Identification and characterization of the Atlantic Salmon Peptide Transporter 1a

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Running title: *Salmo salar* Peptide Transporter 1a

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Abbreviations

CDS, coding sequence; ChrLG16, chromosomal linkage group 16; Chrssa16, chromosome ssa16; Chrssa25, chromosome ssa25; Dmt1, Divalent metal transporter 1; GDV, Genome Data Viewer; Gly-Sar, glycyl-sarcosine; I/V, current/voltage; I_{max}, maximal transport current; $K_{0.5}$, apparent substrate affinity (i.e. apparent concentration of peptide that yields one-half of I_{max}); MNE, Mean Normalized Expression; MS222, tricaine methanesulfonate; Nramp1, natural resistance-associated macrophage protein 1; Nramp2, natural resistance-associated macrophage protein 2; PepT1, Peptide Transporter 1 protein; *pept1a*, *peptide transporter 1a* gene; PepT1a, Peptide Transporter 1a protein; *pept1b* or *pept1*, *peptide transporter 1b* gene; PepT1b, Peptide Transporter 1b protein; PepT2, Peptide Transporter 2 protein; PepT2-like, Peptide Transporter 2-like protein; qPCR, quantitative real-time PCR; Slc15a1 or SLC15A1, Solute carrier family 15 member 1 protein; *slc15a1a*, *solute carrier family 15 member 1a* gene; Slc15a1a, Solute carrier family 15 member 1a protein; *slc15a1b*, *solute carrier family 15 member 1b* gene; Slc15a1b, Solute carrier family 15 member 1b protein; Slc15a2, Solute carrier family 15 member 2 protein; Slc15a2-like, Solute carrier family 15 member 2-like protein; TEVC, Two-Electrode Voltage Clamp; TSA, Transcriptome Shotgun Assembly; UTR, untranslated region; WGD, whole genome duplication.

Abstract

Peptide Transporter 1 (PepT1) mediates the uptake of dietary di/tripeptides in vertebrates. But, in teleost fish gut more than one PepT1-type transporter might operate, due to teleost-specific whole gen(om)e duplication event(s) occurred during evolution. Here, we describe a novel teleost di/tripeptide transporter, i.e. the Atlantic salmon (*Salmo salar*) Peptide Transporter 1a (PepT1a; or Solute carrier family 15 member a1, Slc15a1a), which is a paralogue (77% similarity, 64% identity at the amino acid level) of the well-described Atlantic salmon Peptide Transporter 1b (PepT1b, *alias* PepT1; or Solute carrier family 15 member 1b, Slc15a1b). Comparative analysis and evolutionary relationships of gene/protein sequences were conducted after *ad hoc* database mining. Tissue mRNA expression analysis was performed by quantitative real-time PCR, while transport function analysis was accomplished by heterologous expression in *Xenopus laevis* oocytes and Two-Electrode Voltage Clamp measurements. Atlantic salmon *pept1a* is highly expressed in the proximal intestine (pyloric caeca ≈ anterior midgut > midgut >> posterior midgut), in the same gut regions as *pept1b* but notably ~5-fold less. Like PepT1b, Atlantic salmon PepT1a is a low-affinity/high-capacity system. Functional analysis showed electrogenic, Na^+-independent/pH-dependent transport, and $K_{0.5}$ values for Gly-Gln of 1.593 mmol/L at pH 7.6 and 0.076 mmol/L at pH 6.5. In summary, we show that a piscine PepT1a-type transporter is functional. Defining the role of Atlantic salmon PepT1a in the gut will help to understand the evolutionary and functional relationships among peptide transporters. Its functional characterization will contribute to elucidate the relevance of peptide transporters in Atlantic salmon nutritional physiology.

Keywords

Di/tripeptide transport(ers), digestive physiology, peptide absorption, whole genome duplication, *Xenopus laevis* oocytes.

Introduction

The intestinal oligopeptide transporter Peptide Transporter 1 (PepT1) plays a highly relevant role in protein nutrition by mediating the uptake of dietary amino acids in the di- and tripeptide (di/tripeptide) form **(16, 52)**. Making up a large fraction of the dietary nitrogen present in the gut after a meal or in between meals, such hydrolytic products derive from proteins of animal, plant and microorganism origin and may be released after degradation by digestive or microbial enzymes during gastrointestinal transit or by microbial fermentation of foods during processing or ripening **(12, 22, 65)**. Notably, many of these di/tripeptides have bioactive properties **(6, 31, 50, 54, 57, 62)**. Others seem to play a role in nutrient sensing and metabolic regulation **(15, 43, 66)**. PepT1 is also responsible for the absorption of orally active peptidomimetic drugs, including β-lactam antibiotics and selected pro-drugs **(7, 34, 45, 46, 52)**.

PepT1 belongs to the Peptide Transporter family **(53)**. Members of this family have been characterized in bacteria, fungi, plants, insects, nematodes, and vertebrates **(14, 19, 28, 53)**. In humans, PepT1 is also known as the Solute Carrier 15 family member A1 (SLC15A1) **(13, 52)**. A detailed analysis of its function on mammalian and avian orthologs revealed that PepT1 operates as a Na^+-independent, H^+-coupled electrogenic symporter **(16, 19)**. In mammalian systems, substrate uptake is coupled to the movement of H^+ down an inwardly-directed electrochemical H^+ gradient that allows directional transport of peptides across the plasma membrane, even against a substrate concentration gradient. The transport responds to both membrane potential and extracellular pH and exhibits a pH optimum varying between 4.5 and 6.5 depending on the net charge of the transported substrate **(13, 16, 52)**. More recently, substantial additional information on PepT1 function has come from studies in lower vertebrates, notably teleost fish **(42, 58, 60)**. Interestingly, the first PepT1-type transporter cloned and functionally characterized from a teleost, the zebrafish (*Danio rerio*), was found to exhibit a unique pH dependence, with neutral to alkaline extracellular pH increasing its maximal transport rate **(59)**. However, later analyses of the European sea bass (*Dicentrarchus labrax*) **(49)**, Atlantic salmon (*Salmo salar*) **(44)** and Antarctic icefish (*Chionodraco hamatus*) **(41)** PepT1 transporters revealed a more

conventional behavior with respect to the pH optimum; i.e., the maximal transport rates – similarly to what occurs in higher vertebrates – were found to be rather independent of the extracellular pH in the alkaline to neutral to slightly acidic range, but were instead activated at more acidic extracellular pH **(40, 41, 44, 49)**. With respect to substrate specificity, teleost PepT1 transporters – similarly to higher vertebrates – mediate the uptake of a variety of di/tripeptides in both neutral and charged form, based on analysis of zebrafish, European sea bass, Atlantic salmon and Antarctic icefish proteins **(32, 40, 44, 60)**.

With the increased availability of genome sequences for several teleosts in databanks, it progressively became evident that teleost PepT1-type proteins were the result of a gene duplication. Initially described in the Oriental weatherfish (*Misgurnus anguillicaudatus*) **(20)**, the concept that a *peptide transporter 1a* (*pept1a*; also designated as *solute carrier family 15 member 1a*, *slc15a1a*) gene occurs in teleost fish genomes beside the *peptide transporter 1b* (*pept1b*, *alias pept1*; also designated as *solute carrier family 15 member 1b*, *slc15a1b*) gene has fully emerged. Consequently, it has also become clear that all the data available so far on the functional transport in teleosts refer to PepT1b-type transporters only **(42, 58, 60)**.

To date, it still remains to validate that PepT1a-type proteins are functional. After the molecular cloning and functional expression of Atlantic salmon PepT1b-type di/tripeptide transporter **(44)**, we hereby report data for Atlantic salmon PepT1a on cloning, analysis of sequence, phylogeny, synteny, tissue expression, and functional characterization, transport kinetics and substrate specificity. To our knowledge, this is the first demonstration that a teleost fish PepT1a, which results from a direct gene duplication event, operates as a di/tripeptide carrier system and is able to transport discrete peptide substrates across membranes along the teleost fish intestinal tract epithelial layer.

Methods

Ethical treatment of animals

The research involving Atlantic salmon was conducted in accordance with regulations by National Animal Research Authority in Norway. The fish were sampled from control tanks in a feeding trial and

did not undergo any special treatment or handling except for sampling. The research involving *Xenopus laevis* was conducted using experimental protocol approved locally by the Committee of the *"Organismo Preposto al Benessere degli Animali"* of the University of Insubria (OPBA-permit no. 02_15) and by the Italian Ministry of Health (permit no. 1011/2015).

Animals and tissue sampling

Atlantic salmon were reared (in accordance with the Norwegian Animal Welfare Act of 12 December 1974, no. 73, § 22 and § 30, amended 19 June 2009) at Cargill Innovation Center (Dirdal, Norway), in sea water (8.7 °C) tanks following standard procedures. The facility (formerly EWOS Innovation) has a general permission to conduct experiments on fish, license number 2016/2835 (24 February 2016) provided by the Norwegian Food Safety Authority. The fish diet was produced by EWOS Innovation AS in Norway (see **Supplemental Table S0** [https://doi.org/10.6084/m9.figshare.9988211]) with a pellet size of 10 mm. The feed was provided to the fish using an automatic feeder 3 times a day. Adult Atlantic salmon (65 weeks old; 895.3±118.7 g wet weight; 38.7±1.7 cm total length; n=6) were euthanized with an overdose of MS222 (tricaine methanesulfonate; Norsk Medisinaldepot AS, Bergen, Norway) on site, and various tissues were collected, promptly frozen in liquid nitrogen and finally stored at -80 °C until subsequent analyses.

In silico *identification and molecular cloning*

The Atlantic salmon PepT1b amino acid sequence corresponding to GenBank Acc. No. NP_001140154.1 was used as a query against the Atlantic salmon genome database available in GenBank, and the nucleotide sequence corresponding to GenBank Acc. No. XM_014172951.1 was identified as *pept1a* mRNA. Specific primers were designed (**Supplemental Fig. S1** [https://doi.org/10.6084/m9.figshare.9729383]; **Supplemental Table S1** [https://doi.org/10.6084/m9.figshare.9729398]).

Total RNA was isolated from the midgut of Atlantic salmon using TRI Reagent (Sigma-Aldrich Italia, Milan, Italy) according to the manufacturer's instructions. cDNA was synthesized from 3 μg of total

RNA using SuperScript III First-Strand Synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA, USA) with Oligo $(dT)_{20}$ primers according to the manufacturer's protocol.

pept1a was amplified using specific primers (**Supplemental Table S1** [https://doi.org/10.6084/m9.figshare.9729398]) and Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) according to the manufacture's protocol. The following thermal program: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 62 °C for 20 s, 72 °C for 1.5 min; and a final step at 72 °C for 2 min was used in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA) thermal cycler. The PCR products were resolved on 1% (w/v) agarose gel, purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into a StrataClone blunt PCR cloning vector pSC-B (Agilent Technologies, La Jolla, CA, USA) following the manufacturer's protocol. Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway) and sequence identity confirmed by tBLASTx analysis against the GenBank database.

Computer analysis

Pairwise alignment of PepT1a and PepT1b protein sequences was performed using Clustal X 2.1 **(27)** with default parameters (Gonnet series matrix, Gap opening penalty 10, Gap extension 0.2) (**Fig. 1**). Alignment was displayed in GeneDoc 2.7 software **(35)** and the percentage of sequence identity and similarity between the paralogue proteins calculated. The exon-intron structure of *pept1a* was retrieved from the GenBank gene annotation and the exon-intron graphic made using the Exon-Intron Graphic Maker online tool (http://wormweb.org/exonintron) (**Supplemental Fig. S2** [https://doi.org/10.6084/m9.figshare.9729392]). The putative transmembrane domains were predicted using TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Potential N-glycosylation sites at the extracellular surface (**Supplemental Fig. S1** [https://doi.org/10.6084/m9.figshare.9729383]) were identified using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). Potential cAMP/cGMP-dependent protein kinase phosphorylation sites and protein kinase C phosphorylation sites at the cytoplasmic surface (**Supplemental Fig. S1** [https://doi.org/10.6084/m9.figshare.9729383]) were predicted using the ScanProsite tool (https://prosite.expasy.org/scanprosite/).

177

178 *Phylogenetic analysis*

179 Orthologs of the Atlantic salmon PepT1a in teleost fish were identified by BLAST analysis against
180 several genomes using the ENSEMBL and NCBI databases. Analogously, closer, e.g. PepT1b, and
181 more distant, e.g. Peptide Transporter 2 (PepT2; Solute carrier family 15 member 2, Slc15a2) and
182 Peptide Transporter 2-like (PepT2-like; Solute carrier family 15 member 2-like, Slc15a2-like), paralogues
183 were identified and included in the list of selected sequences. Only full-length sequences with high blast
184 scores were considered (Acc. Nos. reported in **Fig. 2**). Multiple alignment was performed on the
185 selected ortholog/paralog proteins using ClustalW (Gonnet series matrix, Gap opening penalty 10, Gap
186 extension 0.2) and a Neighbor-Joining tree built using MEGA7 **(26)**.

187

188 *Short-range gene linkage and syntenic relationships*

189 Synteny analysis was performed by using the Genome Data Viewer (GDV) tool at the NIH U.S.
190 National Library of Medicine, NCBI. The Atlantic salmon ICSASG_2 genome assembly (RefSeq Acc.
191 No. GCF_000233375.1; GenBank Acc. No. GCA_000233375.4; Submitter: International Cooperation
192 to Sequence the Atlantic salmon genome; Annotation Release: 100; Release Date: 22 September 2015)
193 (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=salmo-salar&group=euteleosteomorpha), and the
194 Northern pike (*Esox lucius*) Eluc_v3 genome assembly (RefSeq Acc. No. GCF_000721915.3; GenBank
195 Acc. No. GCA_000721915.3; Submitter: Ben K. Koop and Jong S Leong; Annotation Release: 102;
196 Release Date: 30 January 2017) (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=esox-
197 lucius&group=euteleosteomorpha) were systematically consulted, and browsed for gene name(s),
198 nucleotide and amino acids sequence(s), accession number(s) related to the searched genes. The
199 Atlantic salmon *pept1a* gene was found to correspond to *LOC106586093*, while the Northern pike
200 *pept1a* gene was found to correspond to *LOC105024756* (**Table 1**).

201

202 *Quantitative real-time PCR analysis (qPCR)*

Total RNA was isolated from several Atlantic salmon tissues as described above. An additional step to avoid genomic DNA contamination was implemented using TURBO DNA-free (Life Technologies, Austin, TX, USA) according to the manufacturer's protocol. DNase-treated total RNA integrity was assessed in all samples using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA was synthesized as described in the section above.

For tissue distribution analysis, specific primers were designed for Atlantic salmon *pept1a* and *pept1b* (**Supplemental Fig. S1** [https://doi.org/10.6084/m9.figshare.9729383]; **Supplemental Table S1** [https://doi.org/10.6084/m9.figshare.9729398]) genes, and *β-actin* (Genbank Acc. No. NM_001123525.1) was used as internal reference gene. Relative quantification was performed using the Mean Normalized Expression (MNE) method of the Q-Gene application **(33, 51)**. The assay efficiency varied between 101-105% (**Supplemental Table S1** [https://doi.org/10.6084/m9.figshare.9729398]) and was determined using a 2-fold cDNA pool dilution series ranging from 200 to 6.25 ng, using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a 20 µl final reaction volume. Reactions for each sample were performed in duplicate using the following PCR conditions: 95 °C for 3 min; 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 20 s, in a CFX 96™ Real Time System (Bio-Rad). Melting curve analysis over a range of 60 to 95 °C (0.5 °C increment, 2 s) allowed detection of primer dimers and/or non-specific products.

Expression in X. laevis *oocytes and electrophysiology*

The open reading frame, from start (ATG) to stop codon, encoding Atlantic salmon PepT1a (GenBank Acc. No. XM_014172951.1) and PepT1b (GenBank Acc. No. NM_0011466882.1) were subcloned in pSPORT1 for *X. laevis* oocyte expression. Both constructs were verified by sequencing.

To improve the expression of Atlantic salmon PepT1a and PepT1b in the membrane of *X. laevis* oocytes, a 3'UTR sequence from rat Divalent metal transporter 1 (rDmt1, *alias* rat *Slc11a2*; GenBank Acc. No. NM_013173.2) was added to the end of the Atlantic salmon PepT1a and PepT1b coding sequence (CDS), as previously reported for *Dictyostelium discoideum* natural resistance-associated macrophage protein 1 (Nramp1) and 2 (Nramp2) **(9)**. The 1725 bp sequence added contains two poly-

adenylation signals and a poly(A) tail at the 3'end. The recombinant plasmids (pSPORT1-asPepT1a and pSPORT1-asPepT1b) were linearized with NotI and purified with Wizard SV Gel and PCR clean-up system (Promega Italia, Milan, Italy), *in vitro* capped and transcribed using T7 RNA polymerase. The purified cRNA was visualized by denaturing formaldehyde-agarose gel electrophoresis and quantified by NanoDrop™ 2000 spectrophotomer (Thermo Fisher Scientific, Monza, Italy). Enzymes were supplied by Promega Italia.

Oocytes were obtained from adult (2-to-5 years old) female *X. laevis* (Envigo, San Pietro al Natisone, Italy). Frogs were anesthetized in MS222 0.10% w/v solution in tap water, and after carefully cleaning the frog abdomen with an antiseptic agent (Providone-iodine 10%), the ovary was removed through laparotomy. The oocytes were treated with 0.5 mg/mL collagenase (Sigma Type IA) in calcium-free ND96 (NaCl 96 mmol/L, KCl 2 mmol/L, $CaCl_2$ 1.8 mmol/L, $MgCl_2$ 1 mmol/L, HEPES 5 mmol/L, pH 7.6) for at least 30 min at 18 °C. After 24 hours at 18 °C in NDE solution (ND96 plus 2.5 mmol/L pyruvate and 0.05 mg/mL gentamycin sulphate), the healthy and full-grown oocytes were injected with 25 ng of cRNA coding for the transporters in 50 nL of water using a manual microinjection system (Drummond Scientific Company, Broomall, PA, USA). The oocytes were then incubated at 18 °C for 3-4 days in NDE before electrophysiological studies **(5)**.

Classic Two-Electrode Voltage Clamp (TEVC) (Oocyte Clamp OC-725B, Warner Instruments, Hamden, CT, USA) was used to record membrane currents under voltage clamp conditions controlled by Clampex 10.2 (Molecular Devices, Sunnyvale, CA, USA). Borosilicate microelectrodes, with a tip resistance of 0.5-4 MΩ, were filled with 3 mol/L KCl. Bath electrodes were connected to the experimental oocyte chamber *via* agar bridges (3% agar in 3 mol/L KCl). The holding potential was kept at -60 mV; the voltage pulse protocol consisted of 10 square pulses from -140 to +20 mV (20 mV increment) of 700 ms each. Signals were filtered at 0.1 kHz, sampled at 200 Hz or 0.5 kHz, and at 1 kHz. Transport-associated currents were calculated by subtracting the traces in the absence of substrate from those in its presence. Data was analyzed using Clampfit 10.2 (Molecular Devices). All figures were prepared with Origin 8.0 (OriginLab, Northampton, MA, USA). The external control solution had the following composition: NaCl 98 mmol/L, $MgCl_2$ 1 mmol/L, $CaCl_2$ 1.8 mmol/L. For pH 6.5 the buffer

solution Pipes 5 mmol/L was used; Hepes 5 mmol/L was used to obtain a pH 7.6. Final pH values were adjusted with HCl or NaOH. The substrate oligopeptides tested were: Gly-Gln, Gly-Sar, Ala-Ala, Gly-Gly-Gly, Gly-Asn, Gly-Pro (Sigma-Aldrich). Every oligopeptide was added at the indicated concentrations (from 0.1 to 30 mmol/L) in the solutions with appropriate pH.

Statistical analysis

For tissue distribution analysis, statistical significance of mRNA levels between tissues was done using one-way ANOVA followed by Tukey's *post hoc* multiple comparison test (differences were considered significant if $P<0.05$). The statistical analysis was conducted in R 3.5.1 **(38)**. For functional analysis, descriptive statistic and logistic fit were applied; number of samples and of batch were reported in each figure.

Results

Sequence analysis

The complete CDS of Atlantic salmon *pept1a* of 2,157 bp encoded a protein of 718 amino acids (**Supplemental Fig. S1** [https://doi.org/10.6084/m9.figshare.9729383]). Atlantic salmon PepT1a and PepT1b amino acid sequences shared 77% similarity and 64% identity (**Fig. 1**). Hydropathy analysis predicted at least 12 potential membrane spanning domains with a large extracellular loop between transmembrane domains IX and X (**Fig. 1**). Structural important motifs such as the PTR2 family proton/oligopeptide symporter signatures were also well conserved in Atlantic salmon PepT1a sequence (amino acid residues 76-100 for signature 1, PROSITE pattern: PS0102; amino acid residues 169-181 for signature 2, PROSITE pattern: PS01023) (**Fig. 1**). Five potential N-glycosylation sites at the extracellular surface, one potential cAMP/cGMP-dependent protein kinase phosphorylation site and one potential protein kinase C phosphorylation site at the cytoplasmic surface were found (**Supplemental Fig. S1** [https://doi.org/10.6084/m9.figshare.9729383]).

Comparative analysis and evolutionary relationships

The evolutionary position of the Atlantic salmon PepT1a was studied by multiple sequence alignment with respect to its closest paralogue Atlantic salmon PepT1b, as well as to orthologues (PepT1a) and more distant paralogues (PepT1b, PepT2 and PepT2-like) from closely related species, which included other three salmoniforms, brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and Arctic char (*Salvelinus alpinus*), two esociforms, Northern pike (*Esox lucius*) and Eastern mudminnow (*Umbra pygmaea*), and the clupeiform Atlantic herring (*Clupea harengus*). The optimal tree from the multiple alignment of the predicted amino acid sequences was generated (**Fig. 2**), indicating that the putative Atlantic salmon PepT1a clustered, as expected, with the PepT1a-type proteins branch. In addition, PepT1b and PepT2 with PepT2-like formed two other distinct clades.

Synteny

As assessed by GDV consulting, the Atlantic salmon *pept1b* gene is located on chromosome ssa16 (Chrssa16), in the genomic region Chrssa16:87,604,364..87,624,485 (complement), while the Atlantic salmon *pept1a* gene is located on chromosome ssa25 (Chrssa25), in the genomic region Chrssa25:15,532,583..15,543,711 (complement). Atlantic salmon *pept1a* consists of 23 exons and 22 introns (**Supplemental Fig. S2** [https://doi.org/10.6084/m9.figshare.9729392]; **Supplemental Table S2** [https://doi.org/10.6084/m9.figshare.9729404]). **Table 1** summarizes the results of a synteny analysis recently performed (August 2018) in which the genomic region encompassing the *pept1a* gene in the Atlantic salmon Chrssa25 was compared to that encompassing the *pept1a* gene in the Northern pike (*Esox lucius*) chromosomal linkage group 16 (ChrLG16). Besides *pept1a*, the four genes upstream and the four downstream *pept1a* were described (**Table 1**). Notably, the Atlantic salmon genomic region Chrssa25:15,472,601..15,820,003 and the Northern pike genomic region ChrLG16:13,993,157..14,269,386 are completely syntenic. Despite the Atlantic salmon genome experienced the salmonid-specific whole genome duplication (WGD) event ~80 million years ago **(3, 17, 30, 37, 39, 48, 61, 64)**, we found no obvious signs of other genes closely or distantly related to *pept1a* and/or *pept1b* in the Atlantic salmon genome (data not shown).

311

312 *Tissue distribution of Atlantic salmon* pept1a *and* pept1b

313 Tissue expression analysis (**Fig. 3**) on the fish gastrointestinal tract (**Fig. 3B**) revealed that Atlantic
314 salmon *pept1a* and *pept1b* share a very similar distribution profile along the alimentary canal and are both
315 highly expressed in the anterior midgut, pyloric caeca and (less abundantly) in the midgut, whereas
316 lower levels of expression were detected in posterior midgut (**Fig. 3A**). Notably, *pept1b* was on average
317 ~5-fold more abundant than *pept1a* (**Fig. 3A**). In all other tissues, only traces of expression of both
318 transporters were observed (**Fig. 3A**).

319

320 *Function*

321 Three days after the injection of 25 ng of cRNA encoding Atlantic salmon PepT1a and PepT1b,
322 oocytes were tested for functional expression (**Fig. 4** and **Fig. 5**). Inward transport currents were
323 recorded in voltage clamp conditions at -60 mV in the presence of Gly-Gln, Ala-Ala and Gly-Gly-Gly 1
324 mmol/L and representative traces are reported in **Fig. 4A**. Atlantic salmon PepT1a, like PepT1b, was
325 electrogenic, with transported di/tripeptides causing inward currents. The recordings showed that in
326 Atlantic salmon PepT1a, in contrast to PepT1b, a decrease in pH from 7.6 to 6.5 increased the
327 amplitude of the current in the presence of all the tested substrates. The transport currents of Atlantic
328 salmon PepT1a and PepT1b were also tested in comparison to the transport currents generated by
329 European sea bass PepT1 (GenBank Acc. No. FJ237043) **(49)** and rabbit PepT1 (GenBank Acc. No.
330 U13707.1) **(4)** (**Fig. 4B**), under the same experimental conditions as in **Fig. 4A**. The mean transport
331 currents amplitude elicited in oocytes expressing the tested proteins confirmed the different pH
332 dependence of the two salmon transporters (**Fig. 4B**). In fact, for Gly-Gln and Ala-Ala, in Atlantic
333 salmon PepT1b, like in rabbit PepT1, the decrease of pH from 7.6 to 6.5 slightly increased (or did not
334 increase) the current at -60 mV; conversely, in Atlantic salmon PepT1a, like in the European sea bass
335 transporter, the currents showed large increases, with different amplitudes according to the substrate
336 tested. When the substrate was Gly-Gly-Gly at pH 7.6, Atlantic salmon PepT1a transport current was
337 drastically reduced (Fig. 4B).

To evaluate the effect of extracellular sodium on the transport activities of the two salmon transporters, the currents elicited by 3 mmol/L Gly-Gln were recorded at different membrane potentials, from -140 mV to +20 mV, at pH 7.6 and with or without sodium (substituted by tetramethylammonium). For both transporters, no differences were noticed in the current amplitude and in the shape of I/V relationship, thus confirming that Atlantic salmon PepT1a and PepT1b are both sodium-independent (**Fig. 4C**).

To define the voltage dependence and substrate apparent affinity of Atlantic salmon PepT1a, the transport currents for Gly-Gln (0.01 to 30 mmol/L) and for Gly-Sar (0.01 to 10 mmol/L) were recorded at two pH values (7.6 and 6.5) and collected from -140 mV to +20 mV (**Fig. 5A**). At -60 mV the measured kinetic parameters ($K_{0.5}$ and I_{max}) for Gly-Gln were 0.076±0.004 mmol/L and -41.317±0.835 nA at pH 6.5 and 1.593±0.166 mmol/L and -49.574±2.128 nA at pH 7.6, while for Gly-Sar were 0.523±0.102 mmol/L and -39.228±4.490 nA at pH 6.5 and 9.215±2.689 mmol/L and -48.844±10.421 nA at pH 7.6. In general, larger currents were recorded at pH 7.6 for both substrates at the maximal substrate concentrations tested. The Atlantic salmon PepT1a relative maximal currents elicited by Gly-Gln and Gly-Sar are reported in **Fig. 5C** and **Fig. 5F**. At voltage values more negative than -100 mV, Gly-Gln relative maximal current was influenced by pH with an increase of current amplitude at 7.6 with respect to 6.5 (**Fig. 5C**). For Gly-Sar, very slight differences in relative maximal current values at the two pH conditions were recorded at all voltages tested (**Fig. 5F**). Atlantic salmon PepT1a affinity for Gly-Gln and Gly-Sar increased with the decrease of pH (**Fig. 5B** and **Fig. 5E**), and at pH 6.5 affinity was only slightly influenced by voltage. For both neutral substrates, $K_{0.5}$ was in the micromolar range at pH 6.5 and in the millimolar range at pH 7.6, that is different from Atlantic salmon PepT1b where pH only slightly influenced $K_{0.5}$ **(44)**. Accordingly, also the transport efficiency, evaluated as the ratio of $I_{max}/K_{0.5}$ values, was largely influenced by pH in Atlantic salmon PepT1a (**Fig. 5D** and **Fig. 5G**), with higher values at pH 6.5 and at more negative voltages (from 0 to -140 mV). Notably, the efficiency of Atlantic salmon PepT1a transport was evidently lower than that obtained for PepT1b (**Table 2**), according to the higher affinity of Atlantic salmon PepT1a for both Gly-Gln and Gly-Sar, particularly at pH 6.5. Data about the I_{max}, $K_{0.5}$ and their ratio in the presence of Ala-Ala, Gly-

Asn, and Gly-Pro at pH 6.5 were also collected and are summarized (compared to Gly-Gln and Gly-Sar) in **Table 3**.

Discussion

In this study, we report for the first time the systematic characterization of a piscine PepT1a-type transporter, the Atlantic salmon PepT1a, and compare it to its closest paralogue, i.e. the Atlantic salmon PepT1b **(44)**. In particular, we demonstrate that Atlantic salmon *pept1a*, which is expressed in the anterior midgut, pyloric caeca and less abundantly in the midgut, is functional and able to mediate the transport of neutral di/tripeptides, such as Gly-Gln, Gly-Sar, Ala-Ala, Gly-Asn, Gly-Pro and Gly-Gly-Gly. Atlantic salmon PepT1a differs from Atlantic salmon PepT1b in terms of transport kinetics, substrate specificity and transport efficiency, while it shares the same rostral-to-caudal expression pattern along the alimentary canal, although at different levels.

Atlantic salmon pept1a *in the context of gen(om)e duplication*

Similar to other teleost fish species, Atlantic salmon has two distinct *pept1*-type genes, namely *pept1a* (*slc15a1a*) and *pept1b* (*slc15a1b*). Comparative analysis of the available genomes from clupeiforms, esociforms and salmoniforms, as well as from more distant teleost fish species (data not shown), suggest that these genes are a result of the teleost-specific WGD event **(39, 61)**. This statement is corroborated by the parallel observation that, differently from PepT1a and PepT1b, Atlantic salmon PepT2 and PepT2-like proteins seem to be encoded by genes resulting from the salmonid-specific WGD event **(3, 17, 30, 37, 48, 64)**.

Why a duplicated *pept1*-type gene set up persists in the teleost genomes is not known yet. But, a significantly higher adaptive flexibility for teleost fish *via* their species-specific repertoire of Slc15-type proteins can be hypothesized since the two PepT1-type di/tripeptide transporters: a) differ in terms of amino acid sequence (Atlantic salmon PepT1a and PepT1b share 77% similarity and 64% identity at the amino acid level while conserving the main PepT1-type functional motifs); b) are coded by similar

genes located in different parts of the genome; c) variably response to external stimuli and/or environmental solicitations (see section below *Tissue expression of Atlantic salmon* pept1a); d) exhibit diverse kinetic properties and functional specificities (see below section *Function of Atlantic salmon* pept1a).

Tissue expression of Atlantic salmon pept1a

In teleost fish, PepT1 is expressed in the gut, but it is also reported in other organs (kidney, liver and spleen), although to a very low extent. In the gut, PepT1 is restricted to the intestine. However, its expression pattern greatly differs among species. For e.g., while in cypriniforms and tetraodondiforms PepT1 is confined to the most proximal portion(s) of the intestine, in gadiforms and cyprinodontiforms it is almost uniformly distributed along the intestinal canal, most distal regions included. Salmoniforms show a steady decrease in PepT1 expression passing from proximal-to-distal adjacent segments of the intestinal canal, although in perciforms, pleuronectiforms and cichliforms, the proximal-to-distal drop of expression along the post-gastric alimentary canal seems steeper than in salmoniforms. Whenever present, the pyloric caeca invariably express PepT1 at very high levels **(58)**. Notably, the spatio-temporal expression of PepT1 intestinal mRNA largely varies during ontogeny, in response to nutritional states (such as food deprivation/refeeding), dietary challenges, and/or environmental conditions (such as in freshwater/seawater adaptation), as well as under certain disease states (such as gut inflammation) **(1, 2, 8, 42, 58, 60)**.

To date, the opinion that in teleost fish PepT1 expression data reflect the levels of expression of a single gene (implicitly meaning *pept1b*) is outdated and needs to be replaced by the view that PepT1a and PepT1b may contemporarily be expressed and operate at the intestinal level. After the first papers in mummichog (*Fundulus heteroclitus macrolepidotus*) **(8)** and Nile tilapia (*Oreochromis niloticus*) **(23)**, in which PepT1a and PepT1b were first analyzed together revealing overlapping expression profiles along the intestine, other studies in Nile and Mozambique tilapia (*Oreochromis mossambicus*) **(10, 11, 21)** and more recently in European sea bass **(25)** fully confirmed the high flexibility of the transporters in the context of gut physiology, their mutual interplay and with PepT2 (which seems to operate downstream the

PepT1a/PepT1b couple along the alimentary canal), and their capacity to respond differentially to various types of external solicitations. In this discussion, it is worth to note that differently from mummichog, in which *pept1a* and *pept1b* seem uniformly distributed along the intestinal tract, in tilapia PepT1a expression largely exceeds that of PepT1b in the proximal intestine, whereas PepT1b expression exceeds that of PepT1a in the mid intestine; thus, the PepT1a-to-PepT1b ratio inverts passing from proximal to mid intestine **(23)**; moreover, in tilapia intestine PepT1a appears more abundantly expressed than PepT1b **(23)**. Such elements may be of reference to define the general organization of the di/tripeptide transporters repertoire along the intestinal tract of other teleost fishes.

The two Atlantic salmon *pept1*-type genes show a similar and overlapping tissue distribution profile. The mRNA distribution of both paralogues is like that described for other teleost fish, including zebrafish **(59)**, grass carp (*Ctenopharyngodon idella*) **(29)**, Oriental weatherfish (*Misgurnus anguillicaudatus*) **(20)** and pufferfish (*Tetraodon nigroviridis*) **(63)**, where *pept1*-type gene expression is confined to the proximal intestine. Also, in the Atlantic salmon very high levels of expression are observed in the pyloric caeca, similarly to what reported previously for this **(44)** and other salmonids, such as the rainbow trout **(24, 36)**. Furthermore, the proximal-to-distal drop of expression along the intestine observed in this study is in agreement with the results obtained for European sea bass **(55)** and gilthead sea bream (*Sparus aurata*) **(56)**. The levels of *pept1*-type mRNA expression in the Atlantic salmon hindgut differs between the present study and the work from Rønnestad and coll. **(44)**. The lower levels of expression obtained in this study may be due to the different life stage (adult *vs* juvenile) and, consequently, to the different rearing environment (seawater *vs.* freshwater), as there is evidence that salinity may play an important role in the regulation of *pept1*-type genes expression **(8, 10, 11, 25)**. However, it could simply be due to technical aspects, as Rønnestad and coll. **(44)** have used primers that are not 100% specific for *pept1b* and could have had simultaneously amplified *pept1a*. The latter observation substantiates the need for a re-evaluation of the previous *pept1*-type mRNA expression studies in teleost fishes.

In this study, we show that in Atlantic salmon the mRNA expression levels of *pept1b* largely exceed those of *pept1a* in the pyloric caeca, anterior midgut and midgut. This contrasts with what was reported

for tilapias but is substantially in line with observations in mummichog **(8, 10, 11, 21, 23)**. In addition, in relation to their mRNA levels we did not observe an inversion of PepT1a-to-PepT1b ratio passing from proximal to mid intestine, unlike to Nile tilapia **(23)**.

The topological expression of both *pept1*-type genes correlates with the intestinal areas that are considered more directly involved in digestion and absorption in the Atlantic salmon and reflect the central role of these genes in Atlantic salmon gut function(s). Identifying similarities and differences in these PepT1-type proteins will allow us to fully understand their physiological role.

Function of Atlantic salmon PepT1a

The experiments presented herein demonstrate that Atlantic salmon PepT1a is functional. Its heterologous expression in *X. laevis* oocytes and experiment conducted keeping the membrane voltage under control allows to record the substrate-induced currents in the presence of neutral 'reference' dipeptides such as Gly-Gln and Gly-Sar, as well as other neutral di/tripeptides such as Ala-Ala, Gly-Asn, Gly-Pro and Gly-Gly-Gly. To our knowledge, this is the first evidence of activity reported for a piscine PepT1a-type transporter.

Our data show that PepT1a is an electrogenic, Na^+-independent, H^+-dependent transporter of di/tripeptides, which essentially operates as a low-affinity/high-capacity system. In this respect, it resembles the other PepT1-type di/tripeptide transporters so far characterized in mammalian, avian and piscine models **(13, 16, 19, 42, 52, 58, 60)**. However, some characteristics that distinguish *in primis* the two Atlantic salmon proteins (PepT1a *vs.* PepT1b) when tested in the presence of the same substrates and under the same experimental conditions clearly emerge from the detailed biophysical and kinetic analysis presented in this study. In summary, it is evident that even if for neutral substrates both transporters share similar relatively low affinity, for e.g., at -60 mV, $K_{0.5}$ for PepT1a between 0.02 and 0.52 mmol/L, and for PepT1b between 0.46 and 0.97 mmol/L, depending on the peptide, and high capacity values **(44)**. PepT1a exhibits higher affinities than PepT1b for certain dipeptides, e.g. Gly-Gln, and it is generally more influenced by external pH and membrane potential. In particular, with the external pH set at 6.5, the affinity values for Gly-Gln and Gly-Sar are only slightly affected (or relatively

unaffected) by membrane potential in PepT1a [e.g. $K_{0.5}$ ~0.52 mmol/L at -60 mV and ~0.69 mmol/L at -120 mV (ratio: 0.76) for Gly-Sar, and $K_{0.5}$ ~0.08 mmol/L at -60 mV and ~0.11 mmol/L at -120 mV (ratio: 0.70) for Gly-Gln], as in PepT1b [e.g. $K_{0.5}$ ~0.50 mmol/L at -60 mV and ~0.41 mmol/L at -120 mV (ratio: 1.21) for Gly-Sar; see also **(44)**]; analogously, similar changes in maximal current values are observed in PepT1a [e.g. I_{max} ~ -39 nA at -60 mV and ~ -109 nA at -120 mV (ratio: 0.36) for Gly-Sar, and I_{max} ~ -41 nA at -60 mV and ~ -121 nA at -120 mV (ratio: 0.34) for Gly-Gln] and PepT1b [e.g. I_{max} ~ -62 nA at -60 mV and ~ -148 nA at -120 mV (ratio: 0.42) for Gly-Sar; see also **(44)**]. Conversely, when the external pH is set at 7.6, both affinity and maximal current are influenced by membrane potential, particularly at more negative values. Moreover, with the external pH set at 7.6, the $K_{0.5}$ values for Gly-Gln and Gly-Sar decrease at more negative membrane voltages in PepT1a [e.g. $K_{0.5}$ ~9.21 mmol/L at -60 mV and ~2.69 mmol/L at -120 mV (ratio: 3.42) for Gly-Sar, and $K_{0.5}$ ~1.59 mmol/L at -60 mV and ~0.51 mmol/L at -120 mV (ratio: 3.15) for Gly-Gln] more than it happens in PepT1b [e.g. $K_{0.5}$ ~1.44 mmol/L at -60 mV and ~0.52 mmol/L at -120 mV (ratio: 2.77) for Gly-Sar; see also **(44)**]; however, similar changes in maximal current values are still observed in PepT1a [e.g. I_{max} ~ -49 nA at -60 mV and ~ -125 nA at -120 mV (ratio: 0.39) for Gly-Sar, and I_{max} ~ -50 nA at -60 mV and ~ -158 nA at -120 mV (ratio: 0.32) for Gly-Gln] and PepT1b [e.g. I_{max} ~ -62 nA at -60 mV and ~ -148 nA at -120 mV (ratio: 0.42) for Gly-Sar; see also **(44)**]. Taken together, all these changes in both I_{max} and $K_{0.5}$ result in a consistent reduction of Atlantic salmon PepT1a transport efficiency ($I_{max}/K_{0.5}$), which is, in all the studied conditions, systematically lower that that recorded for Atlantic salmon PepT1b.

Conclusions

To summarize, the Atlantic salmon PepT1a is functional and operates at the intestinal level in the same post-gastric portions of the intestine where PepT1b acts. Expression in other tissues is much lower. At the moment, the functional role of PepT1a in other tissues is not known.

Our findings clearly indicate that Atlantic salmon gut is equipped with two functional transport systems for the uptake of di/tripeptides. Whether or not the two transport systems share physiological roles (nutrient absorption and/or molecule sensing), cellular localization in the gut epithelium, sub-cellular

localization in the gut epithelial cells, and type of regulation remain open questions for future studies, as well as how they differentially respond to various external stimuli/environmental solicitations (such as nutrients, diets, salinity and temperature).

Acknowledgements

We thank Dr. Ann-Elise O. Jordal, Dr. Anders Aksnes and Dr. Mali B. Hartviksen for technical help during sampling, and Dr. Ann-Elise O. Jordal for technical assistance during gene expression analysis. Finally, we thank Dr. Anders Aksnes for providing fish for sampling.

Grants

Supported by Regional Research Fund West (RFFV; 247978 SalmoFeedPlus) and Cargill.

Disclosures

No conflict of interest, financial or otherwise.

Author contributions

A.S.G., F.V., E.B., I.R. and T.V. conceived and designed research; A.S.G., F.V., R.C., A.B and E.B. performed experiments; A.S.G., F.V., R.C., K.M., A.B., E.B. and T.V. analyzed data; A.S.G., F.V., R.C., K.M., A.B., E.B., I.R. and T.V. interpreted results of experiments; A.S.G., F.V., R.C., E.B. and T.V. prepared figures; A.S.G., F.V., R.C., E.B., I.R. and T.V. drafted manuscript; A.S.G., F.V., K.M., A.B., E.B., I.R. and T.V. edited and revised manuscript; E.B., I.R. and T.V. had primary responsibility for final content. All authors read and approved the final version of the manuscript.

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706 **Figures**

707

708 **Fig. 1. Pairwise alignment between Atlantic salmon PepT1a (Slc15a1a) and PepT1b (Slc15a1b)**
709 **amino acid sequences.**

710 The alignment was obtained by using ClustalX 2.1 **(27)** and edited using GeneDoc 2.7 software **(35)**.
711 The predicted (https://prosite.expasy.org/scanprosite/) conserved PTR2 family proton/oligopeptide
712 symporters signatures (motif 1 - PROSITE pattern PS01022 - amino acid residues 76-100; and motif 2
713 - PROSITE pattern PS01023 - amino acid residues 169-181) are colored in purple. In the amino acid
714 sequence, putative transmembrane domains are named I to XII.

715

716 **Fig. 2. Evolutionary relationships of PepT1(Slc15a1)- and PepT2(Slc15a2)-type transporters in**
717 **teleost fish.**

718 The evolutionary history was inferred using the Neighbor-Joining method **(47)**. The optimal tree with
719 the sum of branch length = 1.76900864 is shown. The percentage of replicate trees in which the
720 associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches
721 **(18)**. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary
722 distances used to infer the phylogenetic tree. The evolutionary distances were computed using the
723 Poisson correction method **(67)** and are in the units of the number of amino acid substitutions per site.
724 The analysis involved 25 amino acid sequences. All positions containing gaps and missing data were
725 eliminated. There were a total of 571 positions in the final dataset. Evolutionary analyses were
726 conducted in MEGA7 **(26)**. Classical protein GenBank accession (Acc.) numbers (Nos.) are indicated
727 for the canonically annotated amino acid sequences, while Transcriptome Shotgun Assembly (TSA)
728 Acc. Nos. are given for those amino acid sequences derived from (a) TSA project(s) by transcript-to-
729 protein sequence translation (*via* ORFfinder; https://www.ncbi.nlm.nih.gov/orffinder/).

730

731 **Fig. 3. Spatial distribution of Atlantic salmon *pept1a* (*slc15a1a*) and *pept1b* (*slc15a1b*).**

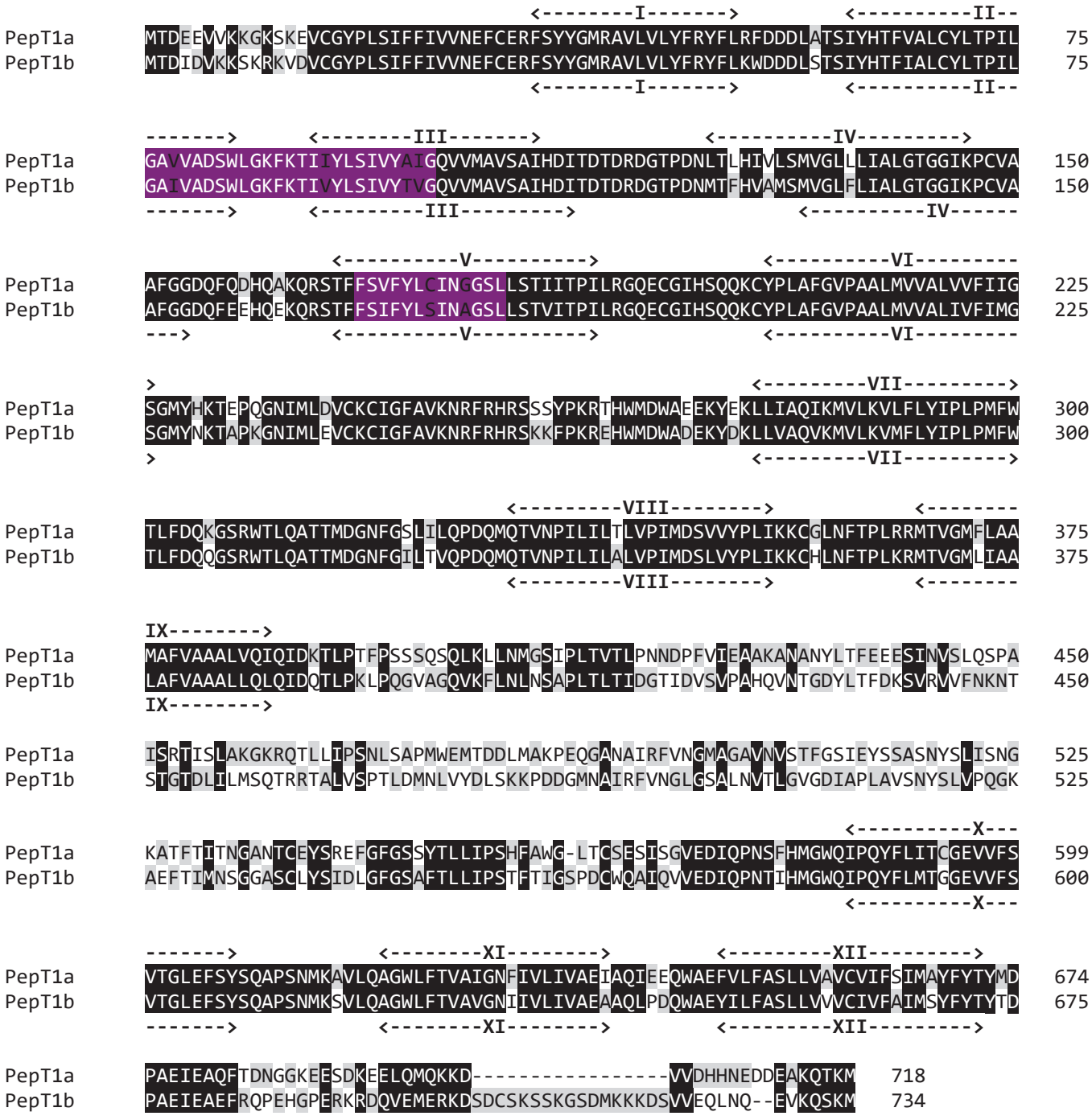
A: *pept1a* (*slc15a1a*) and *pept1b* (*slc15a1b*) mRNA levels in Atlantic salmon tissues as assessed by qPCR. Results are presented as means ± SEM of the normalized expression (MNE) of *pept1a* and *pept1b* mRNA (using *β-actin* as the reference gene; n = 6 for all tissues). Statistical significance of mRNA levels between tissues (one-way ANOVA followed by Tukey's *post hoc* multiple comparison test; $P<0.05$) is detailed in **Supplemental Table S3** [https://doi.org/10.6084/m9.figshare.9729407]. *B*: A representative picture of the Atlantic salmon gastrointestinal tract. AST, anterior stomach; PST, posterior stomach; AMG, anterior midgut; PC, pyloric caeca, MG, midgut; PMG, posterior midgut; AHG, anterior hindgut; PHG, posterior hindgut.

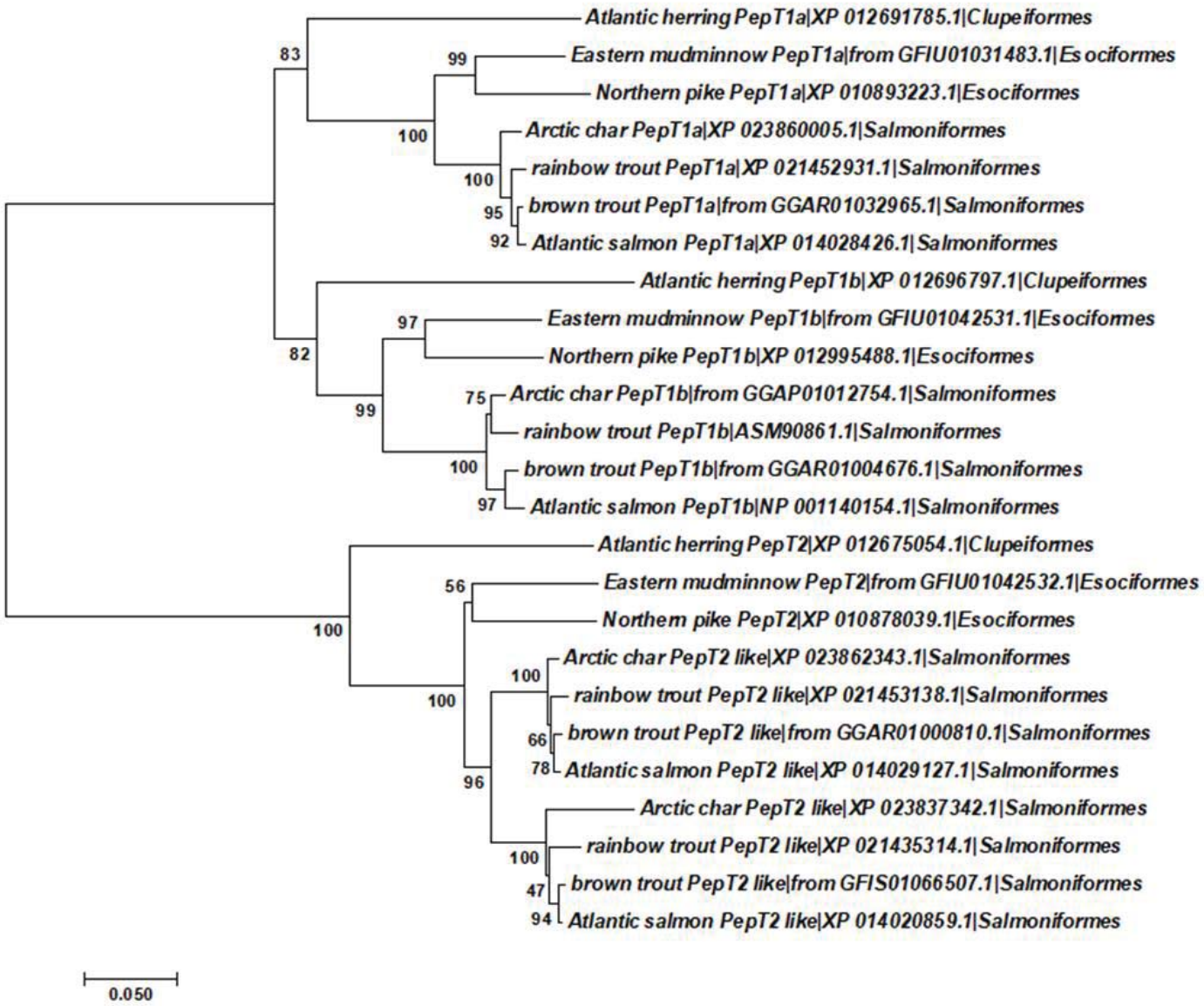
Fig. 4. Transport activity and pH dependence of Atlantic salmon PepT1a (Slc15a1a) and PepT1b (Slc15a1b).

A: Representative traces of transport currents in PepT1b (left) and PepT1a (right) heterologously expressed in *Xenopus laevis* oocytes. The currents in the presence of the substrates (1 mmol/L) are indicated by the gray bars and were recorded at the holding potential (V_h) of -60 mV and at pH 6.5 and 7.6. *B*: Means of transport-associated currents at two pH conditions, in PepT1-type transporters [rabbit (rbPepT1), European sea bass (sbPepT1), and Atlantic salmon (asPepT1a and asPepT1b)]. From the top, the transport current elicited by Gly-Gln (GQ), Ala-Ala (AA) and Gly-Gly-Gly (GGG) (1 mmol/L) at -60 mV at pH 6.5 (light gray) and 7.6 (dark gray); current values are reported as means ± SEM from 3 to 7 oocytes (actual sample sizes for bar graphs, in terms of total number of oocytes per group: rbPepT1, 3 for each pH and substrates; sbPepT1, 3 for each substrate at pH 6.5 and 4 for each substrate at pH 7.6; asPepT1b, 5 at pH 6.5 and 7 at pH 7.6; asPepT1a, 5 at pH 6.5 and 6 at pH 7.6) from 2 to 4 batches (two sample *t*-test; * $P<0.05$, ** $P<0.01$ and *** $P<0.001$). *C*: Current-voltage relationships of transport-associated currents in asPepT1a (gray) and asPepT1b (black), in the presence of 3 mmol/L Gly-Gln in sodium (Na) saline buffer (square) and tetramethylammonium (TMA) saline buffer (empty circle) at pH 7.6. Values are means ± SEM from 9 to 12 oocytes from two batches in each group. The transport-associated current values reported in *B* and *C* were obtained by subtracting the current recorded in the absence of the substrate from that in its presence.

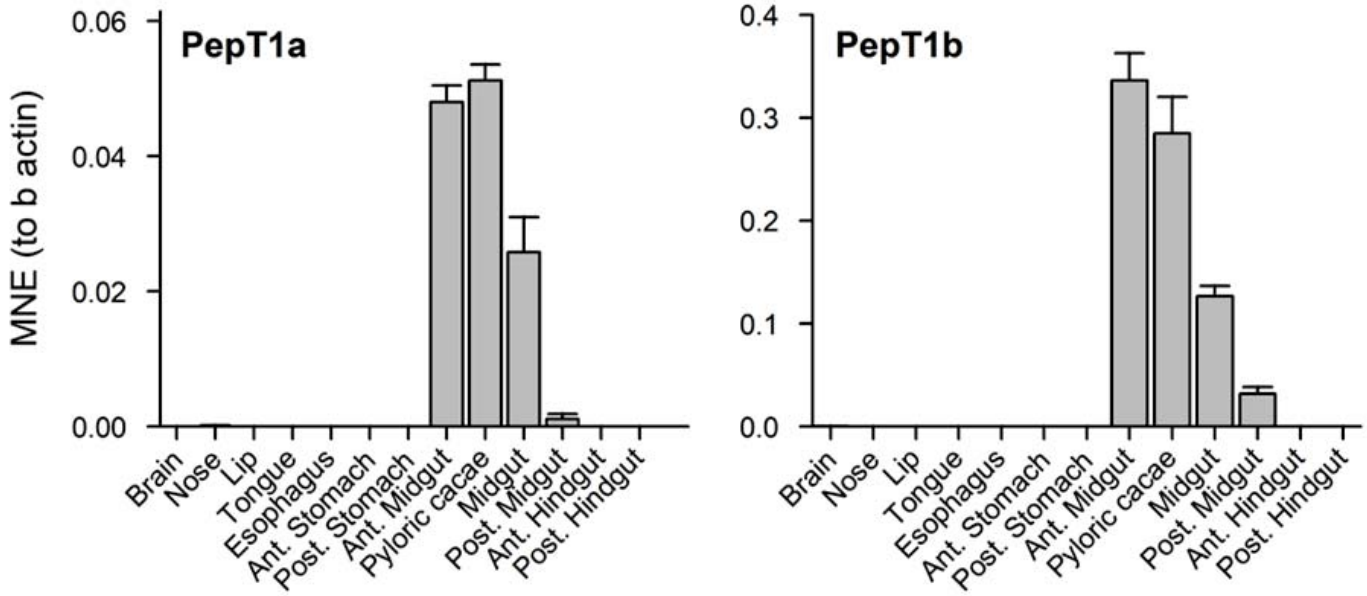
Fig. 5. Dose response analysis: $K_{0.5}$, I_{max} and transport efficiency of Atlantic salmon PepT1a (Slc15a1a).

Kinetic relationships were evaluated in the presence of Gly-Gln (*B*, *C*, *D*) and Gly-Sar (*E*, *F*, *G*). *A*: I/V relationships were obtained by subtracting the current traces in the absence from those in the presence of the indicated amounts of Gly-Gln or Gly-Sar, at pH 6.5 and 7.6. The current values were subsequently fitted with the logistic equation $\left[I_0 = \frac{-I_{max}}{1+([S]/K_{0.5})} + I_{max}\right]$ to obtain $K_{0.5}$, i.e. the substrate concentration that elicits half of the maximal current (I_{max}), at each indicated voltage and at pH 6.5 (square) and 7.6 (triangle). *B*: *E*, Plot of the $K_{0.5}$ values at each voltage and pH condition tested; the inserts (*Bb*, *Ee*) are enlargements of $K_{0.5}$ at pH 6.5. *C*, *F*: Plot of the I_{max} values at each voltage and pH condition tested. *D*, *G*: Plot of the transport efficiency, evaluated as the ratio of $I_{max}/K_{0.5}$ values at each voltage and pH condition tested.





A



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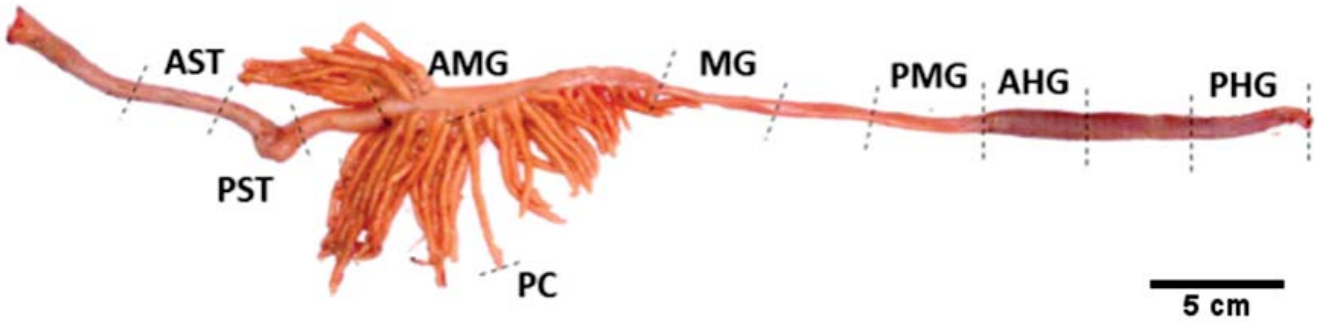

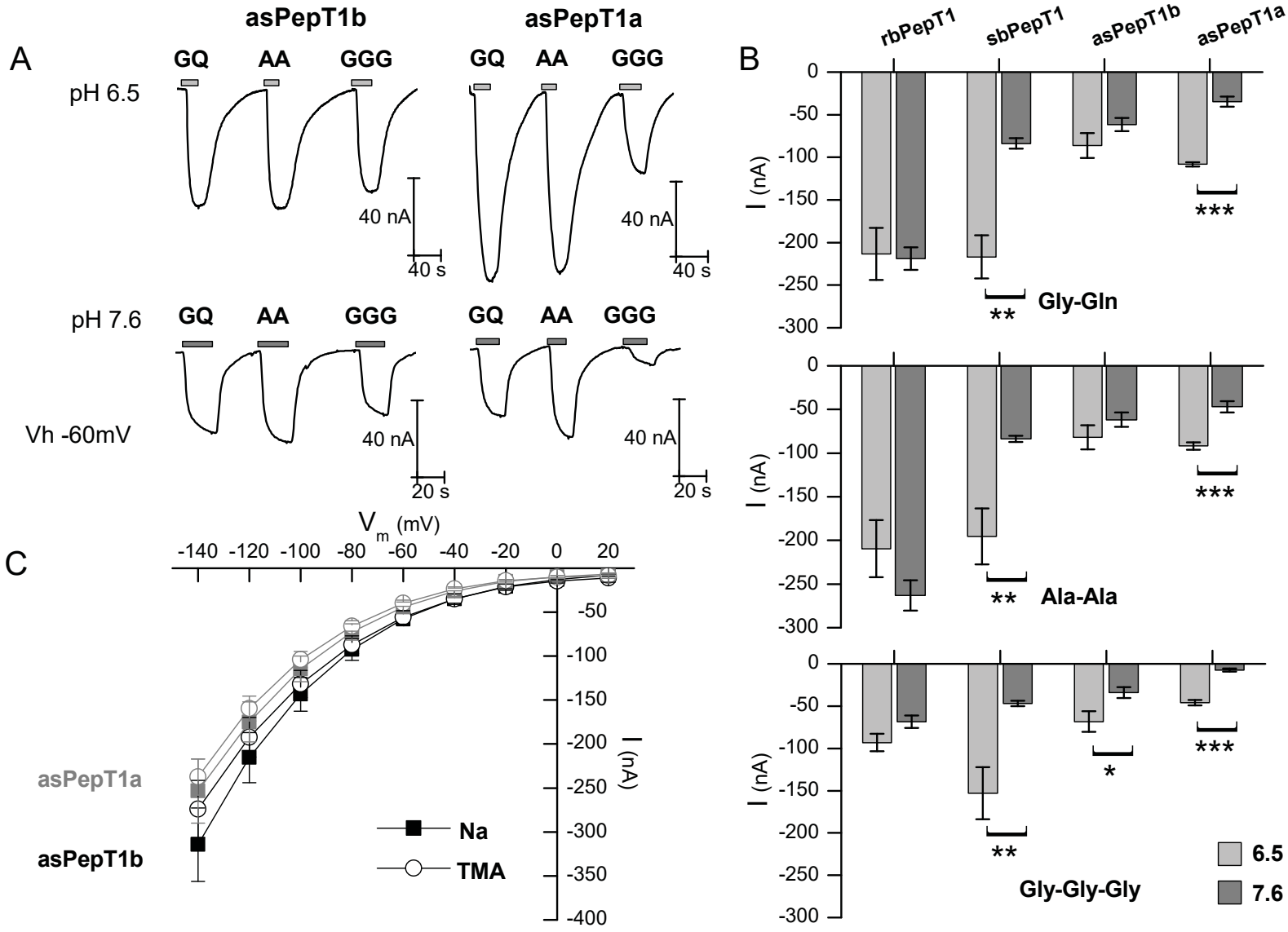

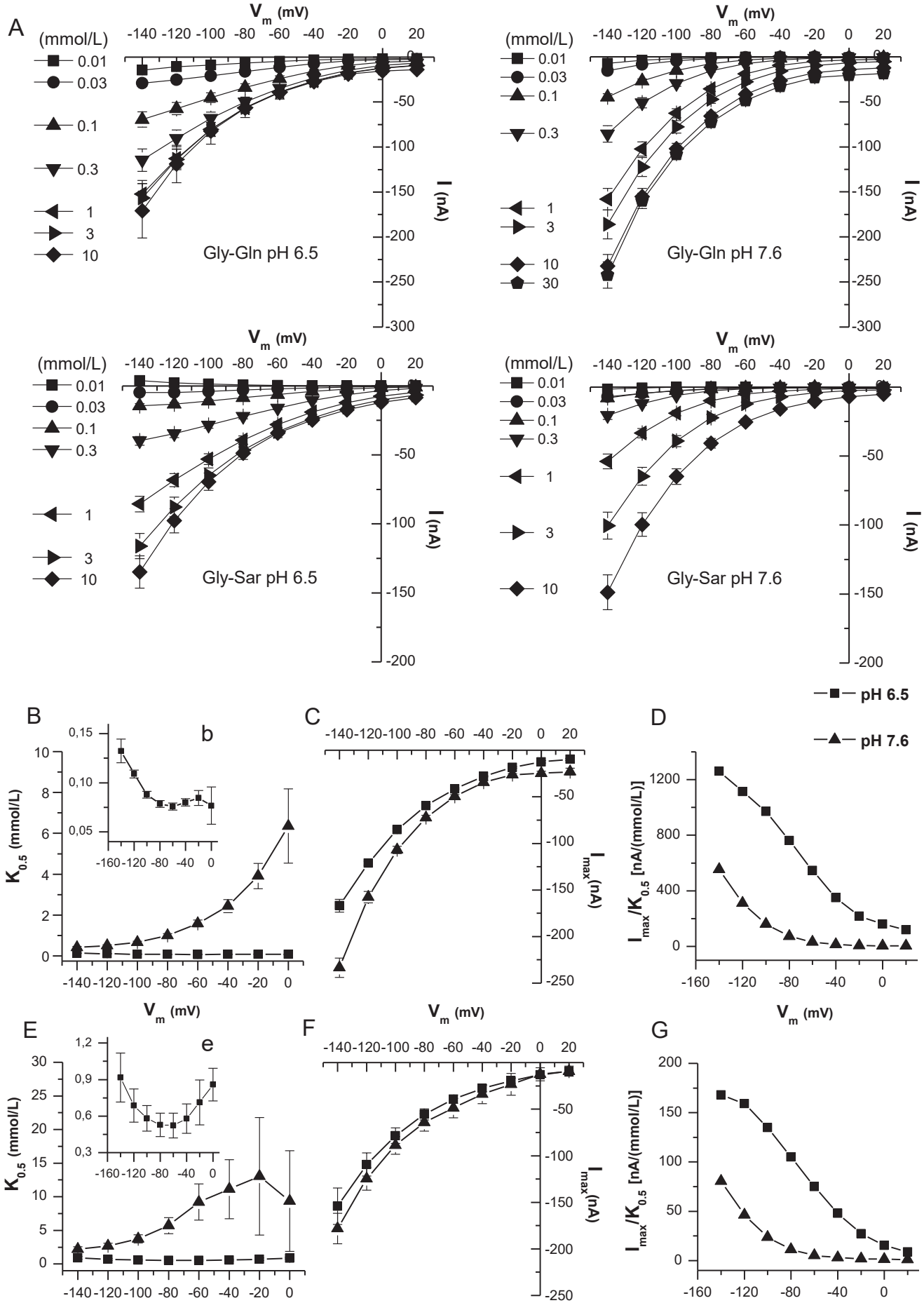

Table 1. Atlantic salmon (*Salmo salar*) *vs.* Northern pike (*Esox lucius*) genomic regions encompassing the *pept1a* (*slc15a1a*) gene.[1]

Atlantic salmon (*Salmo salar*)					Northern pike (*Esox lucius*)				
Chromosome[a] *GenBank Acc. No.*	*Gene name*	*Genomic region (location)*	*mRNA sequence GenBank Acc. No.*	*Protein sequence GenBank Acc. No.*	*Linkage Group*[b] *GenBank Acc. No.*	*Gene name*	*Genomic region (location)*	*mRNA sequence GenBank Acc. No.*	*Protein sequence GenBank Acc. No.*
ssa25 NC_027324.1	*LOC106586257* G-protein coupled receptor 1-like	Chrssa25: 15,472,601..15,475,918	XM_014173332.1	XP_014028807.1	LG16 NC_025983.3	*LOC105024751* muscleblind-like protein 2a	ChrLG16: 13,993,157..14,034,547	XM_020054959.1, XM_010894871.2, XM_010894877.3, XM_010894869.2, XM_010894875.2, XM_010894886.2, XM_010894874.2, XM_010894882.2, XM_010894878.2, XM_010894884.2, XM_010894887.2, XM_010894892.2, XM_020054961.1, XM_020054963.1, XM_020054962.1, XM_010894880.2, XM_010894885.2, XM_010894890.2, XM_020054964.1, XM_010894894.2, XM_010894895.2, XM_010894883.2, XM_010894889.2, XM_010894888.2, XM_010894893.2, XM_020054960.1	XP_019910518.1, XP_010893173.1, XP_010893179.1, XP_010893171.1, XP_010893177.1, XP_010893188.1, XP_010893176.1, XP_010893184.1, XP_010893180.1, XP_010893186.1, XP_010893189.1, XP_010893194.1, XP_019910520.1, XP_019910522.1, XP_019910521.1, XP_010893182.1, XP_010893187.1, XP_010893192.1, XP_019910523.1, XP_010893196.1, XP_010893197.1, XP_010893185.1, XP_010893191.1, XP_010893190.1, XP_010893195.1, XP_019910519.1
	LOC106586260 NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial-like	Chrssa25: 15,479,274..15,485,467	XM_014173336.1	XP_014028811.1		*rap2a* RAP2A, member of RAS oncogene family	ChrLG16: 14,035,160..14,053,640	XM_010894899.2	XP_010893201.1
	ino80d INO80 complex subunit D	Chrssa25: 15,485,819..15,498,511	XM_014173333.1, XM_014173334.1, XM_014173335.1	XP_014028808.1, XP_014028809.1, XP_014028810.1		*farp1* FERM, ARH/RhoGEF and pleckstrin domain protein 1	ChrLG16: 14,068,258..14,132,421	XM_010894902.3, XM_010894901.3, XM_010894900.3	XP_010893204.1, XP_010893203.1, XP_010893202.1
	stk24 serine/threonine kinase 24	Chrssa25: 15,498,768..15,516,219 (complement)	XM_014173337.1	XP_014028812.1		*dock9* dedicator of cytokinesis 9	ChrLG16: 14,132,904..14,212,226	XM_020054814.1, XM_010894912.3, XM_010894913.3, XM_020054821.1, XM_020054818.1, XM_020054809.1, XM_020054816.1, XM_020054817.1, XM_020054811.1, XM_020054819.1, XM_010894910.3, XM_020054810.1, XM_010894907.3, XM_020054820.1, XM_010894905.3, XM_020054812.1, XM_020054813.1, XM_020054815.1, XM_010894911.2	XP_019910373.1, XP_010893214.1, XP_010893215.1, XP_019910380.1, XP_019910377.1, XP_019910368.1, XP_019910375.1, XP_019910376.1, XP_019910370.1, XP_019910378.1, XP_010893212.1, XP_019910369.1, XP_010893209.1, XP_019910379.1, XP_010893207.1, XP_019910371.1, XP_019910372.1, XP_019910374.1, XP_010893213.1
	LOC106586093 solute carrier family 15 member 1-like (ref. *pept1a*/*slc15a1a*)	Chrssa25: 15,532,583..15,543,711 (complement)	XM_014172951.1	XP_014028426.1		*LOC105024756* solute carrier family 15 member 1 (ref. *pept1a*/*slc15a1a*)	ChrLG16: 14,212,413..14,222,383	XM_013137842.2, XM_010894921.3	XP_012993296.1, XP_010893223.1
	LOC106586262 dedicator of cytokinesis protein 9-like	Chrssa25: 15,543,947..15,666,060 (complement)	XM_014173356.1, XM_014173345.1, XM_014173354.1, XM_014173347.1, XM_014173349.1, XM_014173340.1, XM_014173338.1, XM_014173352.1, XM_014173341.1, XM_014173344.1, XM_014173353.1, XM_014173343.1, XM_014173348.1, XM_014173342.1, XM_014173351.1, XM_014173355.1, XM_014173346.1	XP_014028831.1, XP_014028820.1, XP_014028829.1, XP_014028822.1, XP_014028824.1, XP_014028815.1, XP_014028813.1, XP_014028827.1, XP_014028816.1, XP_014028819.1, XP_014028828.1, XP_014028818.1, XP_014028823.1, XP_014028817.1, XP_014028826.1, XP_014028830.1, XP_014028821.1		*LOC105024757* serine/threonine-protein kinase 24	ChrLG16: 14,225,799..14,236,569	XM_010894922.3	XP_010893224.1
	LOC106586263 FERM, RhoGEF and pleckstrin domain-containing protein 1-like	Chrssa25: 15,666,625..15,739,918 (complement)	XM_014173359.1, XM_014173360.1, XM_014173357.1, XM_014173358.1, XM_014173363.1, XM_014173362.1	XP_014028834.1, XP_014028835.1, XP_014028832.1, XP_014028833.1, XP_014028838.1, XP_014028837.1		*ino80d* INO80 complex subunit D	ChrLG16: 14,242,290..14,251,633 (complement)	XM_010894923.2, XM_010894924.2	XP_010893225.1, XP_010893226.1
	LOC106586264 ras-related protein Rap-2a	Chrssa25: 15,770,903..15,784,293 (complement)	XM_014173364.1	XP_014028839.1		*LOC105024759* NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	ChrLG16: 14,252,299..14,264,563 (complement)	XM_010894925.3, XM_020054994.1, XM_010894927.3	XP_010893227.1, XP_019910553.1, XP_010893229.1
	LOC106586265 muscleblind-like protein 2a	Chrssa25: 15,788,454..15,820,003 (complement)	XM_014173366.1, XM_014173370.1, XM_014173375.1, XM_014173365.1, XM_014173367.1, XM_014173376.1, XM_014173378.1, XM_014173379.1, XM_014173369.1, XM_014173368.1, XM_014173380.1, XM_014173372.1, XM_014173373.1, XM_014173374.1, XM_014173377.1	XP_014028841.1, XP_014028845.1, XP_014028850.1, XP_014028840.1, XP_014028842.1, XP_014028851.1, XP_014028853.1, XP_014028854.1, XP_014028844.1, XP_014028843.1, XP_014028855.1, XP_014028847.1, XP_014028848.1, XP_014028849.1, XP_014028852.1		*gpr1* G protein-coupled receptor 1	ChrLG16: 14,265,544..14,269,386 (complement)	XM_010894930.2, XM_010894931.2, XM_020054996.1, XM_010894929.3	XP_010893232.1, XP_010893233.1, XP_019910555.1, XP_010893231.1

Four genes upstream and four genes downstream *pept1a* are represented. Atlantic salmon chromosome ssa25, in the genomic region Chrssa25:15,472,601..15,820,003, and Northern pike linkage group LG16, in the genomic region ChrLG16: 13,993,157..14,269,386, are fully syntenic.

[a]Ref. Assembly Salmo salar:ICSASG_v2 (GCF_000233375.1) (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=salmo-salar&group=euteleosteomorpha)

[b]Ref. Assembly Esox lucius:Eluc_V3 (GCF_000721915.3) (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=esox-lucius&group=euteleosteomorpha)

Table 2. The transport efficiency in Atlantic salmon PepT1a (Slc15a1a) and PepT1b (Slc15a1b).[1]

	$I_{max}/K_{0.5}$							
	Gly-Gln				**Gly-Sar**			
	-60 Mv		**-120 mV**		**-60 Mv**		**-120 mV**	
	7.6	**6.5**	**7.6**	**6.5**	**7.6**	**6.5**	**7.6**	**6.5**
	nA/(mmol/L)							
PepT1a	28.13	545.6	337.82	1114	5.3	75.06	46.37	159.14
PepT1b	111	1062.78	937.52	3728	75.34	372.9	645.04	1414

[1] Transport efficiencies obtained for Gly-Gln and Gly-Sar from dose-response experiments as a ratio between I_{max} and $K_{0.5}$ values collected in voltage clamped oocytes at -60 mV and -120 mV and perfused with pH 6.5 and 7.6 solutions.

Table 3. Kinetic parameters of the inwardly directed transport of selected di/tripeptides *via* the Atlantic salmon PepT1a (Slc15a1a).[1-3]

Substrate[4]	$K_{0.5}$	I_{max}[5]	$I_{max}/K_{0.5}$	Oocytes/Batches
	mmol/L	*nA*	*nA/(mmol/L)*	*n/n*
Gly-Gln	0.076±0.004	-41.317±0.835	546	9-12/2
Gly-Sar	0.523±0.102	-39.228±4.490	75	9/3
Ala-Ala	0.024±0.005	-18.404±0.759	736	8/2
Gly-Asn	0.237±0.106	-39.486±7.099	167	9/2
Gly-Pro	0.317±0.118	-26.479±5.219	84	7/2

[1] Values are expressed as means ± SEM of n oocytes (each oocyte represents an independent observation).
[2] Kinetic parameters ($K_{0.5}$ and I_{max}) were measured in Two-Electrode Voltage Clamp (TEVC) experiments; *Xenopus laevis* oocytes were voltage clamped at -60 mV and perfused with solutions at pH 6.5.
[3] Kinetic parameters ($K_{0.5}$ and I_{max}) were calculated by least-square fit to the logistic equation (**Fig. 5**).
[4] All amino acids were L-type, except for Gly- and Sar-containing peptides, which do not have L- or D-form.
[5] I_{max} values are expressed as the percentage of Gly-Gln I_{max} in the same experiment.