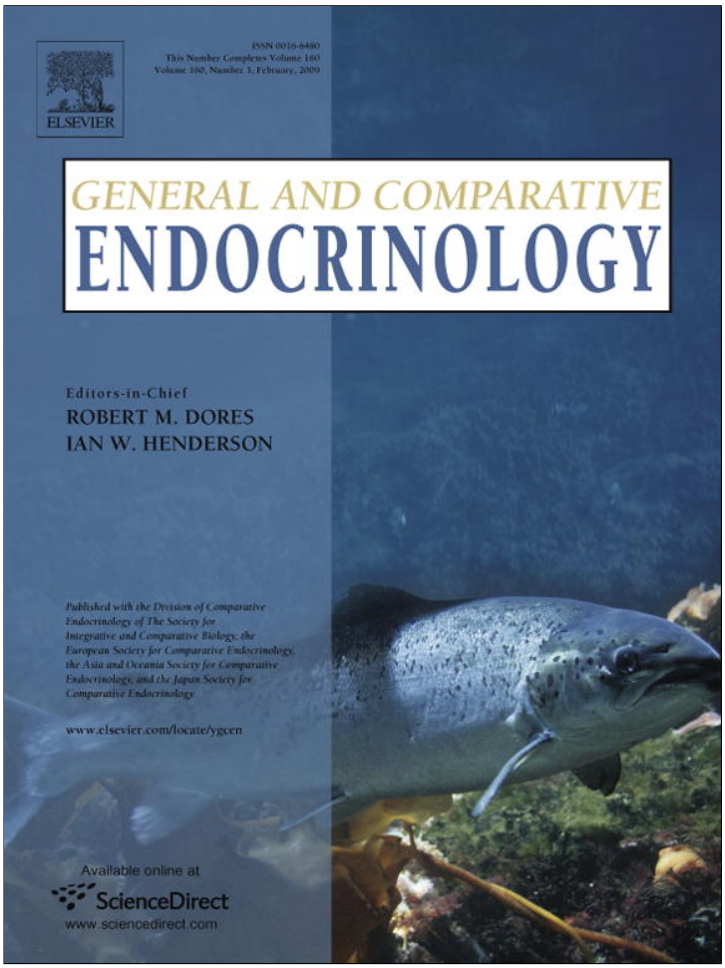

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Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (*Salmo salar*): Molecular cloning and tissue expression

Koji Murashita [a,b], Tadahide Kurokawa [a], Tom Ole Nilsen [b], Ivar Rønnestad [b,*]

[a] Tohoku National Fisheries Research Institute, Fisheries Research Agency, Shinhama 3-27-5, Shiogama, Miyagi 985-0001, Japan
[b] Department of Biology, University of Bergen, NO-5020 Bergen, Norway

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ABSTRACT

Gastrointestinal (GI) peptide hormones, ghrelin (GHRL), cholecystokinin (CCK), and peptide YY (PYY) genes were identified in Atlantic salmon, *Salmo salar*. Full-length cDNAs encoding two isoforms of GHRL (GHRL-1 and GHRL-2), two isoforms of CCK (CCK-L and CCK-N) and peptide YY (PYY) cDNA were obtained. The GHRL-1 and GHRL-2 genes encoded proteins of 111- and 108-amino acids, respectively. Both types of GHRL were mainly expressed in the stomach, but also weakly expressed in the pyloric caeca, mid-gut, adipose tissue, and testis. The CCK-L and CCK-N genes encoded preproproteins of 132- and 140-amino acids, respectively. Both types of CCK were strongly expressed in the brain and comparatively weakly expressed in other tissues, including the digestive tract. In the digestive tract, CCK-L was mainly expressed in the pyloric caeca and hind-gut, while CCK-N was only expressed in the pyloric caeca. The PYY gene encoded for 97-amino acid residues and was mainly expressed in the brain and anterior part of the intestine, including the pyloric caeca. In an experiment, we demonstrated that 6 days starvation led to, increased GHRL-1 mRNA levels in the GI tract (stomach), while there no significant changes in expression levels for the other hormones in the GI tract. This suggests an orexigenic role for GHRL-1 in Atlantic salmon. These data contribute to elucidate the functional relationships among teleost gastrointestinal peptide hormones.

1. Introduction

The gastrointestinal (GI) tract is the largest endocrine organ in vertebrates and produces around 30 different peptide hormones. These hormones act on several tissues including the GI tract itself, exocrine glands and the central nervous system (CNS) (Mendieta-Zerón et al., 2008). Almost all of the GI hormones are sensitive to gut nutrient content and some of them play an important role in control of appetite and food ingestion (Konturek et al., 2004; Murphy and Bloom, 2004).

Cholecystokinin (CCK), secreted by the proximal intestine, is one of the anorexigenic GI peptides (Raybould, 2007). The C-terminal octapeptide (CCK-8) is well conserved among vertebrates except for a single substitution of the sixth amino acid from the C-terminal position (Johnsen, 1998). The tyrosine sulfated form has the highest biological activity (Himick et al., 1993; Nielsen et al., 1998; Chandra and Liddle, 2007). The physiological role of CCK is not only in regulation of food intake and satiation but also in digestion where it has been described as playing key roles in the regulation of the intestinal phase. CCK stimulates the exocrine pancreas and discharge of bile from the gallbladder and also affects smooth muscle contraction in the jejunum and pyloric sphincter (Ramirez and Farrar, 1970; Shiratori et al., 1986). Peptide YY (PYY), which is produced in the distal intestine, is known to act as an antagonistic peptide for CCK in the control of pancreatic exocrine function (Walsh, 1994). PYY is a peptide hormone belonging to the neuropeptide Y (NPY) family that consists of 36 amino acids. Though NPY is well known to have a strong orexigenic function in the hypothalamus (Schwartz et al., 2000), PYY inhibits food intake, and reduces weight gain (Moran, 1955; Batterham et al., 2002; Challis et al., 2003; Moran et al., 2005). In contrast to CCK and PYY, ghrelin (GHRL), which is mainly expressed in the stomach, is the only orexigenic GI peptide that has been isolated to date. While the orexigenic function of GHRL has been well described in mammals, it is still uncertain whether it plays a similar role in teleosts (Jönsson et al., 2007). GHRL was first identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) in rat and human (Kojima et al., 1999). The first four amino acid residues (GSSF) with the *n*-octanoyl modification in the third serine residue in mature GHRL are considered to be the active core of the biological activity of GHRL (Bednarek et al., 2000).

We report here the cloning of full-length cDNAs that encode for the GHRL, CCK and PYY of Atlantic salmon (*Salmo salar*). Atlantic salmon is one of the most important aquaculture species in cold-water regions, and has been targeted for studies of physiology of

* Corresponding author. Fax: +47 55 58 96 67.
E-mail address: ivar.ronnestad@bio.uib.no (I. Rønnestad).

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growth, digestion, and energy homeostasis. While there is a high level of understanding of how GHRL, CCK and PYY affect these processes in mammals, knowledge of their roles in teleosts is still very fragmentary. The basic information regarding GI peptides in Atlantic salmon contributes to the elucidation of the physiological relationships of fish gut peptide hormones.

There is a growing use of vegetable ingredients in aquafeeds due to limitations in the availability of available marine protein sources. One of the main problems that arise when fish are fed high levels of plant proteins is reduced growth. This has been partially explained by a reduced appetite and lower voluntary feed intake level. For this reason, it is important to elucidate the physiological mechanisms involved in the regulation of appetite, feed intake, and satiety in important aquaculture species like Atlantic salmon.

2. Materials and methods

2.1. Animals and samples

For cDNA cloning and analysis of tissue distribution, Atlantic salmon (*S. salar*), average body weight 44.7 g, were reared at the Bergen High-Technology Centre (Bergen, Norway) in indoor tanks supplied with a continuous flow of fresh water at 8 °C. The fish were fed a commercial pellet diet (EWOS innovation, Bergen, Norway). At sampling four males and four females were killed with an overdose of MS-222, and tissues were collected and stored in RNAlater (Ambion, Austin, TX, USA) at −20 °C until RNA isolations were performed.

The spatial distribution of the hormones in the GI tract was characterized on a male Atlantic salmon (body weight: 975 g) reared under commercial conditions in a sea cage (EWOS innovation, Lønningdal, Norway). The fish was killed by a blow to the head and the digestive tract was rapidly removed and dissected into 10 sections for regional analysis of the mRNA expression for each gene in each segment. Three segments were from the stomach and pyloric caeca, three from the mid-gut and three from the hind-gut. The dissected gut segments were immediately immersed in liquid nitrogen and stored at −80 °C until RNA isolations were performed.

To evaluate the effect of the starvation, fed, and starved group of Atlantic salmon (average body weight 44.3 g) was sampled at the Bergen High-Technology Centre. Five fish (fed control) were sampled from a feeding tank with continuous feeding using a feeder machine. Subsequently, the feeder machine was stopped for 6 days, and then five fish were sampled as a starved group. The fish were killed with an overdose of MS-222, and tissues of whole brain, stomach, and pyloric caeca were collected and stored in RNAlater at −20 °C until RNA isolations were performed.

2.2. Cloning of salmon GHRL, CCK-L, CCK-N, and PYY

Total RNA was isolated from whole brain, stomach and pyloric caeca using TRI reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. The isolated total RNA was treated with DNase using Turbo DNase (Ambion). First-strand cDNA of whole brain, stomach, and pyloric caeca with 3′- or 5′-adaptors added was synthesized using SMART RACE cDNA Construction Kit (BD Biosciences Clontech, Palo Alto, CA, USA) for rapid amplification of cDNA ends (RACE) PCR. In order to obtain the full-length salmon GHRL, CCK-L, CCK-N, and PYY sequences, 3′- and 5′-RACE PCR were performed. The adaptor-supplemented cDNA from stomach, brain, and pyloric caeca was used as a template DNA for GHRL, CCKs and PYY RACE PCR, respectively. The RACE PCR products were obtained from single PCR for 5′-RACE of GHRL, 3′-RACE of CCK-L, 3′- and 5′-RACE of CCK-N and from nested RACE PCRs for 3′-RACE of GHRL, 5′-RACE of CCK-L, 3′- and 5′-RACE of PYY. Primers for salmon GHRL were designed on the basis of the sequence of rainbow trout, *Oncorhynchus mykiss* GHRL-1 available from GenBank Accession No. AB096919 (3′-RACE outer, Ss GHRL Fw1; 3′-RACE inner, Ss GHRL Fw2; 5′-RACE, Ss GHRL Rv1, Table 1). Primers for salmon CCK-L, CCK-N, and PYY were designed on the salmon EST data which similar to trout CCK-L, trout CCK-N, and teleost PYY sequences (EST Accession Nos. CCK-L, DW531819; CCK-N, DY738277; PYY, DY7-16773) (3′-RACE for CCK-L, Ss CCK-L Fw1; 5′-RACE outer for CCK-L, Ss CCK-L Rv1; 5′-RACE inner for CCK-L, Ss CCK-L Rv2; 3′-RACE for CCK-N, Ss CCK-N Fw1; 5′-RACE for CCK-N, Ss CCK-N Rv1; 3′-RACE outer for PYY, Ss PYY Fw1; 3′-RACE inner for PYY, Ss PYY Fw2; 5′-RACE outer for PYY, Ss PYY Rv1; 5′-RACE inner for PYY, Ss PYY Rv2, Table 1). The RACE products were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pCR 4-TOPO vector (Invitrogen). The inserts were sequenced at the University of Bergen Sequencing Facility (Bergen, Norway). From the RACE PCR for GHRL, two different isoform sequences were obtained; these were named GHRL-1 and GHRL-2 on the basis of their sequential similarity to rainbow trout GHRL-1 and GHRL-2, respectively. Furthermore, the two types of salmon CCK sequences obtained were named CCK-L and CCK-N, due to their sequential similarity to rainbow trout CCK-L and CCK-N.

2.3. Structural analysis

Multiple sequence alignments were generated using the GENETYX program (GENETYX Co., Tokyo, Japan). The cleavage site of the signal peptide was estimated using the SignalP Ver. 3.0 program (http://www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree based on the amino acid sequences was constructed by the neighbor-joining method of the Clustal W (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) (Thompson et al., 1994) and MEGA 3.1 program (http://www.megasoftware.net/index.html) (Kumar et al., 2004). The *Tyr* sulfation site in deduced CCK amino acid sequences was predicted by the SulfoSite program (http://sulfosite.mbc.nctu.edu.tw/index.php). Secondary and tertiary protein structures of salmon PYY were estimated using the SWISS-MODEL automated protein modeling server (http://swissmodel.expasy.org//SWISS-MODEL.html) (Schwede et al., 2003), based on the human PYY Protein Data Bank (PDB) structure file 2dez.pdb, in order to compare structural similarities of human and salmon PYY proteins.

2.4. Tissue distributions of GHRL-1, GHRL-2, CCK-L, CCK-N, and PYY

The DNase-treated total RNA of the whole brain, pituitary, eye, gill, liver, stomach, pyloric caeca, mid-gut, heart, kidney, intraperitoneal adipose tissue, belly flap, skin, white muscle, red muscle, and gonad was prepared from four males and four females fish as described above (Section 2.2), and first-strand cDNA was synthesized from the total RNA using oligo (dT) primer with a reverse transcription kit (Invitrogen, Carlsbad, CA, USA). The tissue distributions of the mRNA of salmon GHRL-1, GHRL-2, CCK-L, CCK-N, and PYY were analyzed by real-time quantitative RT-PCR (qPCR) using SYBR Green assays (Chromo 4 System, Bio-Rad Laboratories Inc., CA, USA) according to the manufacturer's instructions. Primers set for the qPCR of GHRL-1, GHRL-2, CCK-L, CCK-N, and PYY were designed in the nucleotide sequence obtained (primer set for GHRL-1, Ss GHRL Fw3, and Ss GHRL Rv2; primer set for GHRL-2, Ss GHRL Fw4 and Ss GHRL Rv2; primer set for CCK-L, Ss CCK-L Fw2, and Ss CCK-L Rv3; primer set for CCK-N, Ss CCK-N Fw2, and Ss CCK-N Rv3; primer set for PYY, Ss PYY Fw3, and Ss PYY Rv3, Table 1). The PCR parameters were 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Atlantic salmon elongation factor 1α (EF1α; GenBank Accession No. AF321836) was also amplified as an internal standard (primer set for EF1α, Ss EF1α Fw1, and Ss EF1α Rv1, Table 1). The sequences of PCR products from the each primer set were confirmed.

Table 1
Primers designed for cloning and expression analysis in the present study.

Name	Sequence (5′–3′)	Use
Ss GHRL Fw1	CTGGCTCTGTGGGCCAAGTCAGTCAGTGC	GHRL 3′-RACE outer
Ss GHRL Fw2	GGCTCCAGCTTCCTCAGCCCCTCCCAG	GHRL 3′-RACE inner
Ss GHRL Fw3	CCAGAAACCACAGGTAAGACAGGGTA	GHRL-1 qPCR and RT-PCR
Ss GHRL Fw4	GCCCCTCCCAGAAACCACAGGGTAAA	GHRL-2 qPCR and RT-PCR
Ss GHRL Rv1	ATCATTCTGCAGTGGCAGTGTCTCCCAG	GHRL 5′-RACE
Ss GHRL Rv2	CTCCTGAAACTCCTCCTCACTCATGG	GHRL-1/-2 qPCR and RT-PCR
Ss CCK-L Fw1	GCCCGTCGCACTCGCAGGATGAGGACAAG	CCK-L 3′-RACE and RT-PCR
Ss CCK-L Fw2	GCGCGAACTACTGGCAAGATTGATA	CCK-L qPCR
Ss CCK-L Rv1	ACGAGCTCCTCTGGAATGATCCTTTCCTGG	CCK-L 5′-RACE outer
Ss CCK-L Rv2	TAGTTCGCGCAGACTGGTGCGGGGGTCC	CCK-L 5′-RACE inner
Ss CCK-L Rv3	TGTCCTTTATCTTGTGGCTGGGACCCG	CCK-L qPCR and RT-PCR
Ss CCK-N Fw1	GGACGACCCCAGTCCTCTCCCCCCCTCC	CCK-N 3′-RACE
Ss CCK-N Fw2	CCTCTGAAGCACGTCTTGAAGCCTAC	CCK-N qPCR
Ss CCK-N Fw3	CCCCCTTCCTCTGAAGCACGTCTTGAAG	CCK-N RT-PCR
Ss CCK-N Rv1	GTCTCCATCTTCTTCTGCCGTATATGGGACG	CCK-N 5′-RACE
Ss CCK-N Rv2	GAGATGAGTCTAGCCAACAGTTCACTGA	CCK-N qPCR
Ss CCK-N Rv3	CTCTTATCGCGGGACAGTGACTGAG	CCK-N RT-PCR
Ss PYY Fw1	GACACAGACACCCGTTGTCTTAACCACCTG	PYY 3′-RACE outer and RT-PCR
Ss PYY Fw2	TACGGTAAGAGGTCCGCCCAGGAGGGAG	PYY 3′-RACE inner
Ss PYY Fw3	ACTACACCGCGCTCAGACACTACATC	PYY qPCR
Ss PYY Rv1	GGAGAGGCGGTGGTGGGAGGCACTGGTCGG	PYY 5′-RACE outer and RT-PCR
Ss PYY Rv2	CCAGGTATTGGTGGACACCTGGGGAGAATAC	PYY 5′-RACE inner
Ss PYY Rv3	TCTCTGGTCTCTCTCTGCATTGTTGCCG	PYY qPCR
SsEF1aFw1	GAGAACCATTGAGAAGTTCGAGAAG	EFIaqPCRand RT-PCR
SsEF1aRv1	GCACCCAGGCATACTTGAAAG	EFIaqPCR
SsEF1aRv2	TGTACCTGTGGGCCGTGTG	RT-PCR

For regional analysis of the intestinal tract, RT-PCR was carried out. The total RNA was extracted and first-strand cDNA was synthesized from 10 intestinal segments as described above (see Fig. 8 and Section 2.1). PCR amplification was performed using gene-specific primers (primer set for GHRL-1, Ss GHRL Fw3, and Ss GHRL Rv2; primer set for GHRL-2, Ss GHRL Fw4, and Ss GHRL Rv2; primer set for CCK-L, Ss CCK-L Fw1, and Ss CCK-L Rv3; primer set for CCK-N, Ss CCK-N Fw3, and Ss CCK-N Rv4; primer set for PYY, Ss PYY Fw1, and Ss PYY Rv1, primer set for EF1α, Ss EF1α Fw1, and Ss EF1α Rv2, Table 1). The PCR parameters were 32 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR products were subjected to agarose gel electrophoresis.

2.5. Effect of starvation on the GHRL-1, GHRL-2, CCK-L, CCK-N, and PYY

The first-strand cDNA of the whole brain, stomach, and pyloric caeca was prepared from fed and starved fish (see the Section 2.1) as described in Section 2.4. The GHRL-1, GHRL-2, CCK-L, CCK-N, and PYY gene expression levels were analyzed by qPCR as described in Section 2.4. The cDNA of stomach was used for GHRL-1 and GHRL-2 analysis, and the cDNA of pyloric caeca and brain was used for CCK-L, CCK-N, and PYY analysis. Results of the effect of starvation were analyzed using Student's *t*-test. Differences between groups were considered to be significant if $p < 0.05$.

3. Results

3.1. Molecular cloning and comparison of the amino acid sequences

3.1.1. GHRL

From the RACE PCR for salmon GHRL, full-length cDNA sequences of two different GHRL isoforms were obtained. Atlantic salmon prepro-GHRL-1 (GenBank Accession No. AB443431) nucleotide sequence was 470 bp in length, and contained a 57 bp of 5′-untranslated region (5′-UTR), a 77 bp of 3′-untranslated region (3′-UTR) and a 336 bp of open reading frame (ORF) encoding a prepro-GHRL-1 with 111 amino acids (Fig. 1). Atlantic salmon prepro-GHRL-2 (461 bp, GenBank Accession No. AB443432) nucleotide encoded a protein with 108 amino acid residues, which was identical to the prepro-GHRL-1 sequence except for the deletion of GTAAGACAG nucleotide, which encodes VRQ amino acid residues (Fig. 1). Both prepro-GHRL-1 and GHRL-2 contained the 26-residue putative signal peptide and 62-residue putative C-terminal peptide. The third serine residue with potential acyl modification was confirmed in active core (GSSF) mature peptide. An amidation signal (G) and typical processing signal (RR) were conserved at the C-terminus of putative mature peptide (Fig. 1). The putative mature region of salmon GHRL-1 and GHRL-2 were identical to trout GHRL-1 and GHRL-2, respectively (Fig. 2A). The 5′-end of the mature GHRL, including the active core sequences, is highly conserved among vertebrates (Fig. 2A). The first glycine residue in the C-terminal region of the mature GHRL, which relates to amidation, was well conserved in teleosts (Fig. 2A). A low similarity (33.3%) with human obestatin coding region was found in salmon prepro-GHRL (Fig. 2A). Phylogenetic analysis showed that salmon GHRL-1 and GHRL-2 were close to trout GHRL-1 and GHRL-2 (Fig. 2B). The teleostean GHRL was divided from mammalian, reptilian, avian, and amphibian GHRL with high boot-strap value.

3.1.2. CCK-L and CCK-N

Full-length cDNA sequences of Atlantic salmon CCK-L and CCK-N were obtained by RACE PCR. The salmon CCK-L cDNA (GenBank Accession No. AB443433) was 668 bp in length, and contained a 110 bp 5′-UTR, a 159 bp 3′-UTR and 373 bp ORF encoding a prepro-CCK-L with 132 amino acids (Fig. 3). The predicted prepro-CCK-L peptide contained a putative signal peptide of 20 amino acids and a 112 amino acid mature peptide that included a C-terminal octapeptide, DYLGWMDF (the sixth amino acid from the C-terminal end was L: leucine). The salmon CCK-N cDNA (GenBank Accession No. AB443434) was 799 bp in length, and contained a 62 bp 5′-UTR, a 306 bp 3′-UTR including polyadenylation signal (AATAAA) and a 421 bp ORF encoding a prepro-CCK-N with 140 amino acids (Fig. 3). The predicted prepro-CCK-N peptide contained a putative signal peptide of 20 amino acids and a 120-amino acid mature peptide including a C-terminal octapeptide, DYNGWMDF (the sixth amino acid from the C-terminal end was N: asparagine). The tyrosine residue at

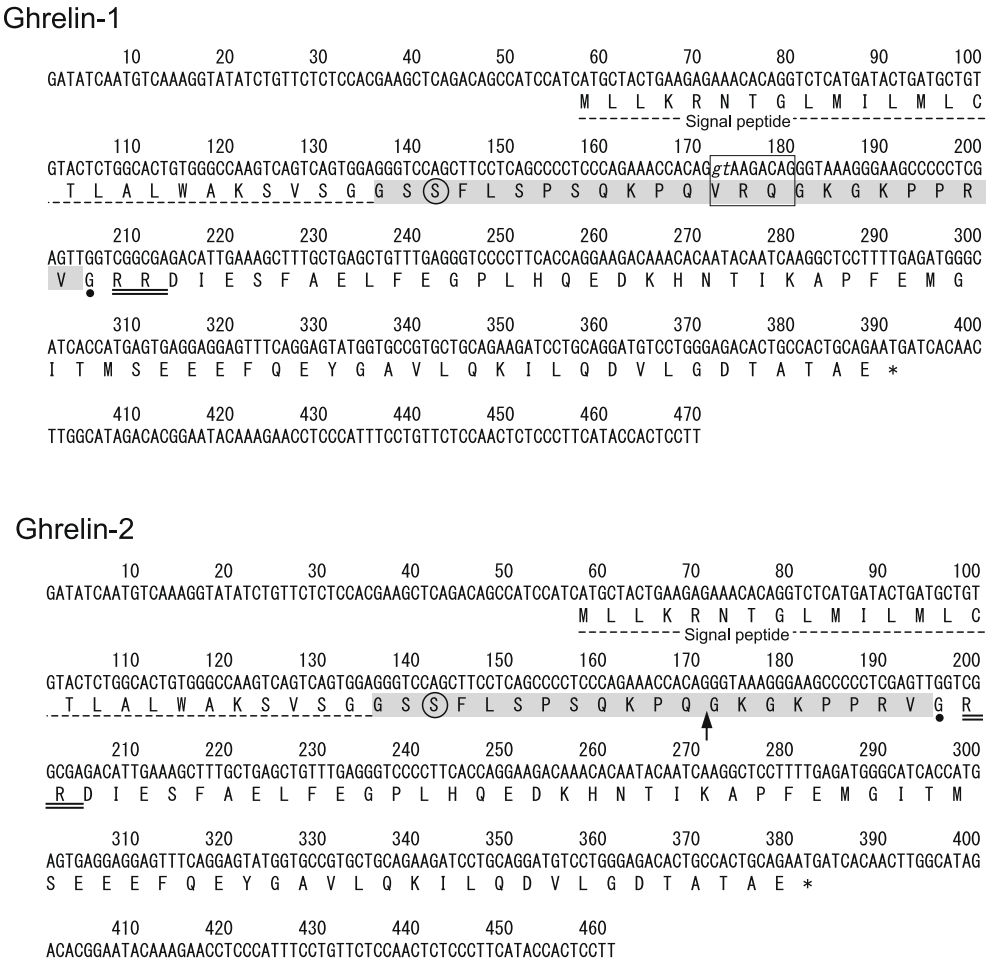

Fig. 1. Nucleotide sequence and deduced amino acid sequence of salmon GHRL-1 and GHRL-2. Amino acids coding for the mature peptide are shaded. The GHRL-1-specific sequence is boxed, and its deletion site in GHRL-2 is arrowed. Double underlining indicates the conserved processing amino acid sites (RR). The third serine residue with potential acyl modification in mature GHRL is circled. The black dot indicates putative *Gly* residues that contribute to C-terminal amide structure. The predicted intron splice donor sequence (*gt*) is indicated by small italics. The asterisk indicates the stop codon.

position seven from the carboxyl terminus with potential sulfate modification was confirmed in the octapeptide region. The processing signals (R or K) are well conserved throughout the vertebrates, except for the rat CCK-58 cleavage site (Fig. 4A). The N-terminal region of the mature CCK peptide is well conserved among vertebrates (Fig. 4A). In the phylogenetic analysis, teleost CCK was separated from the non-teleost cluster (Fig. 4B). The teleost cluster comprised two CCK branches, and the salmon CCK-L and CCK-N were grouped with the fish CCK2 and CCK1 subfamilies, respectively.

3.1.3. PYY

The Atlantic salmon PYY cDNA (GenBank Accession No. AB443435) was 605 bp in length, and contained a 131 bp 5′-UTR, a 180 bp 3′-UTR and 294 bp ORF encoding a prepro-PYY peptide with 97 amino acids (Fig. 5). The predicted prepro-PYY peptide contained a putative signal peptide of 28-amino acid and a 36-amino acid mature peptide. The three proline and two tyrosine residues (*Pro*$^{2/5/8}$ and *Tyr*$^{20/27}$), which construct a PP-fold of NPY family, were conserved in salmon PYY. A potential processing signal (KR) was found at the C-terminus of the putative mature peptide. The processing signal and mature peptide are highly conserved among vertebrates (Fig. 6A). Three-dimensional (3D) structural modeling predicts a high degree of conservation of a tertiary structure between human and salmon PYY (Fig. 6B). In the phylogenetic analysis, teleost PYY was divided from the non-teleost cluster (Fig. 6C). The teleost cluster comprised two PYY branches (PYYa and PYYb), and the salmon PYY was grouped with the fish PYYa subfamily.

3.2. Tissue distribution of salmon GHRL, CCK, and PYY

Tissue distributions of salmon GHRL (-1 and -2), CCK (-L and -N) and PYY were analyzed by qPCR. The data are expressed as average of the analyses of four males and four females, since there did not appear to be gender differences in the tissue distributions of all of these genes. GHRL-1 and GHRL-2 were mainly expressed in the stomach, but were also weakly expressed in the pyloric caeca and adipose tissue (slightly expression level was detected in the brain, mid-gut and testis) (Fig. 7). CCK-L was most strongly expressed in the brain and eye, and weakly expressed in the pituitary, gill, pyloric caeca, mid-gut, heart, skin and ovary (slightly expression level was detected in the stomach, adipose tissue, and belly flap) (Fig. 7). CCK-N was mainly expressed in the brain, and weakly expressed in the pituitary, eye, gill, pyloric caeca (slightly expression level was detected in the stomach, skin, and ovary) (Fig. 7). PYY was mainly expressed in the brain, pyloric caeca, and mid-gut, and weakly expressed in the eye, stomach, adipose tissue, testis, and ovary (slightly expressed in the pituitary, gill, and heart) (Fig. 7).

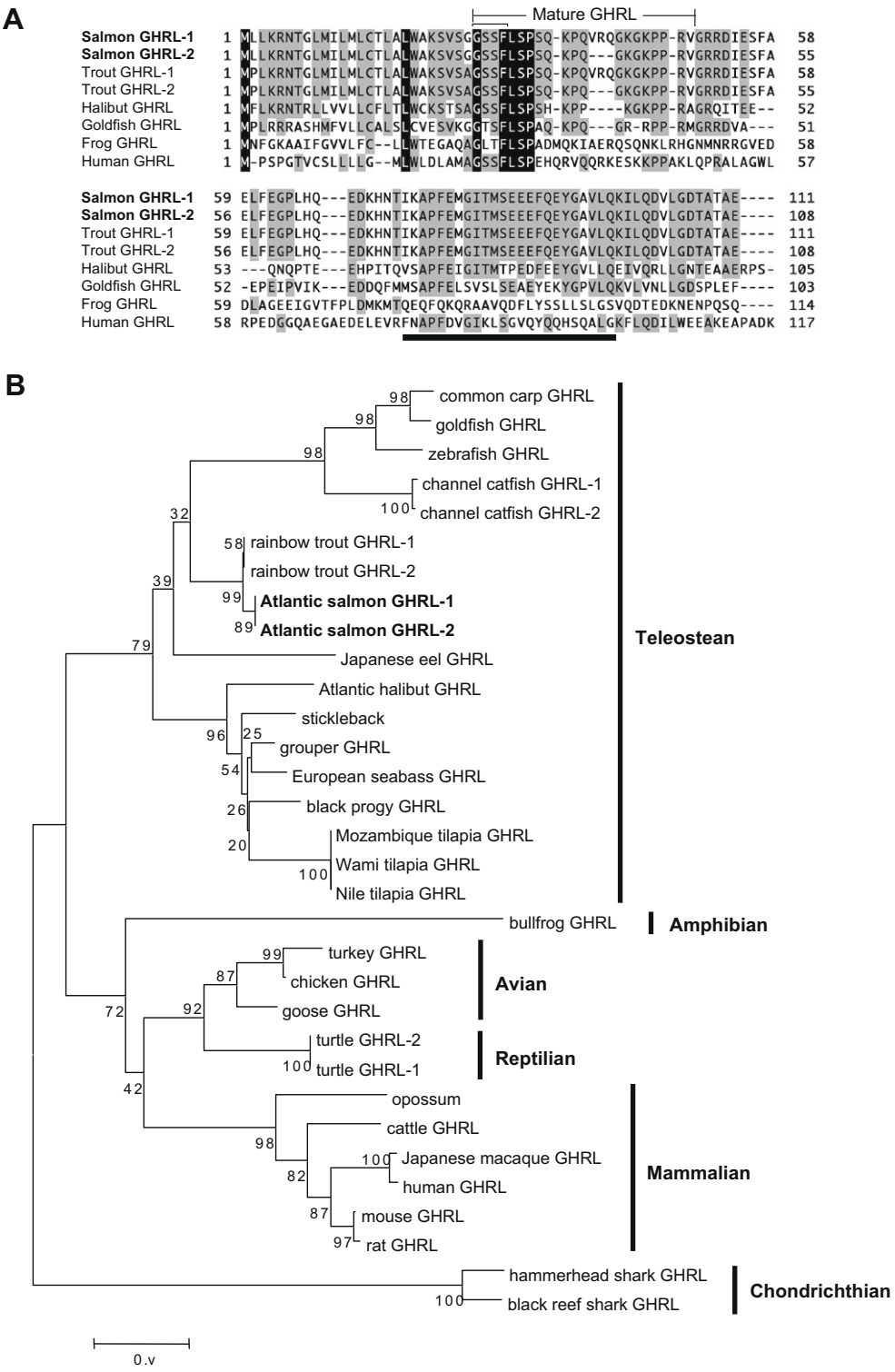

Fig. 2. Molecular characterization of salmon GHRL. (A) Alignment of the amino acid sequences of GHRL-1 and GHRL-2. The active core of mature GHRL is boxed. The region corresponding to the obestatin peptide in human is indicated by a thick line below the aligned sequences. (B) Phylogenetic analysis of GHRL amino acid sequences. The scale bar indicates the substitution rate per residue. Numbers at nodes indicate the bootstrap value, as percentages, obtained for 1000 replicates. Gene/protein IDs (GenBank Accession No. or Ensembl ID) are as follows; Mozambique tilapia (BAC55160), Nile tilapia (BAC65152), Wami tilapia (ABN13418), stickleback (ENSGACG00000000762, Ensembl Stickleback ver. 50.1 g), grouper (ABC69146), European seabass (ABG49130), black porgy (AAV65508), Atlantic halibut (ABS30388), rainbow trout (-1, BAD02979 and -2, BAD02981), Japanese eel (BAB96565), channel catfish (-1, BAE03197, and -2, BAE03198), zebrafish (NP_001077341), common carp (BAF95542), goldfish (AAN16215), turkey (AAR90091), chicken (AAP56234), turtle (-1, BAD29730 and -2, BAD29731), opossum (XP_001375677), cattle (AAK18612), Japanese macaque (BAG16757), human (AAQ89412), mouse (AAI32231), rat (BAA89370), bullfrog (BAB71718), goose (AAQ56122), hammerhead shark (BAF33104), black reef shark (BAF33105).

In addition to the expression analysis in the main body organs, regional analysis of the gene expression along the GI tract by RT-PCR was also performed. These data demonstrated that GHRL-1 and GHRL-2 were expressed in all parts of stomach (segments 1–3), but not in other parts of the intestine by 32 cycles of RT-PCR though low expression was detected by qPCR in pyloric caeca

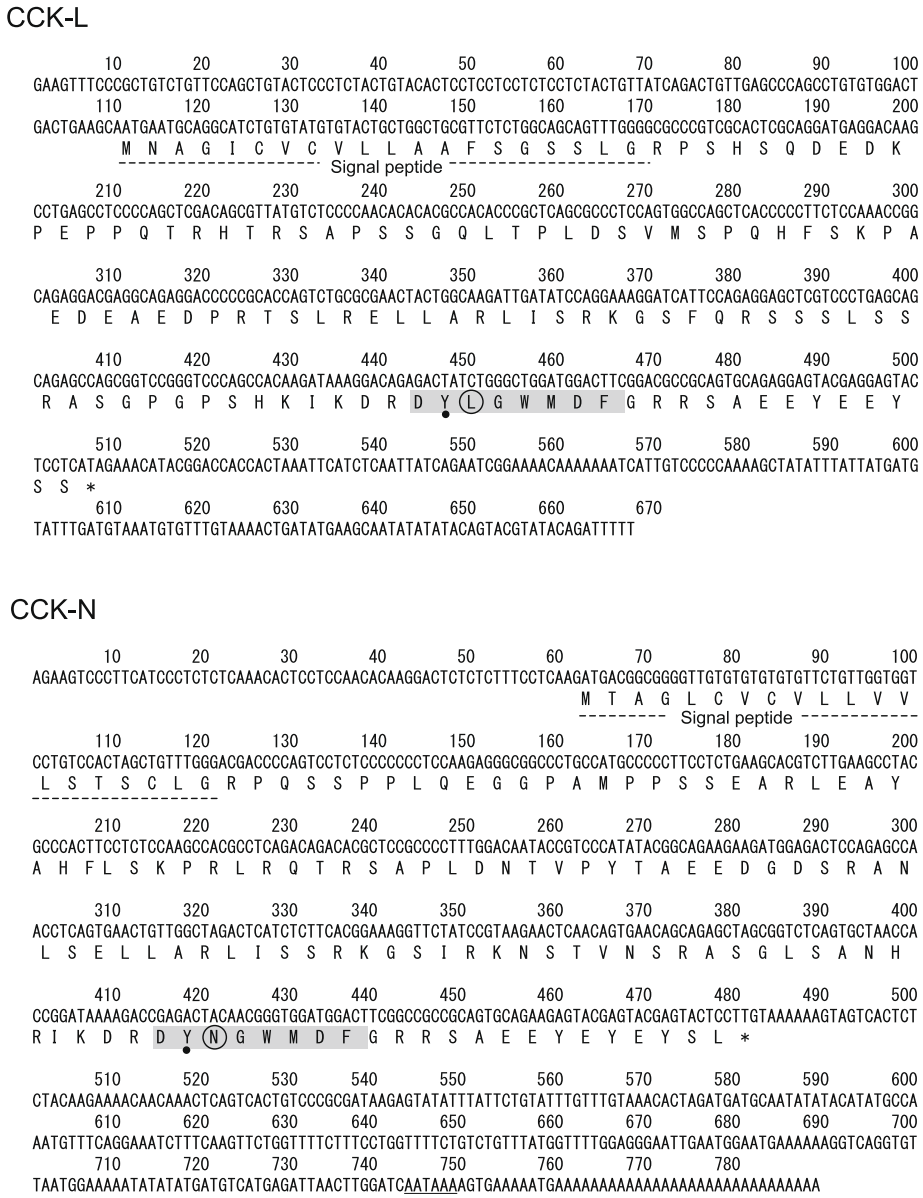

Fig. 3. Nucleotide sequence and deduced amino acid sequence of salmon CCK-L and CCK-N. Amino acids coding for the octapeptides are shaded. Single underlining indicates the polyadenylation signal (AATAAA). The substituted third amino acid residues in the octapeptides are circled. The black dot indicates *Tyr* sulfation site that predicted by the SulfoSite program (http://sulfosite.mbc.nctu.edu.tw/index.php). The asterisk indicates the stop codon.

(Fig. 8). CCK-L was found in all parts of the intestine except for the stomach (segments 1–3). The expression of CCK-L was clearly higher in the pyloric caeca (segment 4) and hind-gut (segments 8–10) compared to the middle part of the intestine (segments 5 and 6, Fig. 8). In contrast to the CCK-L, CCK-N expression was observed only in the pyloric caeca and with a weak band (Fig. 8). PYY expression was observed in all the intestinal segments (segments 4–10), but the levels of expression in the hind-gut (segments 8–10) were clearly lower than those of the pyloric caeca and mid-gut segments (segments 4–7, Fig. 8).

3.3. Effect of starvation on the GHRL-1, GHRL-2, CCK-L, CCK-N and PYY

Though the GHRL-2 gene expression levels in stomach did not change significantly, the GHRL-1 gene expression levels were increased after 6 days of starvation (Fig. 9). The brain CCK-L and CCK-N gene expression levels were decreased by starvation, but those of pyloric caeca levels did not changed significantly. The PYY expression levels did not change by starvation in both of the pyloric caeca and the brain.

4. Discussion

This study identified full-length cDNA sequences for GHRL-1, GHRL-2, CCK-L, CCK-N, and PYY in Atlantic salmon. Comparative analysis of the sequences obtained clearly indicates that they are the respective GHRL, CCK, and PYY gene homologs in Atlantic salmon. In the rainbow trout GHRL gene, an alternative splicing site has been found at the second intron, which results in the production of two types of GHRL (rtGHRL and des-VRQ-rtGHRL) (Kaiya et al., 2003). The salmon GHRL-1 and GHRL-2 were quite similar to those of trout, and the deduced mature peptide sequences were identical in both of species. Furthermore, in our preliminary data, an exon–intron boundary site was found in Atlantic salmon at the same position as the trout GHRL second intron (data not shown). These data indicate that salmon GHRL-1 and GHRL-2 are

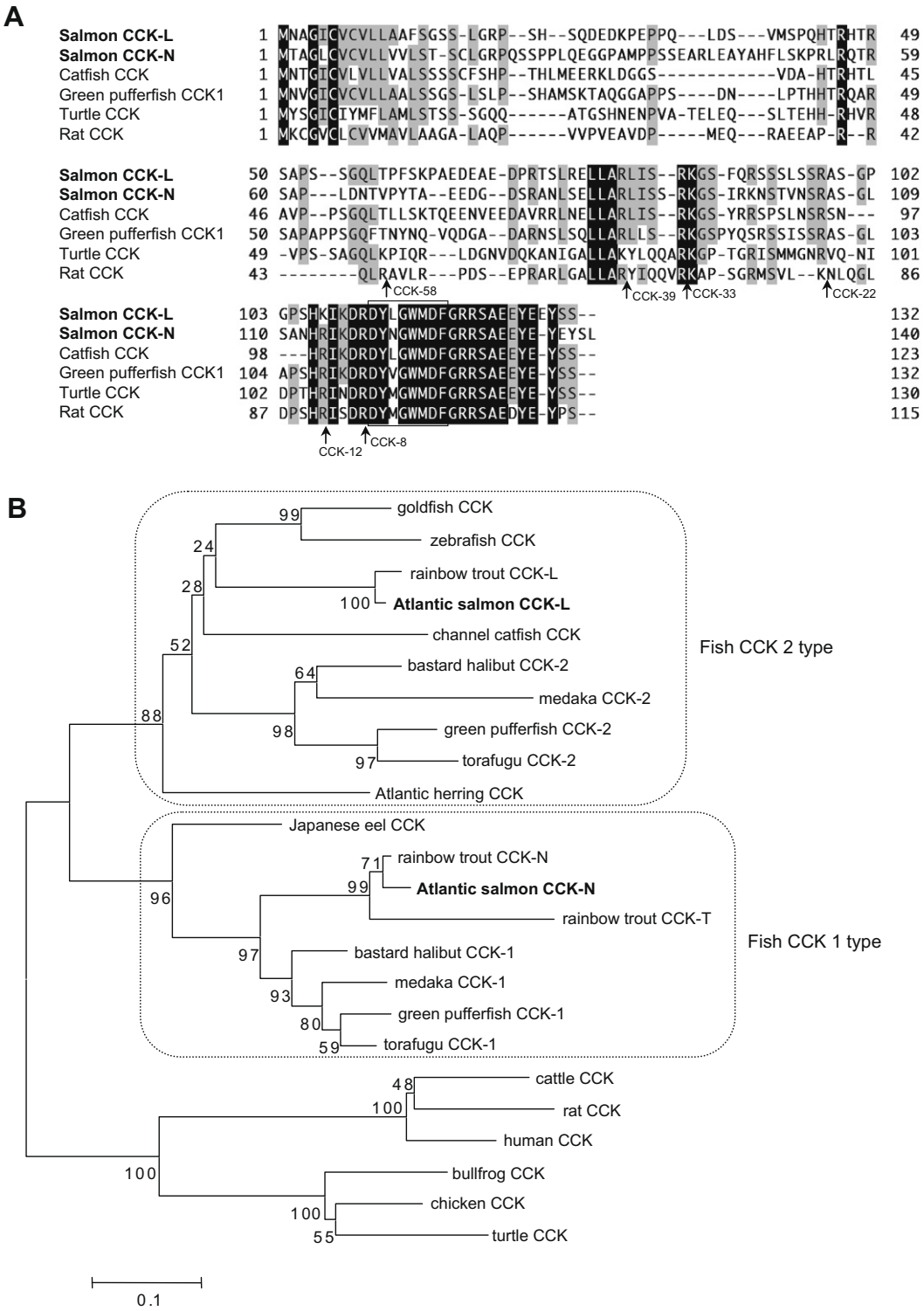

Fig. 4. Molecular characterization of salmon CCK. (A) Alignment of the amino acid sequences of CCK. The octapeptide is boxed. The rat proteolytic cleavage site is arrowed. (B) Phylogenetic analysis of CCK amino acid sequences. The scale bar indicates the substitution rate per residue. Numbers at nodes indicate the bootstrap value, as percentages, obtained for 1000 replicates. Gene/protein IDs (GenBank Accession No. or Ensembl ID) are as follows; rainbow trout (L-, CAA09907; T-, CAA09906; N-, CAA09809), bastard halibut (-1, BAA23734; -2, BAC44892), green pufferfish (-1, BAC44894; -2, BAC44895), channel catfish (BE212760), goldfish (O93464), zebrafish (XP_001346140), Atlantic herring (AAQ17201), Japanese eel (BAD01500), cattle (AAI14185), rat (NP_036961), human (AAA53094), bullfrog (CAA69676), chicken (CAB62203), turtle (CAA09334), torafugu (-1, ENSTRUP00000034252; -2, ENSTRUP00000004505, Ensembl Fugu ver. 50.4j), medaka (-1, ENSORLP00000007034 and -2, ENSORLP00000007487, Ensembl Medaka ver. 50.1f).

splicing variants of the same gene as seen in trout GHRL. Very recently, more two different salmon GHRL mRNA isoforms have been uploaded on the GenBank (*S. salar* preproghrelin-1: EU513378 and *S. salar* preproghrelin-2: EU513379), which means salmon has at least four different GHRL mRNA isoforms. So far, two different GHRL mRNA isoforms have been identified in rainbow trout (Kaiya

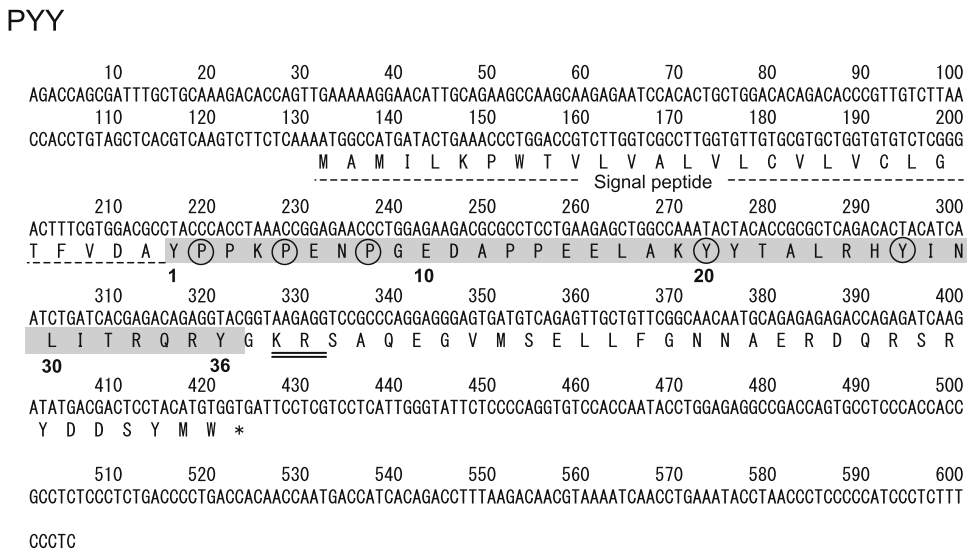

Fig. 5. Nucleotide sequence and deduced amino acid sequence of salmon PYY. Amino acids coding for the mature peptide are shaded. The three proline and two tyrosine residues (*Pro*$^{2/5/8}$ and *Tyr*$^{20/27}$), which construct a PP-fold of NPY-family, are circled. Double underlining indicates the conserved processing amino acid site (KR). The asterisk indicates the stop codon.

et al., 2003), channel catfish, *Ictalurus punctatus* (Kaiya et al., 2005) and red-eared slider turtle, *Trachemys scripta elegans* (Kaiya et al., 2004). Atlantic salmon is the first species in which more than two different GHRL mRNA isoforms have been identified. There is one substitution of amino acid residue at position 103 in the preproghrelin-1 (EU513378) compared with GHRL-1 (*Val*^{103} to *Ile*^{103}), and 3′-UTR is different to GHRL-1 and GHRL-2. The amino acid sequence for preproghrelin-2 (EU513379) is identical to GHRL-2, but the 3′-UTR was different to GHRL-1 and GHRL-2. Recently, a new hormone, obestatin, was found to be an anorexigenic peptide derived from the GHRL gene (Zhang et al., 2005; Lagaud et al., 2007), although its anorexigenic effect remains controversial (Gourcerol et al., 2007; Gourcerol and Tache, 2007). Atlantic salmon GHRL prepropeptide has a low similarity to mammalian obestatin, which compares well with previous reports in black porgy, *Acanthopagrus schlegeli* (Yeung et al., 2006) and Atlantic halibut, *Hippoglossus hippoglossus* (Manning et al., 2008). No reports exist regarding the isolation of obestatin in non-mammalian species, and further studies are required to reveal whether obestatin exists in non-mammalian species.

GHRL is primarily produced in the GI tract in all vertebrates that have been studied to date (Kaiya et al., 2008). The present study has shown that salmon GHRL-1 and GHRL-2 have the same tissue distribution pattern. It is interesting to note that GHRL was not only expressed in the GI tract (stomach, pyloric caeca and midgut) but also in the adipose tissue, although at low levels of expression. Atlantic salmon is the first species in which expression of GHRL in adipose tissue has been demonstrated, although its function in adipose tissue is unknown.

Recently, the CCK gene has been identified in several fish species, and two types of CCK have been reported in bastard halibut, *Paralichthys olivaceus*, green pufferfish, *Tetraodon nigroviridis* and zebrafish, *Danio rerio* (Kurokawa et al., 2003). In addition to these published sequences in the aforementioned species, we also found two types of CCK gene by mining available databases for torafugu, *Takifugu rubripes*, and medaka, *Oryzias latipes*; scaffold_12 (locations 888950–890370) and scaffold_383 (locations 14518–15499) of the Ensemble Fugu database (ver. 50.4j), Chromosome 11 (locations 13551588–13553993) and Chromosome 16 (locations 11275328–11276322) of the Ensemble Medaka database (ver. 50.1f) (http://www.ensembl.org/index.html) (Fig. 4B). We have also obtained two types of CCK sequences (CCK-N and CCK-L) in Atlantic salmon by cDNA cloning, and these belong to the fish CCK-1 and CCK-2 subfamily in phylogenetic analysis. However, in rainbow trout, three types of CCK (CCK-L, CCK-N, and CCK-T) have been identified (Jensen et al., 2001). Another type of CCK (-T) may therefore possibly exist in Atlantic salmon, since it belongs to the salmonidae, like rainbow trout, and also since both CCK-L and CCK-N were highly conserved between these species.

In mammals, six different lengths of CCK peptides have been isolated (CCK-58, -39, -33, -22, -12, -8), and it is agreed that these peptides are generated by sequential proteolytic cleavage at arginine or lysine (Beinfeld, 2003; Chandra and Liddle, 2007). In fish, such analyses have only been performed in rainbow trout, and these studies have isolated three different lengths of CCK peptide (CCK-21 in CCK-L, CCK-8 in CCK-L/-N/-T, and CCK-7 in CCK-L). A sulfate modification was observed on the tyrosine residue at position seven from the carboxyl terminus in rainbow trout CCK. It is known that sulfation increases the activity of CCK in the gastrointestinal tract by a factor of about 100 (Chandra and Liddle, 2007). The SulfoSite program predicted a potential tyrosine sulfation in Atlantic salmon CCK-L and CCK-N on the same position as trout CCKs. Moreover, processing signals (R or K) were found in salmon CCK-L and CCK-N at almost the same positions as the rat cleavage sites, with the exception of the CCK-58 site, which was not found in salmon. These observations suggest that salmon also has a different length of sulfated CCKs than that found in mammals and trout.

CCK is distributed in both the brain and GI tract in vertebrates (Johnsen, 1998), including fish (Kurokawa et al., 2003; Rojas-García and Rønnestad, 2002). Analysis of tissue distribution by qPCR demonstrated that salmon CCK-L and -N were mainly expressed in the brain, and brain CCK mRNA levels were markedly higher than those of the GI tract. The high abundance of CCK in the brain is also observed in yellowtail, *Seriola quinqueradiata* (Murashita et al., 2006) and rainbow trout (CCK-N and CCK-T) (Jensen et al., 2001). A high level of mRNA expression of CCK was found in the eye as well as the brain. Expression of CCK in the eye has previously been reported in mammals, and is probably found throughout the sensory system (Troger et al., 2007). In fish, regional analysis of CCK expression in the intestinal tract has been much studied, although most studies refer to the larval and postlarval stages, where the aim has been to

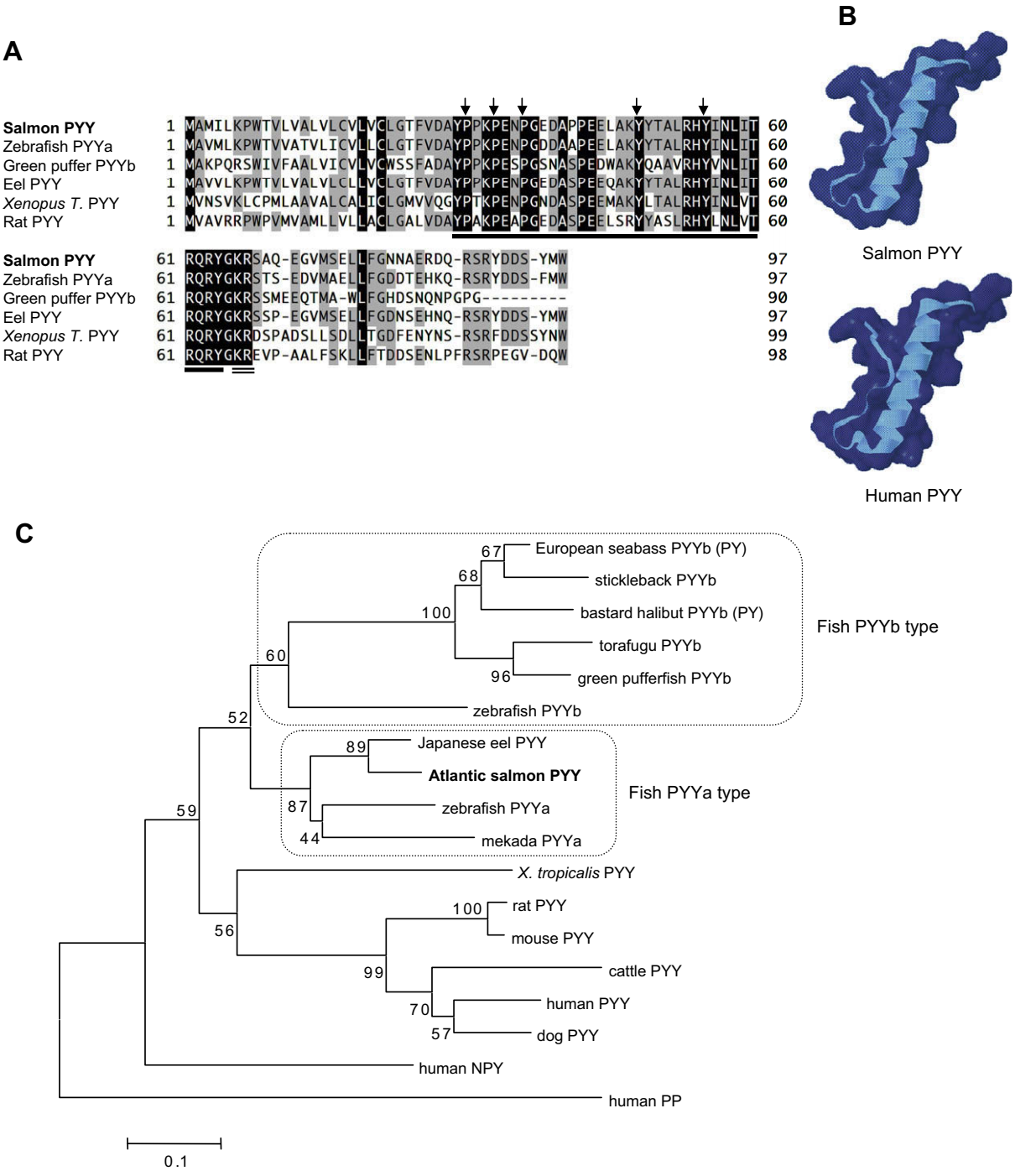

Fig. 6. Molecular characterization of salmon PYY. (A) Alignment of the amino acid sequences of CCK. Heavy underlining indicates the mature PYY. Double underlining indicates sites of conserved processing amino acid (KR). The three proline and two tyrosine residues, which construct a PP-fold of NPY-family, are arrowed. (B) Secondary and tertiary protein structures were modeled using the SWISS-MODEL automated protein modeling server based on human PYY Protein Data Bank structure file 2dez.pdb. (C) Phylogenetic analysis of PYY amino acid sequences. Scale bar indicates the substitution rate per residue. Numbers at nodes indicate the bootstrap value, as percentages, obtained for 1000 replicates. Gene/protein IDs (GenBank Accession No., Ensembl ID or gene location) are as follows; European seabass (PYYb, AJ005380), stickleback (PYYb, ENSGACP00000013144, Ensembl Stickleback ver. 50.1 g), zebrafish (PYYb, XP_001333222; PYYa, ENSDARG00000053449, Ensembl Zebrafish ver. 50.7d), bastard halibut (PYYb, Q90WF4), torafugu (PYYb, ENSTRUP00000042076, Ensembl Fugu ver. 50.4j), green pufferfish (PYYb, SCAF14738, 4.33 Mb, Ensembl *Tetraodon* ver. 50.8), Japanese eel (PYY, BAD01501), medaka (PYYa, ENSORLP00000021809, Ensembl Medaka ver. 50.1f), *X. tropicalis* (PYY, ENSXETP00000007337, Ensembl *X. tropicalis* ver. 50.41j), Rat (PYY, BAE46747), mouse (PYY, CAM23255), cattle (PYY, AAI02124), human (PYY, CAG46926; NPY, AAA59944; PP, AAH40033), dog (PYY, XP_848846). The annotations of PYYb for European seabass and bastard halibut were renamed from PY in accordance with the suggestion of Sundström et al. (2008). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

describe the ontogeny of the digestive tract and functional capacity at onset of exogenous feeding (Kamisaka et al., 2005; Rønnestad et al., 2007). These studies have revealed that in fish with a rotated gut at first feeding, CCK is mainly expressed in the anterior intestine, while CCK-producing cells were widely distributed all over the mid-gut in fish with straight guts (Rønnestad et al., 2007). Although not directly comparable, juvenile and adult Atlantic salmon have a coiled gut with two turns,

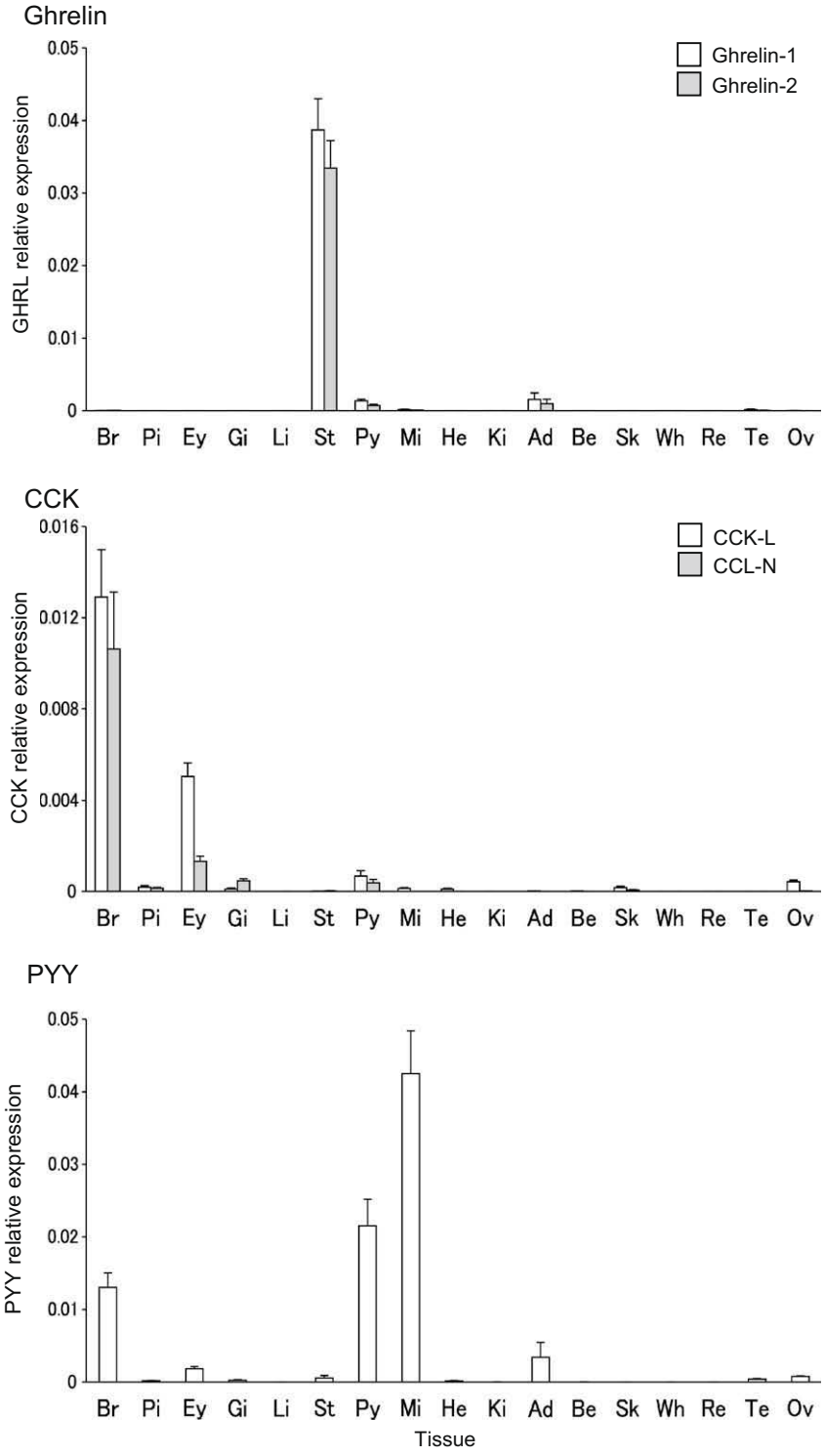

Fig. 7. Tissue distribution of GHRL (-1 and -2), CCK (-L and -N) and PYY mRNA in Atlantic salmon using qPCR and normalized against EF1α as an internal standard. The results were expressed as relative levels of expression (copy numbers of target gene/copy numbers of EF1α gene). Error bars represent standard error of the mean (n = 7–8, Te and Ov: n = 4). Br, brain; Pi, pituitary; Ey, eye; Gi, gill; Li, liver; Py, pyloric caeca; Mi, mid-gut; He, heart; Ki, kidney; Ad, adipose tissue; Be, belly flap; Sk, skin; Wh, white muscle; Re, red muscle; Te, testis; Ov, ovary.

and it appears that CCK-N, which is mainly expressed in the pyloric caeca, compares best with the expression pattern in the GI tract as seen in larva with a rotated gut. The localization of CCK expression in the anterior mid-gut agrees well with the CCK's classical postprandial roles in feedback control of digestion, which require sensing of the composition of the intestinal chyme and optimization of the digestion process by releasing bile and pancreatic secretions, and regulation of the pyloric sphincter.

For CCK-L, there was a unique expression pattern, characterized by high expression in the pyloric caeca and hind-gut (Fig. 8). A unique CCK expression pattern was also observed in rainbow trout

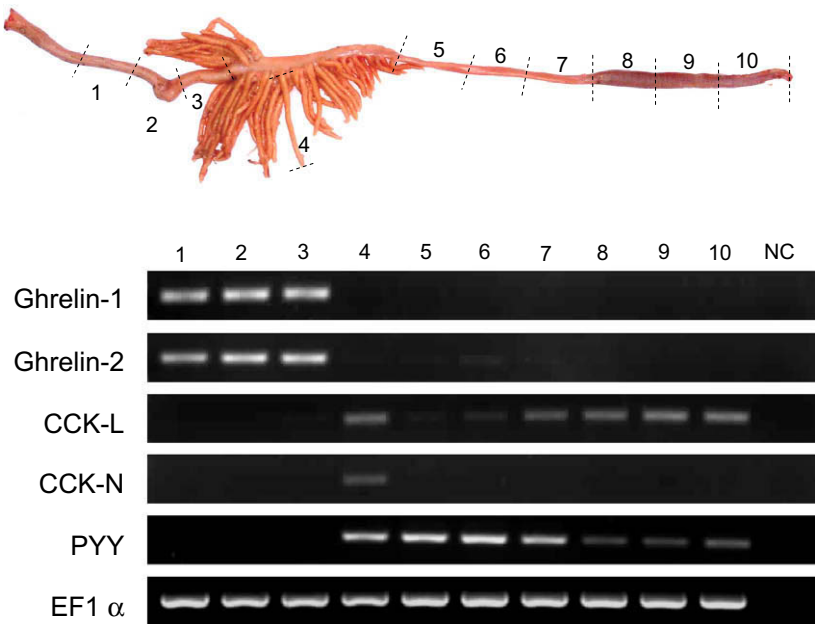

Fig. 8. Spatial distribution of Atlantic salmon GHRL (-1 and -2), CCK (-L and -N) and PYY in GI tract by RT-PCR. 1–3, stomach; 4, pyloric caeca; 5–7, three adjacent regions of mid-gut; 8–10, three adjacent regions of hind-gut, NC, negative control (no template). The EF1α fragment was also amplified to confirm the steady-state level of expression of the housekeeping gene.

CCK-L and CCK-T, but it was different from the salmon CCK-L pattern: rainbow trout CCK-L was mainly expressed in the stomach, anterior intestine and hind-gut, and trout CCK-T was mainly expressed in the pyloric caeca and middle part of the intestine (Jensen et al., 2001). Thus, there may be species-specific differences in the pattern of CCK expression in the GI tract in salmonids. It should be noted that the pattern of expression observed in these reports only includes a single sampling point and the expression of any mRNA may be dynamic and for the genes analyzed here, be influenced by a range of factors including diurnal variations, feeding status and dietary composition. However, the different patterns of expression of CCK-L and CCK-N in salmon intestine were observed in the same individual, supporting the notion of different roles in the GI tract.

An interesting feature which has recently been described in mammals is that vagal CCK receptors are involved in mediating an anti-inflammatory reflex pathway and that enteral lipids may act to stimulate this pathway (Raybould, 2007). It is possible that CCK also has this novel function in the GI tract of teleosts. This hypothesis is of particular interest in farmed aquaculture species such as Atlantic salmon, because the introduction of plant-based dietary components, which have a different composition of protein as well as lipids, has led to problems of enteritis in the digestive tract (Uran et al., 2008).

The NPY-related peptides consist of four peptides; NPY, PYY, pancreatic polypeptide (PP) and pancreatic peptide Y (PY). PP has been identified in all classes of tetrapods, and PY has been reported as a fish-specific peptide. Recently, however, Sundström et al (2008) proposed renaming "PY" as "PYYb", based on gene duplication analysis. We thus refer to PY as PYYb in this article on the basis of their suggestions (Fig. 6C). The three proline and two tyrosine residues ($Pro^{2/5/8}$ and $Tyr^{20/27}$) construct a PP-fold structure of NPY family (Cerdá-Reverter and Larhammar, 2000), and the structure is believed to be maintained in solution (Conlon, 2002). These proline and tyrosine residues are conserved in salmon PYY (Figs. 5 and 6A). The tertiary structure was also strongly conserved between human PYY and salmon PYY (Fig. 6B).

The salmon PYY was mainly expressed in the brain, pyloric caeca and mid-gut, and was present but more weakly expressed in some other tissues, including the eyes, stomach, adipose tissue and gonads (7). Some similarities exist with the expression pattern observed in torafugu PYY; torafugu PYYa and PYYb were mainly expressed in the brain and intestine (Sundström et al., 2008). Compared to the intraperitoneal adipose tissue, no PYY or (GHRL) expression was observed in belly flap which one of the most lipid rich parts of the body. There may possibly be different function between the intraperitoneal and body lipid.

In mammals, PYY is primary produced in the distal intestine and colon (Walsh, 1994). However, in yellowtail, PYYb (called PY in the article) was mainly expressed in the anterior part of the intestine (Murashita et al., 2006). In the present study, salmon PYY was mainly expressed in the pyloric caeca and the anterior part of the intestine (8). Currently available data thus all agree that PYY in fish GI tract seems to be mainly expressed in the anterior part of the intestine.

In mammals, GHRL increases food intake, and CCK/PYY decreases appetite (Cummings and Overduin, 2007). Although GHRL injection increases and CCK injection decreases food intake in goldfish (reviewed in Volkoff and Peter, 2006), GHRL, CCK, and PYY levels have not been analyzed on same samples in fish, especially on GI tract samples. Therefore, as a first step to understand the functional relationship of GHRL, CCK and PYY in fish, we examined the GHRL-1, GHRL-2, CCK-L, CCK-N, and PYY mRNA levels in fed and starved Atlantic salmon. In goldfish, 7 days starvation increased GHRL serum level and mRNA expression in the gut (Unniappan et al., 2004). In the present study, we found similar result in salmon stomach; GHRL-1 expression levels were increased by the 6 days starvation, which suggest that the GHRL might possibly have orexigenic function in Atlantic salmon as seen in goldfish. However, in rainbow trout, GHRL does not seem to stimulate appetite through a peripheral action, since intraperitoneal GHRL injection did not affect food intake and plasma GHRL levels decreased during fasting (Jönsson et al., 2007). Further studies are necessary to describe the correlation between mRNA and plasma levels of GHRL and at present the starvation response and orexigenic function of GHRL

in salmonids is still unclear. In fish, intestinal gene expression levels of CCK and PYY have been measured on the same samples only in yellowtail (Murashita et al., 2006, 2007, 2008), and the opposite patterns of CCK and PYY mRNA expression were found in fed and starved yellowtail; CCK decreased and PYYb increased by 3 days starvation (Murashita et al., 2006). However, in the present study, we could not find significant differences in intestinal expression levels of CCK or PYY between fed and 6 days starved salmon, while the starvation increased CCK-L/-N expression levels in the brain. Thus, effect of starvation on CCK and PYY in the GI tract seems to be species-specific at least after 3–6 days starvation. It should be mentioned that in this experiment, the fish had been sampled on only two time points (fed and 6 days starvation) and GHRL, CCK and PYY expression levels might be influenced by nutritional condition, diurnal variations and fasting duration. Especially, there might be big differences on response to starvation between salmonids and other teleost species since salmonids species can survive long periods of food deprivation. Therefore, to better understand the functional relationship of GI peptides, post-prandial, diurnal variations, and long-term (more than 6 days, Fig. 9) starvation/re-feeding experiments should be performed.

In conclusion, the Atlantic salmon GI tract peptides, GHRL-1, GHRL-2, CCK-L, CCK-N, and PYY have been identified and characterized with respect to their expression in various tissues. These sequences have permitted the analysis of the gene expression levels of GHRL, CCK, and PYY in Atlantic salmon. This will be a useful tool for studies of the GI-tract hormones in fish, especially for a better understanding of the complex physiological processes involved in the regulation of digestion, the GI-tract-brain pathway in the control of food intake and possibly also the role played by the GI tract hormones in inflammation.

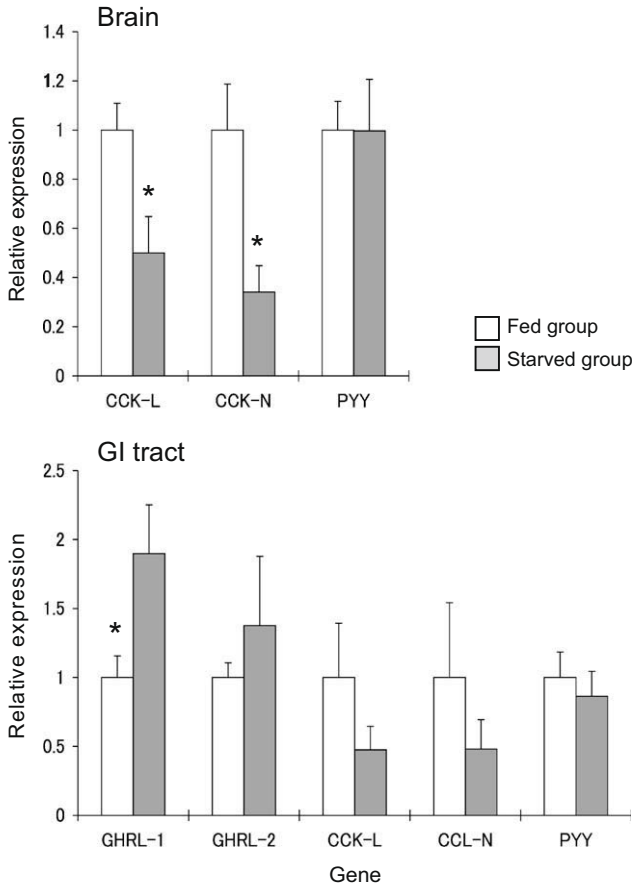

Fig. 9. Effect of 6 days of starvation on GHRL-1, GHRL-2, CCK-L, CCK-N and PYY. After standardization by EF1α, the gene expression levels were normalized as the average of fed group fish expression levels = 1. $^{*}p < 0.05$, significant differences between the groups. Error bars represent standard error of the mean ($n = 5$ fish).

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