Gut glucose metabolism in rainbow trout: implications in glucose homeostasis and glucosensing capacity

Sergio Polakof†*, Rosa Álvarez‡ and José L. Soengas†

Laboratorios de Fisioloxía Animal† and Bioloxía Celular‡, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía, Universidade de Vigo, 36310 Vigo, Spain.

Running title: gut glucose metabolism in trout

Corresponding author:
*Dr. Sergio Polakof
Laboratorio de Fisioloxía Animal
Facultade de Bioloxía
Edificio de Ciencias Experimentais
Universidade de Vigo
E-36310 Vigo
Spain
Tel. +34-986 812 564
Fax +34-986 812 556
E-mail spolakof@uvigo.es
Abstract

The main objective of the present study was to evaluate the relative contribution of the intestine to glucose homeostasis in rainbow trout. In a first set of *in vivo* experiments trout were subjected to oral glucose treatments alone or in combination with insulin injections to assess changes in glucose-related enzymes activities, metabolite levels, and mRNA levels. Rainbow trout gut displays an important glucose metabolism includes the ability to store glucose as glycogen (mostly in the muscle layers) and a large capacity to oxidize glucose. This constitutes a surprising result for a carnivorous fish. In a second set of *in vivo* experiments, trout received an oral amino acid solution alone or in combination with insulin injection to determine if other factors besides fasting could regulate gluconeogenesis in intestine. The results confirm the absence of regulation of gluconeogenesis in trout gut, which does not respond to hormones, glucose, lactate or amino acid changes, either *in vivo* or *in vitro*. We also fully characterized gut glucose metabolism *in vitro*. We observed that a large amount of glucose is oxidized to lactate supporting the importance of glucose in gut metabolism. Moreover, we corroborate the minor actions of insulin in trout gut while other hormones like GLP-1 and C-peptide appear to be major hormonal regulators of glucose metabolism in fish gut. Finally, we obtained the first evidence for the existence of a glucosensing mechanism in the midgut of this carnivorous species.

Key words: trout, intestine, glucose metabolism, glucosensing, lactate
Introduction

Glucose homeostasis in carnivorous fish species like rainbow trout (*Oncorhynchus mykiss*) remains under debate (13, 28). The main tissue controlling glucose homeostasis in fish is the liver, as main site for glucose production and storage (10). Other organs are also involved in glucose homeostasis, either as glucose producers like kidney (52) or as glucose users like muscle (21). Fish gastrointestinal tract (GIT) constitutes the first organ that has access to dietary nutrients and therefore it would be not surprising that its metabolism be strongly dependent on the type of nutrient absorbed, as well as be also influenced by diet and nutritional status (35), in a way similar to that observed in mammals (25).

In fish, whereas nutrient transport by GIT has been widely studied, and is similar to that observed in other vertebrates (5), much less is known about GIT metabolism. Thus, Soengas and Moon in black bullhead (48) or Stokes and Fromm in rainbow trout (50) assessed glucose utilization, while Mommsen et al. (27) were focussed on enzyme zonation in trout, tilapia, and copper rockfish GIT. Finally, Kirchner et al. (18) have shown that the gluconeogenic enzymes in trout GIT are unaffected by changes in dietary carbohydrate/protein ratio, with those changes being in part responsible for the persistent postprandial hyperglycemia occurring in this species. On the other hand, while in mammals GIT is known to be an insulin-sensitive organ (8) and its metabolism is regulated by numerous hormonal factors, in fish the only hormonal effects reported to date were those of black bullhead enterocytes (insulin, glucagon, and GLP-1) (48), and tilapia GIT (cortisol) (27). In mammals, diabetes is accompanied by alterations in the physiological function of the intestine, including increased glucose absorption, glycolytic rates, and glycogen deposition (1, 2). The fact that a carnivorous fish like rainbow trout is considered as a non-insulin dependent diabetes species (28), make trout intestine an attractive model for glucose metabolism studies.

In mammals glucose is the most important nutrient regarding energy supply, and as in other tissues (45), glucosensing capacities have been also reported in GIT cells, including enterocytes, enteroendocrine cells, and enteric neurons (38). Although the mechanism remain to be fully elucidated, some of the actors involved in this function are much likely the same than those present in the glucosensing mechanism of pancreatic β-cells (45), like glucokinase (GK) or ATP-dependent potassium channels (K_{ATP}), while others seem to be more GIT-specific, such as SGLTs (9, 29). Even though glucose is not the main energy substrate for a carnivorous fish, a glucosensing mechanism similar to that described in mammals has been demonstrated in key tissues of rainbow trout that rely on glucose for signal or energy...
purposes such the pancreatic and brain tissues (40). However, no data is available to date about the possible existence of a glucosensing system in fish GIT, and information about enteroendocrine and enteric neurons in fish is scarce (3, 32).

Thus, the main objective of the present study was to evaluate the relative contribution of the intestine to the glucose homeostasis in rainbow trout. In a first set of in vivo experiments trout were subjected to oral glucose treatments alone or in combination with insulin injections in order to assess changes in glucose-related enzymes activities, metabolite levels, and mRNA levels. Based on the lack of response of the gluconeogenic pathway to dietary changes described by Kirchner et al. (18), in a second set of in vivo experiments trout received an oral amino acid solution alone or in combination with insulin injection. We also carried out different sets of in vitro experiments to fully characterize gut glucose metabolism, and to respond several questions arisen from our in vivo studies. Accordingly, a first set of experiments was focussed on hormone regulation and lactate metabolism whereas a second set was focussed on the potential of amino acids to alter gluconeogenic capacity of intestine. Finally, we also aimed to obtain, for the first time in fish, evidence for the existence of a glucosensing mechanism in the midgut of this carnivorous fish species, and, accordingly, we have identified by molecular and immunohistochemical techniques the presence of potential actors in that putative mechanism.

Materials and methods

Fish

Rainbow trout (Oncorhynchus mykiss Walbaum) were obtained from a local fish farm (Soutorredondo, Spain). Fish were maintained for 1 month in 100 litre tanks under laboratory conditions and 12L:12D photoperiod in dechlorinated tap water at 16 °C. Fish mass was 145 ± 7 g. Fish were fed once daily (09.00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Segovia, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). 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.

In vivo experiments
Oral glucose administration. Following 1 month acclimation period, fish were randomly assigned to 100 litre experimental tanks, and each tank was randomly assigned to one of 4 experimental treatments. Fish were lightly anaesthetized with MS-222 (50 mg·l⁻¹) buffered to pH 7.4 with sodium bicarbonate, and weighed. Then, 8 fish per group received both oral (O) 10 mL·kg⁻¹ body mass and intraperitoneal (IP) 5 ml·kg⁻¹ treatments, consisting in: i) control group: tank water O + saline solution IP; ii) insulin group: tank water O + insulin solution (Sigma, 2 IU·kg⁻¹) IP; iii) glucose group: glucose solution (Sigma, 1 g·mL⁻¹) O + saline solution IP; iv) insulin + glucose group: glucose solution (Sigma, 1 g·mL⁻¹) O + insulin solution (Sigma, 2 IU·kg⁻¹) IP. Blood and midgut samples were taken 6 h after treatment using fish fasted for 24 h before treatment to ensure basal hormone levels were achieved. Concentrations and times were selected based on studies carried out previously in fish (42, 47).

Oral amino acid administration. Fish were acclimated, distributed, anaesthetized and weighed as above. Then, 8 fish per group received both oral (O) 10 mL·kg⁻¹ body mass and intraperitoneal (IP) 5 ml·kg⁻¹ treatments, consisting in: i) control group: tank water O + saline solution IP; ii) amino acid group: amino acid solution (Aminoplasmal L-12.5, Braun Medical, Spain) O + saline solution IP; iii) amino acid + insulin group: amino acid solution O + insulin solution (Sigma, 2 IU·kg⁻¹) IP. Sampling was carried out as above.

In vitro experiments

Every morning of an experiment, fish were dipnetted from the tank, anaesthesized with MS-222 (50 mg·l⁻¹) buffered to pH 7.4 with sodium bicarbonate, euthanized by decapitation and weighed. The midgut was removed, cleaned from surrounding vessels and fat, opened, and excised in small pieces (60-80 mg). The small pieces were rinsed with modified Cortland medium (141 mM NaCl; 3.5 mM KCl, 4.5 mM NaHCO₃, 1.0 mM CaCl₂, 1.0 mM MgSO₄, 3.0 mM Na₂HPO₄, 10.0 mM HEPES, 50 U·ml⁻¹ penicillin, and 50 mg·ml⁻¹ streptomycin sulphate, pH 7), sliced in chilled Petri dishes, placed in a chilled Petri dish containing 100 ml of modified Cortland medium·g⁻¹ tissue, and gassed with 0.5% CO₂/99.5% O₂. The tissue was finely minced and mixed and then placed in 48-well culture plates with 100 ml of modified Hanks’medium·g⁻¹ tissue and gassed with 0.5% CO₂/99.5% O₂, and incubated (unless another indication) at 16ºC for 6h. The number of independent experiments carried out for enzyme activities was three (N=3) for treatments and ten (N=10) for controls, whereas a similar number of experiments was carried out to assess tissue metabolites.
Glucose incubations. Control wells contained modified Cortland medium with 0, 5 or 20 mM D-glucose (Sigma). Treated wells contained medium at the same glucose concentration and one of the selected agents or hormones related to glucose metabolism. These included (final concentration): bovine insulin (10^{-8} M, Sigma), human glucagon (10^{-8} M, Sigma), rat GLP-1 (10^{-9} M, 1-37; Tocris Bioscience), rat C-peptide (10^{-9} M, Genosys), cytochalasin B (inhibitor of glucose transport through GLUTs) from Helminthosporium dematioidem, (10 µM, Sigma) and phlorizin (inhibitor of glucose transport through SGLTs; 1 mM, Sigma). In a second set of incubations, two agents related to lactate metabolism were also utilized, such as (final concentration): an inhibitor of lactate dehydrogenase (50 mM sodium oxamate), and an inhibitor of the monocarboxylic acid transporter (1 mM α-cyano-4-hydroxy cinnamate; 4-CIN). All reagents were dissolved in modified Cortland medium, except for cytochalasin B and phlorizin (5% ethanol), and 4-CIN (0.5% ethanol). No effects on the parameters assessed were observed due to the vehicle alone (data not shown). After 6h incubation, tissues were quickly removed, frozen in liquid nitrogen, and stored at -80°C until assay.

Amino acid incubations. Control wells contained modified Cortland medium with 5 mM D-glucose. Treated wells contained medium at the same glucose concentration and Aminoplasmal L-12.5 (Braun Medical, Spain) serial dilutions: 1:20 (1x), 1:8 (2.5x), and 1:4 (5x). Osmolality was corrected with NaCl. After 6h incubation, tissues were quickly removed, frozen in liquid nitrogen, and stored at -80°C until assay.

Assessment of metabolite levels and enzyme activities

Plasma and tissue glucose and lactate levels were determined enzymatically using commercial kits (Spinreact, Spain) adapted to a microplate format.

Samples 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^{-1} potassium bicarbonate). The homogenate was centrifuged, and the supernatant used to assay tissue metabolites. Tissue glycogen levels were assessed using the method of Keppler and Decker (17). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit.

Samples for enzyme activities were homogenized by ultrasonic disruption with 9 vols ice-cold-buffer consisting of 50 mmol·l^{-1} Tris (pH 7.6), 5 mmol·l^{-1} EDTA, 2 mmol·l^{-1} 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged and
the supernatant 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 pre-established protein concentration, omitting the substrate in control wells (final volume 265-295 µl), and allowing the reactions to proceed at 20 °C for pre-established times (3-10 min). Enzyme activities are expressed in terms of mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method (46) with bovine serum albumin (Sigma, USA) as standard. Enzyme analyses were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. FBPase (EC 3.1.3.11), G6Pase (EC 3.1.3.9), GSase (EC 2.4.1.11), γ-amylase (EC 3.2.1.3.), LDH (EC 1.1.1.27), Low Km HK (EC 2.7.1.1), GK (EC 2.7.1.2), AlaAT (EC 2.6.1.1), GDH (EC 1.4.1.2), and PK (EC 2.7.1.40) activities were estimated as described previously (30, 43).

Gene expression analysis by real-time quantitative RT-PCR

Total RNA was extracted from rainbow trout midgut using TRIzol reagent (Invitrogen). One µg total RNA was reverse transcribed into cDNA using the M-MLV reverse transcriptase enzyme (Promega) and random primers (Promega). Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ™ (BIO-RAD, Hercules, CA, USA). Analyses were performed on 10 µl of the diluted cDNA using the iQ™ SYBR® Green Supermix (BIO-RAD, Hercules, CA, USA), in a total PCR reaction volume of 25 µl, containing 200 nM of each primer. For GK (AF135403), G6Pase (tcay0019b.d.18_3.1.s.om.8.1-1693), GLUT2 (AF321816), SGLT-1 (AY210436), and β-actin (NM_001124235.1) qPCR was performed using primers previously described (11, 42). Primers for MCT1, LDH-chain A and LDH-chain B were designed to overlap an intron if possible (Primer3 software) using sequences data from Oncorhynchus mykiss (Sigenae Database) kindly provided by Dr. Stéphane Panserat (INRA, St-Pée-sur-Nivelle, France), as follows: MCT-1 (forward: AGGCTTGGAAGCTAGCTTCA, reverse: AGCCACGCAGCAGTGAACA) (Sigenae: CX251492.p.om.7), LDH-chain A (forward: TCCGTGCTAGGTGTGCTGTGA, reverse: ACACCACTGCTCCCCGACAG) (Sigenae: BE859108.p.om.7), LDH-chain B (forward: TTCCACGTTAGGGTATGGA, reverse: TGGCACTAGGGCGCTCTTTAC) (Sigenae: CA357739.p.om.7). Relative quantification of the target gene transcript was done using β-actin gene expression as reference (34), which was stable expressed in this
experiment. Thermal cycling was initiated with incubation at 95°C for 90s using hot-start iTaq™ DNA polymerase activation; 35 steps of PCR were performed, each one consisting of heating at 95°C for 20s for denaturing, and at specific annealing and extension temperatures. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/s from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Relative quantification of the target gene transcript with the β-actin reference gene transcript was made following the Pfaffl method with the Relative Expression Software tool (REST©) (37). This mathematical algorithm computes an expression ratio based on q-PCR efficiency and the crossing point deviation of the unknown sample versus a control group: 

\[ R = \frac{\left( E_{\text{target gene}} \right)^{\Delta CT_{\text{target gene}}} \text{(mean control - mean unknown sample)}}{\left( E_{\text{EF1α}} \right)^{\Delta CT_{\text{EF1α}}} \text{(mean control - mean unknown sample)}} \]

where \( E \) is PCR efficiency determined using a standard curve of cDNA serial dilutions (cDNA dilutions from 1/32 up to 1/512) and \( \Delta CT \) is the crossing point deviation of an unknown sample versus a control.

For conventional PCR, amplification of cDNA was achieved with an initial denaturation at 94°C followed by 35 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min) and extension (72°C for 1 min) followed by a final extension period of 10 min at 72°C before termination. PCR was carried out in a 20-µl total volume and included 1x PCR buffer, 0.2 mM dNTP, 2 mM MgCl₂, 1 U Taq polymerase (Invitrogen), 0.2 µM forward and reverse primers, and 1 µL of cDNA. Primers for Kir6.x-like and SUR-like were previously described (41). The PCR products were subjected to electrophoresis in a 1.5% agarose gel. The size of each PCR product was established by comparing with a 50-base step DNA ladder (Promega). Quantification of PCR products was performed by densitometric analysis of ethidium bromide-stained gels using gel documentation system and analysis software.

**Immunohistochemical procedure**

Fish were anesthetized with MS-222, their midgut extracted. For histochemistry, small pieces were fixed by immersion for 24 h at 4°C in Bouin fluid, were the paraffin-embedded after dehydration treatment. Then sections of 6-12 µm in thick, were stained using the periodic acid-Schiff (PAS) to differentiate glycogen. Control sections were incubated, before staining, in amylase.

For immunohistochemistry, small pieces of intestinal tissues were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate-buffered saline, (PBS) at pH 7.4, for 24 h at 4°C.
Midgut pieces were then cryoprotected in 30% sucrose and were embedded in Tissue-Tek OCT compound (Sakura). Transversal sections (20 µm-thick) were made on a cryostat, and treated with 3% H2O2 for 30 min and 0.1% bovine serum albumin (BSA) for 1 h, to inhibit the endogenous peroxidase activity and non-specific reactivity, respectively. Next, the sections were then incubated overnight at room temperature in a humid chamber with the following primary antisera raised: GCK (rabbit polyclonal, St. Cruz Biotechnology); SGLT-1 (rabbit polyclonal IgG, Millipore), and GLP-1 (rabbit polyclonal IgG, St Cruz Biotechnology). In all the optimal dilution were 1:100 in PBS. After several washes in PBS, the sections were then incubated subsequently for 1 h in biotynylated anti-rabbit IgG (Vector) diluted 1:100 in PBS and in ABC-kit (Vector) diluted 1:100 in PBS. Finally, the peroxidase reactions was developed in a solution of 3,3′-diaminobenzidine (Sigma) (0.003% in Tris-HCl 0.1M, pH 7.4) and H2O2 (0.01%). Same developed sections were counterstained with Mayer’s hematoxylin solution. In a control group designed to confirm the specificity of immunostaining, the primary antiserum was omitted and no immunoreactivity was observed. Rat gut samples were used as positive controls. For attempt colocalization experiments, sections were first incubated with primary antibodies and then incubated with the secondary specific FITC-and TRITC-conjugated antibodies diluted 1:400. Other control group of preparations were incubated with antibodies against neuronal markers, acetylated tubulin (AcT), human neuronal protein (Hu) and calretinin (CR) on basis of previous studies in fish and mammals (32, 33). Slides were observed and photographed under an Olympus microscope (BX51) and digitally photographed (Olympus DP71).

Results

In vivo glucose oral administrations. Plasma parameters as well as metabolite levels and enzyme activities in midgut of rainbow trout subjected to oral administration of glucose combined with insulin are shown in Figures 1 and 2. As expected, 6h after oral glucose administration, trout were hyperglycemic (Fig. 1A), exhibiting plasma levels ~25 mM. On the contrary, fish receiving intraperitoneally insulin administration showed a mild hypoglycemia, with levels about 3 mM. When both glucose and insulin were co-administered fish remained hyperglycemic, although the degree of hyperglycemia (~17 mM) was lower than in those receiving only glucose treatment. Plasma lactate levels (Fig. 1B) were only altered by insulin treatment, exhibiting those fish lower lactate levels than the control group. After insulin administration, no changes were noticed in either glucose (Fig. 1C) or glycogen (Fig. 1D).
levels in midgut. However, when fish received glucose alone or combined with insulin injection, both parameters increased. It is worth mentioning that the increase was higher for free glucose levels (~10-fold) than for glycogen (~3-4-fold). We found that lactate levels in midgut (Fig. 1E) were higher than the control in all the other treatments, but especially with the combination of insulin and glucose, increasing up to 3-fold more than the saline-sham group. Concerning enzymes related with glucose phosphorylation and oxidation, insulin seems not to regulate their activity, since GK, low Km HK, and PK (Figs. 2A, B, I) remained unaltered by the treatment with the hormone alone. However, when fish received orally glucose alone or together with insulin injection we found enhanced glucose phosphorylation and oxidation through glycolysis. Enzymes related with the gluconeogenic potential were differentially regulated: while FBPase activity (Fig. 2C) remained unaltered by any treatment, we found that G6Pase activity (Fig. 2D) was strongly (~4-fold) stimulated by glucose (alone or in combination with insulin), while the stimulation found with insulin alone was minor. Glycogen-related enzymes were affected by both insulin and glucose: while total GSase activity (Fig. 2E) was enhanced by insulin alone and in combination with glucose comparing to the control group, γ-amylase (Fig. 2F) was also stimulated by insulin alone, but in contrast inhibited by the oral glucose treatment (alone or with insulin injection). Finally, LDH activity (Fig. 2G) was enhanced by all of them, especially by glucose alone.

After oral glucose treatments, mRNA levels of some key proteins involved in glucose and lactate metabolism were also determined (Table 1). Concerning glucose transport we found that while no altered mRNA levels of GLUT2 were found, increased SGLT1 mRNA levels were obtained after glucose (alone or with insulin) and insulin treatments. GK mRNA levels were increased after glucose treatment, but decreased when insulin was injected. However, the combination of both resulted in a synergistic effect, increasing mRNA levels up to 40-fold higher than the control group. For G6Pase mRNA levels we found that while glucose stimulates its expression (alone or in combination with insulin) the hormonal treatment alone had an inhibitory effect on mRNA G6Pase levels. On the other hand, MCT-1 mRNA levels were increased by glucose treatment, especially in combination with insulin. Similarly, LDH-A mRNA levels were also enhanced by glucose alone and with insulin injection, while LDH-B transcript levels were down-regulated by the same treatments.

In vivo amino acid oral administrations. Plasma and midgut metabolite levels as well as midgut enzymes activities are shown in Figure 3. Fish receiving oral administration of amino acid mix (Aminoplasmal) were hypoglycemic (Fig. 3A) compared with those receiving only tank water. Moreover, fish receiving also insulin injection with the amino acid treatment
displaying lower glycemia than the other two groups. In contrast to glycemia, plasma lactate levels (Fig. 3B) were differentially regulated by treatments: while the amino acid mix administration caused a decrease in this parameter, the combination with insulin enhanced lactate levels with respect to the control. Midgut glucose (Fig. 3C) and glycogen (Fig. 3D) levels were changed in similar ways when fish were subjected to treatments with decreased levels when the oral amino acid treatment was combined with the insulin injection. In contrast to those results in glucose-related metabolites, G6Pase activity (Fig. 3F) changed in the other direction, being stimulated by insulin plus amino acid treatment when compared with control fish. Other parameters, like midgut lactate levels (Fig. 3E) or FBPase activity (Fig. 3G) were unaffected by the treatments. Finally, activity of enzymes related with amino acid catabolism, like GDH (Fig. 3H) or AlaAT (Fig. 3I) were affected differentially depending on treatments: GDH activity was only increased with the combination of amino acids and insulin, while in contrast AlaAT was stimulated when only the amino acid mix was administrated orally.

In vitro glucose incubations. The first set of experiments with glucose incubations was done in combination with different hormones involved in glucose metabolism, and is shown in Figure 4. We found that glycogen levels (Fig. 4A) in midgut pieces increased in parallel with the amount of glucose in the medium, from 0 to 20 mM. As a general trend, we observed that insulin and C-peptide treatment increase glycogen levels, especially at high glucose concentrations, while glucagon seems to have the opposite effect on the level of this metabolite. For GLP-1 incubations we found a dual effect, with increased glycogen levels at 0 and 5 mM glucose, but decreased at high glucose concentration. Lactate levels (Fig. 4B) in these midgut pieces seem to follow the glucose concentration in the medium, increasing its concentration in a clear dose-dependent manner with respect to glucose in the wells. This trend was not affected by hormone treatments, although some of them (GLP-1, C-peptide) decrease lactate levels with respect to its control, especially at 5 mM glucose. Both GK (Fig. 4C) and HK (Fig. 4D) activities were affected in similar ways by glucose and hormone treatments. Both increased with the amount of glucose present in the medium, although low Km HK activity seems to be saturated already at 5 mM of glucose, while GK responds also to the higher glucose concentration. GK activity was stimulated by glucagon, but in contrast inhibited by GLP-1 (undetectable levels with 20 mM of glucose) and C-peptide treatments. Low Km HK activity was mainly inhibited by all the hormones tested, especially at high glucose concentrations. However, when the medium was free of glucose, some of the hormones were able to increase HK activity, such as insulin and C-peptide. PK activity (Fig. 4E) increased with the presence of glucose in the medium, but the increase was similar with 5
or 20 mM of glucose. Some of the treatments, like insulin, glucagon or C-peptide increased PK activity when glucose was not in the medium, although at 5 and 20 mM glucose most of the treatments resulted in decreased PK activities. FBPase activity (Fig. 4F) in control group was inhibited by 20 mM glucose. Concerning treatments, C-peptide showed a strong inhibitory effect on FBPase activity at all glucose concentrations; the other hormones assessed enhanced the activity of the enzyme with respect to the control group especially at 20 mM glucose. LDH (Fig. 4G) activity was unaffected by the glucose treatment in the control group. However, LDH was inhibited by the C-peptide treatment independently of the glucose concentration.

Another set of experiments was carried out in order to test the transport capacities of the midgut pieces, and thus several metabolic parameters were assessed after incubations with 5 or 20 mM glucose in the presence of two well known blockers of glucose carriers (cytochalasin B for GLUTs and phlorizin for SGLTs) (Table 2). Concerning metabolite levels, free glucose and lactate levels in the tissue decreased at both glucose concentrations for the two treatments assessed. However, glycogen levels remained unaltered. No low Km HK or GK activities were detected at 5 mM glucose when transport was blocked by phorizin. However, under the other conditions, lower activities than in the control group were found, except for HK with cytochalasin B treatment that increased. Similarly, PK, LDH and FBPase activities were generally reduced by the blocking of glucose transport, or in some cases remained unchanged.

Data presented in Figure 5 show lactate studies carried out with midgut pieces incubated with different glucose concentrations at different times. We found that after adding 5 mM glucose to the medium, lactate content in the tissue (Fig. 5A) arose up to 2.5-3 mM and remained stabilized at those values during the 6 hours of the study. On the other hand, lactate levels increased linearly in the medium (Fig. 5B) during the same period of time ($R^2= 0.9917$). After this study, pieces were incubated for 6h with different agents related to lactate metabolism at 3 glucose concentrations. Thus, lactate levels in the tissue (Fig. 5C) increased following glucose concentration in the medium, from 0 to 20 mM glucose. This lactate production (Fig. 5C) was clearly diminished when oxamate was added to the medium, while an important increase (up to 2-fold) was noticed when 4-CIN was added. Lactate levels in the medium also increased when glucose levels increased; however, when oxamate or 4-CIN were added to the treatment, the amount of lactate released decreased (Fig. 5D). We carried out several calculations regarding the percentage of lactate produced/exported by tissue, which are shown in Figures 5E, F, and G. When the medium was free of glucose, the % of
lactate exported was higher than that produced (Fig. 5E), while in the presence of 5 or 20 mM of glucose, this ratio was the opposite (Fig. 5F, G). When 4-CIN was added to the medium the amount of lactate in the tissue increased, while the exported lactate was reduced, especially if the medium was free of glucose. When oxamate was used as treatment, the amount of lactate released was always higher than that present in the tissue, especially when the medium was free of glucose. Finally, LDH activity ($V_{\text{max}}$) (Fig. 5H) was measured in order to correlate its activity with the lactate production. LDH activity was not affected by the presence of glucose in the medium. However, its activity correlates with the glucose concentration in the medium when oxamate was in the medium, although activities were lower than the control at 0 mM glucose, and higher at 5 mM or 20 mM glucose. LDH activity was only lower than the control group when glucose was not added to the medium. Since inhibition of LDH by oxamate is competitive, the LDH $K_m$ was calculated for this treatment: when glucose was present in the medium, oxamate always increased LDH $K_m$.

**In vitro amino acid incubations.** Results obtained from midgut pieces incubated with 5 mM glucose and increasing amino acid concentration in the medium are shown in Figure 6. Lactate levels (Fig. 6A) in the tissue were only affected by amino acid at 2.5x concentration, showing higher levels than the control (0x). Glycogen levels (Fig. 6B) were more affected by treatment, and lower levels than in the control group were found when the amino acid concentration was 1x or 2.5x. Concerning enzymes activities, while FBPase activity (Fig. 6C) was inhibited as amino acid levels increased in the medium, GDH (Fig. 6D) and AlaAT (Fig. 6E) increased with amino acid concentration (the second in a dose-dependent manner).

In the upper panel of Figure 7 gene expression of main proteins involved in the glucosensing mechanism in mammals and fish are shown. The histological (lower panel) study shows the muscle and epithelial layers of midgut strongly stained following the PAS techniques (Fig. 7A). However, the PAS-staining in the muscle layer disappeared after treatment with amylase, indicating the presence of glycogen (Fig. 7B). As shown in fig 7C, the high glycogen accumulation was observed in the cytoplasm of muscle cells. Immunohistochemical tests (lower panel) with antiserum against GK and GLP-1 revealed scarce spindle-shaped positives endocrines cells (Fig. 7 D, G). Positive cells were observed (distributed) in the upper half of the intestinal folds (mucosal folds). In addition immunoreactivity for GK is present in nerve cells and fibers in the submucosa and in the connective axis of the mucosal folds i.e. lamina propria (Fig. 7 E, F). The immunoreactivity for GLP-1 and SGLT1 was always localized in the absorptive cells or enterocytes (Fig. 7 H, I) and in subtle elements (unknown cells) present in the lamina propria.
Discussion

While the contribution of liver, kidney or muscle to glucose homeostasis in carnivorous fish is well known, the relative contribution of the intestine remains to be elucidated. In mammals, it is well known that the gut can contribute to the control of glucose homeostasis by its high glycolytic capacity and a recently described gluconeogenic function (22, 23).

Glucose metabolism in trout gut in vivo. Glucose homeostasis in fish has been often studied using oral glucose challenges (28), but in most of those studies the role of the intestine in glucose homeostasis was ignored. Here we present data demonstrating that the trout gut is a metabolically active tissue, able to transport, use and store glucose. The metabolic changes we found suggest that trout gut is able to regulate its own glucose homeostasis.

After subjecting trout to glucose oral treatment (alone or combined with IP-injected insulin), we found a global metabolic response in gut characterized by increased glycogen and glucose levels, increased glycolytic potential, and unaltered gluconeogenic potential. Both glucose and glycogen levels in midgut paralleled the changes found in glycemia, indicating that the tissue was taking up large amounts of glucose and that some of that glucose is stored in the same tissue, most likely in muscle cells. This is the first time in which this function is attributed to a lower vertebrate’s intestine. Our findings also showed that most of glucose storage is taking place in the intestinal muscle layer, probably for local use during contraction (39). Moreover, glycogen was also found in the enteric nervous system, probably in enteric glial cells (45), that catabolize it to provide lactate. This lactate could be forwarded to neighbouring neurons, a metabolic cooperation between astrocytes and neurons as found in the mammalian brain (4), and probably also in trout brain (43). Glycogen synthase activities were especially sensitive to insulin treatments rather than to glucose administration. Unlike mammalian gut (39), we were unable to find glycogen phosphorylase (GPase) activity in rainbow trout gut, although our findings (Fig. 2F) supported the presence of γ-amylase in trout gut, replacing GPase function, as it does in carp liver (30).

The large increase in free glucose levels in gut after oral glucose treatment suggests also that a large amount of glucose could be oxidized. Accordingly, we found increased potentials for glucose phosphorylation and glycolysis when glycemia rose. All those changes agree with the important glycolytic potential of the small intestine in mammals (1, 49), supporting the hypothesis that fish gut is a major glycolytic tissue also in trout and that a key
role in glucose homeostasis could be expected (22). The fact that lactate levels, LDH activity and mRNA levels, as well as MCT-1 mRNA levels increased only under hyperglycemia, suggests that the enhanced glycolytic potential and lactate production are strongly glucose-dependent. Our results on G6Pase activity and mRNA levels are consistent with the alternative mechanism of glucose transport in mammalian intestine. This involves glucose export to the blood independent of GLUT2 but dependent on phosphorylation by HKs and further dephosphorylation by G6Pase (51). The increased activity and mRNA levels of GK in the trout gut could be explained by the necessity of phosphorylating high amounts of glucose for the above mentioned mechanism.

The main contribution of small intestine in mammals to glucose homeostasis is through glucose production (22). In our study G6Pase activity and mRNA levels seem to be more related to glucose export into the blood stream, than to glucose production de novo. We found no regulation of FBPase activity by glucose, insulin or amino acids. This is consistent with the lack of regulation in gluconeogenesis enzymes reported by Kirchner et al (18) in trout fed diets with or without carbohydrates.

Our results suggest a minor effect of insulin on glucose metabolism in trout gut. The lack of changes in the activities of glucose phosphorylating enzymes suggest that the enhanced lactate production was not due to an increased glucose uptake from the lumen, consistent with the hypoglycemia experienced by insulin-injected fish (48). While in mammals the intestine is an insulin-sensitive organ where insulin has a major role in glucose metabolism (8, 16), this seems to not be the case in trout, where only minor changes were noticed.

**Glucose metabolism in trout gut in vitro.** Due to the complexity of the intestine metabolism in vivo, several sets of in vitro experiments were carried out to elucidate the mechanisms underlying the metabolic changes observed. Even considering that metabolic rates are lower than those of transport rates (48), our findings showed that glucose metabolism in midgut pieces is strongly dependent on glucose transport because specific glucose transport blockers elicited a decrease in glucose concentration, glucose phosphorylation and oxidation, and lactate production.

Because some of the results obtained with the insulin treatment could be due to insulin-induced hypoglycemia, midgut pieces were incubated in the presence of insulin and other glucose metabolism-related hormones, such as glucagon, GLP-1 and C-peptide (Fig. 4). In parallel with the increased glucose concentration in the medium, there was an enhancement of glucose phosphorylation and storage as glycogen, similar to the results obtained in vivo.
after oral glucose treatment. However, data about glycogen regulation in fish intestine is lacking, and its function remains to be elucidated. Although changes in HK and PK activities correlated with glucose levels in the medium, a saturated response was found at 20 mM glucose. Similar changes were found in lactate levels, showing that the excess of phosphorylated glucose not stored as glycogen was likely oxidized through glycolysis. These results support the hypothesis that, as in mammals (1), glucose might make a significant contribution to the energy requirement of the trout intestine.

Under our in vitro conditions, we found that most of the effects observed in vivo were reproduced (G5 and G5 plus insulin), such as the lack of changes in glycogen levels, and GK, PK, FBPase activities, and increased LDH activity. The minor actions of insulin alone in fish intestine are not surprising (48), but contrast with those observed in mammals (54), suggesting that gut glucose metabolism in fish and mammals are differently regulated by insulin. The most relevant effect of glucagon in midgut was the marked glycogenolytic action when glucose was present in the medium. This agrees the major effect described in fish hepatocytes (25), and is consistent with the presence of glucagon receptors in fish intestine (6). However, glycogen stores in trout gut are weak, suggesting that its physiological relevance might be related to a local use, as in skeletal muscle (21). The stimulation of GK activity by glucagon when glucose was in the medium was inconsistent with changes obtained in both glycogen levels and glycolytic potential. Also, the decreased glycolytic potential and higher FBPase activity after glucagon treatment agrees with the scarce glucagon actions reported in the mammalian gut (19) and fish enterocytes (48). Our in vitro results with GLP-1 support information available concerning extrahepatic actions of GLP-1 (25), such as the presence of GLP-1 receptors (55) and the decreased glucose oxidation potential in fish enterocytes (48).

Moreover, most of the actions exerted by GLP-1 in trout are similar to those of glucagon, including increased glycogenolysis and gluconeogenesis, and decreased glycolysis. This supports the idea that in fish gut GLP-1 is overlapping glucagon actions, in a way similar to that observed in black bullhead enterocytes (48) and in fish liver (25). C-peptide actions in fish are almost unknown (26). In the present study the strongest metabolic effect (besides those found after glucose treatment) were those noticed after C-peptide incubations. Unlike in mammalian tissues where C-peptide stimulates glucose transport and utilization (53), in the present study its main actions seem to be of anabolic nature (decreased glycolytic potential). Moreover, glycogen storage was also enhanced when glucose was present in the medium. Because C-peptide in mammals has been shown to interact with insulin receptors (14), and
most of the results observed in the present study are similar to those of insulin (but even more potent) we can hypothesize that in fish intestine C-peptide might exert insulin-like actions. However, more studies are needed to confirm this hypothesis and to explain why a synergistic effect was noticed.

An interesting result obtained in our in vivo studies was the positive correlation between plasma glucose levels and lactate production in intestine. However, because glucose oxidation could be enhanced in enterocytes as an artefact of tissue isolation in vitro, results must be interpreted with caution. Nevertheless, we show clearly that most of this lactate seems to be produced through the glycolytic pathway from glucose, which is relevant for a carnivorous fish like rainbow trout. In addition, some of the components involved in lactate metabolism, such as LDH and MCT-1, were characterized at the molecular level for the first time in fish. We corroborate that the main substrate for this lactate production was glucose, because we found increased lactate production with glucose concentration in the medium and was stable production when other substrates (like amino acids) are available. Because FBPase activity was not modified by glucose treatments we suggest that this lactate is exported to the plasma in vivo to be utilized by other tissues (like the liver) rather than being converted into glucose in situ, as in mammals (24).

The aim of the last set of in vitro incubations was to obtain more information about the putative ability of the trout gut for using amino acids as gluconeogenic substrates (Fig. 6). Despite the decreased FBPase activity (probably due to higher protein content in those treatments in which more amino acids were available rather than to a decreased activity) the lack of regulation in vitro for FBPase activity could be related to its high activity, as in mammals, where this characteristic makes FBPase a non-limiting enzyme for gluconeogenesis. However, no G6Pase or PEPCK activities were detected in our in vitro conditions.

Potential glucosensing capacity of trout gut. In mammalian intestine, sensing of luminal glucose can be accomplished by different cell types (38), including enterocytes, enteroendocrine cells (K and L), and components of the enteric nervous system. The glucosensing ability in mammalian intestine includes key components of the pancreatic glucosensing system (36, 44, 45), and is involved in functions like GLP-1 and GIP secretion. We provide the first evidence in a non-mammalian vertebrate species for the presence in intestine of glucosensing components, including GLUT2, SGLT-1, GK, SUR-like and Kir-like transcripts. Some of those proteins have also been related to glucosensing machinery in other trout tissues like brain regions and endocrine pancreas (40, 41). In mammals the first
Step of intestinal glucosensing involves SGLT-1 and GLUT2 (7). In trout intestine we found both GLUT2 and SGLT-1 transcripts, and also SGLT-1 immunoreactivity was detected in enterocytes and occasionally in enteroendocrine cells. In mammals, studies addressing GK presence in the gut are scarce (15, 36, 44), and its involvement in glucosensing is under debate in K and L cells whereas is clear in GLUTag cells (12). Although the involvement of GK in gut trout glucosensing remains to be demonstrated, the fact that GK gene expression and activity were enhanced in midgut of hyperglycemic trout, and that GK was localized in enteroendocrine cells constitutes the first evidence for such function in putative enteroendocrine cells of trout gut. In rodents and humans K<sub>ATP</sub> components (SUR1 and Kir6.2) were found in both L and K-cells (31, 36, 44). These glucosensing components have been demonstrated to participate in GIP secretion in K-cells (36) and to regulate the sensitivity of L-cells to luminal glucose (44). As far as we are aware, this is the first time in which K<sub>ATP</sub> channel components are found in fish intestine, although their involvement in glucosensing in enteroendocrine cells of trout gut should be addressed in future experiments in which K<sub>ATP</sub>-expressing cells could be isolated from the whole gut.

Although the involvement of enteric neurons in the reception of luminal glucose might be indirect (38), glucose has been shown to directly influence the activity of enteric neurons in mammals though the mechanism underlying this ability remains under debate. Ma and Kirchgessner (20) have detected (as in the present study) transcripts for GLUT2, GK and K<sub>ATP</sub> subunits in rat mucosa and enteric neurons. They present immunolocalization for GK protein in endocrine cells, neurons and varicose nerve fibers of the gut. The fact that in trout gut GK protein was also localized in equivalent cell types strongly support the hypothesis of glucosensing neurons in trout gut. The presence of other components of the glucosensing system in enteric neurons should be addressed in future studies.

Conclusions and perspectives. Glucose homeostasis in carnivorous fish species like rainbow trout has been studied for a long time, and the basis of the so called ‘glucose intolerance’ is still debated (28). Rainbow trout gut displays an important glucose metabolism based on an ability to store glucose as glycogen as well as to a large capacity to oxidize glucose. This is surprising for a fish whose natural diet contains no more than 1% carbohydrates. That a large amount of glucose is oxidized to lactate supports the importance of glucose in gut metabolism, and agrees with the metabolic characteristics of mammalian intestine. Supporting what other authors suggested with dietary trials (18), we confirm the absence of regulation for gluconeogenesis in trout gut, which does not respond to hormone treatments or to changes in glucose, lactate or amino acid levels, either in vivo or in vitro. On
the other hand, we corroborate the minor actions of insulin in trout gut, an insulin-sensitive tissue in mammals, while other hormones like GLP-1 and C-peptide emerge as major hormonal regulators of fish gut glucose metabolism. We present preliminary evidence for the presence of a glucosensor system in trout gut, similar to that described in mammals (45) and in other trout tissues (40). The components involved in such putative function include SGLT-1, GK and K_ATP. GK and SGLT-1 have been localized in specific cells types similar to those involved in glucosensing in mammalian intestine, for example those involved in GLP-1 or GIP secretion, and glucose detection and control of food intake. Further studies are needed to elucidate the precise role of those glucosensing components in specific cell types from trout gut.

Acknowledgements

This study was supported by research grants from Ministerio de Educación y Ciencia and European Fund for Regional Development (AGL2007-65744-C03-01/ACU), Xunta de Galicia (Consolidación e estructuración de unidades de investigación competitivas, INCITE09ENA310002ES), and Universidade de Vigo (Contrato-Programa con grupos de investigación consolidados). S. Polakof was recipient of a postdoctoral fellowship from the Xunta de Galicia (Program Ángeles Alvariño). The authors would like to acknowledge Diego Rodriguez (B. Braun Medical S.A., Spain) for kindly provide us Aminoplasmal® L-12.5 samples, and Dr Stéphane Panserat (INRA St Péé-sur-Nivelle, France) for provide us trout sequences for primer design.
Figure legends

Figure 1. Plasma glucose (A) and lactate (B) levels, and midgut glucose (C), glycogen (D) and lactate (E) levels in 24h fasted trout receiving intraperitoneally 5 mL·kg\(^{-1}\) of saline solution and orally 10 mL·kg\(^{-1}\) of tank water (Con), intraperitoneally 2 IU·kg\(^{-1}\) of insulin and orally tank water (Ins), intraperitoneally saline solution and orally 1g·mL\(^{-1}\) glucose (Glu), and intraperitoneally 2 IU·kg\(^{-1}\) of insulin and orally 1g·mL\(^{-1}\) glucose (Ins+Glu). Each value is the mean ± SEM of n = 8 animals per treatment and time. Different letters indicate significant differences (\(P<0.05\); two-way ANOVA) among groups.

Figure 2. Glucokinase (GK) (A), Low \(K_m\) hexokinase (HK) (B) fructose-1,6-biphosphatase (FBPase) (C), glucose-6-phosphatase (G6Pase) (D), total glycogen synthetase (GSase) (E), \(\gamma\)-amylase (F), pyruvate kinase (PK) (G) and lactate dehydrogenase (LDH) (H) activities in 24h fasted trout receiving intraperitoneally 5 mL·kg\(^{-1}\) of saline solution and orally 10 mL·kg\(^{-1}\) of tank water (Con), intraperitoneally 2 IU·kg\(^{-1}\) of insulin and orally tank water (Ins), intraperitoneally saline solution and orally 1g·mL\(^{-1}\) glucose (Glu), and intraperitoneally 2 IU·kg\(^{-1}\) of insulin and orally 1g·mL\(^{-1}\) glucose (Ins+Glu). Enzyme activities are expressed as mU·mg\(^{-1}\) protein (except U·mg\(^{-1}\) protein for FBPase, LDH and PK). Each value is the mean ± SEM of n = 8 animals per treatment and time. Different letters indicate significant differences (\(P<0.05\); two-way ANOVA) among groups.

Figure 3. Plasma glucose (A) and lactate (B) levels, and midgut glucose (C), glycogen (D) and lactate (E) levels, and glucose-6-phosphatase (G6Pase) (F) fructose-1,6-biphosphatase (FBPase) (G), glutamate dehydrogenase (GDH) (H), and alanine aminotransferase (AlaAT) (I) activities in 24h fasted trout receiving intraperitoneally 5 mL·kg\(^{-1}\) of saline solution and orally 10 mL·kg\(^{-1}\) of tank water (Con), intraperitoneally saline solution and orally Aminoplasmal L-12.5 (AA), and intraperitoneally 2 IU·kg\(^{-1}\) of insulin and orally Aminoplasmal L-12.5 (AA+Ins). Each value is the mean ± SEM of n = 8 animals per treatment and time. Different letters indicate significant differences (\(P<0.05\); two-way ANOVA) among groups.

Figure 4. Glycogen (A) and lactate (B) levels, and glucokinase (GK) (C), low \(K_m\) hexokinase (HK) (D), pyruvate kinase (PK) (E), fructose-1,6-biphosphatase (FBPase) (F) and lactate dehydrogenase (LDH) (G) activities in rainbow trout midgut pieces incubated \textit{in vitro} for 6 h
at 16 °C in modified Cortland medium containing 0, 5 or 20 mM glucose alone (control) or with 10⁻⁸ M bovine insulin, 10⁻⁸ M human glucagon, 10⁻⁹ M rat GLP-1, and 10⁻⁹ M rat C-peptide. Each value is the mean ± S.E.M. of ten (control) or 3 (treatments) independent experiments. *, Significantly different from control within each glucose concentration (P<0.05); #, Significantly different from groups incubated without glucose at the same treatment (P<0.05); †, significantly different from groups incubated with 5 mM glucose at the same treatment (P<0.05). When necessary values were log transformed prior to statistical analysis.

Figure 5. (A) Time course of lactate levels in midgut pieces of rainbow trout incubated in vitro for 6 h at 16 °C in modified Cortland medium containing 5 mM glucose. (B) Time course of lactate released to the medium by midgut pieces of rainbow trout incubated in vitro for 6 h at 16 °C in modified Cortland medium containing 5 mM glucose. Lactate levels in tissue (C) and released to the medium (D) after incubating midgut pieces of rainbow trout for 6 h at 16 °C in modified Cortland medium containing 0, 5 or 20 mM glucose alone (control) or 1 mM α-cyano-4-hydroxy cinnamate and 50 mM sodium oxamate. In Figs. 5E, F, and G we show the percentage of lactate in the tissue or exported to the medium after incubating midgut pieces of rainbow trout for 6 h at 16 °C in modified Cortland medium containing 0 (E), 5 (F) or 20 (G) mM glucose alone (control) or 1 mM α-cyano-4-hydroxy cinnamate and 50 mM sodium oxamate. Lactate dehydrogenase (LDH) (H) activity in midgut pieces of rainbow trout incubated in vitro for 6 h at 16 °C in modified Cortland medium containing 0, 5 or 20 mM glucose alone (control) or 1 mM α-cyano-4-hydroxy cinnamate and 50 mM sodium oxamate. Km values are shown in the annex table.

Figure 6. Lactate (A) and glycogen levels (B), and fructose-1,6-biphosphatase (FBPase) (C), glutamate dehydrogenase (GDH) (D), and alanine aminotransferase (AlaAT) (E) activities in midgut pieces of rainbow trout incubated in vitro for 6 h at 16 °C in modified Cortland medium containing 5 mM glucose alone (0x) or serial dilutions of Aminoplasmal L-12.5: 1:20 (1x), 1:8 (2.5x), and 1:4 (5x).

Figure 7. Upper panel: Semi-quantitative gene expression of proteins involved in glucosensing: GLUT2, SGLT1, GK, Kir-like and SUR-like. Lower panel: histological sections of midgut. Glycogen accumulation mostly in muscle layers shown with periodic acid-Schiff (PAS) staining (A). After digestion in amylase, the PAS-staining is present only in
epithelial mucous cells (B). High magnification of glycogen accumulation in the cytoplasm of muscle cells (C). Immunoreactivity for GK (D, E, F), GLP-1 (G, H) and SGLT1 (I) is shown. Immunoreactivity is clearly visible in endocrine cells for GK and GLP-1 (D, G), and in enterocytes for GLP1 and SGLT-1 (H, I). GK-immunoreactivity was detected in nerve cells and fibers localized in submucosa and lamina propria of mucosal folds (D, F). Note the weak immunoreactivity to GLP-1 and SGLT1 found in the epithelials folds. (H, I). Scale Bar: 250 µm (A,B), 25 µm (C-H), 50 µm (I).
Table 1. Effects of intraperitoneal administration of saline solution or insulin in fish (n=6) receiving orally water tank or glucose solution on level of expression of mRNA levels of encoding midgut genes.

<table>
<thead>
<tr>
<th></th>
<th>Glucose oral</th>
<th>Insulin IP</th>
<th>Insulin IP-Glucose oral</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUT2</td>
<td>-1.12</td>
<td>-1.04</td>
<td>-1.42</td>
</tr>
<tr>
<td>SGLT1</td>
<td>+1.79*</td>
<td>+1.96*</td>
<td>+1.59*</td>
</tr>
<tr>
<td>GK</td>
<td>+8.47*a</td>
<td>-13.07*b</td>
<td>+42.37*c</td>
</tr>
<tr>
<td>G6Pase</td>
<td>+3.00*a</td>
<td>-2.47*b</td>
<td>+6.74*c</td>
</tr>
<tr>
<td><strong>Lactate metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT1</td>
<td>+1.84*ab</td>
<td>+1.02a</td>
<td>+2.70*b</td>
</tr>
<tr>
<td>LDH-chain A</td>
<td>+2.86*a</td>
<td>+3.05a</td>
<td>+6.20*b</td>
</tr>
<tr>
<td>LDH-chain B</td>
<td>-1.60*</td>
<td>-1.23</td>
<td>-1.71*</td>
</tr>
</tbody>
</table>

GLUT2 (glucose facilitative transporter type 2) SGLT-1 (sodium-dependent glucose cotransporter type 1), GK (glucokinase), G6Pase (glucose 6-phosphatase), MCT-1 (monocarboxylate transporter type 1), LDH-A (lactate dehydrogenase, chain A), LDH-B (lactate dehydrogenase, chain B). Results are expressed as fold variation of the saline-injected treated group and were analyzed by one-way ANOVA followed by Student–Newman–Keuls comparison test. *Significant different from the control group (saline IP-tank water oral) ($P<0.05$). Different letters indicate significant difference among treatment groups.
Table 2. Enzymes activities and metabolites levels in midgut pieces of rainbow trout incubated in vitro for 6 h at 16°C in modified Cortland medium containing 5 or 20 mM glucose alone (control) or with 1 mM phlorizin or 10 µM cytochalasin B. Each value is the mean ± SEM of 10 (control) or 3 (treatments) independent experiments. Data is expressed as percentage of increase (+) or decrease (-) vs. pieces incubated with glucose alone. Each value is the mean ± S.E.M. of ten (control) or 3 (treatments) independent experiments. *, Significantly different from control within each glucose concentration (P<0.05) (t-Student). When necessary values were log transformed prior to statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Glucose 5 mM</th>
<th>Glucose 20 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phlorizin</td>
<td>Cytochalasin B</td>
</tr>
<tr>
<td>Glucose</td>
<td>-287*</td>
<td>-139*</td>
</tr>
<tr>
<td>Glycogen</td>
<td>+184</td>
<td>+219</td>
</tr>
<tr>
<td>Lactate</td>
<td>-436*</td>
<td>-223*</td>
</tr>
<tr>
<td>HK</td>
<td>n.d.</td>
<td>+130*</td>
</tr>
<tr>
<td>GK</td>
<td>n.d.</td>
<td>-145*</td>
</tr>
<tr>
<td>PK</td>
<td>-217*</td>
<td>-179</td>
</tr>
<tr>
<td>LDH</td>
<td>-143*</td>
<td>-140*</td>
</tr>
<tr>
<td>FBPase</td>
<td>-132*</td>
<td>-162*</td>
</tr>
</tbody>
</table>
References


Figure 1

A - Intestine lactate levels (μmol·g⁻¹ wet wt)
B - Intestine glucose levels (μmol·g⁻¹ wet wt)
C - Plasma glucose levels (mM)
D - Intestine glycogen levels (μmol glycosyl units·g⁻¹ wet wt)

Treatment:
- Con
- Ins
- Glu
- Ins+Glu
Figure 3

- Plasma glucose levels (mM)
  - A: Comparison of plasma glucose levels across different treatments.

- Intestine glucose levels (μmol·g⁻¹ wet wt)
  - C: Comparison of intestine glucose levels across different treatments.

- Intestine lactate levels (μmol·g⁻¹ wet wt)
  - E: Comparison of intestine lactate levels across different treatments.

- G6Pase activity (mU·mg⁻¹ protein)
  - F: Comparison of G6Pase activity across different treatments.

- FBPase activity (U·mg⁻¹ protein)
  - G: Comparison of FBPase activity across different treatments.

- GDH activity (U·mg⁻¹ protein)
  - H: Comparison of GDH activity across different treatments.

- AlaAT activity (U·mg⁻¹ protein)
  - I: Comparison of AlaAT activity across different treatments.
Figure 4
Figure 6
Polakof et al. Figure 7