

RESEARCH ARTICLE

Glucose metabolism ontogenesis in rainbow trout (*Oncorhynchus mykiss*) in the light of the recently sequenced genome: new tools for intermediary metabolism programming

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ABSTRACT

The rainbow trout (*Oncorhynchus mykiss*), a carnivorous fish species, displays a 'glucose-intolerant' phenotype when fed a high-carbohydrate diet. The importance of carbohydrate metabolism during embryogenesis and the timing of establishing this later phenotype are currently unclear. In addition, the mechanisms underlying the poor ability of carnivorous fish to use dietary carbohydrates as a major energy substrate are not well understood. It has recently been shown in trout that duplicated genes involved in glucose metabolism may participate in establishing the glucose-intolerant phenotype. The aim of this study was therefore to provide new understanding of glucose metabolism during ontogenesis and nutritional transition, taking into consideration the complexity of the trout genome. Trout were sampled at several stages of development from fertilization to hatching, and alevins were then fed a non-carbohydrate or a high-carbohydrate diet during first feeding. mRNA levels of all glucose metabolism-related genes increased in embryos during the setting up of the primitive liver. After the first meal, genes rapidly displayed expression patterns equivalent to those observed in the livers of juveniles. *g6pcb2.a* (a glucose 6-phosphatase-encoding gene) was up-regulated in alevins fed a high-carbohydrate diet, mimicking the expression pattern of *gck* genes. The *g6pcb2.a* gene may contribute to the non-inhibition of the last step of gluconeogenesis and thus to establishing the glucose-intolerant phenotype in trout fed a high-carbohydrate diet as early as first feeding. This information is crucial for nutritional programming investigations as it suggests that first feeding would be too late to programme glucose metabolism in the long term.

KEY WORDS: Ontogenesis, Duplicated genes, Gluconeogenesis, Carnivorous fish, Nutritional programming

INTRODUCTION

The rainbow trout (*Oncorhynchus mykiss*) belongs to a high trophic level and is thus considered to be a strictly carnivorous species metabolically adapted for high catabolism of proteins and low utilization of dietary carbohydrates. Glucose is highly important for specific functions and for tissues such as the brain, heart and gills in this species (Polakof et al., 2012). Despite this, the rainbow trout is considered to be a glucose-intolerant species, displaying persistent hyperglycaemia after intake of a high-carbohydrate diet. It is therefore thought to have modified or lost some of its glucose

metabolism capacities (Bergot, 1979a,b; Polakof et al., 2011b; Skiba-Cassy et al., 2013). Investigations have been carried out since the 1990s to try to understand the mechanisms underlying the poor ability of carnivorous fish to use dietary carbohydrates (Caruso and Sheridan, 2011; Cowey et al., 1977; Enes et al., 2009; Hung and Storebakken, 1994; Kamalam et al., 2012; Krasnov et al., 2001; Mommsen and E., 1991; Moon, 2001; Planas et al., 2000; Polakof et al., 2011a, 2012). Recent analysis of the newly sequenced rainbow trout genome has allowed identification of new genes that may contribute to the hyperglycaemic phenotype in trout (Marandel et al., 2015). Moreover, the study demonstrated the indisputable value of considering the complexity of the genome and the fate of duplicated genes after the salmonid-specific fourth whole-genome duplication event (Ss4R) to improve the understanding of the phenotype observed. Such duplication may lead to adaptive innovation via the conservation of duplicated genes available for the evolution of new functions (Berthelot et al., 2014). Indeed, two paralogous genes out of five encoding gluconeogenic glucose 6-phosphatase (G6pc) found in the rainbow trout genome exhibited the same expression pattern as genes encoding glycolytic glucokinase (Gck) in trout fed a high-carbohydrate diet. The authors suggested that under hyperglycaemic conditions these genes may together establish a futile glucose/glucose 6-phosphate cycle that could contribute to plasma glucose enrichment. To fully understand how the neo- or sub-functionalization of the newly identified duplicated glucose metabolism-related genes could lead to or at least be one factor involved in the poor use of dietary carbohydrates in rainbow trout, it is important to consider their transcriptional behaviour during the ontogenesis of hepatic functions and during early nutrition, i.e. during the transition from endogenous to exogenous feeding. Such studies might also be highly valuable in designing new research into nutritional strategies. In aquaculture, such investigations are carried out to improve the sustainability of aquaculture production of rainbow trout by replacing fish meal in aquafeeds with plant-based diets naturally rich in carbohydrates (Panserat and Kaushik, 2010). Nutritional programming, a currently promising nutritional strategy, is based on early-life modifications induced during a 'sensitive' period by environmental stimuli (strictly nutritional or not, such as temperature, hypoxia, etc.) that result in long-term modifications in metabolism, especially glucose metabolism, later in life (Jia et al., 2012; Metges et al., 2014; Ozanne et al., 1996; Patel and Srinivasan, 2002). Several studies have demonstrated that one decisive point in this concept is that the susceptibility of organisms is limited to a critical ontogenic period, also known as a window of metabolic plasticity, during which an environmental stimulus may affect metabolism in the long-term (for review, see McMillen and Robinson, 2005). Determining the ontogenesis of glucose metabolism-related genes potentially involved in the glucose-intolerant phenotype in trout could thus

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help to time when best to apply the environmental stimulus in nutritional programming investigations.

The aim of the present study was to analyse glucose metabolism genes, i.e. genes belonging to the two main glucose-related pathways: glycolysis and gluconeogenesis (Fig. S1), to: (i) identify and characterize the evolution of the duplicated glycolysis genes in rainbow trout (i.e. liver phosphofructokinase and liver pyruvate kinase); (ii) determine the expression pattern of duplicated glucose metabolism-related genes, i.e. glycolytic genes (glucokinase paralogs, *gcka* and *gckb*; liver phosphofructokinase paralogs, *pfkla* and *pfklb*; liver and red blood cell pyruvate kinase, *pkbr*) and gluconeogenic genes (cytosolic and mitochondrial phosphoenol pyruvate carboxykinases *pck1* and *pck2*, respectively; fructose 1,6-bisphosphatase 1 paralogs *fbp1a*, *fbp1b1* and *fbp1b2*; glucose 6-phosphatase paralogs *g6pca*, *g6pcb1.a*, *g6pcb1.b*, *g6pcb2.a* and *g6pcb2.b*), and their corresponding overall enzyme activity at critical developmental stages in liver ontogenesis; and (iii) study the expression pattern of these genes during nutritional transition from endogenous to exogenous feeding in trout fed a non- or a high-carbohydrate diet.

MATERIALS AND METHODS

Ethics statement

The experiment was carried out in strict accordance with EU legal frameworks related to the protection of animals used for scientific purposes (directive 2010/63/EU) and guidelines of the French legislation governing the ethical treatment of animals (decree no. 2001-464, 29 May 2001). It was approved by the Direction Departementale des Services Veterinaires (French Veterinary Services) for the carrying out of animal experiments (INRA 2002–36, 14 April 2002). The INRA experimental station is certified for animal experimentation under permit no. A64.495.1 by the French Veterinary Services, the relevant authority.

Fish diets

Two experimental feeds for rainbow trout alevins, i.e. no-CHO (diet without carbohydrate) and high-CHO (diet with very high carbohydrate), were prepared in our own facilities (INRA, Donzacq, Landes, France) as extruded pellets. Glucose and gelatinized starch were included as the carbohydrate sources; protein originated from fish meal, and dietary lipids from fish oil and fish meal (Table 1). The two diets had similar amounts of lipids. The large increase in dietary carbohydrate content (~60%) in the high-CHO diet was compensated for by a smaller proportion of protein (~20%). No carbohydrates were added to the no-CHO diet, which contained ~60% crude protein.

Fish and experimental design

Spawns were fertilized synchronously with *neomale sperm* and reared in separate tanks at 8°C at the INRA experimental facilities, Lees-Athas, France. Fish were sampled according to spawn origin before fertilization (oocyte) and then during development according to Vernier (1969) at stages 2, 5, 6, 7, 8, 10, 12, 15, 22 and 23. Endogenous feeding alevins (unfed fish) were sampled at 384°D (degree days, Vernier stage 31). Alevins at 616°D (Vernier stage 35) still exhibiting small yolk reserves were divided into two tanks. Each group was fed a first meal with the no-CHO or high-CHO diet at 09:00 h and killed 3 h after the meal (corresponding to the postprandial period in alevins). Finally, 87 day old alevins (696°D at 8°C), characterized by complete resorption of the yolk sac (Vernier stage 37) and maintained on a continuous feeding regime of several meals a day, were killed 3 h following the final feeding as

Table 1. Formulation and proximate composition of the two experimental diets (no-CHO and high-CHO)

| | No-CHO | High-CHO |
|--------------------------------------|--------|----------|
| Ingredients (%) | | |
| Fish meal ¹ | 85.9 | 21.5 |
| Fish oil ² | 10.1 | 14.5 |
| Starch ³ | 0 | 40 |
| Glucose ⁴ | 0 | 20 |
| Vitamin mix ⁵ | 1 | 1 |
| Mineral mix ⁶ | 1 | 1 |
| Alginate ⁷ | 2 | 2 |
| Proximate composition | | |
| DM (% diet) | 90.35 | 90.90 |
| Crude protein (% DM) | 58.37 | 19.50 |
| Crude lipid (% DM) | 17.46 | 16.00 |
| Gross energy (kJ g ⁻¹ DM) | 21.73 | 21.60 |
| Ash (% DM) | 20.16 | 6.10 |
| Carbohydrates (% DM) | <1.0 | 58.4 |

DM, dry matter.

¹Sopropeche, Boulogne-sur-Mer, France; ²Sopropeche, Boulogne-sur-Mer, France; ³gelatinized corn starch (Roquette, Lestrem, France); ⁴D-(+)-glucose (Sigma-Aldrich, G7021); ⁵supplying (kg⁻¹ diet): 60 IU DL- α -tocopherol acetate, 5 mg sodium menadione bisulphate, 15,000 IU retinyl acetate, 3000 IU DL-cholecalciferol, 15 mg thiamine, 30 mg riboflavin, 15 mg pyridoxine, 0.05 mg vitamin B12, 175 mg nicotinic acid, 500 mg folic acid, 1000 mg inositol, 2.5 mg biotin, 50 mg calcium pantothenate and 2000 mg choline chloride; ⁶supplying (kg⁻¹ diet): 2.15 g calcium carbonate (40% Ca), 1.24 g magnesium oxide (60% Mg), 0.2 g ferric citrate, 0.4 mg potassium iodide (75% I), 0.4 g zinc sulphate (36% Zn), 0.3 g copper sulphate (25% Cu), 0.3 g manganese sulphate (33% Mn), 5 g dibasic calcium phosphate (20% Ca, 18% P), 2 mg cobalt sulphate, 3 mg sodium selenite (30% Se), 0.9 g potassium chloride and 0.4 g sodium chloride; and ⁷Louis François, Marne-la-Vallée, France.

described above. Care was taken to maintain the same daily time frame before samplings to avoid potential circadian effects, known to affect metabolism in rainbow trout (Bolliet et al., 2000). Fish were therefore consistently sampled at 12:00 h for each ontogenetic stage investigated in this study, as previously described (Mennigen et al., 2013). Embryos were directly snap-frozen, whereas alevins were killed by terminal anaesthetization by bathing in benzocaine prior to pooling and storage in liquid nitrogen. The samples were stored at -80°C until analysed.

Analysis of diets

The chemical composition of the diets was analysed using the following procedures: dry matter was analysed after drying at 105°C for 24 h, lipid content was obtained by petroleum ether extraction (Soxtherm), protein content (N \times 6.25) was obtained by the Kjeldahl method after acid digestion, gross energy was measured in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany), and ash content was measured by incinerating the samples in a muffle furnace for 6 h at 600°C.

Body composition analysis of oocytes, embryos and endogenous feeding alevins

Endogenous and glucose content were analysed in 600 mg of pooled fish (oocytes, embryos from stage 2 to stage 23 and endogenous feeding alevins, stage 31). Glycogen content was determined by a hydrolysis technique previously described by Good et al. (1933). Each sample was ground in 1 mol l⁻¹ HCl (VWR, USA). An aliquot was saved at this stage to measure the free glucose content. After 10 min centrifugation at 10,000 g, free glucose was measured using the Amplite™ Fluorimetric Glucose Quantitation Kit (AAT Bioquest®, Inc., USA) according to the manufacturer's instructions. The remaining ground tissue was boiled at 100°C for 2.5 h and then

Table 2. Primer sequences and accession numbers for qPCR analysis

| Gene | Forward primer (5'→3') | Reverse primer (5'→3') | Accession number (Genoscope or Sigenae) |
|--------------------------------|------------------------|------------------------|---|
| <i>pfkla</i> (<i>sc7584</i>) | GATCCCTGCCACCATCAGTA | GTAACCACAGTAGCCTCCCA | GSONMG00009459001 |
| <i>pfklb</i> (<i>sc8651</i>) | AGTGCTCGCTGTAAGGTCTT | GTGATCCGGCCTTTCTGAAC | GSONMG00001975001 |
| <i>pklr</i> | CCATCGTCGCGGTAACAAGA | GCCCTGGCCTTTCCTATGT | ContigAF246146.s.om.10 |

neutralized by 5 mol l⁻¹ KOH (VWR, USA). The pH of the solution was then adjusted to 7.4 and total glucose (free glucose+glucose obtained from glycogen hydrolysis) was measured using the same kit as before. Glycogen content was evaluated by subtracting free glucose levels.

Total lipid content was determined in duplicate by the sulphophosphovanilun method described by Barnes and Blackstock (1973) using a fish oil standard (Sopropeche, Boulogne-sur-Mer, France) instead of cholesterol.

Total protein content was also measured in duplicate by using the Kjeldahl method as for diet analysis.

In silico analysis

Orthologous *pfkl* and *pklr* genes in the rainbow trout genome (Berthelot et al., 2014) were identified in the *Oncorhynchus mykiss* genome browser (Genoscope: <http://www.genoscope.cns.fr/trout/>) extracted from the SIGENAE database (<http://www.sigenae.org>) using the BLAST tool (all accession numbers are given in Table 2). Sequences from other species were collected from the Ensembl Genome database (Ensembl release 77, October 2014: <http://www.ensembl.org>). The sequence analyser tool from the EMBL Pfam software (<http://pfam.xfam.org>) was used to confirm sequence identities. Genomicus software, v01.01 (<http://www.genomicus.biologie.ens.fr/genomicus-trout-01.01/cgi-bin/search.pl>) was used to confirm the identities of the *pfkl* genes.

Phylogenetic analysis of *pklr* (Fig. 1) was performed using the MEGA package v6 software (Tamura et al., 2013), as previously described (Marandel et al., 2015). The phylogenetic tree, based on deduced full-length amino acid sequences, was produced using the neighbour-joining (NJ) method and confirmed by the minimum evolution method (data not shown). The reliability of the inferred trees was estimated using the bootstrap method with 1000 replications. The *Petromyzon marinus* *pk* (Ensembl Accession ENSPMAP00000000233) protein sequence was used to root the tree. The evolutionary distances were computed using the Poisson correction method (Zuckermandl, 1965) and expressed in units of the number of amino acid substitutions per site. The analysis involved 10 amino acid sequences. All positions containing gaps

and missing data were eliminated, leaving a total of 127 positions in the final dataset.

New gene annotations were allocated according to ZFIN Nomenclature guidelines (<http://zfin.org/>). All sequences and deduced partial amino acids are given in Fig. S2.

Total RNA extraction and cDNA synthesis

Relative gene expression was determined by quantitative real-time RT-PCR. Samples were homogenized using Precellys[®]24 (Bertin Technologies, Montigny-le-Bretonneux, France): (1) in 7 ml tubes containing Trizol reagent (Invitrogen, Carlsbad, CA, USA) and 2.8 mm ceramic beads for 2×30 s, separated by 15 s, at 5500 rpm for oocytes and embryos (30 per sample and per spawn); and (2) in 2 ml tubes containing Trizol reagent (Invitrogen) and 2.8 mm ceramic beads for 2×20 s, separated by 15 s, at 5500 rpm for alevins (1 alevin per tube, 6 alevins per spawn/diet for mixed and exogenous feeding fish). Luciferase control RNA (Promega), 10 pg per 1.9 mg of embryo/alevin or oocyte, was added to each sample to allow for data normalization during early development as previously described (Desvignes et al., 2011; Marandel et al., 2012). Total RNA was then extracted according to the manufacturer's instructions. Total RNA (1 µg) was used for cDNA synthesis. The Super-Script III RNase H-Reverse transcriptase kit (Invitrogen) was used with random primers (Promega, Charbonnières, France) to synthesize cDNA.

Real-time RT-PCR

The primer sequences used in real-time RT-PCR assays for gluconeogenic and *gcka* and *gckb* gene analysis were as previously described (Marandel et al., 2015). Primers newly designed to study the *pfkl* and *pklr* genes are listed in Table 2. They were validated on a pool of cDNA and amplified products were sequenced systematically. The protocol conditions for real-time RT-PCR have been published previously (Seilliez et al., 2011). For real-time RT-PCR assays, 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 (1:25), 0.12 µl of each primer (10 µmol l⁻¹), 3 µl Light Cycler 480 SYBR[®] Green I Master mix

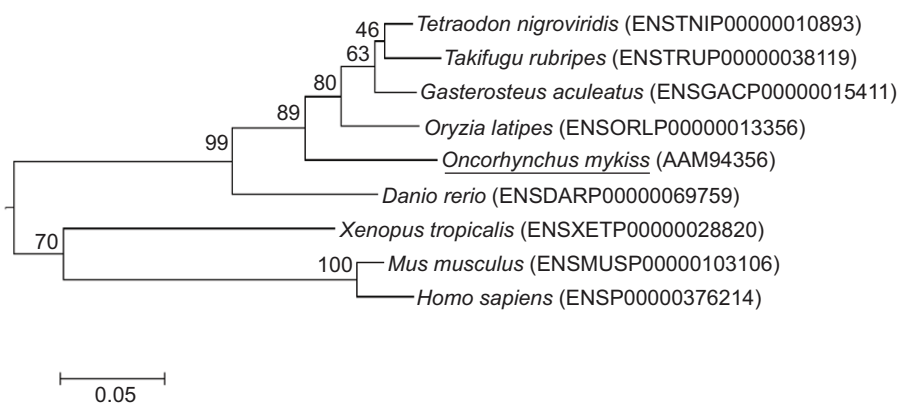


Fig. 1. Phylogenetic analysis of Pklr (liver and red blood cell pyruvate kinase). Deduced amino acid sequences were aligned using MUSCLE software (Edgar, 2004). Phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA) software v6.0 (Tamura et al., 2013). The phylogenetic tree was built using the neighbour-joining (NJ) method. The reliability of the inferred tree was estimated using the bootstrap method with 1000 replications. The lamprey *pklr* sequence (*Petromyzon marinus*, Ensembl ID: ENSPMAP00000000233) was used to root the tree. Protein accession numbers are given in parentheses.

and 0.76 μ l 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 program (15 s at 95°C; 10 s at 60°C). Melting curves were monitored systematically (temperature gradient 1.1°C/15 s from 65 to 97°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, respectively) and negative controls (reverse transcriptase- and cDNA template-free samples, respectively). Data were subsequently normalized to the exogenous luciferase transcript

abundance in samples diluted at 1:25 using the ΔC_t method (Pfaffl, 2001) as previously described (Marandel et al., 2012).

Overall enzyme activity

Embryos at stages 6, 15, 22 and 23, and alevins at stage 31 (see ‘Fish and experimental design’, above) used to assess enzyme activity were ground in 8 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 (P2714; Sigma, St Louis, MO, USA) and centrifuged for 10 min at 900 *g* at 4°C. Assays for Gck and Fbp activity were performed on the recovered supernatants. For Pfk1 and Pklr, additional centrifugation was performed (20 min at 10,000 *g* at 4°C)

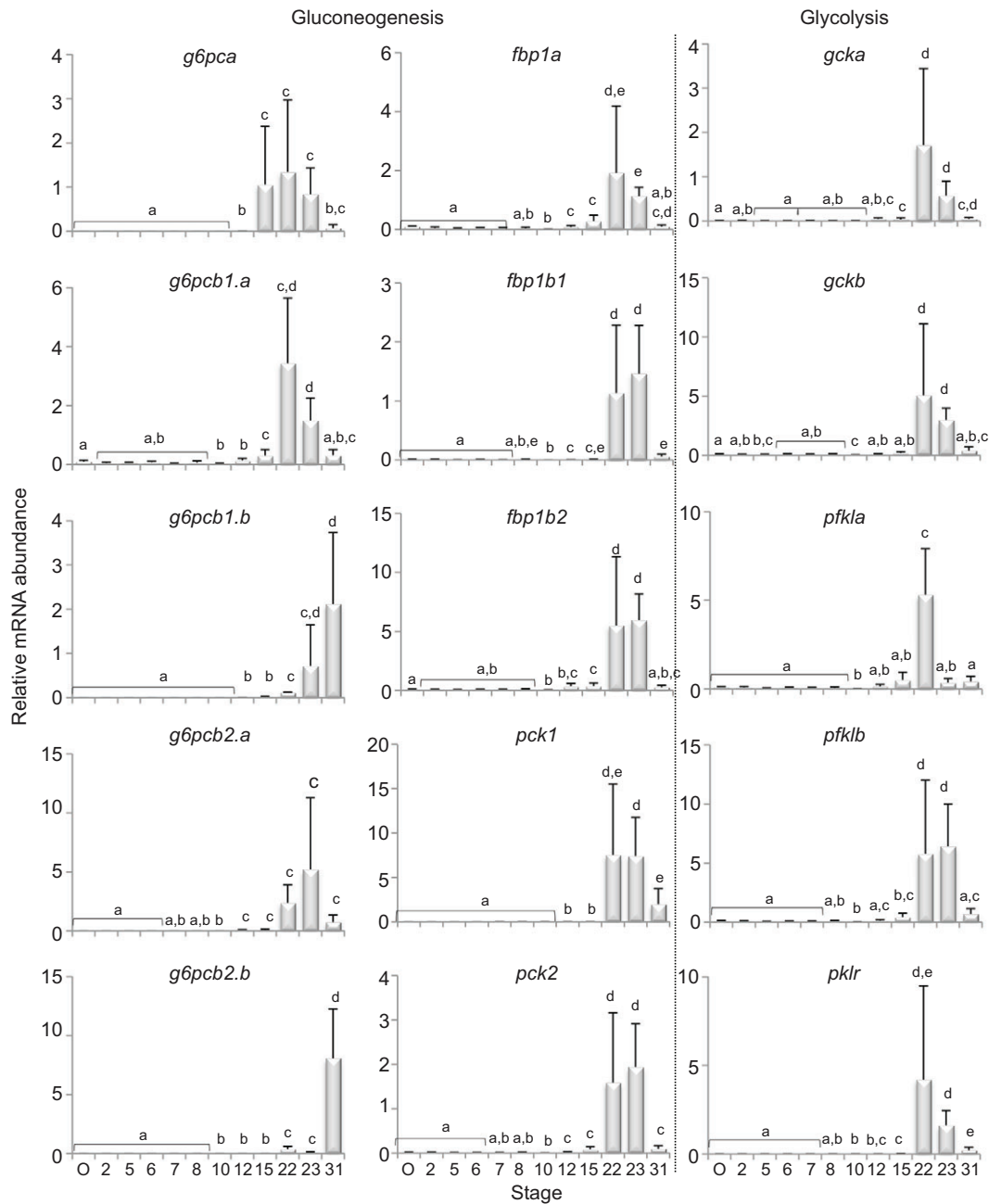


Fig. 2. Expression pattern of gluconeogenic and glycolytic genes during development. Data are the relative abundance of gluconeogenic (left) and glycolytic (right) genes during development. Embryos were sampled according to Vernier (1969) at stages O (oocyte), 2, 5, 6, 7, 8, 10, 12, 15, 22 and 23, and 31 (alevin). For all stages, gene expression level was normalized by the abundance of exogenous luciferase RNA. Data are expressed as means \pm s.e.m. ($N=3$ pools of embryos, one pool of 30 embryos per spawn). Different letters indicate significant differences between conditions ($P<0.01$).

Table 3. Overall enzyme activity

| Stage | Gluconeogenesis (U g ⁻¹) | | | Glycolysis (U g ⁻¹) | | |
|-------|--------------------------------------|------------|-------------|---------------------------------|-------------|--------------|
| | G6pc | Fbp | Pck | Gck | Pfkl | Pklr |
| 6 | 0.01±0.00 | n.d. | n.d. | n.d. | 0.04±0.01 | 0.50±0.06 |
| 15 | 0.01±0.00 | n.d. | 0.01±0.00 | n.d. | 0.02±0.00 | 0.41±0.09 |
| 22 | 0.01±0.00 | n.d. | 0.01±0.00 | n.d. | 0.03±0.02 | 0.41±0.04 |
| 23 | 0.01±0.00 | n.d. | 0.03±0.01* | n.d. | 0.07±0.01* | 0.41±0.12 |
| 31 | 0.03±0.01* | 0.09±0.00* | 0.48±0.07** | n.d. | 3.86±1.30** | 100.19±8.08* |

Embryos were sampled according to Vernier (1969) at stages 6, 15, 22, 23 and 31. Data are expressed as means±s.d. (N=3 pools of embryos, one pool per spawn) in U g⁻¹ of tissue. Asterisks indicate significant differences between conditions (P<0.01).

and supernatants were used for enzyme assays. For G6pc and Pck, 1 min of sonic disruption was applied before additional centrifugation as above. The enzymes assayed were: high-*K_m* hexokinase (Gck), as described by Panserat et al. (2000a), Pklr and Pck following the protocol of Kirchner et al. (2003), Pfkl according to Metón et al. (2004), G6Pase according to Alegre et al. (1988), and Fbp as described by Tranulis et al. (1996). Enzyme activity was measured in duplicate at 30°C following variation of absorbance of nicotinamide adenine dinucleotide phosphate at 340 nm. The reactions were started by the addition of the specific substrate; a Power Wave X (BioTek Instrument) plate reader was used. Water was used as a blank for each sample. One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 mol of substrate per minute at 30°C. Enzyme activity was expressed per gram of tissue.

Statistical analysis

Normality of distributions was assessed using the Shapiro–Wilk test. Data were then analysed by a Kruskal–Wallis non-parametric test followed by a Tukey test as *post hoc* analysis. Data were analysed using the R software (v3.1.0)/R Commander Package.

RESULTS

In silico analysis of rainbow trout *pfkl* and *pklr* genes

Using the Genomicus software (v01.01), three rainbow trout predicted *pfkl* genes were identified (Fig. S2), two of which on the scaffold 7584 (Genoscope accession no. GSONMG00009460001 and GSONMG00009459001) were syntenic to the zebrafish *pfkla* gene (Ensembl accession no. ENSDARG0000060504) and one of which, on the scaffold 8651 (Genoscope accession no. GSONMG00001975001), was syntenic to the *pfklb* zebrafish gene

(Ensembl accession no. ENSDARG0000099755). Using the sequence analyser tool from the EMBL Pfam software (<http://pfam.xfam.org>), we confirmed that these two rainbow trout sequences were related to a phosphofructokinase-encoding gene. Amino acid sequences deduced from these rainbow trout predicted genes were shorter than the corresponding zebrafish sequences and were thus not appropriate for phylogenetic analysis. Indeed, scaffold 7584 sequences corresponded to exons 2–5 of the zebrafish *pfkla* (Fig. S3A) and scaffold 8651 sequences corresponded to exons 15–17 of the zebrafish *pfklb* (Fig. S3B), suggesting that these two scaffolds were too short to cover the lengths of these genes as a whole. This analysis was therefore complemented by investigating the EST (expressed sequence tags) rainbow trout Sigenae database (www.sigenae.org). One EST contig (FYV3OTN01AZR97.s.om.10; Fig. S3A) was shown to correspond to exons 7–22 of the zebrafish *pfkla*, demonstrating that the two automatically predicted genes (GSONMG00009460001 and GSONMG00009459001) on scaffold 7584 were in fact only one. Similarly, we found an EST contig (FYV3OTN02GK8A7.s.om.10; Fig. S3B) astride exons 1–5 of the zebrafish *pfklb*, suggesting that the corresponding orthologous gene in rainbow trout was partially due to the incomplete assembling of the rainbow trout genome at this locus.

No *pklr* orthologous gene was found in the rainbow trout genome (either using Genomicus software or by direct BLAST against the rainbow trout genome) but an EST contig (Sigenae, AF246146.s.om.10) sharing high sequence homology (around 75%) with teleost *pklr* genes was identified. A phylogenetic analysis was performed to confirm its identity and this showed that the contig identified grouped with vertebrate *pklr* orthologues (Fig. 1). Moreover, Pfam analysis confirmed that the trout sequence was related to a pyruvate kinase-encoding gene. In conclusion, our *in silico* analysis identified two

Table 4. Body composition of embryos and hatched alevins

| Stage | DM | Proteins (%DM) | Lipids (%DM) | Carbohydrates (%DM) |
|--------|------------|----------------|--------------|---------------------|
| Oocyte | 37.73±2.75 | 69.55±1.70 | 29.62±1.77 | 0.34±0.03 |
| 2 | 33.79±0.42 | 69.42±1.19 | 29.6±2.34 | 0.31±0.06 |
| 5 | 33.94±0.53 | 69.19±1.39 | 29.66±2.88 | 0.31±0.04 |
| 6 | 33.99±0.14 | 69.27±1.44 | 29.62±2.44 | 0.31±0.04 |
| 7 | 34.19±0.21 | 69.46±1.03 | 29.79±1.77 | 0.31±0.06 |
| 8 | 33.52±0.51 | 69.89±1.75 | 30.05±1.52 | 0.32±0.07 |
| 10 | 34.01±0.21 | 69.10±1.00 | 29.87±2.84 | 0.31±0.04 |
| 12 | 33.62±0.30 | 69.17±1.20 | 30.04±2.55 | 0.30±0.03 |
| 15 | 34.10±0.30 | 69.22±1.06 | 30.96±2.71 | 0.31±0.03 |
| 22 | 33.76±0.20 | 69.09±1.06 | 29.96±2.05 | 0.21±0.03 |
| 23 | 33.06±0.31 | 69.59±1.43 | 29.69±2.83 | 0.23±0.03 |
| 31 | 31.29±0.28 | 68.97±1.75 | 31.59±2.61 | 0.32±0.02 |

DM, dry matter.

Embryos were sampled according to Vernier (1969) at stages O (oocyte), 2, 5, 6, 7, 8, 10, 12, 15, 22 and 23, and 31 (alevin). Data are expressed as means±s.d. (N=3 pools of embryos, one pool per spawn). Values in bold show a decrease from levels at other stages.

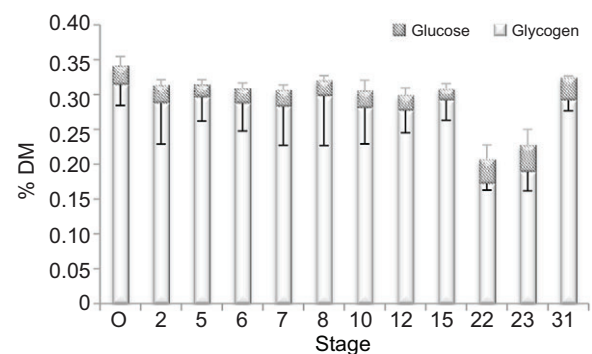


Fig. 3. Glucose and glycogen content in embryos and hatched (stage 31) alevins. Glucose and glycogen content expressed as percentage of dry matter (DM). Embryos were sampled according to Vernier (1969) at stages O (oocyte), 2, 5, 6, 7, 8, 10, 12, 15, 22 and 23, and 31 (alevin). Data are expressed as means±s.e.m. (N=3 pools of embryos, one pool per spawn).

paralogous *pfkl* genes in the rainbow trout genome, one orthologous to the zebrafish *pfkla* (scaffold 7584) and the other to the zebrafish *pfklb* (scaffold 8651, GSONMG00001975001), and one *pklr* orthologous gene (Sigenae, AF246146.s.om.10).

Expression analysis and overall enzymatic activity of glucose metabolism-related gene products during embryo development

Real-time PCR was performed to determine the stage-specific expression of rainbow trout glucose metabolism-related genes during embryogenesis and in hatched alevins. Stages were defined on the basis of the Vernier development table (Vernier, 1969) and chosen to target developmental periods of interest for glucose metabolism study [i.e. oocyte to stage 8, before embryonic genome activation (EGA); stage 10, EGA; stages 12 and 15, epiboly period; stage 22, primitive liver; stage 23, primitive hepatic portal vein, and stage 31, hatched embryo/endogenous feeding period]. This

analysis showed (Fig. 2) that mRNA levels of all glucose metabolism genes increased after EGA (stage 10) to reach a maximum level at stages 22 and 23, except for *g6pcb1.b* and *g6pcb2.b*, the mRNA levels of which rose until stage 31.

The overall activity of all key glucose metabolism enzymes (gluconeogenesis: G6pc, Fbp, Pck; glycolysis: Gck, Pfk1, Pk1r) were also measured at critical stages of development: before EGA (stage 6), after EGA (stage 15), during liver ontogenesis (stages 22 and 23) and shortly after hatching (stage 31). The enzymatic activity of Gck remained undetected during development and at hatching (Table 3), whereas enzymatic activity significantly increased from stage 23 for Pck and Pfk1 and at stage 31 for G6pc, Fbp and Pk1r.

Body composition during embryo development

In order to follow the relative consumption of macronutrients in the embryo and the yolk reserves during development, protein, lipid and carbohydrate (i.e. glucose and glycogen) levels were measured. No

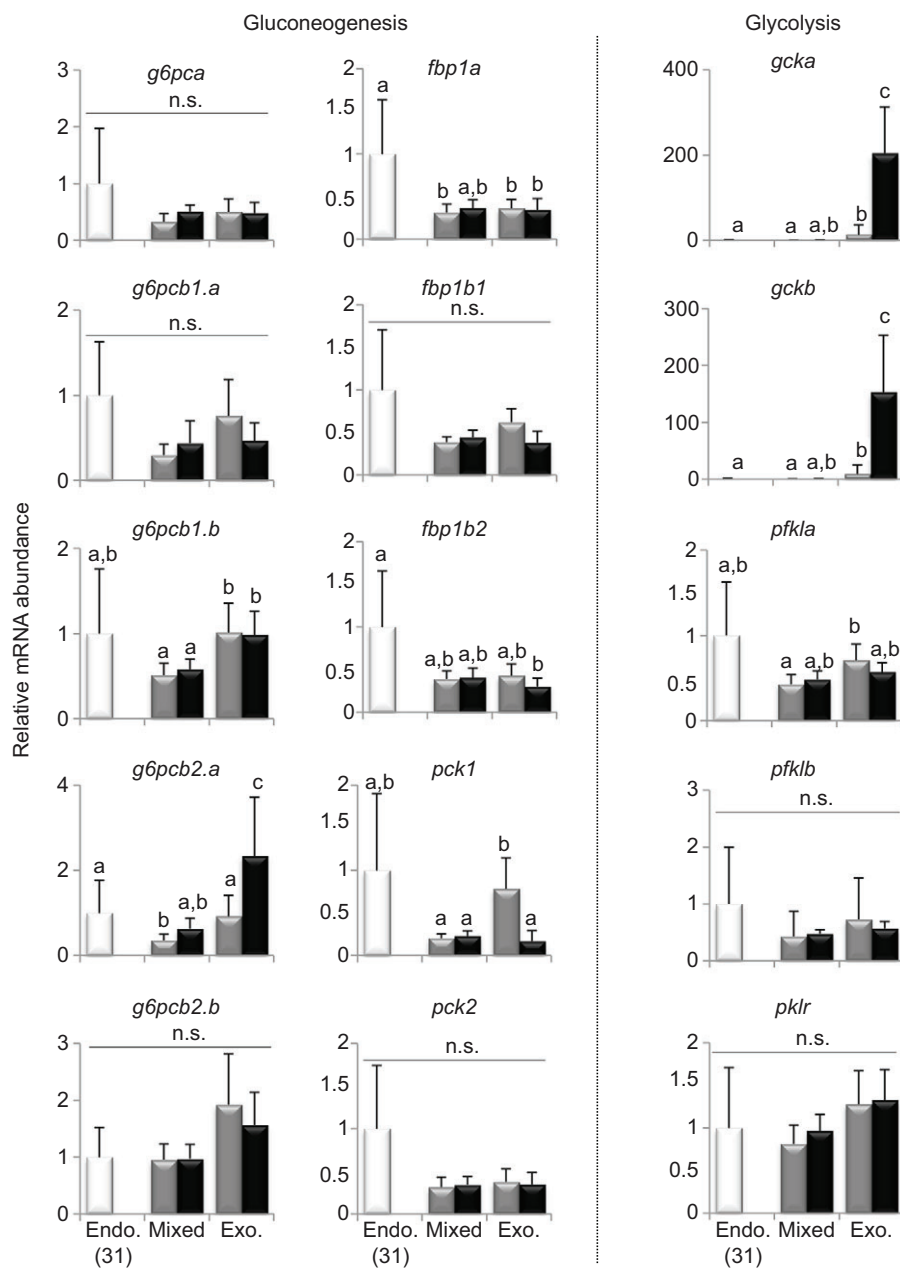


Fig. 4. Expression pattern of gluconeogenic and glycolytic genes during nutritional transition.

The relative abundance of mRNA of gluconeogenic (left) and glycolytic (right) genes in strictly endogenous feeding alevins (white bars), and mixed feeding (3 h after first meal) and exogenous feeding alevins fed with the no-CHO diet (grey bars) or the high-CHO diet (black bars). For all stages, gene expression level was normalized by the abundance of exogenous luciferase RNA. Data are expressed as means \pm s.e.m. ($N=9$ alevins, 3 alevins per spawn). Different letters indicate significant differences between conditions ($P<0.01$).

statistically significant variation in lipid or protein levels was noted during development (Table 4). Although the overall carbohydrate content of embryos (Table 4, Fig. 3) was not significantly modified during development, it seemed to decrease at stages 22 and 23, as did glycogen content (Fig. 3).

Analysis of expression of glucose metabolism-related genes during nutritional transition

Examination of the expression of glucose metabolism-related genes (Fig. 4) revealed that for half of the genes (*g6pca*, *g6pcb1.a*, *g6pcb2.b*, *fbp1b1*, *pck2*, *pfklb* and *pklr*) there was no difference in expression pattern whatever the nutritional source used by alevins (yolk reserves versus exogenous feed) or diet (no-CHO versus high-CHO), irrespective of the metabolic pathway considered (i.e. gluconeogenesis or glycolysis).

For alevins fed the no-CHO diet, mRNA levels of glycolytic *gcka*, *gckb* and *pfkla* genes remained stable at the mixed feeding stage compared with strictly endogenous feeding alevins and then increased at the exogenous feeding stage. The same expression pattern was followed by gluconeogenic *g6pcb1.b* and *pck1* genes, whereas the *fbp1b2* mRNA level remained stable during the nutritional transition in alevins fed the no-CHO diet from the first meal. Finally, mRNA levels of gluconeogenic *g6pcb2.a* and *fbp1a* genes decreased at the mixed feeding stage and only the *g6pcb2.a* mRNA level increased at the exogenous feeding stage, whereas the *fbp1a* mRNA level remained unchanged.

Intake of dietary carbohydrates by fed alevins (i.e. high-CHO diet) had no effect on mRNA levels of any genes at the mixed feeding stage or on *pfkla*, *g6pcb1.b*, *fbp1a* and *fbp1b2* mRNA levels at the exogenous feeding stage. However, at the exogenous feeding stage the *pck1* mRNA level decreased whereas *g6pcb2.a* and *gck* genes were up-regulated. Finally, the *fbp1b2* mRNA level decreased between the endogenous and exogenous feeding stages in alevins fed the high-CHO diet.

DISCUSSION

A considerable amount of research has been undertaken over several decades to elucidate the role of environmental factors (mainly nutritional and hormonal) in glucose metabolism and their impact on the utilization of dietary carbohydrates in trout (Bergot, 1979a,b; Kirchner et al., 2003, 2008, 2005; Moon, 2001; Panserat and Kaushik, 2010; Panserat et al., 2000a,b, 2002, 2001, 2014; Polakof et al., 2012, 2009; Seiliez et al., 2011; Skiba-Cassy et al., 2013). However, the importance of carbohydrate metabolism during embryogenesis and the timing of the setting up of the glucose-intolerant phenotype in trout are not clear. In addition, mechanisms underlying the poor ability of carnivorous fish to use dietary carbohydrates as a major energy substrate remain poorly understood. A recent study emphasized that the fate of duplicated genes after Ss4R may participate in the initiation of the glucose-intolerant phenotype in juvenile trout fed a high-carbohydrate diet (Marandel et al., 2015). This new genomic point of view following the recent sequencing of the rainbow trout genome should provide new pathways to investigate impaired glucose metabolism in this species. The aim of the investigations carried out in this study was to provide original understanding of glucose metabolism during ontogenesis, taking into consideration the complexity of the trout genome.

mRNA levels increase during ontogenesis of the primitive liver

Increases in mRNA levels of both gluconeogenic and glycolytic genes occurred at stage 22 or 23, corresponding to the setting up of

the primitive liver and the primitive hepatic portal vein, respectively (Vernier, 1969). Indeed, gluconeogenic genes are mainly expressed in the liver as well as *gck* genes. But an increase in glycolytic gene expression could also occur in other glycolytic tissues, such as muscle or brain, the development of which has been completed at these stages (22 or 23). This increase was correlated with a slight decrease in total carbohydrate content of embryos, whereas total proteins and lipids remained constant and at levels previously described in trout embryos (Craik and Harvey, 1984; Smith, 1957). Shortly after the embryos hatched (stage 31), the carbohydrate load increased again slightly, mainly due to an increase in glycogen, at the same time as the glucose metabolism-related enzyme activity (G6pc, Pck, Pflk and Pklr) became detectable. Such an accumulation of glycogen after stage 23 has previously been described in embryos, together with an increase in G6pc activity (Vernier and Sire, 1976). We confirmed these findings and demonstrated that gluconeogenic Pck activity occurred in the same pattern as G6pc activity. Such activation of the gluconeogenic pathway might serve to produce glucose to supply glycogenogenesis, as free glucose storage at this stage is low and probably just used to support brief outbursts of activity. Indeed, Terner (1968) proposed that glycogen stored during late embryogenesis originates from glucose made from glycerol liberated by the utilization of triglycerides. The early metabolic role of the liver might thus be more related to the control of plasma lipid concentration, a necessary role for the use of yolk storage, than to glycaemic regulation. Overall, Pflk and Pklr glycolytic activity was greatly increased after hatching (stage 31), probably to provide energy to sustain motor function or basal metabolism in alevins. However, overall Gck activity was undetected at any stage analysed, suggesting that the glucose concentration potentially released by gluconeogenesis at stage 31 was too weak to counteract the low affinity of this hexokinase for glucose. However, Gck was certainly translated and stored in cells in an inactive form (Cornish-Bowden and Ca'ardenas, 2004) or combined with the GCK regulatory protein (Baltrusch et al., 2004) as both *gck* genes were transcribed early (at stages 22 and 23) and then turned off later at stage 31.

Finally, the early increase in mRNA levels of glucose metabolism-related genes (at stage 22/23) followed by the detection of enzyme activity suggested that glucose metabolism might be essential at the end of embryonic development and for the production of energy but could also reflect a phase of preparation for the catabolism of dietary nutrients at first feeding, as already proposed for digestive enzymes (Zambonino Infante and Cahu, 2001).

Studying duplicated glucose metabolism-related genes: New insights into nutritional transition

Our study also aimed to investigate changes in the expression of glucose metabolism-related genes in the light of the recent genome sequencing during nutritional transition in alevins. Indeed, during the first feeding period, trout alevins undergo a rapid ontogenic switch in nutritional strategy, passing successively through an endogenous nutrition period dependent exclusively on yolk reserves, then to a mixed feeding period after the first meal and before yolk resorption, and finally to exclusively exogenous nutrition (Vernier, 1969).

When analysing expression data first as a whole (Fig. 4), we found that paralogous genes (i.e. mammalian *G6pc*, *Fbp1*, *Gck* and *pfkl* co-orthologues, respectively) displayed divergent patterns. These results confirmed that a sub- or neo-functionalization of these genes occurred after their duplication, as previously proposed by Marandel et al. (2015).

In alevins fed the no-CHO diet from the first meal, gluconeogenic genes displayed a constant expression pattern during nutritional transition (*g6pca*, *g6pcb1.a*, *g6pcb2.b*, *fbp1b1*, *fbp1b2* and *pck2*), down-regulation at the first meal followed by an increase in mRNA level at the exogenous feeding stage not exceeding the level at the endogenous feeding stage (*g6pcb1.b*, *g6pcb2.a* and *pck1*), or down-regulation from the first meal (*fbp1a*). Thus, taking into consideration all duplicated genes encoding gluconeogenic enzymes, our results suggested maintenance of an active gluconeogenic pathway during nutritional transition and no inhibition in alevins utilizing an exogenous diet, as previously proposed by Mennigen et al. (2013). There was a decrease in the mRNA levels of two genes involved in the last step of glucose release at the first meal, i.e. *g6pcb1.b* and *g6pcb2.a*. At this stage, the glycogen stored from yolk glycerol through gluconeogenesis during embryo development (see paragraph above) stabilized (Vernier and Sire, 1976). It can be hypothesized that this stabilization leads to a fleeting down-regulation of *g6pc* paralogous genes at the first meal. At first feeding, the liver, which governs lipid regulation during embryogenesis with the use of yolk reserves, regulates glycaemia (Vernier and Sire, 1976). The conservation of an active gluconeogenic pathway in exogenous feeding alevins as well as in juveniles (Marandel et al., 2015; Seiliez et al., 2011) fed without dietary carbohydrates thus made it possible to sustain endogenous glucose demands for the maintenance of normoglycaemia (Covey et al., 1981; French et al., 1981; Walton and Covey, 1979, 1983). Moreover, genes involved in the glycolytic pathway were maintained (*pfkl* paralogues and *pklr* gene) or only weakly increased expression (*gck* genes) with advancing nutritional transition, confirming the observations of Mennigen et al. (2013).

In contrast, and as expected with a high-carbohydrate content diet (Geurden et al., 2014; Marandel et al., 2015; Seiliez et al., 2011), mRNA levels of both *gck* genes were greatly increased in exogenous feeding alevins. However, at the first meal, trout fed the high-CHO diet did not display additional expression regulation compared with trout fed the no-CHO diet for either *gck* genes or any other genes analysed. In contrast, at the exogenous feeding stage, most of the gluconeogenic genes already displayed a similar expression pattern to that previously described in the livers of juveniles (Marandel et al., 2015). For instance, *fbp1b2* and *pck1* were down-regulated in alevins fed the high-CHO diet compared with those fed the no-CHO diet, whereas *g6pcb2.a* was surprisingly up-regulated, as in the livers of juveniles (Marandel et al., 2015), thus displaying the same expression pattern as *gck* genes. The latter result suggests that the *g6pcb2.a* gene may contribute to the non-inhibition of the last stage of gluconeogenesis and thus may be involved in the establishment of the glucose-intolerant phenotype in trout fed a high-carbohydrate diet as early as first feeding.

New tools for programming investigation

Interestingly, all our results demonstrated that the regulation of glucose metabolism-related genes by dietary carbohydrates at the transcript level, and particularly the atypical level of the *g6pcb2.a* gene, took place very early during first feeding. This information is of particular interest in the context of nutritional programming. Indeed, Geurden et al. (2014) did not succeed in programming the gluconeogenesis genes in the livers of trout subjected to a high-carbohydrate nutritional stimulus during first feeding in the long term. The discovery of new G6pc-encoding genes, i.e. *g6pcb.2* genes (Marandel et al., 2015), and their early atypical transcriptional regulation by dietary carbohydrates suggested that first feeding is

too late to programme glucose metabolism-related genes in the liver by nutritional stimulus in the long term. In other words, it seems that the first feeding period did not correspond to a window of metabolic plasticity, during which a nutritional stimulus may impact on the expression of liver glucose metabolism-related genes or at least expression of gluconeogenic genes in the long term (for review, see McMillen and Robinson, 2005). In order to impact on this metabolism in the long term, another window of plasticity has to be defined before the first feeding period. However, such an investigation entails considering a non-nutritional stimulus such as hypoxia, known to affect glucose metabolism (Osumek et al., 2014; Zhong and Mostoslavsky, 2010), and having greater understanding of the transcription patterns of glucose metabolism-related genes during ontogenesis and before nutritional transition. Previous studies carried out in zebrafish (Rocha et al., 2013, 2015) have revealed that metabolic programming could occur following a non-nutritional stimulus such as nutrient injection, applied during late embryogenesis and before first feeding. The information obtained in this study will thus assist identification of potential windows of plasticity and the timing of application of the environmental stimulus. Indeed, in the light of our results during ontogenesis, it would be a good option to apply a stimulus around or before stages 22/23 in order to affect the expression of glucose metabolism-related genes, as mRNA levels of all genes increased during this period of development. A hypoxic stimulus could be of interest in this context as hypoxia is known to affect glucose metabolism (Osumek et al., 2014; Zhong and Mostoslavsky, 2010).

The results presented in this study demonstrate that glucose metabolism-related genes are regulated early by dietary carbohydrates just as they are in the livers of juvenile trout. These findings are also highly original because, despite the existence of at least one gene in other teleost genomes (such as zebrafish), *g6pcb.2* has never previously been studied during ontogenesis. In addition to the expression pattern of the above genes during ontogenesis, we provide here new perspectives for intermediary metabolism programming, particularly the choice of the nature of the stimulus and the timing of its application. Finally, this study also confirms that taking into consideration the complexity of the rainbow trout genome, particularly all duplicated genes fixed in the trout genome after Ss4R, is highly important in understanding the physiological phenotype in this species.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

L.M. and S.P. designed the study. L.M. managed the study, performed *in silico*, expression and statistical analysis, and wrote the manuscript. V.V. performed enzyme activity analysis. A.S. performed body and diet composition analysis. E.P.-J. performed assays on extraction of mRNA of embryos. L.M., V.V., E.P.-J., A.S. and S.P. contributed to manuscript correction.

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Supplementary information

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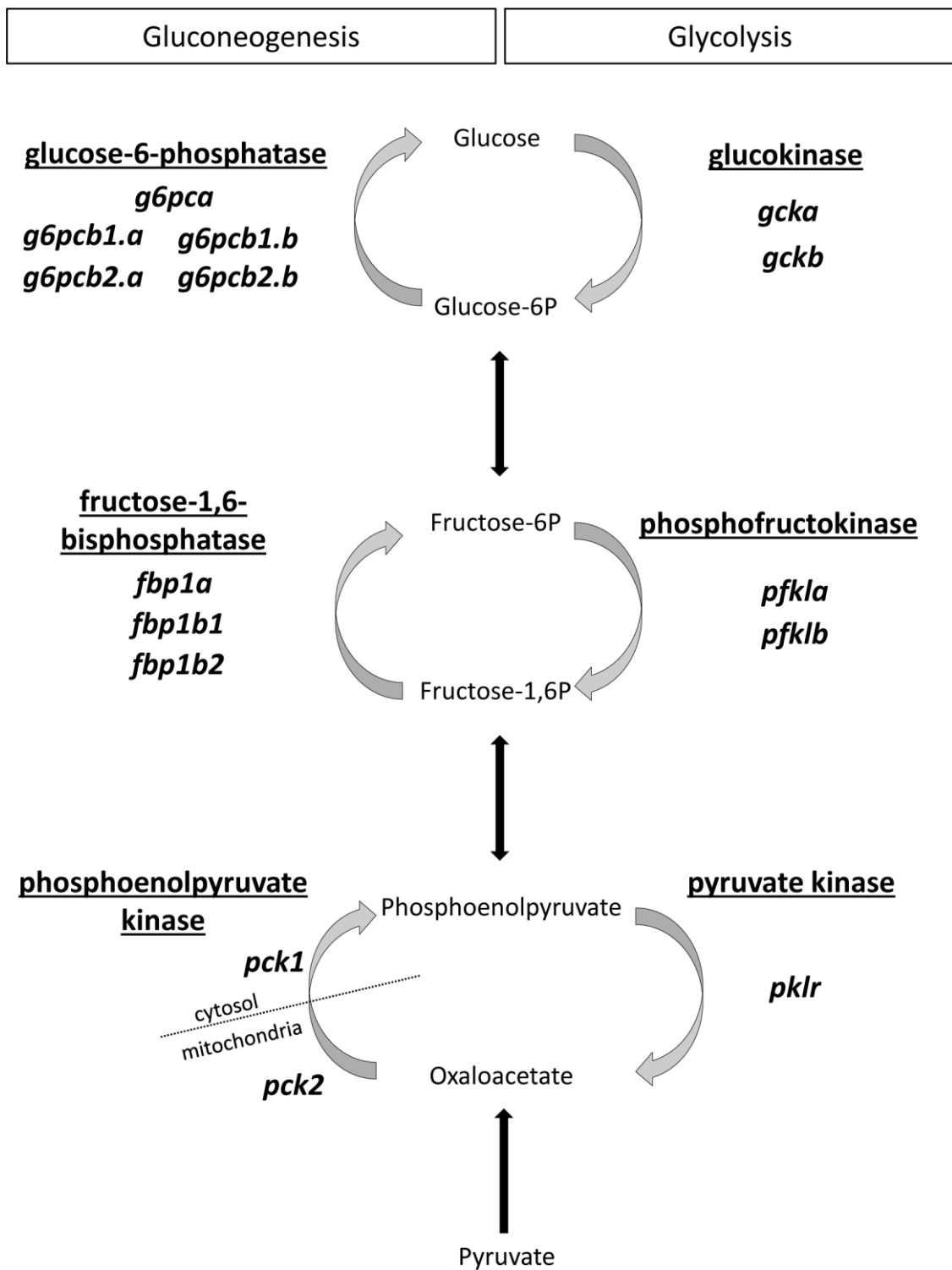


Fig. S1. Gluconeogenesis and glycolysis pathway description.

pfkla

>GSONMG00009459001 scaffold_7584 (*pfkla*)

AACCCTTCCCAGTACTTGCATGCAGAAGATCGTGGACACCATCAACAAGCATAATATCCAGGCTC
TGCTGATCGTTGGGGGATTTGAGGCGTACGAAGGCGTGCTGGAGTTATATGATGCTCGCGGTAG
CTTTGACGAGTTGTGTATTCCCATGGTGGTATCCCTGCCACCATCAGTAACAACGTACCCGGGA
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TCAACCTCCAGGACCTAAAGGTGAAAACCAGGCTGCTGCTGGCATAGCACCTTATCCCTACTCT
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> GSONMG00009459001 sc_7584 deduced partial amino acids

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> FYV3OTN01AZR97.s.om.10:1:2407:1

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> GSONMG00001975001 sc_8651 deduced partial amino acids sequence

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pklr

>AF246146.s.om.10 (*pklr*)

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CGGCATGAACATAGGAAAGGCCAGGGGCTTCTTCAAGACAGGTGACATGGTGTGCTGGTGGACC
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ATGTGGAGATGAAGTACATAAACAACAACAGTTTAGAGATTACGATGAAAAGTCAATTTTCC
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> AF246146.s.om.10 (*pklr*) deduced partial amino acids sequence

AEQSDVANAVLDGADCVMLSGETAKGLFPVESVAMMHSICREAEAAIFQQQLFEELRRLTPLSNDPT
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Fig. S2. Coding and deduced amino acid sequences of the newly identified genes.

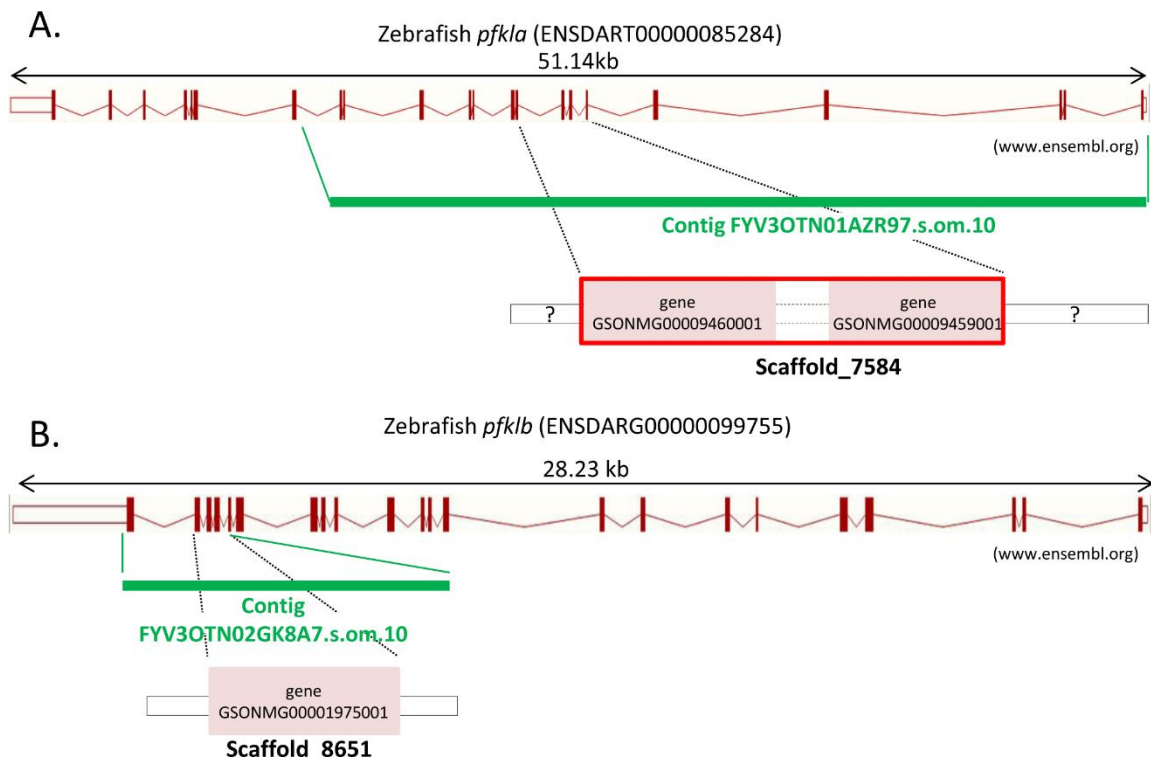


Fig. S3. Identification of rainbow trout *pfkl* predicted genes in databases.