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Expression Pattern of Peptide and Amino Acid Genes in Digestive Tract of Transporter Juvenile Turbot (*Scophthalmus maximus* L.)

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Abstract Turbot (*Scophthalmus maximus* L.), a carnivorous fish species with high dietary protein requirement, was chosen to examine the expression pattern of peptide and amino acid transporter genes along its digestive tract which was divided into six segments including stomach, pyloric caeca, rectum, and three equal parts of the remainder of the intestine. The results showed that the expression of two peptide and eleven amino acid transporters genes exhibited distinct patterns. Peptide transporter 1 (PepT1) was rich in proximal intestine while peptide transporter 2 (PepT2) was abundant in distal intestine. A number of neutral and cationic amino acid transporters expressed richly in whole intestine including B^0-type amino acid transporter 1 (B^0AT1), L-type amino acid transporter 2 (LAT2), T-type amino acid transporter 1 (TAT1), proton-coupled amino acid transporter 1 (PAT1), y^+L-type amino acid transporter 1 (y^+LAT1), and cationic amino acid transporter 2 (CAT2) while ASC amino acid transporter 2 (ASCT2), sodium-coupled neutral amino acid transporter 2 (SNAT2), and y^+L-type amino acid transporter 2 (y^+LAT2) abundantly expressed in stomach. In addition, system $b^{0,+}$ transporters (rBAT and $b^{0,+}$AT) existed richly in distal intestine. These findings comprehensively characterized the distribution of solute carrier family proteins, which revealed the relative importance of peptide and amino acid absorption through luminal membrane. Our findings are helpful to understand the mechanism of the utilization of dietary protein in fish with a short digestive tract.

Key words turbot; digestive tract; amino acid; peptide; transporter; expression pattern

1 Introduction

Carnivorous fish require high dietary protein for protein synthesis and homeostasis maintenance (Halver and Hardy, 2002; Kimball and Jefferson, 2002). After protein digestion, peptides and amino acids (AAs) are efficiently absorbed by epithelial cells along the digestive tract (Webb, 1990). During this process, di- and tri-peptides and free amino acids are transported by specific transporters (Bröer, 2008; Gilbert *et al*., 2008), which act as carriers and nutrient sensors for cellular AA supply and homeostasis (Taylor, 2014).

As the (alimentary tract length)/(body length) ratio of carnivorous fish is small (Kramer and Bryant, 1995), high rate of absorption is demanded. Although peptide and AA transporters have been extensively studied in human and rodents, much less information is available in fish. Previous studies showed a variety of peptide transporter 1 (PepT1) expression pattern and function in teleosts (Liu *et al*., 2013; Rønnestad *et al*., 2007, 2010), yet the distribution of PepT2 has not been defined in fish digestive tract. Furthermore, functional studies have confirmed the existence of various types of AA transporters in fish (Storelli *et al*., 1989), but the gene expression profile of these proteins have not been determined so far. It is well-known that AA transporters exhibit substrate specificity, while PepT1 can potentially transport all di- and tri-peptides (Gilbert *et al*., 2008). In teleost fish, especially in carnivorous fish, the relative importance of peptide and AA absorption in digestive tract is not clearly established.

Essential AAs for fish growth include both neutral and cationic AAs, which are absorbed by peptide transporters, as well as neutral and cationic AA transporters in digestive tract. AA transport activities are frequently referred as 'systems', the 'neutral system' transporting all neutral AAs and the 'basic system' transporting cationic amino acids together with cysteine (Bröer, 2008). In the present study, we mainly investigated the 'neutral system' and 'basic system' transporters. Turbot (*Scophthalmus maximus* L.), a carnivorous fish species, was chosen as the animal model because it demands high dietary protein/AAs requirement and represents an economically valuable aquacultured species (Lee *et al*., 2003; Cho *et al*.,

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2005). We focus on studying the expression pattern of major transporter genes for essential amino acids and two peptide transporters of turbot digestive tract. To our knowledge, this is the first investigation for assessing the expression pattern of these transporter genes in gastrointestinal tract in all fish species.

2 Materials and Methods

2.1 Fish Rearing

All experimental plans were approved by the Animal Care Committee of Ocean University of China. Twenty juvenile turbot (*Scophthalmus maximus* L., 18–20 g in body weight) were obtained and acclimated in the experimental station of Haiyang Fish Farm (The yellow sea fisheries co., LTD, Haiyang, China) for one week in order to obtain the consistent levels of metabolites. Turbot were acclimated in one tank filled with 500 L sand-filtrated seawater, and fed with commercial diet (Great Seven Bio-tech, China; crude protein, 53%; crude lipid, 11%) twice a day (07:00 and 19:00, respectively) to apparent satiation. The temperature of seawater was maintained at (18±1)℃. The seawater was changed at a speed of approximately 1.5 L min^{-1}.

2.2 Sample Collection

After acclimation, fish were fasted for 48 hours in order to obtain the homogeneous metabolic response (Seiliez *et al*., 2011). Before dissection, fish were anesthetized with eugenol (1:10000) and sacrificed by cervical section. All dissections were performed on ice. The digestive tract was divided into six segments consisting of stomach, pyloric caeca, rectum, and three equal parts of the remainder of the intestine (Fig.1A). Each segment was rinsed thoroughly with cold phosphate-buffer saline (PBS) (2 mmol L^{-1} Na_2HPO_4, 8 mmol L^{-1} Na_2HPO_4, 145 mmol L^{-1} NaCl, pH 7.2) to empty any leftover content (Bakke *et al*., 2010). Twelve fish were randomly sampled. Tissues from every four fish were mixed as one sample. In total, 3 replicates were conducted each assay (*n*=3). Segments were immediately dissected, frozen in liquid nitrogen and kept at −80℃ till analysis.

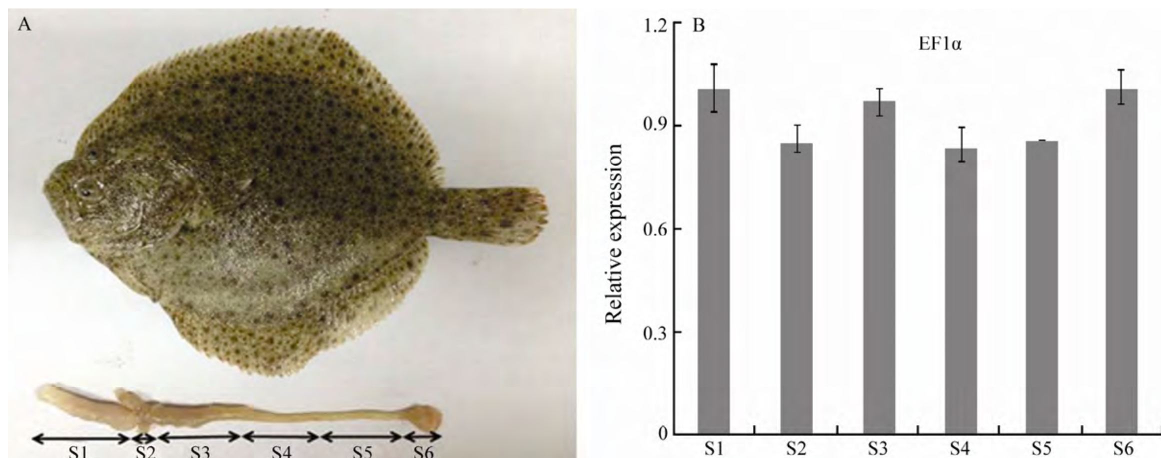

Fig.1 (A) Juvenile turbot digestive tract: stomach (S1), pyloric caeca (S2), three adjacent equal parts intestinal segments starting after the pyloric area (S3–S5), and rectum (S6). (B) Regional distribution of reference gene EF1α mRNA in the digestive tract of juvenile turbot. Values are means ± SE. (*n*=3). Bars with different letters were significantly different by Tukey's test ($P<0.05$).

2.3 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from 50 to 100 mg frozen tissue using Trizol reagent (Invitrogen, USA). RNA was reversely transcribed to cDNA by PrimeScript®RT Kit With gDNA Eraser (Perfect Real Time) (TAKARA, Japan). Subsequently, 900 ng total RNA in a 20 μL reaction mixture was reversely transcribed into cDNA following the instructions.

Partial sequences of target genes were obtained through a degenerate PCR strategy. Specific primers for each gene were designed using the primer design software Primer 5. All primer sequences were listed in Table 1. Quantitative PCR analyses were carried out using a real-time PCR kit with an SYBR Green fluorophore (TAKARA, Japan) according to the manufacturer's instructions. qRT-PCR reactions were implemented using a Mastercycler ep Realplex PCR system (Eppendorf, German) (Zuo *et al*., 2012). Results were normalized to the reference elongation factor-1-alpha (EF1α) gene for quantification. No change in EF1α gene expression was observed in our study (Fig.1B). The quantity of target mRNAs was calculated using the comparative cycle threshold (Ct) values method expressed as $2^{-(\Delta\Delta Ct)}$ (Bunpo *et al*., 2009).

2.4 Statistical Analysis

Results were expressed as means±SE. SPSS 16.0 was used for all statistical analysis. All data were subjected to one-way analysis of variance (ANOVA) and difference between the means was tested by Tukey's multiple range test. The level of significance was chosen at $P<0.05$.

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Table 1 Primer sequences used for real-time quantitative (qRT-PCR)

Gene	Forward primer	Reverse primer
PepT1/SL15A1	GCATCCACACCCAGCAGAAG	GTCCTCAGCCCAGTCCATCC
PepT2/SL15A2	CAAGGAGAATGTCAGCGAGAGG	AACAACCAGAGCCACGACCAT
B^0AT1/SLC6A19	AGACTCTCAACACCTCCGAAGC	AGCCTTTCCTGTGGTCTCAATCC
LAT2/SLC7A8	TGCCTCGTGCCATCTTCATC	GCTCCAGCAAAGAACAATCTCG
PAT1/SLC36A1	TCAGTGACAACATCAAGCAGGTG	GAAGGCGGGCAGGAAGAAGAG
ASCT2/SLC1A5	ACCTTGATCGCCTCGTCCATC	CATCTGTGCCGTTCCTTGTAACC
SNAT2/SLC38A2	TGCTGCTGGTGACGCTCTTC	CAGGTGTCCTCGCTGTAGTCC
TAT1/SLC16A10	TCTCCCATCGTCAGCGTCTTC	CTGCCAGCCGTCACAATGC
y^+LAT1/SLC7A7	TGTGACGTTTGCGGACCAG	GACGGGAGTGTAGCGGAAGAC
y^+LAT2/SLC7A6	TGCCCATCGTCACCATCAT	AGCACGACAAAGCCACAGC
$b^{0,+}$AT/SLC7A9	GGGCTTTGGGCTTATGATGGATG	TGGAGACAACAGCAGTTCAGTGG
rBAT/SLC3A1	CAATCGGGTGACTGTGTTTGGG	TCATCTCTTGGCGGACTTGTGG
CAT-2/SLC7A2	TGCTGCTGTTCGTGACCATCTC	AGGTTCCAGAAATGCCATAAGGG
EF1α	TCATTGGCCATGTCGACTCC	ACGTAGTACTTGGCGGTCTC

Notes: GenBank Accession No.: EF1α, AF467776.1. Partial sequences of target genes in turbot were obtained through a degenerate PCR strategy in this study.

3 Results

3.1 Expression Pattern of Peptide Transporter Genes

The expression of two kinds of peptide transporter genes were determined in six digestive tract segments of juvenile turbot including stomach (S1), pyloric caeca (S2), proximal intestine (S3), middle intestine (S4), distal intestine (S5) and rectum (S6) and illustrated in Fig.2. PepT1 gene expressed markedly lower in S1 than in other segments. The expression of PepT1 gene peaked at S3 while that of PepT2 gene peaked in S5 and S6 where the abundance of PepT2 gene transcripts was several hundred folds higher than that in S1 through S3. The abundance of PepT1 gene transcripts was markedly higher than that of PepT2 gene in all tissue segments. In addition, the expression of PepT1 gene in intestine was higher than that of amino acid transporter genes.

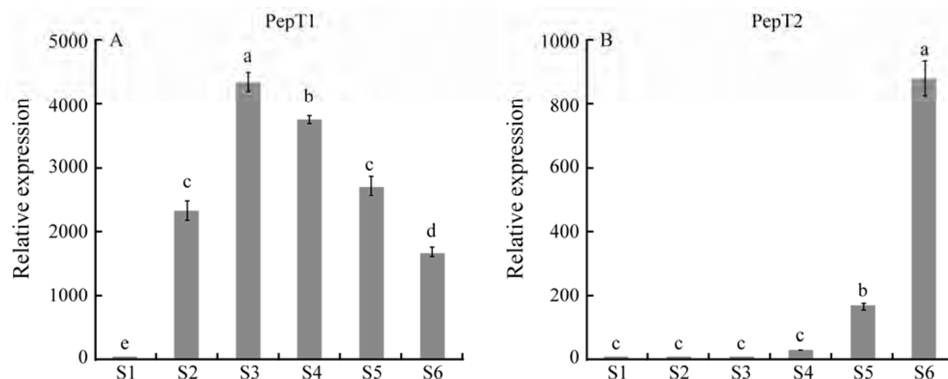

Fig.2 Regional distribution of peptide transporters mRNA in the digestive tract of juvenile turbot. (A) peptide transporter 1 (PepT1); (B) PepT2. Transcription levels were normalized by the reference gene EF1α. All treatments were normalized to the expression levels of PepT2 S1. Values are means ± SE (n=3). Bars with different letters are significantly different by Tukey's test ($P < 0.05$).

3.2 Expression Pattern of Neutral Amino Acid Transporter Genes

The expression of six kinds of neutral amino acid transporter genes were determined in six digestive tract segments of juvenile turbot and illustrated in Fig.3. The expression of SNAT2 and ASCT2 genes peaked at S1, while that of the abundance of B^0AT1, LAT2, TAT1, and PAT1 gene transcripts were markedly lower in S1. The abundance of B^0AT1 gene transcripts remained constant from S3 to S6, which was significant higher than that in S1 and S2. The expression of LAT2 gene increased from S1 to S6 and peaked in S6. The abundance of TAT1 gene transcripts were enriched in S3 and S4. The expression of PAT1 gene peaked in S5, which higher than that any other segments. Among six neutral amino acid transporters, the abundance of these gene transcripts were summarized as B^0AT1 > LAT2 > TAT1 > PAT1 > SNAT2 > ASCT2.

3.3 Expression Pattern of Cationic Amino Acid Transporter Genes

The expression of four kinds of cationic amino acid transporter genes were determined in six digestive tract segments of juvenile turbot and illustrated in Fig.4. The

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expression of $b^{0,+}$AT gene was low in stomach and proximal intestine (S1 through S4), while peaked in hindgut (S5 through S6). The expression of rBAT gene, the heaven chain of $b^{0,+}$AT, showed a gradual increase from S1 to S6. The abundance of y^{+}LAT1 gene transcripts was concentrated in intestine (S3–S6), while that of y^{+}LAT2 gene peaked in stomach (S1). In addition, the expression of y^{+}LAT1 gene was much higher than that of y^{+}LAT2 from S2 to S6. The abundance of CAT2 gene transcripts reached to its lowest level in S1 and S2 whereas peaked in S6.

4 Discussion

Peptide transport system is considered as a fast and energetically efficient way of nutrient absorption (Gilbert *et al.*, 2008). In the present study, the expression of a set of peptide and AA transporter genes of turbot digestive tract including stomach, pyloric caeca, intestine and rectum were analyzed. We observed that PepT1 gene expressed highly in proximal intestine and then steadily weakened from the proximal to the distal. A similar pattern was described in sea bass (Terova *et al.*, 2009). However, its expression was strictly confined to the proximal portions of intestine in carps (Liu *et al.*, 2013) and zebrafish (Verri *et al.*, 2003), and ubiquitous in Atlantic cod (Rønnestad *et al.*, 2007). Our findings reinforced the notion that the expression pattern of PepT1 gene in digestive tract was fish species specific.

The preceding intestinal segments may be important for the absorption of dietary proteins in form of di- and tri-peptides in turbot. The expression of PepT1 gene in the distal intestinal segments indicated that PepT1 may also act on final absorption of residual luminal peptides. Furthermore, we found that PepT1 gene expression was identical in pyloric caeca and the distal intestinal segment, which may be explained by the retrograde peristalsis mechanism moving chyme in a posteriorto-anterior direction from the adjacent proximal intestine into pyloric caeca (Rønnestad *et al.*, 2000). Therefore, the regulation of peptide absorption in pyloric caeca might be in line with intestine rather than stomach. Interestingly, the similar

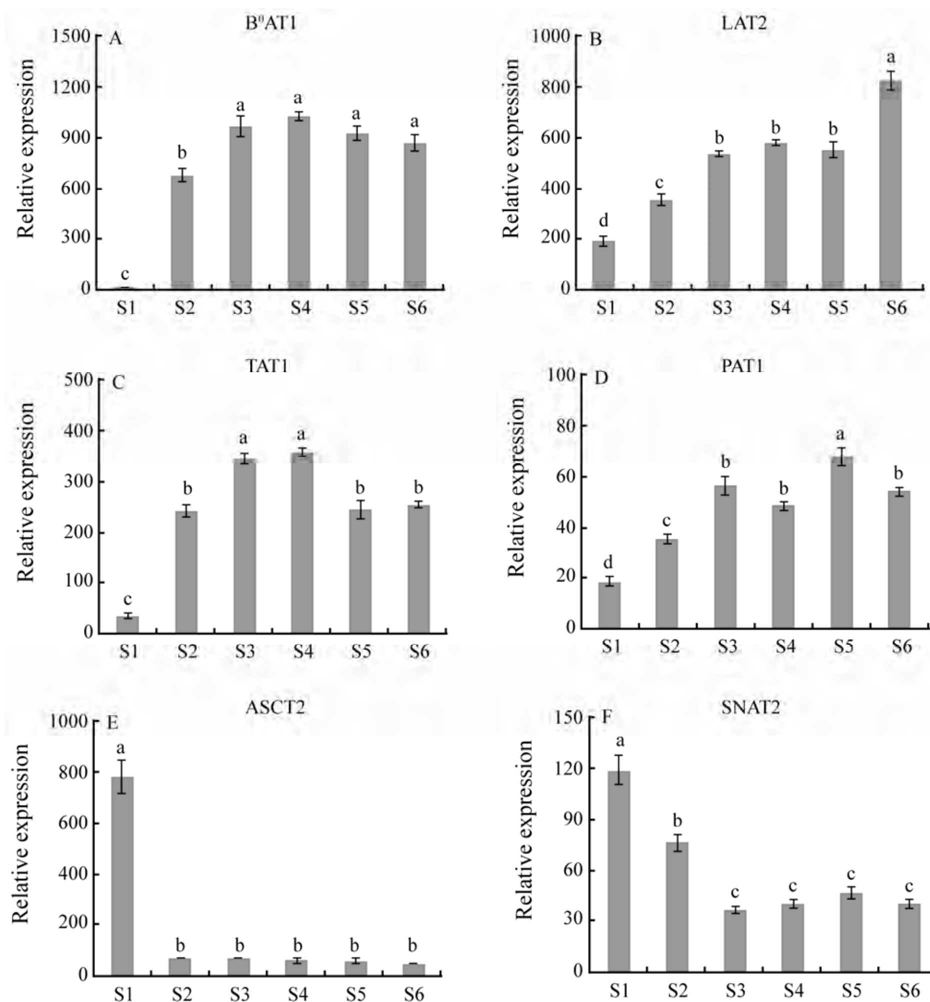

Fig.3 Regional distribution of neural amino acid transporters mRNA in the digestive tract of juvenile turbot. (A) B^0-type amino acid transporter 1 (B^0AT1); (B) L-type amino acid transporter 2 (LAT2); (C) T-type amino acid transporter 1 (TAT1); (D) Proton-coupled amino acid transporter1 (PAT1); (E) System ASC amino acid transporter 2 (ASCT2); (F) System A amino acid transporter (SNAT2). Transcription levels were normalized by the reference gene EF1α. All treatments were normalized to the expression levels of PepT2 S1. Values are means ± SE (n=3). Bars with different letters are significantly different by Tukey's test (P < 0.05).

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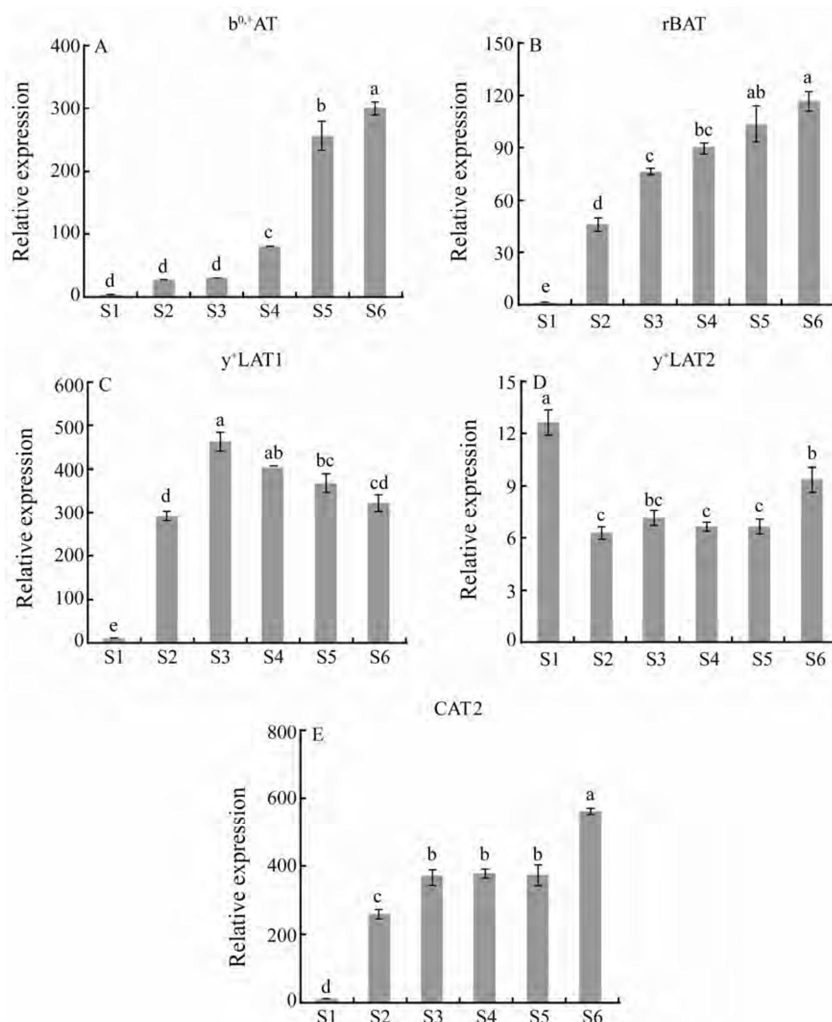

Fig.4 Regional distribution of cationic amino acid transporters mRNA in the digestive tract of juvenile turbot. (A) $b^{0,+}$-type amino acid transporter ($b^{0,+}$AT); (B) Related to $b^{0,+}$ amino acid transporter (rBAT); (C) y^+L-type amino acid transporter 1 (y^+LAT1); (D) y^+LAT2, (E) Cationic amino acid transporter-2 (CAT2). Transcription levels were normalized by the reference gene EF1α. All treatments were normalized to the expression levels of PepT2 S1. Values are means ± SE (n=3). Bars bearing with different letters are significantly different by Tukey's test ($P<0.05$).

results were reported in sea bass (Terova *et al*. 2009) and Atlantic cod (Bakke *et al*., 2010) too, which indicates that the pyloric caeca play an important role in peptide absorption similar as intestine in teleost.

In addition, expression level of PepT1 gene was hundreds folds higher than that of PepT2 gene, indicating that PepT1 was the dominant carrier of di- and tri-peptides in fish intestine (Ostaszewska *et al*., 2010). PepT1 gene expression level was also higher than that of all AA transporter genes examined in this study, which suggested that PepT1 may play a vital role in amino acid absorption in turbot. Yet this needs further exploration at protein level in order to illustrate its functional importance. The high peptide absorption potential in turbot means that the fish prefers to use protein rich feed, such as fishmeal, that can be digested into small peptides, but not the protein, such as plant protein, that releases less small peptide (Savoie *et al*., 2005, 1989). In contrast to PepT1, the expression of PepT2 gene was strictly confined to distal intestine, a pattern similar to what was reported in rabbit (Döring *et al*., 1998). In mammals, PepT2 was reported to be associated with the enteric glial cells and macrophages in the neuromuscular layer of gastrointestinal tract (Rühl *et al*., 2005). Ostaszewska and cooperator (2010) reported that unlike the abundance of PepT1 gene transcripts in intestine of common carp, the expression of PepT2 gene was very low and it was barely regulated by different diets. In this respect, the role of PepT2 in fish should be similar to the mammals.

The transportation of AAs from digestive tract into bloodstream needs functional cooperation of transporters across the intestine epithelial cell layer (Bröer, 2008). B^0AT1 is the major apical neutral AA transporter (Bröer *et al*., 2004), while LAT2 and TAT1 are mainly involved in basolateral trans-epithelial AA efflux (Kim *et al*., 2002; Verrey, 2003). These transporters cooperate in maximal uptake of neutral AAs from intestinal lumen to blood. Our results showed that the expression level of neutral AA transporters including B^0AT1, LAT2 and TAT1 remained constantly high in the whole intestine. In contrast, the expression level of B^0AT1 and LAT2 was either low or not detectable in distal intestine in mammals (Dave *et al*.,

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2004; Romeo *et al*., 2006). Therefore, the ubiquitous high expression of these AA transporter genes can be beneficial for maximizing AA absorption in a very short digestive tract in turbot.

Similar to the expression pattern in mammals (Avissar *et al*., 2001), ASCT2 and SNAT2 genes were mainly expressed in stomach which is not considered as a major AA absorption tissue in fish. ASCT2 mediates transport of small neutral AAs, yet interacts with anionic AAs at low pH (Utsunomiya-Tate *et al*., 1996). SNAT2 displays H^+-dependent activity and is responsible for basolateral uptake of glutamine from circulation into intestine (Mackenzie and Erickson, 2004). ASCT2 and SNAT2 are known to provide quick responses to AA deprivation (Gaccioliy *et al*., 2006) and AA availability (Pinilla *et al*., 2010). High level of ASCT2 and SNAT2 in stomach could provide a quick sensing and response to dietary AA quality.

The transport of cationic AA (CAA) across the epithelium is mediated by apical (system $b^{0,+}$) and basolateral (system y^+L, system y^+) transporters (Bröer, 2008) including $b^{0,+}AT1$, y^+LAT1 and CAT2, which are abundant in turbot intestine. More importantly, system $b^{0,+}$ genes ($b^{0,+}AT$ and rBAT formed heterodimer) rich expressed in distal segments of intestine to ensure the effective and complete absorption of dietary CAAs. The combined action of $b^{0,+}AT$ and y^+LAT1 cause cross-epithelial CAA absorption into blood, while counter-flowed neutral AAs is re-taken by apical transporter B^0AT1 (Wolfram *et al*., 1984).

In the present study, we found that PepT1, as well as most of the AA transporters including B^0AT1, LAT2, TAT1, PAT1, y^+LAT1, $b^{0,+}AT$/rBAT, and CAT2 were highly expressed along the whole post-gastric digestive tract, while little or none expression level was detected in colon of higher vertebrates such as murine (Dave *et al*., 2004). With the cooperation of peptide and amino acid transporters, turbot have a great capacity to absorb high level of dietary protein in such short intestinal tract. It has been reported that the transcription of transporters in intestine is regulated by dietary nutrition composition (Kamalam *et al*., 2013; Ostaszewska *et al*., 2010). Indeed, study in Atlantic cod demonstrates that substitution of 30% dietary fish meal by whole fish hydrolysate (FH), retenate after ultrafiltration of FH, nanofiltered retenate of FH, or a mix of FAAs in diet can cause different regional expression profiles of PepT1. However, preceding intestinal segments are still the main PepT1 expression region no matter which diet is used (Bakke *et al*., 2010). The regional distribution of transporters in our study may not be directly related to the commercial diet of juvenile turbot. However, the results will provide insight into the fundamental information about sensitive peptide and amino acid transporters and appropriate digestive tract segments in turbot, which can serve to further explorations.

In conclusion, cellular transport of small peptides and AAs is a key step in digestive assimilation. Our data demonstrates that peptide and AA transporter genes express along the turbot digestive tract with distinct patterns to ensure maximum protein absorption. Further studies on the physiological coordination and regulation of these transporters should aid to improve nutrient utilization in teleosts.

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