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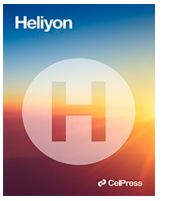

Immunomodulatory role of vasoactive intestinal peptide and ghrelin in *Oncorhynchus mykiss*

Carolina Muñoz-Flores [a], Francisco J. Roa [a], Paulina Saavedra [a], Pablo Fuentealba [a], María F. Starck [a], Leonardo Ortega [a], Raquel Montesino [a], Ariel Valenzuela [b], Allisson Astuya [c], Natalie Parra [a], Iván González-Chavarría [a], Oliberto Sánchez [d], Jorge R. Toledo [a], Jannel Acosta [a,*]

[a] *Laboratorio de Biotecnología y Biofármacos, Departamento de Fisiopatología, Facultad de Ciencias Biológicas, Universidad de Concepción, Víctor Lamas 1290, P.O. Box 160-C, Concepción, Chile*

[b] *Laboratorio de Piscicultura y Patología Acuática, Departamento de Oceanografía, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Victor Lamas 1290, P.O. Box 160-C, Concepción, Chile*

[c] *Laboratorio de Genómica Marina y Cultivo Celular, Departamento de Oceanografía y COPAS Sur-Austral, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Victor Lamas 1290, P.O. Box 160-C, Concepción, Chile*

[d] *Laboratorio de Biofármacos Recombinantes, Departamento de Farmacología, Facultad de Ciencias Biológicas, Universidad de Concepción, Víctor Lamas 1290, P.O. Box 160-C, Concepción, Chile*

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ABSTRACT

Neuropeptides are a group of peptides derived from precursor proteins synthesized in neuronal and nonneuronal cells. The classical functions of neuropeptides have been extensively studied in mammals, including neuromodulation in the central nervous system, molecular signaling in the peripheral nervous system, and immunomodulation associated mainly with anti-inflammatory activity. In contrast, in teleosts, studies of the immunomodulatory function of these neuropeptides are limited. In *Oncorhynchus mykiss*, vasoactive intestinal peptide (VIP) mRNA sequences have not been cloned, and the role of VIP in modulating the immune system has not been studied. Furthermore, in relation to other neuropeptides with possible immunomodulatory function, such as ghrelin, there are also few studies. Therefore, in this work, we performed molecular cloning, identification, and phylogenetic analysis of three VIP precursor sequences (prepro-VIP1, VIP2 and VIP3) in rainbow trout. In addition, the immunomodulatory function of both neuropeptides was evaluated in an *in vitro* model using the VIP1 sequence identified in this work and a ghrelin sequence already studied in *O. mykiss*. The results suggest that the prepro-VIP2 sequence has the lowest percentage of identity with respect to the other homologous sequences and is more closely related to mammalian orthologous sequences. VIP1 induces significant expression of both proinflammatory (IFN-γ, IL-1β) and anti-inflammatory (IL-10 and TGF-β) cytokines, whereas ghrelin only induces significant expression of proinflammatory cytokines such as IL-6 and TNF-α.

* Corresponding author. Laboratorio de Biotecnología y Biofármacos Departamento de Fisiopatología Facultad de Ciencias Biológicas Universidad de Concepción, Victor Lamas 1290, P.O. Box 160-C, Concepción, Chile.
E-mail address: janacosta@udec.cl (J. Acosta).

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1. Introduction

Neuropeptides are a large group of peptides that play a key role in communication among the central nervous system, peripheral nervous system, and immune system [1]. All neuropeptides are processed from precursor proteins that are proteolytically cleaved and typically exert their effects on target cells through the specific binding and activation of G protein-coupled receptors (GPCRs) [2–7].

Neuropeptides have been identified in bilaterian animals, which comprise two superphyla: deuterostomes (e.g., vertebrates such as tetrapods and teleost fishes) and protostomes (e.g., mollusks and nematodes) [2]. However, the roles of neuropeptides have been more thoroughly studied in mammalian models. Neuromodulation in the central nervous system and molecular signaling in the periphery, such as peptide hormones, have been the most studied classical functions of neuropeptides in mammals [5,8,9]. On the other hand, direct communication between the neuroendocrine system and immune system has also been described. Some immune cells, including macrophages and lymphocytes, can produce neuropeptides in response to inflammatory and antigenic signals. These neuropeptides, which function as cytokine-like factors, act at the autocrine/paracrine level on specific receptors of immune cells [6]. Most neuropeptides with immunomodulatory functions show anti-inflammatory activity, and among them are the neuropeptides ghrelin (GHRL) and vasoactive intestinal peptide (VIP) [6,10].

The immunomodulatory functions of ghrelin and VIP have been extensively studied in mammals. Ghrelin is a neuropeptide synthesized mainly by enteroendocrine cells, with an important role in the regulation of appetite, growth hormone, gut motility, cardiovascular function, memory, and energy homeostasis, among others [11]. In the innate immune system, ghrelin acts on macrophages and induces an anti-inflammatory state (M2 macrophages). Furthermore, in the adaptive immune system, this peptide inhibits type 1 T helper (Th1) cells and increases the polarization of type 2 T helper (Th2) cells and regulatory T cells, which contributes to reducing the levels of proinflammatory cytokines and increasing the levels of anti-inflammatory cytokines, respectively [11,12]. Ghrelin has also been reported to increase phagocytic and bactericidal activity in neutrophils [13]. On the other hand, VIP is synthesized by neurons, endocrine cells and immune cells [14]. Its biological functions involve bronchodilation, smooth muscle contraction, regulation of secretory processes, and motility of the gastrointestinal tract, among others [15,16]. Importantly, VIP is a sequence that derives from another precursor named prepro-VIP, which is structurally composed of a signal peptide, a sequence of a bioactive hormone called peptide histidine methionine (PHM) in humans or peptide histidine isoleucine (PHI) in other vertebrates, and the VIP sequence [17]. Regarding the immune function of endogenous VIP, a vital immunoregulatory role similar to ghrelin has been described. This role includes the promotion of Th2 cell development and the inhibition of Th1 differentiation [18]. In macrophages, VIP inhibits the production of proinflammatory cytokines and promotes the induction of anti-inflammatory cytokines [15,19]. Additionally, direct antimicrobial properties of VIP have been described through the disruption of endosomal-lysosomal vesicles that result in metabolic failure of *T. brucei* [7,20].

Ghrelin has been identified in several species of teleosts, including salmonids such as *O. mykiss* (rainbow trout) and *S. salar* (Atlantic salmon). For these species, 2 isoforms of the ghrelin gene were cloned (GHRL-1 GHRL-2) [21,22]. The function of ghrelin in fish is commonly related to the regulation of energy metabolism and appetite [23–25]. In contrast, studies regarding its immunomodulatory function are limited. A study carried out on head kidney leukocytes (HKLs) in rainbow trout showed that ghrelin stimulates superoxide production in phagocytic leukocytes, which is dependent on the growth hormone secretagogue receptor (GHS-R) pathway [26]. On the other hand, in a recent study performed with rainbow trout HKLs, it was observed that the immune response mediated by this neuropeptide could be regulated both by central regulation of the somatotropic axis and by cytokine/chemokine signaling pathways [27]. Furthermore, in an *in vivo* assay in hybrid Nile tilapia (*O. niloticus* x *O. aureus*), ghrelin administration increased the survival rate after *A. hydrophila* infection, which could be related to the modulation of the immune response observed, manifested as stimulation of the production of reactive oxygen species [28] and changes in the expression of pro- and anti-inflammatory cytokines in different tissues [28]. VIP, on the other hand, has been identified in some teleosts, including rainbow trout. However, in this salmonid species, only a peptide was identified from a stomach extract, which has not been cloned [29]. Regarding the function of VIP in teleosts, its role in the regulation of appetite and in the control of cardiac baroreflex functions has been studied [30,31]. Finally, some studies have described a possible immunomodulatory function of VIP in teleosts. In a study carried out in the species *Paralichthys olivaceus*, an increase and a decrease in VIP expression in the spleen and head kidney were observed when an *in vivo* challenge with *Edwardsiella tarda* was performed [32]. On the other hand, in research on the species *Oreochromis niloticus*, it was concluded that VIP would present several immunological functions, such as decreasing the expression of pro-inflammatory cytokines and increasing the expression of anti-inflammatory cytokines, reducing the expression of genes involved in inflammation signaling and protection against *Streptococcus agalatiae* infection [33].

Additionally, the identification of new VIP genes or isoforms, in addition to the study of possible immunomodulatory functions of VIP and ghrelin, could suggest their potential application to stimulate the immune system of salmonids at the aquaculture industry level to offer a therapeutic or prophylactic alternative to the conventional use of antibiotics and antivirals. Accordingly, from predicted VIP sequences deposited in databases, 3 VIP sequences were cloned and identified in rainbow trout. Subsequently, peptide sequences were analyzed and aligned with homologous sequences from other species for domain identification and analysis of their phylogenetic relationship. Finally, to evaluate the possible immunomodulatory function of one of the identified VIP sequences, in addition to ghrelin, real-time PCR assays were performed to analyze cytokine expression in RTS11 cells stimulated with the neuropeptides. Therefore, in this work, we cloned and identified for the first time 3 VIP sequences in *O. mykiss* and evaluated the potential immunomodulatory function of VIP and ghrelin in an *in vitro* model of this species.

Table 1
Primer sequences used in conventional PCR for predicted rainbow trout VIP sequences.

Gene name		Sequence 5′-3′	Amplicon size (bp)	NCBI access number
VIP1	Forward	AGACGCTGACCAGCGAAGA	629	XM_021580727.2
	Reverse	GACTGATTGAGGTAATCGCACCAG		
VIP2	Forward	GAGATGACACCGTGGACAGC	652	XM_021557364.2
	Reverse	GTCTGGCTTTGCCGTTCGTT		
VIP3	Forward	CAGCCACAAAGCGTTTCAAG	563	XM_036986363.1
	Reverse	GACGATTGAGGTCGTCGCA		

VIP1: *vasoactive intestinal peptide 1*, **VIP2**: *vasoactive intestinal peptide 2* and **VIP3**: *vasoactive intestinal peptide 3*.

2. Materials and methods

2.1. Fish maintenance

Unvaccinated rainbow trout weighing approximately 200 g were obtained and maintained in the unit of Marine Biotechnology, Faculty of Natural and Oceanography Science, University of Concepcion. The fish were screened for health conditions and certified to be free of the most prevalent pathogens prior to the experiments. The specimens were maintained under a 12:12 h light:dark cycle in single-pass flow-through tanks supplied with filtered, ultraviolet-treated saltwater. The rainbow trout were fed once daily to satiation with a commercial diet (Micro 200, EWOS).

Four specimens were sacrificed by overexposure to benzocaine (20 %), and the head kidney and gut were aseptically removed. The tissues were kept in RNAlater (Invitrogen) at −80 °C until use.

All the animals used in this study were treated under the Biosecurity Regulations and Ethical Protocols approved by the Universidad de Concepción Ethics Committee, as required by Chilean Regulatory Entities: Agencia Nacional de Investigación y Desarrollo (ANID) and Servicio Nacional de Pesca y Acuicultura (SERNAPESCA).

2.2. Molecular cloning of VIP sequences

Three predicted VIP sequences of *O. mykiss* deposited in the Nucleotide database of NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/) were selected, which have the accession codes XM_021580727.2, XM_021557364.2 and XM_036986363.1. From these sequences, primers were designed for the amplification of mature mRNA by conventional PCR (Table 1).

From rainbow trout head kidney and gut samples, total RNA was purified using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The concentration and purity of the RNA were measured using a Synergy HTX microplate reader (BioTek Instruments, USA), and the integrity of the RNA was checked on a 1 % agarose gel. RNA samples were treated with DNase I (Invitrogen) using 1 IU/μg RNA. Subsequently, cDNA synthesis was performed from 1 μg RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) and random hexanucleotides as primers. The cDNA and RNA samples were stored at −20 °C and −80 °C, respectively.

For conventional PCRs, the commercial SapphireAmp® Fast PCR Master Mix kit (Takara) was used in a final volume of 20 μl, containing 10 μl of the master mix, 0.8 μl of each primer (5 μM each), 1.6 μl of cDNA template and 6.8 μl of molecular biology water. The following thermal cycling protocol was used: 1) polymerase activation for 3 min at 95 °C, 2) denaturation for 30 s at 95 °C, 3) annealing for 45 s at 55 °C (VIP1), 57 °C (VIP2) and 53 °C (VIP3), 4) extension for 40 cycles for 45 s at 72 °C, and 5) final extension for 5 min at 72 °C. No template controls were considered for each reaction mixture. Visualization of PCR products was performed by 1 % agarose electrophoresis with 0.001 % propidium iodide.

Subsequently, for cloning, PCR products were extracted from the agarose matrix with the GeneJET Gel Extraction kit (Thermo Scientific) and cloned into the pGEM-T-Easy vector (Promega) using the T4 DNA Ligase enzyme (New England Biolabs). The volume of insert required in the ligation reaction was determined according to the following formula: ng insert = ((50 ng vector x kb insert)/kb vector). The chemocompetent strain of *E. coli* TOP 10 bacteria was transformed with the ligation product of each reaction, and from the selected clones, plasmid purification and subsequent restriction analysis were performed. Finally, the inserts obtained from the restriction assay were purified from agarose gels and sequenced to corroborate the identity of the respective cloned VIP inserts.

2.3. Bioinformatics sequence analysis

A multiple sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) from the predicted amino acid sequences of rainbow trout VIP1, VIP2 and VIP3 preproproteins. The alignment included prepro-VIP sequences from other representative teleost and mammalian species. InterPro (https://www.ebi.ac.uk/interpro/) and SMART (http://smart.embl-heidelberg.de) tools were used for protein domain identification, and SignalP 6.0 (https://services.healthtech.dtu.dk/service.php?SignalP) for signal peptide identification. In addition, the percentage identity of *O. mykiss* VIP sequences compared to the other sequences was calculated using the program Jalview version 2.11.2.0.

A multiple sequence alignment was performed from the predicted or cloned coding nucleotide sequences for VIP of different species, including the predicted sequences for rainbow trout. This alignment was performed with the Clustal Omega tool, and a

Table 2
Primer sequences used in qRT−PCR for stimulation assays in RTS11 cells.

Gene name		Sequence 5′-3′	Amplicon size (bp)	NCBI access number
IL-1β	Forward	GCTGGAGAGTGCTGTGGAAGA	73	XM_014170479.2
	Reverse	TGCTTCCCTCCTGCTCGTAG		
IFN-γ	Forward	CCGTACACCGATTGAGGACT	133	XM_045698694.1
	Reverse	GCGGCATTACTCCATCCTAA		
IL-6	Forward	GCGGAACCAACAGTTTGTGG	71	NM_001124657.1
	Reverse	CCTGGTGCTGTGAGAACGAT		
TNF-α	Forward	AGGTTGGCTATGGAGGCTGT	173	NM_001124357.1
	Reverse	TCTGCTTCAATGTATGGTGGG		
IL-8	Forward	ATTGAGACGGAAAGCAGACG	136	NM_001124362.1
	Reverse	CGCTGACATCCAGACAAATCT		
IL-10	Forward	CGACTTTAAATCTCCCATCGAC	69	NM_001245099.1
	Reverse	GCATTGGACGATCTCTTTCTT		
TGF-β	Forward	GGGAGACAACACAAGGTGGAG AATGGACAAGAACATGGAGAGACA	99	XM_021591332.2
	Reverse			
EF1-α	Forward	GTGACACCGAAACTAAGCGAC	110	BT072490.1
	Reverse	TGTAGATCAGATGGCCGGTG		

IL-1β: *interleukin-1β*, **IFN-γ**: *interferon-γ*, **IL-6**: *interleukin-6*, **TNF-α**: *tumor necrosis factor-α*, **IL-8**: *interleukin-8,* **IL-10**: *interleukin-10,* **TGF-β**: *transforming growth factor-β* and **EF1-α**: *elongation Factor 1-α*.

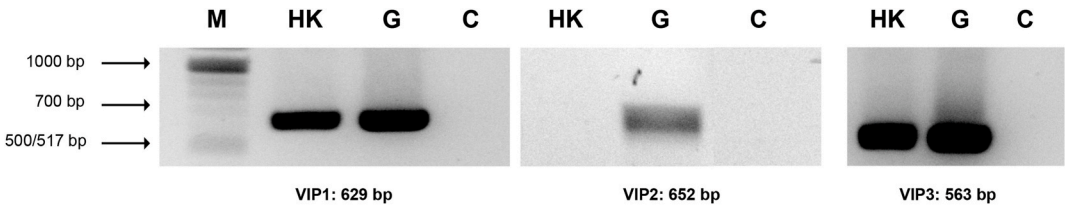


Fig. 1. Amplification of mature mRNA sequences of rainbow trout VIPs. Conventional PCR products were obtained with the expected sizes for VIP1, VIP2 and VIP3. M: 100 bp DNA Ladder (New England Biolabs). HK: head kidney. G: gut. C: PCR control without cDNA template (no template control). Images of complete gels are shown in Supplementary Material 1.

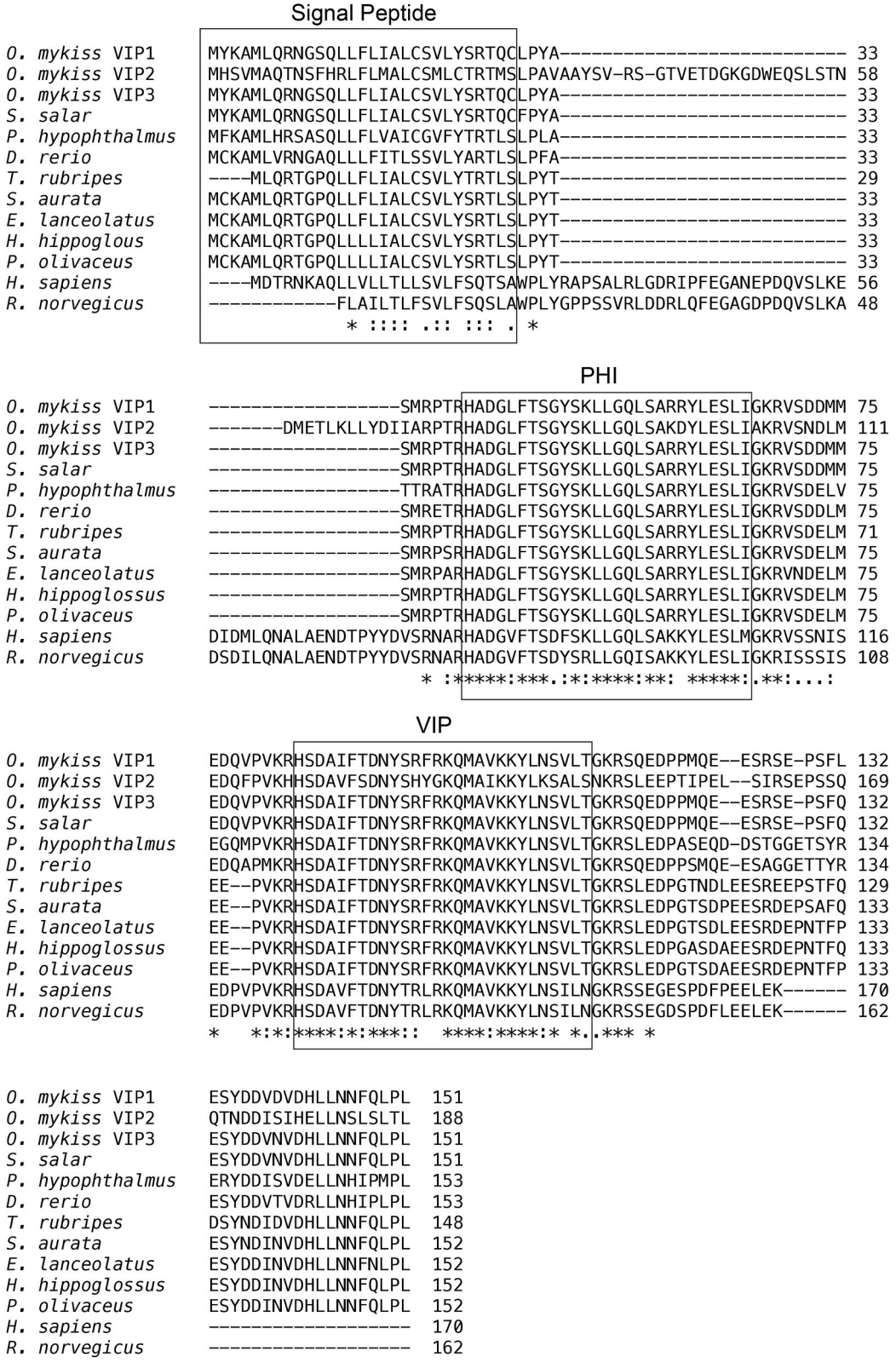
phylogenetic tree was constructed with the Molecular Evolutionary Genetic Analysis 11 (MEGA11) program using the neighbor-joining (NJ) method with 1000 bootstrap trials.

2.4. In vitro cytokine induction assay by qRT−PCR

The rainbow trout monocyte/macrophage cell line RTS11 was maintained in Leibovitz Medium (L-15, Gibco) supplemented with 10 % fetal bovine serum (FBS, HyClone), penicillin (100 IU/mL, Gibco), streptomycin (100 μg/mL, Gibco) and L-glutamine (2 mM, Gibco) and incubated at 18 °C.

For *in vitro* stimulation assays, two peptides chemically synthesized by GenScript company (https://www.genscript.com/) were used. The lyophilized peptides were stored at −20 °C and dissolved in PBS (pH 7.5) before use. The two peptides synthesized were 1) VIP1/VIP3 studied in this work (HSDAIFTDNYSRNYSRFRKQMAVKKKNSVLT), which corresponds to the peptide sequence previously isolated from the rainbow trout gut [29]. 2) Ghrelin (GSSFLSPSQPSQKKKPQVRQGKPPRVG) [21] from *O. mykiss* is another model neuropeptide with possible immunomodulatory function. Therefore, RTS11 cells were incubated with 20 nM of each peptide for 4 and 8 h, and then total RNA extraction was performed using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The concentration and purity of the RNA were measured using a Synergy HTX microplate reader (BioTek Instruments, USA).

Real-time quantitative reverse transcription PCR (qRT−PCR) was performed using Brilliant II SYBR® Green QRT-PCR Master Mix, 1-Step (Agilent, USA) in a final volume of 11 μl, containing each reaction: 2 μl of RNA template (100 ng/μl), 5 μl of Master Mix, 0.5 μl of mix primer (forward and reverse, 5 μM each), 3.1 μl of nuclease-free water and 0.4 μl of reverse transcriptase. The reaction mixtures were incubated in the AriaMx Real-Time PCR System (Agilent, USA) using the following conditions: reverse transcription at 50 °C for 30 min, hot start activation of the polymerase at 95 °C for 10 min, denaturation at 95 °C for 15 s, and annealing/extension at 58 °C for 30 s for 40 cycles. It was completed with a dissociation or melting curve at 95 °C for 15 s, 59 °C for 1 min, and 95 °C for 15 s. Specific primers were used to study the following genes: interleukin-1β (IL-1β), interferon-γ (IFN-γ), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (Table 2). The results were analyzed by $2^{-\Delta\Delta CT}$ relative quantification, and the comparative threshold cycle values were normalized to elongation Factor 1-α (EF1-α) mRNA. One-way analysis of variance (ANOVA) was performed for multiple comparisons, comparing each experimental condition with the negative control (Dunnett's multiple comparison test). Statistical significance was defined as a value of $p < 0.05$. The construction of graphs of the experimental information, as well as the statistical analyses, were carried out with the GraphPad Prism 8 program.



(caption on next page)

Fig. 2. Multiple alignments of prepro-VIP mature proteins from *O. mykiss* and other representative species. In the alignment, sequences corresponding to the signal peptide, PHI peptide and VIP peptide are framed. In addition, "*" indicates positions with a single fully conserved residue, ":" indicates conservation between residues with highly similar properties, and "." indicates conservation between residues with weakly similar properties. Gaps (indicated with "-") were considered to achieve complete alignment of all sequences. The sequences of the other teleosts and mammals correspond to *S. salar* (NCBI Reference Sequence: XP_014010319.1), *P. hypophthalmus* (XP_026795265.1), *D. rerio* (NP_001108025.2), *T. rubripes* (XP_011601347.1), *S. aurata* (XP_030296899.1), *E. lanceolatus* (XP_033468632.1), *H. hippoglossus* (XP_034463973.1), *P. olivaceus* (XP_019946092.1), *H. sapiens* (NP_003372.1) and *R. norvegicus* (NP_446443.1).

3. Results

3.1. Molecular identification of rainbow trout VIP sequences

Products of the expected size were amplified in head kidney and gut samples for both VIP1 and VIP3. For VIP2, a product of the expected size was obtained only for gut samples (Fig. 1). PCR products of the expected sizes were subsequently purified from the gel, used as inserts for cloning and finally sent for sequencing. Sequencing confirmed the identity of the cloned VIP1, VIP2 and VIP3 sequences.

3.2. Multiple sequence alignment and phylogenetic tree

The multiple sequence alignment shows that the most conserved residues are found mainly in the PHI and VIP domains of mature prepro-VIP proteins, as well as to a lesser extent in signal peptide sequences (Fig. 2). The mature prepro-VIP sequences with the highest conservation and the highest percentage identity between them correspond to VIP1 (*O. mykiss*), VIP3 (*O. mykiss*) and VIP from *S. salar*. On the other hand, the VIP sequences of VIP1 (*O. mykiss*) and VIP3 (*O. mykiss*) are 100 % identical to each other and with the rest of the VIP sequences of the other teleost species analyzed. The VIP sequence of VIP2 (*O. mykiss*) shows 67.86 % identity with the rest of the teleost and mammalian sequences. Similarly, the PHI sequences of VIP1 and VIP3 of *O. mykiss* are 100 % identical to each other and the rest of the PHI sequences of the other teleost species. The PHI sequence of VIP2 (*O. mykiss*) is 92.59 % identical to the other teleost sequences (Table 3).

The phylogenetic tree was classified into 2 main branches. The first branch includes the VIP1 and VIP3 sequences of *O. mykiss* and VIP of *S. salar*. The second main branch is divided into two subbranches: one that groups most of the teleost sequences analyzed and one that groups the mammalian sequences with VIP2 of *O. mykiss*. Therefore, VIP2 of *O. mykiss* shows a closer phylogenetic relationship with the mammalian VIP sequences than with the VIP1 and VIP3 sequences of *O. mykiss* (Fig. 3).

3.3. Effect of neuropeptides on cytokine expression in RTS11 cells

Ghrelin treatments significantly increased the expression of the cytokines IL-6 and TNF-α at 8 h of stimulation (Fig. 4B and E). The greatest induction of a 1.9-fold increase was observed with IL-6 expression (Fig. 4B). On the other hand, VIP1 treatments significantly increased IFN-γ, IL-10 and TGF-β expression at 4 h of stimulation (Fig. 4A, F, 4G) and increased IL-1β expression at 8 h poststimulation (Fig. 4C). The greatest increase in VIP1-mediated cytokine induction corresponded to IL-1β and TGF-β expression (1.6-fold each) (Fig. 4C and G). It was observed that both ghrelin and VIP1 induced a significant decrease in IFN-γ expression at 8 h poststimulation (Fig. 4A). For both neuropeptides, no changes in IL-8 expression were observed (Fig. 4D).

4. Discussion

In the early evolution of vertebrates, 2 duplication events of the complete genome occurred [34], and a third event occurred in the ancestor of teleost fish [35,36]. In addition, a fourth round of duplication has been described in the salmonid and common carp lineage [37,38]. These tetraploidization events may result in the duplication of genes with partitioned functions from the ancestral gene (subfunctionalization), leading to increased evolutionary plasticity [39]. Therefore, it is postulated that in teleosts, and especially in salmonids and cyprinids, these events could have expanded the families of neuroendocrine peptides and GPCRs [2], which correlates with the finding of several VIP sequences in rainbow trout.

While in a study of *O. mykiss,* a VIP sequence obtained from stomach extracts was identified and structurally characterized [29], in this work, for the first time, 3 prepro-VIP sequences were identified and cloned from *O. mykiss* named VIP1, VIP2 and VIP3, which are all expressed in the gut. In addition, VIP1 and VIP3 are expressed in the head kidney.

The expression of these cloned sequences in the gut is consistent with what has been studied in mammals and teleosts. In mammals, VIP is expressed in neurons of the central and peripheral nervous systems and is stored and released from nerve fibers that innervate different organs, including the intestine and immune organs such as the bone marrow [15]. In addition, VIP is expressed in immune cells such as T cells and B cells and is induced by stimulation with lipopolysaccharide (LPS) and proinflammatory cytokines [40]. In teleosts, few studies have cloned prepro-VIP sequences, since the first studies on VIP were based on the extraction, identification and functional studies of this neuropeptide in tissues such as the brain, kidney, pancreas and intestine of different species [41–45]. In *P. olivaceus,* a prepro-VIP sequence was cloned, and its expression was determined in tissues such as the brain, intestine, stomach, pyloric ceca, spleen and heart [46]. Regarding expression in the head kidney, in the study of cloning and expression of VIP in *P. olivaceus*, it was observed that the expression of prepro-VIP in the anterior kidney is induced after infection with *E. tarda*, in contrast

Table 3
Percentage identity of prepro-VIP mature proteins, PHI peptide and VIP peptide of *O. mykiss* and other representative species.

Prepro – VIPMATUREPROTEIN	*O. mykissVIP1*	*O. mykissVIP2*	*O. mykissVIP3*	*S. salar*	*D. rerio*	*T. rubripes*	*H. hippoglossus*	*P. hypophthalmus*	*E. lanceolatus*	*S. aurata*	*P. olivaceus*	*H. sapiens*	*R. norvegicus*
***O. mykiss* VIP1**		51.06	98.68	98.01	81.7	84.67	84.42	73.86	83.12	84.42	84.42	53.49	51.94
***O. mykiss* VIP2**			51.6	51.06	51.9	50.54	48.42	47.09	47.89	49.74	48.42	45.29	44.17
***O. mykiss* VIP3**				99.34	81.7	84.67	85.71	73.86	83.77	85.71	85.06	53.49	51.94
VIP SEQUENCE	***O. mykiss VIP1***	***O. mykiss VIP2***	***O. mykiss VIP3***	***S. salar***	***D. rerio***	***T. rubripes***	***H. hippoglossus***	***P. hypophthalmus***	***E. lanceolatus***	***S. aurata***	***P. olivaceus***	***H. sapiens***	***R. norvegicus***
***O. mykiss* VIP1**		67.86	100	100	100	100	100	100	100	100	100	82.14	82.14
***O. mykiss* VIP2**			67.86	67.86	67.86	67.86	67.86	67.86	67.86	67.86	67.86	67.86	67.86
***O. mykiss* VIP3**				100	100	100	100	100	100	100	100	82.14	82.14
PHI SEQUENCE	***O. mykiss VIP1***	***O. mykiss VIP2***	***O. mykiss VIP3***	***S. salar***	***D. rerio***	***T. rubripes***	***H. hippoglossus***	***P. hypophthalmus***	***E. lanceolatus***	***S. aurata***	***P. olivaceus***	***H. sapiens***	***R. norvegicus***
***O. mykiss* VIP1**		92.59	100	100	100	100	100	100	100	100	100	77.78	77.78
***O. mykiss* VIP2**			92.59	92.59	92.59	92.59	92.59	92.59	92.59	92.59	92.59	81.48	81.48
***O. mykiss* VIP3**				100	100	100	100	100	100	100	100	77.78	77.78

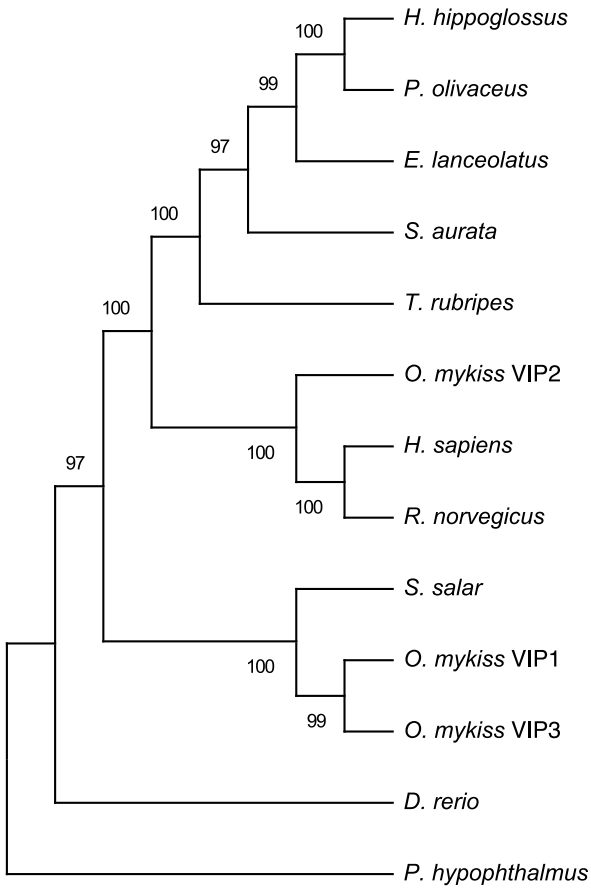


Fig. 3. Phylogenetic analysis of VIP nucleotide sequences of *O. mykiss* and other representative species. A phylogenetic tree was constructed using the neighbor-joining method. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The numbers on the branch indicate the frequencies for 1000 bootstrap analyses. The sequences of the other teleosts and mammals correspond to *S. salar* (NCBI Reference Sequence: XM_014154844.2), *P. hypophthalmus* (XM_026939464.2), *D. rerio* (NM_001114553.3), *T. rubripes* (XM_011603045.2), *S. aurata* (XM_030441039.1), *E. lanceolatus* (XM_033612741.1), *H. hippoglossus* (XM_034608082.1), *P. olivaceus* (XM_020090533.1), *H. sapiens* (NM_003381.4) and *R. norvegicus* (NM_053991.1).

to the expression in the intestine, which is constitutive [32]. Therefore, the absence of prepro-VIP2 expression in *O. mykiss* head kidney in this study could be related to possible inducible expression by an infectious agent, as observed in *P. olivacerus*; however, this must be corroborated with further expression studies in response to pathogen challenges.

In addition, the analysis of the cloned prepro-VIP sequences shows that the VIP1 and VIP3 sequences are 100 % identical among them and the other teleost sequences analyzed, as well as with the PHI sequence. Related to the above, the complete prepro-VIP sequences of *O. mykiss* (VIP1 and VIP3) and the sequence of *S. salar* present a higher percentage of identity, higher than 98 %. However, the prepro-VIP2 and VIP2 sequences of rainbow trout have the lowest identity concerning the other homologous genes. These findings correlate with the phylogenetic analysis, where VIP2 of *O. mykiss* is found within the cluster of sequences related to mammals and other teleosts. This suggests that the duplication of the prepro-VIP genes in *O. mykiss* gave rise to 2 classes: one related to the VIP sequences of salmonids, with expression in the intestine and head kidney (VIP1 and VIP3), and another class related to mammalian sequences and other teleosts, with expression in the intestine and not in the head kidney (VIP2). Similar results were obtained in the analysis of two zebrafish VIP sequences, where one class of sequence is structurally and phylogenetically related to the mammalian VIP sequence, while the other sequence is related to teleosts [32].

In this work, we also studied the possible immunomodulatory function of one of the cloned sequences, VIP1. We also include the study of another neuropeptide that has been identified in *O. mykiss*, ghrelin, whose role in the immune response in this species has also only recently been studied [27]. The results of this study suggested that the stimulation of RTS11 cells with ghrelin significantly increased the expression of pro-inflammatory cytokines such as IL-6 and TNF-α after 8 h of treatment. On the other hand, VIP1 treatments significantly increased the expression of IFN-γ, IL-1β, IL-10 and TGF-β, with peaks at different treatment times. Thus, ghrelin induced increased expression of pro-inflammatory cytokines, whereas VIP1 increased the expression of both pro- and anti-inflammatory cytokines.

The immunomodulatory function of these neuropeptides has been extensively studied in mammals, where VIP and ghrelin induce a

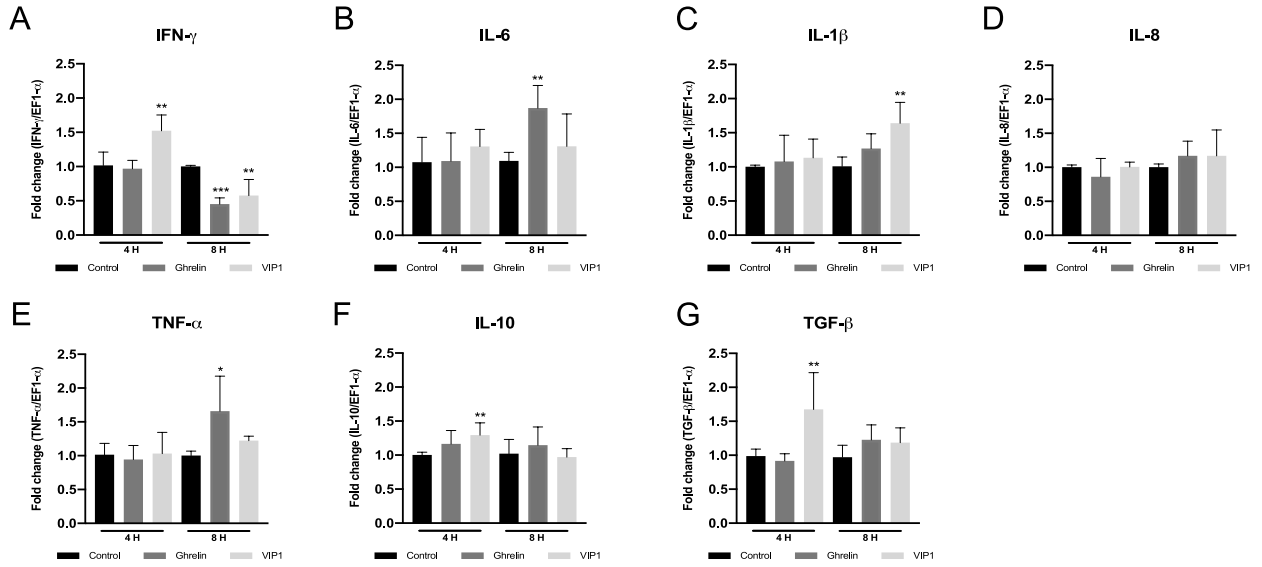


Fig. 4. Stimulation of RTS11 cells with neuropeptides and analysis of relative cytokine expression. RTS11 cells were stimulated with ghrelin (GHR) and VIP1 at concentrations of 20 nM each for 4 and 8 h. The relative expression of the cytokines IFN-γ (A) IL-6 (B), IL-1β (C), IL-8 (D), TNF-α (E), IL-10 (F) and TGF-β (G), normalized to EF1-α gene expression, was evaluated. Statistical analyses were performed comparing each condition with the control using ordinary one-way ANOVA with Dunnett's multiple comparisons tests. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

decrease in pro-inflammatory cytokines and chemokines and induce an increase in the expression of anti-inflammatory cytokines such as IL-10 and TGF-β [10] in the innate immune response. This response induces an anti-inflammatory state in macrophages (M2) and inhibits proinflammatory macrophages (M1) [11,15,16]. Furthermore, in mammals, VIP promotes IL-10 expression in macrophages stimulated with LPS [19]. In contrast, in teleosts, there is little information on the immunomodulation of these neuropeptides. In one study, ghrelin was injected into hybrid tilapia infected with *Aeromonas hydrophila*, and the relative expression of different cytokines in different organs was analyzed. In general, an increase in IL-1β and TGF-β expression was observed, while TNF-α expression increased in the spleen and decreased in the kidney [28]. In another study, ghrelin stimulation in *O. mykiss* HKLs induced a significant increase in pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-8, IL-1β, and IL-6 at different times. However, a significant increase in TGF-β at 24 h poststimulation was also observed. In a study performed in a model of *O. niloticus* infected with *S. agalactiae* and subsequently treated with VIP, the relative expression of cytokines in different tissues was analyzed. Treatment with VIP for 6 h increased the relative expression levels of anti-inflammatory cytokines such as IL-10 and TGF-β in different tissues. In addition, there was also a significant increase in the expression of pro-inflammatory cytokines such as IL-1β and TNF-α at 6 h of treatment in the head kidney [33]. Therefore, these studies suggest that in teleosts, VIP and ghrelin induce both pro- and anti-inflammatory responses, different from those observed in mammals. This dual behavior in fish has also been observed in the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), which although in mammals has an anti-inflammatory function [47,48], pro-inflammatory properties have been observed in teleosts [49–51]. Finally, our results showed that ghrelin only induces the expression of pro-inflammatory cytokines, in contrast to the study performed in hybrid tilapia where the induction of pro- and anti-inflammatory cytokines was described [28]. This difference could be related to the fact that in the abovementioned study, fish infected with a pathogen and treated with ghrelin were used as an animal model, without considering in the analyses only treatment with the neuropeptide (without infection). Therefore, bacterial challenge could modulate the ghrelin-induced inflammatory response differently.

In conclusion, in this work, we cloned 3 *O. mykiss* prepro-VIP sequences for the first time, two of which (VIP1 and VIP3) have an identity greater than 98 % and the same identity pattern in tissues. The cloned sequence VIP2 has a low percentage of identity with the other salmonid sequences and is more closely related to the homologous mammalian sequences. In addition, the immunomodulation induced by treatment with VIP1 and another neuropeptide identified in rainbow trout, ghrelin, was functionally studied in an *in vitro* model. Our results suggest that both neuropeptides induce the expression of proinflammatory cytokines, while VIP1 further modulates the anti-inflammatory response. Finally, rainbow trout have been described as susceptible to infection by aquatic pathogens such as *Piscirickettsia salmonis* [52], as well as opportunistic pathogens such as *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Yersernia ruckeri* [53–55]. Prophylactic and therapeutic strategies are limited, so these infections lead to production losses at the industrial level, which can also have environmental consequences such as antibiotic resistance [52,56]. Therefore, the study of immunomodulatory peptides appears to be an emerging prophylactic or therapeutic application in aquaculture, which could have application potential for reducing the incidence of infections [57–59]. Therefore, the neuropeptides ghrelin and VIP from rainbow trout are proposed as peptides with potential application as immunostimulants.

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Carolina Muñoz-Flores: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francisco J. Roa:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paulina Saavedra:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pablo Fuentealba:** Methodology, Investigation, Data curation, Conceptualization. **María F. Starck:** Methodology, Investigation, Data curation, Conceptualization. **Leonardo Ortega:** Methodology, Investigation, Formal analysis, Conceptualization. **Raquel Montesino:** Supervision, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Ariel Valenzuela:** Validation, Supervision, Resources, Formal analysis, Data curation, Conceptualization. **Allisson Astuya:** Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Natalie Parra:** Methodology, Investigation, Formal analysis, Conceptualization. **Iván González-Chavarría:** Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Oliberto Sánchez:** Supervision, Resources, Project administration, Formal analysis, Conceptualization. **Jorge R. Toledo:** Validation, Supervision, Resources, Formal analysis, Data curation, Conceptualization. **Jannel Acosta:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23215.

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