating immune responses that have to be triggered in each tissue. For this reason, we firstly analyzed the transcriptional levels of AMPs in the NNV target tissue, the brain, obtaining interesting differences. We observed that in NNV-infected fish the AMPs gene expression in the brain tended to decrease. However, the expression levels of *hamp2, nkl* and *defb1* was up-regulated in the pretreated groups, constituting a strong difference between both conditions that might explain the partial protection observed. Similarly, the pattern of AMPs expression is also modulated by Hamp and Dic upon NNV infection in the HK and muscle suggesting that the effects of these AMPs extent along tissues. Although our data and other (Valero et al., 2020a) point to the fact that the AMP pathway is relevant against NNV infection, other mechanisms are also involved in the anti-NNV response in European sea bass (Chaves-Pozo et al., 2012; Valero et al., 2015b). For this reason, we wanted to get deeper knowledge about other immune mechanisms.

It has been documented that an exacerbated inflammatory response in brain during NNV infection cause the death to the infected fish (Chiang et al., 2017; Krasnov et al., 2013; Montes et al., 2010; Poisa-Beiro et al., 2008). Although NNV triggered inflammation in the brain, as indicated by elevated *il6* transcription, pretreatment with AMPs abrogated the exacerbated and pathogenic inflammation with a down-regulation of *il6* expression along with the up-regulation of the anti-inflammatory molecule *il10*. To fulfill this, the main players in the regulation of the inflammatory response, macrophages and neutrophils, were restored in the brain from AMP-treated and NNV-infected fish, as indicated by the transcription of *csf1r* and *mpo*, respectively. In addition, the systemic inflammation induced by NNV, studied in the HK, was significantly reduced by the AMP treatments evidenced by the

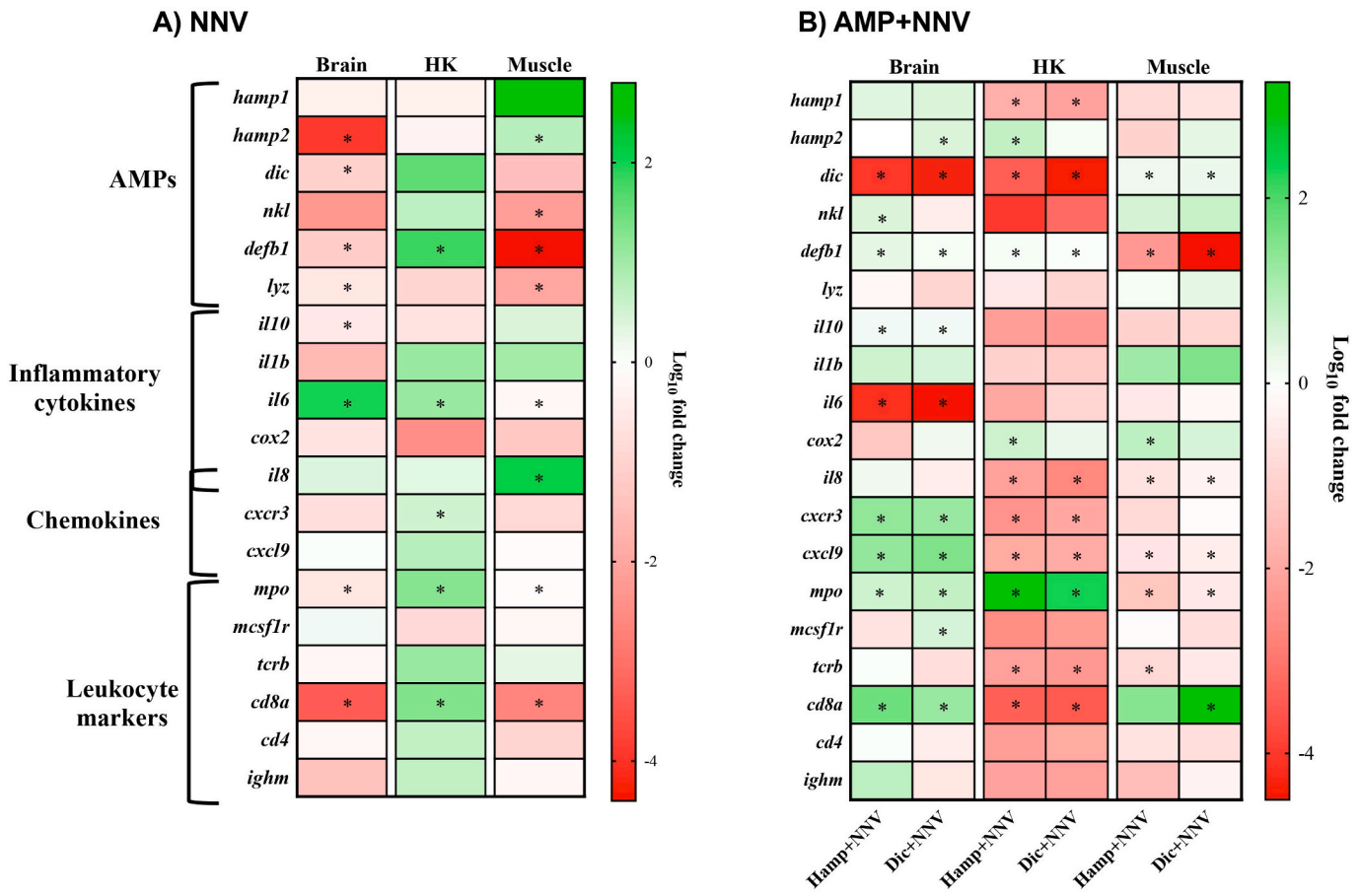

Fig. 5. Hamp and Dic peptides restore the immunoregulation provoked by NNV infection. Heatmap of immune-related gene transcription in the brain, head-kidney (HK) and muscle from European sea bass juveniles intramuscularly injected with PBS, Hamp or Dic peptides (~1 μg peptide per g of fish) and 1 day later intramuscularly infected with nodavirus (NNV; $TCID_{50}$/fish = 5.6×10^6). A mock group was injected twice with only PBS. **A.** Transcription in NNV-infected group respect to the mock group. Transcription was corrected by the *ef1a* and *l13a* expression in each sample. Data are expressed as the $\log_{10}$ fold change respect to the mock group. Asterisks indicate significant differences due to the NNV infection ($p \leq 0.05$). **B.** Transcription in AMP-treated and NNV-infected groups respect to the NNV-infected group. Transcription was corrected by the *ef1a* and *l13a* expression in each sample. Data are expressed as the $\log_{10}$ fold change respect to the NNV-infected group. Asterisks indicate significant differences due to the AMP pre-treatment ($p \leq 0.05$). Gene abbreviations are described in Table 2.

transcriptional levels of *cxcr3*, *il6* and *il8*, which, remaining at similar values than in the non-infected group, were greatly down-regulated comparing with the PBS + NNV group. This fact demonstrates the role of AMPs on controlling the inflammatory responses as previously described (Drayton et al., 2021; Kang et al., 2017; Luo and Song, 2021) and point to Hamp and Dic as excellent agents for its use in aquaculture. On the other hand, several other inflammatory markers remained unaltered suggesting that even if these peptides possess anti-inflammatory properties, they maintain key functions for the resolution of the infective processes (Drayton et al., 2021).

One of the inflammatory markers, which levels of expression were up-regulated in the brain of AMPs pretreated and NNV-infected fish, was Cxcl9, a chemokine scarcely studied in teleost but well-characterized in mammals. Cxcl9 is involved in immunoregulation by attracting immune cells as macrophages or T-cells, namely $CD8^+$ cells, and it is induced by IFN (Corbera-Bellalta et al., 2016; Lieberman et al., 2020; Wang et al., 2021). In this sense, the dropped levels of *cd8a* gene expression in brain upon infection are restored in Hamp and Dic pre-treated fish, perhaps thanks to the increased *cxcl9* transcription. In addition, several leucocyte gene markers (mainly *mcsf1r* and *cd8a*) decreased in HK and increased in the brain in AMP pretreated and infected fish, suggesting that their mobilization is triggered by AMP pretreatment upon infection. This mobilization of leucocytes probably plays a pivotal role in the inflammatory resolution but also in the cell-mediated cytotoxic response (Castro et al., 2014; Fischer et al., 2013; Jung et al., 2020; Somamoto et al., 2013, 2014). In vaccination studies, up-regulation of *cd8a* has been related to the increase of survival (Buonocore et al., 2019; López-Vázquez et al., 2023; Valero et al., 2016) as also occurs in this work, suggesting that this cell-type might contribute to the proper clearance of NNV induced by the synthetic Hamp and Dic peptides.

5. Conclusions

In conclusion, the preventive administration of Hamp and Dic prior to NNV infection, significantly reduced the mortality rates associated to this disease though failed to modify viral replication or titers. The increase of survival might be due to the primed immune status before infection as well as to the restoration of the anti-inflammatory status and leucocyte trafficking in the brain upon NNV infection. Therefore, the preventive protective actions of Hamp and Dic peptides seem to be more dependent on the immunomodulatory actions than on the direct lytic or agglutinative properties of those AMPs. For these reasons, Hamp and Dic synthetic peptides can be postulated as excellent tools to deal with NNV outbreaks in fish farms.

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All authors have read and agreed to the published version of the manuscript.

CRediT authorship contribution statement

Laura Cervera: Data curation, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Marta Arizcun:** Funding acquisition, Methodology, Supervision, Writing – review & editing. **Luis Mercado:** Investigation, Methodology, Writing – review & editing. **Elena Chaves-Pozo:** Funding acquisition, Supervision, Writing – review & editing, Methodology. **Alberto Cuesta:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.740592.

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