Comparative Biochemistry and Physiology, Part A 155 (2010) 107–114

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

The effect of peptide absorption on PepT1 gene expression and digestive system hormones in rainbow trout (*Oncorhynchus mykiss*)

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ARTICLE INFO

Article history:
Received 25 August 2009
Received in revised form 12 October 2009
Accepted 14 October 2009
Available online 22 October 2009

Keywords:
Gastrin/CCK
Gene expression
Leptin
PepT1
Rainbow trout
Real time-PCR

ABSTRACT

The present study evaluates the effect of protein source (dipeptides, free amino acids, and intact protein) on development and growth of Salmonid fish alevin. Specifically, we follow the expression of oligopeptide transporter protein PepT1 in the intestine of rainbow trout (*Oncorhynchus mykiss*). Fish were fed exogenously one of four diets: three formulated (lysyl–glycine dipeptide supplemented diet — PP, free lysine and glycine supplemented diet — AA, control diet with no lysine — CON) or commercial starter (Aller Futura — AF). Fish increased mean body weight 8 fold with PP- and AA-supplemented diets resulting in significantly higher weight gain than fish fed CON. Statistical analysis revealed a significant increase in relative PepT1 expression of fish fed experimental diets. Immunohistochemical staining with PepT1 antibody showed the presence of the transporter protein in the brush border membrane of the proximal intestinal enterocytes of fish from all experimental groups. Leptin immunoreactivity occurred not only in the gastric glands but also in proximal intestine and pyloric caeca of fish fed PP, AA and AF diets. Leptin immunoreactivity was also observed in hepatocyte cytoplasm and pancreatic acinar cells. Gastrin/CCK immunoreactive cells were present in the proximal intestine and pyloric caeca.
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1. Introduction

Morphological, physiological and biochemical adaptation of the digestive system, as well as regulation of metabolic rate maximizes feeding rate and efficiency in juvenile fish (Dabrowski et al., 2003). When larval tissues exceed 50% protein content, they show high amino acid demand to cover anabolic requirement (Dabrowski, 1986). Therefore, the availability of amino acids for protein synthesis becomes critical.

An accelerated rate of amino acid absorption occurs when short peptides are transported by PepT1, present in the apical membrane of enterocytes (Daniel, 2004). Expression of this transporter protein is regulated by amino acids and peptides (Shiraga et al., 1999), as well as growth factors (Nielsen et al., 2001).

Peptide transporters are membrane proteins responsible for selective transport of dipeptides and tripeptides across the enterocyte membrane (Chen et al., 2005). One of these proteins, the PepT1, occurs in the small intestine epithelium. The PepT1 gene was described for the first time in rat (Fei et al., 1994), and 1 year later PepT1 was also sequenced in human (Liang et al., 1995). Sequences are also available for zebrafish *Danio rerio* and *Chionodraco hamatus* (Verri et al., 2003; Maffia et al., 2003) and cod (*Gadus morhua*) (Amberg et al. 2008). The PepT1 protein in higher vertebrates is consistent in length, approximately 700 amino acids, while in most lower vertebrates it is usually longer (Liang et al., 1995; Miyamoto et al., 1996; Fei et al., 2000; Rønnestad et al., 2007). In comparison to higher vertebrates both the protein and nucleotide sequence of the PepT1 transporter protein in fish are conserved (Ostaszewska et al., 2009).

Oligopeptide carrier proteins are a large family of peptide transporters (PTR) (Steiner et al., 1995). They are less studied in lower vertebrates, including fish, than in mammals. Studies of peptide transporters may provide information about their molecular structure, function, and the process of peptide transport in vertebrates. This group of proteins include PepT1 showing high expression in vertebrate intestine (Daniel, 2004; Rønnestad et al., 2007). In teleost fish, and in higher vertebrates peptide transport is strongly stimulated by the inside the negative transmembrane electric potential (Thamotharan et al., 1996; Maffia et al., 1997). Similarly seen in mammals (Daniel, 2004), the structure and function of PepT1 in the teleost fish *D. rerio* revealed the protein to be active, under conditions of low-affinity/high-capacity, but it also depends on the extracellular pH (Verri et al., 2003).

In mammals expression of PepT1 can be regulated by hormones like insulin (Meredith and Boyd, 2000), epidermal growth factor (Nielsen

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doi:10.1016/j.cbpa.2009.10.017

et al., 2001), leptin and thyroid hormone (Buyse et al., 2001; Ashida et al., 2002). Similarly in fish leptin, cholecystokinin and gastrin may have regulatory effects on the developmental regulation of intestinal transport.

The aim of the present study was to evaluate the effect of protein sources (dipeptides, free amino acids and intact protein) on growth, development and expression of PepT1 oligopeptide transporter in the intestine of rainbow trout juveniles. Additionally the location and expression of PepT1, leptin and gastrin/CCK in fish digestive tract (tissues, cells and subcellular compartments) were evaluated.

2. Materials and methods

2.1. Experimental design

Rainbow trout alevins (average body mass: 0.13 ± 0.02 g; total body length: 24.42 ± 1.67 mm) were obtained after complete yolk sac resorption from the Department of Salmonid Fish Culture of Inland Fisheries Institute in Rutki. The experiment was carried out in the Division of Ichthyobiology and Fisheries, Warsaw University of Life Sciences. The fish were stocked (50 fish/tank) into 16 tanks (four experimental groups, each in four replicates) of 20 L in volume. The recirculation system incorporated mechanical and biological filtration as well as UV irradiation. The average water temperature was 17.5 ± 1.4 °C, pH 6.9 ± 0.06, dissolved oxygen concentration 8.27 ± 0.39 mg/L, the level of NH_4^+ 0.24 ± 0.08 mg/L, and PO_4^{3-} 0.49 ± 0.5 mg/L. The fish were maintained in 12 h light:12 h dark photoperiod regime.

The fish were fed four diets of different composition: three formulated diets (Lys-Gly), dipeptide based diet (PP), free lysine and glycine based diet (AA), control diet without lysine supplement (CON), and a commercial salmonid starter Aller Futura (AF) (Aller Aqua, Poland). The formulated diets were developed and made in the Aquaculture Laboratory, Ohio State University (Table 1). The total fish biomass in each tank was evaluated weekly. The following feeding regime was applied during 12 h light: days 1–7: every hour (12 h biomass), days 8–14: every 2 h (12 h) (3% biomass), days 15–28: every 2 h (12 h) (5% biomass).

Table 1
Composition of feeds used in this experimental study.

Diet					
Lys-Gly dipeptide based diet (PP)		Free lysine and glycine based diet (AA)		Control diet (CON)	
Ingredient	% in diet	Ingredient	% in diet	Ingredient	% in diet
Fish meal	12.4	Fish meal	12.4	Fish meal	12.4
Wheat gluten[a]	37	Wheat gluten[a]	37	Wheat gluten[a]	37
Wheat	17.18	Wheat	17.18	Wheat	17.18
Fish	5	Fish	5	Fish	5
Lecithin	15	Lecithin	15	Lecithin	15
Mineral mix	3	Mineral mix	3	Mineral mix	3
Vitamin mix	4	Vitamin mix	4	Vitamin mix	4
Lysine–gycine[d]	3.4	Lysine[e]	2.1	Lysine[e]	0
Cysteine[b]	0.2	Glycine[e]	1.3	Glycine[e]	1.3
Arginine	0.65	Cysteine	0.2	Glutamate[c]	2.1
Methionine	0.33	Arginine	0.65	Cysteine	0.2
Threonine	0.29	Methionine	0.33	Arginine	0.65
Ca-mono P	1.5	Threonine	0.29	Methionine	0.33
Vitamin C	0.05	Ca-mono P	1.5	Threonine	0.29
		Vitamin C	0.05	Ca-mono P	1.5
				Vitamin C	0.05

[a] MP Biomedicals-Solon-OH.
[b] Hara (2006); most potent olfactory stimulating amino acid.
[c] Hughes (1985); non-toxic level of glutamic acid.
[d] MP Biomedicals.
[e] Bachem, NY.

2.2. Tissue collection and preparation for relative expression measurement

Intestinal samples for gene expression evaluation were collected before the first feeding (0), and then on days 7, 14, 21 and 28 of the experiment. The intestines were dissected from three fish for each experimental group ($n = 3 \times 4$). The tissue samples were washed in 1.1% NaCl, and stored at −80 °C in RNA*later* (Ambion, Austin, TX, USA), according to the producer's instruction, until RNA isolation.

2.3. Total RNA isolation and cDNA synthesis

Total RNA was isolated using a microcolumn kit, according to the recommended procedure (A&A Biotechnology, Gdynia, Poland). To eliminate contamination of RNA with DNA, the samples were digested with DNase I, according to the manufacturer's instruction (AppliChem, Darmstadt, Germany). Concentration (ng/μL) and purity ($A_{260\ nm}/A_{280\ nm}$) of the isolated RNA was measured using Nanodrop® (Thermo Scientific, Wilmington, DE, USA). Additionally, the level of RNA degradation was evaluated in 1% denaturing agarose gel (AppliChem, Darmstadt, Germany). Total RNA (1 μg) was then used for the reverse transcriptase, followed by polymerase chain reaction (RT-PCR) to synthesize cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The RT-PCR reaction was carried out according to the manufacturer's recommendation.

2.4. Quantitative real time-PCR analysis

Quantitative real time polymerase chain reaction (qRT-PCR) was carried out using Kappa® SYBR® Green QRT-PCR Master Mix Kit (Kappa Laboratories, Boston, MA, USA), in the Opticon Mini thermocycler (Bio-Rad Laboratories). The qRT-PCR was started with a preliminary matrix denaturation for 5 min at 94 °C, followed by 40 cycles of: denaturation at 95 °C for 15 s, primers binding at 60 °C for 20 s, and synthesis of qRT-PCR specific product at 72 °C for 40 s. All samples were processed in three replicates. Additionally, each plate contained control samples (without matrix). Specificity of qRT-PCR products was verified using the melting curve.

Primers for the rainbow trout PepT1 gene amplification were designed according to a sequence of 384 base pairs (bp) in length obtained from degenerated primers. The sequence was deposited in the GenBank (access no. EU853718). We surveyed β-actin (access no. AJ438158), Elongation Factor 1-α (access no. AF498320.1), and 18 S rRNA (access no. AF308735) as potential internal control genes for RT-PCR (data not shown).

The rainbow trout β-actin gene (access no. AJ438158) was used as the reference. The sequence of the primers used in the present experiment is shown in Table 2. The relative expression was calculated according to Pfaffl's method (Pfaffl, 2001). The C_T value obtained for "0" samples (collected before first feeding) was used as the reference.

2.5. Immunohistochemistry analysis for PepT1, leptin, and gastrin/CCK

Eight fish were collected from each experimental group on day 28 of the experiment for immunohistochemical analysis. Fish were anesthetized using 1:5000 MS-222 solution (pH 7.5 adjusted with $NaHCO_3$), and preserved in Bouin's solution and 4% buffered formaldehyde. The

Table 2
Sequence of the primers used for qRT-PCR.

Gene	Primer	Sequence (5′→3′)	Primers length (bp)	Product length (bp)
PepT1	PepT1 F	CCTGTCAATCAACGCTGGT	19	161
	PepT1 R	CACTGCCCATAATGAACACG	20	
β-actin	β-actin F	ACTGGGACGACATGGAGAAG	20	190
	β-actin R	GAGGCGTACAGGGACAACAC	20	

Table 3
Antibodies and dilutions used in immunohistochemical analyses.

Antibody	Origin	Dilution
PEPT1 (H-235) rabbit polyclonal antibody	Santa Cruz Biotechnology, sc-20653	1:200
Ob (A-20) rabbit polyclonal antibody (leptin)	Santa Cruz Biotechnology, sc-842	1:100
Gastrin/CCK rabbit polyclonal antibody	Novocastra, NCL-GASp	1:100

samples were embedded in Paraplast (Sigma, Poole, Dorset, UK), and cut using the microtome Leica RM2265 (Leica Microsystems, Nussloch, Germany) into 5 μm sections. The samples for PepT1, leptin and gastrin staining were washed in xylene to remove paraffin, hydrated in alcohol series, and incubated in 3% H_2O_2 to inhibit endogenous peroxidase activity. Then, the samples were immersed in Tris buffer of pH 8.0 (Sigma), and incubated with antibodies (Table 3). For leptin and gastrin/CCK detection tissue sections were incubated with Labeled Polymer for 30 min, and then with DAB (EnVision+ System HRP, DAKO Cytomation, Carpinteria, CA, USA), until the color change. The nuclei were stained with Harris hematoxylin. The samples were preserved with DPX Moutant for histology (Fluka, Milwaukee, WI, USA). The primary antibodies were incubated at appropriate dilution (Table 3), at room temperature for 1 h. A negative control was used (sections incubated without the primary antibody) for immunohistochemical staining. Then preparations for PepT1 visualization were incubated with secondary antibodies goat anti-rabbit IgG-FITC (sc-2012, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Observations were made using a Nikon Eclipse 90i microscope connected with Nikon Digital Sight DS-U1 camera (Nikon Corporation, Tokyo, Japan). Micrographs were taken using NIS-Elements AR 2.10 software (Nikon Corporation). The samples stained with fluorescein isothiocyanate (FITC) were viewed using the confocal microscope Olympus FV-1000 equipped with FV10-ASW 1.4 software (Olympus Polska, Warsaw, Poland).

2.6. Western blot PepT1 analysis

Proximal intestines were homogenized in RIPA solution (Tris pH 7.5 10 mM, NaCl 150 mM, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/mL PMSF, 0.05 mg/mL aprotinin, 5 μg/mL pepstatin A, 10 μg/mL leupeptin, 1 mM sodium orthovanadate) using the POLYTRON PT-MR 2100 apparatus (Kinematica AG, Littau, Switzerland). The obtained homogenate was centrifuged at 4 °C for 20 min at 16,000*g* (Eppendorf Centrifuge 5804R, Eppendorf, Hamburg, Germany), and supernatant separated. To measure the protein concentration Lowry's method was used (Total Protein Kit, Micro Lowry, Peterson Modification, Sigma). Equal quantities of protein (75 μg) from each sample were denatured using denaturing buffer Laemmli (Bio-Rad Laboratories) at 100 °C for 5 min. PepT1 protein homogenates were subjected to the unidirectional separation on horizontal 10% polyacrylamide gel (100 V, for 4 h) in appropriate buffer, using Mini-Protean Tetra Cell Electrophoresis Cell and Accessories (Bio-Rad Laboratories). The electrotransfer was done in the same equipment but at 100 V for 1 h at 4 °C, again in appropriate buffer. The proteins were transferred on 0.45 μm nitrocellulose with filter papers (Bio-Rad Laboratories). The membranes were washed in PBS-T solution and blocked with 5% skim milk solution. The membrane was incubated with rabbit polyclonal antibodies anti-PepT1 in 1:200 dilution (Santa Cruz Biotechnology) (Table 3) for 1 h, at room temperature. Goat anti-rabbit secondary antibodies were then applied, conjugated with alkaline phosphatase at the concentration 1:30,000 (Sigma), and incubated for 45 min at room temperature. After washing the membranes with PBS-T buffer, the detection of labeled protein was carried out by the addition of substrate for alkaline phosphatase (AP Color Development, Bio-Rad Laboratories). The membranes were archived in the Gel Doc Xr apparatus (Bio-Rad Laboratories).

2.7. Statistical analysis

Arithmetic means and standard deviations were calculated for survival, body mass and length, and relative expression for each experimental group. Significant differences among the groups were identified using one-way ANOVA (Statgraphics Plus 4.1, Statpoint Technologies, Warrenton, VA, USA), with LSD test being applied in such cases. The results are shown in graphs (Statistica 8.0, StatSoft, Tulsa, OK, USA), and tables (arithmetic means, and differences significant at $p<0.05$). The fish survival was calculated as an average of 4 replicates.

3. Results

3.1. Alevin/juvenile growth and survival

Fish fed the AF diet showed the highest survival (87.9 ± 4.2%), while the lowest (74.0 ± 4.3%) occurred in the CON group. The other

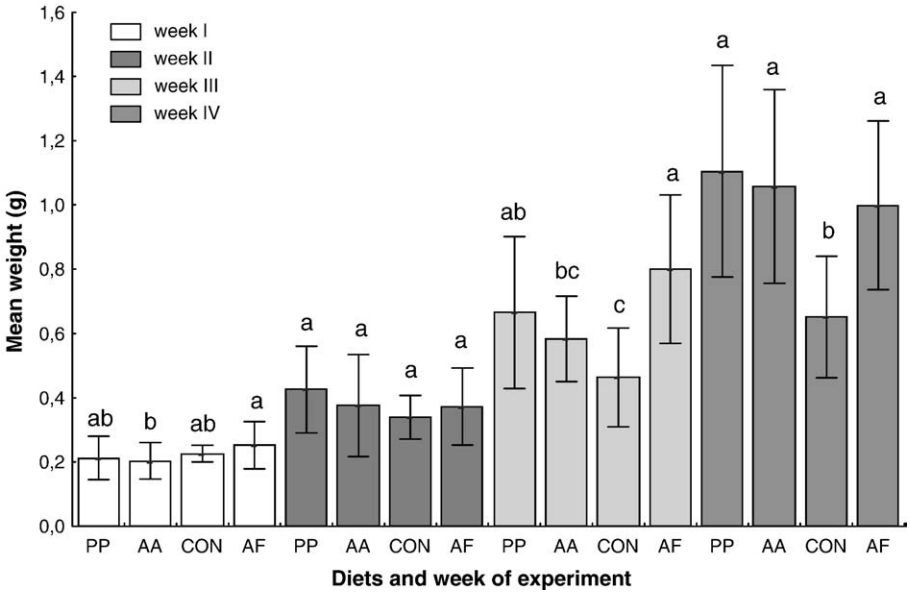


Fig. 1. Growth rate of rainbow trout fed PP, AA, CON and AF diets. Means with different letters are statistically different ($p<0.05$).

experimental groups showed survival of 82.5 ± 4.4%, and 86.0 ± 4.0%, in PP and AA respectively.

After 4 weeks of experimental feeding, fish from PP, AA, and AF groups showed relatively high mean body mass (Fig. 1), compared to those fed CON diet which were significantly smaller.

3.2. Analysis of PepT1 expression in fish intestine tissues

Similar values of C_T β-actin during the experiment indicate a proper choice of reference gene (Fig. 2). PepT1 expression levels were normalized to the expression of β-actin.

PepT1 gene expression in fish was observed already before the first feeding (PepT1 $C_T = 18.14 \pm 0.58$; C_T β-actin $= 13.37 \pm 0.49$). After the first week of experimental feeding, PepT1 expression did not differ among the dietary groups. In the second week, the expression significantly increased in the AA and CON groups compared to the PP and AF groups. The highest value of PepT1 expression over the experimental period (204.9%) occurred in fish fed CON diet, while the lowest in AF group (44.05%). In fish fed PP and AA diets, the increase in PepT1 expression was 163.55% and 102.90%, respectively (Fig. 3).

During the experimental period, a statistically significant increase in the relative PepT1 expression was observed in fish fed formulated diets PP, AA and CON. In fish fed AF, the increase in relative expression of PepT1 was low and constant over the experimental period (Fig. 3).

3.3. Spatial PepT1 visualization

The Western blotting of proximal intestine of rainbow trout showed a single protein band of ca. 80 kDa (Fig. 4) which suggests a

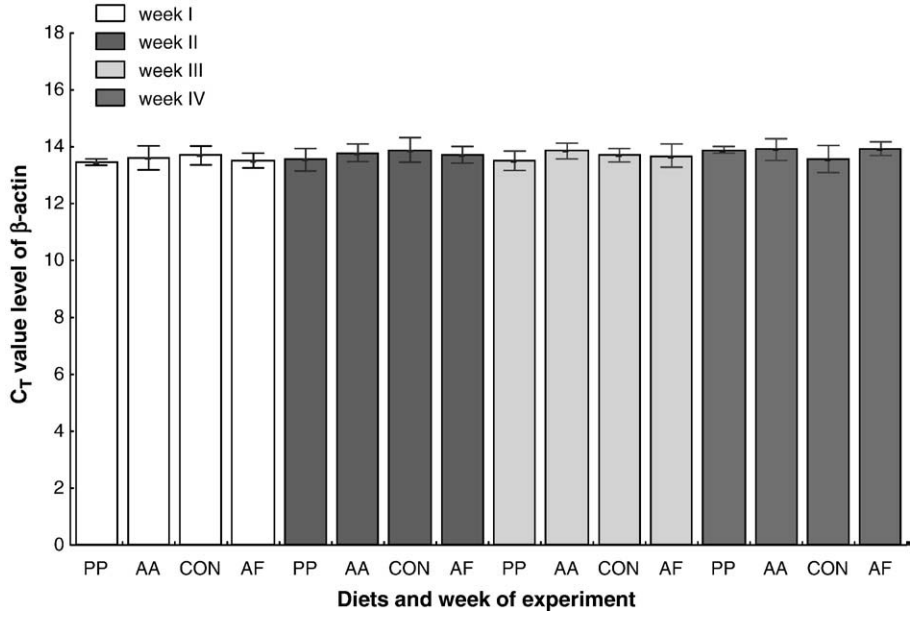


Fig. 2. The C_T values for the reference β-actin gene in the intestine of rainbow trout fed PP, AA, CON and AF diets. Mean with different letters are statistically different ($p < 0.05$).

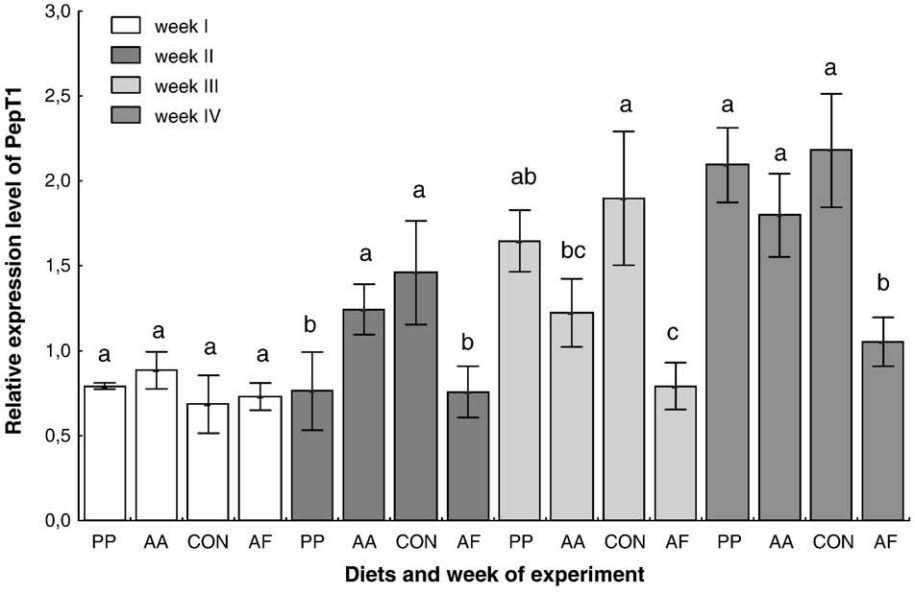


Fig. 3. The changes in relative PepT1 expression during the experiment in the intestine of rainbow trout alevins/juveniles fed diets of PP, AA, CON and AF. The results are expressed as means ± SD. Data in the same row with the same superscripted letter do not differ significantly ($p < 0.05$).

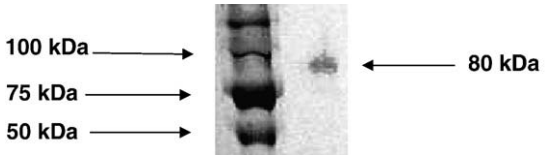


Fig. 4. Western blotting of proximal intestinal mucosa of rainbow trout showed a single protein band ca. 80 kDa protein.

cross-reaction of the commercial antibody of rabbit PepT1 with the PepT1 protein (ca. 80 kDa) of the rainbow trout.

The immunohistochemical PepT1 reaction was observed in the apical region of the proximal intestine folds of fish from all experimental groups. The brush border of the proximal intestine was most immunoreactive in the PP group (Fig. 5A), while weaker reaction occurred in the brush border of AA, CON and AF fed fish (Fig. 5B). In the posterior intestine, no PepT1 immunoreactivity was found. Only in fish fed PP and AA diets a weak PepT1 immunoreactivity was observed in the folds of basal enterocytes.

3.4. Spatial leptin visualization

In fish fed PP, AA and AF diets a strong leptin-like immunoreactivity was shown in the brush border of proximal intestine (Fig. 6A) and pyloric caeca, while in fish fed CON this activity was significantly reduced (Fig. 6B).

Additionally, in fish fed PP and AA diets leptin-like immunoreactive staining was also observed in the nuclear regions of posterior intestinal enterocytes. A strong leptin-like immunoreactivity was also detected in gastric gland and stomach epithelium of fish from PP, AA and AF groups (Fig. 6C), contrary to the CON group in which staining was much weaker (Fig. 6D).

Hepatocyte cytoplasm of fish in all dietary treatments possessed leptin-like immunoreactivity, similarly to cytoplasm of pancreatic acinar cells (Fig. 6E, F).

3.5. Spatial gastrin/CCK visualization

In the proximal intestine and pyloric caeca, the gastrin/CCK immunoreactive cells were observed in fish of all experimental groups, particularly in the pyloric region. The number of gastrin/CCK immunoreactive cells increased in the region where the caeca opens into the intestine. In fish fed PP, AA and AF diets (Fig. 7A) a much higher number of gastrin/CCK immunoreactive cells were observed comparing to the fish fed CON diet (Fig. 7B). In the posterior intestine epithelium no gastrin/CCK immunoreactivity occurred.

4. Discussion

In the present study fish survival was over 80%, except for the control (CON) group in which survival was lower (74.0 ± 4.3%). Also the body mass of fish fed PP, AA, and AF diets, were significantly higher comparing to the CON group that indicates good fish condition.

The highest relative expression of the PepT1 was observed in the last week of the experiment in fish fed CON, PP and AA diets, while in AF group it was the lowest decreased. The PepT1 expression in rainbow trout increased in AA and CON groups beginning from the second week, while in the other groups — from the third week of exogenous feeding. In cod fed zooplankton the increase of PepT1 expression also increased after the onset of exogenous feeding (Amberg et al., 2008). Rats showed the maximum PepT1 expression 3–5 days post birth, before a sharp decline was observed (Shen et al., 2001).

In rainbow trout intestine the PepT1 mRNA expression occurred before exogenous feeding commenced which was also observed in zebrafish *D. rerio* (Verri et al., 2003), cod, *G. morhua* (Amberg et al., 2008), as well as in rat *Rattus norvegicus* (Shen et al., 2001) and chicken, *Gallus gallus* (Chen et al., 2005).

In the present study, the high level of relative PepT1 expression was observed in fish fed diets containing supplements of dipeptides and free amino acids, as well as in those fed control diet deficient in essential lysine. The results obtained by various authors showed that expression and activity of the PepT1 transporter protein increased with the level of dietary protein and peptides (Shiraga et al., 1999; Chen et al., 2005). High expression of PepT1 in the rainbow trout intestine during its early development is likely to be induced by dipeptides and free amino acids. Thamotharan et al. (1998) reported that addition of Gly-Sar dipeptides to the medium of human Caco-2 cells induced a 3 fold increase of PepT1-mRNA expression. In rat, a diet enriched with Gly-Sar induced a 6 fold increase in PepT1-mRNA expression (Hindlet et al., 2007). Activity and expression of PepT1 probably depends on availability, composition and concentration of peptides, as well as the presence in the intestinal lumen of other components that may affect absorption dynamics (Gilbert et al., 2008). According to Gilbert et al. (2008), uptake of free amino acids may be indirectly regulated by PepT1 activity. Wenzel et al. (2001) proved that uptake of dipeptides stimulates amino acid absorption which may explain the increase of PepT1 expression in fish fed AA supplemented diet. These responses reinforce the conclusion of Daniel (2004) who summarized previous experiences as indicative of free amino acid inhibitory role on peptidase activity and thus helpful to bypass hydrolysis, becoming substrates for PepT1.

High level of expression of PepT1 carrier protein in the intestine of fish fed CON diet can be explained by the deficiency of lysine which is considered a primary limiting amino acid in fish and higher vertebrates. Dietary deficiency, and essential amino acid imbalance of this diet is also

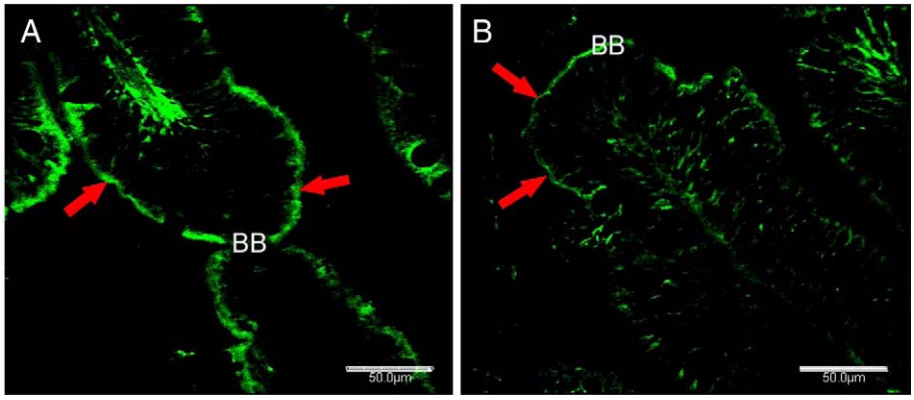


Fig. 5. Immunofluororeactivity localization of PepT1 (red arrows) in the intestinal brush border (BB). (A) Strong signal in IR brush border of proximal intestine of fish fed with a PP diet. (B) A weaker signal in the brush border of proximal intestine of fish fed with an AF diet. Bars: 50 µm.

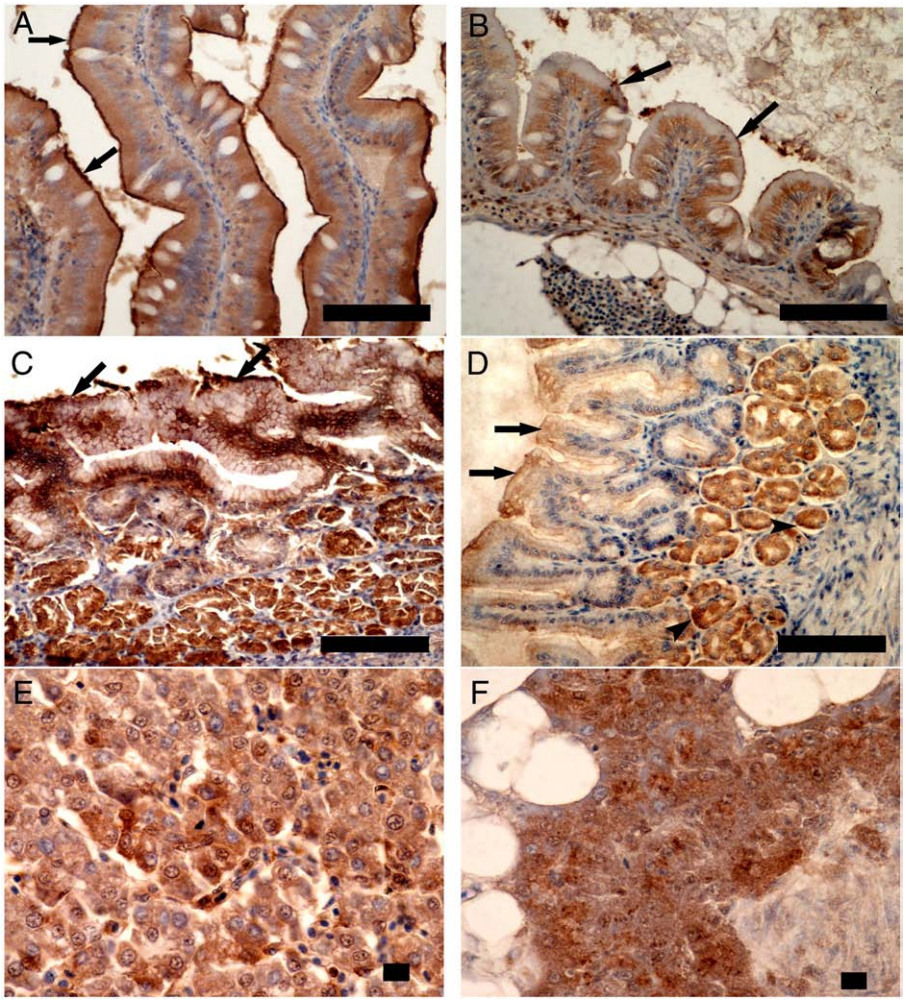


Fig. 6. Section of the intestinal fold of the proximal intestine: (A) Leptin-like immunoreactivity in the brush border of the intestine of fish fed with a PP diet (arrows). (B) Leptin-like immunoreactivity in the brush border of the intestine of fish fed with an AF diet (arrows). Section of stomach. (C) Leptin-like immunoreactivity in the cytoplasm of epithelial cells (arrows) and cytoplasm of gastric glands (arrowheads) of fish fed with a PP diet. (D) Leptin-like immunoreactivity in the cytoplasm of epithelial cells (arrows) and cytoplasm of gastric glands (arrowheads) of fish fed with a CON diet. Bars: 100 μm. (E) Section of liver. Leptin-like immunoreactivity in the cytoplasm of hepatocytes. (F) Section of pancreas. Leptin-like immunoreactivity in the cytoplasm of pancreatic acinar cells. Bars: 10 μm.

indicated by low growth rate and survival. Shiraga et al. (1999) fed rats protein-free, protein, Gly-Phe, Gly-Sar, Lys-Phe and Asp-Lys diets. Their results suggest that the up-regulation of dipeptide transport activity was caused by transcriptional activation of the PepT1 gene, due to the dietary protein (specific amino acids and dipeptides). It is known that short-term starvation and malnutrition cause up-regulation of the PepT1 transporter (Ogihara et al., 1999; Ihara et al., 2000). Unbalanced diet or even short-term starvation induced pathological changes in the intestine (Ostaszewska et al., 2006). In the present study morphological changes in the intestine of fish fed CON diet such as shortening of intestinal folds indicating malnutrition (Ostaszewska et al., 2009). The increase in PepT1 expression in fish fed CON diet might have been a compensatory reaction to the reduced intestine absorptive surface. During starvation or nutritional shortage the need for absorption of endogenous amino acids and peptides increases, which in turn increase the expression of ASCT2, EAAC1, NBAT and PepT1 transporters (Howard

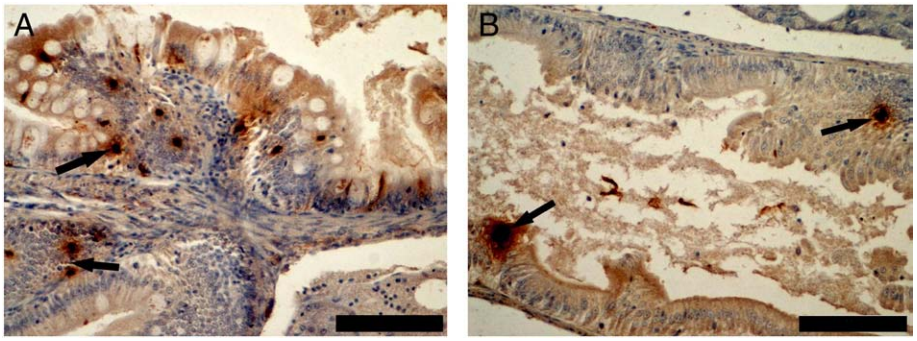


Fig. 7. Sections of the region where pyloric caeca open into the intestine. (A) Numerous endocrine cells immunoreactive to the gastrin/CCK antibody (arrows) in fish fed PP diet. (B) Low numbers of endocrine cells immunoreactive to the gastrin/CCK antibody (arrows) in fish fed CON diet. Bars: 100 μm.

et al., 2004). Howard et al. (2004) observed elevated expression of carrier proteins in the ileum of rats with no oral food intake, and their nutritional status was maintained with total parenteral nutrition.

Cloning of the mammalian PepT1 for the first time (Fei et al., 1994) was followed by immunocytochemical detection of the PepT1 protein in small intestine (Ogihara et al., 1996, 1999). The brush border of proximal intestine mucosal folds was strongly labeled for PepT1 in PP fed fish, while in other groups a weaker reaction occurred. The results of immunohistochemical staining for PepT1 were closely related to the results of gene expression obtained from qRT-PCR. The PepT1 mRNA expression was also observed in the proximal intestine of four day old zebrafish larvae, rats and chicken (Verri et al., 2003; Shen et al., 2001; Chen et al., 2005). Expression of PepT1 in cod larvae was observed in the entire digestive system, except for esophagus and sphincter (Amberg et al., 2008).

The posterior intestine of rainbow trout alevins/juveniles showed no PepT1 expression. In juvenile cod lower PepT1 expression was observed in the posterior intestine compared to the anterior (Rønnestad et al., 2007).

The distribution and expression of PepT1 in the small intestine of new born rats was concentrated in the cytoplasm of subapical and basolateral enterocytes. However, in adult rats the expression is exclusively in the brush border of enterocytes (Hussain et al., 2002).

Feeding is the main regulator of peptide transport (Ogihara et al., 1999; Ihara et al., 2000), but there is little information on hormonal and neurotransmitter-mediated control of peptide absorption. Leptin is a key factor regulating food uptake and energetic homeostasis in mammals (Murashita et al., 2008) and fish (de Pedro et al., 2006). In the present study, leptin was found in the intestine, pyloric caeca, epithelium of stomach and gastric glands, liver and pancreas. Available data on the physiological role of leptin in fish are unclear and frequently contradictory. Our results aligned with Bosi et al. (2004) indicating the presence of leptin in various parts of fish digestive tract (eg. cytoplasm of epithelial gastric gland cells of trout) whereas Muruzábal et al. (2002) did not.

Hepatocyte cytoplasm of all feeding groups showed leptin-like IR. Unlike mammals where adipose tissue is the main site of leptin synthesis (Zhang et al., 1994), the liver seems to play more important role in fish. Leptin was observed in liver of green sunfish (*Lepomis cyanellus*), bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), white crappie (*Pomoxis annularis*), channel catfish (*Ictalurus punctatus*), rainbow trout (Johnson et al., 2000), puffer fish (*Takifugu rubripes*) (Kurokawa et al., 2005) and common carp (*Cyprinus carpio*) (Huising et al., 2006). In mammals, leptin is produced not only by adipose cells but also by nonadipose tissues, including the stomach (Sobhani et al., 2000). In fish gastric leptin is most likely also produced, secreted into blood and into gastric lumen, similarly as in rat (Bado et al., 1998; Buyse et al., 2001).

It was also suggested that leptin secretion into the intestine lumen activates the brush border PepT1 transporter protein (Guilmeau et al., 2003; Hindlet et al., 2009). Leptin may induce translocation of peptide transporters, and regulate activity and expression of intestinal PepT1 (Hindlet et al., 2007). Therefore, this line of research on PepT1–leptin interaction proves to be also extremely potential in fish.

Strong leptin immunoreactivity of the proximal intestine brush border of fish fed PP and AA supplemented diets was similar to rats fed dipeptides Gly-Sar and leptin in which a 25% increase in PepT1 expression, and increase in PepT1 protein levels were observed. During dipeptide feeding, concentrations and activity of leptin in jejunum increased, inducing a considerable increase in body mass (Hindlet et al., 2007). A weak leptin immunoreactivity in the brush border of fish fed CON diet explains the poorer growth of these fish, but it does not explain a high PepT1 gene expression. Further studies are necessary to clarify the mechanisms of this reaction. It is hypothesized that, gastric leptin in fish may control dietary protein absorption by a modulatory effect on PepT1 activity and expression, as seen in mammals (Buyse et al., 2001; Hindlet et al., 2007).

Proteins, peptides and free amino acids are strong stimulators of gastrin secretion (Le Drean et al., 1998). Many authors suggest that fish gastrin and CCK-8 occur in the same endocrine cells of the pyloric part of the stomach and pyloric caeca (García Hernández et al., 1994; Reinecke et al., 1997). However, Bosi et al. (2004), using a double immunofluorescent staining, detected gastrin and CCK-8 in brown trout (*Salmo trutta*) endocrine cells of pyloric caeca and proximal intestine, while in the stomach gastrin alone was present. In the middle intestine only CCK-8 was found.

In the present study of rainbow trout juveniles endocrine gastrin/CCK immunoreactive cells were observed in the proximal intestine and pyloric caeca, particularly in the pyloric region in all experimental groups. A high number of gastrin/CCK positive cells in the pyloric region of trout stomach was also observed by Barrenechea et al. (1994). No gastrin/CCK occurred in the stomach of trout juveniles, which confirmed the results obtained by Holmgren et al. (1982). However, the gastrin/CCK positive cells were found in the stomach of other fish species, namely *Ambloplites rupestris* and *Lepomis gibbosus* (Reifel et al., 1983). Kurokawa et al. (2003) observed gastrin expression in flounder (*Paralichthys olivaceus*) intestine, and concluded that it was the site of gastrin production in fish.

In fish fed CON diet the number of gastrin/CCK positive cells was low which indicates a reduced gastrin secretion. This suggests that lack of dietary lysine may exert an inhibitory effect on gastric acid secretion. According to Kurokawa et al. (2003), gastrin stimulates gastric acid secretion, and probably regulates gastric functions in fish.

In conclusion, this is the first study of PepT1 expression regulation by dietary treatment in fish.

Acknowledgement

The present work was financed by Scientific Research Committee grant N31103032/2256.

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