Aquaculture 290 (2009) 110–115

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Expression of brush border enzymes and transporters in the intestine of European sea bass (*Dicentrarchus labrax*) following food deprivation

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ARTICLE INFO

Article history:
Received 25 December 2008
Received in revised form 9 February 2009
Accepted 10 February 2009

Keywords:
Gene expression
Intestine
Brush-border enzymes
Food deprivation transporters
Transporters

ABSTRACT

Studies on aquacultured fish nutrition have focused solely on performance and the activity of the brush-border enzymes, while the molecular aspects have barely been addressed. Nevertheless, the expression of genes encoding enzymes and transporters of the brush-border membrane is crucial for the final stages of digestion and absorption of nutrients through the epithelial luminal membrane of the intestine. To establish a molecular tool for such studies, primers for the European sea bass intestinal genes were designed utilizing evolutionarily conserved DNA sequences for the following intestinal enzymes and transporters: maltase, APN, PepT1 and Na^+/K^+-ATPase. The derived European sea bass cDNA fragments showed 64–92% homology to other animal species and enabled us to study the differences in their expression. The mRNA expression of these genes in pyloric caeca and the upper and lower intestinal sections were compared in fed fish vs conditions of food deprivation for 3, 7, 14 and 21 days. The mRNA expression examined using real time PCR of APN, maltase and PepT1 in all the intestinal tract sections, was the highest after 7 days of food deprivation. Na^+/K^+-ATPase showed a different pattern of expression in each section of the intestine. Significant positive linear correlations between APN, maltase and PepT1 mRNA expression were detected in all but day 3 post food deprivation. The cDNA fragments isolated in this study provide a novel molecular tool for revealing new aspects in fish nutrition and can provide additional information regarding the pattern of expression of intestinal genes following long-term fasting in fish.

1. Introduction

The nutrients digested and absorbed together with electrolytes through the intestine epithelium are utilized to fulfill the metabolic needs of the body. These processes, which include the final stages of digestion and absorption that take place in the brush-border region of the intestine, are a result of the activity of the digestive enzymes.

Periods of nutrient deprivation are common in many fish life cycles and these appear to be tolerated by many species (Navarro et al., 1997; Olivereau and Olivereau, 1997; Belanger et al., 2002). Cultured fish can also experience such situations as a result of several factors such as starvation prior to slaughter (to ensure that the fish have an empty gut) or under conditions of reproduction or overcrowding.

The gastrointestinal tract is the initial organ system which is affected by changes in nutrient intake, and it also displays the most rapid and dramatic changes in a state of nutrient deprivation. These changes may bring about intestinal electrolyte imbalances and also affect mucosal structure and function, which, in turn, may alter the processing of food when feeding resumes (Ferraris and Carey, 2000).

Among the brush-border digestive enzymes (BBDE) are the disaccharidases, such as maltase–glucoamylase (MGA; EC 3.2.1.20); sucrase–isomaltase (SI; EC 3.2.1.48/10); and peptide hydrolases, such as amino peptidase N (APN; EC 3.4.11.2). It is known that MGA and SI are responsible for 20 and 80% of maltase activity, respectively (Nichols et al., 1998). Maltase hydrolyses maltose into its component parts, i.e. two molecules of α-glucose. APN is probably the most abundant peptidase, which removes N-terminal amino acids sequentially from oligopeptides (Sitrin, 1996). The absorption of peptides is mediated predominantly by a single peptide transporter coined peptide transporter 1 (PepT1), while absorption of amino acids involves participation of several transport systems. Adibi (2003) has shown that in vitro, PepT1 can potentially transport 400 di-peptides and 8000 tri-peptides produced from the digestion of dietary and body proteins. A wide range of di- or tri-peptides, regardless of their molecular weight, electrical charge or hydrophobicity, can be transported by bovine and chicken PepT1, demonstrating the importance of peptide transport in domestic animals (Pan et al., 2001; Chen et al., 2002; Van et al., 2005).

Another transporter of great importance is the sodium/potassium transporter (Na^+/K^+-ATPase), which is located on the basolateral

Abbreviations: APN, amino peptidase N; BBM, brush-border membrane; BBDE, brush-border digestive enzymes; MGA, maltase-glucoamylase; SI, sucrase–isomaltase; Na^+/K^+-ATPase, sodium/potassium transporter; PC, pyloric caeca; PepT1, peptide transporter 1.

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doi:10.1016/j.aquaculture.2009.02.008

membrane, and maintains the homeostasis of Na^+ and K^+ ions in eukaryotic cells. These ionic gradients formed by the Na^+/K^+-ATPase are critical in regulating osmotic balance, cell volume, cytoplasmic pH (through the Na^+/H^+ exchanger), and Ca^{+2} levels (by action of Na^+/Ca^{+2} exchanger), as well as Na^+-coupled transport of nutrients and amino acids into the cells (Panayiotidis et al., 2006).

Although the small intestine is the site of the major regulatory mechanisms that may be altered by reductions in nutrient intake, minimal information is available on the mechanisms which regulate the digestion and assimilation processes that occur in the intestine during fasting. Indeed, previous studies have documented BBDE activity under different food manipulation conditions (Harpaz and Uni, 1999; Krogdahl et al., 1999; Harpaz et al., 2005a,b; Hakim et al., 2006; Tibaldi et al., 2006), but the molecular aspects of the intestinal digestion and absorption have not yet been examined.

In the present study, primers were adapted from evolutionarily conserved DNA sequences for the following brush-border membrane (BBM) enzymes and intestinal transporters: maltase (the same primer for MGA and SI), APN, PepT1 and Na^+/K^+-ATPase. The resulting cDNA fragments showed 68 to 87% homology to other animal species and were used to determine mRNA expression in the intestine of fed and food-deprived European sea bass.

The results obtained in this study show for the first time the fluctuations in the intestinal genes expression and correlations in their expression following prolonged fasting.

2. Materials and methods

2.1. Isolation and sequencing of cDNA fragments of MGA/SI, APN, Na+/K+-ATPase, PepT1 and 18S genes

The intestinal tract (pyloric caeca [PC] and intestine) was isolated from five European sea bass (*Dicentrarchus labrax*) and homogenized in TRI-REAGENT (100 mg tissue/2 mL) according to the manufacturer's protocol (Molecular Research Center Cincinnati, OH, USA). The RNA obtained served as the source for the RT-PCR. RNA quality was determined by spectrophotometry using the ratio of absorbance at 260 and 280 nm and then quantified. A comparison of several published sequences of MGA/SI, APN, Na^+/K^+-ATPase, PepT1 and 18S genes enabled us to identify common regions, from which the relevant primers were chosen: Primers for 18S were designed by comparing conserved regions of 18S rRNA from rainbow trout, bullet tuna (*Auxis rochei*), rockcod (*Notothenia coriiceps neglecta*), and tilapia (*Oreochromis mossambicus*) published in GenBank, under accession nos. AF308735, AB193747, AF518192 and AF497808, respectively. Primers for maltase were designed by comparing conserved MGA mRNA regions from chicken, mouse, cattle and humans and by juxtaposing conserved SI mRNA regions from mouse, cattle and humans (GenBank accession nos. for MGA: XM422811, XR004953, XM594452 and AF016833, respectively; for SI: XM143332, XM580476 and BC116453, respectively). Primers for APN were designed by comparing conserved APN mRNA regions from winter flounder (*Pseudopleuronectes americanus*), chicken, cattle and humans (GenBank accession nos. AF012465, NM204861, NM001074144 and X13276, respectively). Primers for PepT1 were designed by comparing conserved PepT1 mRNA regions from zebra fish, chicken, cattle and humans published in GenBank under accession nos. AY300011, AY029615, XM599441 and AM005073, respectively. Primers for Na^+/K^+-ATPase were designed by comparing conserved Na^+/K^+-ATPase mRNA regions from zebra fish, rainbow trout and chicken published in GenBank under accession nos. BC063936, AY319390, U82549 and J03230, respectively.

The selected primers (Table 1) were subjected with intestinal European sea bass RNA to RT-PCR with the Promega Access RT-PCR System (Madison, WI) according to their technical bulletin #TB220: first-strand cDNA synthesis −45 °C for 45 min (for reverse transcription), 94 °C for 2 min (AMV RT inactivation and RNA/cDNA/primer denaturation); second-strand synthesis and amplification −40 cycles of: 30 s at 94 °C for denaturation, 1 min at 53 °C for primer annealing for all of the genes except for APN which was annealed for 1 min at 58 °C , and 2 min at 68 °C, followed by final extension cycle of 7 min at 68 °C.

The RT-PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide, excised from the gel and purified with a DNA isolation system (DNA Isolation Kit; Biological Industries, Kibbutz Beit Haemek, Israel).

The isolated products were sequenced using automated Applied Biosystem 373A DNA sequencer (Foster City, CA USA). Nucleic-acid sequences were analyzed using Invitrogen Vector NTI advance 10 software (Informax Inc., Bethesda, MD USA). Sequences of the different products were compared and their homology calculated with EMBL-EBI ClustalW software.

2.2. Fish, experimental tanks and rearing conditions

The European sea bass (*D. labrax*) used for this study were brought from Tirat Zvi Aqua farm (Tirat Zvi, Israel) and acclimated to our holding system for several weeks prior to being tested. The average initial weight of the fish participating in the experiment was 121.06 ± 4.7 g and there were no significant differences in the total biomass of each tank among the experimental groups at the onset of the experiment ($P>0.05$).

Five groups of 6 fish each were put into five plastic tanks (100 L each) in a closed fresh water re-circulating system with a central bio-filter and were kept under standard conditions considered favorable for these fish, i.e. a dissolved-oxygen level of 90 to 100% saturation, water temperature was 26 °C ± 1 °C and there were no measurable levels of total ammonia (measured as NH_4^+) and nitrite. The culture system was set indoors and the light source was natural photoperiod enhanced with florescent light providing a light intensity of 1200 lx during the day hours. No food was given to the fish during the experiment.

Table 1
Primers and probes used for isolation of *D. labrax* BBDE and transporters gene fragments cDNA (A) and real time PCR (B).

Gene	GenBank ID	A		B		
		Sense/antisense (5′–3′)	Length	Sense /antisense (5′–3′)	Probe (5′–3′)	Length
Maltase	AM419039	GAACAGCAATGCCATGGAGG / GACTACATGGACCGTCAGCT	297	CCTGTTCTACCTGCCTATTGG / ATACAGAGACATGAGGGAAGC	CGCCAACGACCAGGAGATAGCAGAT	101
APN	AM419036	GAGCTGGCTCATATGTGGTT / CAGTCCTCAGGATGCTCTCA	300	TGTGGTTCGGGAACTTGGT / CTTTGCCACCTATGTCTCTTATCT	GGTGGTGGAATGACTTGTGGCTCAATG	82
PepT1	AM419037	GCTTGTTACCCACTGGCCTTTG / AGATGCAGACGGTGAACGCCAT	407	GGACTGGGCTGAGGAGAAA / TTCCTCTACATCCCTCTTCCC	AGGTGAAGATGGTGCTGAAGGTGCT	88
ATPase	AM419034	CCAAGCAAGCAGCTGACATGA / CAGCAGGGGATGAAGAACAAG	629	CCTGCTGGAcGACAACTTTG / TGAAGAAgTCCATCGCCTACA	CGTTACTGGAGTGGAAGAAGGTCGTCTG	89
18S	AM419038	TTCGTATTGTGCCGCTAGAGGT/ TGGTGCATGGCCGTTCTTA	406	CGCTAGAGGTGAAATTCTTGGA / GATCAGATACCGTCGTAGTTCC	ACGAAAGCGAAAGCATTTGCCAAGAA	125

2.3. Treatments

The experiment included five treatment groups: Fish which were slaughtered at the beginning of the experiment (day 0) – the control; and groups of food deprived fish which were slaughtered at 3, 7, 14 and 21 days after the beginning of the experiment.

2.4. Sampling

On days 0, 3, 7, 14 and 21 from the beginning of the experiment, each group of six fish were sacrificed using a scalpel to sever the spine. The entire digestive tract was removed and carefully cleaned of adipose tissue. Krogdahl and Bakke-McKellep (2005) have shown that long starvation of fish (2 days and above) results in a significant effect on the BBDE activity. In the present study all fish from the control group were sampled prior to feeding, i.e. they were food deprived only on the actual day they were sampled to ensure empty digestive tracts. When necessary, remaining food residues were gently squeezed out. The digestive system was then divided into PC, upper and lower sections. The dissected sections were immediately stored in liquid nitrogen and transferred to a −80 °C freezer for RNA extraction and gene expression analyzing.

2.5. Fish weight

The daily weight loss rate was calculated as:

$$\frac{|(\ln \text{final weight} - \ln \text{initial weight})|}{\text{Days of food deprivation}} * 100$$

2.6. Real Time PCR

Total RNA was extracted from all the samples and then reverse-transcribed to produce cDNA in a 20-μL volume containing 1 μg of extracted RNA. Reverse transcription was carried out using an EZ-First strand cDNA synthesis kit for RT-PCR according to the manufacturer's protocol (Biological Industries, Beit Haemek, Israel). The reaction was performed at 70 °C for 10 min followed by 60 min at 42 °C and 15 min at 70 °C. Nested Taqman primers and probes were designed from the sequenced gene fragments for Real Time PCR by PrimerDesign Ltd (Millbrook Technology Campus, Southampton, Hants, UK) (Table 1). The TaqMan probes were labeled at the 5′ end with the FAM reporter dye. Real-time PCR was performed on a Stratagene MX 3000P instrument. The 20 μL PCR reaction mixture was composed of 5 μL of the cDNA diluted 1:100, 10 μL of Absolute blue QPCR low Rox mix (Thermo Fisher Scientific Inc. Waltham, MA 02454), 4 μL of water, and 1 μL of the primer and probe mix (1 pmol forward and reverse each). All polymerase chain reactions were done in duplicate in ABgene PCR plates closed with Absolute QPCR seals (Thermo Fisher Scientific Inc. Waltham, MA 02454) under the following conditions: 95 °C for 15 min and 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. Standard curves were also generated to determine the efficiency of amplification by pooling undiluted cDNA from each day (all sections) and diluting the pooled cDNA to dilutions of 1:5, 1:25, 1:125, 1:625, 1:3125. Cycle threshold (Ct) values were calculated for each sample automatically by the MXpro software. The Ct values of 18S ware not significantly different ($P>0.05$) among different days and sections and therefore gene expression levels were determined by analyzing the resulting Ct values for each sample, normalizing to the level of 18S expression for the same RNA sample. The lower the Ct value, the higher the expression level. The n-fold change was calculated relative to that on day 0 using the $\Delta\Delta C_t$ method of Pfaffl (2001) including the efficiencies for both the experimental gene and 18S (internal controls).

2.7. Statistical analysis

Statistical analysis was carried out using the JMP software (SAS Institute, 1994). All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using one-way ANOVA for daily weight loss rate (%) and a two-way ANOVA for gene expression with days of food deprivation and intestine sections as the main effects, and their interaction, followed by Tukey Kramer HSD test. The correlation coefficients, the strength of the linear relationships between each pair of response (Y) variables was tested by Correlations Multivariate. Pair-wise correlation relationships were examined using Pearson. P values less than 0.05 were considered significant.

3. Results

3.1. Isolation of MGA/SI, APN, Na^+/K^+-ATPase, PepT1 and 18S gene fragment cDNA

Maltase. A single 297-bp fragment was obtained after amplification by RT-PCR using primers which were designed for MGA and SI (Table 1). The isolated *D. labrax* maltase fragment (accession no. AM419039) was 69, 70, 70 and 73% homologous, respectively, to cattle, chicken, human, and mouse MGA genes, while it was 64, 68 and 68% homologous, respectively, to cattle, mouse and human SI genes.

APN. A single 300-bp fragment was obtained following amplification by PCR using primers which were designed for this gene (Table 1). The APN fragment isolated from *D. labrax* enterocytes (accession no. AM419036) was 69, 70, 72 and 74% homologous, respectively, to APN genes of chicken, cattle, winter flounder and humans.

Na^+/K^+-ATPase. A single 629-bp fragment was obtained following PCR amplification using the relevant primers (Table 1). The fragment isolated from *D. labrax* intestine (accession no. AM419034) was 79, 83, 87, and 92% homologous to the chicken, zebra fish, rainbow trout, and *O. mossambicus* Na^+/K^+-ATPase genes, respectively.

PepT1. A single 407-bp fragment was obtained by PCR using the relevant primers (Table 1). The *D. labrax* PepT1 fragment (accession no. AM419037) showed 72, 74, 74 and 76% homology to the corresponding cattle, chicken, human and zebra fish mRNA sequences, respectively.

3.2. Fish weight

As was expected, fish lost weight during the experiment (Fig. 1) with the highest daily weight percent loss, during the first 3 days of the experiment (1.9%). The daily weight percent loss after 14 and

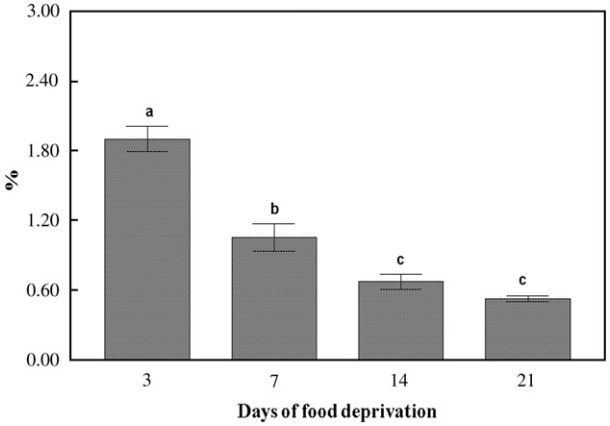

Fig. 1. Daily weight loss rate of *D. labrax* following 3, 7, 14 and 21 days of food deprivation. Values are means ± SEM. Different letters represent significant differences among the days of food deprivation ($P<0.05$).

21 days of food deprivation significantly decreased (0.67 and 0.52%, respectively) in comparison to fish which were starved for 3 or 7 days.

3.3. mRNA expression of intestinal genes during food deprivation

In this study, we evaluated the expression of 4 genes which are located in the intestinal tract during a food deprivation period of 21 day. The Ct ratios (gene Ct:18S Ct) for each of the genes on each of the days of food deprivation in the three sections of the intestinal tract (pyloric caeca, upper and lower intestine) are shown in Table 2. No interaction was found between the two main effects (days of food deprivation and intestine sections) for most of genes. Thus, unless otherwise indicated, the following results apply to the mRNA expression in the entire intestinal tract for all the examined days' comparisons and to the mRNA expression in all the examined days for intestinal section comparisons.

The results show that the duration of food deprivation had a significant effect on the expression of Maltase, APN and PepT1 (Table 2A and B). The Ct ratio of these genes decreased significantly from day 0 to day 7 (increase in fold change expression; Fig. 2A, B and C) with a significant difference in Ct ratio between day 0 and day 7. From day 7 to day 14 there was a significant increase in Ct ratio (decrease in fold change expression, Fig. 2A, B and C). The Ct ratios on days 0, 14 and 21 were not significantly different for all of these genes. Comparisons of the different sections of the intestinal tract showed the highest significant Ct ratio (the lowest expression) for APN in the PC while the highest significant Ct ratio for PpeT1 was found in the lower intestinal section. There were no significant differences in the Ct ratio of maltase among the different intestinal sections (Table 2A and B).

The Ct ratio of Na+/K+-ATPase was significantly influenced by the interaction between the two main effects, days of food deprivation and intestinal sections. The Ct ratio of Na^+/K+-ATPase in the PC fluctuated with significantly lower Ct ratio on days 0 and 7 compared with days 3 and 14. The highest significant Ct ratio was found on day 14. In the upper intestine there were no significant differences in the Ct ratio among the different days. In the lower intestine, Ct ratio on day 21 was significantly higher compared with all but day 3. Among the different intestinal sections, on days 3 and 14, the significantly highest Ct ratio was found in the PC compared with the lower intestinal tract section on day 3 and with both the lower and upper intestinal tract sections on day 14. On days 0, 7 and 21 there were no significant differences in the Na+/K+-ATPase Ct ratio among the different intestinal sections (Table 2B).

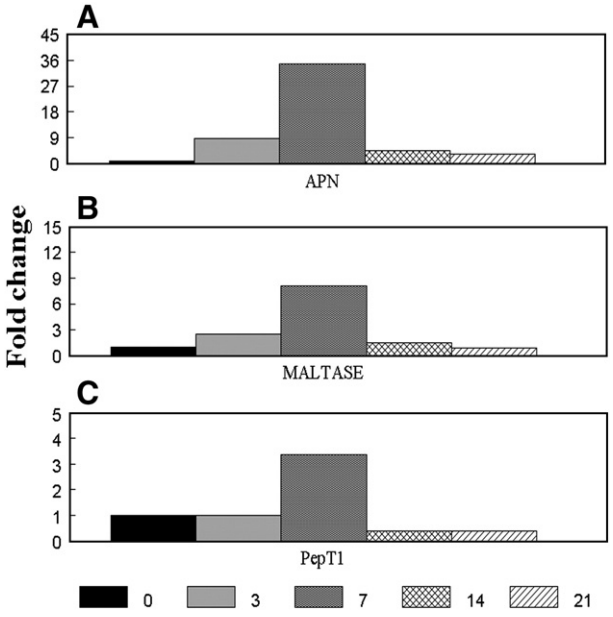

Fig. 2. The n-fold changes among the days of food deprivation for APN (A), Maltase (B) and PepT1 (C) calculated relative to day 0 using the ΔΔCt method of Pfaffl (2001) including the efficiencies for both the experimental gene and 18S (internal controls).

The expression of genes encoding enzymes and transporters of the brush-border membrane is crucial for the ability to digest and absorb food. We therefore set out to determine whether there are correlations among the genes expression of the BBDE and transporters i.e. maltase, APN and PepT1 during days of food deprivation. Indeed, correlation analysis between these genes mRNA expression in the intestinal tract of fish that were not food deprived (day 0) revealed positive significant associations. After 3 days of food deprivation there were weak insignificant correlations among these genes expression. Significant positive correlations were observed again from day 7 of food deprivation and they stayed significantly positive until the end of the experiment. It can be seen that the highest correlations among the

Table 2
The Ct ratios (gene Ct: 18S Ct) for APN and Maltase (A) and PepT1 and ATPase (B) in each of the days of food deprivation in three sections of *D. labrax* intestinal tract (pyloric caeca, upper and lower intestine).

Day / digestive tract section	0	3	7	14	21	Average	0	3	7	14	21	Average
A												
Target gene	APN						Maltase					
Pyloric caeca	1.64 (±0.036)	1.46 (±0.021)	1.37 (±0.026)	1.64 (±0.055)	1.68 (±0.038)	1.54 (±0.031)^{a}	1.41 (±0.040)	1.20 (±0.020)	1.17 (±0.024)	1.39 (±0.041)	1.39 (±0.054)	1.31 (±0.022)^{a}
Upper intestine	1.63 (±0.098)	1.48 (±0.024)	1.31 (±0.031)	1.42 (±0.057)	1.48 (±0.026)	1.47 (±0.029)^{b}	1.30 (±0.062)	1.26 (±0.062)	1.17 (±0.062)	1.20 (±0.062)	1.31 (±0.062)	1.25 (±0.027)^{a}
Lower intestine	1.55 (±0.075)	1.48 (±0.034)	1.44 (±0.047)	1.47 (±0.047)	1.56 (±0.041)	1.50 (±0.023)^{ab}	1.22 (±0.062)	1.32 (±0.013)	1.15 (±0.024)	1.26 (±0.041)	1.35 (±0.042)	1.26 (±0.023)^{a}
Average	1.61 (±0.046)^{A}	1.48 (±0.014)^{BC}	1.37 (±0.022)^{C}	1.50 (±0.036)^{AB}	1.57 (±0.029)^{AB}		1.31 (±0.038)^{A}	1.26 (±0.015)^{AB}	1.16 (±0.024)^{B}	1.28 (±0.031)^{A}	1.35 (±0.026)^{A}	
B												
Target gene	PepT1						ATPase					
Pyloric caeca	1.28 (±0.006)	1.22 (±0.027)	1.09 (±0.023)	1.37 (±0.051)	1.26 (±0.044)	1.24 (±0.025)^{b}	1.12 (±0.043)^{Ca}	1.34 (±0.029)^{Ba}	1.14 (±0.010)^{Ca}	1.48 (±0.043)^{Aa}	1.26 (±0.026)^{BCa}	1.27 (±0.020)
Upper intestine	1.27 (±0.055)	1.25 (±0.028)	1.06 (±0.039)	1.17 (±0.043)	1.23 (±0.047)	1.19 (±0.027)^{b}	1.19 (±0.065)^{Aa}	1.28 (±0.043)^{Aab}	1.16 (±0.035)^{Aa}	1.15 (±0.032)^{Ab}	1.32 (±0.042)^{Aa}	1.22 (±0.032)
Lower intestine	1.32 (±0.050)	1.26 (±0.027)	1.24 (±0.036)	1.37 (±0.068)	1.45 (±0.041)	1.33 (±0.022)^{a}	1.14 (±0.044)^{Ba}	1.20 (±0.014)^{ABb}	1.19 (±0.023)^{Ba}	1.20 (±0.040)^{Bb}	1.34 (±0.037)^{Aa}	1.21 (±0.023)
Average	1.27 (±0.029)^{A}	1.24 (±0.015)^{A}	1.13 (±0.030)^{B}	1.30 (±0.039)^{A}	1.32 (±0.036)^{A}		1.15 (±0.029)	1.27 (±0.023)	1.16 (±0.013)	1.28 (±0.042)	1.31 (±0.022)	

Capital letters represent significant differences ($P<0.05$) among days of food deprivation. Small letters represent significant differences ($P<0.05$) among digestive tract sections.

Table 3
Correlations among BBDE and transporters.

Days of food deprivation	Maltase \ APN	Maltase \ PePt1	APN \ PepT1
0	0.95*	0.68*	0.74*
3	0.21	0.28	0.19
7	0.92*	0.63*	0.60*
14	0.95*	0.83*	0.89*
21	0.91*	0.70*	0.82*

*correlations with asterisk are significant ($P<0.05$).

tested genes expression occurred after 14 days of food deprivation (Table 3).

4. Discussion

In the present study, the transcriptomic aspects of BBE digestion and absorption processes in the intestines of starved fish were investigated. The isolation, for the first time, of gene fragments coding for functional genes in the *D. labrax* enterocytes enabled us to study their expression under the nutritional condition of food deprivation.

Nucleotide-sequence comparison analysis of the maltase, APN, PepT1, and Na^+/K^+-ATPase gene fragments showed 64 to 92% homology to other animal species, emphasizing the "conserved nature" of these genes despite the evolutionary divergence.

Previous studies on the influence of food manipulation on intestinal physiology in fish relied mainly on the use of biochemical assays. Such methods are known to have limitations, as exemplified by Krogdahl et al. (1999) who found that in Atlantic salmon, fasting causes an immediate decrease in maltase activity, a temporary increase during the second week and an overall lower activity during the 60 days of the experiment, compared to the control-fed group. The increase in enzyme activity was explained by the fact that during fasting, non-enzyme proteins may be mobilized from the gut, thereby partially masking changes in enzyme activity (Krogdahl et al., 1999). Therefore, the use of a molecular tool, such as the one developed in this study, is likely to add pivotal information regarding the changes in BBDE and transporters as they adapt to nutritional manipulations.

When food is withheld, an adaptive response rapidly occurs. In rats, starvation causes an atrophic state and after a short period of fasting (2–3 days) the mucosal alterations are characterized by villus hypoplasia, reduced mucosal mass, DNA synthesis and crypt cell production rate (Brown et al., 1963; Clarke, 1975). Habold et al. (2005) described three periods during fasting which can be established through the calculation of specific body mass loss versus time of fasting (Daily weight loss rate). The first phase (phase I) is characterized by a sharp decrease in the daily weight loss rate. This phase is a rapid period of adaptation. At phase II this value reaches a steady rate. This second phase which is a long phase of economizing is marked by an increase in epithelium apoptosis. The third phase shows a rapid increase of daily weight loss rate. At this phase proliferation and enterocyte migration along the crypt villus axis occurs. The daily weight loss rate for *D. labrax* in our study after 21 days of food deprivation was not significantly different from day 14 and therefore, it can be assumed that these fish, after 21 days of food deprivation, are still in phase II. The expression of APN, maltase and PepT1 increased significantly after 7 days of food deprivation but then decreased again. In rats the second phase lasts for the first 5 days of fasting (Habold et al., 2007) and during this time there is an increase in the expression of APN and PepT1 (Ogihara et al., 1999; Ihara et al., 2000; Naruhashi et al., 2002; Habold et al., 2007) most prominently in the upper region (Naruhashi et al., 2002). The mRNA level of SI was not significantly different in the intestine of 5 day food deprived rats (Naruhashi et al., 2002). The differences between the genes mRNA expression of rats and the genes mRNA expression found in the present study can be explained by the fact that fish, unlike terrestrial animals, can undergo long periods of fasting during their life. Aranda et al. (2001) postulated that changes in metabolic status, in food deprived fish, are noticed later than in mammals, these changes rapidly occurring after a few days in the latter. In fish, compared with terrestrial animals, the second phase period might last for longer and therefore, their evolved biological ways of coping with food deprivation might be somewhat different from those of the terrestrial animals.

The active transport of ions (principally Na^+, Cl^-, and HCO^-) across the small intestinal epithelium provides the electrical and chemical forces that drive the coupled absorption of nutrients as well as the net absorption or secretion of water. Although it is known that fasting induces a shift from a neutral state or net absorptive flux to a state of higher secretion (Ferraris and Carey, 2000), the extent to which fasting induced changes in ion and fluid transport during food deprivation is not clear. One of the factors which might affect ion and fluid transport during fasting is the increase in the activity of the basolaterally located Na^+K^+-ATPase pump (Ferraris and Carey, 2000). In *D. labrax*, fasting had the main effect on the mRNA expression of ATPase in the PC with fluctuations in its expression. The increase from day 3 to day 7 and then the decline in Na^+/K^+-ATPase mRNA expression in this section occurred parallel to those of APN, maltase and PepT1. It can be assumed that along the digestive tract the mechanisms which deal with the alternations in BBM potential during fasting might be different in each of the digestive tract sections, while Na^+/K^+-ATPase transporter is mainly activated in the PC interdependent of the biological occurrence in the enterocytes. It should be mentioned that the fish in this experiment were raised in fresh water and since the BBE activity is affected by the salinity of the environment (Harpaz et al., 2005a) their expression is probably different for European sea bass raised in salt water.

A positive linear correlation between APN, maltase and PepT1 mRNA expressions was found throughout the experiment except for day 3. These positive liner correlations might point towards a compensation mechanism rendering available nutrients energy from food or internal reserves in a more efficient manner availing them for growth (e.g. compensatory growth) and physiological maintenance. The low level of digestive enzymes and transporters mRNA expression coupled with the low genes expression correlations found on day 3 might indicate that in fish, the adaptation of the intestine to cope with starvation is longer.

We examined three different sections of the intestine known for their different digestion capacities. The highest activity of the digestive enzymes is usually found in the PC section (Harpaz and Uni, 1999; Harpaz et al., 2005a,b; Tibaldi et al., 2006).However, the current research shows that for all the days examined, the expression level of APN mRNA was the lowest in the PC and for maltase mRNA expression it was the same in all the sections. The PC is an exclusive organ of fish and further research on different fish is needed to clarify this issue.

The present study demonstrates that the quantification of intestinal maltase, APN, PEPT1, and Na^+/K^+-ATPase mRNA expression provides an important tool for studying fish nutrition. The mRNA expression of the tested genes increased significantly after 7 days of food deprivation but then it decreased again. During the fasting period, after day 3, maltase, APN, PEPT1 showed a high significant positive linear correlation between the mRNA genes expression, which increased up to day 14 and then decreased. The fluctuations in the genes expression and correlations observed here can indicate that periodical rhythms occur in the digestive tract of fishes, which cope with continuously food deprivation. More research is needed on fasting fish, using this tool, in order to gain more information about the regulatory mechanisms which are activated with long-term effects of malnutrition and fasting in fish.

The results obtained in this study can provide a new molecular tool for studies on fish nutrition and shed light on the regulatory mechanisms which are activated and their long-term effects on the intestine following prolonged fasting in fish.

Acknowledgment

The authors wish to thank PrimerDesign Ltd (Millbrook Technology Campus, Southampton, Hants, UK) for designing Real Time PCR primers and probs.

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