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RESEARCH ARTICLE

High or low dietary carbohydrate:protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout

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ABSTRACT

Based on the concept of nutritional programming in mammals, we tested whether an acute hyperglucidic–hypoproteic stimulus during first feeding could induce long-term changes in nutrient metabolism in rainbow trout. Trout alevins received during the five first days of exogenous feeding either a hyperglucidic (40% gelatinized starch + 20% glucose) and hypoproteic (20%) diet (VLP diet) or a high-protein (60%) glucose-free diet (HP diet, control). Following a common 105-day period on a commercial diet, both groups were then challenged (65 days) with a carbohydrate-rich diet (28%). Short- and long-term effects of the early stimuli were evaluated in terms of metabolic marker gene expressions and intestinal microbiota as initial gut colonisation is essential for regulating the development of the digestive system. In whole alevins (short term), diet VLP relative to HP rapidly increased gene expressions of glycolytic enzymes, while those involved in gluconeogenesis and amino acid catabolism decreased. However, none of these genes showed persistent molecular adaptation in the liver of challenged juveniles (long term). By contrast, muscle of challenged juveniles subjected previously to the VLP stimulus displayed downregulated expression of markers of glycolysis and glucose transport (not seen in the short term). These fish also had higher plasma glucose (9 h postprandial), suggesting impaired glucose homeostasis induced by the early stimulus. The early stimulus did not modify the expression of the analysed metabolism-related microRNAs, but had short- and long-term effects on intestinal fungi (not bacteria) profiles. In summary, our data show that a short hyperglucidic–hypoproteic stimulus during early life may have a long-term influence on muscle glucose metabolism and intestinal microbiota in trout.

KEY WORDS: Nutritional programming, Rainbow trout, Carbohydrates, Protein, Metabolism

INTRODUCTION

Prenatal or early nutritional neonatal events exerted during critical developmental windows may result in permanent changes in postnatal growth potential, health and metabolic status in mammals (Burdge and Lillycrop, 2010; Lucas, 1998; Patel and Srinivasan, 2002; Patel et al., 2009; Metges et al., 2014; Duque-Guimarães and Ozanne, 2013; Devaskar and Thamotharan, 2007). It has been suggested that this process of developmental plasticity utilizes the early nutritional cues to prepare individual phenotypes to better match the predicted future nutritional environment (Gluckman et al., 2005). Possible biological mechanisms for 'imprinting' the nutritional event until adulthood in mammalian vertebrates comprise adaptive changes in gene expression pattern or cellular phenotype (epigenetic phenomenon), nutrient-sensitive signalling pathways and adaptive clonal selection, which may be transmitted to future offspring (Lucas, 1998; Symonds et al., 2009; Waterland and Jirtle, 2003; Gut and Verdin, 2013). Experimental data on the concept of nutritional programming in fish are limited and some first knowledge stems from recent studies dealing with the possibility of altering the functioning of long-chain fatty acid desaturation in European seabass (*Dicentrarchus labrax*) (Vagner et al., 2007; Vagner et al., 2009), the use of dietary carbohydrates in rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] (Geurden et al., 2007) or zebrafish (*Danio rerio*) (Fang et al., 2014; Rocha et al., 2014) and the acceptance and use of plant-based feed in rainbow trout (Geurden et al., 2013). Taken together, the results all showed particular long-term effects on molecular markers or on growth, because of the acute early-life exposure to the imposed nutritional stimulus. This appears promising as a prospect for altering nutrient use by nutritional programming, even in carnivorous species such as trout, which in their natural habitat are not confronted with such important dietary change.

Rainbow trout is a species of a high trophic level whose natural feed is rich in protein (>40%) and very poor in carbohydrates (<1%) (Panserat et al., 2013). In order to improve the sustainability of the aquaculture production of trout (and of other farmed fish), it is essential to develop alternative aquafeeds without fishmeal and fish oil as ingredients (Naylor et al., 2009). Fishmeal can be replaced as a protein source by plant ingredients naturally rich in carbohydrates (Panserat and Kaushik, 2010). Unfortunately, rainbow trout exhibit a poor utilization of dietary carbohydrates, characterized by reduced growth and prolonged postprandial hyperglycemia when carbohydrate inclusion exceeds 20% of the diet (Polakof et al., 2012; Wilson, 1994). Important research has been undertaken to establish the fate of dietary carbohydrates at a metabolic and physiological level in fish (Polakof et al., 2012). These studies focused mainly on the role of dietary factors as modulators of glucose utilization and on the expression of key enzymes of the intermediary metabolism (Enes et al., 2011; Panserat et al., 2002; Skiba-Cassy et al., 2013). However, the mechanisms underlying the relatively poor ability of fish to utilize dietary carbohydrates as a major energy-yielding substrate remain poorly understood. Rainbow trout also has a high dietary protein requirement, i.e. more than 35% of diet dry matter (NRC, 2011), probably linked to persistent amino acid catabolism for energy purposes (Kaushik and Seiliez, 2010). Thus, new

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List of abbreviations

6PFK-L	6-phosphofructokinase, isoform from liver
6PFK-M	6-phosphofructokinase, isoform from muscle
18S	18S ribosomal RNA
DGGE	denaturing gradient gel electrophoresis
EF1α	elongation factor-1, isoform alpha
FBPase	fructose1,6-biphosphatase
G6Pase	glucose-6-phosphatase (two isoforms)
GK	glucokinase
GLUT1	glucose transporter 1
GLUT2	glucose transporter 2
GLUT4	glucose transporter 4
GDH1	glutamate dehydrogenase 1
GDH2	glutamate dehydrogenase 2
GDH3	glutamate dehydrogenase 3
HK1	hexokinase 1
NBAT	Na^+-dependent amino acid transporters
PAST	paleontological statistics software package
PEPCK	phosphoenol pyruvate carboxykinase, mitochondrial isoform
PEPT1	H^+-dependent peptide transporter
PK-L	pyruvate kinase, isoform from liver
PK-M	pyruvate kinase, isoform from muscle
RT-PCR	reverse transcription polymerase chain reaction
SDH	serine dehydratase
SGLT1	sodium-dependent glucose cotransporter 1

nutritional strategies that may overcome the metabolic (nutritional) bottlenecks of poor use of dietary glucose as an energy source and of the high protein requirement should be explored. In this respect, the concept of nutritional programming appears promising. Indeed, numerous studies in (omnivorous) mammals have shown that modifying macronutrient (carbohydrates, proteins, lipids) intakes at early life stages can cause long-term modifications to metabolism, especially glucose metabolism (Ozanne et al., 1996; Patel and Srinivasan, 2002; Burdge and Lillycrop, 2010; Waterland and Jirtle, 2003; Metges et al., 2014; Jia et al., 2012). To the best of our knowledge, no such data are available for strict mammalian carnivores such as the cat *Felis catus* or mink *Mustela vison*. Early nutritional events can also modify the expression of specific microRNAs playing a role in developmental programming (Zhang et al., 2009) as well as the composition of gut microbiota in fish larvae (Delcroix et al., 2014). The early microbial colonisation may affect body composition, digestion and metabolic homeostasis, as seen in newborn infants (Nauta et al., 2013).

In fish, a nutritional stimulus during stages of high metabolic plasticity, such as embryogenesis or early larval development, can be applied at the egg stage [e.g. egg glucose injection (Rocha et al., 2014) or maternal nutrient transfer (Fernández-Palacios et al., 1995; Fernández-Palacios et al., 1997)] or at the onset of exogenous feeding (Geurden et al., 2007; Vagner et al., 2007; Vagner et al., 2009; Fang et al., 2014). Indeed, a previous study in trout by Geurden et al. (Geurden et al., 2007) has shown that a hyperglucidic stimulus at the onset of feeding had a permanent effect on carbohydrate digestive enzymes at the juvenile stage, indicating a positive long-term physiological change induced by the first-feeding stimulus in rainbow trout. However, none of the metabolic parameters related to glucose utilization (e.g. gluconeogenesis, glycolysis) was altered by the early stimulus in the trout, even not in the short term, which we hypothesized to be due its short duration (only 3 days) or to the carbohydrate source (dextrin). In contrast, and following an experimental procedure similar to the one applied in rainbow trout (Geurden et al., 2007), a recent study with zebrafish revealed long-lasting changes in the expression of genes involved in glucose intestinal transport and in glucose metabolism (Fang et al., 2014). As it is uncertain whether this difference in response is species-related (omnivorous feeding habit as well as faster larval development and higher rearing temperature in zebrafish compared with trout), we decided to boost the early stimulus in the present study with rainbow trout.

Therefore, in order to further test the premise of altering nutrient use in juvenile rainbow trout by nutritional programming, we now intensified the early hyperglucidic stimulus using highly digestible carbohydrates (a mix of pure glucose and gelatinized starch) and by extending the first-feeding stimulus duration (5 days). The strong increase in carbohydrates was adjusted for by a concomitant decrease in dietary protein. Thus, the main objective of the present work was to assess the short- and long-term effects of first-feeding rainbow trout alevins with a high level of carbohydrates and a low level of protein on metabolic programming with respect to: (1) the regulation of genes involved in glucose and amino acid digestion, transport and/or metabolism, (2) the expression of specific microRNAs involved in metabolism and (3) the composition of the gut microbiota, whose composition and metabolites are influenced by the diet, with potential epigenetic consequences (Mischke and Plösch, 2013). In order to assess the long-term effect of the early stimulus, we performed a challenge test during which juvenile rainbow trout were fed a diet marginally adequate in protein [36%, the recommended dietary protein for rainbow trout being 38% of diet (NRC, 2011)] but rich in carbohydrates (28%). It was hypothesized that the nutritional stimulus applied at the first-feeding stage (alevins of <200 mg) would induce persistent adaptive physiological changes in juvenile trout (fish of ~20 g), thus improving their capacity to cope with high dietary carbohydrate loads.

RESULTS

We first studied the short-term (5 days) effects of feeding trout alevins with diets containing a high (HP) or very low (VLP) protein/carbohydrate ratio. This early first-feeding period (stimulus) was followed by a common intermediate rearing period during which all fish were fed a commercial diet. During the final phase, we performed a dietary challenge by feeding the alevins a carbohydrate-rich diet for 65 days in order to reveal a possible metabolic programming due to the nutritional stimuli at first-feeding.

Short-term effects of intake of VLP and HP diets in rainbow trout alevins

Rainbow trout alevins (around 100 mg) were fed for 5 days with the two experimental HP and VLP diets. At the end of the treatment, no differences in the percentages of survival (87% versus 85.5% for HP and VLP fish, respectively) were detected between the two dietary groups, indicating the absence of an early selection process.

Analyses of gene expression for glucose and amino acid metabolic proteins, miRNAs and analysis of microbial profiles have been performed in whole body of alevins 3 h after the last meal (Figs 1, 2, Tables 1, 2). The intake of VLP diets by trout alevins induced higher gene expression of glycolytic enzymes involved in glucose phosphorylation (GK and HK1), but not of the other glycolytic actors (PKs and 6PFKs) (Fig. 1). By contrast, the expression of all the analysed genes coding for gluconeogenic enzymes (G6Pase, FBPase and PEPCK) was significantly lower in alevins fed the VLP diet (Fig. 1). Moreover, alevins fed the VLP diet showed a lower expression of genes involved in amino acid catabolism (GDHs and SDH) (Fig. 1). No short-term diet effect was detected for the genes coding proteins involved in glucose and

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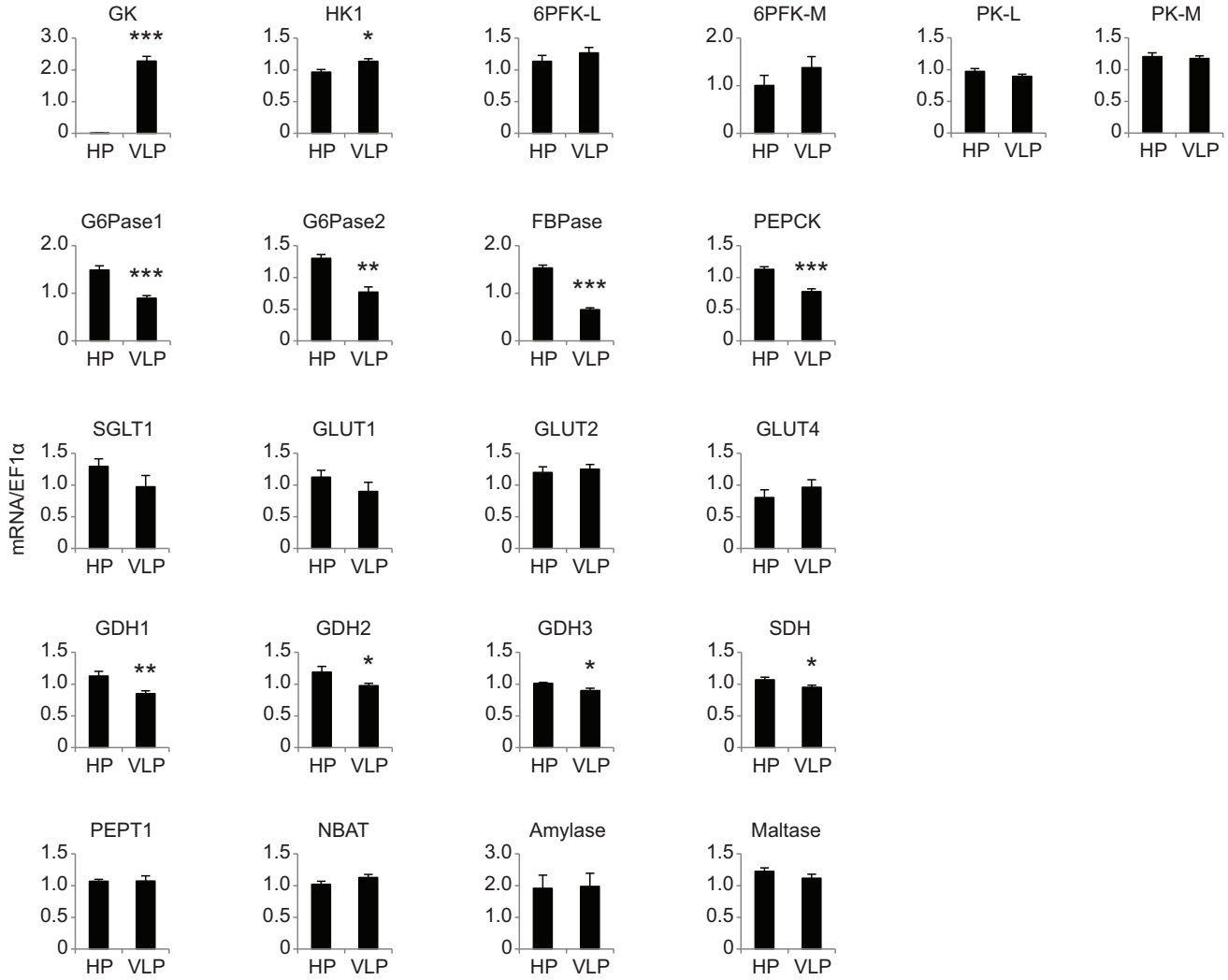

Fig. 1. Short-term immediate effects of intake of diets with low (VLP) and high (HP) protein/carbohydrate ratio in rainbow trout alevins. Genes involved in carbohydrate digestion (AMY and MAL), protein and glucose transport (PEPT1, SGLT1 and GLUTs), glucose metabolism (glycolysis and gluconeogenesis) and amino catabolism (GDHs and SDH) were analysed. Gene expression analyses were performed on total RNA extracted from whole larval body, sampled 3 h after the last meal. Data represent means ± s.e.m. Statistical differences in gene expression (normalised by the reference EF1α gene) between samples were evaluated in group means by Student's *t*-test (R-software); asterisks indicate that the target gene is differentially expressed in VLP fish compared with HP fish (*P<0.05; **P<0.01; ***P<0.001).

amino acid transport (SGLT1, GLUTs, PEPT1 and NBAT), nor for those involved in carbohydrate digestion (amylase and maltase) (Fig. 1).

For the first time in fish, we also analysed miRNA gene expression (miRNAs 29, 107, 33 and 143) in relation to differences in dietary macronutrient intake. The expression of the studied miRNAs was not different in alevins fed the HP versus the VLP diet (Fig. 2).

We finally studied the short-term effect of feeding the two experimental diets on the microbial profiles in alevins (Tables 1, 2). The bacterial and fungal profiles were compared separately between the two dietary groups (Table 1). Though the overall dissimilarity and diversity were relatively high in both of the taxonomic domains, the fungal profiles were significantly distinct between the two groups, unlike the bacterial profiles. The main fungal taxa that contributed to the dissimilarity between groups could not be identified from the denaturing gradient gel electrophoresis (DGGE) gel, and only three moulds were identified, each one accounting for 1.6–2% of the overall dissimilarity (*Cladosporium* sp., *Dothideomycetes* and *Pseudocercospora* sp., by increasing order of SIMPER contribution). The bacterial profiles were strongly dominated by five taxa of γ-Proteobacteria, and more specifically by *Escherichia* sp. (Table 2).

Intermediate grow-out phase

After the 5-day nutritional stimulus, rainbow trout were fed commercial trout feed for 105 days. During this pre-challenge period (days 0–105), we did not find any significant effect of the early VLP and HP diets at first feeding on the growth performances of the fish (feed intake, growth rate or feed efficiency) (Table 3).

Long-term effects of the early nutritional stimulus in rainbow trout juveniles: challenge with a diet rich in carbohydrates

For the last step, juvenile trout (approximately 17 g) were challenged to eat a diet rich in carbohydrates (28%) for 65 days. No effects of the early nutritional history were detected on growth, feed intake or feed efficiency (Table 3).

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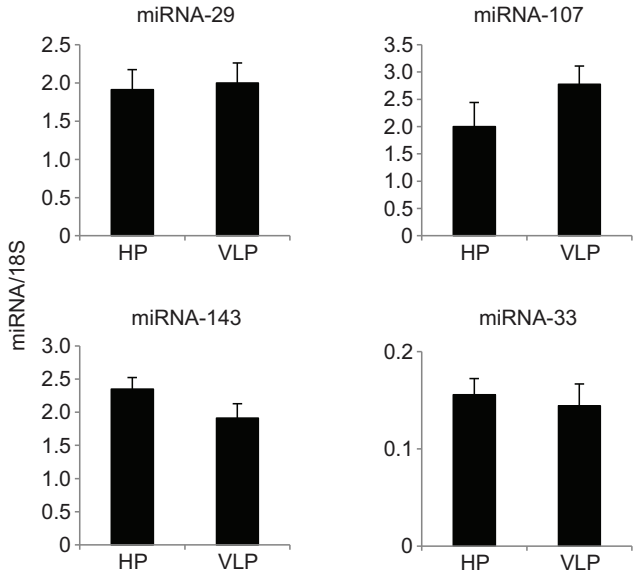

Fig. 2. Short-term immediate effects of intake of diets with low (VLP) and high (HP) protein/carbohydrate ratio in rainbow trout alevins on miRNA expressions. Selected miRNAs involved in regulation of metabolism were analysed: miRNA-29 (regulation of insulin signaling), miRNA-33 (regulation of liver proliferation), miRNA-107 (regulation of insulin signaling) and miRNA-143 (reguilation of fat tissue proliferation). Gene expression analyses were performed on total RNA extracted from whole larval body, sampled 3 h after the last meal. Data represent means ± s.e.m. Statistical differences in gene expression (normalised by the reference 18S gene) between samples were evaluated using Student's *t*-test (R software). No differences were found.

The major plasma metabolites linked to carbohydrate intake (glucose and triglyceride levels, which may reflect bioconversion of glucose into lipids through the hepatic lipogenesis) were measured 2, 6 and 9 h after the last meal (Table 3). There was no significant effect of the early stimulus on the level of circulating triglycerides. We noted a significant effect on plasma glucose of juvenile trout at 9 h after the last meal, being higher in fish subjected previously to the VLP stimulus.

In the challenged juvenile trout, we first analysed the parameters (metabolism and microbiota) that had been found to respond differently according to the early stimulus (see 'Short-term effects of intake of VLP and HP diets in rainbow trout alevins'). However, at this stage, no more differences were detected in the expression of glycolytic enzymes (GK and HK), gluconeogenic enzymes (G6Pase, FBPase and PEPCK) and amino acid catabolising enzymes (GDHs) in the liver of juveniles (Fig. 3), as confirmed by enzymatic activity analyses (Table 4). Secondly, we analysed in the challenged fish the genes that were not found to be regulated by the diet at the first-feeding stage. The majority of these genes were not affected by differences in nutritional history. In contrast, muscle of challenged juveniles showed a significant difference in the expression of some parameters of glucose metabolism, with a significant decrease of muscle Glut4, HK and PK-M gene expressions in the (pre-treated) VLP fish (Fig. 3). Even though these effects were not confirmed at the enzymatic level (Table 4), our data suggest the existence of a metabolic programming in juvenile rainbow trout, which may contribute to the differences in glycemia between pre-treated HP and VLP fish (Table 3). The fungal profiles associated with intestinal mucosae were characterised by low indices of dissimilarity and diversity, but they remained significantly affected by the initial dietary manipulation (Table 1). The bacterial profiles appeared more diverse and dissimilar, without significant differences related to the early feeding. The main contributors to microbial dissimilarity could not be identified from the DGGE gels, but Firmicutes (*Staphylococcus* sp. and *Clostridium* sp.) were retrieved among the subdominant bacteria, in addition to Enterobacteriaceae.

DISCUSSION

Most fish species are quite different from terrestrial livestock species (e.g. pigs, chickens and cows) in that they have evolved by eating diets high in protein and low in carbohydrates. In order to modify the nutrient use in rainbow trout, a fish species known to be glucose intolerant (Polakof et al., 2012) and requiring high-protein feed (Panserat et al., 2013), we tested a new nutritional strategy in fish based on the concept of metabolic programming in mammals. The trout alevins were fed from first-feeding (at mouth opening) diets with low or high carbohydrate/protein ratio and then challenged to eat a high-carbohydrate diet at the later juvenile stage. Compared with mammals, trout alevins (larvae) constitute a powerful and unique model with which to try to modify metabolism because of the very high plasticity (Pittman et al., 2013, Mennigen et al., 2013) and the direct acceptability of exogenous solid feed at this developmental stage. Indeed, drastic changes in metabolic gene expression occur in first-feeding rainbow trout, during the transition from endogenous (yolk-sac) to exogenous feeding, as illustrated for some genes involved in carbohydrate utilisation (Geurden et al., 2007) and for the expression of miRNAs that may contribute to the ontogenetic metabolic changes (Mennigen et al., 2013).

Short-term effects of the early nutritional stimuli

The 5 days of the first-feeding stimulus with the hyperglucidic and hypoproteic feed were sufficient to induce clear effects on metabolic gene expression in the trout alevins without a negative effect on survival. Indeed, VLP relative to HP feeding enhanced the expression of genes coding for glycolytic enzymes (GK and HK1; at least for the first step involved in glucose phosphorylation) and

Table 1. Dissimilarity in microbial profiles induced by diet VLP or HP during the first 5 days of exogenous feeding (early stimulus) in rainbow trout alevins (short-term effect, 3 h after the last meal) and in intestinal mucus of juvenile trout at the end of the challenge period (long-term effect, 6 h after the last meal)

	ANOSIM			
Microbial profile	*R*	*P*	SIMPER overall average dissimilarity (%)	Band richness
Bacteria (alevins)	0.04	$0.21^{n.s.}$	57.1	7.3±4.1
Fungi (alevins)	0.15	0.02*	56.0	8.4±4.1
Bacteria (juveniles)	0.06	$0.17^{n.s.}$	61.4	9.4±3.0
Fungi (juveniles)	0.10	0.04*	32.7	2.8±1.4

Comparison of the Bray–Curtis similarity between the two dietary groups by ANOSIM (*significant dissimilarity; n.s., not significant), SIMPER overall average dissimilarity and mean band richness (not significantly different between dietary groups).

Table 2. Main contributors to Bray-Curtis dissimilarity between the bacterial profiles associated with rainbow trout alevins (short-term effect) fed the two experimental diets (HP and VLP; 3 h after the last meal) for 5 days (SIMPER analysis, overall average dissimilarity: 57.1%)

		Mean relative abundance (%)			
DGGE rank	Contribution to dissimilarity (%)	VLP	HP	Identification	Similar GenBank ID
23	15.5	35.8	49.6	*Escherichia* sp. ATB	JQ989156
21	7.8	14.9	5.3	*Shewanella* sp. ATF	EU290154
26	6.4	9.2	6.6	*Aeromonas* sp. ATA	JX899616
18	4.5	5.5	9.2	*Vibrio* sp. ATE	KC579367
29	3.1	5.7	1.3	γ-Proteobacteria ATR	AB435574

The taxonomic units were identified from identical sequences in GenBank.

decreased expression of all analysed genes involved in gluconeogenesis (G6Pase, FBPase and PEPCK) and amino acid catabolism (GDHs and SDH). These data confirm the molecular regulation of glucose metabolism by carbohydrates in trout alevins similar to that in mammals (Pilkis and Granner, 1992). It should be noted that such nutritional regulation, especially that of gluconeogenesis, is less evident in juvenile and adult rainbow trout (Panserat et al., 2000; Panserat et al., 2001; Polakof et al., 2012), underlining the plasticity of the metabolic responses at this early life stage in trout. These short-term molecular changes also agree with recent findings in zebrafish (an omnivorous species) fed for 5 days from first feeding with a high level (50%) of carbohydrates (Fang et al., 2014). The present data thus open perspectives for a nutritional programming strategy in rainbow trout. Indeed, the short-term regulation of these parameters of glucose metabolism was not observed in a previous study with trout alevins (Geurden et al., 2007), which may be related to the duration of the stimulus (5 days versus 3 days previously) or the type of carbohydrate intake (glucose and gelatinized starch versus dextrin previously). An effect of the duration of the first-feeding stimulus was also seen for zebrafish, in which the gluconeogenic genes G6Pase and PEPCK were, as in the present study, downregulated at the end of the 5-day stimulus, whereas the reverse was seen at the end of the 3-day stimulus (Fang et al., 2014).

Moreover, the present study confirms the efficient regulation of amino acid catabolism (GDHs and SDH) by the (drastic) changes in dietary protein at the early alevin stage, as seen previously in adult trout (Skiba-Cassy et al., 2013; Figueiredo-Silva et al., 2013). We must note here the absence of regulation of genes involved in the transport of glucose, amino acids and small peptides (SGLT1, GLUTs, NBAT and PEPT1) and in carbohydrate digestion (amylase and maltase). The latter was unexpected as the hyperglucidic diet contained a source of complex carbohydrates requiring digestive breakdown (gelatinized starch) and as digestive enzyme expression was highly upregulated after an early 3-day dextrin stimulus in studies with both trout (Geurden et al., 2007) and with zebrafish (Fang et al., 2014). On the whole, the present short-term feeding stimulus showed that the use of digestible starch and glucose has an immediate and drastic effect on intermediary metabolism at a molecular level in trout alevins.

We also analysed the early nutritional response of some specific miRNAs involved in metabolism regulation (Dumortier et al., 2013), taking into consideration the role of microRNAs in nutritional programming (Zhang et al., 2009). We previously found that the transition from endogenous to exogenous feeding altered the expression of the studied miRNAs in trout alevins [(Mennigen et al., 2013) except for miRNA-107]. However, the drastic change in macro-nutrient intake at first feeding did not modify their expression level in the present study.

Table 3. Effect of feeding either the VLP or HP diet during the first 5 days of exogenous feeding (early stimulus) on rainbow trout body mass, specific growth rate (SGR), feed intake and feed efficiency (body mass gain/dry matter feed intake) during the intermediate (days 0–105) and challenge (days 105–170) phases, and on postprandial plasma glucose and triglycerides at the end of challenge

	Early stimulus		
	VLP	HP	*P*
Intermediate pre-challenge period (days 0–105)			
Body mass day 21 (g fish^{-1})	0.36±0.02	0.38±0.02	0.34
Final body mass (g fish^{-1})	17.3±0.9	17.2±0.9	0.90
SGR (% d^{-1})	4.91±0.05	4.90±0.05	0.90
Feed intake (g fish^{-1})	12.4±0.6	12.5±0.6	0.75
Feed efficiency	1.39±0.10	1.37±0.05	0.73
Challenge period (days 105–170)			
Final body mass (g fish^{-1})	51.2±0.9	50.6±2.0	0.65
SGR (% d^{-1})	4.24±0.17	4.27±0.12	0.85
Feed intake (g fish^{-1})	31.8±2.0	31.4±2.1	0.82
Feed efficiency	0.95±0.04	0.95±0.04	0.93
Postprandial plasma metabolites (day 170)			
Plasma glucose (2 h, mmol l^{-1})	13.0±4.7	13.5±5.6^{a}	0.83
Plasma glucose (6 h, mmol l^{-1})	9.4±3.7	10.3±4.8^{a,b}	0.67
Plasma glucose (9 h, mmol l^{-1})	10.8±3.3	7.7±1.4^{b}	0.02*
Plasma triglycerides (2 h, g l^{-1})	3.98±1.26^{x}	2.76±1.21^{x}	0.05
Plasma triglycerides (6 h, g l^{-1})	8.60±3.47^{y}	6.77±1.74^{y}	0.18
Plasma triglycerides (9 h, g l^{-1})	6.47±2.37^{y}	6.27±3.65^{y}	0.89

Data represent means ± s.d. (n=3 replicate tanks/treatment and n=9 fish/treatment for plasma metabolites). Different superscripts within a column indicate a postprandial time effect on the plasma metabolites. *Significant difference related to the early stimulus.

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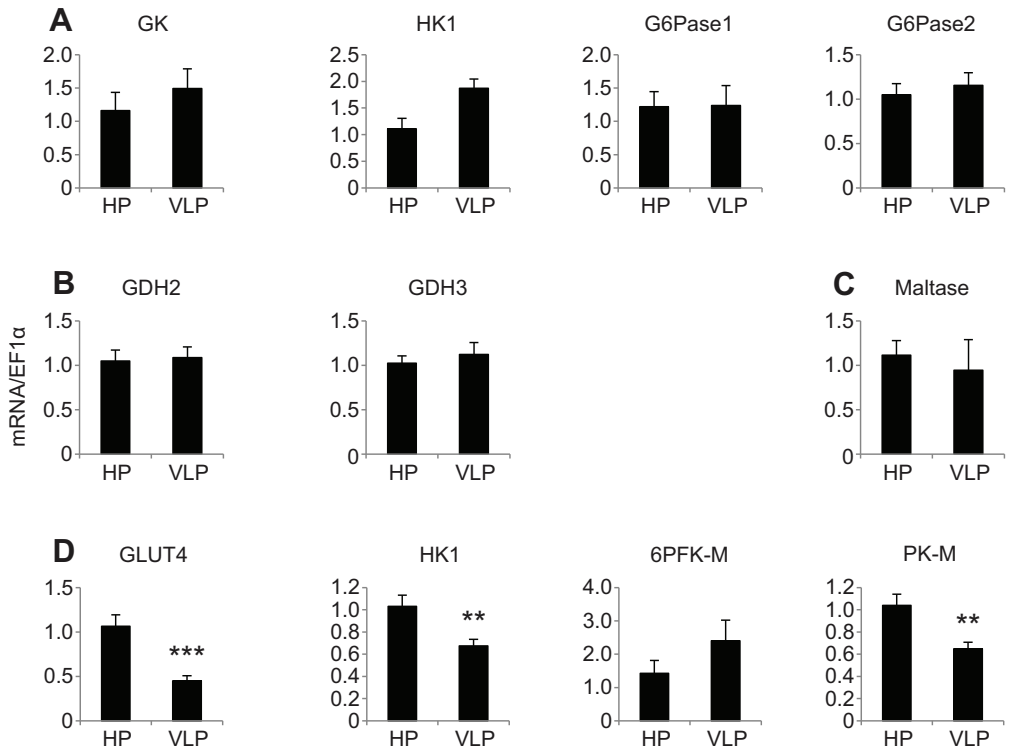

Fig. 3. Long-term effects induced by the early 5-day feeding of diets with low (VLP) and high (HP) protein/carbohydrate ratio on gene expression in rainbow trout juveniles fed the challenge diet for 65 days. (A,B) Liver; (C) intestine; (D) muscle. Genes were selected based on their importance in glucose metabolism and on the short-term immediate effect (see Fig. 1). The expression of the other genes previously tested in alevins was not differentially affected by the early stimulus (data not shown). Gene expression analyses were performed on samples (n=6 per group) taken 6 h after the last meal. Data represent means ± s.e.m. Statistical differences in gene expression (normalised by the reference EF1α gene) between samples were evaluated using Student's t-test (R software); asterisks indicate that the target gene is differentially expressed in VLP fish compared with HP fish (**P<0.01; ***P<0.001).

This study analyses for the first time in fish the imprinting effect of a change in diet composition at first feeding as well as its long-term effect on the possible programming of intestinal fungal microbiota, which can impact metabolism and health (Huffnagle and Noverr, 2013; Iliev and Underhill, 2013). The present DGGE analysis did not allow the identification of yeast, but we suspect their prevalence among the unidentified fungal taxa. We used the reverse transcription (RT)-PCR-DGGE to focus on the active part of microbiota, as recommended by Navarrete et al. (Navarrete et al., 2012) in rainbow trout. These analyses showed significant differences in the fungal profiles between the two groups of alevins after the first 5 days of feeding. A previous study with culture-dependent methods pointed out the importance of early colonisation by yeast in the gut of rainbow trout for the maturation of the digestive system (Waché et al., 2006). In human gut microbiome, the increased abundance of *Candida* due to high-carbohydrate diets was related to its contribution to starch digestion (Hoffmann et al., 2013). The presence of *Candida* was also reported in the intestine of rainbow trout (Gatesoupe, 2007). The high level of starch in the conditioning diet could thus stimulate the activity of similar amylolytic microbes. Although further taxonomical investigation is required, these preliminary data demonstrate the feasibility of modifying the microbiome at first feeding by high carbohydrate and low protein intake.

Table 4. Effect of feeding either the VLP or HP diet during the first 5 days of exogenous feeding (early stimulus) on enzyme activities in rainbow trout liver and muscle at the end of the challenge period (9 h after the last meal)

	Early stimulus		
Enzyme	HP	VLP	P
Liver			
GK	1.22±0.53	1.99±1.60	0.2
HK	0.68±0.34	0.56±0.25	0.4
G6Pase	47.2±7.4	46.9±7.3	0.9
GDH	102.2±29.7	96.1±28.1	0.7
Muscle			
HK	0.74±0.64	0.97±0.51	0.4
PK	9.71±2.57	1.20±4.55	0.2

Values are means ± s.d. (n=9 in all groups). Values are specific activities expressed in mUI mg^{-1} protein except for PK, for which values are expressed in UI mg^{-1} protein.

Long-term effects of the early nutritional stimuli

During the 105-day intermediate period, when fed the commercial trout feed, both groups showed similar growth. At this stage, no effect of the early nutritional history (VLP and HP diets) was noted on growth-related parameters, which we ascribed to the optimal (non-extreme) composition of the commercial feed, which was low in carbohydrates (10–15%) and high in protein (54–56%). Thus, in order to evaluate the initial hypothesis, we challenged the juvenile trout with a diet rich in carbohydrates (28%) and marginal in dietary protein (36%) to study the long-term outcome of the early stimulus.

However, similar to the intermediate period, the 9-week challenge did not reveal any difference in growth, feed intake or efficiency of feed utilization. This indicates that the early hyperglucidic stimulus did not provoke a long-lasting positive adaptive response, which we had anticipated as advantageous for growth in trout when exposed again, at later life stages, to high carbohydrates. Yet, we observed a significant effect on glucose homeostasis. As expected, the high level of gelatinized starch in the challenge diet (28%) induced a postprandial hyperglycemia in all trout [>2 g l^{-1}, compared with the basal glycemia of ~0.8–1 g l^{-1} (Polakof et al., 2012)]. Yet, 9 h after feeding, HP-fed fish had significantly lower plasma glucose (1.39 g l^{-1}) than VLP-fed fish (1.95 g l^{-1}). The finding that the reduced glycemia was seen in HP but not in VLP fish confirms the lack of a positive effect of the early hyperglucidic stimulus. However, it highlights the existence of a nutritional programming induced by the early nutritional event because both groups of fish were raised identically, except for the imposed changes in protein

and carbohydrate supply at first feeding. Of interest, the lower glycemia in HP fish was associated with higher gene expression for Glut4, HK and PK-M in muscle, known to play an important role in the regulation of glucose homeostasis in mammals (Abdul-Ghani and DeFronzo, 2010). The observation that the latter genes (Glut4 gene, HK gene and PK-M gene) were not detected as differentially expressed at the early first-feeding stage favours the occurrence of an indirectly programmed regulation of the persistent expression of these glucose-related genes (e.g. altered hormonal levels, such as insulin) rather than a direct programming effect on the target genes. By contrast, the metabolic genes differentially expressed at the early stimulus stage (glucose metabolism: GK, G6Pase, FBPase and PEPCK; and protein metabolism: GDHs and SDH) were no longer differentially expressed in liver at the juvenile stage, as opposed to data from a recent study with zebrafish, which observed a long-term programming effect for some of these metabolic markers (Fang et al., 2014).

Another interesting long-term result concerns the effect of the early feeding on the intestinal fungi, whose profiles, differently expressed in alevins, remained differently expressed in juveniles. As revealed by RT-PCR-DGGE, the active fungal community associated with the intestinal mucus in juveniles, although dissimilar and less diverse compared with that associated with the alevins, still depended on the initial dietary manipulation. To our knowledge, these findings show for the first time the capability of inducing microbial programming by early nutrition in fish. Wong et al. (Wong et al., 2013) found a core of intestinal bacteria that were resistant to dietary changes in rainbow trout. However, several other studies demonstrated the impact of feed on gut microbiota in rainbow trout, especially linked to the introduction of vegetable ingredients (Desai et al., 2012; Heikkinen et al., 2006; Mansfield et al., 2010; Merrifield et al., 2009; Navarrete et al., 2012). In our study, the high dissimilarity between individual bacterial profiles does not allow us to confirm the hypothetical core microbiota in rainbow trout, but DGGE can detect only the most abundant taxa. Further studies are thus needed to investigate in depth the long-term effects of early dietary treatments on gut microbes. This requires not only taxonomic characterisation, but also functional investigation of the microbiome. Future studies should also attempt to understand the mechanisms of interaction with gene expression on a long-term scale in the host, especially those linked to metabolism.

The higher blood glucose at 9 h after feeding suggests a less efficient regulation of postprandial glycemia in rainbow trout juveniles previously fed with the low-protein high-carbohydrate feed. Studies in mammals likewise report impaired glucose homeostasis at adulthood due to protein restriction or carbohydrate excess at early life (Patel et al., 2009; Ozanne et al., 1996; Fernandez-Twin et al., 2003). Still, such comparison requires specific caution because ‘normal’ proportions of both macronutrients in diets of (omnivorous) mammals strongly differ from those in diets of (carnivorous) fish. Moreover, a majority of these studies focused on fetal programming via the mother’s diet and few focused on dietary modifications at the immediate postnatal life because of the difficulty in raising newborn sucklings away from the mother. In any event, be it prenatally or postnatally, a high carbohydrate supply has been associated with alterations in insulin secretion, insulin resistance and impaired glucose metabolism often characterised by higher gluconeogenic output in adult mammals (Patel and Srinivasan, 2002; Patel et al., 2009). Analogous observations have been made in offspring following maternal protein restriction or intrauterine growth retardation, displaying glucose intolerance (Frantz et al., 2012; Duque-Guimarães and Ozanne, 2013; Kongsted et al., 2014), increased hepatic glucose output, reduced glucose phosphorylation (Desai et al., 1995; Ozanne et al., 1996) and altered glucose uptake by muscle skeletal cells as reflected by reduced Glut4 expression (Thamotharan et al., 2005; Devaskar and Thamotharan, 2007). In mammals, these are common signs of defects in glucose metabolism related to insulin resistance (Abdul-Ghani and DeFronzo, 2010). In our study, as mentioned above, the juvenile VLP trout subjected previously to the early hyperglucidic stimulus did not show higher expression or activity of gluconeogenic enzymes in liver, but muscle Glut4, HK and PK-M mRNA were clearly reduced, in line with the ‘programmed’ glucose-intolerant mammalian model. However, the physiological relevance of this observation, in particular the extent to which these changes contribute to the higher glycemia in the juvenile VLP trout, remains uncertain. This is because of the lack of difference in HK or PK enzyme activity in the muscle as well as their overall low expression in trout skeletal muscle, a feature suggested to explain the overall poor use of glucose by this large-body-mass tissue in trout (Hemre et al., 2002; Panserat et al., 2013). A second aspect that deserves specific attention concerns the changes in macronutrient supply during the stimulus period. In our study, as in several studies on metabolic programming in mammals (Devaskar and Thamotharan, 2007), we balanced one macronutrient against another. It is thus impossible to identify the exact causal factor, namely the early protein restriction, the early carbohydrate excess or a combination of both, responsible for the altered muscle gene expression and plasma glucose homeostasis observed in the juvenile VLP trout.

Conclusions

In summary, our data show that a short hyperglucidic hypoproteic stimulus during early life of rainbow trout may have a long-term influence on the expression of genes involved in glucose utilisation in muscle (glucose trasnport and catabolism) as well as the gut fungal microbiota. These data hence clearly demonstrate the possibility of nutritional programming in rainbow trout. The mechanisms (epigenetic, development of organs, clonal selection of cells/bacteria) supporting our observations must be further studied. Finally, it should be noted that the early stimulus did not induce the anticipated positive long-term metabolic effect. For that reason, further research is clearly needed before the possible large-scale implementation of this innovative feeding strategy, aimed at programming the improved use of specific aquafeed ingredients in fish.

MATERIALS AND METHODS

Fish diets

Two experimental diets for rainbow trout alevins, namely HP (high-protein based diet without carbohydrate; high protein:carbohydrates ratio) and VLP (very low level of protein diet with very high level of carbohydrates; low protein:carbohydrate ratio), were prepared in our own facilities (INRA, Donzacq, Landes, France) as extruded pellets. Glucose and gelatinized starch were included as the carbohydrate sources, protein came from fishmeal and dietary lipid from fish oil and fishmeal (Table 5). The two diets had similar amounts of lipids. The large increase in dietary carbohydrate content (65%) in the VLP diet was compensated for by a decreased proportion of protein (20%), which is below the 37% protein requirement of rainbow trout (NRC, 2011). No carbohydrates were added to the HP diet, which contained 60% crude protein. After the nutritional stimulus (first feeding), rainbow trout were fed a standard trout commercial diet during the intermediary period (T-3P classic, Skretting, France; Table 5). Finally, a challenge diet (Table 5) was used for the last part of the experiment corresponding to a diet rich in carbohydrates (28%) and marginal in protein (36%).

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Table 5. Formulation and proximate composition of the two experimental diets used during the first-feeding stimulus (VLP and HP), the challenge phase and the intermediate rearing period (commercial feed)

				Commercial feed[f] (mm)		
Diet	VLP	HP	Challenge	0.8	1.1	1.8
Ingredients (%)						
Fish meal[a]	28.5	94	52			
Fish oil[b]	7.5	2	14			
Starch[c]	40	0	30			
Glucose	20	0	0			
Vitamin mix[d]	1	1	1			
Mineral mix[e]	1	1	1			
Alginate	2	2	2			
Proximate composition						
Dry matter (DM; % diet)	93.2	89.7	89.9	86.7	92	91.7
Crude protein (% DM)	19.9	60	35.7	63.8	60	56.2
Crude lipid (% DM)	9.9	11.3	16.6	18.6	17.4	19.6
Gross energy (kJ g^{-1} DM)	17.8	20.3	19.7	25	23.7	24.4
Ash (% DM)	5.1	13.8	8	9.5	8.7	7.3
Carbohydrates (% DM)	65.1	<1.0	27.6	10.9	11.4	15.4

[a]Sopropeche, Boulogne-sur-Mer, France.
[b]North Sea fish oil, Sopropeche.
[c]Gelatinized corn starch, Roquette, Lestrem, France.
[d]Supplied the following (kg^{-1} diet): DL-a tocopherol acetate 60 IU, sodium menadione bisulphate 5 mg, retinyl acetate 15,000 IU, cholecalciferol 3000 IU, thiamin 15 mg, riboflavin 30 mg, pyridoxine 15 mg, vitamin B_{12} 0.05 mg, nicotinic acid 175 mg, folic acid 500 mg, inositol 1000 mg, biotin 2.5 mg, calcium panthotenate 50 mg, choline chloride 2000 mg.
[e]Supplied the following (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, potassium chloride 0.9 g, sodium chloride 0.4 g.
[f]Skretting, France (different pellet sizes). Exact list of ingredients not available.

Fish and experimental design

The experiments were carried out within the clear boundaries of EU legal frameworks relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU) and of the French legislation governing the ethical treatment of animals (Décret no. 2001-464, 29 May 2001). Rainbow trout embryos were initially reared at the INRA experimental facilities of Lees-Athas, France (8°C water temperature). They hatched at 44 days post-fertilization. At 64 days post-fertilization, the trout alevins were transferred to the experimental facilities at INRA, Donzacq, France, and distributed into 50 l tanks (18°C, oxygenated spring water). Following emergence, the first-feeding trout alevins (~150 mg body mass), still having small yolk reserves, were randomly divided into two groups and fed with either HP or VLP diets (triplicate tanks per diet; 450 fish per tank, 50 l per tank) during 5 days (Table 5) and killed 3 h after the last meal. Care was taken to maintain the same daily time frame before sampling to avoid potential circadian effects, which is known to affect metabolism in rainbow trout (Bolliet et al., 2000). In all cases, fish were terminally anaesthetized by bathing in benzocaine prior to storage in liquid nitrogen. The samples were then stored at −80°C until they were utilized for molecular (gene and miRNA expressions) analysis (n=9 fish per group, 3 per tank). Six fish per dietary group were stored individually in RNA stabilization reagent (RNAlater®) to be further analysed for microbial profiles, after overnight incubation at 4°C and storage at −80°C.

After the nutritional stimulus, trout were fed for 105 days with a commercial trout feed (Skretting, France) (Table 1), during which growth, feed intake and feed efficiency (body mass gain/dry matter feed intake) were monitored. Fish were allocated to new tanks (1 m^2) at a size of ~4 g (3 tanks per dietary group, n=200 fish per tank). During the final dietary challenge (3 tanks per dietary group, n=100 fish per tank), all fish were fed the challenge diet for 65 days in order to test the existence of nutritional programming in trout caused by the early nutritional stimulus.

At the end of the challenge, fish were anaesthetized by excess of anaesthetic (benzocaine), killed by cervical section and sampled at 2, 6 and 9 h after the last meal (n=9 per dietary history group at each sampling time, 3 fish per tank). Blood was quickly removed from the caudal vein using heparinized syringes and then centrifuged (3000 ***g***, 5 min). The recovered plasma was immediately frozen and stored at −20°C until analysis. Liver, midgut and a sample of dorsal white muscle were dissected and immediately frozen in liquid nitrogen, while the intestine was incubated in RNAlater®. All samples were stored at −80°C pending analyses.

Analytical methods

The chemical composition of the diets was analysed using the following procedures: dry matter after drying at 105°C for 24 h, lipid content by petroleum ether extraction (Soxtherm), protein content (N×6.25) by the Kjeldahl method after acid digestion, gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany), ash content by incinerating the samples in a muffle furnace at 600°C for 6 h, and starch content by enzymatic method (InVivo Labs, France). Plasma glucose (Glucose RTU, bioMérieux, Marcy l'Etoile, France) and triglyceride (Triglycerides PAP 150, bioMérieux) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer.

Relative quantification of mRNA and miRNA

Relative gene expression of mRNA and miRNA was determined by quantitative real-time RT-PCR on RNAs extracted from whole body (alevins) or tissue (juveniles). The extraction of total RNA from whole alevins or tissues in juveniles (liver and muscle) was performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. An amount of 1 µg of total RNA was used for cDNA synthesis. The NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen) or the SuperScript III RNaseH-Reverse Transcriptase Kit (Invitrogen) with random primers (Promega, Charbonniéres, France) was used according to the manufacturer's protocol to synthesize cDNA (n=9 for each time point) for miRNA and mRNA, respectively.

The primer sequences used in the real-time RT-PCR assays as well as the protocol conditions of the assays for mRNA of glucose and amino acid metabolic genes (Kamalam et al., 2012; Kamalam et al., 2013) and miRNAs (Mennigen et al., 2012) have previously been published. The transcripts analysed were glucokinase (GK; EC 2.7.1.2), hexokinase 1 (HK1; EC 2.7.1.1), 6-phosphofructo-1-kinase (6PFK; EC 2.7.1.11) and pyruvate kinase

(PK; EC 2.7.1.40) for glycolysis; glucose-6-phosphatase (G6Pase; EC 3.1.3.9), fructose1,6-bisphosphatase (FBPase; EC 3.1.3.11) and phosphoenol pyruvate carboxykinase (PEPCK; EC 4.1.1.32) for gluconeogenesis; sodium-dependent glucose transporter 1 (SGLT1), and facilitative glucose transporters (GLUT1, GLUT2 and GLUT4) for glucose transporters; glutamate dehydrogenase (GDH, EC 1.4.1.2) and serine dehydratase (SDH, EC 4.3.1.17) for amino acid catabolism; Na^+-dependent amino acid transporters (NBAT) and H^+-dependent peptide transporter (PEPT1) for amino acid transporters; and amylase (EC 3.2.1.1) and maltase (EC 3.2.1.20) for carbohydrate digestion. The selected miRNA are miRNA-29 and miRNA-107 (regulation of insulin signaling), miRNA-33 (regulation of liver proliferation and glucose metabolism) and miRNA-143 (regulation of adipocyte proliferation and hepatic glucose metabolism) (Dumortier et al., 2013; Ramírez et al., 2013).

For real-time RT-PCR assays of transcripts of metabolic genes and miRNAs, the Roche Lightcycler 480 system was used (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 of diluted cDNA template, 0.12 µl of each primer (10 µmol l^{-1}), 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 60–64°C), according to the primer set used. Melting curves were systematically monitored (temperature gradient at 1.1°C 10 s^{-1} from 65 to 94°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (reverse transcriptase- and cDNA template-free samples). The gene expression assays for the metabolic genes have been described in the publications previously cited for primer sequences. Briefly, the PCR protocol was initiated at 95°C for 3 min for initial denaturation of the cDNA and hot-start DNA polymerase activation and continued with 35 cycles of a two-step amplification programme (20 s at 95°C; 20 s at 56–60°C), according to the primer set used. Melting curves were systematically monitored (temperature gradient at 0.5°C 10 s^{-1} from 55 to 94°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each real-time RT-PCR run included replicate samples and controls as described above. For the expression analysis of both miRNA and mRNA, relative quantification of target gene expression was performed using the ΔC_t method (Pfaffl, 2001). The relative gene expression of 18S and ef1α were used for the normalization of measured miRNAs and mRNAs, respectively, as their relative expression did not significantly change over sampling time (data not shown). In all cases, PCR efficiency (E) was measured by the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.8 and 2.2.

Enzyme activities

Tissues (liver or muscle) used to assess enzyme activities was ground in 10 volumes of ice-cold buffer at pH 7.4 (50 mmol l^{-1} Tris, 5 mmol l^{-1} EDTA, 2 mmol l^{-1} DTT and a protease inhibitor cocktail; Sigma-Aldrich, St Louis, MO, USA; P2714). After homogenization, 1 min of sonic disruption was applied to the samples kept on ice. Then, homogenates were centrifuged at 4°C and supernatants were used immediately for enzyme assays. Enzymes assayed were: high-K_m hexokinase (GK) and low-K_m hexokinase (HK) as described by Panserat et al. (Panserat et al., 2000), G6Pase from Alegre et al. (Alegre et al., 1988), PK following the protocol of Kirchner et al. (Kirchner et al., 2003), and GDH described by Gomez-Requeni et al. (Gómez-Requeni et al., 2003). The enzyme activity was measured at 30°C in duplicate following the variation of absorbance of NADPH at 340 nm. The reactions were started by the addition of the specific substrate; a Power Wave X (BioTek Instrument, Inc.) plate reader was used. A blank with water instead of the substrate was run for each sample. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 µmol of substrate per minute at 30°C. Enzyme activity was expressed per milligram of soluble protein. Protein concentration was measured in triplicate according to Bradford (Bradford, 1976), using a protein assay kit (Bio-Rad, München, Germany) with BSA as a standard.

Microbial analyses

After thawing, the alevins were removed from the tubes with RNAlater® and immediately dissected in sterile Petri dishes on ice. The head and the dorso-posterior section were separated from the abdominal section, which was retained for microbial analysis. The intestinal sections from the juveniles were also placed in sterile Petri dishes on ice after thawing, to squeeze out the gut mucosae and the associated microbiota with a metallic spatula. The samples were immediately collected with sterile tweezers, and immersed in Extract-All® (Eurobio) for RNA extraction and subsequent RT-PCR-DGGE, as described for bacterial profiling by Lamari et al. (Lamari et al., 2013). Fungi were analysed using the primers NL1GC and LS2 (Verdugo Valdez et al., 2011) with the same PCR conditions as for bacteria. The PCR products were individually deposited on seven gels for DGGE, which were arranged to compare separately the fungal and bacterial profiles of either the alevins or the intestinal mucosae. The method allowed us to discriminate the bacterial phylotypes after their partial sequences of 16S and 26S rRNA for bacteria and fungi, respectively. The sequences could not be systematically read, but some bands were extracted from the DGGE gels for bacterial sequencing, when feasible (Lamari et al., 2013).

Statistical analysis

Data are presented as means ± s.d. The effect of the early feeding stimulus (HP versus VLP diets) on the growth and feed intake data, plasma metabolites, enzyme activities and gene expression was tested using an unpaired two-tailed Student's t-test (R software, R Commander package). The bacterial community profiles were analysed with PAST (Hammer et al., 2001), the dissimilarities between the profiles of the dietary groups were evaluated with Bray–Curtis indices compared by analysis of similarities and similarity percentage (Clarke, 1993). For all statistical analyses, the level of significance was set at $P<0.05$.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Conception and design of the study: I.G., D.M., J.Z. and S.P. Execution of the study: I.G., J.M., E.P.J., V.V., T.C. and J.G. Interpretation of the findings published: I.G., J.G., J.Z., S.S. and S.P. Drafting of the article: S.P. and I.G. Final responsibility of manuscript content: S.P.

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