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In vivo modulation of the rainbow trout (*Oncorhynchus mykiss*) immune response by the human alpha defensin 1, HNP1

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Abstract Recent studies have demonstrated that the synthetic human defensin-α1, also designated as human neutrophil peptide 1 (HNP1), not only has in vitro antiviral activity against viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus, but can also modulate some immune activities of rainbow trout (*Oncorhynchus mykiss*) head kidney leucocytes. However, none of these HNP1 properties have been analysed in vivo so far. Thus, in the current work, we have studied the in vivo immunomodulatory capacity of HNP1 on the rainbow trout immune system as a first approach to evaluate the possible use of this family of antimicrobial peptides (AMPs) to increase fish resistance by enhancing non-specific defence mechanisms. The intramuscular injection of synthetic HNP1 induced the transcript expression of genes encoding both pro-inflammatory cytokines (IL-1β, TNF-α1 and specially IL-8) and CC chemokines (CK5B, CK6 and CK7A) as well as of the genes related to type I interferon (IFN) production (Mx1, Mx2, Mx3 and IFN regulatory factor 3, IRF-3) in different trout tissues (muscle, head kidney and blood). Furthermore, the chemotactic capacity of HNP1 towards trout leucocytes has been clearly revealed. All together, these results demonstrate that in vivo HNP1 is active across species and can modulate fish immune responses. Therefore, in a moment when most pathogens have developed resistance to commonly used antibiotics, natural antimicrobial peptides with interspecific activity, such as HNP1, might prove to be useful model molecules for the development of novel therapeutic agents that exhibit both microbicidal and immunoenhancing capabilities.

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Introduction

Despite advances in prevention, diagnosis, and treatment, infectious diseases continue to challenge the aquaculture industry. Due to the limitations of vaccine-based immunization strategies and the increasing resistance of microbes to existing antibiotics, research related to innate host defence mechanisms that are not dependant on specific recognition of individual antigens offers a promising field to search for new antibiotics or immunostimulants. In this context, defensins, are now considered as model molecules for the development of novel therapeutic agents that exhibit both microbicidal and immunoenhancing capabilities.

Defensins are cationic antimicrobial peptides (AMPs) with β-sheet structures stabilised by three intramolecular disulphide bonds [1,2]. Mammalian defensins are classified into α, β, or θ depending on the distribution of the disulphide bonds between their six conserved cysteine residues. Human α-defensins, also designated as human neutrophil peptides (HNP1, HNP2, HNP3 and HNP4), are synthesised as prepropeptides, and therefore contain an amino-terminal signal sequence, an anionic propiece and a carboxy-terminal mature peptide of approximately 30 amino acid [2]. Alpha-defensins are mainly produced by neutrophils, as part of their granule content, but they can also be produced by other cell types such as NK cells [3]. These molecules not only exhibit antimicrobial properties, but are also capable of modulating the immune response; thus they constitute very good candidates for therapy or for use as adjuvants in vaccination.

Due to their high homology, HNP1-3 are usually studied as a group although differences in their microbicidal [4] and immunoregulatory activities have been reported [5]. HNPs can influence various mechanisms of the innate immune response; thus they have been shown to modulate the production of chemokines, such as interleukin 8 (IL-8) [6,7], apart from being chemoattractant for some cell types themselves [5]. HNPs also regulate NK-mediated cytotoxicity, although the mechanism of action is not entirely clear yet [3]. Furthermore, HNPs can also modulate the adaptive immune response and have been shown to enhance specific antibody and cellular responses [8—10], acting as effective adjuvants. The mechanism through which HNPs exert their adjuvant activity has not been fully elucidated yet in mammals, and many hypothesis such as direct modulation of lymphocyte responses or modulation of antigen presenting cell function, through enhanced chemotaxis, have been postulated [11]. In fact, there is even some controversy dealing with the hypothesis that the main role of HNPs in vivo is the immunomodulation since their microbicidal effects is abrogated in the presence of serum and albumin [12].

Using a database mining approach, genes encoding β-defensin-like peptides have been recently discovered in three non-salmonid fish species (zebrafish, puffer fish, and tetraodon) [13] and rainbow trout (GenBank accession nos. AM286737, AM282656, AM282655 and AM282657), but biological activity has been only shown for the trout β-defensin-like peptide [14]. To date, although there is no evidence of α-defensins in fish, their presence can not be excluded until more expressed sequence databases and genome sequences become available. Moreover, the in vitro inhibition of viral hemorrhagic septicemia rhabdovirus (VHSV) infectivity by HNP1 as well as the induction of transcript expression of genes related to interferon (IFN) production and other immune-related genes (interleukin 1β, IL-1β and inducible nitric oxide synthase, iNOS) after ex vivo treatment of rainbow trout head kidney leukocytes with HNP1 has been recently demonstrated [15], suggesting the presence of α-defensins in fish.

While awaiting for the discovery of fish α-defensins and following the in vitro results previously found in fish with HNP1, in this work, we have evaluated the effect of HNP1 on the levels of expression of genes relevant to the early inflammatory response (IL-1β; IL-8 and tumour necrosis factor α1, TNF-α1), chemokines belonging to the CC family such as CK5B, CK6 and CK7A, genes related to IFN production (Mx1, Mx2, Mx3 and interferon regulatory factor 3, IRF-3), to macrophage activation (iNOS and IFN-γ) or to antigen presentation (major histocompatibility complex, MHC-I and MHC-II) in rainbow trout. In order to establish if there is a direct effect of defensins on fish leukocyte chemotaxis, we have also studied the capacity of HNP1 to attract trout leukocytes.

These results can be useful towards the understanding of how antimicrobial peptides work in fish, and might help elucidate whether α-defensin homologues are likely to be present in fish. Moreover, due to the effectiveness of DNA vaccination in fish [16], and the possibility of incorporating molecular adjuvants in these plasmids in the form of gene sequences within the vaccine plasmid, it is not outrageous to further investigate the effects of the incorporation of sequences that code for these human defensins which not only are immunostimulatory, but also exert antiviral effects against VHSV [15].

Materials and methods

Fish

Rainbow trout (*Oncorhynchus mykiss*) of approximately 8—10 cm obtained from Lillogen (Leon, Spain) were maintained in 50 L tanks at the Miguel Hernandez University (Elche, Spain) laboratory at 14 °C with a re-circulating water system using water from the fish farm. Fish were fed daily until satiated with a commercial diet (Trow, Leon, Spain). Prior to the experiments, fish were acclimatised to laboratory conditions for 2 weeks.

Peptides

Synthetic human alpha-Defensin-1 (HNP1) (ACYCRIPACIAGERRYGTCIYQGRLWAFCC-NH_2, disulfide bonds: 2—30, 4—19 and 9—29) was purchased from Peptides International (Louisville, KY, USA). The purity of the peptide was >98%. The mature sequence of Limanda (*Limanda limanda*) pleurocidin (LmPle) (GWKKWFKKATHVGKHVGKAALDAYL) [17] was used as a control to verify that the effects of HNP1 on the trout immune response were specific of HNP1 and not only due to the injection of a foreign peptide. Synthetic LmPle was obtained from Diverdrugs (Diverdrugs

S.A., Barcelona, Spain). The purity of peptide was >95%. Both HNP1 and pleurocidin were reconstituted to a final concentration of 1 μg μl^{-1} in sterile distilled water and stored until used in suitable aliquots at −20 °C.

Injection of HNP1 and LmPle into rainbow trout muscle

To determine the effect of HNP1 on the rainbow trout immune system, fish were divided into 3 groups (12 fish each). One group was intramuscularly injected with the human defensin HNP1 (1 μg in 100 μl of phosphate buffered saline, PBS, per fish) another one was injected with LmPle (1 μg in 100 μl of phosphate buffered saline, PBS, per fish) and the last one was mock-injected with the same volume of PBS. At days 1, 3, and 7 post-injection four trout from each group were sacrificed by overexposure to MS-222, muscle and head kidney removed and blood extracted from the caudal vein.

cDNA synthesis

Total RNA of the different tissues was extracted using Trizol (Invitrogen, UK). Individual organs were homogenized in 1 ml of Trizol in an ice bath, and mixed with 200 μl of chloroform. The suspension was then centrifuged at 12,000 × *g* for 15 min. The clear upper phase was aspirated and placed in a clean tube. Five hundred μl of isopropanol were then added, and the samples were again centrifuged at 12,000 × *g* for 10 min. The RNA pellet was washed with 75% ethanol, dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C.

Two μg of RNA were used to obtain cDNA using the Superscript II reverse transcriptase (Invitrogen, UK). Briefly, RNA was incubated with 1 μl of oligo (dT)12−18 (0.5 μg ml^{-1}) and 1 μl 10 mM deoxynucleotide triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of Superscript II reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min, and the resulting cDNA was diluted in a 1:5 proportion with DEPC-treated water and stored at −20 °C.

PCR of immune genes

All amplification reactions were performed using 0.5 μl dNTP mix (10 mM each), 0.2 μl Taq polymerase (5 units μl^{-1}, Invitrogen, UK), 2.5 μl Taq 10× buffer, 0.75 μl $MgCl_2$ 50 mM, 0.5 μl of each primer (50 μM) and 1 μl of cDNA in a final volume of 25 μl. First, a PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with all samples as a positive control for RT-PCR, since GAPDH is constitutively expressed in all organs. These PCR products also allowed the verification that equivalent amounts of cDNA were present in the different samples and therefore amplifications of the different immune genes were comparable among samples. A negative control in which no cDNA was added was included in all PCR reactions.

Primers used for gene amplification, and the sizes of the different PCR products are shown in Table 1. All PCRs were carried out in a Perkin Elmer 2720 cycler and amplification conditions always consisted of a denaturing step of 94 °C for 5 min followed by the different specific cycling conditions shown in Table 1 followed by a final extension of 7 min at 72 °C. For each gene, after optimising the conditions following protocols described previously and referenced in Table 1, at least two PCRs with different number of cycles were performed in order to determine at which point of the amplification differences were evident among samples. Once the optimal number of cycles (Table 1) was determined, all samples were amplified twice to verify the results. The PCR products (8 μl) were visualized on a 2% agarose gel stained with ethidium bromide. Samples that were to be compared were always run in the same agarose gel. A 100 bp ladder was used as a size marker. The intensity of the amplification bands was estimated using Image Gauge v. 4.0 software (Fujifilm, Barcelona, Spain). Semi-quantitative analysis of mRNA transcription for each gene was performed relative to the GAPDH expression of the same sample using the formula: intensity of target gene band/intensity of its corresponding GAPDH band. Data were then analysed using Student's *t*-test comparing values obtained in mock-injected fish and fish injected with HNP1. Differences were considered statistically significant at $p < 0.05$.

Blood leukocyte isolation

Blood leukocytes were isolated following the method previously described [18]. Briefly, blood obtained from the tail vein was diluted 5 times with Leibovitz medium (L-15, Gibco, Invitrogen, UK) supplemented with penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), heparin (10 units ml^{-1}) and 2% foetal calf serum (FCS, Gibco). The resulting cell suspension was placed onto 51% Percoll density gradients. The gradients were centrifuged at 500 × *g* for 30 min at 4 °C. The interface cells were collected and washed twice at 500 × *g* for 5 min in L-15 containing 0.1% FCS. The viable cell concentration was determined by Trypan blue exclusion. Cells were resuspended in L-15 with 5% FCS at a concentration of 5 × 10^5 cells ml^{-1}.

Chemotaxis experiments

The capacity of HNP1 to induce specific migration in trout blood leukocytes was studied using 96-well chemotaxis chambers (Neuroprobe, Gaithersburg, MD, USA) in which the different concentrations of HNP1 (0.1, 1 and 2 μg ml^{-1}) were diluted in L-15 medium to make a final volume of 30 μl. Controls consisted in L-15 medium alone. Blood leukocytes (30 μl of a suspension containing 5 × 10^6 cells ml^{-1}) were dispensed in the upper chamber, separated by a 3 μm polycarbonate membrane. After 60 min of incubation at 20 °C, the number of cells that had migrated to the bottom wells was estimated using CellTiter 96 (Promega, Madison, WI, USA) according to manufacturer's instructions. This assay is based on the bioreduction of an MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopehyl)-2H-tetrazolium] into a coloured formazan product soluble in culture medium which can be then estimated by its absorbance at 490 nm.

Table 1 Primer sequences, sizes of PCR products and amplification conditions for the different genes studied

Gene	Primers	Size of PCR product (bp)	N° of cycles	Cycling conditions	Reference
GAPDH	F: 5′ ATGTCAGACCTCTGTGTTGG 3′ R: 5′ TCCTCGATGCCGAAGTTGTCG 3′	514 bp	32	94 °C 30 s 58 °C 30 s 72 °C 1 min	[25]
IL-1β	F: 5′ AGGGAGGCAGCAGCTACCACAA 3′ R: 5′ GGGGGCTGCCTTCTGACACAT 3′	353 bp	35	94 °C 30 s 60 °C 30 72 °C 30 s	[26]
IL-8	F: 5′ GAATGTCAGCCAGCCTTGTC 3′ R: 5′ TCCAGACAAATCTCCTGACCG 3′	226 bp	35	94 °C 30 s 60 °C 30 s 72 °C 30 s	[20]
TNF-α1	F: 5′ TTCGGGCAAATATTCAGTCG 3′ R: 5′ GCCGTCATCCTTTCTCCACT 3′	433 bp	10 25	94 °C 1 min 60 °C 1 min 72 °C 20 s 94 °C 1 min 60 °C 1 min 72 °C 20 s + 1 s per cycle	[27]
CK5B	F: 5′ TTTGCTGATCGTCAGATACCC 3′ R: 5′ GGACCATGACTGCTCTCTCTG 3′	315 bp	37	94 °C 20 s 55 °C 20 s 72 °C 30 s	[23]
CK6	F: 5′ CGAATCTGCTCTGACACTTCC 3′ R: 5′ TGGTGAGTTGTTGACCATTGA 3′	219 bp	37	94 °C 20 s 55 °C 20 s 72 °C 30 s	[23]
CK7A	F: 5′ TCTGCAGGTGTCATTAAGTTGG 3′ R: 5′ TCTTTGTGGTGAAAATCAGTGC 3′	139 bp	37	94 °C 20 s 55 °C 20 s 72 °C 30 s	[23]
Mx1	F: 5′ ATGCCACCCTACAGGAGATGAT 3′ R: 5′ TAACTTCTATTACATTTACTATGCAA 3′	421 bp	37	94 °C 30 s 52 °C 30 s 72 °C 30 s	[24]
Mx2	F: 5′ ATGCCACCCTACAGGAGATGAT 3′ R: 5′ GGAAGCATAGTAACTTTATTATAAC 3′	400 bp	37	94 °C 30 s 52 °C 30 s 72 °C 30 s	[24]
Mx3	F: 5′ ATGCCACCCTACAGGAGATGAT 3′ R: 5′ CCACAGTGTACATTTAGTTG 3′	381 bp	37	94 °C 30 s 52 °C 30 s 72 °C 30 s	[28]
IRF-3	F: 5′ GTCCCTCTTTAGCACAAGTC 3′ R: 5′ GGTGGAGCAGTTCACAAATG 3′	690 bp	35	94 °C 20 s 60 °C 20 s 72 °C 20 s	CB515644
iNOS	F: 5′ CATACGCCCCCAACAAACCAGTGC 3′ R: 5′ CCTCGCCTTCTCATCTCCAGTGTC 3′	746 bp	40	94 °C 1 min 62 °C 1 min 72 °C 2 min	[27]
IFN-γ	F: 5′ GTGAGCAGAGGGTGTTGATG 3′ R: 5′ GATGGTAATGAACTCGGACAG 3′	251 bp	40	94 °C 20 s 60 °C 20 s 72 °C 20 s	[29]
MHC-I	F: 5′ CAGTGTCTCTGCTCCAGAAGG 3′ R: 5′ TCAGAACCTCGATGAAGTCCTT 3′	263 bp	28—32	94 °C 30 s 55 °C 30 s 72 °C 30 s	[30]
MHC-II	F: 5′ ATGTCGATGCCAATTGCCTTCTA 3′ R: 5′ TGTCTTGTCCAGTATGGCGCT 3′	336 bp	28	94 °C 30 s 57 °C 30 s 72 °C 30 s	[27]

Results

Effect of HNP1 on the expression of pro-inflammatory cytokines

The effect of HNP1 on the expression of different pro-inflammatory cytokines was studied in order to evaluate the potential use of defensins as adjuvants in fish (Fig. 1). In the head kidney, HNP1 produced no effect on IL-1β and TNF-α1 expression, while it significantly increased IL-8 at day 1 post-injection. In the blood, however, a moderate increase of IL-1β was observed at day 3. For TNF-α1, we observed a significant decrease of the constitutive levels of expression at day 7. In the muscle, there was a strong induction of all three cytokines.

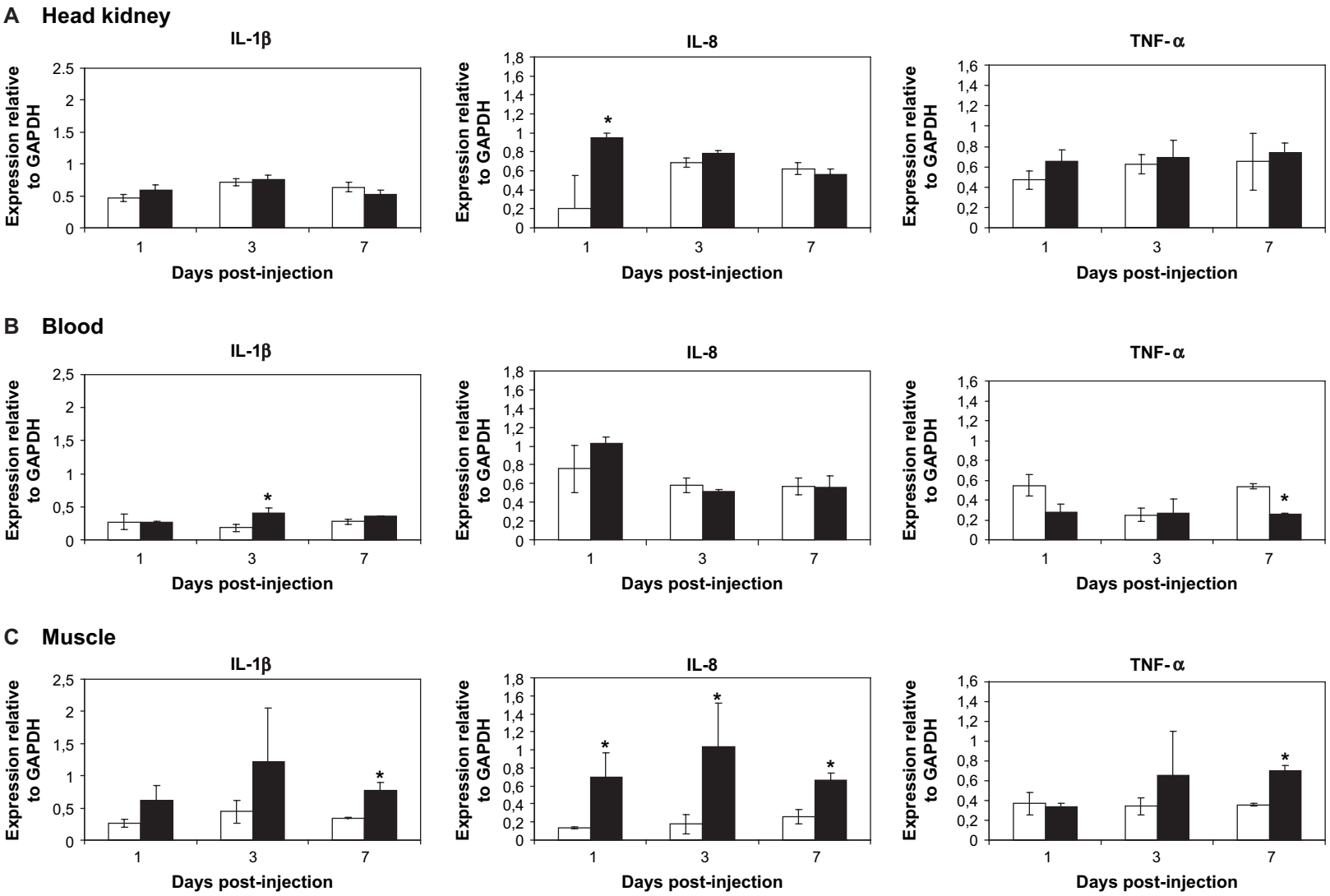

Figure 1 Effect of HNP1 on the expression of pro-inflammatory cytokines: IL-1β, IL-8 and TNF-α1. Levels of expression of the different cytokines were assayed by semi-quantitative RT-PCRs in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (black bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression ± SD for four individuals from each group. *Expression significantly different from that observed in mock-injected controls ($p < 0.05$).

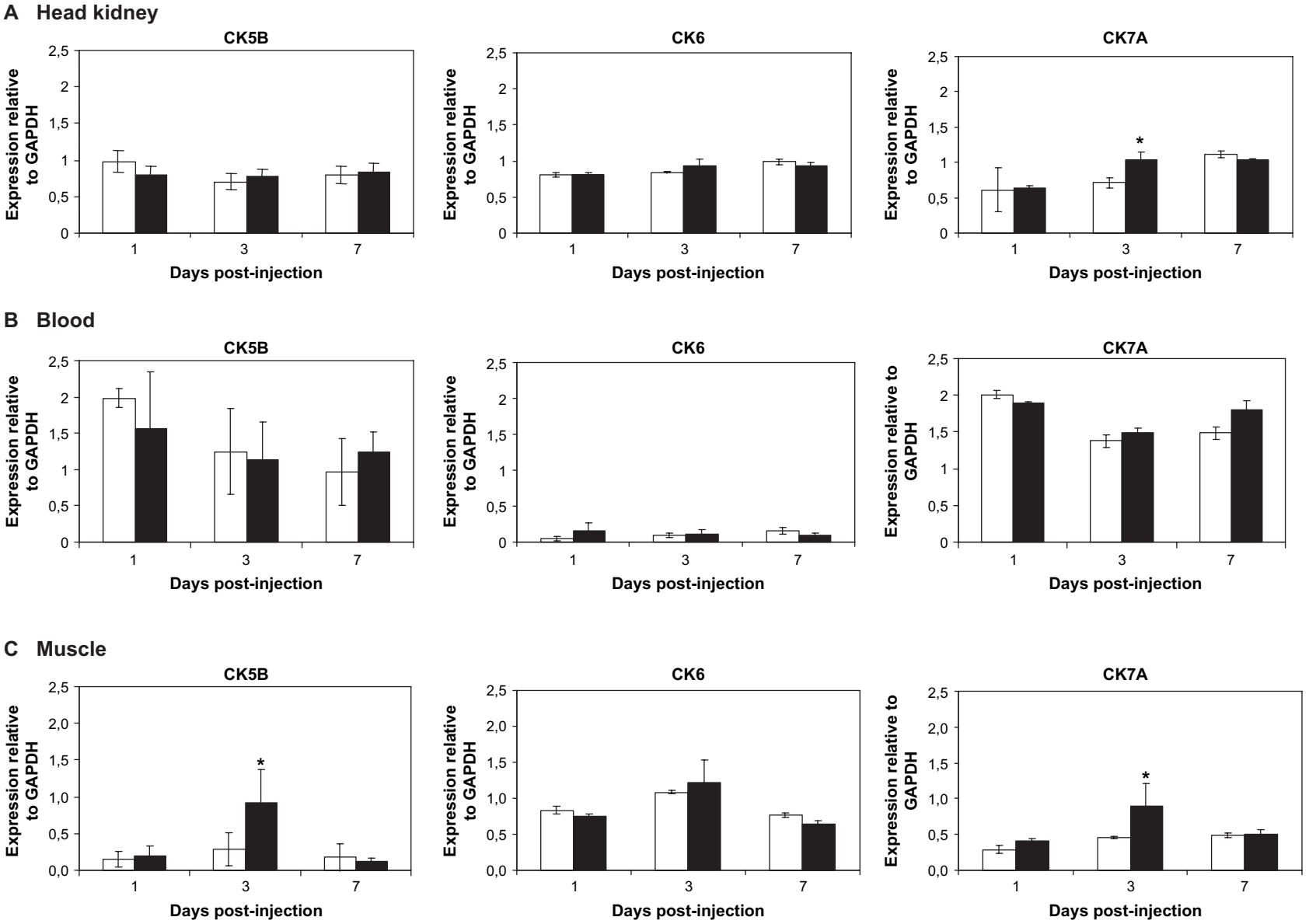

Figure 2 Effect of HNP1 on the expression of the CC chemokines CK5B, CK6 and CK7A. Expression was studied in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (black bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression ± SD for four individuals from each group. *Expression significantly higher than that observed in mock-injected controls ($p < 0.05$).

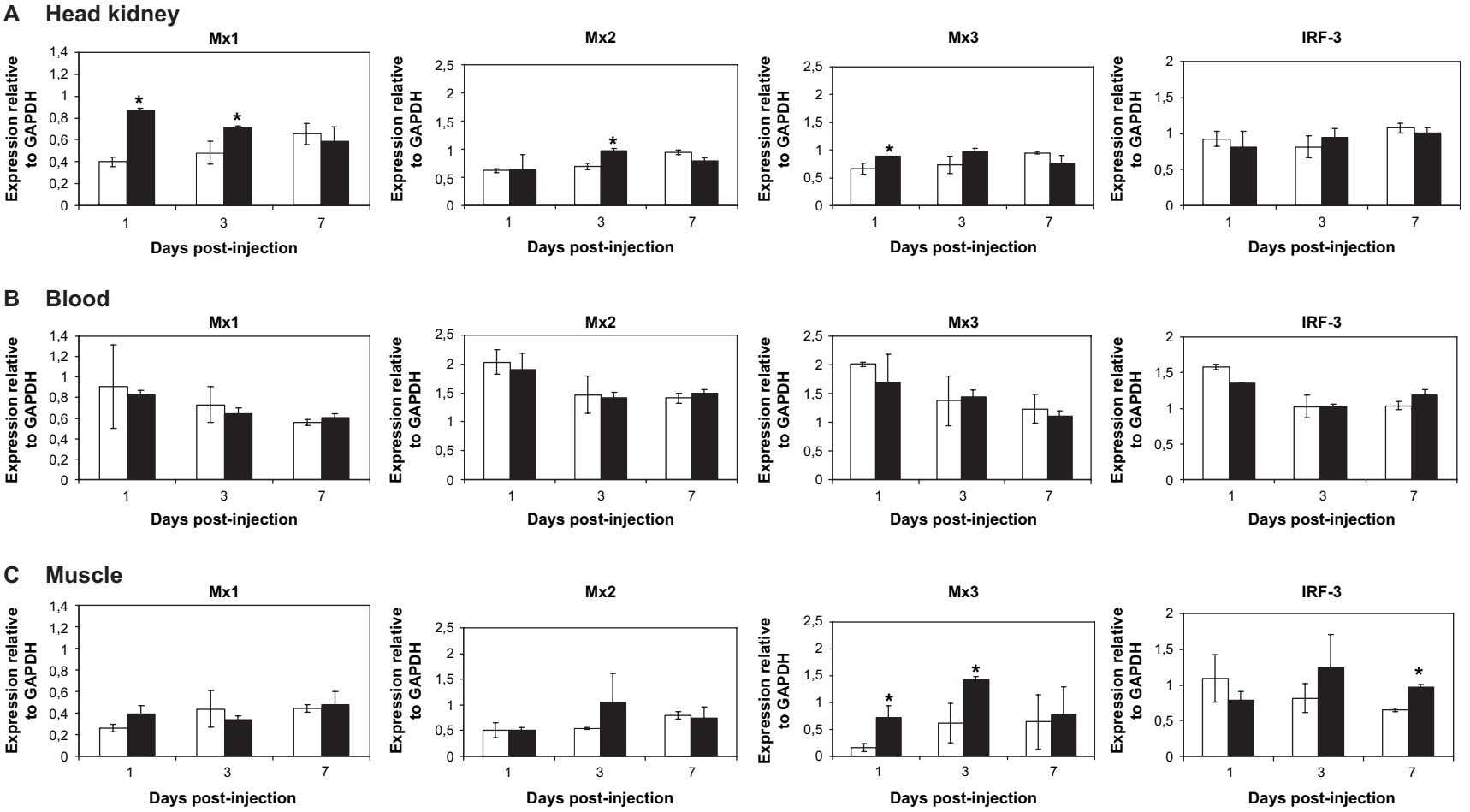

Figure 3 Effect of HNP1 on the expression of genes related to IFN production: Mx1, Mx2, Mx3 and IRF-3. Expression was studied in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (black bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression ± SD for four individuals from each group. *Expression significantly higher than that observed in mock-injected controls ($p < 0.05$).

Effect of HNP1 on the expression of CC chemokines

We also studied the effect of HNP1 administration on the expression of chemokines belonging to the CC family such as CK5B, CK6 and CK7 (Fig. 2). In the head kidney, only the levels of expression of CK7A were increased in treated animals. The levels of expression found in blood were not altered by HNP1, whereas in the muscle, a strong induction of both CK5B and CK7A transcription was observed at day 3.

Effect of HNP1 on the expression of molecules related to the IFN system

The effects of HNP1 on the expression of IFN-induced genes was evaluated through the analysis of the transcript expression of the three trout Mx isoforms and IRF-3 (Fig. 3). In the head kidney, the three Mx isoforms were significantly induced in response to HNP1, while no effect on IRF-3 expression was observed. In the blood, neither of the IFN-related genes studied was altered. In the muscle, Mx3 was the only isoform induced. IRF-3 was also significantly induced in this tissue after 7 days post-injection.

Effect of HNP1 on the expression of molecules related to macrophage functions

The injection of HNP1 did not have a significant effect on the expression of genes related to macrophage activation such as iNOS or IFN-γ in the head kidney nor in blood (Fig. 4). However, a significant up-regulation of iNOS was observed in the muscle at day 3 post-injection.

Effect of HNP1 on the expression of MHC genes

Concerning the effect of HNP1 injection on the expression of MHC genes, only MHC-I was significantly up-regulated when compared to the controls and only in muscle and only on day 7 (Fig. 5).

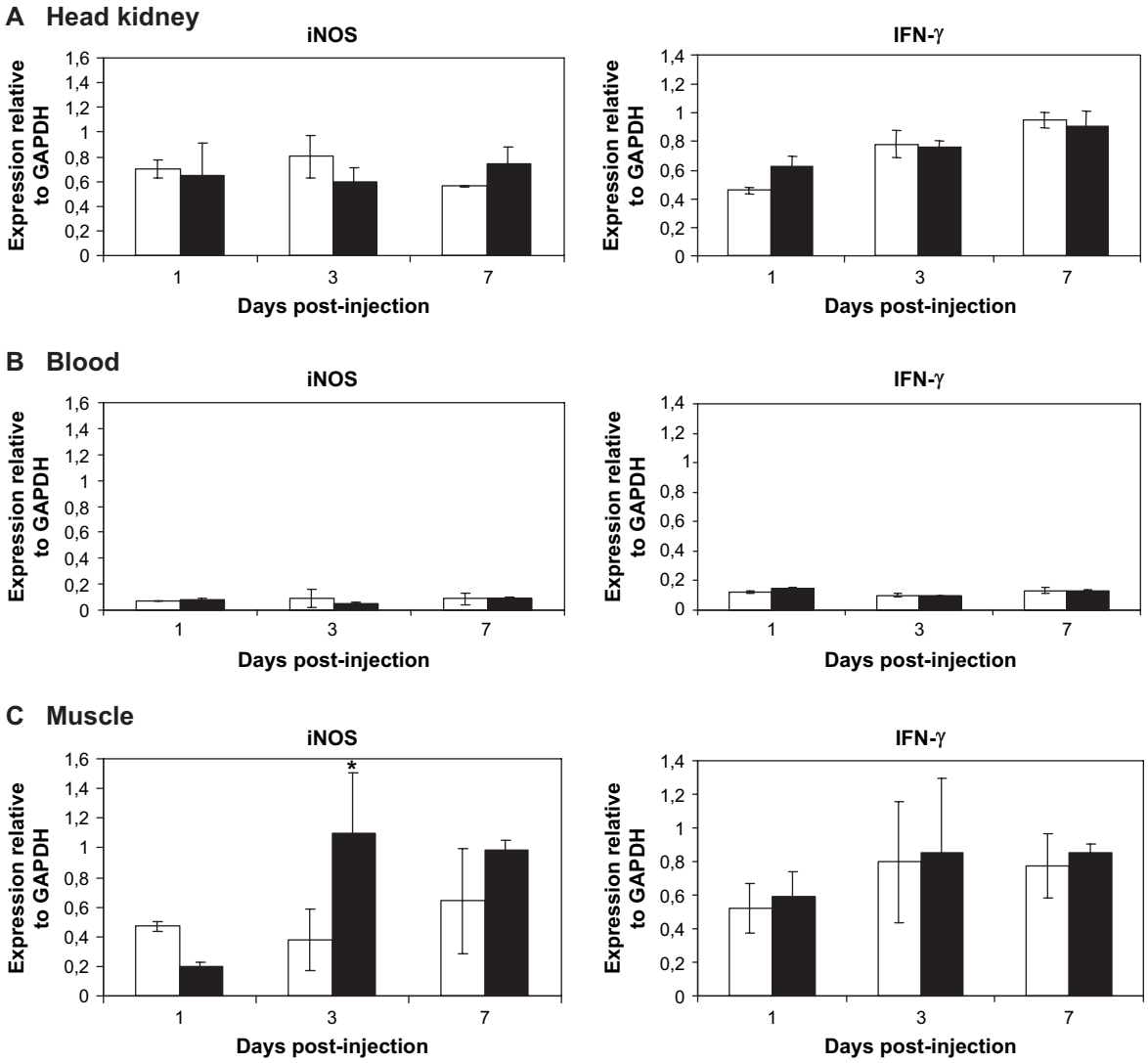

Figure 4 Effect of HNP1 on the expression of genes implicated in macrophage activation: iNOS and IFN-γ. Expression was studied in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (black bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression $\pm$ SD for four individuals from each group. *Expression significantly higher than that observed in mock-injected controls ($p < 0.05$).

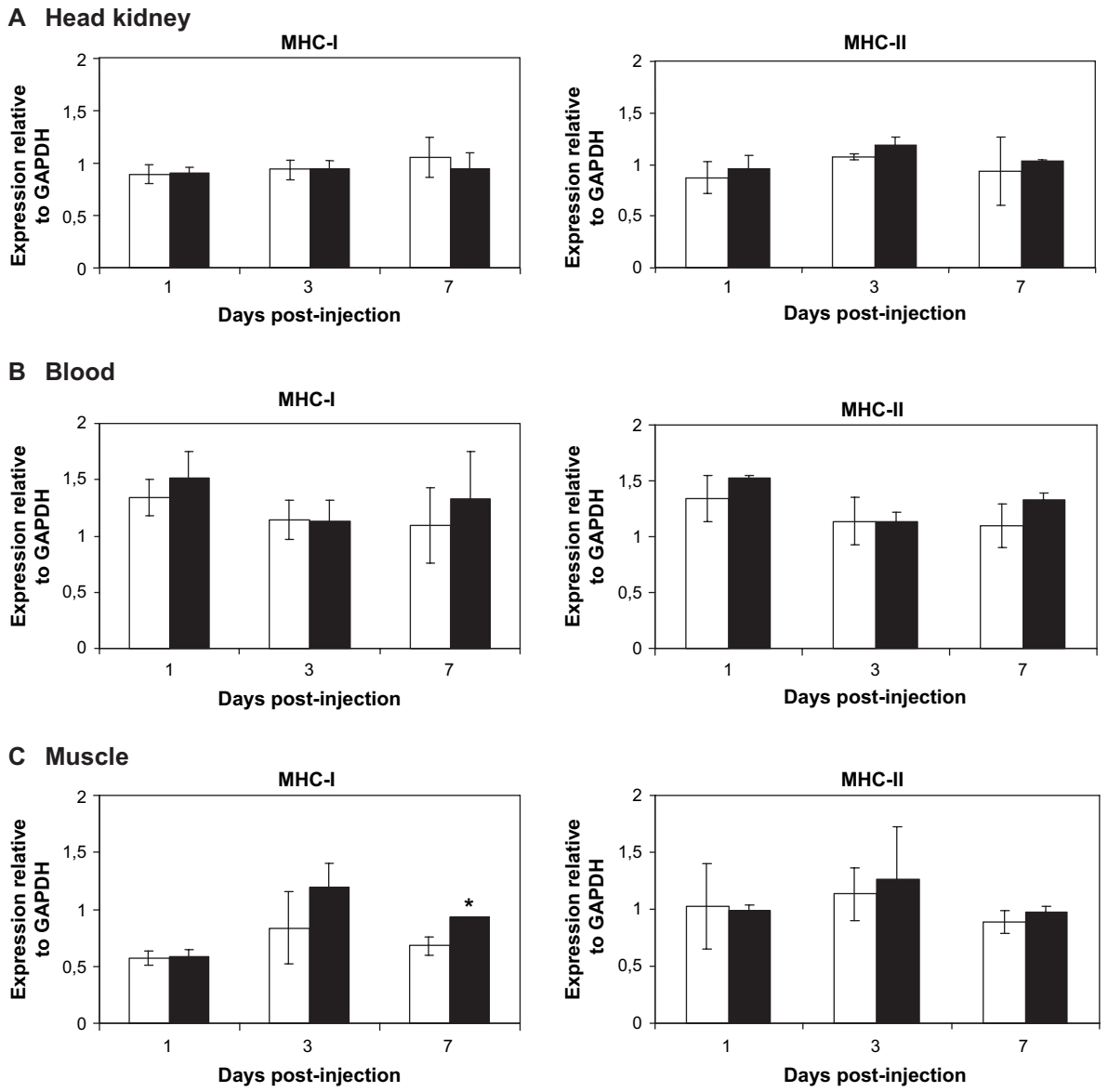

Figure 5 Effect of HNP1 on the expression of MHC genes. Expression was studied in the head kidney (A), blood (B) and muscle (C) of trout intramuscularly injected with 1 μg HNP1 (white bars) or mock-injected (white bars) at days 1, 3 and 7 post-injection. Data are presented as mean relative expression ± SD for four individuals from each group. *Expression significantly higher than that observed in mock-injected controls ($p < 0.05$).

Chemoattractant capacity of HNP1

To elucidate a possible direct effect of defensins in fish leukocyte chemotaxis, we studied if HNP1 could specifically attract trout blood leukocytes. First, a high range of HNP1 concentrations was assayed in order to establish the optimal HNP1 doses. Once we determined that the optimal HNP1 doses for this assay ranged from 0.1 and 2 μg ml^{-1} HNP1, we performed the assay in leukocytes belonging to three different trout (Fig. 6). We found a significant migration of leukocytes when compared to controls towards 0.1 and 1 μg ml^{-1} HNP1.

Discussion

In the current study, using human HNP1, we have evaluated for the first time in fish the immunomodulatory capacity of a defensin in vivo. We have demonstrated that HNP1 is active across species in vivo and has diverse immunomodulatory properties in fish, in addition to its established in vitro antiviral activity against VHSV [15].

HNP1 was able to modulate the expression of many genes related to the innate immune response (genes encoding pro-inflammatory cytokines, chemokines, IFN-stimulated genes, etc.) in rainbow trout, not only in the muscle (site of injection) but for some genes also in the blood and head kidney. Since the presence of HNP1 is not expected in the head kidney after intramuscular injection, the effects of HNP1 seen on this organ should be part of a systemic response to this α-defensin. Because the biological effects of AMPs, similarly to their expression and secretion, are often induced by inflammatory stimuli and are influenced by the physiological setting, including the concentration of the peptide,

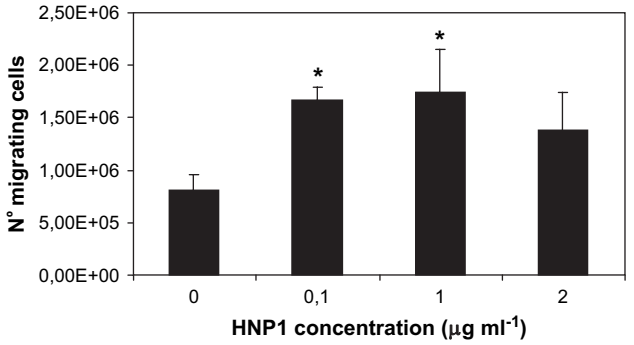

Figure 6 Migration of trout blood leukocytes towards HNP1. Chemotactic activity towards HPN1 was assayed in 96-well chemotaxis chambers. The different dilutions of HPN1 (30 μl) were placed in the lower chambers, while 30 μl of the cell suspensions (5×10^6 cells ml^{-1}) were placed in the upper wells. After 60 min of incubation at 20 °C, the number of cells that had migrated to the bottom chambers were counted using CellTiter 96 (Promega). After the addition of 5 μl of CellTiter96 per well, the plate was incubated at 37 °C for 2 h. After that time, the optical density at 492 nm was determined. The number of cells was estimated using a standard curve with known cell concentrations. Results are presented as the mean number of cells that had migrated ± SD ($N = 3$).

the cellular environment and soluble components of the extracellular milieu, we also analysed the in vivo effect of LmPle, a cationic AMP of 25 amino acid member of a larger family of AMPs present in flatfish, on the expression of these immune genes. Of all genes studied, LmPle only produced a significant up-regulation of the levels of expression of pro-inflammatory cytokines (data not shown), thus, we can conclude that most of the effects of HNP1 are an exclusive response to the HNP1.

Regarding the expression of pro-inflammatory cytokines (IL-1β, TNF-α1 and IL-8) HNP1 strongly increased all three cytokines in the muscle, and also in the blood in the case of IL-1β, and in the head kidney in the case of IL-8. In mammals the effect of HNPs on IL-1β expression is controversial [7], and it seems to be dependant on the cell type. In our work, we observed an up regulation of IL-1β in response to HNP1, however, we also observed a significant increase of IL-1β in response to LmPle (data not shown) which confirms previous results showing induction of IL-1β by Ple in RTS11 cells (trout macrophages) [19]. Thus, it seems that the effect that HNP1 produces on IL-1β and TNF-α1 genes, are mostly part of an inflammatory response to a foreign peptide. The levels of expression of IL8 induced by HNP1 were, however, significantly stronger, in agreement with what had been previously reported in mammals [6,7]. Although IL-8 is characteristic of the early immune response and it belongs to the CXC family of chemokines [20], this cytokine can be classified within the pro-inflammatory cytokines as well as within chemokines. This ''dual character'' of IL8 could be underlying the differential effect of HNP1 on the IL-8 induction related to that observed for IL-1β and TNF-α1. There is a close relation between antimicrobial peptides and chemokines, and although controversial, it has been proposed that some antimicrobial peptides evolved from chemokines [21], since some chemokines have some antimicrobial activity [22], and many antimicrobial peptides have chemoattractant capacity [5]. To investigate whether any relation between HNP1 and chemokines can observed in fish, we also analysed the effect of HNP1 on the expression of other chemokines belonging to the CC family: CK5B, CK6 and CK7A. In rainbow trout, two forms (A and B) are found for CK5 and CK7 [23] but their biological significance is still unknown. For this study, we chose only one of the isoforms for each of these genes. HNP1 was also capable of increasing the levels of expression of two of the three CC chemokines studied, CK5B and CK7A, mostly in the muscle, although for CK7A, some effect was also visible in the head kidney. CK5B and CK7A, are homologues of the mammalian RANTES (regulated on activation, normal T cells expressed and secreted) and MCP (monocyte chemotactic protein), respectively. It seems that this effect on CC chemokines is specific for HNP1, since other peptides such as LmPle or even VHSV (Jana Montero, personal communication) failed to induce their expression. In this context, we thought it was important to study whether HNP1 by itself was chemotactic for fish leucocytes, as in mammals. We found that HNP1 significantly attracted trout blood leukocytes. Again, this demonstrates that antimicrobial peptides play a major role in chemotaxis, in part indirectly, by the activation of other chemokines, and directly by being chemotactic themselves.

Since the in vitro inhibition of VHSV by HNP1 is, at least in part, mediated by a type I IFN-antiviral response [15], we evaluated the effect of HNP1 injection on the expression of IFN-related genes such as the different Mx isoforms found in rainbow trout and IRF-3. In the head kidney, all three Mx isoforms were induced, while only Mx3 was significantly induced in the muscle. Preferential induction of the Mx3 isoform in muscle cells regardless of the IFN inductor used has been recently reported [24]. Previous studies had showed that other antimicrobial peptides such as cecropin and Ple were not able to increase the levels of expression of Mx genes in RTS11 cells [19], thus again, the capacity of HNP1 to modulate the expression of genes related to the IFN system, seems exclusive to HNP1.

Concerning genes related to the macrophage response, only iNOS was significantly up-regulated in the muscle. Studies performed in head kidney leucocytes in vitro also demonstrated an increased iNOS expression in response to HNP1 [14]. More work should be done to determine if the NO released plays a role in the microbicidal activity of defensins. As occurred in response to cecropin and Ple [19], MHC-II gene transcription was not altered by HNP1, but a modest induction of MHC-I genes, probably with an unknown biological significance, was observed in the muscle.

In conclusion, we have demonstrated for the first time in fish that human α-defensins such as HNP1, are able to modulate the cytokine response in vivo, having the most relevant effects on genes related to IFN production and chemokines, since other effects seen for example on pro-inflammatory genes and MHC are most probably due to non-specific responses to a foreign peptide. Moreover, the chemoattractant capacity of a defensin has been established for the first time in fish. Regardless of their possible biological significance, the immunostimulant effect of HNP1 on fish immune response is clearly of interest from an immunotherapeutic and vaccinology perspective.

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