New insights into the nutritional regulation of gluconeogenesis in carnivorous rainbow trout (Oncorhynchus mykiss): a gene duplication trail

Lucie Marandel, Iban Seiliez, Vincent Véron, Sandrine Skiba-Cassy, and Stéphane Panserat
Institut National de la Recherche Agronomique (INRA), Nutrition, Metabolism and Aquaculture Unit (UR1067), Saint-Pée-sur-Nivelle, France
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Marandel L, Seiliez I, Véron V, Skiba-Cassy S, Panserat S. New insights into the nutritional regulation of gluconeogenesis in carnivorous rainbow trout (Oncorhynchus mykiss): a gene duplication trail. Physiol Genomics 47: 253–263, 2015. First published April 21, 2015; doi:10.1152/physiolgenomics.00026.2015.—The rainbow trout (Oncorhynchus mykiss) is considered to be a strictly carnivorous fish species that is metabolically adapted for high catabolism of proteins and low utilization of dietary carbohydrates. This species consequently has a “glucose-intolerant” phenotype manifested by persistent hyperglycemia when fed a high-carbohydrate diet. Gluconeogenesis in adult fish is also poorly, if ever, regulated by carbohydrates, suggesting that this metabolic pathway is involved in this specific phenotype. In this study, we hypothesized that the fate of duplicated genes after the salmonid-specific 4th whole genome duplication (Ss4R) may have led to adaptive innovation and that their study might provide new elements to enhance our understanding of gluconeogenesis and poor dietary carbohydrate use in this species. Our evolutionary analysis of gluconeogenic genes revealed that pck1, pck2, fbp1a, and g6pca were retained as singletons after Ss4r, while g6pbc1, g6pbc2, and fbp1b ohnolog pairs were maintained. For all genes, duplication may have led to sub- or neofunctionalization. Expression profiles suggest that the gluconeogenesis pathway remained active in trout fed a no-carbohydrate diet. When trout were fed a high-carbohydrate diet (30%), most of the gluconeogenic genes were non- or downregulated, except for g6pbc2 ohnologs, whose RNA levels were surprisingly increased. This study demonstrates that Ss4R in trout involved adaptive innovation via gene duplication and via the outcome of the resulting ohnologs. Indeed, maintenance of ohnologous g6pbc2 pair may contribute in a significant way to the glucose-intolerant phenotype of trout and may partially explain its poor use of dietary carbohydrates.

gluconeogenesis; carbohydrates; trout; gene duplication; carnivorism; adaptation

THE RAINBOW TROUT (Oncorhynchus mykiss) is considered to be a strictly carnivorous fish species with nutritional needs linked to this specific dietary regime. Indeed, carnivorous species naturally require a diet consisting of high protein and moderate fat content but with minimal amounts of carbohydrate. The rainbow trout, with other carnivores such as the cat, mink, and carnivorous teleosts (for instance European seabass, Dicentrarchus labrax), are thus metabolically adapted for high catabolism of proteins and low utilization of dietary carbohydrates. They are therefore considered to be a “glucose-intolerant” species due mainly to persistent hyperglycemia after intake of carbohydrate-enriched meal(s) or glucose tolerance tests (cat (20, 51); trout (2, 3, 43, 53); and several teleosts, reviewed by Polakof et al. 2012 (44)). Moreover, another adaptive carnivorous trait is a higher rate of hepatic gluconeogenesis (48, 54, 56, 60, 63) than omnivorous species, allowing them to sustain endogenous glucose demands, i.e., maintenance of normoglycemia and production of energy using gluconeogenic amino acids and fat rather than dietary carbohydrates (9, 12, 61, 62, 65). Probably as a result of no dietary carbohydrate requirements, gluconeogenesis in adult fish is also poorly, if ever, regulated by carbohydrates (19, 23, 29, 33–35, 37).

Several studies performed in rainbow trout have shown that biochemical pathways (25, 32, 33, 40, 57) and control mechanisms (10, 30, 42, 44, 45) of glucose metabolism are conserved (41). Nevertheless, the plasticity of this system differs from one carnivorous species to another. For instance, the differential nutritional regulation of glucokinase (Gck, enzymatic activity and gene expression) across carnivorous species [reviewed by Panserat et al. 2014 (36)] is an indisputable illustration of this fact. Indeed, cats have a very low functional Gck with no capacity to be inducible by dietary carbohydrates, whereas in rainbow trout both expression and enzymatic activity of Gck are well upregulated by such a diet. Species thus adapt to carnivorism by differential physiological strategies.

In teleosts, radiation was preceded by three rounds of whole genome duplication (WGD) (teleost-specific 3rd WGD or Ts3R), believed to be involved in the evolution and diversification of teleost fish. An additional round of WGD then occurred in salmonids [Ss4R salmonid-specific 4th WGD (4, 27)]. WGD may represent a major evolutionary event in the study of adaptive mechanisms to carnivorism in rainbow trout. Indeed, after WGD, several scenarios can occur: the duplicated genes can be lost or fixed and maintained with three distinct outcomes, i.e., neofunctionalization, subfunctionalization, and conservation of function (16). Whole genome duplication via gene duplication is thus a source of genetic novelty and can lead to adaptive innovation. In the study of the gluconeogenic pathway, Seiliez et al. [2011 (52)] were the first to grasp this feature of rainbow trout by identifying a second gene encoding a glucose-6-phosphatase (called g6pase-2) in EST databases (expressed sequence tag, SIGENAE, http://www.sigenae.org) and showing that both genes displayed differential expression patterns in trout fed a high-carbohydrate diet. While the original gluconeogenic pathway in rainbow trout has been well documented by relating measurements of enzyme activity or gene expression gathered in EST databases, this metabolic pathway has never been considered to our knowledge from a genomic point of view, probably due to the unavailability of the genome sequence. Analysis of the recently sequenced rainbow trout genome (4) can now shed new light on processes.
that contribute to the hyperglycemia and poor utilization of dietary carbohydrates in this species.

The aims of this study, focusing on the liver (the main gluconeogenic organ) in rainbow trout, were to characterize 1) the evolutionary history of specific hepatic-expressed gluconeogenic genes in teleosts, i.e., cytosolic phosphoenol-pyruvate carboxykinase 1 and mitochondrial phosphoenol-pyruvate carboxykinase 2 (PCK1 and PCK2), fructose-1,6-bisphosphatase 1 [FBP1 (15, 58)] and glucose-6-phosphatase 1 [G6PC (28)] and 2) their expression profile under different nutritional conditions (i.e., fasted, non- or high-carbohydrate diet fed, refasted).

MATERIALS AND METHODS

Ethical issues and approval. Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare (Décret 2001-464, 29 May 2001 and Directive 2010/63/EU, respectively). Fish were killed by concussion/blow to the skull, and death was confirmed by exsanguination. No anesthetic was used to avoid bias in analysis of enzymatic activity. This protocol and the project as a whole were approved by the French National Consultative Ethics Committee.

Fish diets. Two experimental diets, i.e., the NoCHO (diet without carbohydrate) and the HighCHO (diet with about 30% carbohydrates), were prepared in our facilities (INRA, Saint-Pée-sur-Nivelle, France) as pressed pellets. The two diets were isoprotein and isoenenergetic (Table 1). Gelatinized starch was included as carbohydrate source, protein was provided by fishmeal, and dietary lipid by fish oil and fish meal. Inclusion of carbohydrates (~30%) in the HighCHO diet was compensated for by a decreased dietary protein level (~40%), which was still above the 37% protein requirement of rainbow trout (NRC, 2011). No carbohydrates were added to the NoCHO diet, which contained ~60% crude protein.

Fish and experimental design. Juvenile rainbow trout (~70 g body mass) were distributed in six tanks (15 fish per tank) and reared at 17°C in the INRA experimental facilities at Saint-Pére-sur-Nivelle, France. After 4 days of total starvation, two fish per tank were killed. Fish were then fed with either the NoCHO or the HighCHO diet (triplicate tanks per diet) twice a day at 2.5% live weight for 4 days and sampled 6 h after the last meal (n = 9 fish per diet, 3 per tank) to monitor the expected hyperglycemic phenotype. Gut content of the sampled animals was systematically checked to confirm that the fish sampled had consumed the diet. The remaining fish were then starved again and killed after 4 days (n = 9 fish per diet, 3 per tank). Blood was removed from the caudal vein via heparinized syringes and centrifuged (3,000 g, 5 min). The plasma recovered was immediately frozen and kept at −20°C until analysis. The fresh liver of each fish was dissected; part was used for enzyme activity analysis, and the remaining part was immediately frozen in liquid nitrogen and then kept at −80°C.

Analytical methods. The chemical composition of the diets was analyzed by the following procedures: 1) dry matter was determined after drying at 105°C for 24 h, 2) protein content (N × 6.25) was determined by the Kjeldahl method after acid digestion, 3) fat was determined by petroleum ether extraction (Soxtherm), 4) gross energy was determined in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany), 5) ash content was determined by incineration of the samples in a muffle furnace at 600°C for 6 h, and 6) starch content was measured by an enzymatic method (InVivo Labs). Plasma glucose levels were determined by using a commercial kit (Biomérieux, Marcy l’Etoile, France) adapted to a microplate format according to the manufacturer’s instructions.

Table 1. Formulation and proximate composition of the two experimental diets used (NoCHO and HighCHO diets) in this experiment

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>NoCHO</th>
<th>HighCHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>90.85</td>
<td>55.9</td>
</tr>
<tr>
<td>Fish oil</td>
<td>5.15</td>
<td>10.1</td>
</tr>
<tr>
<td>Starch</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Alginate</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Proximate composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (DM), %</td>
<td>95.46</td>
<td>94.69</td>
</tr>
<tr>
<td>Crude protein, % DM</td>
<td>61.30</td>
<td>39.08</td>
</tr>
<tr>
<td>Crude lipid, % DM</td>
<td>18.86</td>
<td>17.70</td>
</tr>
<tr>
<td>Gross energy, kJ/g DM</td>
<td>22.05</td>
<td>21.76</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td>17.73</td>
<td>11.38</td>
</tr>
<tr>
<td>Carbohydrates, % DM</td>
<td>&lt;1.0</td>
<td>28.4</td>
</tr>
</tbody>
</table>

NoCHO, no-carbohydrate diet; HighCHO, high-carbohydrate diet. 1Spro- opeche, Boulogne-sur-Mer, France. 2Fish oil; Spropeche, Boulogne-sur-Mer, France. 3Gelatinized corn starch; Roquette, Les trem, France. 4Supplied the following (kg diet): tri-a-tocopherol acetate 60 IU; sodium menadione bisulphate 5 mg; retinyl acetate 15,000 IU; cholecalciferol 3,000 IU; thiamin 15 mg; riboflavin 30 mg; pyridoxine 15 mg; vitamin B12 0.05 mg; nicotinic acid 175 mg; phosphate 5 mg; retinyl acetate 15,000 IU; DL-cholecalciferol 3,000 IU; thiamin 15 mg. 5Supplied the following (kg 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 carbonate (40% Cu) 0.3 g, manganese sulphate (33% Mn) 0.3 g, dicalcium 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. 6Louis François, Marne-la-Vallée, France.
Fig. 1. Phylogenetic analysis of phosphoenol-pyruvate carboxykinase (pck2 and pck1, A), fructose-1,6-bisphosphatase 1 (fbp1, B), and glucose-6-phosphatase (g6pc, C). Phylogenetic analyses were performed using MEGA package version 5 software (55), as previously described (13). The phylogenetic trees were built by the neighbor-joining (NJ) method. The reliability of the inferred trees was estimated by the bootstrap method with 1,000 replications. Branchiostoma floridae Pck (GenBank accession XP_002593974), Oikopleura dioica Fbp (GenBank accession CBY10016), and Latimeria chalumnae G6pc (Ensembl accession ENSLACG00000022601) protein sequences were used to root trees for Pck (A), Fbp1 (B), and G6pc (C) phylogenetic analysis, respectively. All accession numbers (from GenBank, Ensembl, or Genoscope databases) are specified in parentheses.
primers (Promega, Charbonnières, France) to synthesize cDNA (n = 9 for each treatment).

Real-time RT-PCR. The primer sequences used in real-time RT-PCR assays are listed in Table 2. For gene targets that had not previously been validated, primers were tested on a pool of cDNA and amplified products were systematically sequenced. The protocol conditions for real-time RT-PCR have been published previously (52). 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, 0.12 μl of each primer (10 μM), 3 μl Light Cycler 480 SYBR Green I Master mix, and 0.76 μl DNase/RNase-free water (5 Prime, 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, 40 s at 60°C). Melting curves were systematically monitored (temperature gradient 1.1°C/10 s from 65–94°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, respectively) and negative controls (reverse transcriptase- and cDNA template-free samples, respectively). For the expression analysis, relative quantification of target gene expression was performed using the ΔCT method (39). The relative gene expression of Rps16 [ribosomal protein 40S S6 (31)] was used for the normalization of measured mRNA as its relative expression did not significantly change over sampling time (data not shown). In all cases, PCR efficiency was measured by the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.89 and 2.0.

Enzyme activity. Fresh livers used to assess enzyme activity were ground in 8 volumes of ice-cold buffer at pH 7.4 (50 mmol/l TRIS, 5 mmol/l EDTA, 2 mmol/l DTT) and a protease inhibitor cocktail (P2714; Sigma, St. Louis, MO) and centrifuged for 10 min at 900 g at 4°C. Assays were performed from the recovered supernatants for Gck and Fbp activity. For G6pc, 1 min of sonic disruption was then applied to the homogenized samples kept on ice, then samples were centrifuged for 20 min at 10,000 g at 4°C and supernatants were used for enzyme assays. For Pck1 and Pck2 activity, to separate the cytosolic and the mitochondrial fractions, additional centrifugation (20 min, 10,000 g at 4°C) was performed on supernatants obtained after first centrifugation at 900 g. After the second centrifugation, supernatants were kept for cytosolic Pck1 assay. The enzymes assayed were: high Km Hexokinase (Gck), as described by Panserat et al. [2000 (32)], G6Pase from Alegre et al. [1988 (1)], Pck following protocol of Kirchner et al. [2003 (22)], and Fbp, described by Tranulis et al. [1996 (59)]. The enzyme activity was measured in duplicate at 30°C following the 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 catalyzed the hydrolysis of 1 μmol of substrate per minute at 30°C. Enzyme activity was expressed per milligram of soluble protein. Protein concentrations were measured in triplicate according to Bradford [1976 (5)], using a protein assay kit (Bio-Rad, Munich, Germany) with bovine serum albumin as a standard.

**RESULTS**

**In silico analysis of gluconeogenic genes.** By analyzing assembly of the rainbow trout genome (4) we identified for the first time several genes sharing high sequence homology with the gluconeogenic zebrafish (*Danio rerio*) and human (*Homo sapiens*) orthologs: two were related to PCK, three to FBP1, and five to G6PC. A phylogenetic analysis, using full-length vertebrate protein sequences, and a syntenic analysis were then performed to clarify the identity of these rainbow trout sequences.

**Table 2. Primer Sequences and accession numbers for qPCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Sigena or Genoscope Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS16</td>
<td>TTTTACGCGGGGACACATGC</td>
<td>GGGTCTGTCATGACCTTG</td>
<td>tcbh0005c.o.13_.5.1.om.4</td>
</tr>
<tr>
<td>gcka</td>
<td>CTGCGCACCTAAGCTCTGT</td>
<td>GCTATGCGGTCTGAGAGAT</td>
<td>GSONMGO0033781001</td>
</tr>
<tr>
<td>gckb</td>
<td>AGTGTCTCTAGAGACGCC</td>
<td>CATTGCACGGCTGACTCCT</td>
<td>GSONMGO0012878001</td>
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<tr>
<td>pck1</td>
<td>ACAATGCGGAGCTGACTGAG</td>
<td>GTATGCTCGAGGTCTAAGGCG</td>
<td>GSONMGO0082468001</td>
</tr>
<tr>
<td>pck2</td>
<td>ACAATGCGGAGCTGACTGAG</td>
<td>GTATGCTCGAGGTCTAAGGCG</td>
<td>GSONMGO0059643001</td>
</tr>
<tr>
<td>fbpl1</td>
<td>AGACAGAAGAGCGACCCG</td>
<td>GATACGACCGGCTGACAACT</td>
<td>GSONMT00001932001</td>
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<tr>
<td>fbplb1</td>
<td>CTGCTCTAGATGAGATGAGAT</td>
<td>GATACGACCGGCTGACAACT</td>
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</tr>
<tr>
<td>fbplb2</td>
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<td>GATACGACCGGCTGACAACT</td>
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<tr>
<td>g6pca</td>
<td>GATCGACCGGCTGACAACT</td>
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<tr>
<td>g6p1.a</td>
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<td>g6p1.b</td>
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<td>g6p2.a</td>
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<tr>
<td>g6p2.b</td>
<td>CTGCGCGAGATCAGCGGGC</td>
<td>ATGCGCGAGATCAGCGGGC</td>
<td>GSONMGO0014864001</td>
</tr>
</tbody>
</table>
Our *Pck* phylogenetic analysis showed that one of the rainbow trout sequences identified (GSONMG00082468001) was grouped with *pck1* vertebrate orthologs, while the other (GSONMG00059643001) was grouped with *pck2* vertebrate orthologs (Fig. 1A). We also found that both rainbow trout genes were located in two distinct syntenic groups, *ccndbp1-psme1* for GSONMG00082468001, conserved around *pck1* and *pck2* loci, respectively, in zebrafish (data not shown). In addition, amino acid sequence identity analysis confirmed that the deduced protein sequence from the GSONMG00082468001 gene shared higher percentage identity with human and zebrafish Pck1 sequences than with Pck2 sequences, while the opposite was true for the amino acid sequence deduced from the GSONMG00059643001 gene (Fig. 3A).

On studying *FBP1* related rainbow trout genes, we showed that two of the three genes identified were co-orthologous to the *fbp1b* gene in zebrafish (Fig. 1B). We therefore arbitrarily annotated these genes *fbp1b1* (GSONMT00063051001) and *fbp1b2* (GSONMT0015701001). *Fbp1b1* and *Fbp1b2* deduced amino acid sequences were highly conserved (>97% identity) and shared ~90% identity with zebrafish *Fbp1b* (Fig. 3B). The size of the scaffold bearing the third rainbow trout *FBP1*-related sequence (GSONMG0001932001) was not sufficient to cover the whole gene length. The length of the deduced amino acid sequence was therefore not appropriate for inclusion in the phylogenetic analysis. However, this truncated trout sequence was submitted to a BLAST search against several sequenced teleost genomes [zebrafish, medaka, *Oryzias latipes*, fugu (*Takifugu rubripes*), and tetraodon (*Tetraodon nigroviridis*)] available in the Ensembl database. In all species, the hit with the highest score matched the *fbp1a* gene (data not shown). Finally, no informative syntenic conservation was found around *fbp1a* or around *fbp1b2* because of the small size of the scaffolds. Nevertheless, *fbp1b1*, borne by a larger scaffold, was located in the *gas1-C9-dab2-thsb4b* syntenic group conserved in the zebrafish, medaka, stickleback (*Gasterosteus aculeatus*), tetraodon, and fugu around the *fbp1b* gene (data not shown).

Our phylogenetic analysis for *G6pc*-related genes revealed that one rainbow trout sequence was orthologous to the zebrafish gene annotated *g6pca.1* (ENSDARP00000116534) in Ensembl (release 77), while the others grouped pairwise with zebrafish genes annotated *g6pca.2* (ENSDARP0000086301) and *g6pcb* (ENSDARP0000128434), respectively. The topology of the phylogenetic tree indicated that the initial annotation of co-orthologous *G6pc* genes in zebrafish was inaccurate. Indeed, the rooting of the branches inside the teleost subtree was more in favor of an initial duplication of the *G6pc* gene into *g6pca* and *g6pcb* before or around teleost radiation, followed by a second duplication of at least *g6pcb* giving rise to *g6pcb1* and *g6pcb2* genes. We therefore reannotated zebrafish co-orthologous *G6pc* genes in our phylogenetic and synteny analysis (*g6pca* as a replacement for *g6pca.1*, *g6pca1* for *g6pca.2*, and *g6pcb2* for *g6pcb*) (Fig. 1C). Moreover, the matrix protein identity (Fig. 3C) confirmed that, except for one *G6pc* (GSONMG00076843001), other rainbow trout *G6pc* amino acid sequences shared pairwise >90% identity, suggesting that duplicated *g6pca1* and duplicated *g6pcb2* genes were retained after *Ss4R* (4) in the rainbow trout genome as *g6pca1* (GSONMG00076841001), *g6pcb1.a* (GSONMG00013076001), and *g6pachb2.b* (GSONMG00014864001). Finally, our syntenic analysis (Fig. 2) showed that *G6pc* in the human and the mouse was located in the *wnk4-cedc56-aoc2-aoc3-aards1-nbr1-tnem160a-ar14d-etr4-mexol* syntenic group. Interestingly, syntenic conservation of *nbr1-tnem160a-ar14d* was found with all chromosomal regions of teleosts [zebrafish, trout, stickleback (*Gasterosteus aculeatus*), fugu, and tetraodon], which carried the duplicated copies of *g6pc* (except around *g6pca2* in zebrafish where the syntenic group was only partially conserved). Similarly, the *etr4-mexol* group was found to be specifically syntenic with the *g6pca-g6pca1* group, while *aoc2* was only syntenic with *g6pcb2* genes, thus confirming the identity of such paralogous genes.

An expected phenotype confirmed by persistent postprandial hyperglycemia and upregulation of gck genes after a high-carbohydrate content diet. Our experimental conditions and diets were designed to arrive at a previously described (52) hyperglycemic phenotype characterized by a steep rise in plasma glucose concentration and a dramatic increase in gck mRNA level and enzyme activity with a high-carbohydrate diet. The carbohydrate content of the HighCHO diet (30%) is higher than those usually used in so-called carbohydrate-rich diets (<20% carbohydrates) for long-term nutrition but was chosen in order to dramatically induce such a phenotype. We therefore monitored glycemia and gck mRNA level and enzyme activity.

The plasmatic glucose analysis showed that plasma glucose levels were significantly higher after a high-carbohydrate diet (HighCHO, Fig. 4A) than in fasted trout (i.e., Fasted or Re-fasted, Fig. 4A) or trout fed the NoCHO diet, which displayed normoglycemia (~0.8 g/l).

As for gluconeogenic genes, we performed an in silico analysis to elucidate the gck evolutionary history. We thus identified two sequences in the rainbow trout genome sharing high homology with the zebrafish gck gene. These two genes were grouped together in the teleost *gck* phylogenetic subtree (Fig. 5) as co-orthologs to teleost and tetrapod gck genes. As no duplication of *gck* in other sequenced teleost genomes was found, we concluded that duplication of *gck* in rainbow trout occurred before or around the *Ss4R*, giving rise to *gcka* (GSONMG00033781001) and *gckb* (GSONMG00012878001) in this species. Compared with fasted trout and fish fed the NoCHO diet, both *gcka* and *gckb* mRNA levels increased dramatically in fish fed the high-carbohydrate diet (Fig. 4B). The overall Gck activity followed the same trend (Fig. 4C), confirming that Gck enzyme activity is a strong indicator of dietary carbohydrate intake.

Taken together, these results confirmed the physiological effects of a high-carbohydrate content diet in trout expected with this study design and as previously described (52).

Expression analysis of gluconeogenic genes. Analysis of gene expression revealed that, although the *pck1* mRNA level was not clearly statistically affected by the nutritional status, it was higher in trout fed the NoCHO diet than in trout fed the HighCHO diet (Fig. 6A). The mRNA level of *pck2* (Fig. 6A) was constant whatever the experimental condition. No change in Pck1 or Pck2 enzyme activity was noted (Fig. 6Da and 6Db). As previously described (21), we found that overall Pck activity in the liver comprised approximately one-third cyto-
sollic activity (Pck1) and two-thirds mitochondrial activity (Pck2) (Fig. 6Da and 6Db).

As for pck2, fbp1a mRNA level remained stable, irrespective of the nutritional status (Fig. 6B). In contrast to fbp1b, the fbp1b1 mRNA level increased in trout fed the NoCHO diet compared with fasted animals (Fig. 6B). However, intake of the HighCHO diet caused a decrease in both fbp1b1 and fbp1b2 paralog mRNA levels (Fig. 6B). The overall Fbp activity (Fig. 6Db) followed the same trend as fbp1b1 and fbp1b2 mRNA levels, with a significant decrease in trout fed the HighCHO diet, but did not follow the increase in fbp1b1 mRNA level in trout fed the NoCHO diet.

The mRNA level of g6pca (Fig. 6C) remained unchanged whatever the nutritional status in trout fed the NoCHO diet, while it decreased in trout fed the HighCHO diet and in refasted fish fed the latter diet. The g6pcb1.b mRNA level followed almost the same pattern as g6pca, whereas the paralogous g6pbc1.a gene displayed a decrease in mRNA level in trout fed the NoCHO diet, with the same fold change as that observed in trout fed the HighCHO diet (Fig. 6C). In striking contrast to all other co-orthologous G6pc genes, the mRNA level of both g6pbc2 paralogues increased in fed trout compared with fasted or refasted fish and to a greater extent when trout were fed the HighCHO diet. Also, the overall G6pc enzyme activity remained unchanged in fasted and fed trout but decreased in refasted trout (Fig. 6Dd).

DISCUSSION

The rapidly increasing number of whole genomes sequenced in model and nonmodel organisms in the last decade (4, 6, 64) makes us seriously consider the DNA sequence information to be a powerful tool for unraveling the mechanisms of environmental adaptation. In this context, the outcome of duplicated genes arising through Ss4R in rainbow trout can lead to adaptive innovation (50), depending on whether they are lost or fixed and maintained (16). Elucidating the evolutionary history of such genes and studying their expression patterns may help to reveal new molecular underpinnings involved in adaptive behavior in rainbow trout. This approach is of particular interest when considering the adaptation of rainbow trout to carnivory, as this has never previously been explored from a genomic point of view. The present study therefore focused on genes encoding key gluconeogenic enzymes in the liver, the genomic point of view. The present study therefore focused on these genes arising through Ss4R in rainbow trout and the adaptive innovation (50), depending on whether they are lost or fixed and maintained (16). Elucidating the evolutionary history of such genes and studying their expression patterns may help to reveal new molecular underpinnings involved in adaptive behavior in rainbow trout. This approach is of particular interest when considering the adaptation of rainbow trout to carnivory, as this has never previously been explored from a genomic point of view. The present study therefore focused on genes encoding key gluconeogenic enzymes in the liver, the genomic point of view. The present study therefore focused on genes arising through Ss4R in rainbow trout and the adaptive innovation (50), depending on whether they are lost or fixed and maintained (16).

In silico analysis of the recently sequenced rainbow trout genome revealed the conservation of several duplicated gluconeogenic genes. The first critical step in elucidating the evolutionary history of gluconeogenic genes was to identify accurately the orthologous genes of mammalian Pck1, Pck2, G6pc, and Fbp1 genes in the newly sequenced rainbow trout genome (4). Using phylogenetic and syntentic analyses we identified for the first time a pck1 gene in the rainbow trout genome that was orthologous to the cytosolic Pck1. Our results also confirm that the pck gene previously sequenced in rainbow trout by Panserat et al. [2001 (34)] was orthologous to the mitochondrial Pck2 gene in other species. For Fbp1, we demonstrated that a duplication of the gene occurred before or around teleost radiation, giving rise to Fbp1a and Fbp1b in teleosts probably as a consequence of the Ts3R. After 4SsR, Fbp1a was retained as a singleton in the rainbow trout genome,
while the \( fbp1b \) ohnologous gene pair (paralogs formed by a WGD event (4)) consisting of \( fbp1b1 \) and \( fbp1b2 \) was preserved. We finally clarified the complex evolutionary history of \( G6pc \) (summarized in Fig. 7), showing that a duplication of this gene occurred before or around teleost radiation, giving rise to \( g6pca \) and \( g6pcb \) in the ancestral teleost genome. The presence of both \( g6pcb1 \) and \( g6pcb2 \) in the genome of a cypriniform (zebrafish) and a salmonidae (rainbow trout), but only \( g6pcb1 \) in Percomorph genomes (medaka, stickleback, fugu, and tetraodon), was then in favor of an additional duplication of \( g6pcb \), at least in the genome of the common ancestor of the Euteleost, Salmonidae, and Ostariophys, followed by a loss of \( g6pcb2 \) in the Percomorph ancestor. Analysis of the rainbow trout genome indicated that \( g6pca \) was retained as a singleton, whereas both ohnologous gene pairs \( g6pcb1.a-g6pcb1.b \) and \( g6pcb2.a-g6pcb2.b \) remained whole after Ss4R. The evolutionary history
Expression analysis of hepatic gluconeogenic genes in trout fed with or without carbohydrates revealed differential paralogous profiles. On considering the different nutritional conditions tested, analyses of the expression of the duplicated gluconeogenic genes revealed that all genes were regulated by nutritional status except pck2 and fbp1a, the mRNA levels of which remained stable whatever the nutritional status. Our results show that none of the genes encoding key gluconeogenic enzymes (except g6pc1.a) were downregulated in trout fed the NoCHO diet. Indeed, compared with fasting pck1, fbp1b2, g6pca, and g6pcb1.b were not regulated, whereas fbp1b1, g6pcb2.a, and g6pcb2.b were upregulated. These expression data, combined with the maintenance of gluconeogenic enzyme activity, suggest that the gluconeogenesis pathway remained active under NoCHO nutrition according to the need for endogenous glucose production to sustain maintenance of normoglycemia due to the dietary habits and physiology of carnivorous species (9, 12, 61, 62, 65). Furthermore, this is consistent with previous in vivo and in vitro studies showing that the gluconeogenesis flux is never turned off in fed carnivorous teleosts (38, 60). Moreover, when trout were submitted to a refasting period we showed that mRNA levels for most of the genes (pck1, fbp1b1, fbp1b2, g6pca, and g6pcb1b) were maintained at the same levels as in fed trout.

By contrast, the HighCHO diet led to the downregulation of most of the gluconeogenic genes (pck1, fbp1b1, fbp1b2, g6pca, and g6pcb1b) by 10.220.32.247 on June 23, 2017 http://physiolgenomics.physiology.org/ Downloaded from g6pcb paralogs. Indeed, the expression profiles of g6pcb2 paralogs mimicked those of gck paralogs and might in part be responsible for the poor regulation of the gluconeogenesis pathway. Such an expression profile of G6pc encoding genes under high-carbohydrate nutrition in rainbow trout has never previously been described, as duplicated g6pcb2 genes had not been identified before. Indeed, we found that primers used in previous studies amplified only g6pca (e.g., primers named “G6Pase” in Refs. 23 and 33 or “g6pase 2” in Refs. 14 and 52) and a conserved region between g6pcb1 ohnologs (primers named “g6pase 1” in Refs. 14 and 52). In addition, it can be hypothesized that the glucose-intolerant phenotype in rainbow trout was accentuated by the increased g6pcb2 gene dosage due to the fixation of both ohnologs in its genome (24, 46). This gene dosage may explain how overall G6pc enzyme activity remained stable in trout fed the HighCHO diet while the mRNA level of three orthologous G6pc genes decreased (g6pca, g6pcb1.a, and g6pcb1.b) compared with trout fed the NoCHO diet. The downregulation of overall G6pc enzyme activity in refasted trout also supports this hypothesis as g6pcb2 mRNA levels decreased dramatically under refasting.

Altogether, our results suggest that the atypical expression profiles of g6pcb2 ohnologs, and probably the resulting enzyme activity, may contribute significantly to the noninhibition of the last step of gluconeogenic flux and to the enrichment of plasma glucose via futile glucose/glucose-6-phosphate cycling.
(60) in trout fed a carbohydrate-enriched diet. Interestingly, in Type 2 diabetes under hyperglycemia and in conditions where endogenous glucose production is minimized, a significant glucose/glucose-6-phosphate activity cycle occurs (49) that is mediated in part by increased G6pase flux (8) but largely dependent on Gck activity. The lesson learned from trout naturally exhibiting this phenotype may thus cast light on defects in metabolism that contribute to diabetes.

Conclusions

The results of this study demonstrate the importance of considering the additional round of WGD occurring in teleosts and in salmonids as major evolutionary events that can lead to adaptive innovation via gene duplication. Indeed, under the HighCHO diet the absence of pkc2 and fbp1a downregulation and, more surprisingly, the increase in mRNA levels of both g6pcb2 ohnologs are new elements that enhance our understanding of the poor use of dietary carbohydrates in rainbow trout. Further studies such as gene knockout assays are now needed to evaluate the involvement of these duplicated genes in the carnivorous glucose-intolerant phenotype of rainbow trout.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.M. and S.P. conception and design of research; L.M. performed experiments; L.M. analyzed data; L.M. interpreted results of DISCLOSURES

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REFERENCES

1. Alegre M, Ciudad CJ, Fillat C, Guinovart JJ. Determination of glu-


2. Bergot F. Effects of dietary carbohydrates and of their mode of distribu-


15. Gautier A, Le Gac F, Lareyre JJ. The gsdf gene locus harbors evolu-


22. Kirchner S, Panserat S, Lim PL, Kaushik S, Ferraris RP. The role of hepatic, renal and intestinal glucogenic enzymes in glucose homeosta-


30. Marcolongo P, Fulceri R, Gamberucci A, Czegle I, Banhegyi G, Benedetti A. Multiple roles of glucose-6-phosphatases in pathophysiol-
Glucose homeostasis is impaired by a paradoxical interaction between metformin and insulin in carnivorous fish: a review. 


Glucokinase-like-enzyme in the liver of Atlantic salmon (Salmo salar). 


Activities of carbohydrate and amino acid metabolizing enzymes from liver of mink (Mustela vison) and preliminary observations on steady state kinetics of the enzymes. 


Comparison of expression of glucokinase gene and activities of enzymes related to glucose metabolism in livers between dog and cat. 


Cloning and characterization of glucose transporter in teleost fish rainbow trout (Oncorhynchus mykiss). 


Activities of enzymes related to glycolysis and gluconeogenesis in the liver of dogs and cats. 


Comparison of the activities of enzymes related to glycolysis and gluconeogenesis in the liver of dogs and cats. 


Glucose metabolism in fish: a review. 


Glucose metabolism in fish: state of the art and trends. 


Functional cloning of hepatic glucose-6-phosphatase catalytic subunit from gilthead sea bream (Sparus aurata): response of its mRNA levels and glucokinase expression to refeeding and diet composition. 


Nutritional regulation of glucokinase: a cross-species story. 


Comparison of expression of glucokinase gene and activities of enzymes related to glucose metabolism in livers between dog and cat. 


Effects of metformin on gene expression in juvenile rainbow trout (Oncorhynchus mykiss). 


Activities of carbohydrate and amino acid metabolizing enzymes from liver of mink (Mustela vison) and preliminary observations on steady state kinetics of the enzymes. 


Comparison of expression of glucokinase gene and activities of enzymes related to glucose metabolism in livers between dog and cat. 