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

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**Polakof S, Míguez JM, Soengas JL.** Dietary carbohydrates induce changes in glucosensing capacity and food intake of rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 295: R478–R489, 2008. First published June 4, 2008; doi:10.1152/ajpregu.00176.2008.—We hypothesize that variations in dietary carbohydrate levels produce changes in glucosensor parameters in previously characterized glucosensing areas (hypothalamus and hindbrain) related with the regulation of food intake of a carnivorous fish species like rainbow trout. Therefore, we fed trout with standard, carbohydrate-free (CF) or high-carbohydrate (HC) diets for 10 days to assess changes in glucosensing system and food intake. Fish fed CF diet displayed hypoglycemia and increased food intake. Fish fed a HC diet displayed hyperglycemia and decreased food intake. Changes in food intake due to dietary carbohydrates were accompanied in hypothalamus and hindbrain of fish fed with HC diet by changes in parameters involved in glucosensing, such as increased glucose, glucose 6-phosphate, and glycogen levels and increased glucokinase (GK), glycogen synthase, and pyruvate kinase activities as well as increased GK and GLUT2 expression. All those results address the first time in fish, despite the relative intolerance to glucose of carnivorous species, that dietary carbohydrates are important regulators of the glucosensing system in carnivorous fish, suggesting that the information generated by this system can be associated with the changes observed in food intake.

Carnivorous fish show an impaired ability to clear excesses in blood glucose levels (9) and therefore have been traditionally considered as relatively glucose-intolerant species (31, 55). However, plasma glucose levels do fluctuate (16), and glucose is also essential for brain function (48), suggesting the existence of a glucosensing system in fish. The mechanism of glucose signaling is well known in mammals requiring glucose uptake by the low-affinity glucose transporter type 2 (GLUT2), glucose phosphorylation by glucokinase (GK), and the subsequent metabolism of glucose through glycolysis to increase the intracellular ATP-to-ADP ratio. This leads to the closure of ATP-dependent K⁺ channels, membrane depolarization, the entry of Ca²⁺, which triggers increased neuronal activity and neurotransmitter secretion in brain regions and insulin release in pancreatic β-cells (27). In several recent studies, we have provided evidence for the presence of a glucosensor system (similar to the mammalian model) in hypothalamus, hindbrain, and Brockmann bodies (BB) of rainbow trout (36, 37).

In mammals, changes in glucose levels detected by glucosensing mechanisms elicit a number of regulatory responses that serve to control blood glucose levels, including regulation of food intake (FI; see Ref. 27). Accordingly, induced hyperglycemia is known to increase FI (42), whereas induced hypoglycemia decreased FI (2, 10). Not surprisingly, the same brain regions involved in glucosensing in mammals are also important in the regulation of feeding behavior (19, 44). Available evidence in fish suggests that the regulation of FI in fish is mediated by a neuronal circuit of hypothalamic origin that integrates orexigenic and anorexigenic endocrine and neurotransmitter signals such as neuropeptide Y (NPY), orexins, galanin, serotonin, bombesin, or cholecystokinin (CCK) among others (25, 54). Because at least hypothalamus is involved in the control of FI in fish, we hypothesize, in a way similar to that reported in mammals (27, 40), that brain glucosensor areas may be involved in the regulation of FI in fish.

Carnivorous teleosts possess a limited capacity to utilize dietary carbohydrates for energy purposes (17, 50, 55). In rainbow trout, digestible carbohydrate contents of more than 20–30% of the diet result in prolonged postprandial hyperglycemia (21) and impaired growth (15). Therefore, a natural way of inducing changes in circulating glucose levels would be using diets with different carbohydrate contents. However, there is almost no evidence in fish regarding the influence of dietary carbohydrates in FI. Thus we hypothesize that: 1) the glucosensor areas described previously in rainbow trout responding to changes in glycemia induced by exogenous treatments should respond to changes in plasma glucose levels induced by the absence/presence of carbohydrates in the diet, and 2) the changes in glycemia due to different carbohydrate intake may be related with changes elicited in FI. Therefore, we fed trout with standard, carbohydrate-free (CF) or high-carbohydrate (HC) diets for 10 days to assess changes in parameters involved in the glucosensing system such as GK activity and expression, GLUT2 expression, glycolytic potential, and glycogen levels to relate them to changes observed in FI.

MATERIALS AND METHODS

**Fish and diets.** Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (Soutorredondo, Spain). Fish were maintained for 1 mo in 100-liter tanks under laboratory conditions at a natural photoperiod in dechlorinated tap water at 14°C. Fish mass was 150 ± 5 g. Fish were fed daily to satiety during the acclimation period with commercial dry fish pellets denoted a standard diet (Table 1). After acclimation, fish were fasted for 24 h and then started to be fed (one time daily) to satiety for 10 days with a standard diet (control) or two formulated experimental diets: a high-carbohydrate/low-protein diet (HC) and a carbohydrate-free/low-protein diet (CF) (Table 1). Crude protein analysis (as N×6.25) was made by nitrogen determination (mass spectrometer VG Autospec M)

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and starch following Thivend et al. (52). The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the Spanish Government (RD 1201/2005) for the use of animals in research and were approved by the Ethics Committee of the University of Vifó (Spain). Experiments were undertaken in March 2007.

**Assessment of FI.** FI was registered for 3 days before treatment (to define basal line data). Next, fish in one-third of the tanks remained to be fed with standard diet, whereas fish in one-third of the tanks started to be fed with HC diet, and fish in the remaining one-third of the tanks started to be fed with CF diet. FI was measured each day for 10 days. Food was supplied in batches of \(20 \text{ g} \) each every 10 min until satiation. After feeding, the uneaten food remaining at the bottom (conical tanks) and feed waste were withdrawn, dried, and weighed. The amount of food consumed by each tank was calculated as the difference from the feed offered (11, 45). FI was registered 3 days before food deprivation in the two groups assessed, with values being approximately the same, and therefore were considered as baseline values for all subsequent assessment of FI. Thus, all values of FI registered after treatments are referred to those of basal values. The results are shown as means ± SE obtained in three different tanks per treatment at each sampling time. Because all fish in tanks were removed for sampling of metabolic parameters on days 1, 2, 3, 5, and 10 (see below), we had fish (and tanks) far in excess for treatments to make sure that FI was assessed at least in three different tanks per treatment at each sampling time. Fish density (0.012 kg/l) remained unchanged throughout the experiment.

**Sampling.** Fish were removed from replicate holding tanks at days 1, 2, 3, 5, and 10 for the assessment of metabolic parameters \((n = 8/diet group at each sampling time). On samplings carried out on days 1, 2, and 3, four additional fish were sampled per treatment and sampling time to assess gene expression in hypothalamus and hind-
brain. Fish were anaesthetized with MS-222 (50 mg/l) buffered to pH 7.4 with sodium bicarbonate and weighed. Blood was obtained by caudal puncture in ammonium-heparinized syringes. Plasma was obtained after centrifugation of the blood (1 min at 10,000 g), deproteinized (6% perchloric acid, PCA), and neutralized (1 mol/l potassium bicarbonate) before freezing in liquid nitrogen and further storage at −80°C until assayed. The liver and Brockman bodies (BB) were removed, freeze-clamped in liquid nitrogen, and stored at −80°C until assayed. The brain was removed and placed on a chilled petri dish, two regions (hypothalamus and hindbrain) were obtained as described previously (49), and each section was freeze-clamped in liquid nitrogen and stored at −80°C until assayed.

Assessment of metabolite levels and enzyme activities. Plasma glucose and lactate levels were determined enzymatically using commercial kits (Spinreact, Spain) adapted to a microplate format. Plasma total α-amino acids were assessed colorimetrically using the ninhydrin method of Moore (32); alanine was used to develop a standard curve. Frozen tissues were quickly minced on a chilled petri dish to very small pieces that (still frozen) were divided into two homogeneous portions to assess enzyme activities and metabolite levels. The tissue portions used to assess metabolite levels were homogenized immediately by ultrasonic disruption in 7.5 vol of ice-cooled 6% PCA and neutralized (using 1 mol/l potassium bicarbonate). The homogenate was centrifuged, and the supernatant was used to assay tissue metabolites. Tissue glycogen levels were assessed using the method of Keppler and Decker (20). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (Biomérieux, Spain). Tissue lactate and total α-amino acid levels were assessed as described above for plasma samples and glucose 6-phosphate levels as previously described (36). Tissue used to assess enzyme activities was homogenized by ultrasonic disruption with 9 vol of ice-cold buffer consisting of 50 mmol/l Tris (pH 7.6), 5 mmol/l EDTA, 2 mmol/l 1,4-dithiothreitol, and a protease inhibitor cocktail (P-2714; Sigma, St. Louis, MO). The homogenate was centrifuged, and the supernatant was used immediately for enzyme assays. Enzyme activities were determined using a microplate reader (SPECTRAFluor; Tecan, Grödig, Austria) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of supernatant (15 µl) at a preestablished protein concentration, omitting the substrate in control wells (final volume 265–295 µl) and allowing the reactions to proceed at 20°C for preestablished times (3–10 min). Enzyme activities are expressed in terms of milligram protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method (47) with BSA (Sigma) as standard. Enzyme analyses were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. Fructose 1,6-bisphosphatase (FBPase), glucose 6-phosphatase (G-6-Pase), glycogen synthase (GSase), glycogen phosphorylase (GPase), glucose 6-phosphatase dehydrogenase (G-6-PDH), glutamate dehydrogenase (GDH), low Kₗ hexokinase (HK), GK, and pyruvate kinase (PK) activities were estimated as described previously (43, 49). All enzymes were assessed for freeze stability, and they were freeze stable. The PK activity ratio assessed in brain regions is the ratio of activities at suboptimal substrate concentration (0.04 mM) to activities at saturating substrate concentration (0.3 mM), as determined from prior kinetic analysis. 6-Phosphofructo 1-kinase (PFK) activity was assessed using (in mM): 100 Tris·HCl (pH 8.25), 50 KCl, 5 MgCl₂, 0.15 NADH, 4 (NH₄)₂SO₄, 10 dithiothreitol, 10 fructose 6-phosphate, 30 glucose 6-phosphate, excess aldolase, excess

Fig. 3. Glucose (A), glycogen (B), glucose 6-phosphate (C), lactate (D), and α-amino acid (E) levels in hypothalamus of rainbow trout fed daily to satiety with a standard, CF, or HC diet for 10 days. Each value is the mean ± SE of n = 8 fish/group. When necessary, values were log transformed before statistical analysis. P < 0.05, significantly different from fish fed with standard diet at the same time (*) and significantly different from fish fed with CF diet at the same time (#). Different letters indicate significant differences (P < 0.05) among sampling times within each diet.
triose phosphate isomerase, excess α-glycerophosphate dehydrogenase, and ATP (omitted for control). 0.3 in liver; 0.2 in hindbrain. Phosphoenolpyruvate carboxykinase (PEPCK) activity was assessed in liver using (in mM): 50 Tris-HCl (pH 7.5), 1 MnCl₂·4H₂O, 20 NaHCO₃, 1.5 PEP, 0.3 NADH, excess malic dehydrogenase, and 1 2’-deoxyguanosine 5’-diphosphate (omitted for control).

RT-PCR analysis of HK-IV/GK and GLUT2 gene expression. Total RNA was extracted from frozen brains using Trizol reagent as recommended by the manufacturer (Invitrogen). The quality and quantity of the isolated RNA was assessed spectrophotometrically. Total RNA (2 μg) was reverse transcribed into first-strand cDNA when primed with random primers (Promega Biotech, Madrid, Spain) using M-MLV reverse transcriptase for 1 h at 37°C by methods recommended by the manufacturer (Promega Biotech). We have assessed the expression of single isoforms of HK-IV/GK and GLUT2 genes. GK cDNA was PCR-amplified using specific primers developed for rainbow trout by Panserat et al. (33): 5’-GATTTGTGTT-GAAGGTGGG-3’ (forward) and 5’-TTCATAGGATGCCCTT-GTC-3’ (reverse); amplification with these primers resulted in a 250-bp product. GLUT2 cDNA was PCR-amplified using specific primers developed for rainbow trout: 5’-GTCCTTTTACCATGGTGTCG-3’ (forward) and 5’-TCAAGAACGAAAGTCGGAGG-3’ (reverse); amplification with these primers resulted in a 222-bp product. The housekeeping gene used to assess the relative cDNA levels of GK/GLUT-2 was rainbow trout 18S. Accordingly, 18S cDNA was amplified by PCR using specific primers developed for rainbow trout: 5’-TCAAGAACGAAAGTCGGAGG-3’ (forward) and 5’-GGACATCTAAGGGCATCACA-3’ (reverse). The PCR reactions were carried out using a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA) in a final volume of 20 μl containing cDNA template (8 μl for GK and GLUT2, and 2 μl for 18S), 1× buffer (50 mM KCl, 20 mM Tris-HCl, and 0.1% Triton X-100), 0.2 mM dNTPs, 1.5 mM MgCl₂, 2 pmol of each primer (forward and reverse), and 1 U of Taq polymerase (Ecogen, Barcelona, Spain). The optimal number of cycles for amplification was established (reactions were terminated in the logarithmic phase of the PCR reaction). Amplification of cDNA was achieved with an initial denaturation at 94°C followed by 35 (HK-IV/GK and GLUT2) or 15 (18S) cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 30 s); a final extension period of 10 min occurred before termination. Negative controls without reverse transcriptase or cDNAs were performed to ensure observed bands were not a result of contamination. The PCR products were subjected to electrophoresis in 1.5% agarose gel. Size of PCR reaction products was established by comparison with a 50-bp DNA step ladder (Promega). Densitometric analysis of the bands of interest was performed using the gel electrophoresis documentation and analysis system EDAS 290 (Kodak) of images captured from ultraviolet transilluminated ethidium bromide-stained gels. Results are shown as arbitrary units and represent the ratio (%) between GK/GLUT-2 and 18S expression. To ensure the bands of interest were in fact trout GK and GLUT2, each band was gel-purified using the GFX PCR DNA and gel band purification kit (Amersham Biosciences) and cloned using Pgm-T Vector Systems II (Promega). White colonies were amplified by PCR using primers T7 and M13 (flanking the insert) and sequenced in both directions using the dRhodamine terminator cycle sequencing kit (Amersham Biosciences) and cloned using Pgm-T Vector Systems II (Promega). The optimal number of cycles for amplification was established (reactions were terminated in the logarithmic phase of the PCR reaction). Amplification of cDNA was achieved with an initial denaturation at 94°C followed by 35 (HK-IV/GK and GLUT2) or 15 (18S) cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 30 s); a final extension period of 10 min occurred before termination. Negative controls without reverse transcriptase or cDNAs were performed to ensure observed bands were not a result of contamination. The PCR products were subjected to electrophoresis in 1.5% agarose gel. Size of PCR reaction products was established by comparison with a 50-bp DNA step ladder (Promega). Densitometric analysis of the bands of interest was performed using the gel electrophoresis documentation and analysis system EDAS 290 (Kodak) of images captured from ultraviolet transilluminated ethidium bromide-stained gels. Results are shown as arbitrary units and represent the ratio (%) between GK/GLUT-2 and 18S expression. To ensure the bands of interest were in fact trout GK and GLUT2, each band was gel-purified using the GFX PCR DNA and gel band purification kit (Amersham Biosciences) and cloned using Pgm-T Vector Systems II (Promega). White colonies were amplified by PCR using primers T7 and M13 (flanking the insert) and sequenced in both directions using the dRhodamine terminator cycle sequencing kit (Applied Biosystems). The reactions were run on an Applied Biosystems automated sequencer model ABI PRISM 310. The resulting sequences were compared with known sequences on GenBank using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/).

Statistics. Comparisons among groups were performed using two-way ANOVA (SigmaStat; SPSS, Chicago, IL) with diet (CF and HC) and sampling day as factors. Where appropriate, values were log transformed before statistical analysis. P < 0.05, significantly different from fish fed with standard diet at the same time (*).
and time as main factors. When necessary, data were log transformed to fulfill the conditions of the analysis of variance. Post hoc comparisons within a factor were made using a Student-Newman-Keul’s test, and differences were considered statistically significant at $P < 0.05$.

RESULTS

Accumulated FI is shown in Fig. 1. Fish fed with a CF diet ate more than fish fed with a standard or HC diet from day 3 to 8. Fish fed with a HC diet ate less than those fed with a standard diet on days 4, 6, and 10.

Plasma metabolite levels are shown in Fig. 2. Fish fed with the HC diet showed higher plasma glucose levels (Fig. 2A) than fish fed with the CF diet from the first day of treatment onwards; values were also higher than those of fish fed a standard diet on days 3, 5, and 10. There were no differences on $\alpha$-amino acid or lactate plasma levels between the three dietary regimes (Fig. 2, B and C). However, fish fed with the HC diet displayed increased lactate levels in the first days.

Glucose, glycogen, and glucose 6-phosphate levels in hypothalamus (Fig. 3, A-C) in fish fed with the HC diet were higher than those of fish fed standard or CF diet; values in fish fed CF diet were also generally lower than those of fish fed a standard diet from days 1 to 5. In lactate levels (Fig. 3D), fish fed with HC diet displayed higher values than fish fed standard diet on days 1, 3, 5, and 10. $\alpha$-Amino acid levels (Fig. 3E) in fish fed HC diet were lower than those of fish fed a standard diet on days 3 and 5 higher on day 10; fish fed a CF diet displayed lower values than fish fed a standard diet on day 5 and higher on day 10. GK, PK (activity ratio), and GSase activities (Fig. 4, B, D, and E) were generally higher in hypothalamus of fish fed with the HC diet than in trout fed with the CF (from day 2 onward) or standard (on days 1, 2, and 10 for GK; on day 5 for PK, and on days 3, 5, and 10 for GSase) diet. The optimal activity of PK (Fig. 4C) in fish fed CF and HC diets was higher than in fish fed standard diet on days 1 and 2. Low $K_m$ HK activity displayed differences between dietary treatments on days 3 and 5; values were lower than those of fish fed standard diet on days 1 and 2 vs. fish fed a CF and HC diet, and lower than fish fed a standard diet on day 3 vs. fish fed HC diet (Fig. 4A).

Metabolite levels in hindbrain are shown in Fig. 5. Glucose, glycogen, and glucose 6-phosphate levels (Fig. 5, A-C) were consistently higher in fish fed with the HC diet than those in trout fed CF and standard diets. In addition, glycogen and glucose 6-phosphate levels showed a different pattern than glucose levels, since levels increased at day 2 and remained higher until day 10. Also, glucose, glycogen, and glucose 6-phosphate levels in fish fed CF diet were generally lower than those of fish fed a standard diet. Lactate levels in fish fed HC diet were higher than those in fish fed standard on days 3, 5, and 10 and higher than fish fed CF diet on days 3 and 10 (Fig. 5D). No major differences were found in $\alpha$-amino acid levels (Fig. 5E). GK, PK (activity ratio), GSase, and PFK activities (Fig. 6, B, D, E, and F) in general showed higher values in hindbrains of fish fed with the HC diet than in trout fed with the standard and CF diets. However, the time course was not the same for all the enzymes: in GK, GSase, and PFK

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**Fig. 5.** Glucose (A), glycogen (B), glucose 6-phosphate (C), lactate (D), and $\alpha$-amino acid (E) levels in hindbrain of rainbow trout fed daily to satiety with a standard, CF, or HC diet for 10 days. Each value is the mean ± SE of $n = 8$ fish/group. When necessary, values were log transformed before statistical analysis. $P < 0.05$, significantly different from fish fed with standard diet at the same time (*) and significantly different from fish fed with CF diet at the same time (#). Different letters indicate significant differences ($P < 0.05$) among sampling times within each diet.
the increase occurred in the first days, maintaining high values until day 10, whereas in PK (activity ratio) the activity started higher and decreased slowly throughout sampling times. The optimal activity of PK in fish fed experimental diets was different than that of fish fed standard diet on days 1 and 3 (Fig. 6C). No significant differences between fish fed with the two diets were observed in the optimal activity of PK and low Km HK activity (Fig. 6, A and C).

Liver metabolite levels are shown in Fig. 7. Glucose levels in liver (Fig. 7A) were significant lower in trout fed with the HC diet than in fish fed with the CF diet; they were also lower than in fish fed standard diet on days 5 and 10. Glycogen levels (Fig. 7B) in fish fed with the HC diet were higher than those of trout fed with the standard and CF diets displaying an increase from day 1 to 10. Glucose 6-phosphate levels were higher in fish fed HC than in fish fed CF on days 2, 3, and 5 (Fig. 7C) and higher than those of fish fed standard diet on days 2 and 3; moreover, levels in fish fed CF diet were lower than those of fish fed standard diet on days 2 and 5. Lactate levels (Fig. 7D) were higher in fish fed HC diet than in fish fed standard (days 2–10) and CF (days 3 and 5) diets. No major differences were observed among diets in α-amino acid levels (Fig. 7E). Enzyme activities in liver are shown in Fig. 8. Fish fed with the HC diet showed higher GK and GSase activities (Fig. 8, B and G) than those fed with the standard and CF diet. GK activity increased from day 1 to 10, whereas GSase activity was higher throughout the experiment. In contrast, fish fed with the HC diet displayed lower activities than fish fed with the CF diet in PEPCK, FBPase, G6Pase, and G-6-Pase activities; values in fish fed standard diet were intermediate (Fig. 8, E, F, H, and I). G-6-Pase and PEPCK activities were similar in fish fed with both diets in the first 3 days, but from day 3 to 10 trout fed with the HC diet displayed lower values. Minor differences were observed in the other enzymes assessed (Fig. 8, C, D, and J).

Metabolite levels and enzymes activities in BB are shown in Fig. 9. Glucose and glycogen levels (Fig. 9, A and B) in trout fed with the HC diet displayed higher values than those fed with the standard and CF diets. No major changes were noticed in lactate levels (Fig. 9C) among diets, whereas a clear increase with time was observed in fish fed with the HC diet. α-Amino acid levels (Fig. 9D) in fish fed the experimental diets were lower than in fish fed standard diet on days 2 and 3; then fish fed with the CF diet displayed higher levels than those fed with the HC diet. As for enzyme activities, values observed in low Km HK activity (Fig. 9E) in fish fed experimental diets were lower than those of fish fed standard diet on days 3–10. GK activity (Fig. 9F) generally displayed higher values in trout fed with HC diet than in those fed with the standard and CF diets, whereas values in fish fed CF diet were also lower than in fish fed standard diet on days 3 and 5. A similar profile was observed in PK activity (Fig. 9G), with higher activities in fish fed HC diet than those fed with the standard and CF diet, whereas fish fed CF diet displayed lower values than in fish fed standard diet on days 3 and 5.

In general, GK and GLUT2 (Fig. 10) expression was higher in fish fed with the HC than in those fed standard or CF diets in hypothalamus, hindbrain, and BB, whereas expression was generally lower in fish fed CF than in fish fed standard diet.
DISCUSSION

Validation of experimental design. Plasma glucose levels increased in fish fed with HC diet with levels being 100% higher than those of fish fed CF diet. Levels of plasma glucose observed in fish fed with HC diet are in agreement with similar studies in the same (6, 22, 51) and other (3) fish species. Glycemia did not change significantly among sampling times in fish fed CF diet and were lower than those observed in the same species when fed with standard levels of carbohydrates in the diet (this study and Refs. 21 and 51). The absence of changes among sampling times is similar to that previously addressed in fish fed CF diets (3, 12, 33).

To further validate the experimental design, we have also assessed several metabolic parameters in liver known to change when fish are fed with diets containing different amounts of carbohydrates, such as: 1) increased glycogen levels in fish fed with HC diet, in agreement with results obtained in the same species (6, 22, 51), whereas levels decreased slowly in fish fed with CF in a way similar to that observed in other studies (3, 12), 2) no changes in low \(K_m\) HK activity, whereas GK activity increased clearly in fish fed with the HC diet, in a way similar to previous studies in rainbow trout (6, 33) and other species (8), 3) the absence of changes with diets or time in the glycolytic (PK and PFK activities) or in the pentose phosphate pathway (lack of changes in G-6-PDH activity; data not shown) potentials, in agreement with previous studies in rainbow trout (13, 18), and 4) inhibition of gluconeogenesis in fish fed with the HC diet and lack of stimulation in those fed with CF diet, also in agreement with comparable studies in the same species (18). Moreover, we have also assessed several metabolic parameters not assessed previously in liver in comparable studies such as the increased GSase and decreased GPase activities in fish fed with the HC vs. those fed CF, which are in agreement with changes display by glycogen levels.

As an additional support for the experimental design, we have also assessed changes in glucosensing capacity of BB, a distinct grouping of pancreatic endocrine cells near the gall bladder (57), to compare them with the known changes occurring in mammalian pancreas (29). In the present study, GK activity and GK and GLUT2 expression in BB were higher in fish fed with the HC diet than in those fed CF diet, supporting that the glucosensor mechanism responded to changes in circulating glucose levels induced by diet, and not only when glucose levels change as result of exogenous glucose administration (36). In addition, glucose and glycogen levels and PK activity in BB changed in parallel with changes in plasma glucose levels in agreement with previous short-term studies in rainbow trout (36) as well as with the mammalian model (29).

Since carbohydrates were mainly replaced by protein in the CF diet, fish fed CF diet ingested more protein. Our experimental conditions do not allow us to distinguish between the potential effects of protein decrease and/or carbohydrate increase. However, the diets differed in carbohydrate content by
~2,000% and in protein by only 25%. Thus the change in carbohydrate levels is about two orders of magnitude greater than that of protein, and therefore changes could be mainly attributed to variations in carbohydrate rather than in protein contents. Moreover, no changes were observed in amino acid and protein levels (in plasma or liver) or GDH activity in liver (although a decrease occurred at day 10 in fish fed with HC).

This absence of changes in parameters involved in amino acid metabolism is similar to that already observed in the same species after feeding diets with different amounts of proteins (22), reinforcing that the experimental design was valid for the assessment of changes due to variations in carbohydrate levels.

**Effects on FI.** Fish submitted to the prolonged hyperglycemia induced by feeding HC diets ate less than those submitted to the hypoglycemia induced by feeding CF diets. This response is similar to that observed in mammals where induced
hyperglycemia resulted in decreased FI (2). In fish literature, there are no other time courses available for FI of fish fed HC diets compared with those fed standard diets. In studies assessing FI only at the end of feeding HC diets for several days, the results obtained provide contradictory results such as the decreased FI in rainbow trout (51) or the absence of changes in rainbow trout (6) and carp (7).

Fish subjected to dietary glucoprivation (CF diet) ate more than those fed with a standard or HC diets from day 3 of experiment onward. This time course is different from that described in mammals in which the increased FI occurring after glucoprivation is known to occur a few (5, 42) hours after feeding and could be related to the relative glucose intolerance of fish. Increases in FI after feeding with a CF diet had been previously observed in the same species before (6, 41) although in those studies no time courses were available since only end-point measurements were carried out.

As a whole, the changes in FI associated with changes in glycemia suggest that fish with less circulating glucose levels ate more than those with high levels of glucose. Therefore, despite the relative intolerance to glucose of carnivorous fish like rainbow trout (31), dietary carbohydrates seem to be able to generate the sufficient changes in glycemia to elicit changes in FI in a way similar to the regulatory capacity of glucose in mammals although delayed in time. This delay is also reflected by the fact that on days 2 and 3 fish fed with the HC diet maintained high glucose levels and as consequence of the progressive and inevitable glucose intake from the diet started to decrease their FI. In this way, from a metabolic point of view, trout displayed a clear compensatory mechanism to avoid the increase in plasma glucose levels, storing the excess glucose as hepatic glycogen in agreement with several previous studies (1, 4, 18, 23, 36).

Glucosensing in hypothalamus and hindbrain. In mammals, changes in plasma glucose levels are detected in the central nervous system (CNS), basically in hypothalamus and brain stem (27). In fish, a glucosensing mechanism has been also described in hypothalamus and hindbrain, responding to...
changes in glucose levels either in vivo (36) or in vitro (37) with changes in GK activity and expression, glucose and glycogen levels, and glycolytic potential similar to those observed in mammalian glucosensor areas (24). We aimed in the present study to assess in those brain regions, for the first time in fish, changes in those parameters involved in glucosensing capacity elicited by a differential carbohydrate intake.

Glucoprivation elicited by CF diet produced marked changes in several metabolic parameters involved in glucosensing. Thus decreased glucose, glucose 6-phosphate, and glycogen levels, as well as GK and GSase activity, and GK and GLUT2 expression in the two glucosensor areas were assessed in brain, i.e., hypothalamus and hindbrain were observed. All those changes are comparable with those previously observed in the same brain regions of the same species after insulin-induced hypoglycemia either at short-term (36) or long-term (S. Polakof J. M. Miguez, and J. L. Soengas, unpublished observation) exposure and occurred at the same time than those changes observed in FI. Therefore, we can state that the fall in glucose levels per se (elicited by the absence of carbohydrates in the diet) and not the effects of insulin are what produced the response of glucosensing systems. GK constitutes the key component of the glucose-sensing mechanism in hypothalamic (30, 56) and hindbrain (39) neurons in mammals. Moreover, inhibition of GK activity is known to stimulate FI in mammals (14). Therefore, we may hypothesize that, in fish, GK activity in hypothalamus and hindbrain is a key mediator in the stimulation of FI due to the reduced plasma and brainin hypothalamus and hindbrain is a key mediator in the glucose levels produced by CF diet. Accordingly, a clear decrease in GK activity and expression occurred in fish fed CF diet compared with those fed standard diet (this study and Ref. 36). This response is similar to that previously observed in the same brain regions when submitting fish to changes in circulating glucose levels through insulin treatment (36). Thus we may suggest that the information generated by the glucosensing mechanism in the CNS of the glucoprived trout could be integrated by the neural network that controls FI in fish, favoring an increase in FI to counteract the hypoglycemia produced by the noncarbohydrate intake, thus preventing glycogen exhaustion and increasing the available glucose pool, as suggested in mammals (38). This similar response is probably reflecting the important metabolic needs of fish brain, which also relies on glucose as fuel (48), consequently making necessary a response to keep glucose levels high enough for brain function.

Furthermore, we observed that trout fed HC diet displayed a 100% increase in levels of plasma glucose compared with fish fed CF diet. Plasma glucose levels did not reach values higher than 10 mM, probably due to the glucose buffering action of liver (31). The increased glycemia was reflected in both hypothalamus and hindbrain by increased levels of glucose, glycogen, and glucose 6-phosphate, as well as by increased GSase and GK activities and increased GK and GLUT2 expression.
All those changes are comparable to those previously observed after short-term hyperglycemic conditions induced by glucose administration (36). Moreover, fish fed HC diet displayed in both brain regions an increased glycolytic potential based on changes in PK and PFK activities, in agreement with the increased glycolytic potential already reported in those brain regions in trout after short-term induced hyperglycemia (36) and the mammalian model of glucose-excited neurons (24) where this increased glycolytic potential is one of the more important steps for the final cell response. The present results are therefore supporting the glucosensor role of hypothalamus and hindbrain (36), which may probably participate in the response generated in FI.

The changes in glucose levels in plasma and brain regions in fish fed HC diet were markedly faster and more important than those of fish fed CF diet, supporting our previous hypothesis (36) that the glucosensing mechanism present in trout brain seems to respond better to hypoglycemia rather than to hyperglycemia. This clear differential response may be related to the relative intolerance to glucose observed in carnivorous fish (31). However, fish fed CF diet showed a sustained hypoglycemia and a concomitant increase in FI that may be related to the glucodependence of fish brain (48), which needs a continuous glucose input for normal function, since local glycogen stores are weak and useful just in a short period postglucopenia.

In mammals, hypothalamic as well as brain stem nuclei play a critical role in integrating the information of absorbed nutrients, the amount of energy stored in the form of fat, and changes in circulating glucose levels to regulate feeding, energy storage, or expenditure (27). This information is transmitted to the brain by hormones produced by gut endocrine cells, such as ghrelin, CCK, glucagon-like protein-1, or peptide YY3-36, by fat (leptin) or pancreatic β-cells (insulin), but also through autonomic nervous connections activated by nutrients like glucose (27). The sites of convergence for those signals are neuronal circuits in the hypothalamus and brain stem (26, 53), which can be directly regulated by leptin, insulin, ghrelin, lipids, and glucose (44). In the present study, the response of the fish glucosensor system in hypothalamus and hindbrain to changes in glycemia induced by dietary carbohydrates occurred simultaneously with changes in FI. Fish hypothalamus is also a site for convergence and integration of signals from the periphery and neighboring neurons where several FI-related hormones are produced, including NPY, CCK, ghrelin, agouti-related protein, cocaine- and amphetamine-related transcript, orexins, pro-opiomelanocortin-derived peptides, galanin, and melanin concentrating hormone (25, 54). Therefore, we can suggest that the information elaborated by the glucosensing neurons in fish, at least in hypothalamus, may be integrated in near or even in the same neurons involved in the regulation of FI as occurs in mammals (27).

Perspectives and Significance

We have demonstrated, for the first time in fish, that the glucosensing system previously characterized in hypothalamus, hindbrain, and BB of rainbow trout (36, 37) respond to changes in glycemia induced by the differential intake of dietary carbohydrates. Those changes in glucosensing parameters are accompanied by concomitant changes in FI similar to those reported in mammals addressing increased FI when glucose levels in the diet fall (30). Both changes in FI and the glucosensing system were characterized by a delayed time of appearance compared with mammals (46) and by showing a greater magnitude in fish subjected to hypoglycemia than in those under hyperglycemic conditions. All those results suggest an important role of dietary carbohydrates as regulators of the glucosensing system in both hypothalamus and hindbrain of carnivorous fish, which can be related to changes observed in FI. On those areas, not only glucose but also any of the orexigenic and anorexigenic factors involved in the control of FI in fish (54), may interact, producing changes in FI in response to changes in plasma glucose levels. Further studies are thus necessary to assess such integration mechanisms and to know which factors are involved.

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