Comparative and evolutionary physiology
JUN 2007; 292 (6) R2275-R2283
http://dx.doi.org/10.1152/ajpregu.00444.2006

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Abstract:

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 utilisation 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). Prior and after the hyperglucidic stimulus, they received a commercial diet until juvenile stage (>10g). Fish which did not experience the hyperglucidic stimuli served as a control. The short and long term effects of the stimuli were evaluated by measuring the expression of five key genes involved in carbohydrate utilisation: α-amylase, maltase (digestion), SGLT1 (intestinal glucose transport), glucokinase (GK) and glucose-6-phosphatase (G6Pase) involved in the utilisation and production of glucose, respectively. The hyperglucidic diet rapidly increased expressions of maltase, α-amylase and GK 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 up regulated in fish which had experienced the hyperglucidic stimulus at first-feeding, confirming the possibility to modify at long term some physiological functions in rainbow trout. In contrast, no persistent molecular adaptations were found for the genes involved in glucose transport or metabolism. Also growth and postprandial glycaemia were unaffected by the stimuli. In summary our data show that a short hyperglucidic stimulus during early trout life may influence permanently carbohydrate digestion.

Keywords: fish nutrition, nutritional programming, carbohydrate digestion; intestinal glucose transport; glucose metabolism

Introduction

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 gained an enormous interest as an alternative for the fishery-dependent fish meal (36). Carnivorous teleosts like rainbow trout, Atlantic salmon, yellowtail, eel or sea bream are however recognized for their inefficiency to use high levels of dietary carbohydrates (35, 55). In rainbow trout, digestible carbohydrate contents of more than 20-30% of the diet result in prolonged postprandial glycaemia (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 however not different from those in herbivorous or omnivorous fish species (22, 31). But the abundance as well as the dietary regulation of the proteins involved in carbohydrate utilization in fish appears to be influenced by the potential variation in carbohydrate supply and thus by the natural feeding habit. Illustrative examples here are the several-fold 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 or cyprinids. Moreover, rainbow trout had no different maltase activities when fed with or without carbohydrates (11) and was found incapable to adjust intestinal glucose transport to dietary supply (10). Also the regulation of hepatic gluconeogenesis is found to be influenced by the natural feeding habit. In omnivorous fish (43), as in non-diabetic mammals (47, 52), gluconeogenesis becomes unnecessary and is switched off when glucose is readily available from dietary sources (37). By 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 retro inhibition 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 differential 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 [glucokinase and phosphoenol pyruvate carboxykinase activities] (15). Although most concentrated on the intra-uterine nutrient supplies, also changes in early neonatal nutrition were 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 prior to weaning resulted in a rapid precocious induction of hepatic glucokinase (20) and an immediate onset of hyperinsulinemia which persisted into adulthood (1). Similarly the precocious increase of pancreatic glucokinase observed in 12-day old suckling rats fed carbohydrate-enriched milk remained in 100-day old rats (2). Also in rat, but at later developmental stages, the ratio of polyunsaturated/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 if dietary carbohydrate utilisation 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 following the complete absorption of yolk reserves (~700 mg fish, exotrophic stage, stimulus 2). The aim was to reveal if, 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 Na+-dependent glucose co-transporter, SGLT1 (SGLT1) and hepatic glucose metabolism (GK and G6Pase, involved in the utilisation 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, in order to reveal molecular changes during the transition to exotrophy what has been little documented in rainbow trout. After a common feeding period on the commercial trout feed, we then analysed the effect of the early hyperglucidic stimulus on the capacity of the juvenile fish (> 10g) to adapt to a carbohydrate-rich diet (25% dextrin). In addition, we verified if the early hyperglucidic stimuli affected the growth of the juveniles or their pre and postprandial glycaemia level.

Material and Methods

Diets

Two experimental diets were prepared (Table 1). Dextrin (partially hydrolyzed starch) was included as 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 2). The second diet is a high-carbohydrate diet (HC-diet, 25% dextrin), which carbohydrate level should not negatively affect growth in salmonids (55). The HC-diet was fed to the juvenile fish during the final challenge test in order to analyze the long-term effect of the early stimulus. A commercial trout diet (Ecostart 18, Biomar, France), moderately rich in carbohydrates (18%, mainly derived from wheat), was fed to the experimental groups outside the VHC-interventions as well as to a control treatment which did not experience the VHC-interventions.

Fish rearing and sampling

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 prior to 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-l tanks supplied with flow-through well water of fairly constant water temperature (16°C ± 1). One group of 80 larvae (unfed yolk-sac larvae) was placed in a closed but aerated aquarium which contained filtered well water in order to avoid the presence of planctonic feed organisms. The fed fish were group-weighed every 3 weeks and counted in order to calculate their average body weight (BW) and to establish the growth curves. During sampling, the fish were anaesthetised with 2-phenoxy-ethanol at the recommended concentration for surgical procedures (0.2 ml/l) and weighed individually.

Very-high-carbohydrate 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 BW) (Figure 1a) and two other groups after yolk exhaustion, i.e. 3 weeks after stimulus 1, during 5 days (“stimulus 2” fish, ~ 700 mg initial BW) (Figure 1b). Two other groups did not undergo the VHC-stimulus (control group fed the commercial diet) (Figure 1c). The diets were carefully distributed by hand (five meals per 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, gastro-intestinal 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 which had experienced the VHC-stimuli.

Transition to exotrophy. In order 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 which remained unfed during the 3 feeding days of stimulus 1 and thus relied exclusively on the yolk reserves as they never ingested any exogenous food (“unfed” fish).

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

Final experiments (juvenile stage). At the end of this common feeding period with the commercial control diet, the three groups received the high-carbohydrate diet (final challenge test, HC-diet, 25% dextrin,) for 5 days (Figure 1). The juvenile fish (12.8 ± 0.7 g) were sacrificed (3h after last meal) and the liver and gastro-intestinal 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 weeks 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) (Figure 1). Prior to and 5.5 h after the glucose loading test, blood was sampled at the caudal vein in order to compare their plasma glucose levels.

Analytical methods

The chemical composition of the diets was analyzed using the following procedures: dry matter after drying at 105°C for 24h, fat by dichloromethane extraction (Soxhlet) and gross energy in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany). Protein content (N*6.25) was determined by the Kjeldahl method after acid digestion. Plasma glucose concentration was determined using the glucose oxidase method in a Beckman glucose analyzer (Beckman II, USA).

Gene expression analysis: real time PCR

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were treated with RQ1 RNase-Free DNase prior to RT-PCR (Promega, Madison, WI), to avoid genomic DNA amplification. 1 µg of total RNA was reverse transcripted to cDNA with the Superscript ^{TM} III RNAse H Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) 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 ESTs database (from National Institute of Agronomic Research INRA - SIGENAE http://ensembl-sigenae.jouy.inra.fr/ or TIGR *Oncorhynchus mykiss* Gene Index http://www.tigr.org/tdb/tgi/). Gene expression levels were determined by real-time RT-PCR, performed by means of the iCycler iQ ^{TM} (BIO-RAD, Hercules, CA, USA). Quantitative PCR analyses for gene expressions were performed on 10 µl of the RT reaction mixture using the iQ ^{TM} SYBR $^{®}$ Green Supermix. The total volume of the PCR reaction was 25 µl, 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). Elongation factor 1 alpha (EF1α was used as reference gene (38). The different PCR products were controlled by sequencing in order 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 90s for hot-start iTaq ^{TM} DNA polymerase activation. 35 steps of PCR were performed, each one consisting of heating at 95°C for 20s for denaturing and at 59°C for 30s for annealing and extension. Following 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 REST© software. This mathematical algorithm, which needs no calibration curve, computes an expression ratio, based on real-time PCR efficiency and the crossing point deviation of the unknown sample *versus* a control group: $R= [(E_{\text{target gene}})^{\Delta CT_{\text{Target gene}}(\text{mean control- mean unknown sample})}] / [(E_{EF1\alpha})^{\Delta CT_{EF1\alpha}(\text{mean control- mean unknown sample})}]$ where E is PCR efficiency determined by standard curve using serial dilutions of cDNA, and ΔCT the

crossing point deviation of an unknown sample versus a control. 2000 random allocations were performed and significant differences were considered at $P<0.05$. The data represent the mean difference in expression between the sample versus the control together with the respective coefficients of variation (CV, %). Growth and plasma glucose data were compared by a 1-way ANOVA (Statistica 5.0, StatSoft, Inc, Tulsa, USA). 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 (Figure 2)

The 3-day first-feeding period induced significant differences in growth between the three groups (Figure 2B, 1-way ANOVA, $p<0.05$). As expected, the body weight of the unfed yolk-sac fish, which never received any exogenous feed, was the lowest (198 ± 13 mg). The highest growth occurred with the commercial diet (control fish, 270 ± 18 mg) which was significantly different than with the hyperglucidic diet (stimulus 1 fish, 255 ± 12 mg), what might be ascribed to the lower protein and lipid level in the VHC-diet (Figure 2b). Three weeks later the inverse was seen as stimulus 1 fish (880 ± 16 mg) now had a higher body weight than the control fish (755 ± 32 mg) (Figure 2C, $p<0.05$). The latter difference disappeared at the following weighing (2.5 weeks later, Figure 2D). At the end of stimulus 2, 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 (Figure 2C). The difference in body weight between both groups was still visible 2.5 weeks later, but not anymore at the following weightings (Figure 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) (Figure 2A). Also feed efficiency (weight gain/feed intake) and survival were not affected by the earlier nutritional interventions.

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).

As compared to 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 down-regulated by a factor 7 and 2, respectively, compared to the unfed group. At a metabolic level, G6Pase gene expression was significantly down-regulated (- 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 gene expression between fish fed the VHC-diet and unfed yolk-sac fish followed the same tendency (data not shown).

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) up regulated (Rest test, $p<0.05$) the transcription of α-amylase and maltase genes (3.9 and 2.3 times respectively) compared to the control larvae fed the commercial diet (Table 4A). 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 nor of the enzyme G6Pase (Table 4A). Following stimulus 2, only maltase was slightly up-regulated in larvae fed the VHC-diet during 5 days compared to the control larvae (Table 4B). The expression of the other studied genes was unaltered by the VHC-diet as compared to the control diet at this later stage of development (stimulus 2).

Long-term effects of VHC-stimuli on candidate gene expression and glycaemia 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 proteins involved in glucose metabolism or transport (GK, G6Pase or SGLT1)

irrespective of the nutritional history: control/ stimulus 1/ stimulus 2 (Table 5A and B). 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 early hyperglucidic stimulus than in the control fish (Table 5A and B) without significant difference associated with the timing of the stimulus (data not shown).

The fasted plasma glucose levels (measured prior to the force–feeding glucose loading test) were similar in the control fish (0.84 ± 0.15 g l^{-1}) to those in the groups which had experienced the short term VHC-stimuli (0.73 – 0.78 g l^{-1}). Postprandial glycaemia levels following the glucose loading increased over 12-fold (9.2 ± 1.0 to 9.8 ± 0.8 g l^{-1}), but were unaffected by the carbohydrate feeding history (ANOVA, $p>0.05$)

Discussion

The natural diet of rainbow trout is poor in carbohydrates. Based on the concept of nutritional programming (33), the current study examined whether a short-term drastic change in early carbohydrate nutrition could induce a long-lasting effect on carbohydrate utilisation 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 oesophagus, 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 three days of feeding. This rapid growth implicates that the transition to exotrophy is accompanied by a drastic change in digestive and metabolic capacities in order to ensure the efficient utilisation 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, prior to 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 digestive functioning at first-feeding is far less developed than 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 utilisation 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 down-regulation of the digestive enzymes was abolished by feeding the hyperglucidic diet, so that fish which 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 4-fold higher α-amylase and 2-fold 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), no such short term dietary response was seen for α-amylase, in contrast to maltase 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 sodium-dependent glucose transporter, SGLT1, and out of the cell by the basolateral Glut-2 carrier (3, 31). As found for the enzymes involved in carbohydrate digestion, the expression of the glucose carrier SGLT-1 also appears to be ontogenetically programmed since its transcripts were found in the unfed larvae and thus prior to 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 in higher vertebrates, mostly omnivores (rat, human), where 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), where rainbow trout were found incapable to adjust intestinal glucose transport to dietary levels in contrast to herbivores (carp) or omnivores (catfish, tilapia) which increased both apical and basolateral membrane glucose transport in relation with the dietary supply as clearly demonstrated in an in vitro study with enterocytes isolated from the omnivorous black bullhead, *Ictalurus melas* (49). The dietary up regulation 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 prior to the start of exogenous feeding. That the appearance of the glucokinase enzyme is not development-dependent but is controlled by the presence of the stimulus corroborate findings in rat (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 current start-feeding fish larvae. Such precocious induction was already seen before in start-feeding carp (*Cyprinus carpio*) larvae 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 up regulation by the hyperglucidic stimulus when applied at the later stage of development (stimulus 2) was unexpected since 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 which energy supply was fully dependent on the catabolism of the vitelline reserves. In rainbow trout yolk, storage of glycogen is too small (<1%) as compared to that of protein and lipid (55 and 45%, respectively) (5) to ensure free glucose supplies (50). Prior to 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 prior to 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 glycaemic regulation and to act upon the presence of exogenous glucose, whatever the quantity (18% up 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 in foregoing studies 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 utilise carbohydrate-rich feed, but also that they were capable to adapt 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 very high dietary carbohydrate intake (only seen for maltase following stimulus 2). Although we have no proof that the observed responses, known to be related with dietary glucose utilisation, are uniquely caused by the higher dietary carbohydrates and not by the lower dietary protein or lipid, the major question was if the short-term physiological plasticity towards 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, due to 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 post-hath 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 underlined by (33), the nutritional programming stimulus has a permanent effect only when applied at a sensitive or critical period in development when there is yet physiological plasticity. In this respect, the observed short-term plasticity in dietary response at first-feeding hence favoured 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 utilisation was confirmed by the current data but only at the digestive level and 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 and, when applied at a later stage (after yolk exhaustion, stimulus 2), only the α-amylase gene was found to be up regulated. 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 glycaemia 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. As this is the first study in fish, a non-mammalian 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 HNF-1α, involved in intestinal tissue specialization and in maltase transcription in mammals (7). The particular implication of HNF-1α. in chromatin remodelling of target genes (48) may lead to different methylation patterns of intestinal stem cells in response to a nutritional stimulus, recording nutritional events and hence producing, all along animal life, differentiated enterocytes pre-programmed for a specific nutrient. 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 as 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. It is also admitted that future studies need to assess the enzyme activities in order to further validate the changes observed in expression. Also the second question on the absence of a long-term effect on glucose transport and hepatic metabolism during the juvenile period necessitates further verification. In this respect, a next feeding trial with first-feeding rainbow trout will compare the long-term outcome of a more severe hyperglucidic stimulus (pure glucose instead of dextrin in order 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).

Conclusion

It is well known that juvenile rainbow experience some difficulties to use high levels of dietary carbohydrates, what might be due to 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) and 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 the glucose transport or metabolism, but successful for the digestive enzymes. The latter as well as 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, what has a 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.

Acknowledgments

This work was supported by the Aquitaine region (N°CCRRDT-2002-0308002C). We acknowledge E Plagnes-Juan and MM Le Gall for their excellent technical assistance. We thank F Terrier, Y Hontang, F Sandres, P Maunas and JP Flouriot for the rearing of the fish in our experimental fish farms (Lées-Athas and Donzacq).

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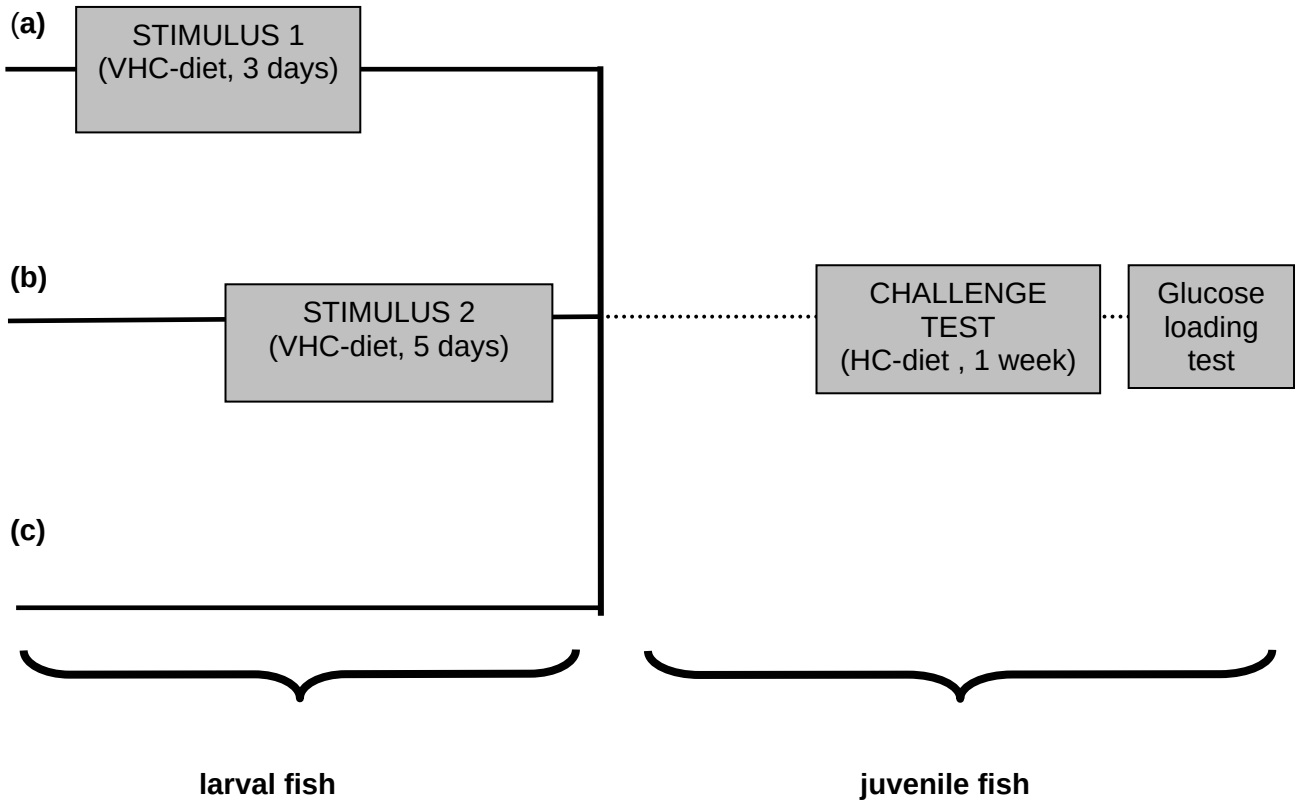

Figure 1: Schematic representation of the experiment from the short-term hyperglucidic stimuli up to the final challenge test in juvenile rainbow trout. Stimulus: fish fed with the very high carbohydrate diet (VHC-diet, 60% dextrin) at the first-feeding stage during 3 days (stimulus 1) **(a)** or after 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 (**…….**). During the final challenge tests, the 3 groups (control and stimulus 1 and 2) received a high-carbohydrate diet (HC-diet, 20% dextrin) and, at a later stage, a high pure glucose load in order to reveal if early feeding history affected carbohydrate utilisation in the juvenile fish.

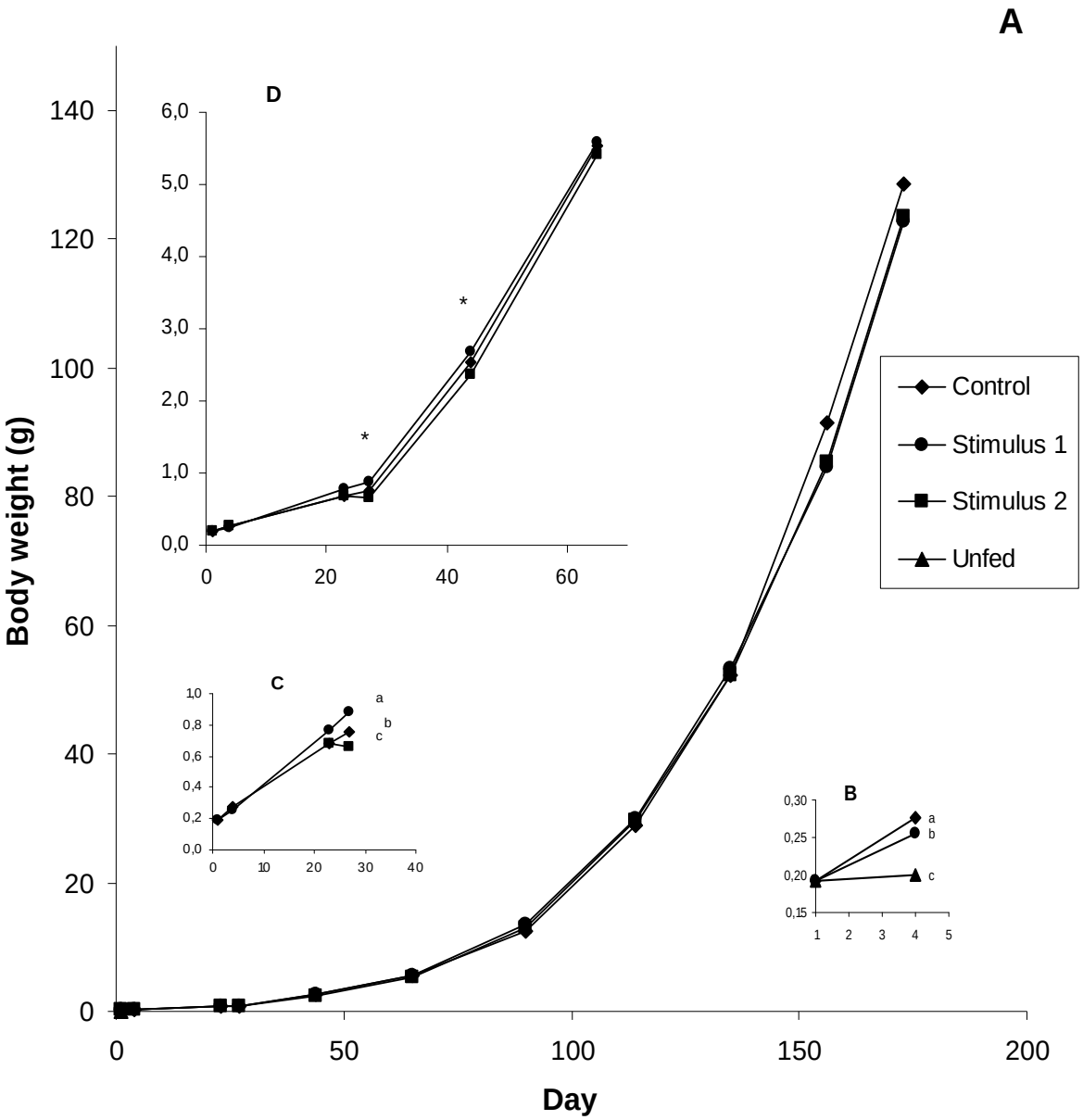

Figure 2: Growth performance of the experimental groups of rainbow trout. Control: fish always fed the commercial diet. Stimulus 1: fish fed the very-high carbohydrate diet at first-feeding during 3 days. Stimulus 2: fish fed the very-high carbohydrate diet after yolk exhaustion during 5 days. Significant 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.

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

	Control diet[1]	VHC-diet	HC-diet
Ingredients%			
Fish meal	n.a.	35	60
Fish oil	n.a.	5	12
Dextrin [2]	n.a.	60	25
Vitamin mix	n.a.	1	1
Mineral mix	n.a.	1	1
Alginate	n.a.	1	1
Proximate composition			
Dry matter (DM, % diet)	92.9	93.2	93.7
Crude protein (% DM)	53.9	26.1	45.5
Crude lipid (% DM)	16.7	6.8	15.2
Gross energy (kJ/g DM)	22.5	19.3	21.4
Ash (% DM)	11.2	6.3	10.6
Carbohydrates[3]	18.1	60.8	28.6

[1] Ecostart 18, Biomar, France

[2] Scharlau, White pure dextrin, CAS No 9004-53-9

[3] calculated: 100% - (lipid% + protein% + ash%)

Table 2: Sequences of primers used in qRT-PCR

Genes	5'-3' forward primer	5'-3' reverse primer
Maltase	GCAGCAGGAATACCCTACGA	GGCAGGGTCCAGTATGAAGA
α-Amylase	ACCGTGGCTTCATTGTCTTC	GTCGGTGTTGCTGATCTTGA
SGLT1	TCTGGGGCTGAACATCTACC	GAAGGCATAACCCATGAGGA
GK	TGAAGGATCAGAGGTGGGTGAT	GAAGGTGAAACCCAGAGGAAGC
G6Pase	TGCCCACTTCCCACACCA	AGCCCACAGCAAAGGAGAG
EF1α	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG

Gene accession numbers for each gene:

Maltase: TC3451 (The TIGR *Oncorhynchus mykiss* Gene Index (RtGI))

α-Amylase: TC87786 (The TIGR *Oncorhynchus mykiss* Gene Index (RtGI))

SGLT1: AY210436 (Genbank)

GK: AF135403 (Genbank)

G6Pase: AF120150 (Genbank)

EF1α: AF498320 (Genbank)

Table 3: Comparison of gene expression between rainbow trout larvae fed the control diet or VHC-diet during three days and the unfed yolk-sac larvae. Gene expression analyses were performed on total RNA extracted from whole body. Statistical differences in gene expression between samples were evaluated in group means by randomisation tests (46) using REST© software: down and up regulation means that target gene is expressed at lower or higher level, respectively, in fed than in unfed yolk-sac larvae. **A- Control *versus* unfed yolk-sac larvae**; **B- VHC-larvae (stimulus 1) *versus* unfed yolk-sac larvae.**

A-

Genes	**Type of regulation**	**CV $_{Control}$**	**CV $_{Unfed}$**	**P-values**
α-Amylase	down-regulation – 7 fold	8.52	0.67	<0.005
Maltase	down-regulation – 2 fold	1.19	0.18	<0.05
SGLT1	up-regulation – 2.2 fold	1.08	0.61	<0.005
GK	switch on [1]	1.13	n.a.	n.a.
G6Pase	down-regulation – 2.5 fold	0.37	0.23	<0.001

Transcript level of target genes normalised with EF1α expressed transcripts.

[1]Switch on: there was no detectable GK gene expression in unfed fish; n.a.: non applicable

B-

Genes	**Type of regulation**	**CV $_{Stimulus\ 1}$**	**CV $_{Unfed}$**	**P-values**
α-Amylase	no regulation	3.55	0.67	0.88
Maltase	no regulation	1.92	0.18	0.93
SGLT1	up-regulation – 2.3 fold	0.49	0.61	<0.001
GK	switch on [1]	0.87	n.a.	n.a.
G6Pase	down-regulation – 2.2 fold	0.46	0.23	<0.001

Transcript level of target genes normalised with EF1α expressed transcripts.

[1]Switch on: there was no detectable GK gene expression in unfed fish; n.a.: non applicable

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 two developmental stages. Statistical differences in gene expression between samples were evaluated in group means by randomisation tests (46) using REST© software: down and up regulation means that the target gene is expressed at a lower or higher level, respectively, in the VHC-fish than in the control fish. **A- at the first-feeding stage (stimulus 1)**, gene expression analyses were performed on total RNA extracted from whole larval body; **B- after the yolk absorption (stimulus 2)**, gene expression analyses were performed on total RNA extracted from whole viscera (liver, gastro-intestinal tract + diffuse pancreas).

A-

Genes	**Type of regulation**	**CV $_{\text{Stimulus 1}}$**	**CV $_{\text{Control}}$**	**P-values**
α-Amylase	up-regulation – 4 fold	3.55	8.52	<0.05
Maltase	up-regulation – 2.2 fold	1.92	1.19	<0.05
SGLT1	no regulation	0.49	1.08	0.72
GK	up-regulation – 1.5 fold	0.87	1.13	<0.05
G6Pase	no regulation	0.46	0.37	0.23

Transcript level of target genes normalised with EF1α expressed transcripts.

B-

Genes	**Type of regulation**	**CV $_{\text{Stimulus 2}}$**	**CV $_{\text{Control}}$**	**P-values**
α-Amylase	no regulation	2.14	6.54	0.23
Maltase	up-regulation – 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 normalised with EF1α expressed transcripts.

Table 5: Comparison of the long-term effect of the hyperglucidic stimulus (VHC-diet) on gastro-intestinal (GI) and hepatic (L) gene expression in juvenile rainbow trout during the final challenge test with the HC-diet. Statistical differences in gene expression between samples were evaluated in group means by randomisation tests (Pfaffl et al., 2002) using REST© software: down and up regulation 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. **A- Long-term effect stimulus 1:** comparison between juvenile trout fed the VHC-diet at first-feeding and control fish. **B- Long-term effect stimulus 2:** comparison between juvenile trout fed the VHC-diet after vitellus resorption and unstressed control fish

A-

Genes	**Type of regulation**	**CV $_{\text{Stimulus 1}}$**	**CV $_{\text{Control}}$**	**P-values**
α-Amylase	up-regulation – 1.7 fold	3.64	4.42	<0.05
Maltase	up-regulation – 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

Transcript level of target genes normalised with EF1α expressed transcripts**.**

B-

Genes	**Type of regulation**	**CV $_{\text{Stimulus 2}}$**	**CV $_{\text{Control}}$**	**P-values**
α-Amylase	up-regulation – 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 normalised with EF1α expressed transcripts.