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Postprandial kinetics of gene expression of proteins involved in the digestive process in rainbow trout (*O. mykiss*) and impact of diet composition

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Abstract The impact of increased incorporation of plant ingredients on diets for rainbow trout was evaluated in terms of gene expression of gastric (gastric lipase, pepsinogen) and intestinal (prolidase, maltase, phospholipase A2) digestive enzymes and nutrient transporters (peptide and glucose transporters), as well as of postprandial levels of plasma glucose, triglycerides and total free amino acids. For that purpose, trout alevins were fed from the start of exogenous feeding one of three different experimental diets: a diet rich in fish meal and fish oil (FM–FO), a plant-based diet (noFM–noFO) totally free from fish meal and fish oil, but containing plant ingredients and a Mixed diet (Mixed) intermediate between the FM–FO and noFM–noFO diets. After 16 months of rearing, all fish were left unfed for 72 h and then given a single meal to satiation. Blood, stomach and anterior intestine were sampled before the meal and at 2, 6 and 12 h after this meal. The postprandial kinetics of gene expression of gastric and intestinal digestive enzymes and nutrient transporters were then followed in trout fed the FM–FO diet. The postprandial profiles showed that the expression of almost all genes studied was stimulated by the presence of nutrients in the digestive tract of trout, but the timing (appearance of peaks) varied between genes. Based on these data, we have focused on the molecular response to dietary factors in the stomach and the intestine at 6 and 12 h after feeding, respectively. The reduction in FM and FO levels of dietary incorporation induced a significant decrease in the gene expression of gastric lipase, GLUT2 and PEPT1. The plasma glucose and triglycerides levels were also reduced in trout fed the noFM–noFO diet. Consequently, the present study suggests a decrease in digestive capacities in trout fed a diet rich in plant ingredients.

Keywords Rainbow trout · Plant-based diet · Nutrient transporters · Digestive enzymes

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Introduction

In the last decade, products of plant origin have increasingly been incorporated into aqua feed as alternative feed ingredients to fish meal (FM) and fish oil (FO) (Naylor et al. 2009). Although the novel diets have been formulated to meet all known requirements and processed to reduce the proportions of anti-nutritional factors of plant origin, the rainbow trout

(*Oncorhynchus mykiss*, a carnivorous fish) is still reluctant to accept plant-based diets. The most recent findings in different carnivorous fish have shown that a high level of replacement of FM and FO by plant products reduces voluntary feed intake (Geurden et al. 2013), feed efficiency (Glencross et al. 2006), protein digestibility (Santigosa et al. 2011), growth rate (Gómez-Requeni et al. 2005; Santigosa et al. 2008; Hua and Bureau 2012) and gut integrity (Glencross et al. 2006). Furthermore, the apparition of most amino acids (essential and non-essential) in the plasma after intestinal absorption is slower in rainbow trout fed protein of plant origin compared to trout fed protein of fish meal origin (Yamamoto et al. 1998; Larsen et al. 2012). In terms of lipids, the incorporation of oil of plant origin in the trout diet reduces plasma cholesterol and low-density lipoproteins (Ingham and Arme 1977; Richard et al. 2006). Given that metabolites enter into the plasma compartment through specific intestinal transporters once the ingested feed has been appropriately hydrolysed into short glucose, monoglycerides and amino acids, it is relevant to study the impact of the replacement of FM and FO by feedstuffs of plant origin on the main plasma metabolites and on gastric and intestinal capacity (digestive enzymes and nutrient transporters).

Understanding of the postprandial (PP) dynamics of gastric and intestinal gene expression in rainbow trout is still limited. However, previous studies in trout have shown the importance of their nutritional status in both gastric and intestinal gene expression. For instance, in a study involving carnivorous white seabream (*Diplodus sargus*), gastric pepsinogen transcription peaked from the twelfth till the twenty-first hour post-feeding (Yúfera et al. 2012). The nature of the diet can also affect the transcription level of intestinal nutrient transporters. PepT1 mRNA abundance was shown to be higher in the omnivorous red crucian carp (*Carassius auratus gibelio* × *Cyprinus carpio*) fed fish meal than in those fed soybean meal (Liu et al. 2014). Starch intake does not enhance the transcription of intestinal glucose transporters GLUT2 in rainbow trout (Kirchner et al. 2008; Kamalam et al. 2013), but is associated with higher expression of Pept1 (Kamalam et al. 2013). Findings regarding the effects of starch on the expression of the glucose transporter SGLT1 are still contradictory in this fish species (Kirchner et al. 2008; Kamalam et al. 2013).

In the study presented here, we first measured in rainbow trout the impact of plant protein and plant oil consumption on plasma metabolites which are, in part, the products of the feed digestion and then, we approach the postprandial molecular regulation of proteins involved on digestion. However, it first seemed crucial to study the postprandial kinetics of gene expression involved in the digestive process in this fish species and in our experimental conditions. The aim was to determine how long after the last meal it would be appropriate to carry out the sampling needed for the study of the GIT response to the alternative diets. We then compared the molecular response of the GIT of trout fed a diet rich in fish meal and fish oil with that of the GIT of trout fed a diet with partial or total replacement of fish meal and fish oil by plant proteins and vegetal oils. The study focused on the gene expression of the main gastric and intestinal digestive enzymes (pepsinogen, gastric lipase, prolidase, maltase and phospholipase) and of the main intestinal nutrient transporters (PEPT1, SGLT1 and GLUT2). Enzyme gene expression was then compared to the digestion end products, i.e. plasma glucose, triglycerides and amino acid levels.

Materials and methods

Fish and diets

The experiment was carried out in accordance with the Guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree N°2001-464, May 29, 2001) and the Ethics Committee of INRA (INRA 2002-36, April 14, 2002).

The fish used in this experiment came from another study supported by the FP7 programme of the European ARRAINA project (Advanced Research Initiatives for Nutrition and Aquaculture) (N°288925) and by the FUI VegeAqua project. As described in Lazzarotto et al. (2014), the aim was to study the long-term effects of diets free of fish meal (FM) and fish oil (FO), from first feeding onwards. Rainbow trout alevins((*Oncorhynchus mykiss*; initial body weight of 0.14 g) produced by the INRA experimental fish farm at Lees Athas (Pyrénées Atlantique, France; natural photoperiod and constant water temperature of 7 °C) were fed from their first meal one of three different experimental diets: a diet rich in fish meal and fish oil (FM–FO), a plant-based diet (noFM–noFO) totally free from fish meal and fish oil, but

containing plant ingredients and a Mixed diet (Mixed) intermediate between the FM–FO and noFM–noFO diets (Table 1). After 7 months of rearing, the fish were transferred to the INRA experimental fish farm of Donzacq (Landes, France; natural photoperiod and constant water temperature of 17 °C) where they were reared for another 8 months, till the final sampling time.

At the end of the study, the trout (15 months old) fed the noFM–noFO diet had significantly lower body weights ($p < 0.001$) than those fed the FM–FO and Mixed diets (248 ± 8 vs. 357 ± 9 and 371 ± 8 g, respectively) and a lower feed intake when expressed as g of feed ingested per fish (3.85 ± 0.01 vs. 5.75 ± 0.08 and 5.62 ± 0.19 g/trout/day, respectively) over the whole period. However, when the feed intake was expressed as percentage of g fish body weight, there was no difference between the dietary treatments (1.46 ± 0.03 % body weight/day). More was there any difference between the treatments in the global feed efficiency (1.07 ± 0.03 g/g). In fact, the growth difference was mainly caused by a transiently lower feed efficiency in trout fed the noFM–noFO diet measured during the rearing at Lees Athas (water temperature: 7 °C), then during the first 3 months of feeding at Donzacq (water temperature: 17 °C).

All diets were manufactured at the Donzacq INRA facility (France) using a twin-screw extruder (Clextral). Diets were formulated to meet the nutritional requirements of rainbow trout (NRC 2011). There were three dietary conditions as already described: the FM–FO, noFM–noFO and Mixed diets. In the noFM–noFO and Mixed diet, in order to avoid exceeding anti-nutritional factor content, we used a blend of different plant ingredients. Synthetic L-lysine, L-methionine, $CaHPO_4.2H_2O$ and soy lecithin were added to the noFM–noFO diet to correct any deficiency in essential amino acids, phosphorous and phospholipid. The three experimental diets were formulated to be as isonitrogenous and isoenergetic as possible, but in reality, although the measured energy content was similar, the protein and fat content levels measured were lower with the FM–FO diet (protein 43.9 % DM, fat 21.8 % DM) than with the Mixed and noFM–noFO diet (protein 46.1–46.3 % DM, fat 24.2–25 % DM) (Table 1). On a digestible basis (calculated data), there was also some differences: from 37 to 41 % DM of digestible proteins and from 18 to 20 kJ/g DM for digestible energy, the diet noFM–noFO diets being

that with the highest digestible protein and energy contents. The dietary level of starch was very different, the highest level (19.3 % DM) was obtained with the FM–FO diet (rich in FM but also containing 30 % whole wheat) and the lowest with the noFM–noFO diet (7.8 % DM). Trouts were fed the experimental diets until visual satiety. Diets were distributed manually, and the number of meals was adjusted according to the physiological needs of the fish: from eight meals per day in alevins to only one meal per day in ongrowing trouts at the end of the study.

Sampling

Fifteen months after their first feeding with the three different diets, all fish were left unfed for 72 h and then given a single meal to visual satiation. The aim of this 72 h of starvation was to obtain the basal levels of plasma metabolites, reached later in fish than in mammals due to slower intestinal transit and longer gastric emptying time at low temperatures compared to endothermic animals.

Samplings were performed at the end of the fasting period (starved) and 2, 6 and 12 h after the single meal (H2, H6 and H12). At each sampling time, nine fish per condition were killed by anaesthetic overdose (benzocaine at 0.06 %). Blood was removed from the caudal vein into heparinized syringes and rapidly centrifuged (3000 g, 5 min); the plasma recovered was then frozen and kept at −20 °C until analysis of the plasma metabolites. In order to study the digestive and absorptive capacity of the GIT, we focused on the main digestive enzymes and transporters present in the pyloric portion of the stomach and in the anterior portion of the intestine. We intentionally discarded the more distal intestinal region because the more anterior region makes a greater contribution to nutrient absorption (Ferraris and Ahearn 1984). The pyloric stomach and midgut were opened lengthwise in order to remove the remaining digested feed. Both were then rinsed in three successive baths of physiological salt solution (NaCl 0.9 %), and the scrubbed mucosa were immediately frozen in liquid nitrogen and kept at −80 °C until analysis.

Analysis of plasma metabolites

Circulating metabolite levels were measured using commercial kits: plasma glucose using the Glucose

Table 1 Ingredient composition and nutrient composition of the three experimental diets

	FM–FO	Mixed	noFM–noFO
Ingredients (%, as fed basis)			
Norwegian herring meal (CP 70; Sopropêche, France)	54.3	30.0	0
Corn gluten meal (CP 60; Inzo, France)	0	10.3	18.0
Wheat gluten (CP 70; Roquette, France)	0	5.0	12.1
Soybean meal (CP 48; Inzo, France)	0	6.3	4.3
Soy protein concentrate (Estrilvo; CP 70; Sopropêche, France)	0	3.5	18.1
White lupin meal (Terrena, France)	0	6.5	5.0
Extruded peas (Aquatex, Sotexpro, France)	0	7.0	2.4
Rapeseed meal (SudOuest Aliment, France)	0	6.3	9.9
Extruded whole wheat (SudOuest Aliment, France)	30.1	7.2	2.8
Fish oil (Southern hemisphere, Sopropêche, France)	13.6	8.0	0
Rapeseed oil (Daudruy, France)	0	8.0	7.3
Linseed oil (Daudruy, France)	0	0	7.4
Palm oil (Daudruy, France)	0	0	3.0
Soy lecithin (Louis François, France)	0	0	2.0
L-lysine (Eurolysine)	0	0.05	1.5
L-methionine (Evonik, Germany)	0	0	0.3
CaHPO4.2H20 (18 %P; 22 % Ca)	0	0	2.6
Attractant mix[a]	0	0	1.5
Mineral premix[b]	1.0	1.0	1.0
Vitamin premix[c]	1.0	1.0	1.0
Analysed macro-nutrient composition			
Dry matter (DM, % diet)	96.3	96.0	96.3
Protein (% DM)	43.9	46.3	46.1
Fat (% DM)	21.8	24.2	25.0
Energy (kJ/g DM)	24.5	23.8	25.3
Ash (% DM)	7.9	6.1	5.7
Starch (% DM)	19.3	11.41	7.8
Nutrients composition on a digestible basis[d]			
Digestible protein (% DM)	38.0	37.1	40.7
Digestible energy (% DM)	17.7	19.0	20.1

DM dry matter

[a] Attractant mix: glucosamine, 0.5 g; taurine, 0.3 g; betaine, 0.3 g; glycine, 0.2 g; alanine, 0.2 g

[b] Mineral premix (g or mg kg^{-1} diet): calcium carbonate (40 % Ca), 2.15 g; magnesium oxide (60 % Mg), 1.24 g; ferric citrate, 0.2 g; potassium iodide (75 % I), 0.4 mg; zinc sulphate (36 % Zn), 0.4 g; copper sulphate (25 % Cu), 0.3 g; manganese sulphate (33 % Mn), 0.3 g; dibasic calcium phosphate (20 % Ca, 18 %P), 5 g; cobalt sulphate, 2 mg; sodium selenite (30 % Se), 3 mg; KCl, 0.9 g; NaCl, 0.4 g (UPAE, INRA)

[c] Vitamin premix (IU or mg kg^{-1} diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3,000 IU; thiamine, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium pantothenate, 50 mg; choline chloride, 2000 mg (UPAE, INRA)

[d] Calculated from ADC values given in Sauvant et al. (2004), NRC (2011) and Feedipedia (Animal feed resources information system; http://www.feedipedia.org)

RTU[TM] kit from BioMerieux (Marcy-l'Etoile, France), plasma triglycerides using the WTRIG050 kit from Sobioda (Montbonnot-Saint-Martin, France) and total plasma free amino acids by the ninhydrin reaction (Moore 1968), with glycine (Sigma) as the standard.

Gene expression analysis by quantitative RT-PCR

Samples were taken from trout fed the FM–FO diet for the study of the kinetics of gene expression. Using the data obtained in the kinetics study, we chose the most appropriate postprandial times to study the impact of replacement of the FM–FO diet by Mixed and noFM–noFO diets.

Total RNA was extracted from the scrubbed mucosa of the pyloric stomach and of the midgut using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations ($n = 6$ for each condition). Concentration (ng/μL) and purity (A260 nm/A280 nm) of the isolated RNA were measured using a NanoDrop, and the integrity of the RNA extracted was evaluated by agarose gel electrophoresis.

Total RNA (1 μg) was then used for the reverse transcription. RNA was reverse transcribed into cDNA using the SuperScriptIII RNaseH-reverse transcriptase kit (Invitrogen) and random primers (Promega, Charbonniéres, France) according to the manufacturers' protocols.

Relative gene expression of mRNA was then determined by quantitative real-time PCR using the Roche Lightcycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France). The assays were performed using a reaction mix of 6 μL per sample, each of which contained 2 μL diluted cDNA template, 0.12 μL of each primer (400 nM), 3 μL Light Cycler 480 SYBR® Green I Master mix and 0.76 μL DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany).

The PCR protocol was initiated at 95 °C for 10 min for initial denaturation of the cDNA and hot-start *Taq* polymerase activation, followed by 45 cycles of a two-step amplification programme (15 s at 95 °C; 40 s at 60 °C). Melting curves were systematically monitored (temperature gradient ranging from 65 to 94 °C) at the end of the last amplification cycle to confirm the production of a single product. Each PCR assay included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (reverse transcriptase- and cDNA template-free samples). The qPCR assay was optimized with a linear standard curve ($R^2 > 0.98$) and checked for congruity across replicates. PCR efficiency for each run was estimated based on the slope of the five-point standard curve obtained with serial dilutions of pooled sample cDNA. PCR efficiency ranging from 1.8 to 2.2 was considered to be acceptable. Gene expression data obtained from qPCR were normalized using the NORMA-gene algorithm, which uses a least squares method to minimize data variability and which does not require the identification and validation of reference genes to calculate a normalization factor without the use of reference genes (Heckmann et al. 2011).

The transcripts analysed in the stomach were pepsinogen (PEPS) for protein luminal digestion and gastric lipase (GLIP) for lipid luminal digestion. The transcripts analysed in the intestine were the peptide transporter Pept1 for peptide transport, prolidase (Prld) for cytosolic peptide digestion, maltase for membrane-bound maltose hydrolysis, the glucose transporters GLUT2 and SGLT1 and phospholipase A2G12a (PhLip) for luminal phospholipid digestion (Table 2). The GLIP, Peps, Prld and PhLip primers were designed using the Primmer3plus browser (http://bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) based on the cDNA sequences obtained from the Sigenae (http://sigenae.org/), Genoscope trout (http://genoscope.cns.fr/trout/) or the Institute for Genome Research (TIGR) genome databases. When it was possible, the primers thus designed were constructed on cDNA sequences that covered two different exons, in order to avoid the amplification of residual DNA from the RNA extraction. Amplicons obtained after PCR were sent for sequencing for validation of each primer. Primers were validated when the sequences obtained matched well with their corresponding cDNA sequence on the ClustalW2 sequence alignment browser (http://ebi.ac.uk/Tools/msa/clustalw2/) and when the blast of the sequence obtained on the NCBI database yielded the expected protein.

Statistical analysis

Statistical analyses on gene expression data and plasma metabolite levels were performed using R.2.14.0. As the body weight of the trout was significantly different between the groups of trout (lower in fish fed the noFM–noFO diet), it was used as covariate in the statistical analysis. Two-way ANCOVA was performed to test whether an

Table 2 Sequence of primers used for qRT-PCR

Organ analysed	Gene	Primer sequence (5′–3′)	Database, Accession number
Stomach	PEPS	F: GTTTCGGTGGGAGGCATCT	Sigeneae, **CX136077.s.om.10**
		R: TGTTTCCGAAAGACACCACA	
Stomach	GLIP	F: TCAACATGACTCGCACACCT	Sigenae, **CB492742.s.om.10**
		R: TTCATGTCCCGGATGTTGTA	
Intestine	PEPT1[a]	F: CCTGTCAATCAACGCTGGT	GenBank, EU853718
		R: CACTGCCCATAATGAACACG	
Intestine	Prolidase	F: GCCCATAAAGAGATCATGAAGC	Genoscope, GSONMT00010279001
		R: CGTTGTTTCCAGTTCCACAG	
Intestine	Maltase[b]	F: GCAGCAGGAATACCCTACGA	TIGR, TC3451
		R: GGCAGGGTCCAGTATGAAGA	
Intestine	GLUT2	F: GTGGAGAAGGAGGCGCAAGT	GenBank, AF321816
		R: GCCACCGACACCATGGTAAA	
Intestine	SGLT1[b]	F: TCTGGGGCTGAACATCTACC	GenBank, AY210436
		R: GAAGGCATAACCCATGAGGA	
Intestine	Phospholipase A2G12	F: TGTGTCATGTAGTAAGGAACCTGAA	Genoscope, GSONMT00024336001
		R: TTTGTAACCATCGCTGCATT	

Primers reported by: [a] Ostaszewska et al. (2010), [b] Geurden et al. (2007)

interaction existed between the diet and the time of sampling ($p < 0.05$). Gene expression data and plasma metabolite levels were first tested for normality and homogeneity of variances by Shapiro–Wilk and Leven tests, respectively. Additional assumptions were controlled for ANCOVA (linearity of the relationship between the variable and the covariate, homogeneity of regression slopes). Data were then submitted to one-way ANCOVA ($p < 0.05$) to test the significance of the effects of diet or time of sampling when appropriate. When the normality and the homogeneity of variance were significant, post hoc comparisons were made using the parametric Newman–Keuls tests, but the nonparametric Mann–Whitney–Wilcoxon test ($p < 0.05$) was used when at least one of the assumptions did not passed.

Results

Evolution of the filling rates of the stomach and intestine before and after a meal

Figure 1 shows the filling rates of the stomach and intestine of the sampled trout. The stomachs and intestines of all the starved fish were totally empty.

Two hours after the meal, the stomachs of almost all fish (about 90 %) were full, with the chyme just starting to fill the intestine: the intestines of the majority (56–78 %) of the trout were already partially filled. Six hours after the meal, the stomachs of many trout (67–78 % of individuals) were still full and the intestines were partially filled (33–67 %) or full (11–67 %). However, the intestines of some individuals (11–22 %) were still empty. Twelve hours after the meal, the stomachs of all fish were still full (67–89 %) or partially filled (11–33 %), and the intestines were also still full (33–56 %) or partially filled (44–67 %).

Impact of nutritional status on the plasma metabolite levels

The levels of plasma metabolites were measured for each experimental diet (noFM–noFO, Mixed and FM–FO diets) at each sampling time (in starved fish, then 2, 6 and 12 h after the meal). There was no significant "Diet*Sampling Time" interaction (two-way ANCOVA; $p = 0.151$, $p = 0.416$, $p = 0.193$ for glucose, triglycerides or amino acids, respectively) for any of the metabolites studied after controlling for the effect of the trouts body weight. However, there
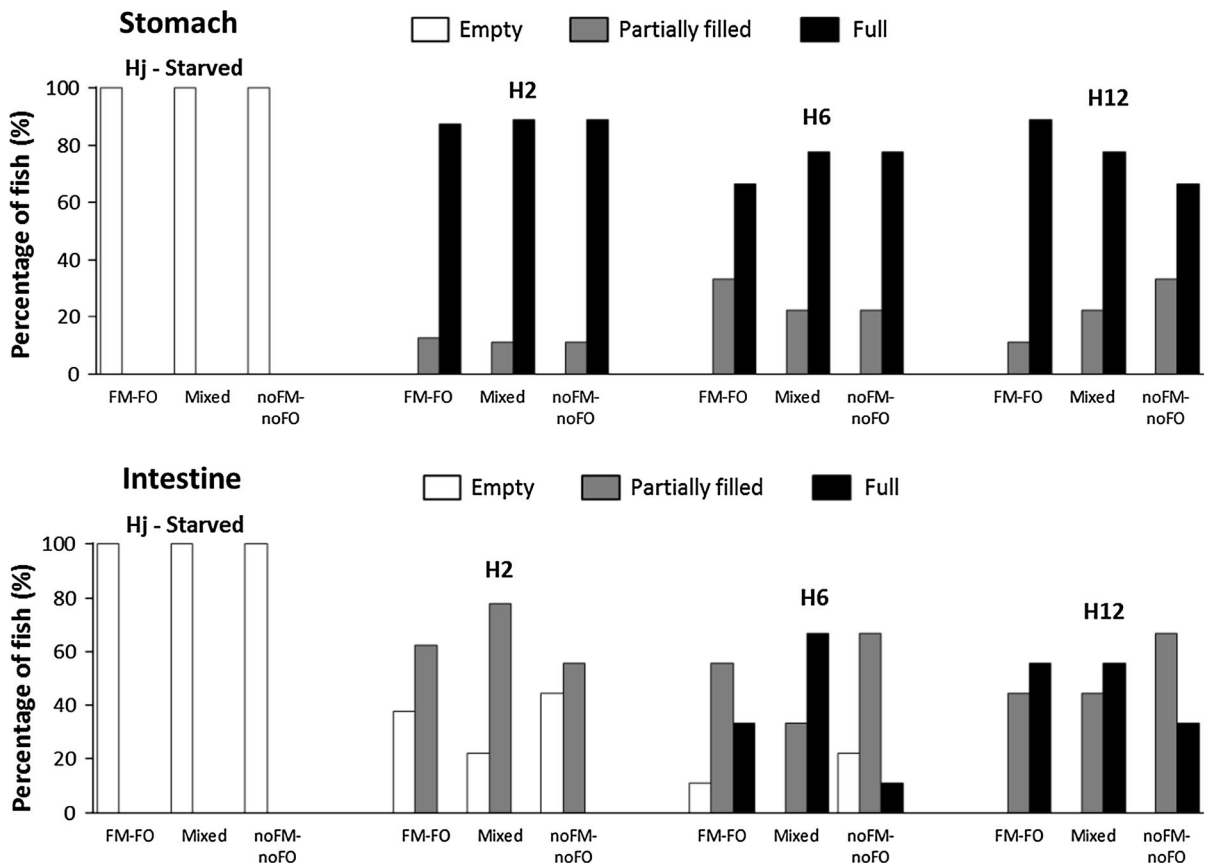
Fig. 1 Evolution of the filling rates of the stomachs and intestines of trout ($n = 9$ per diet and sampling time) fed the different diets (FM–FO, Mixed and noFM–noFO) at four different sampling times (starved, then 2, 6, and 12 h after the last meal)

were some significant "diet" and "time" effects for some of the metabolites (Fig. 2).

When the evolution of the plasma glucose levels over time was considered (Fig. 2a), there were significant changes only with the Mixed diet ($p = 0.046$), with a significant peak observed 6 h after the meal (one-way ANCOVA with control for the effect of the trouts body weight). However, although there was a significant "diet" effect ($p = 0.031$) only 12 h after the meal, the plasma glucose level being significantly lower in trout fed the noFM–noFO diet compared to the others, the pattern of evolution over time seemed different according to the diet. The basal plasma (in starved fish) glucose level was slightly higher in trout fed the FM–FO diet than that observed with the Mixed and noFM–noFO diets. The glucose level then increased but later (from 12 h after the meal) than that observed in the trout fed the Mixed diet (from 2 h after the meal). The plasma glucose level remained

low in trout fed the noFM–noFO diet, i.e. in a normal range, throughout the postprandial period.

The evolution of the plasma triglyceride levels over the postprandial period appeared similar in trout fed the FM–FO and Mixed diets (Fig. 2b), increasing with time and presenting a peak 12 h after the meal, although it was significant ($p = 0.009$) only with the Mixed diet. In contrast, the triglyceride levels remained low over time in trout fed the noFM–noFO diet and they were significantly lower than those observed in the FM–FO and Mixed diet trout 12 h after the meal ($p = 0.049$).

The pattern of evolution of the total amino acid levels over time (Fig. 2c) was different from that observed with the other metabolites. Indeed, the basal level (H0) was significantly lower in trout fed the FM–FO diet compared to the others ($p = 0.009$). The plasma amino acids levels then increased during the postprandial period although it was not significant in

Fig. 2 Plasma glucose (**a**), triglycerides (**b**) and total amino acid (**c**) levels of trout ($n = 9$ per diet and sampling time) fed different diets (FM–FO, Mixed and noFM–noFO) at four different sampling times (starved, then 2, 6, and 12 h after the last meal). For each diet, values (means ± SE) *without common letters* over the four sampling times are significantly different, while *asterisks* indicate significant differences detected between the three dietary treatments at each sampling time ($p < 0.05$; two-way ANCOVA using the fish body weight as covariate, and Newman–Keuls test as post doc test)
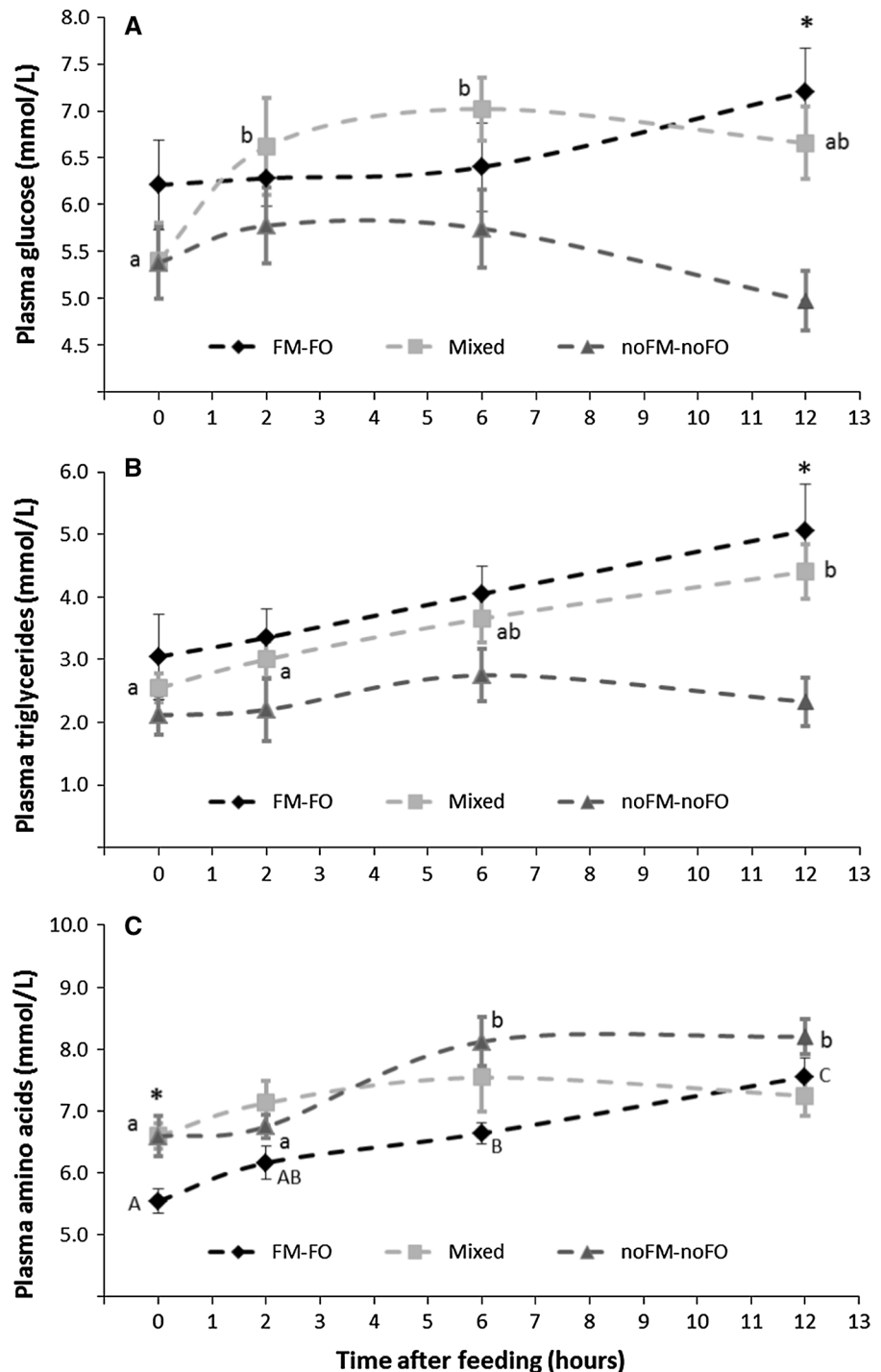
trout fed the Mixed diet. In trout fed the noFM–noFO diet, a significant peak was observed from 6 h after the meal ($p = 0.0006$), while it appeared later in the FM–FO-fed trout, i.e. from 12 h after the meal ($p = 0.00008$).

Postprandial kinetics of the expression of genes involved in the digestive process

Comparative analysis of the expression of genes involved in the digestive utilization of feed measured

at four postprandial times (fasted, 2 PP, 6 PP and 12 PP) in trout fed the FM–FO diet showed that the expression of some genes measured in the stomach and in the proximal intestine were significantly affected by nutritional status, even after controlling for the effect of the trouts body weight (covariate) (Fig. 3).

The expression of the three studied genes involved in the digestion and absorption of carbohydrates (Fig. 3a) in proximal intestine increased over the postprandial period, although it was not significant in the case of the maltase gene, maybe because of an higher inter-individual variation ($p = 0.072$, $p = 0.0001$ and

Fig. 3 Postprandial kinetics of the expression of genes involved in the digestion and absorption of carbohydrates (**a**), lipids (**b**) and protides (**c**) in the stomach and proximal intestine of rainbow trout fed a fish meal-based diet (FM–FO) at four different times (starved, then 2, 6, and 12 h after the last meal). For each gene, means (±SE, $n = 6$) *without common superscript letters* are significantly different, while *asterisks* indicate significant differences detected between genes at each sampling time ($p < 0.05$; one-way ANCOVA using the fish body weight as covariate, and Newman–Keuls test as post hoc test)
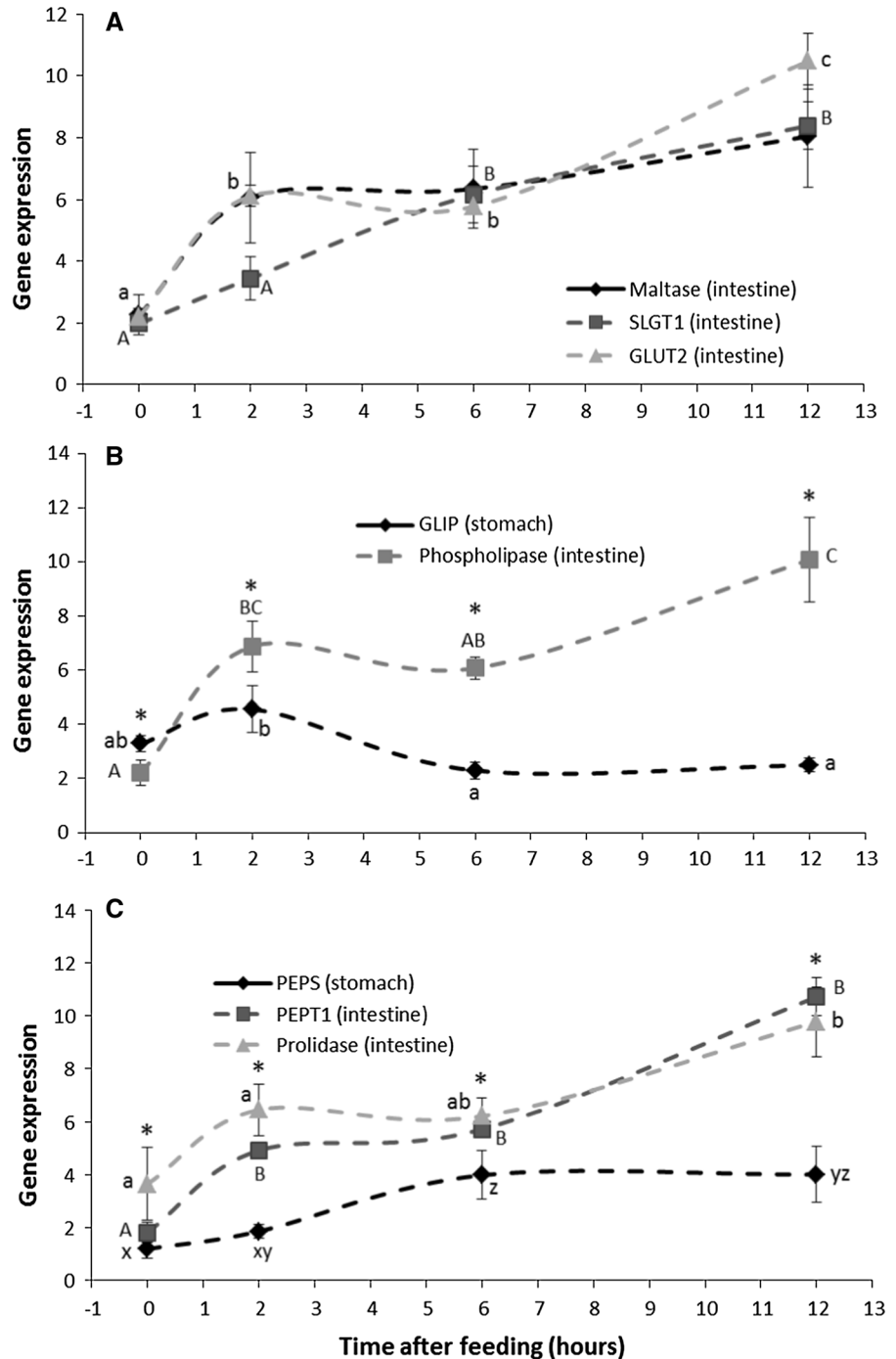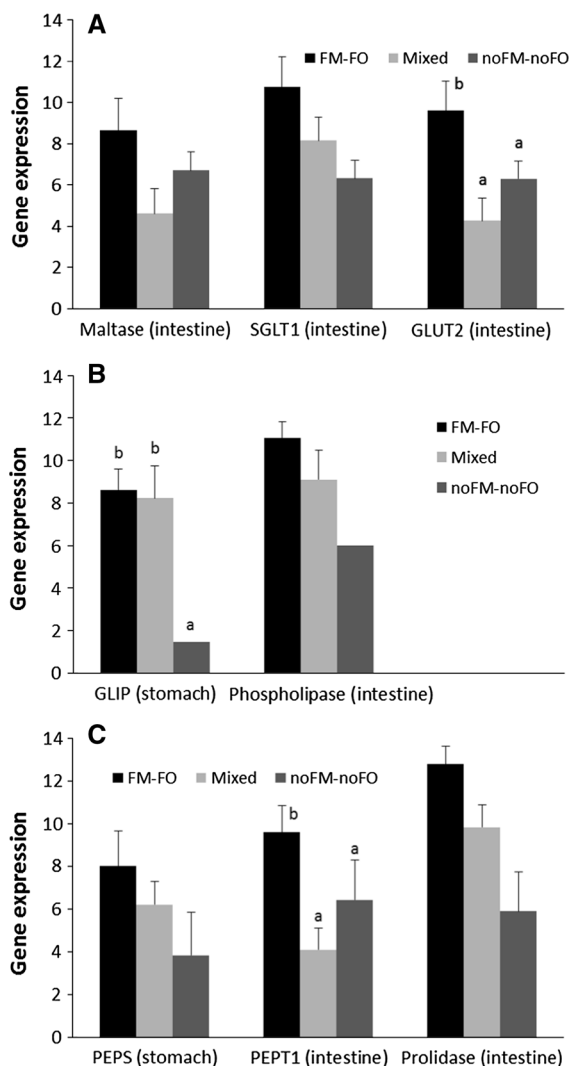
Fig. 4 Effect of feed composition on the expression of genes involved in the digestion and transport of carbohydrates (**a**), lipids (**b**) and protides (**c**) in the stomach (sampled 6 h after the meal) and proximal intestine (sampled 12 h after the meal) of rainbow trout fed a fish meal-based diet (FM–FO), a Mixed diet (Mixed) or a plant-based diet (noFM–noFO). For each gene, means (±SE, $n = 6$) *without common superscript letters* are significantly different ($p < 0.05$; one-way ANCOVA using the fish body weight as covariate, and Newman–Keuls test as post hoc test)

$p < 0.00001$ for maltase, SGLT1 and GLUT2, respectively). The gene expression of the brush border enzyme maltase and of the apical membrane transporter SGLT1 increased early after the meal (from 2 PP), while it started to increased later (from 6 PP) in the case of the basal membrane transporter GLUT2. The highest

values were observed at the latest sampling time (12 PP) for these three genes.

The maximum expression of the gene involved in lipid digestion in the stomach (GLIP) was recorded very early, i.e. only 2 h after the meal ($p = 0.023$) (Fig. 3b). In the intestine, the gene expression of phospholipase ($p = 0.003$) started to increase also early after the meal (2 PP), but the maximum expression was observed at the latest sampling time (12 PP). The basal level of expression of GLIP in the stomach was significantly higher ($p = 0.003$) than that of the gene coding for phospholipase, but the situation is reversed after the meal ($p < 0.0001$, $p < 0.000001$ and $p = 0.0002$ at 2 PP, 6 PP and 12 PP, respectively).

Concerning the digestion and absorption of protides (Fig. 3c), surprisingly, the expression of the PEPS-encoding gene in the stomach was significantly higher only 6 and 12 h PP compared to its expression level in starved fish ($p = 0.002$) and remained significantly lower than that of the intestinal genes (PEPT1 and Prolidase) during the postprandial period ($p < 0.00001$, $p < 0.000001$, $p < 0.00001$ at 2 PP, 6 PP and 12 PP, respectively. The postprandial kinetic of the expression of the brush border membrane transporters (PepT1) and of the enterocyte cytosolic digestive enzyme (prolidase) were similar and both affected by the nutritional status of the trouts ($p < 0.000001$ and $p = 0.047$, respectively): the levels of expression started to increase already 2 h after the meal and the maximum were observed at the last sampling time (12 PP).

Effects of diet composition on the gastro-intestinal expression of genes involved in the digestive process

In accordance with the results of the kinetics study, the effects of diet composition were studied on the gene expression of all the digestive enzymes studied and on the nutrient transporters from the intestine at 12 PP. The gene expression of PEPS in the stomach was studied at 6 PP. The choice of the right time to study GLIP gene expression was more difficult because, although the kinetics study showed that the maximum level of gene expression was reached 2 h after the meal that seemed very early after feed intake. As a consequence, expression of the GLIP gene was studied twice, i.e. at 2 and 6 h after the meal.

The expression of almost all genes (excepted PEPS) was significantly impacted by the diets composition when the trouts body weight was not used as covariate in the statistical analyse. However, when the effect of body weight was controlled, the "diet" effect concerned a lower number of genes (Fig. 4).

Among the three studied genes involved in the digestion and absorption of glucides (Fig. 4a), only the expression of the intestinal transporter GLUT2 was significantly affected by the diet composition ($p = 0.003$): the FM–FO diet induced a higher GLUT2 expression than the Mixed and noFM–noFO diets. For lipids digestion (Fig. 4b), only the expression in the stomach of GLIP was affected by the diet composition, but only 6 h after the meal ($p = 0.0005$): it was significantly more highly expressed in trout fed the FM–FO and Mixed diets than in trout fed the noFM–noFO diet. At 2 PP, no significant difference was observed ($p = 0.66$) (data not shown). Concerning the protides digestion and absorption (Fig. 4c), PEPT1 gene transcription in the intestine was significantly enhanced in trout fed the FM–FO diet compared to those fed the Mixed and the noFM–noFO diets ($p = 0.007$). The expression of the two other genes was not significantly impacted by the diet.

Discussion

The study presented here addressed the impact of the nature of the diet (with greater or lesser quantities of fish meal and fish oil) on the concentration of the main plasma metabolites in rainbow trout and then focused on the gene expression of important digestive enzymes and nutrient transporters. We therefore first defined the most appropriate time to sample the stomach and the intestine for measurement of the gene expression of the digestive enzymes and nutrient transporters investigated here. Targeted genes (gastric and intestinal digestive enzymes: PEPS, GLIP, prolidase, maltase and phospholipase; intestinal nutrient transporters: PEPT1, SGLT1, GLUT2) were chosen since they contribute to the digestion and absorption of the main feed components: carbohydrates, lipids and proteins.

Kinetics of digestion

The time the digesta remains in the different gastrointestinal compartments in rainbow trout depends on the trout body weight, the rearing water temperature and the dryness of the pellets (Fauconneau et al. 1983; Ruohonen et al. 1997), but also most probably the size of the meal. In rainbow trouts weighing 300–400 g reared at 10–13 °C, when they receive a single meal (commercial diet, ration of 2 % of their body mass) after a 1 week of fasting, the chyme leaves the stomach and starts to fill the anterior intestine 8 h after the meal and the intestine finishes emptying at 72 h (Bucking and Wood 2006). In our study, with trout weighing 250–370 g reared at 17 °C, the chyme had already filled the intestine 2 h after a single meal in the majority of individuals, and this compartment was still full or at least partially filled 12 h after the meal in all fish. Obviously, the higher water temperature in our study led to a higher chyme transfer speed between the stomach and the intestine. However, as the last sampling was performed 12 h after the meal, it was not possible in our study to determine whether transit of the chyme would also have been shorter in the intestine at 17 °C than at 10–13 °C.

This study demonstrated that the expression of all the gastrointestinal genes studied were related to the nutritional status of the trout and the existence of temporal dynamics, as already suggested in the white seabream (Yúfera et al. 2012) and in the red crucian carp (Liu et al. 2014). In stomach, a peak of expression of the gastric lipase (GLIP) gene occurred rapidly, from the second hour PP, and was already complete 6 h after the meal, whereas the stomach was still full or partially filled with chyme in all trout 12 h PP. There was also an increase in pepsinogen (PEPS) gene expression, but this occurred later. Indeed, it occurs from 6 h PP and is not fully complete 12 h PP. Yúfera et al. (2012) reported a peak of PEPS gene expression occurring even later in white seabream juveniles (*Diplodus sargus*) reared at 20 °C, between 14 and 19 h PP. As the stomach is the first part of the gastrointestinal tract filled by the feed, the interval between feed intake and the peak of PEPS gene expression seems long. Although PEPS and GLIP are synthesized by the same gastric cells, in vitro studies performed on human gastric explants suggest that the PEPS and GLIP genes are regulated differently in the human gastric mucosa (Basque and Ménard 2000). It has been suggested that regulatory peptides, such as glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK), may influence the secretion and/or activity of GLIP in humans (Wøjdemann et al. 1999; Borovicka et al.

2000), and it has been hypothesized that, as in mammals, the gene expression of PEPS is stimulated in fish by a hormone, i.e. gastrin. Gastrin is released by the stomach (pyloric part) and intestinal endocrine cells in response to the presence of feed in the lumen (Rust 2002).

In the proximal intestine, gene expression of all the proteins studied (prolidase, maltase, phospholipase, peptide transporter PEPT1 and glucose transporters GLUT2 and SGLT1) had already started to increase 2 h after the meal and the maximum levels were observed at the last sampling time (12 h PP). This timing seemed to be in good agreement with the filling rate of the intestine. Santigosa et al. (2008) reported a peak in intestinal alkaline protease activity much earlier (3 h after a meal) in 120–150 g rainbow trout reared at the same temperature as in our study (17 °C) and fed different diets, but no peak of activity was observed for α-amylase. It is difficult to compare the gene expression of one enzyme (prolidase) and the activity of a family of enzymes (proteases). However, it is surprising that the highest level of gene expression observed in our study occurred after the peak activity reported by Santigosa et al. (2008). The difference in fish size might be one explanation.

The postprandial profiles of expression of all the studied genes evidenced that these expressions were stimulated by the presence of nutrients in the digestive tract of rainbow trout and suggested that the secretion of the corresponding digestive proteins would be also stimulated. This is in good agreement with the data of Castro et al. (2016) in juvenile sea bass (*Dicentrarchus labrax*) sampled 2, 6 and 12 h after the meal. Indeed they demonstrated that the activity of the intestinal amylase, lipase, alkaline phosphatase, total alkaline protease and trypsin are stimulated by the feed intake from 2 h PP. The enzyme activities were very low 24 h post-feeding, indicating that digestive processes were already completed.

In plasma, amino acids, glucose and triglyceride concentrations were significantly higher from about 6 h PP for the two first nutrients and about 12 h PP for the latter. These results suggest that the supply of amino acids and glucose from feed digestion occurs earlier than the supply of triglycerides from dietary fat digestion, although the timing of gene expression of the different enzymes and transporters involved in their digestion and absorption was similar in the intestine. However, the lipids could only not be visible

so early in the plasmatic compartment as they took the lymph circulation way. In addition, although we analysed intestinal digestion and absorption efficiency in our study through measurement of the plasma metabolites, it should be borne in mind that the dynamics of the plasma metabolites are not the only consequence of intestinal absorption, but are also related to the tissue metabolic utilization of the nutrients.

Impact of the nature of the diet on digestion

Following the results of the kinetics study, the impact of partial or total replacement of fish meal with protein and lipids of plant origin was studied on the gene expression of certain digestive enzymes and nutrient transporters in the stomach at 2 and 6 h PP and in the intestine at 12 h PP.

At the end of the study, the trout fed the noFM–noFO diet had significantly lower body weights than those fed the FM–FO and Mixed diets. It is why the body weight of the fish was used as a covariate in the statistical analyses performed. The size of the last meal distributed up to satiety to the trout was not measured in our study. However, feed intake measured during the rearing period at the Donzacq fish farm was lower in fish fed the noFM–noFO diet when expressed as g of feed ingested per fish. But when the feed intake was expressed as percentage of g of fish body weight, there was no difference between the dietary treatments. The following discussion is based on the hypothesis that the size of the last meal reflected, for each diet, the same pattern.

It is important to note that the feed efficiency and growth rates were also similar during the last period of the experiment, whatever the diet. It seems that the trout finally adapted to the noFM–noFO diet after a transitory period of lower feed intake and feed efficiency, although this adaptation did not result in compensatory growth. Indeed, trout fed this diet presented lower final body weights.

In our study, as suggested by their plasma levels, the replacement of fish meal by plant ingredients did not seem to reduce the rate of absorption of total amino acids: the evolution of the plasma total amino acid levels over time was similar whatever the diet and the basal levels (in starved fish) were even significantly higher in trout fed the noFM–noFO and the Mixed diets than in those fed the FM–FO diet. These higher

basal levels may have resulted from higher levels of protein in the noFM–noFO diet (46.1 % DM) and Mixed diet (46.3 % DM) than in the FM–FO diet (43.9 % DM). However, the gene expression of the proteins involved in the digestion and transport of protides seemed to follow the opposite pattern: the lowest values were observed with the diets rich in plant ingredients, although it was significant only for the gene expression of the transporter PEPT1. Santigosa et al. (2008) observed a much stronger decrease in postprandial alkaline protease activity (especially trypsin) in trout fed a diet rich in plant protein ingredients, and they suggested that this lowest protease activity was caused by the presence of plant protease inhibitors. It is possible that the plant-based diets used in our study contained lower concentration of these anti-nutritional factors than those of Santigosa et al. (2008), as a result of plant selection and/or technologic improvements.

Vegetable oil added to the noFM–noFO diet might have reduced intestinal lipid absorption, as our results showed significantly lower plasma levels of triglycerides in trout fed the noFM–noFO diet than in trout fed the Mixed or FM–FO diet, despite a slightly higher fat level in the noFM–noFO diet (25 % DM in the noFM–noFO diet versus 24.5 and 23.8 % DM in the FM–FO and Mixed diets, respectively). The impaired intestinal triglyceride absorption in trout fed the noFM–noFO diet may be in part explained by the lower GLIP expression. This hypothesis is in agreement with the previously reported reduction in GLIP activity in mammals fed compounds of plant origin (cocoa procyanidins, tea polyphenols, hydroxytyrosol and carnosic acid) (Romo Vaquero et al. 2012). It would be interesting to evaluate the impact of a plant-based diet on intestinal lipase gene expression in order to assess whether the same effect occurs at the intestinal level. In addition, it is known that the smaller chain length and the lower degree of unsaturation of the oil in fish fed with vegetal oil lead to an accumulation of lipid droplets in the enterocytes instead of the physiological formation of chylomicron (Olsen et al. 1999, 2000; Caballero et al. 2002).

Phospholipids are an important part of the cell biomembrane and need to be provided in the fish diet in order to sustain good growth performance (Coutteau et al. 1997), especially in fry. Vegetal sources (soy lecithin for instance) do not provide the same phospholipids and fatty acids as animal sources and then

have been shown to affect both growth performance and digestive enzyme activities (Azarm et al. 2013). However, in our study, the gene expression of intestinal phospholipase tended to decrease with decreasing levels of FM and FO in the diet, but it was not significant. Obviously, the replacement of marine phospholipids by vegetal phospholipids had no significant impact on the gene expression of this enzyme.

The evolution of the plasma glucose concentrations differed according to the diet ingested, but obviously this effect is more likely to be related to the dietary starch levels. In the 72-h starved fish, the glucose plasma level of trout previously fed the FM–FO diet, the richest in starch (19 % DM), was slightly higher compared to those previously fed the other diets and slightly higher than the basal glycaemia (about 5 mmol/l) as determined by Polakof et al. (2012). The glycaemia of the FM–FO trout then remained stable over a longer period than that observed in the fish fed the Mixed diet containing 11 %DM of digestible starch. It seems that these fish had higher basal glycaemia levels than the others and were able to maintain glycaemia for longer. This may have been the result of adaptation processes resulting from their early exposure to high levels of digestible dietary carbohydrate. However, after a while, the amounts of starch ingested became so high that fish started to become hyperglycaemic. Over the same postprandial period, the glycaemia level of the trout fed the noFM–noFO diet remained low (close to the basal glycaemia), probably because of the low dietary digestible starch content (8 %). These results are in good agreement with those of Polakof et al. (2012) and Geurden et al. (2014) which showed that early exposure to a diet rich in digestible carbohydrate (40 %) appeared to modify the regulatory glycaemia capacity of rainbow trout. The lower ingestion of digestible starch in trout fed the noFM–noFO diet may also explain the lower SGLT1 and GLUT2 gene expression observed in these trout. Consequently, diet composition seemed to affect intestinal glucose absorption.

Limitations in the study

An impact of the noFM–noFO diet on plasma nutrient concentrations was observed in our study as well as an impact on the expression of different genes encoding

proteins involved in the digestive process. However, it is difficult to rely on these findings to the past nutritional history and past phenotype of the trout. Indeed, as the fish were unfed for 72 h before sampling, it is not possible to eliminate the possibility that prolonged fasting could modify intestinal physiology in fish and result in a modified digestive process when fish are fed again: a kind of adaptation to starvation-inducing compensatory growth. In most cases, compensatory growth has been achieved through hyperphagia, an increase in appetite, and sometimes the feed efficiency can be improved (see review by Azodi et al. 2015). Thus, the link between the digestive capacity we analysed after the fasting period and the feed efficiency we measured with data over the whole trout lifespan must be interpreted with caution.

An objective of this study was to choose the most appropriate timing to study the PP gene expression and this choice was made on the FM–FO diet. However, as the kind of diet can strongly influence the digestion time and transit, it is therefore possible that the time chosen for the FM–FO diet was not the most appropriate for the other two diets, especially the noFM–noFO diet.

Moreover, we hypothesize the presence of digestive enzymes and nutrient transporters through their mRNA abundance, but measurement of the activity of the enzymes studied, and measurement of the protein abundance and transport capacity of the transporters studied, is necessary to evaluate the full digestive capacities of this fish. Indeed, the function of these digestive proteins can be modulated at a transcriptional level and/or later at a post-transcriptional level by hormones, anti-nutritional factors, the chemical and physical environment in the chyme, etc.

Finally, we chose to focus our analysis on the expression of genes encoding intestinal enzymes and transporters in the anterior part of the intestine, since its contribution to nutrient transport has been shown to be greater than the contribution of the more distal region in rainbow trout (Dabrowski and Dabrowska 1981) and more generally in carnivorous fish (Ferraris and Ahearn 1984). However, the distal segments of the intestine also have a role in the digestive process as they have been reported to ensure the absorption of intact proteins (McLean et al. 1999) and reabsorption of endogenous components (Sire and Vernier 1992). Moreover, a later study on trout fed a plant-based diet containing fewer digestible feedstuffs revealed that certain amino acids that are not absorbed in the anterior part of the intestine can be absorbed later in the distal segments (Santigosa et al. 2008), but without any certainty that the delayed absorption by the distal region of the intestine can effectively compensate for the impaired absorption observed in the anterior region of the intestine.

Conclusions and future perspectives

The control and regulation of the digestive process so that all the various processes occur in a coordinated and timely way is complex. The secretion of digestive juices, coordination of muscle contractions to mix and move the bolus and activation of enzymes and intestinal transporters must all occur in an appropriate and measured process. In our study, the postprandial filling rate of the stomach and intestine, the gene expression of the proteins (enzymes and transporters) involved in feed digestion and resulting metabolite plasma levels seemed to follow coherent dynamics. Obviously, the maximum levels of expression of most of the studied genes and of plasma metabolites were not reached 12 h after the meal. It should be interesting to extend the postprandial sampling period in order to observe the return to basal levels.

In our study, a partial or total replacement of FM and FO by plant ingredients induced a significant reduction in the gene expression of a digestive enzyme (gastric lipase which contributes to lipids digestion) and of two intestinal transporters (GLUT2 located on the basal membrane of enterocytes and which transports glucose to reach the lymph system, and PEPT1 located on apical membrane of enterocytes and which mediates the transport of dietary di- and tripeptides into the cytosol). Plasma metabolites levels were also affected by these dietary changes: lower postprandial concentrations of glucose and triglycerides, but a higher basal concentration of amino acids. These data are not enough to clearly define the impact of the nature of the diet to the efficiency of the digestion and absorption of nutrients. It is only a first glance. It would be highly valuable now to extend this study to the activity of the digestive enzymes and transporters at least.

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