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

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Running head : The atypical regulation of gluconeogenesis : a gene duplication trail

Abstract

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 utilisation of dietary carbohydrates. This species consequently has a “glucose-intolerant” phenotype manifested by persistent hyperglycaemia 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 hypothesised 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 carbohydrates use in this species. Our evolutionary analysis of gluconeogenic genes revealed that *pck1*, *pck2*, *fbp1a* and *g6pca* were retained as singletons after Ss4r while *g6pcb1*, *g6pcb2* and *fbp1b* ohnologue pairs were maintained. For all genes, duplication may have led to sub- or neo-functionalisation. Expression profiles suggested 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 down-regulated, except for *g6pbc2* ohnologues the RNA levels of which were surprisingly increased. This study demonstrated that Ss4R in trout involved adaptive innovation *via* gene duplication and *via* the outcome of the resulting ohnologues. Indeed, maintenance of ohnologuous *g6pcb2* pair may contribute in a significant way to the “glucose-intolerant” phenotype of trout and may partially explain its poor use of dietary carbohydrates.

Keywords

gluconeogenesis, carbohydrates, trout, gene duplication, carnivorism, adaptation

Introduction

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 utilisation of dietary carbohydrates. They are therefore considered to be a glucose intolerant species due mainly to persistent hyperglycaemia after intake of carbohydrate-enriched meal(s) or glucose tolerance tests (cat (18, 48); trout (1, 2, 40, 50); several teleosts, reviewed by Polakof *et al.*, 2012 (41)). Moreover, another adaptive carnivorous trait is a higher rate of hepatic gluconeogenesis (45, 51, 53, 56, 59) than omnivorous species, allowing them to sustain endogenous glucose demands, *i.e* maintenance of normoglycaemia and production of energy using gluconeogenic amino acids and fat rather than dietary carbohydrates (7, 10, 57, 58, 61). Probably as a result of no dietary carbohydrate requirements, gluconeogenesis in adult fish is also poorly, if ever, regulated by carbohydrates (17, 20, 26, 30-32, 34).

Several studies performed in rainbow trout have shown that biochemical pathways (22, 29, 30, 37, 54) and control mechanisms (8, 27, 39, 41, 42) of glucose metabolism are conserved (38). 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 (33)) is an indisputable illustration of this

76 fact. Indeed, cats have a very low functional Gck with no capacity to be inducible by
77 dietary carbohydrates whereas in rainbow trout both expression and enzymatic
78 activity of Gck are well up-regulated by such a diet. Species thus adapt to carnivorism
79 using differential physiological strategies.

80 In teleosts, radiation was preceded by three rounds of whole genome duplication
81 (WGD)(teleost-specific 3rd WGD or Ts3R), believed to be involved in the evolution
82 and diversification of teleost fish. An additional round of WGD then occurred in
83 salmonids (Ss4R salmonid-specific 4th WGD, (3, 24)). WGD may represent a major
84 evolutionary event in the study of adaptive mechanisms to carnivorism in rainbow
85 trout. Indeed, after whole genome duplication, several scenarios can occur: the
86 duplicated genes can be lost or fixed and maintained with three distinct outcomes, *i.e.*
87 neofunctionalisation, subfunctionalisation, and conservation of function (14). Whole
88 genome duplication *via* gene duplication is thus a source of genetic novelty and can
89 lead to adaptive innovation. In the study of the gluconeogenic pathway, Seiliez *et al.*
90 (2011,(49)) were the first to grasp this feature of rainbow trout by identifying a
91 second gene encoding a glucose-6-phosphatase (called *g6pase-2*) in EST databases
92 (Expressed Sequence Tag, SIGENAE, <http://www.sigenae.org>) and showing that both
93 genes displayed differential expression patterns in trout fed a high carbohydrate diet.

94 While the original gluconeogenic pathway in rainbow trout has been well documented
95 by relating measurements of enzyme activity or gene expression gathered in EST
96 databases, this metabolic pathway has never been considered to our knowledge from a
97 genomic point of view, probably due to the unavailability of the genome sequence.

98 Analysis of the recently sequenced rainbow trout genome (3) can now shed new light
99 on processes which contribute to the hyperglycaemia and poor utilisation of dietary
100 carbohydrates in this species.

101 The aims of this study, focusing on the liver (the main gluconeogenic organ)
102 in rainbow trout, were to characterise i) the evolutionary history of specific hepatic-
103 expressed gluconeogenic genes in teleosts, *i.e. cytosolic phosphoenol-pyruvate*
104 *carboxykinase 1 and mitochondrial phosphoenol-pyruvate carboxykinase 2 (PCK1*
105 *and PCK2), fructose-1,6-bisphosphatase 1 (FBP1; (13, 55)) and glucose-6-*
106 *phosphatase 1 (G6PC; (25))* and ii) their expression profile under different nutritional
107 conditions (*i.e* fasted, non- or high-carbohydrate diet fed, re-fasted).

108 **Glossary**

109 EST: Expressed Sequence Tag

110 HighCHO: high carbohydrate content diet

111 NADPH: Nicotinamide adenine dinucleotide phosphate

112 NoCHO: diet containing no carbohydrate

113 WGD: whole genome duplication

114 Ss4R: salmonid-specific 4th WGD

115 Ts3R: teleost-specific 3rd WGD

116 **Materials and Methods**

117 **Ethical issues and approval**

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

125 **Fish diets**

126 Two experimental diets, *i.e.*, the NoCHO (diet without carbohydrate) and the
127 HighCHO (diet with about 30% carbohydrates), were prepared in our facilities
128 (INRA, Saint-Pée-sur-Nivelle, France) as pressed pellets. The two diets were
129 isolipidic and isoenergetic (Table 2). Gelatinized starch was included as carbohydrate
130 source, protein was provided by fishmeal and dietary lipid by fish oil and fish meal.
131 Inclusion of carbohydrates (~30%) in the HighCHO diet was compensated for by a
132 decreased dietary protein level (~40%), which was still above the 37% protein
133 requirement of rainbow trout (NRC, 2011). No carbohydrates were added to the
134 NoCHO diet, which contained ~60% crude protein.

135 **Fish and experimental design**

136 Juvenile rainbow trout (~70g body mass) were distributed in six tanks (15 fish per
137 tank) and reared at 17°C in the INRA experimental facilities at Saint-Pée-sur-Nivelle,
138 France. After four days of total starvation, two fish per tank were killed. Fish were
139 then fed with either the NoCHO or the HighCHO diet (triplicate tanks per diet) twice
140 a day at 2.5% live weight for four days and sampled 6h after the last meal (n=9 fish
141 per diet, 3 per tank) in order to monitor the expected hyperglycaemic phenotype. Gut
142 content of the sampled animals was systematically checked to confirm that the fish
143 sampled had consumed the diet. The remaining fish were then starved again and killed
144 after four days (n=9 fish per diet, 3 per tank). Blood was removed from the caudal
145 vein using heparinized syringes and centrifuged (3000 g, 5 min). The plasma
146 recovered was immediately frozen and kept at -20°C until analysis. The fresh liver of
147 each fish was dissected, part was used for enzyme activity analysis and the remaining
148 part was immediately frozen in liquid nitrogen and then kept at -80°C.

149 **Analytical methods**

150 The chemical composition of the diets was analysed using the following procedures:

151 1) dry matter was determined after drying at 105°C for 24 h, 2) protein content
152 (N×6.25) was determined by the Kjeldahl method after acid digestion, 3) fat was
153 determined by petroleum ether extraction (Soxtherm), 4) gross energy was determined
154 in an adiabatic bomb calorimeter (IKA, Heitersheim Griebheimer, Germany), 5) ash
155 content was determined by incinerating the samples in a muffle furnace at 600°C for
156 6h, and 6) starch content was measured by an enzymatic method (InVivo Labs,
157 France). Plasma glucose levels were determined by using a commercial kit
158 (Biomérieux, Marcy l'Etoile, France) adapted to a microplate format according to the
159 manufacturer's instructions.

160 ***In silico* analysis**

161 Orthologous *Pck1*, *Pck2*, *Fbp1* and *G6pc* genes in the rainbow trout genome (3) were
162 collected from the SIGENAE database (<http://www.sigenae.org>) using the BLAST
163 tool (all accession numbers are given in brackets in Figure 1). Sequences from other
164 species were collected from the Ensembl Genome database (Ensembl release 77 -
165 October 2014, <http://www.ensembl.org>) or Genbank. The Genomicus software
166 program, version 01.01 ([http://www.genomicus.biologie.ens.fr/genomicus-trout-](http://www.genomicus.biologie.ens.fr/genomicus-trout-01.01/cgi-bin/search.pl)
167 [01.01/cgi-bin/search.pl](http://www.genomicus.biologie.ens.fr/genomicus-trout-01.01/cgi-bin/search.pl)) was used to confirm their identities by establishing syntenic
168 analysis (Fig.2).

169 Phylogenetic analyses were performed using the MEGA package version 5 software
170 (52), as previously described (11). The phylogenetic trees, based on full-length amino
171 acid deduced sequences, were built using the Neighbor-Joining (NJ) method and
172 confirmed by the Minimum evolution method (data not shown). The reliability of the
173 inferred trees was estimated using the bootstrap method with 1,000 replications.

174 *Branchiostoma floridae* Pck (Genbank accession XP_002593974), *Oikopleura dioica*

175 Fbp (Genbank accession CBY10016) and *Latimeria chalumnae* G6pc (Ensembl
176 accession ENSLACG00000022601) protein sequences were used to root trees for *Pck*
177 (Fig.1A), *Fbp1* (Fig.1B) and *G6pc* (Fig.1C) phylogenetic analysis, respectively.
178 New gene annotations were allocated according to ZFIN Nomenclature guidelines
179 (<http://zfin.org/>).
180 Percentage Identity Matrices established among paralogous teleost sequences (Table
181 1) were calculated with Clustal 2.1 using the MUSCLE interface (9).

182 **Total RNA extraction and cDNA synthesis**

183 Relative hepatic gene expression was determined by quantitative real-time RT-PCR.
184 Samples were homogenised using Precellys®24 (Bertin Technologies, Montigny-le-
185 Bretonneux, France) in 2mL tubes containing Trizol reagent (Invitro-gen, Carlsbad,
186 CA, USA) and 2.8 millimeter ceramic beads, 2 x 10 seconds, separated by 15 seconds
187 off, at 5,000 rpm. The extraction of total RNA was then performed according to the
188 manufacturer's instructions. Total RNA (1µg) was used for cDNA synthesis. The
189 Super-Script III RNase H–Reverse transcriptase kit (Invitro-gen) was used with
190 random primers (Promega, Charbonnières, France) to synthesize cDNA (n = 9 for each
191 treatment).

192 **Real time RT-PCR**

193 The primer sequences used in real-time RT-PCR assays are listed in Table 3. For gene
194 targets that had not previously been validated, primers were tested on a pool of cDNA
195 and amplified products were systematically sequenced. The protocol conditions for
196 real-time RT-PCR have been published previously (49). For real-time RT-PCR
197 assays, the Roche Lightcycler 480 system was used (Roche Diagnostics, Neuilly-sur-
198 Seine, France). The assays were performed using a reaction mix of 6 µl per sample,
199 each of which contained 2 µl of diluted cDNA template, 0.12 µl of each primer (10

200 μM), 3 μl Light Cycler 480 SYBR® Green I Master mix and 0.76 μl DNase/RNase
201 free water (5 Prime GmbH, Hamburg, Germany). The PCR protocol was initiated at
202 95°C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase
203 activation, followed by 45 cycles of a two-step amplification programme (15s at
204 95°C; 40s at 60°C). Melting curves were systematically monitored (temperature
205 gradient 1.1°C/10 s from 65-94°C) at the end of the last amplification cycle to
206 confirm the specificity of the amplification reaction. Each PCR assay included
207 replicate samples (duplicate of reverse transcription and PCR amplification,
208 respectively) and negative controls (reverse transcriptase- and cDNA template-free
209 samples, respectively). For the expression analysis, relative quantification of target
210 gene expression was performed using the ΔCT method (36). The relative gene
211 expression of *Rps16* (ribosomal protein 40S S6, (28)) was used for the normalization
212 of measured mRNA as its relative expression did not significantly change over
213 sampling time (data not shown). In all cases, PCR efficiency was measured by the
214 slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency
215 values ranged between 1.89 and 2.0.

216 **Enzyme activity**

217 Fresh livers used to assess enzyme activity were ground in 8 volumes of ice cold
218 buffer at pH 7.4 (50mmol/l TRIS, 5mmol/l EDTA, 2 mmol/l DTT and a protease
219 inhibitor cocktail (Sigma, St Louis, MO; P2714) and centrifuged for 10 min at 900g at
220 4°C. Assays were performed from the recovered supernatants for Gck and Fbp
221 activity. For G6pc, one minute of sonic disruption was then applied to the
222 homogenized samples kept on ice, then samples were centrifuged for 20 min at
223 10,000g at 4°C and supernatants were used for enzyme assays. For Pck1 and Pck2
224 activity, in order to separate the cytosolic and the mitochondrial fractions, additional

centrifugation (20 minutes, 10,000g at 4°C) was performed on supernatants obtained after first centrifugation at 900g. After the second centrifugation, supernatants were kept for cytosolic Pck1 assay. The pellets containing the mitochondrial fraction were recovered in 500µL of ice cold buffer at pH 7.4 and sonicated as described above. The enzymes assayed were: high Km Hexokinase (Gck), as described by Panserat *et al.* (2000, [25]), G6Pase from Alegre *et al.* (1988, [62]), Pck following protocol of Kirchner *et al.* (2003, [63]) and Fbp, described by Tranulis *et al.* (1996, [64]). The enzyme activity was measured in duplicate at 30°C following the variation of absorbance of NADPH (Nicotinamide adenine dinucleotide phosphate) at 340nm. The reactions were started by the addition of the specific substrate; a Power Wave X (BioTek Instrument, Inc.) 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 1µmol 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, [65]), using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

Statistical analysis

Normality of distributions was assessed using the Shapiro-Wilk test. Data were then analyzed by a Kruskal-Wallis non-parametric test following by a Tukey test as a post-hoc analysis. Data were analysed using the R software (v.3.1.0)/R Commander package.

Results

***In silico* analysis of gluconeogenic genes**

By analysing assembly of the rainbow trout genome (3) we identified for the first time several genes sharing high sequence homology with the gluconeogenic zebrafish

250 (*Danio rerio*) and human (*Homo sapiens*) orthologues: two were related to *PCK*, three
251 to *FBP1*, and five to *G6PC*. A phylogenetic analysis, using full-length vertebrate
252 protein sequences, and a syntenic analysis were then performed to clarify the identity
253 of these rainbow trout sequences.

254 Our *Pck* phylogenetic analysis showed that one of the rainbow trout sequences
255 identified (GSONMG00082468001) was grouped with *pck1* vertebrate orthologues
256 while the other (GSONMG00059643001) was grouped with *pck2* vertebrate
257 orthologues (Fig.1A). We also found that both rainbow trout genes were located in
258 two distinct syntenic groups, *ccndbp1-psme1* for GSONMG00082468001 and *dcaf11-*
259 *psme1* for GSONMG00059643001, conserved around *pck1* and *pck2* loci,
260 respectively, in zebrafish (data not shown). In addition, amino acid sequence identity
261 analysis confirmed that the deduced protein sequence from the
262 GSONMG00082468001 gene shared higher percentage identity with human and
263 zebrafish Pck1 sequences than with Pck2 sequences, while the opposite was true for
264 the amino acid sequence deduced from the GSONMG00059643001 gene (Table 1-A).

265 On studying *FBP1* related rainbow trout genes, we showed that two of the
266 three genes identified were co-orthologous to the *fbp1b* gene in zebrafish (Fig.1B).
267 We therefore arbitrarily annotated these genes *fbp1b1* (GSONMT00063051001) and
268 *fbp1b2* (GSONMT00015701001). Fbp1b1 and Fbp1b2 deduced amino acid sequences
269 were highly conserved (more than 97% identity) and shared approximately 90%
270 identity with zebrafish Fbp1b (Table 1-B). The size of the scaffold bearing the third
271 rainbow trout *FBP1*-related sequence (GSONMG00001932001) was not sufficient to
272 cover the whole gene length. The length of the deduced amino acid sequence was
273 therefore not appropriate for inclusion in the phylogenetic analysis. However, this
274 truncated trout sequence was submitted to a BLAST search against several sequenced

275 teleost genomes (zebrafish, medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*) and
 276 tetraodon (*Tetraodon nigroviridis*)) available in the Ensembl database. In all species,
 277 the hit with the highest score matched the *fbp1a* gene (data not shown). Finally, no
 278 informative syntenic conservation was found around *fbp1a* or around *fbp1b2* because
 279 of the small size of the scaffolds. Nevertheless, *fbp1b1*, borne by a larger scaffold,
 280 was located in the *gas1-C9-dab2-thsb4b* syntenic group conserved in the zebrafish,
 281 medaka, stickleback (*Gasterosteus aculeatus*), tetraodon and fugu around the *fbp1b*
 282 gene (data not shown).

283 Our phylogenetic analysis for *G6pc*-related genes revealed that one rainbow
 284 trout sequence was orthologous to the zebrafish gene annotated *g6pca.1*
 285 (ENSDARP00000116534) in Ensembl (release 77) while the others grouped pairwise
 286 with zebrafish genes annotated *g6pca.2* (ENSDARP00000086301) and *g6pcb*
 287 (ENSDARP00000128434), respectively. The typology of the phylogenetic tree
 288 indicated that the initial annotation of co-orthologous *G6pc* genes in zebrafish was
 289 inaccurate. Indeed the rooting of the branches inside the teleost sub-tree was more in
 290 favour of an initial duplication of the *G6pc* gene into *g6pca* and *g6pcb* before or
 291 around teleost radiation, followed by a second duplication of at least *g6pcb* giving rise
 292 to *g6pcb1* and *g6pcb2* genes. We therefore re-annotated zebrafish co-orthologous
 293 *G6pc* genes in our phylogenetic and syntenic analysis (*g6pca* as a replacement for
 294 *g6pca.1*, *g6pcb1* for *g6pca.2*, and *g6pcb2* for *g6pcb*) (Fig.1C). Moreover, the matrix
 295 protein identity (Table 1-C) confirmed that, except for one *G6pca*
 296 (GSONMG00076843001), other rainbow trout *G6pc* amino acid sequences shared
 297 pairwise more than 90% identity, suggesting that duplicated *g6pcb1* and duplicated
 298 *g6pcb2* genes were retained after Ss4R (3) in the rainbow trout genome as *g6pcb1.a*
 299 (GSONMG00076841001), *g6pcb1.b* (GSONMG00066036001), *g6pcb2.a*

(GSONMG00013076001) and *g6pcb2.b* (GSONMG00014864001). Finally, our syntenic analysis (Fig.2) showed that *G6pc* in the human and the mouse was located in the *wnk4-ccdc56-aoc2-aoc3-aards1-nbr1-tmem106a-arl4d-etv4-meox1* syntenic group. Interestingly, syntenic conservation of *nbr1-tmem106a-arl4d* 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 *g6pcb2* in zebrafish where the syntenic group was only partially conserved). Similarly, the *etv4-meox1* group was found to be specifically syntenic with the *g6pca-g6pcb1* group, while *aoc2* was only syntenic with *g6pcb2* genes, thus confirming the identity of such paralogous genes.

An expected phenotype confirmed by persistent post-prandial hyperglycaemia and up-regulation of *gck* genes after a high carbohydrate content diet

Our experimental conditions and diets were designed to arrive at a previously described (49) hyperglycaemic phenotype characterised by a steep rise in plasma glucose concentration and a dramatic increase in glucokinase (*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 rich-carbohydrate called diets (<20% carbohydrates) for long term nutrition but was chosen in order to dramatically induce such a phenotype. We therefore monitored glycaemia and glucokinase mRNA level and enzyme activity.

The plasmatic glucose analysis showed that plasma glucose levels were significantly higher after a high carbohydrate diet (HighCHO, Fig.3A) than in fasted trout (*i.e.* Fasted or Refasted, Fig.3A) or trout fed the NoCHO diet which displayed normoglycaemia (~0.8g/L).

As for gluconeogenic genes, we performed an *in silico* analysis in order to elucidate the *gck* evolutionary history. We thus identified two sequences in the rainbow trout

326 genome sharing high homology with the zebrafish *gck* gene. These two genes were
327 grouped together in the teleost *gck* phylogenetic sub-tree (Fig.4) as co-orthogues to
328 teleost and tetrapod *gck* genes. As no duplication of *gck* in other sequenced teleost
329 genomes was found, we concluded that duplication of *gck* in rainbow trout occurred
330 before or around the Ss4R, giving rise to *gcka* (GSONMG00033781001) and *gckb*
331 (GSONMG00012878001) in this species. Compared with fasted trout and fish fed the
332 NoCHO diet, both *gcka* and *gckb* mRNA levels increased dramatically in fish fed the
333 high carbohydrate diet (Fig.3B). The overall Gck activity followed the same trend
334 (Fig.3C), confirming that Gck enzyme activity is a strong indicator of dietary
335 carbohydrate intake.

336 Taken together, these results confirmed the physiological effects of a high
337 carbohydrate content diet in trout expected with this study design, and as previously
338 described (49).

339 **Expression analysis of gluconeogenic genes**

340 Analysis of gene expression revealed that, although the *pck1* mRNA level was not
341 clearly statistically affected by the nutritional status, it was higher in trout fed the
342 NoCHO diet than in trout fed the HighCHO diet (Fig.5A). The mRNA level of *pck2*
343 (Fig.5A) was constant whatever the experimental condition. No change in Pck1 or
344 Pck2 enzyme activity was noted (Fig.5D-a and 5D-b). As previously described (19),
345 we found that overall Pck activity in the liver comprised approximately one third
346 cytosolic activity (Pck1) and two thirds mitochondrial activity (Pck2) (Fig.5D-a and
347 5D-b).

348 As for *pck2*, *fbp1a* mRNA level remained stable, irrespective of the nutritional status
349 (Fig.5B). In contrast to *fbp1b2*, the *fbp1b1* mRNA level increased in trout fed the
350 NoCHO diet compared to fasted animals (Fig. 5B). However, intake of the HighCHO

351 diet caused a decrease in both *fbp1b1* and *fbp1b2* paralogue mRNA levels (Fig.5B).
352 The overall Fbp activity (Fig.5D-c) followed the same trend as *fbp1b1* and *fbp1b2*
353 mRNA levels, with a significant decrease in trout fed the HighCHO diet, but did not
354 follow the increase in *fbp1b1* mRNA level in trout fed the NoCHO diet.
355 The mRNA level of *g6pca* (Fig.5C) remained unchanged whatever the nutritional
356 status in trout fed the NoCHO diet while it decreased in trout fed the HighCHO diet
357 and in re-fasted fish fed the latter diet. The *g6pcb1.b* mRNA level followed almost the
358 same pattern as *g6pca* whereas the paralogous *g6pcb1.a* gene displayed a decrease in
359 mRNA level in trout fed the NoCHO diet, with the same fold change as that observed
360 in trout fed the HighCHO diet (Fig.5C). In striking contrast to all other co-
361 orthologous *G6pc* genes, the mRNA level of both *g6pcb2* paralogues increased in fed
362 trout compared to fasted or re-fasted fish, and to a greater extent when trout were fed
363 the HighCHO diet. Also, the overall G6pc enzyme activity remained unchanged in
364 fasted and fed trout but decreased in re-fasted trout (Fig.5D-d).

365 Discussion

366
367 The rapidly increasing number of whole genomes sequenced in model and
368 non-model organisms in the last decade (3, 4, 60) makes us seriously consider the
369 DNA sequence information to be a powerful tool for unravelling the mechanisms of
370 environmental adaptation. In this context, the outcome of duplicated genes arising
371 through Ss4R in rainbow trout can lead to adaptive innovation (47), depending on
372 whether they are lost or fixed and maintained (14). Elucidating the evolutionary
373 history of such genes and studying their expression patterns may help to reveal new
374 molecular underpinnings involved in adaptive behaviour in rainbow trout. This
375 approach is of particular interest when considering the adaptation of rainbow trout to

376 carnivorism as this has never previously been explored from a genomic point of view.
377 The present study therefore focused on genes encoding key gluconeogenic enzymes in
378 the liver, the atypical nutritional regulation of the gluconeogenesis pathway being one
379 trail to explain the “glucose-intolerant” phenotype of this carnivorous species.

380 ***In silico* analysis of the recently sequenced rainbow trout genome revealed the**
381 **conservation of several duplicated gluconeogenic genes**

382 The first critical step in elucidating the evolutionary history of gluconeogenic
383 genes was to identify accurately the orthologous genes of mammalian *Pck1*, *Pck2*,
384 *G6pc* and *Fbp1* genes in the newly sequenced rainbow trout genome (3). Using
385 phylogenetic and syntenic analyses we identified for the first time a *pck1* gene in the
386 rainbow trout genome which was orthologous to the cytosolic *Pck1*. Our results also
387 confirmed that the *pck* gene previously sequenced in rainbow trout by Panserat *et al.*
388 (2001, (31)) was orthologous to the mitochondrial *Pck2* gene in other species. For
389 *Fbp1*, we demonstrated that a duplication of the gene occurred before or around
390 teleost radiation, giving rise to *Fbp1a* and *Fbp1b* in teleosts probably as a
391 consequence of the teleost-specific 3rd WGD (Ts3R). After 4SsR, *fbp1a* was retained
392 as a singleton in the rainbow trout genome while the *fbp1b* ohnologous gene pair
393 (paralogues formed by a WGD event, (3)) consisting of *fbp1b1* and *fbp1b2* was
394 preserved. We finally clarified the complex evolutionary history of *G6pc*
395 (summarised in Fig.6), showing that a duplication of this gene occurred before or
396 around teleost radiation, giving rise to *g6pca* and *g6pcb* in the ancestral teleost
397 genome. The presence of both *g6pcb1* and *g6pcb2* in the genome of a cypriniform
398 (zebrafish) and a salmonidae (rainbow trout), but only *g6pcb1* in Percomorph
399 genomes (medaka, stickleback, fugu and tetraodon), was then in favour of an
400 additional duplication of *g6pcb*, at least in the genome of the common ancestor of the
401 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 of gluconeogenic genes confirmed that genes retained as 2R (two rounds of WGD occurring at the base of vertebrates) and Ts3R ohnologues were more likely to have been retained in duplicated copies after Ss4R in rainbow trout (for instance *g6pcb* and *fbp1b*, in contrast to *pck1* or *pck2* that were retained as singletons after Ts3R (47)) as demonstrated by Berthelot *et al.* (2014, (3)). These authors also suggested that WGD involved the loss of one gene copy of ohnologous gene pairs by gene fractionation (loss of functioning DNA sequence, (23)). This process may be the origin of the loss of one *pck1*, *pck2*, *g6pca* and *fbp1a* ohnologue in rainbow trout after Ss4R.

Overall analysis of hepatic expression profiles of gluconeogenic genes suggested sub- or neo-functionalisation of ohnologous genes after Ss4R

An overall analysis of expression profiles of the previously described gluconeogenic genes revealed that both genes of each identified ohnologue pair (i.e., *fbp1b1/fbp1b2*, *g6pcb1.a/g6pcb1.b* and *g6pcb2.a/g6pcb2.b*) displayed similar expression patterns, indicating that they belonged to the HC (“high correlation”) group defined by Berthelot *et al.* (2014, (3)). Moreover, these convergent expression patterns were associated with a high percentage of identity of the ohnologue coding region (*fbp1b1/fbp1b2*, 93.6%; *g6pcb1.a/g6pcb1.b*, 92.4% and *g6pcb2.a/g6pcb2.b*, 96.4%) suggesting that these sequences were submitted to a high selective pressure, as proposed by Berthelot *et al.* (2014, (3)). From a paralogous genes point of view, our results showed that *g6pcb* duplication may have led to sub- or neo-functionalisation because *g6pcb1* ohnologues and *g6pcb2* ohnologues displayed opposite expression profiles. Nonetheless, since *g6pcb2* expression has to our knowledge never been studied in other species (especially not in zebrafish), we cannot conclude that this sub-

428 or neo-functionalisation occurred only in rainbow trout nor whether it occurred before
429 salmonid radiation. Similarly, *fbp1* duplication was probably followed by sub- or neo-
430 functionalisation of *fbp1a* and *fbp1b* ohnologues which probably still occurred after
431 Ts3R. Indeed, whole mount *in situ* hybridisation data available for zebrafish embryos
432 demonstrates that *fbp1a* mRNA is more related to lens whereas *fbp1b* mRNA is
433 located, amongst other organs, in the liver and in the gut (5, 44). Finally, the sub-
434 functionalisation of *pck1* and *pck2* highlighted by our results has long been suggested
435 (15, 16).

436 **Expression analysis of hepatic gluconeogenic genes in trout fed with or**
437 **without carbohydrates revealed differential paralogous profiles**

438 On considering the different nutritional conditions tested, analyses of the
439 expression of the duplicated gluconeogenic genes revealed that all genes were
440 regulated by nutritional status except *pck2* and *fbp1a*, the mRNA levels of which
441 remained stable whatever the nutritional status. Our results showed that none of the
442 genes encoding key gluconeogenic enzymes (except *g6pcb1.a*) were down-regulated
443 in trout fed the NoCHO diet. Indeed, compared to fasting *pck1*, *fbp1b2*, *g6pca* and
444 *g6pcb1.b* were not regulated whereas *fbp1b1*, *g6pcb2.a* and *g6pcb2.b* were up-
445 regulated. These expression data, combined with the maintenance of gluconeogenic
446 enzyme activity, suggested that the gluconeogenesis pathway remained active under
447 NoCHO nutrition according to the need for endogenous glucose production to sustain
448 maintenance of normoglycaemia due to the dietary habits and physiology of
449 carnivorous species (7, 10, 57, 58, 61). Furthermore, this is consistent with previous
450 *in vivo* and *in vitro* studies showing that the gluconeogenesis flux is never turned off
451 in fed carnivorous teleosts (35, 56). Moreover, when trout were submitted to a re-
452 fasting period we showed that mRNA levels for most of the genes (*pck1*, *fbp1b1*,
453 *fbp1b2*, *g6pca* and *g6pcb1b*) were maintained at the same levels as in fed trout.

454 By contrast, the HighCHO diet led to the down-regulation of most of the
455 gluconeogenic genes (*pck1*, *fbp1b1*, *fbp1b2*, *g6pca*, *g6pcb1.a* and *g6pcb1.b*),
456 suggesting that, as in mammals, dietary carbohydrates are involved in the inhibition of
457 expression of gluconeogenic genes. In addition, dietary carbohydrates seemed to
458 down-regulate overall Fbp1 enzyme activity. These findings suggested that the
459 potential mechanism to minimize gluconeogenic flux in the presence of dietary
460 carbohydrates exists in the trout. However, and surprisingly, mRNA levels of *g6pcb2*
461 orthologues dramatically increased in trout fed the HighCHO diet in contrast to other
462 *G6pc* paralogues. Indeed, the expression profiles of *g6pcb2* ohnologues mimicked
463 those of *gck* ohnologues and might in part be responsible for the poor regulation of
464 the gluconeogenesis pathway. Such an expression profile of *G6pc* encoding genes
465 under high carbohydrate nutrition in rainbow trout has never previously been
466 described, as duplicated *g6pcb2* genes had not been identified before. Indeed, we
467 found that primers used in previous studies amplified only *g6pca* (e.g., primers named
468 “*G6Pase*” in (30) and (20) or “*g6pase 2*” in (49) and (12)) and a conserved region
469 between *g6pcb1* ohnologues (primers named “*g6pase 1*” in (49) and (12)). In
470 addition, it can be hypothesised that the “glucose-intolerant” phenotype in rainbow
471 trout was accentuated by the increased *g6pcb2* gene dosage due to the fixation of both
472 ohnologues in its genome (21, 43). This gene dosage may explain how overall *G6pc*
473 enzyme activity remained stable in trout fed the HighCHO diet while the mRNA level
474 of three orthologous *G6pc* genes decreased (*g6pca*, *g6pcb1.a* and *g6pcb1.b*)
475 compared to trout fed the NoCHO diet. The down-regulation of overall *G6pc* enzyme
476 activity in re-fasted trout also supports this hypothesis as *g6pcb2* mRNA levels
477 decreased dramatically under re-fasting.

478 Altogether, our results suggest that the atypical expression profiles of *g6pcb2*
479 ohnologues, and probably the resulting enzyme activity, may contribute significantly
480 to the non-inhibition of the last step of gluconeogenic flux and to the enrichment of
481 plasma glucose *via* futile glucose/glucose-6-phosphate cycling (56) in trout fed a
482 carbohydrate-enriched diet. Interestingly, in Type 2 diabetes under hyperglycaemia
483 and in conditions where endogenous glucose production is minimized, a significant
484 glucose/glucose-6-phosphate activity cycle occurs (46) that is mediated in part by
485 increased G6pase flux (6) but largely dependent on glucokinase activity. The lesson
486 learned from trout naturally exhibiting this phenotype may thus cast light on defects
487 in metabolism that contribute to diabetes.

488 **Conclusions**

489 The results of this study demonstrated the importance of considering the additional
490 round of WGD occurring in teleosts and in salmonids as major evolutionary events
491 which can lead to adaptive innovation *via* gene duplication. Indeed, under the
492 HighCHO diet the absence of *pck2* and *fbp1a* down-regulation and, more surprisingly,
493 the increase in mRNA levels of both *g6pcb2* ohnologues are new elements to enhance
494 our understanding of the poor use of dietary carbohydrates in rainbow trout. Further
495 studies such as gene knock-out assays are now needed to evaluate the involvement of
496 these duplicated genes in the carnivorous “glucose-intolerant” phenotype of rainbow
497 trout.

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Disclosures

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Authors' 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. I.S., S. S.-C., V.V. and S. P. contributed to the manuscript correction.

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Figure captions

Figure 1 - Phylogenetic analysis of *pck1* and *pck2* (A), *fbp1* (B) and *g6pc* (C)

Phylogenetic analyses were performed using MEGA package version 5 software (52), as previously described (11). The phylogenetic trees were built using the Neighbor-Joining (NJ) method. The reliability of the inferred trees was estimated using 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 brackets.

Figure 2 - Conserved synteny around the *g6pc* loci in mammals and teleosts

Data were collected using Genomicus software version 01.01, and *g6pc* genes were annotated by ourselves according to our phylogenetic analysis following ZFIN Nomenclature guidelines. Black crosses indicate loss of the *g6pcb2* gene in medaka, stickleback, fugu and tetraodon genomes. Ch., chromosome; sc., scaffold, cont., contig; Gp., group.

Figure 3 – Physiological confirmation of expected phenotypes depending on the nutritional status and diet

Plasma levels of glucose (A), relative abundance of *glucokinase a* (*gcka*) and *glucokinase b* (*gckb*) mRNA (B), and overall glucokinase activity (C) in fasted (white bars), fed with NoCHO (grey bars) or HighCHO (black bars) diets and re-fasted trout after NoCHO or HighCHO diet. Data are expressed as means $\pm$ SE (n = 9). Different letters indicate significant differences between conditions (P < 0.01).

Figure 4 – Phylogenetic analysis of *gcka* and *gckb*

Phylogenetic analyses were performed using MEGA package version 5 software (52), as previously described (11). The phylogenetic tree was built using the Neighbor-

754 Joining (NJ) method. The reliability of the inferred trees was estimated using the
755 bootstrap method with 1,000 replications. The *Branchiostoma japonicum*, (GenBank
756 ID: AGW32307) protein sequence was used to root the tree. All accession numbers
757 (from Ensembl or Genoscope databases) are specified in brackets.

758

759 **Figure 5 – Relative expression of paralogous gluconeogenic genes and**
760 **corresponding overall enzyme activity**

761 Relative abundance of *pck1* and *pck2* mRNA (A), *fbp1* paralogues (B) and *g6pcb*
762 paralogues (C) and respective related overall enzyme activity (D) in fasted trout
763 (white bars), trout fed with the NoCHO (grey bars) or the HighCHO (black bars) diet
764 and re-fasted trout after NoCHO or HighCHO diet. Data are expressed as means $\pm$ SE
765 (n = 9). Different letters indicate significant differences between conditions (P <
766 0.01).

767 **Figure 6 – Model depicting the evolution of *g6pc* genes in teleosts**

768 *G6pc* (grey) duplicated before or around teleost radiation giving rise to *g6pca* (green)
769 and *g6pcb* (brown). *g6pcb* was then duplicated at least in the common ancestor of
770 Euteleost, Salmonidae and Ostariophys into *g6pcb1* (dark yellow) and *g6pcb2*
771 (orange). Around salmonid radiation *g6pcb1* and *g6pcb2* were duplicated (*g6pcb1a* in
772 light yellow, *g6pcb1b* in brown, *g6pcb2a* in red and *g6pcb2b* in pink). *g6pcb2* was
773 not maintained in the percomorph ancestor.

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780 Tables

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A.		Hs-PCK1	Dr-Pck1	Om-Pck1	Hs-PCK2	Dr-Pck2
Hs-PCK1						
Dr-Pck1	73,63					
Om-Pck1	75,56	79,94				
Hs-PCK2	70,74	69,18	70,14			
Dr-Pck2	67,04	66,29	68,12	67,36		
Om-Pck2	68,01	68,55	69,24	68,1	86,93	

B.		Hs-FBP1	Dr-Fbp1a	Dr-Fbp1b	Om-Fbp1b1
Hs-FBP1					
Dr-Fbp1a	79,53				
Dr-Fbp1b	79,53	88,72			
Om-Fbp1b1	76,85	84,27	89,02		
Om-Fbp1b2	77,74	86,05	90,5	97,33	

C.		Hs-G6PC	Dr-G6pca	Om-G6pca	Dr-G6pcb1	Om-G6pcb1.a	Om-G6pcb1.b	Dr-G6pcb2	Om-G6pcb2.a
Hs-G6PC									
Dr-G6pca	55,27								
Om-G6pca	54,15	71,92							
Dr-G6pcb1	64,12	58,17	58,17						
Om-G6pcb1.a	62,54	57,83	60,57	81,97					
Om-G6pcb1.b	62,43	58,29	61,14	80,56	92,72				
Dr-G6pcb2	53,8	51,14	51,15	61,08	59,04	57,79			
Om-G6pcb2.a	59,26	54,57	53,43	61,89	70,66	70	69,71		
Om-G6pcb2.b	58,12	54	52,15	61,03	68,09	67,14	67,43	94,3	

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783 **Table 1 – Matrix protein identity of phosphoenol-pyruvate carboxykinase (A),**
784 **fructose-1,6-bisphosphatase (B) and glucose-6-phosphatase (C) among zebrafish,**
785 **rainbow trout and the human**

786 For accession numbers referred to phylogenetic analysis (Fig.1). Dr, *Danio rerio*; Hs,
787 *Homo sapiens*; Om, *Oncorhynchus mykiss*.

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		Diets	
		NoCHO	HighCHO
	Ingredients %		
	Fish meal ¹	90.85	55.9
	Fish oil ²	5.15	10.1
	Starch ³	0	30
	Vitamin mix ⁴	1	1
	Mineral mix ⁵	1	1
	Alginate ⁶	2	2
	Proximate composition		
	Dry matter (DM, % diet)	95.46	94.69
	Crude protein (% DM)	61.30	39.08
	Crude lipid (% DM)	18.86	17.70
	Gross energy (kJ/g DM)	22.05	21.76
	Ash (% DM)	17.73	11.38
	Carbohydrates (% DM)	<1.0	28.4
	¹ Sopropêche, Boulogne-sur-Mer, France		
	² fish oil; Sopropêche, Boulogne-sur-Mer, France.		
	³ Gelatinized corn starch (Roquette, Lestrem, France)		
	⁴ Supplied the following (kg-1 diet): DL-α tocopherol acetate 60 IU, sodium menadione bisulphate 5 mg, retinyl acetate 15000 IU, DLcholecalciferol 3000 IU, thiamin 15 mg, riboflavin 30 mg, pyridoxine 15 mg, vit. B12 0.05 mg, nicotinic acid 175 mg, folic acid 500 mg, inositol 1000 mg, biotin 2.5 mg, calcium pantothenate 50 mg, choline chloride 2000 mg.		
	⁵ 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.		
	⁶ Louis François, Marne-la-Vallée, France.		

Table 2 - Formulation and proximate composition of the two experimental diets used (NoCHO and HighCHO diets) in this experiment.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Signae or Genoscope Accession number
<i>RS16</i>	TTTCAGGTGGCGAAACATGC'	GGGGTCTGCCATTCACCTTG	tcbk0005c.o.13_5.1.om.4
<i>gcka</i>	CTGCCACCTACGTCTGT	GTCATGGCGTCCTCAGAGAT	GSONMG00033781001
<i>gckb</i>	TCTGTGCTAGAGACAGCCC	CATTTTGACGCTGGACTCCT	GSONMG00012878001
<i>pck1</i>	ACAGGGTGAGGCAGATGTAGG	CTAGTCTGTGGAGGTCTAAGGGC	GSONMG00082468001
<i>pck2</i>	ACAATGAGATGATGTGACTGCA	TGCTCCATCACCTACAACCT	GSONMG00059643001
<i>fbp1a</i>	GACAGAGGACGACCCGTG	GTACTGACCGGGTCCAACAT	GSONMT00001932001
<i>fbp1b1</i>	CTCTCAAGAACCTCTACAGCCT	TCAGTTCTCCCGTTCCTTC	GSONMT00063051001
<i>fbp1b2</i>	ATCAGCAGGAATAGGTCGCG	CCTCCTCCAGCACGAATCTC	GSONMT00015701001
<i>g6pca</i>	GATGGCTTGACGTTCTCCT	AGATCCAGGAGAGTCCTCC	GSONMG00076843001
<i>g6pcb1.a</i>	GCAAGGTCAAAGATCAGGC	GCCAATGTGAGATGTGATGGG	GSONMG00076841001
<i>g6pcb1.b</i>	GCTACAGTGCTCTCCTTCTG	TCACCCCATAGCCCTGAAA	GSONMG00066036001
<i>g6pcb2.a</i>	ATCGGACAATACACAGAACT	CAACTGATCTATAGCTGCTGCCT	GSONMG00013076001
<i>g6pcb2.b</i>	CCTCTGCTCTTCTGACGTAG	TGTCCATGGCTGCTCTCTAG	GSONMG00014864001

Table 3 - Primer Sequences and accession numbers for qPCR analysis

A.

B.

C.

D.