

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

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#### insights into the nutritional regulation New of 1 gluconeogenesis in carnivorous rainbow trout 2 (Oncorhynchus mykiss): a gene duplication trail 3 4 5 Lucie Marandel<sup>§</sup>, Iban Seiliez, Vincent Véron, Sandrine Skiba-Cassy, Stéphane Panserat

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- 24 **Running head** : The atypical regulation of gluconeogenesis : a gene duplication trail
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## 27 Abstract

28 The rainbow trout (Oncorhynchus mykiss) is considered to be a strictly carnivorous 29 fish species that is metabolically adapted for high catabolism of proteins and low 30 utilisation of dietary carbohydrates. This species consequently has a "glucose-31 intolerant" phenotype manifested by persistent hyperglycaemia when fed a high-32 carbohydrate diet. Gluconeogenesis in adult fish is also poorly, if ever, regulated by 33 carbohydrates, suggesting that this metabolic pathway is involved in this specific 34 phenotype. In this study, we hypothesised that the fate of duplicated genes after the salmonid-specific 4<sup>th</sup> whole genome duplication (Ss4R) may have led to adaptive 35 36 innovation and that their study might provide new elements to enhance our 37 understanding of gluconeogenesis and poor dietary carbohydrates use in this species.

38 Our evolutionary analysis of gluconeogenic genes revealed that *pck1*, *pck2*, *fbp1a* and 39 g6pca were retained as singletons after Ss4r while g6pcb1, g6pcb2 and fbp1b 40 ohnologue pairs were maintained. For all genes, duplication may have led to sub- or 41 neo-functionalisation. Expression profiles suggested that the gluconeogenesis 42 pathway remained active in trout fed a no carbohydrate diet. When trout were fed a 43 high-carbohydrate diet (30%), most of the gluconeogenic genes were non- or down-44 regulated, except for *g6pbc2* ohnologues the RNA levels of which were surprisingly 45 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 "glucoseintolerant" phenotype of trout and may partially explain its poor use of dietary carbohydrates.

## 51 Keywords

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

### 53 Introduction

54 The rainbow trout (Oncorhynchus mykiss) is considered to be a strictly 55 carnivorous fish species with nutritional needs linked to this specific dietary regime. 56 Indeed, carnivorous species naturally require a diet consisting of high protein and 57 moderate fat content but with minimal amounts of carbohydrate. The rainbow trout, 58 with other carnivores such as the cat, mink and carnivorous teleosts (for instance 59 European seabass, *Dicentrarchus labrax*), are thus metabolically adapted for high 60 catabolism of proteins and low utilisation of dietary carbohydrates. They are therefore 61 considered to be a glucose intolerant species due mainly to persistent hyperglycaemia 62 after intake of carbohydrate-enriched meal(s) or glucose tolerance tests (cat (18, 48); 63 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, 64 65 53, 56, 59) than omnivorous species, allowing them to sustain endogenous glucose 66 demands, *i.e.* maintenance of normoglycaemia and production of energy using 67 gluconeogenic amino acids and fat rather than dietary carbohydrates (7, 10, 57, 58, 68 61). Probably as a result of no dietary carbohydrate requirements, gluconeogenesis in 69 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 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 up-regulated by such a diet. Species thus adapt to carnivorism 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 salmonids (Ss4R salmonid-specific 4<sup>th</sup> WGD, (3, 24)). WGD may represent a major 83 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 charbohydrate
- 113 WGD: whole genome duplication
- 114 Ss4R: salmonid-specific 4<sup>th</sup> WGD
- 115 Ts3R: teleost-specific 3<sup>rd</sup> WGD

## 116 Materials and Methods

### 117 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 anaesthetic 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.

### 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 ( $\sim$ 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

Juvenile rainbow trout (~70g body mass) were distributed in six tanks (15 fish per 136 137 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  $-20^{\circ}$ 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  $-80^{\circ}$ 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  $(N \times 6.25)$  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 Gribheimer, 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-167 01.01/cgi-bin/search.pl) was used to confirm their identities by establishing syntenic 168 analysis (Fig.2).

Phylogenetic analyses were performed using the MEGA package version 5 software (52), as previously described (11). The phylogenetic trees, based on full-length amino acid deduced sequences, were built using the Neighbor-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 1,000 replications. *Branchiostoma floridae* Pck (Genbank accession XP\_002593974), *Oikopleura dioica* 

- 175 Fbp (Genbank accession CBY10016) and Latimeria chalumnae G6pc (Ensembl
- accession ENSLACG0000022601) 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 guidelines179 (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 millimiter 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, Charbonniéres, France) to synthesize cDNA (n = 9 for each 191 treatment).

### 192 Real time RT-PCR

The primer sequences used in real-time RT-PCR assays are listed in Table 3. 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 (49). 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  $\mu$ l per sample, each of which contained 2  $\mu$ l of diluted cDNA template, 0.12  $\mu$ l 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  $\Delta CT$  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

225 centrifugation (20 minutes, 10,000g at 4°C) was performed on supernatants obtained 226 after first centrifugation at 900g. After the second centrifugation, supernatants were 227 kept for cytosolic Pck1 assay. The pellets containing the mitochondrial fraction were 228 recovered in 500µL of ice cold buffer at pH 7.4 and sonicated as described above. The 229 enzymes assayed were: high Km Hexokinase (Gck), as described by Panserat et al. 230 (2000, [25]), G6Pase from Alegre et al. (1988, [62]), Pck following protocol of 231 Kirchner et al. (2003, [63]) and Fbp, described by Tranulis et al. (1996, [64]). The 232 enzyme activity was measured in duplicate at 30°C following the variation of 233 absorbance of NADPH (Nicotinamide adenine dinucleotide phosphate) at 340nm. The 234 reactions were started by the addition of the specific substrate; a Power Wave X 235 (BioTek Instrument, Inc.) plate reader was used. Water was used as a blank for each 236 sample. One unit of enzyme activity was defined as the amount of enzyme that 237 catalysed the hydrolysis of 1µmol of substrate per minute at 30°C. Enzyme activity 238 was expressed per milligram of soluble protein. Protein concentrations were measured 239 in triplicate according to Bradford (1976, [65]), using a protein assay kit (Bio-Rad, 240 München, Germany) with bovine serum albumin as a standard.

### 241 Statistical analysis

242 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
- 245 package.

## 246 **Results**

### 247 *In silico* analysis of gluconeogenic genes

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

(*Danio rerio*) and human (*Homo sapiens*) orthologues: 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.

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 identity (Table 1-C) confirmed that, protein 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 g6pacb2.b (GSONMG00014864001). Finally, our 300 301 syntenic analysis (Fig.2) showed that G6pc in the human and the mouse was located 302 in the wnk4-ccdc56-aoc2-aoc3-aards1-nbr1-tmem106a-arl4d-etv4-meox1 syntenic 303 group. Interestingly, syntenic conservation of *nbr1-tmem106a-arl4d* was found with 304 all chromosomal regions of teleosts (zebrafish, trout, stickleback (Gasterosteus 305 *aculeatus*), fugu and tetraodon) which carried the duplicated copies of g6pc (except 306 around *g6pcb2* in zebrafish where the syntenic group was only partially conserved). 307 Similarly, the *etv4-meox1* group was found to be specifically syntenic with the *g6pca*-308 g6pcb1 group, while *aoc2* was only syntenic with g6pcb2 genes, thus confirming the 309 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

312 Our experimental conditions and diets were designed to arrive at a previously 313 described (49) hyperglycaemic phenotype characterised by a steep rise in plasma 314 glucose concentration and a dramatic increase in glucokinase (gck) mRNA level and 315 enzyme activity with a high carbohydrate diet. The carbohydrate content of the 316 HighCHO diet (30%) is higher than those usually used in rich-carbohydrate called 317 diets (<20% carbohydrates) for long term nutrition but was chosen in order to 318 dramatically induce such a phenotype. We therefore monitored glycaemia and 319 glucokinase mRNA level and enzyme acitivity.

320 The plasmatic glucose analysis showed that plasma glucose levels were significantly

- 321 higher after a high carbohydrate diet (HighCHO, Fig.3A) than in fasted trout (i.e.
- 322 Fasted or Refasted, Fig.3A) or trout fed the NoCHO diet which displayed
- 323 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.

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 (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).

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

diet caused a decrease in both *fbp1b1* and *fbp1b2* paralogue mRNA levels (Fig.5B).
The overall Fbp activity (Fig.5D-c) 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.

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

carnivorism 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 atypical nutritional regulation of the gluconeogenesis pathway being one
trail to explain the "glucose-intolerant" phenotype of this carnivorous species.

# *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 382 383 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 PckI. 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 consequence of the teleost-specific 3<sup>rd</sup> WGD (Ts3R). After 4SsR, *fbp1a* was retained 391 392 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 genome. The presence of both g6pcb1 and g6pcb2 in the genome of a cypriniform 397 398 (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 402 Percomorph ancestor. Analysis of the rainbow trout genome indicated that *g6pca* was 403 retained as a singleton whereas both ohnologous gene pairs g6pcb1.a-g6pcb1.b and 404 g6pcb2.a-g6pcb2.b remained whole after Ss4R. The evolutionary history of 405 gluconeogenic genes confirmed that genes retained as 2R (two rounds of WGD 406 occurring at the base of vertebrates) and Ts3R ohnologues were more likely to have 407 been retained in duplicated copies after Ss4R in rainbow trout (for instance *g6pcb* and 408 *fbp1b*, in contrast to *pck1* or *pck2* that were retained as singletons after Ts3R (47)) as 409 demonstrated by Berthelot et al. (2014, (3)). These authors also suggested that WGD 410 involved the loss of one gene copy of ohnologous gene pairs by gene fractionation 411 (loss of functioning DNA sequence, (23)). This process may be the origin of the loss 412 of one *pck1*, *pck2*, *g6pca* and *fbp1a* ohnologue in rainbow trout after Ss4R.

## 413 Overall analysis of hepatic expression profiles of gluconeogenic genes 414 suggested sub- or neo-functionalisation of ohnologous genes after Ss4R

415 An overall analysis of expression profiles of the previously described 416 gluconeogenic genes revealed that both genes of each identified ohnologue pair (i.e., 417 fbp1b1/fbp1b2, g6pcb1.a/g6pcb1.b and g6pcb2.a/g6pcb2.b) displayed similar 418 expression patterns, indicating that they belonged to the HC ("high correlation") 419 group defined by Berthelot et al. (2014, (3)). Moreover, these convergent expression 420 patterns were associated with a high percentage of identity of the ohnologue coding 421 region (*fbp1b1/fbp1b2*, 93.6%; *g6pcb1.a/g6pcb1.b*,92.4% and *g6pcb2.a/g6pcb2.b*, 422 96.4%) suggesting that these sequences were submitted to a high selective pressure, as 423 proposed by Berthelot et al. (2014, (3)). From a paralogous genes point of view, our 424 results showed that *g6pcb* duplication may have led to sub- or neo-functionalisation 425 because  $g\delta pcb1$  ohnologues and  $g\delta pcb2$  ohnologues displayed opposite expression 426 profiles. Nonetheless, since g6pcb2 expression has to our knowledge never been 427 studied in other species (especially not in zebrafish), we cannot conclude that this sub428 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  $pck^2$  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 g6pcb2479 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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## 505 **Disclosures**

506 The authors declare that there are no conflicts of interests.

## 507 Authors' contributions

- 508 L.M and S.P designed the study. L.M managed the study, performed *in silico*,
- 509 expression and statistical analysis, and wrote the manuscript. V.V performed enzyme
- 510 activity analysis. I.S., S. S.-C., V.V. and S. P. contributed to the manuscript
- 511 correction.

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## 727 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),

- 730 as previously described (11). The phylogenetic trees were built using the Neighbor-
- 731 Joining (NJ) method. The reliability of the inferred trees was estimated using the
- 732 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)
- 735 protein sequences were used to root trees for Pck (A), Fbp1 (B) and G6pc (C)
- phylogenetic analysis, respectively. All accession numbers (from Genbank, Ensembl
- 737 or Genoscope databases) are specified in brackets.

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

739 Data were collected using Genomicus software version 01.01, and *g6pc* genes were

- 740 annotated by ourselves according to our phylogenetic analysis following ZFIN
- 741 Nomenclature guidelines. Black crosses indicate loss of the *g6pcb2* gene in medaka,
- 742 stickleback, fugu and tetraodon genomes. Ch., chromosome; sc., scaffold, cont.,
- 743 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).

### 751 Figure 4 – Phylogenetic analysis of gcka and gckb

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

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

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#### 759 Figure 5 - Relative expression of paralogous gluconeogenic genes and 760 corresponding overall enzyme activity Relative abundance of *pck1* and *pck2* mRNA (A), *fpbp1* paralogues (B) and *g6pcb* 761 762 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)

- and *g6pcb* (brown). *g6pcb* was then duplicated at least in the common ancestor of
- Euteleost, Salmonidae and Ostariophys into g6pcb1 (dark yellow) and g6pcb2
- (orange). Around salmonid radiation *g6pcb1* and *g6pcb2* were duplicated (*g6pcb1a* in
- light yellow, *g6pcb1b* in brown, *g6pcb2a* in red and *g6pcb2b* in pink). *g6pcb2* was
- not maintained in the percomorph ancestor.
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## 780 Tables

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Α.	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
В.	Hs-FBP1	Dr-Fbp1a	Dr-Fbp1b	Om-Fbp1b1	
				•	
INS-FOF1				·	
Dr-Fbp1a	79,53			·	
Dr-Fbp1a Dr-Fbp1b	79,53 79,53	88,72			
Dr-Fbp1a Dr-Fbp1b Om-Fbp1b1	79,53 79,53 76,85	88,72 84,27	89,02		

С.	Hs-G6PC	Dr-G6pca	<i>Om-</i> 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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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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	Γ	Diets	
	NoCHO	HighCHO	
Ingredients %			
Fish meal <sup>1</sup>	90.85	55.9	
Fish oil <sup>2</sup>	5.15	10.1	
Starch <sup>3</sup>	0	30	
Vitamin mix <sup>4</sup>	1	1	
Mineral mix <sup>5</sup>	1	1	
Alginate <sup>6</sup>	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	

806 <sup>1</sup> Sopropeche, Boulogne-sur-Mer, France

807 <sup>2</sup> fish oil; Sopropeche, Boulogne-sur-Mer, France.

808 <sup>3</sup> Gelatinized corn starch (Roquette, Lestrem, France)

<sup>4</sup> Supplied the following (kg-1 diet): DL-a 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 panthotenate 50 mg, choline chloride 2000 mg.

<sup>5</sup> 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

817 chloride 0.4 g.

818 <sup>6</sup> Louis François, Marne-la-Vallée, France.

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

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Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Sigenae or Genoscope Accession number
RS16	TTTCAGGTGGCGAAACATGC'	GGGGTCTGCCATTCACCTTG	tcbk0005c.o.13_5.1.om.4
gcka	CTGCCCACCTACGTCTGT	GTCATGGCGTCCTCAGAGAT	GSONMG00033781001
gckb	TCTGTGCTAGAGACAGCCC	CATTTTGACGCTGGACTCCT	GSONMG00012878001
pck1	ACAGGGTGAGGCAGATGTAGG	CTAGTCTGTGGAGGTCTAAGGGC	GSONMG00082468001
pck2	ACAATGAGATGATGTGACTGCA	TGCTCCATCACCTACAACCT	GSONMG00059643001
fbp1a	GACAGAGGACGACCCGTG	GTACTGACCGGGTCCAACAT	GSONMT00001932001
fbp1b1	CTCTCAAGAACCTCTACAGCCT	TCAGTTCTCCCGTTCCCTTC	GSONMT00063051001
fbp1b2	ATCAGCAGGAATAGGTCGCG	CCTCCTCCAGCACGAATCTC	GSONMT00015701001
д6рса	GATGGCTTGACGTTCTCCT	AGATCCAGGAGAGTCCTCC	GSONMG00076843001
g6pcb1.a	GCAAGGTCCAAAGATCAGGC	GCCAATGTGAGATGTGATGGG	GSONMG00076841001
g6pcb1.b	GCTACAGTGCTCTCCTTCTG	TCACCCCATAGCCCTGAAA	GSONMG00066036001
g6pcb2.a	ATCGGACAATACACACAGAACT	CAACTGATCTATAGCTGCTGCCT	GSONMG00013076001
g6pcb2.b	CCTCTGCTCTTCTGACGTAG	TGTCCATGGCTGCTCTCTAG	GSONMG00014864001

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831 Table 3 - Primer Sequences and accession numbers for qPCR analysis

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