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S. Polakof, R. Alvarez and J. L. Soengas

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Dietary carbohydrates induce changes in glucosensing capacity and food intake of rainbow trout

S. Polakof, J. M. Miguez and J. L. Soengas

Am J Physiol Regulatory Integrative Comp Physiol, August 1, 2008; 295 (2): R478-R489.

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Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles

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Geurden I, Aramendi M, Zambonino-Infante J, Panserat S. Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. *Am J Physiol Regul Integr Comp Physiol* 292: 2275–2283, 2007. First published February 15, 2007; doi:10.1152/ajpregu.00444.2006.—Based on the concept of nutritional programming in higher vertebrates, we tested whether an acute hyperglucidic stimulus during early life could induce a long-lasting effect on carbohydrate utilization in carnivorous rainbow trout. The trout were fed a hyperglucidic diet (60% dextrin) at two early stages of development: either at first feeding (3 days, *stimulus 1*) or after yolk absorption (5 days, *stimulus 2*). Before and after the hyperglucidic stimulus, they received a commercial diet until juvenile stage (>10 g). Fish that did not experience the hyperglucidic stimuli served as controls. The short- and long-term effects of the stimuli were evaluated by measuring the expression of five key genes involved in carbohydrate utilization: α -amylase, maltase (digestion), sodium-dependent glucose cotransporter (SGLT1; intestinal glucose transport), and glucokinase and glucose-6-phosphatase, involved in the utilization and production of glucose, respectively. The hyperglucidic diet rapidly increased expressions of maltase, α -amylase, and glucokinase in *stimulus 1* fish and only of maltase in *stimulus 2* fish, probably because of a lower plasticity at this later stage of development. In the final challenge test with juveniles fed a 25% dextrin diet, both digestive enzymes were upregulated in fish that had experienced the hyperglucidic stimulus at first feeding, confirming the possibility of modification of some long-term physiological functions in rainbow trout. In contrast, no persistent molecular adaptations were found for the genes involved in glucose transport or metabolism. In addition, growth and postprandial glycemia were unaffected by the stimuli. In summary, our data show that a short hyperglucidic stimulus during early trout life may permanently influence carbohydrate digestion.

fish nutrition; nutritional programming; carbohydrate digestion; intestinal glucose transport; glucose metabolism

CARBOHYDRATES IN DIETS OF farmed fish are added either directly as a relatively cheap source of energy or indirectly as a by-product of plant proteins, which has gained an enormous interest as an alternative for the fishery-dependent fish meal (36). Carnivorous teleosts such as rainbow trout, Atlantic salmon, yellowtail, eel, and sea bream are, however, recognized for their inefficiency to use high levels of dietary carbohydrates (35, 55). In rainbow trout, digestible carbohydrate

contents of >20–30% of the diet result in prolonged postprandial glycemia (6, 35, 55) and impaired growth (4, 23, 29).

The general mechanisms for the digestion, absorption, and metabolism of glucose and starch-like substances in carnivorous fish are not different from those in herbivorous or omnivorous fish species (22, 31). However, the abundance and the dietary regulation of the proteins involved in carbohydrate utilization in fish appear to be influenced by the potential variation in carbohydrate supply and thus by the natural feeding habit. Illustrative examples here are the severalfold lower activities of pancreatic α -amylase (E.C. 3.2.1.1) (24) and of intestinal brush-border membrane carbohydrases (disaccharidases) like maltase (E.C. 3.2.1.20) (12), as well as the lower abundance of glucose transporters (12) in carnivores relative to omnivores and herbivores such as tilapia, catfish, and cyprinids. Moreover, rainbow trout showed no differences in maltase activities when fed a diet with or without carbohydrates (11) and were found incapable of adjusting intestinal glucose transport to dietary supply (10). In addition, the regulation of hepatic gluconeogenesis is found to be influenced by the natural feeding habit. In omnivorous fish (43), as in nondiabetic mammals (47, 52), gluconeogenesis becomes unnecessary and is switched off when glucose is readily available from dietary sources (37). In contrast, in rainbow trout, mRNA levels and activities of gluconeogenic enzymes [such as glucose-6-phosphatase (G6Pase) (E.C. 3.1.3.9)] remained persistently high without retroinhibition by dietary glucose (39, 40, 42), despite a mammalian-type regulation for hepatic hexokinase IV [glucokinase (GK) (E.C. 2.7.1.1)] (41). Collectively, the above data clearly illustrate the poor adaptation of carnivorous rainbow trout to deal with high dietary carbohydrate loads.

Several studies in mammals and humans showed that dietary influences exerted at critical developmental stages early in life may have long-term consequences on physiological functions in later life (19, 33, 44). This phenomenon, known as nutritional programming, is largely studied in mammalian models for the understanding of some particular adult disease such as the metabolic syndrome or diabetes (1, 2, 9, 19). Possible biological mechanisms for storing the nutritional programming event until adulthood include adaptive changes in gene expression (epigenetic phenomenon), preferential clonal selection of adapted cells in programmed tissues, and programmed differ-

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ential proliferation of tissue cell types (27, 33, 54). Modifications of adult glucose metabolism due to early nutritional events were reported in numerous studies. In rat, temporary exposure to increased levels of insulin during gestation was shown to cause glucose intolerance in the progeny (21), whereas prenatal dietary protein restrictions induced lifetime changes in hepatic glucose metabolism (GK and phosphoenolpyruvate carboxykinase activities) (15). Although most have concentrated on the intrauterine nutrient supplies, changes in early neonatal nutrition were also found to have life-long consequences on carbohydrate uptake and metabolism. For instance, the use of an artificially high-carbohydrate milk formula in suckling rats before weaning resulted in a rapid precocious induction of hepatic GK (20) and an immediate onset of hyperinsulinemia, which persisted into adulthood (1). Similarly, the precocious increase of pancreatic GK observed in 12-day-old suckling rats fed carbohydrate-enriched milk remained in 100-day-old rats (2). Also in rats, but at later developmental stages, the ratio of polyunsaturated to saturated fatty acids in the weaning and postweaning diet was found to alter the normal ontogeny of intestinal glucose absorption (51).

The objective of our study was to examine whether dietary carbohydrate utilization in rainbow trout can be modified (improved) by a mechanism related to metabolic programming. It was hypothesized that an acute nutritional stimulus in early life may improve the ability of the juvenile trout to cope with dietary carbohydrates. For this, the trout received a hyperglucidic diet (60% dextrin) during a few days at two developmental stages: either at first feeding (~190 mg fish, transition to exogenous feeding, *stimulus 1*) or after the complete absorption of yolk reserves (~700 mg fish, exotrophic stage, *stimulus 2*). The aim was to reveal whether, on a molecular basis, a particular long-term effect of the early-feeding stimulus could be distinguished. The analyses concerned the molecular expression of target proteins involved in carbohydrate digestion (α -amylase and maltase), intestinal sodium-dependent glucose cotransporter (SGLT1), and hepatic glucose metabolism (GK and G6Pase, involved in the utilization and production of glucose, respectively). We first compared the immediate short-term outcome of the hyperglucidic stimuli with that of a commercial control feed. Unfed yolk sac larvae were included in the analysis of the short-term effects of *stimulus 1* to reveal molecular changes during the transition to exotrophy, which has been little documented in rainbow trout. After a common feeding period with commercial trout feed, the effect of the early hyperglucidic stimulus on the capacity of the juvenile fish (>10 g) to adapt to a carbohydrate-rich diet (25% dextrin) was analyzed. In addition, we verified whether the early hyperglucidic stimuli affected the growth of the juveniles or their pre- and postprandial glycemic levels.

MATERIALS AND METHODS

Diets

Two experimental diets were prepared (Table 1). Dextrin (partially hydrolyzed starch) was included as a carbohydrate source. The increase in dietary dextrin was accompanied by decreased levels of fish oil and fish meal (Table 1). The first diet, a very-high-carbohydrate diet (VHC diet; 60% dextrin), was used during the two acute nutritional interventions (*stimulus 1* and *stimulus 2*). The second diet was a high-carbohydrate diet (HC diet; 25% dextrin); this carbohydrate level did not negatively affect growth in salmonids (55). The HC diet

Table 1. *Formulation and proximate composition of the control diet and the two experimental diets*

	Control Diet	VHC Diet	HC Diet
<i>Ingredient, %</i>			
Fish meal	NA	35	60
Fish oil	NA	5	12
Dextrin	NA	60	25
Vitamin mix	NA	1	1
Mineral mix	NA	1	1
Alginate	NA	1	1
<i>Proximate composition</i>			
Dry matter, %diet	92.9	93.2	93.7
Crude protein, %dry matter	53.9	26.1	45.5
Crude lipid, %dry matter	16.7	6.8	15.2
Gross energy, kJ/g dry matter	22.5	19.3	21.4
Ash, %dry matter	11.2	6.3	10.6
Carbohydrates	18.1	60.8	28.6

Control diet is Ecostart 18 (Biomar). Dextrin is from Scharlau (white pure dextrin, CAS no. 9004-53-9). Composition of carbohydrate calculated as 100% - (%lipid + %protein + %ash). NA, not applicable; VHC, very high carbohydrate; HC, high carbohydrate.

was fed to juvenile fish during the final challenge test to analyze the long-term effect of the early stimulus. A commercial trout diet (Ecostart 18, Biomar), moderately rich in carbohydrates (18%, mainly derived from extruded wheat), was fed to the experimental groups outside the VHC interventions and to a control treatment that did not experience the VHC interventions.

Fish Rearing and Sampling

The experiment was conducted following the *Guidelines of the National Legislation on Animal Care* of the French Ministry of Research. Fertilized rainbow trout (*Oncorhynchus mykiss*) eggs were obtained from a commercial fish farm (Sarrance, Viviers de France, France) and hatched at the INRA Lées-Athas experimental fish farm (France) at 7.5°C. Four days before the first feeding, the larvae were transferred to the INRA experimental fish farm at Donzacq (France) for the feeding experiments. Six groups of 450 larvae each ($n = 2$ groups per dietary treatment) were placed in 60-liter tanks supplied with flow-through well water of fairly constant water temperature (16°C \pm 1). One group of 80 larvae (unfed yolk sac larvae) was placed in a closed but aerated aquarium that contained filtered well water to avoid the presence of planctonic feed organisms. The fed fish were group weighed every 3 wk and counted to calculate their average body weight and to establish the growth curves. During sampling, the fish were anesthetized with 2-phenoxyethanol at the recommended concentration for surgical procedures (0.2 ml/l) and weighed individually.

VHC feeding interventions (hyperglucidic stimuli). Two groups of rainbow trout experienced the VHC stimulus at first feeding during 3 days (*stimulus 1* fish, ~190 mg initial body wt) (Fig. 1A) and two other groups after yolk exhaustion, i.e., 3 wk after *stimulus 1*, during 5 days (*stimulus 2* fish, ~700 mg initial body wt) (Fig. 1B). Two other groups did not undergo the VHC stimulus (control group fed the commercial diet) (Fig. 1C). The diets were carefully distributed by hand (5 meals/day) until visual satiation. At the end of the VHC-feeding periods (3 h after last meal), fish were sampled randomly, and whole larval bodies (*stimulus 1*, $n = 6$ samples of 5 fish each) or dissected viscera (liver, gastrointestinal tract plus diffuse pancreatic cells) (*stimulus 2*, $n = 6$ samples of 3 fish each) were quickly frozen in liquid nitrogen and stored at -80°C before molecular analyses. Samples were also taken from the control groups under similar conditions as the fish that had experienced the VHC stimuli.

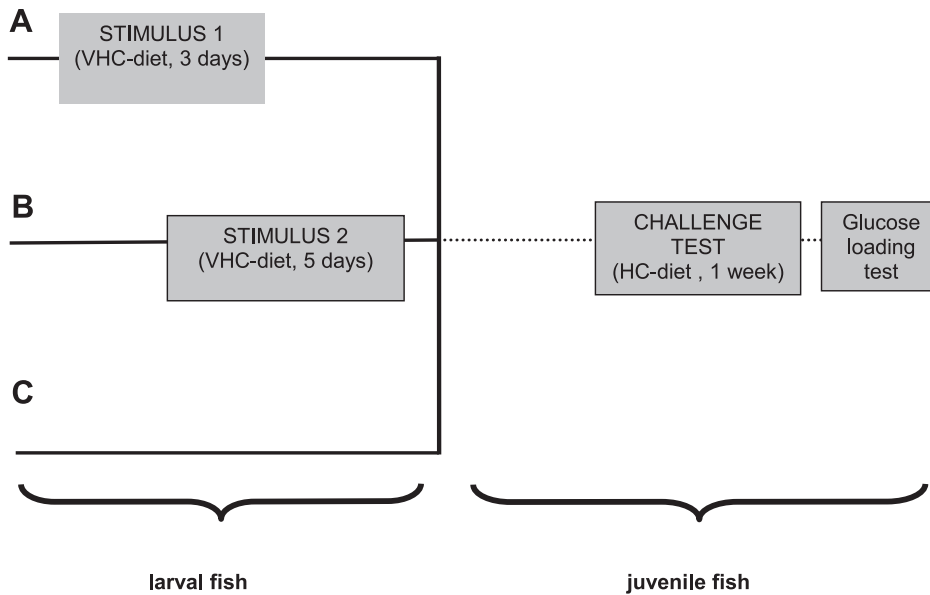


Fig. 1. Schematic representation of the experiment from the short-term hyperglucidic stimuli up to the final challenge test in juvenile rainbow trout. Fish were fed with the very high carbohydrate diet (VHC diet; 60% dextrin) at the first-feeding stage during 3 days (*stimulus 1*; A) or were fed after yolk absorption during 5 days (*stimulus 2*; B). Outside the indicated VHC feeding period, the fish received a commercial control diet. The latter diet was given to the control groups throughout the entire period (C). After the acute nutritional stimuli, the 3 fish groups were fed the same commercial diet until they reached >10 g (dotted line). During the final challenge tests, the 3 groups (control and stimulus 1 and 2) received a high-carbohydrate diet (HC diet; 25% dextrin) and, at a later stage, a high, pure glucose load to reveal whether early feeding history affected carbohydrate utilization in the juvenile fish.

Transition to exotrophy. To document the transition from endotrophy to exotrophy, *stimulus 1* and first-feeding control samples were compared with unfed yolk sac larvae ($n = 6$ samples of 5 fish each) taken from the group that remained unfed during the 3 feeding days of *stimulus 1* and thus relied exclusively on the yolk reserves because they never ingested any exogenous food ("unfed" fish).

Intermediate feeding period. During the 12 or 8.5 wk after *stimulus 1* or *stimulus 2*, respectively, the commercial trout diet (Ecostart 18, Biomar) was fed by hand (until visual satiation) in three (first 3 wk after the stimuli) or two (the rest of the trial) meals per day. The commercial diet was also given to the control fish and before *stimulus 2*.

Final experiments (juvenile stage). At the end of this common feeding period with the commercial control diet, the three groups received the HC diet (final challenge test, 25% dextrin) for 5 days (Fig. 1). The juvenile fish (12.8 ± 0.7 g) were killed (3 h after last meal), and the liver and gastrointestinal tract (plus diffuse pancreatic cells) were sampled ($n = 6$ per treatment), frozen in liquid nitrogen, and stored at -80°C for molecular analyses. In larger fish (90 ± 3.5 g), 24 wk after first feeding, 24-h-fasted juvenile rainbow trout were force fed with gelatin capsules filled with 1.2 g of D-glucose ($n = 8$ per treatment) (Fig. 1). Before and 5.5 h after the glucose loading test, blood was sampled at the caudal vein to compare plasma glucose levels.

Analytic Methods

The chemical composition of the diets was analyzed by the following procedures: dry matter after drying at 105°C for 24 h, fat by

dichloromethane extraction (Soxhlet), and gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Griebheimer, Germany). Protein content (determined nitrogen $\times 6.25$) was determined by the Kjeldahl method after acid digestion. Plasma glucose concentration was determined with the glucose oxidase method in a Beckman glucose analyzer (Beckman II).

Gene Expression Analysis: Real-Time PCR

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA samples were treated with RQ1 RNase-free DNase before RT-PCR (Promega, Madison, WI), to avoid genomic DNA amplification. Total RNA ($1 \mu\text{g}$) was reverse transcribed to cDNA with the Superscript III RNase H reverse transcriptase kit (Invitrogen) using oligo(dT) primers.

Known trout α -amylase, maltase, SGLT1, GK, and G6Pase gene sequences were obtained from GenBank (http://www.genome.ad.jp/htbin/www_bfind?dna-today) or expressed sequence transcript databases (from National Institute of Agronomic Research, <http://ensembl-sigenae.jouy.inra.fr/>, or from The Institute for Genomic Research *Oncorhynchus mykiss* gene index, <http://www.tigr.org/tdb/>). Gene expression levels were determined by real-time RT-PCR, performed by means of the iCycler iQ (Bio-Rad, Hercules, CA). Quantitative PCR analyses for gene expressions were performed on $10 \mu\text{l}$ of the RT reaction mixture using the iQ SYBR green supermix. The total volume of the PCR reaction was $25 \mu\text{l}$, containing 200 nM of primers. Specific trout gene primers were chosen overlapping an intron when possible (data not shown) using Primer3 software: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Table 2). Elon-

Table 2. Sequences of primers used in quantitative RT-PCR

Genes	5'-3' Forward Primer	5'-3' Reverse Primer
Maltase	GCAGCAGGAATACCCTACGA	GCCAGGGTCCAGTATGAAGA
α -Amylase	ACCGTGGCTTCATTGTCTTC	GTCCGTGTTGCTGATCTTGA
SGLT1	TCTGGGGCTGAACATCTACC	GAAGGCATAACCCATGAGGA
GK	TGAAGGATCAGAGGTGGGTGAT	GAAGGTGAAACCCAGAGGAAGC
G6Pase	TGCCCACTTCCCACACCA	AGCCACAGCAAAGGAGAG
EF1 α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG

Gene accession numbers for each gene are as follows: for maltase, TC3451 [The Institute for Genomic Research (TIGR) *Oncorhynchus mykiss* gene index]; for α -amylase, TC87786 (The TIGR *Oncorhynchus mykiss* gene index); for sodium-dependent glucose cotransporter (SGLT1), AY210436 (GenBank); for glucokinase (GK), AF135403 (GenBank); for glucose-6-phosphatase (G6Pase), AF120150 (GenBank); and for elongation factor 1 α (EF1 α), AF498320 (GenBank).

gation factor 1 α (EF1 α) was used as the reference gene (38). The different PCR products were controlled by sequencing to confirm the nature of the amplicon. Negative controls (samples without reverse transcriptase, samples without RNA) were included for each reaction. Thermal cycling was initiated with the incubation at 95°C for 90 s for hot-start iTaq DNA polymerase activation. Thirty-five steps of PCR were performed, each one consisting of heating at 95°C for 20 s for denaturing and at 59°C for 30 s for annealing and extension. After the final cycle of the PCR, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 94°C).

Statistical Analysis

Statistical differences in gene expression between control and sample were evaluated by randomization tests (46) using the Relative expression software tool (REST). This mathematical algorithm, which needs no calibration curve, computes an expression ratio (R), based on real-time PCR efficiency and the crossing-point deviation of the unknown sample vs. a control group: $R = [(E_{\text{target gene}})^{\Delta CT_{\text{target gene}}(\text{mean control} - \text{mean unknown sample})}] / [(E_{\text{EF1}\alpha})^{\Delta CT_{\text{EF1}\alpha}(\text{mean control} - \text{mean unknown sample})}]$, where E is PCR efficiency determined by standard curve using serial dilutions of cDNA and ΔCT is the crossing-point deviation of an unknown sample vs. a control. Two thousand random allocations were performed, and significant differences were considered at $P < 0.05$. The data represent the mean difference in expression between the sample vs. the control together with the respective coefficients of variation (%). Growth and plasma glucose data were compared by one-way ANOVA (Statistica 5.0; StatSoft, Tulsa, OK). The Newman-Keuls multiple-range test was used to compare means in case of a significant effect ($P < 0.05$).

RESULTS

Short- and Long-Term Effects of the VHC Stimuli on Growth Parameters

The 3-day first-feeding period induced significant differences in growth between the three groups (Fig. 2B; 1-way ANOVA, $P < 0.05$). As expected, the unfed yolk sac fish, which never received any exogenous feed, had the lowest body weight (198 ± 13 mg). The highest growth occurred with the commercial diet (control fish, 270 ± 18 mg); this result was significantly different from results with the hyperglucidic diet (*stimulus 1* fish, 255 ± 12 mg), possibly because of the lower protein and lipid levels in the VHC diet (Fig. 2B). Three weeks later, the inverse was seen: *stimulus 1* fish (880 ± 16 mg) now had a higher body weight than the control fish (755 ± 32 mg) (Fig. 2C; $P < 0.05$). The latter difference disappeared at the next weighing (2.5 wk later; Fig. 2D). At the end of *stimulus 1*, the fish fed the hyperglucidic diet (*stimulus 2* fish, 660 ± 27 mg) were significantly smaller than the control fish (755 ± 32 mg), probably because of the lower protein or lipid in the VHC diet (Fig. 2C). The difference in body weight between both groups was still visible 2.5 wk later but not anymore at the subsequent weighings (Fig. 2D). The final growth performances of the two experimental groups (*stimulus 1*, *stimulus 2*) were not different from those of the control group fed the commercial diet during the whole experimental period (172 days) (Fig. 2A). In addition, feed efficiency (weight gain/feed intake) and survival were not affected by the earlier nutritional interventions.

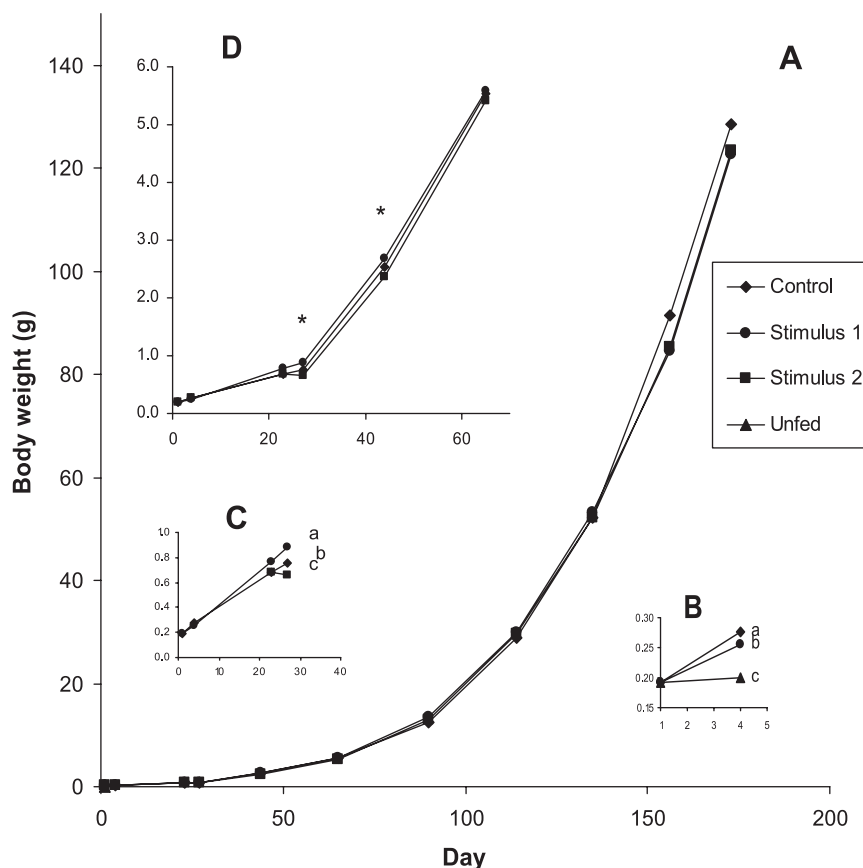


Fig. 2. Growth performance of the experimental groups of rainbow trout. Control: fish always fed the commercial diet. *Stimulus 1*: fish fed VHC diet at first feeding during 3 days. *Stimulus 2*: fish fed VHC diet after yolk exhaustion during 5 days. Significant differences are indicated by different letters (1-way ANOVA, $P < 0.05$). A: whole feeding trial. B: focus on *stimulus 1*. C: focus on *stimulus 2*. D: following *stimulus 2*. *Significant differences between *stimulus 2* and control.

Candidate Gene Expressions

The data are presented in three parts. The first part concerns the transition from endogenous to exogenous feeding (Table 3), the second part the short-term effects of the early VHC stimuli (Table 4), and the third part the long-term effect of the early VHC stimuli in juvenile fish (*stimuli 1 or 2* vs. control fish; Table 5) on the selected target genes.

Transition to exotrophy (control or stimulus 1 fish vs. unfed yolk sac larvae). Compared with the unfed yolk sac larvae, the 3-day first-feeding period with the commercial diet induced significant changes in transcript levels of all studied genes (REST test; $P < 0.05$) (Table 3). The expression of α -amylase and maltase was downregulated by a factor of 7 and 2, respectively, compared with the unfed group. At the metabolic level, G6Pase gene expression was significantly downregulated (-2.5 times), whereas GK, absent in the unfed yolk sac larvae, was highly induced. Transcripts of the glucose transporter SGLT1, present in the unfed larvae, increased 2.2-fold by feeding the commercial control diet (Table 3). Observed changes in SGLT1, GK, G6Pase gene expression between fish fed the VHC diet and unfed yolk sac fish followed the same tendency (Table 3).

Short-term effects of the VHC stimuli on candidate gene expression at the early feeding stages (stimuli 1 or 2 vs. control fish). Feeding the VHC diet during 3 days from mouth opening (*stimulus 1*) upregulated (REST test, $P < 0.05$) the transcription of α -amylase and maltase genes (3.9 and 2.3 times, respectively) compared with the control larvae fed the commercial diet (Table 4). Also the GK gene expression was increased (1.5 times) by the VHC diet (REST test, $P < 0.05$). The VHC stimulus did not modify the gene expression of the intestinal glucose transporter SGLT1 or the enzyme G6Pase (Table 4). After *stimulus 2*, only maltase was slightly upregulated in larvae fed the VHC diet during 5 days compared with

Table 3. Comparison of gene expression between rainbow trout larvae fed the control diet or VHC diet during 3 days and the unfed yolk-sac larvae

Gene	Type of Regulation	CV _{control}	CV _{unfed}	P Value
<i>Control vs. unfed yolk sac larvae</i>				
α -Amylase	Downregulation of 7-fold	8.52	0.67	<0.005
Maltase	Downregulation of 2-fold	1.19	0.18	<0.05
SGLT1	Upregulation of 2.2-fold	1.08	0.61	<0.005
GK	Switch on	1.13	NA	NA
G6Pase	Downregulation of 2.5-fold	0.37	0.23	<0.001
Gene	Type of Regulation	CV _{stimulus 1}	CV _{unfed}	P Value
<i>VHC larvae (stimulus 1) vs. unfed yolk sac larvae</i>				
α -Amylase	No regulation	3.55	0.67	0.88
Maltase	No regulation	1.92	0.18	0.93
SGLT1	Upregulation of 2.3-fold	0.49	0.61	<0.001
GK	Switch on	0.87	NA	NA
G6Pase	Downregulation of 2.2-fold	0.46	0.23	<0.001

Transcript level of target genes was normalized with EF1 α -expressed transcripts. Gene expression analyses were performed on total RNA extracted from whole body. Down- and upregulation means that target gene is expressed at lower or higher level, respectively, in fed than in unfed yolk sac larvae. "Switch on" means there was no detectable GK gene expression in unfed fish. Statistical differences in gene expression levels between samples were evaluated in group means by randomization tests (46) using relative expression software tool (REST). CV, coefficient of variation.

Table 4. Comparison of the short-term effect of the hyperglucidic stimuli (VHC diet) on gene expressions in larvae fed the VHC diet or the commercial diet (control) at 2 developmental stages

Gene	Type of Regulation	CV	CV _{control}	P Value
<i>At the first-feeding stage (stimulus 1)</i>				
α -Amylase	Upregulation of 4-fold	3.55	8.52	<0.05
Maltase	Upregulation of 2.2-fold	1.92	1.19	<0.05
SGLT1	No regulation	0.49	1.08	0.72
GK	Upregulation of 1.5-fold	0.87	1.13	<0.05
G6Pase	No regulation	0.46	0.37	0.23
<i>After the yolk absorption (stimulus 2)</i>				
α -Amylase	No regulation	2.14	6.54	0.23
Maltase	Upregulation of 1.3-fold	1.41	1.43	<0.05
SGLT1	No regulation	0.60	1.16	0.64
GK	No regulation	11.37	1.87	0.20
G6Pase	No regulation	5.24	1.21	0.08

Transcript level of target genes was normalized with EF1 α -expressed transcripts. At the first-feeding stage (*stimulus 1*), gene expression analyses were performed on total RNA extracted from whole larval body. After the yolk absorption (*stimulus 2*), gene expression analyses were performed on total RNA extracted from whole viscera (liver, gastrointestinal tract + diffuse pancreas). Down- and upregulation means that the target gene is expressed at a lower or higher level, respectively, in the VHC fish than in the control fish. Statistical differences in gene expression between samples were evaluated in group means by randomization tests (46) using REST software.

the control larvae (Table 4). The expression of the other studied genes was unaltered by the VHC diet compared with the control diet at this later stage of development (*stimulus 2*).

Long-term effects of VHC stimuli on candidate gene expression and glycemia at the juvenile stage. During the final HC-challenge test, the three groups of juvenile rainbow trout received for 5 days the HC diet containing 25% dextrin. There were no significant differences in transcript levels of the

Table 5. Comparison of the long-term effect of the hyperglucidic stimulus (VHC diet) on gastrointestinal and hepatic gene expression in juvenile rainbow trout during the final challenge test with the HC diet

Gene	Type of Regulation	CV	CV _{control}	P Value
<i>Long-term effect of stimulus 1</i>				
a-Amylase	Upregulation of 1.7-fold	3.64	4.42	<0.05
Maltase	Upregulation of 1.5-fold	1.81	2.43	<0.005
SGLT1	No regulation	1.16	4.88	0.47
GK	No regulation	3.30	15.41	0.16
G6Pase	No regulation	1.67	5.39	0.77
<i>Long-term effect of stimulus 2</i>				
α -Amylase	Upregulation of 1.8-fold	5.15	4.42	<0.05
Maltase	No regulation	1.34	2.43	0.45
SGLT1	No regulation	5.82	4.88	0.65
GK	No regulation	1.25	15.41	0.51
G6Pase	No regulation	1.98	5.39	0.51

Transcript level of target genes was normalized with EF1 α -expressed transcripts. Down- and upregulation means that the target gene is expressed at a lower or higher level, respectively, in the juveniles, which underwent the early VHC stimulus, than in the control fish. For long-term effect of *stimulus 1*, comparison was between juvenile trout fed the VHC diet at first-feeding and control fish. For long-term effect of *stimulus 2*, comparison was between juvenile trout fed the VHC diet after vitellus resorption and unstressed control fish. Statistical differences in gene expression between samples were evaluated in group means by randomization tests (46) using REST.

proteins involved in glucose metabolism or transport (GK, G6Pase, or SGLT1) irrespective of the nutritional history: control/*stimulus 1*/*stimulus 2* (Table 5). In contrast, maltase (*stimulus 1*) and α -amylase (*stimulus 1* and *stimulus 2*) genes were expressed at higher levels in the juveniles, which had experienced the earlier hyperglucidic stimulus than the control fish (Table 5), without significant difference associated with the timing of the stimulus (data not shown).

The control fish (0.84 ± 0.15 g/l) had fasting plasma glucose levels (measured before the force-feeding glucose loading test) similar to those in the groups that had experienced the short-term VHC stimuli (0.73 – 0.78 g/l). Postprandial glycemia levels following the glucose loading increased over 12-fold (9.2 ± 1.0 to 9.8 ± 0.8 g/l) but were unaffected by the carbohydrate feeding history (ANOVA, $P > 0.05$).

DISCUSSION

The natural diet of rainbow trout is poor in carbohydrates. On the basis of the concept of nutritional programming (33), in this study, we examined whether a short-term drastic change in early carbohydrate nutrition could induce a long-lasting effect on carbohydrate utilization in rainbow trout.

Short-Term Changes Related to the Transition to Exotrophy and to the Hyperglucidic Stimulus at First Feeding (Stimulus 1) or After Yolk Exhaustion (Stimulus 2)

Fish larvae cannot ingest exogenous food when they hatch and thus exclusively depend on the yolk reserves. After the opening of the esophagus, first-feeding fish have an extremely high growth capacity as illustrated here by the 40% body weight increase of the control fish during the first 3 days of feeding. This rapid growth implies that the transition to exotrophy is accompanied by a drastic change in digestive and metabolic capacities to ensure the efficient utilization of exogenous feed (25). That several of these early adaptive changes are preset by genetical determinants (56) is shown here by the analysis of the unfed yolk sac larvae.

A first example concerns the relatively high-transcript level of both enzymes involved in carbohydrate digestion, pancreatic α -amylase and intestinal maltase, before the initiation of exogenous feeding. Early gene expressions or enzyme activities of α -amylase and maltase have been reported before in carnivorous marine fish larvae, which have digestive functioning at first feeding that is far less developed than shown in rainbow trout (13, 14, 16, 34, 45, 56). The predisposition of carnivorous larval fish to digest starch-like substances is not fully understood, especially when considering the low-carbohydrate content of the zooplanktonic prey organisms (14). It however highlights a genetically programmed plasticity in the variety of feed source utilization at the onset of feeding. At later larval stages, transcript levels of both digestive enzymes generally decrease (13, 56), as confirmed here by the lower expression in larvae fed the control diet than in the unfed fish. Interestingly, this hard-wired downregulation of the digestive enzymes was abolished by feeding the hyperglucidic diet, so that fish that underwent the early stimulus (*stimulus 1*) had as high expression levels as the unfed yolk sac larvae. This capacity of first-feeding rainbow trout to adapt digestive enzyme synthesis to the dietary carbohydrate load is also clearly illustrated by the fourfold higher α -amylase and twofold higher maltase gene

levels with the hyperglucidic diet than with the control diet (*stimulus 1*) and is consistent with the early indications in young rainbow trout (30) that α -amylase and maltase enzyme activity responses occurred within a few days. When applying the hyperglucidic stimulus at the later stage of development after yolk exhaustion (*stimulus 2*), we observed no such short-term dietary response for α -amylase, in contrast to that shown with maltase, in which gene expression was 30% higher with the hyperglucidic than with the control diet.

In fish, monosaccharides cross the brush-border membrane by simple diffusion or by the aid of specific transporters, similar to mechanisms described in mammals (31). D-Glucose in rainbow trout is actively transported into the enterocyte by the apical SGLT1 and out of the cell by the basolateral GLUT2 carrier (3, 31). As found for the enzymes involved in carbohydrate digestion, the expression of the glucose carrier SGLT1 also appears to be ontogenetically programmed because its transcripts were found in the unfed larvae and thus before the presence of a luminal glucose cue. In fish, there is no information on the molecular regulation of SGLT1 during early development, but our data agree with those observed in higher vertebrates, mostly omnivores (rat, human), in which SGLT1 transcripts were detected before weaning and even before birth (17). Furthermore, the ingestion of exogenous feed rapidly increased the SGLT1 gene transcripts, confirming the high adaptive capacity of the young intestinal cells. This first-feeding-enhanced transcription of SGLT1 in the rainbow trout larvae was not fully expected because dietary adaptation of intestinal glucose transport normally appears to be determined by the potential variation in carbohydrate supply of the natural diet of the organism (17, 18). This was also seen in adult fish (10, 17); that is, rainbow trout were found to be incapable of adjusting intestinal glucose transport to dietary levels. This is in contrast to herbivores (carp) or omnivores (catfish, tilapia), which increased both apical and basolateral membrane glucose transport in relation to the dietary supply, as clearly demonstrated in an in vitro study with enterocytes isolated from the omnivorous black bullhead, *Ictalurus melas* (49). The dietary upregulation of the SGLT1 gene noted during the transition to exotrophy was however not amplified by the hyperglucidic stimulus (*stimulus 1* or *2*).

In contrast, no transcripts of GK, the first enzyme of glycolysis, were found before the start of exogenous feeding. That the appearance of the GK enzyme is not development dependent but is controlled by the presence of the stimulus corroborates findings in rats (20). In the latter study, the GK gene, normally expressed only at weaning, could be precociously induced by feeding a carbohydrate-enriched milk to suckling rat pups (20). Similarly, the exogenous diets rapidly induced the molecular expression of the GK gene (probably the hepatic isoform) in the present first-feeding fish larvae. Such precocious induction was already seen before in first-feeding carp (*Cyprinus carpio*) larvae, which are known to tolerate high levels of carbohydrates (40). Moreover, at this early developmental stage (*stimulus 1*), GK mRNA abundance was proportional to the level of carbohydrates (18 or 60%), indicating a very quick adaptation of the carnivorous rainbow trout to the utilization of exogenous glucose (39). The absence of GK gene upregulation by the hyperglucidic stimulus when applied at the later stage of development (*stimulus 2*) was unexpected because GK normally responds very well in juveniles (39, 41).

G6Pase, involved in the production of glucose (by gluconeogenesis and glycogenolysis), was highly expressed in the unfed larvae in which the energy supply was fully dependent on the catabolism of the vitelline reserves. In rainbow trout yolk, storage of glycogen is too small (<1%), compared with that of protein and lipid (55 and 45%, respectively) (5), to ensure free glucose supplies (50). Before feeding, the larvae are believed to use triglyceride-derived glycerol and gluconeogenic amino acids from the remaining yolk as substrates for glucose synthesis (28). In a study on the evolution of G6Pase enzyme activities during rainbow trout embryonic development, first activities were seen before hatching, which then increased slightly up to first feeding (53). The 2.5-fold inhibition of the G6Pase gene expression induced by first feeding shows the capacity of the larvae to carry out glycemic regulation and to act on the presence of exogenous glucose, whatever the quantity (from 18 to 60%). This rapid response contrasts with the failure of dietary carbohydrates to suppress hepatic glucose output in rainbow trout observed here at the later stage of development or as shown before at the juvenile or adult stage (42).

In summary, the data not only show that the larvae at onset of feeding were prepared to digest, absorb, and utilize carbohydrate-rich feed but also that they were capable of adapting the molecular synthesis of some of the above proteins to the dietary carbohydrate load. Indeed, although no regulation of G6Pase and SGLT1 genes was detected, the first-feeding trout larvae (following *stimulus 1*) had higher maltase, α -amylase, and GK mRNA levels, reflecting the acute adaptation to the VHC intake (only seen for maltase following *stimulus 2*). Although we have no proof that the observed responses, known to be related to dietary glucose utilization, are uniquely caused by the higher dietary carbohydrates and not by the lower dietary protein or lipid, the major question was whether the short-term physiological plasticity toward dietary carbohydrates seen at this early-feeding stage would persist in the juvenile fish.

Long-Term Effects of the Early Hyperglucidic Stimuli in Juvenile Rainbow Trout

In mammals, nutritional programming of glucose metabolism was found to occur by either prenatal (15, 21) or postnatal (1, 44) interventions. In fish, because of the experimental difficulty to modify the macronutrient composition of the yolk reserves, especially of carbohydrate and protein, by maternal nutrition (26), we chose to apply the hyperglucidic stimulus at two early posthatching feeding stages. The absence of a negative effect of the acute nutritional stimuli on the final growth or survival of the fish confirms the potential of this type of approach in rainbow trout even at the first-feeding stages. As shown previously (33), the nutritional programming stimulus has a permanent effect only when applied at a sensitive or critical period during development when there is still physiological plasticity. In this respect, the observed short-term plasticity in dietary response at first feeding favors the possibility of nutritional programming at this early phase of development.

The a priori expectation that the early hyperglucidic stimulus might exert a persistent positive effect on carbohydrate utilization was confirmed by the present data but only at the digestive level, and this confirmation was dependent on the

timing of the stimulus. When applied at first feeding (*stimulus 1*), expressions of both the α -amylase and maltase genes were found to be increased in the juvenile fish during the final challenge test; when applied at a later stage (after yolk exhaustion, *stimulus 2*), only the α -amylase gene was found to be upregulated. In contrast, the stimuli did not provoke persistent molecular adaptations of the (intestinal) transport or (hepatic) metabolism of glucose in the juveniles. Also, their plasma glycemic levels after the glucose loading test were not visibly affected by the earlier nutritional experience, suggesting the absence of metabolic adaptation of glucose homeostasis mechanisms to dietary glucose. Because this is the first study in fish, a nonmammalian vertebrate, several questions still await answers. A first question concerns the mechanism by which the gene expression of both digestive enzymes is altered. For the enterocyte-specific gene maltase, it would be of interest to examine the role of the specific transcription factors Cdx2 or hepatocyte nuclear factor-1 α , which are involved in intestinal tissue specialization and in maltase transcription in mammals (7). The particular implication of hepatocyte nuclear factor-1 α in chromatin remodeling of target genes (48) may lead to different methylation patterns of intestinal stem cells in response to a nutritional stimulus; in recording nutritional events, differentiated enterocytes programmed for a specific nutrient are produced all along animal life. Concerning pancreatic α -amylase, to our knowledge, there are no previous data on the nutritional programming of exocrine pancreas development (in contrast to endocrine pancreas), whereas acute adaptations to the diet have been largely documented (8). Another important point here concerns the tissue specificity of the α -amylase gene in fish. Our study detected α -amylase gene expression in liver (data not shown), similar to findings in rat, where α -amylase was reported to be involved in hepatic glycogen metabolism (32). The latter might explain the apparent contradiction between the absence of a short-term effect (RNA extract including hepatic tissue) and the presence of a long-term effect (no hepatic tissue) on the α -amylase gene in fish from *stimulus 2*. Future studies may also be needed to assess the enzyme activities to further validate the changes observed in expression. The second question on the absence of a long-term effect on glucose transport and hepatic metabolism during the juvenile period also needs further verification. In this respect, a next feeding trial with first-feeding rainbow trout is needed to compare the long-term outcome of a more severe hyperglucidic stimulus (pure glucose instead of dextrin to bypass the step of carbohydrate digestion and to obtain higher plasma glucose and putatively higher effects at the metabolic level) with that of a completely negative control (no sugars).

In conclusion, it is well known that juvenile rainbow trout experience some difficulties with high levels of dietary carbohydrates, perhaps because of the low dietary adaptation of carbohydrate digestion (11, 24) or intestinal glucose transport (10, 17), the insufficient induction of hepatic GK to store glucose in excess as glycogen or lipid (41), or the absence of inhibition of G6Pase as last enzyme of hepatic glucose production (42). Our assay to improve dietary carbohydrate utilization in rainbow trout by the concept of nutritional programming, for the first time in fish, appeared to be ineffective for glucose transport or metabolism but successful for digestive enzymes. The latter result and the absence of harmful effects of these stimuli on fish growth or survival highlight a promising

new study area on the investigation of nutritional programming of glucose utilization in fish, which may have practical relevance from an aquaculture perspective. A more focused study with pure dietary glucose given during a longer period at first feeding may help to elucidate whether glucose transport or metabolism can be persistently altered in rainbow trout.

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