

## RESEARCH ARTICLE

# ACTH-stimulated cortisol release from head kidney of rainbow trout is modulated by glucose concentration

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### SUMMARY

To assess the hypothesis that cortisol release in rainbow trout is modulated by glucose levels, we first evaluated cortisol release [basal and adrenocorticotrophic hormone (ACTH)-regulated] by head kidney tissue superfused with medium reflecting hypoglycaemic, normoglycaemic or hyperglycaemic conditions. Next, cortisol release from head kidney fragments in static incubations was assessed in parallel with changes in parameters related to cortisol synthesis (mRNA abundance of StAR, P450scc, 3 $\beta$ HSD and 11 $\beta$ H) and the GK-mediated glucosensing mechanism (levels of glycogen and glucose, activities of GK, GSase and PK, and mRNA levels of GK, GLUT-2, Kir6.x-like and SUR-like). We then evaluated the effects of two inhibitors of glucose transport, cytochalasin B and phlorizin, on cortisol production and glucosensing mechanisms. The ACTH-induced release of cortisol proved to be modulated by glucose concentration such that increased release occurs under high glucose levels, and decreased ACTH-stimulated cortisol release occurs when glucose transport is inhibited by cytochalasin B. The release of cortisol can be associated with increased synthesis as enhanced mRNA abundance of genes related to cortisol synthesis was also noted in high glucose medium. Specific GK immunoreactivity in the cortisol-producing cells (not in chromaffin cells) further substantiates GK-mediated glucosensing in cortisol production. In contrast, no changes compatible with those of glucose levels and cortisol release/synthesis in the presence of ACTH were noted for any other putative glucosensor mechanisms based on LXR, SGLT-1 or Gnat3. These combined results are the first evidence for a mechanism in fish linking the synthesis and release of a non-pancreatic hormone like cortisol with circulating glucose levels. The relationship was evident for the regulated (ACTH-dependent) pathway and this suggests that under acute stress conditions glucose is important for the regulation of cortisol synthesis and release.

Key words: rainbow trout, head kidney, cortisol, glucose.

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### INTRODUCTION

Cortisol is the main steroid produced in fish head kidney, which harbours the inter-renal cells, homologues of the mammalian adrenal zona fasciculata cells. In fish, cortisol plays an important role in energy homeostasis, growth and osmoregulation (Mommsen et al., 1999). The main secretagogue for cortisol is adrenocorticotrophic hormone (ACTH) released from the anterior pituitary; ACTH release, in turn, is controlled by corticotropin releasing factor (CRF) produced by the hypothalamus (Wendelaar Bonga, 1997). The main proteins involved in the regulation of the corticosteroidogenic pathway (Payne and Hales, 2004) comprise steroidogenic acute regulatory (StAR) protein, cytochrome P450 cholesterol side-chain cleavage (P450scc), 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD) and 11 $\beta$ -hydroxylase (11 $\beta$ H).

In previous studies in rainbow trout hypothalamus, hindbrain and Brockmann bodies, we demonstrated the existence of a glucosensor mechanism dependent on glucokinase (GK), glucose facilitative transporter type 2 (GLUT-2) and the ATP-dependent inward rectifier potassium channel ( $K_{ATP}$ ) (for review, see Polakof et al., 2011). This glucosensor system is adjusted under stress conditions, like those associated with high stocking density, resulting in its inability

to respond to changes in circulating glucose levels (Conde-Sieira et al., 2010a; Conde-Sieira et al., 2010b). The adjustment of the glucosensor in central areas (hypothalamus and hindbrain) under stress conditions is apparently mediated by CRF as treatment with this neuropeptide affects the sensitivity of the glucosensing mechanism in a similar way to that observed under stress conditions (Conde-Sieira et al., 2011). In these studies we observed a very complex interaction among cortisol levels, glycaemia and stress (Conde-Sieira et al., 2010a; Conde-Sieira et al., 2010b). Therefore, we hypothesized that cortisol synthesis and release in inter-renal tissue may be modulated by circulating glucose levels, i.e. that some type of glucosensor mechanism is operational in inter-renal cells of rainbow trout, in a similar way to that characterized in pancreatic  $\beta$ -cells, where the glucosensor system is related to insulin release. In other studies carried out in rainbow trout, plasma cortisol levels decreased under conditions known to produce hypoglycaemia such as food deprivation (Pottinger et al., 2003; Polakof et al., 2007a), whereas plasma cortisol levels increased under hyperglycaemic conditions such as post-feeding (Bry, 1982; Holloway et al., 1994) or exercise (Nielsen et al., 1994). A recent study carried out in zebrafish provided direct evidence for a relationship between

cortisol levels and glycaemia as it was found that levels of whole-body cortisol were dependent on glucose (Powers et al., 2010). In mammals, cortisol secretion from the adrenal cortex has also been hypothesized to be related to glycaemic conditions (Andrews et al., 2002), though evidence obtained to date is not conclusive as cortisol levels do not change under hyperglycaemic conditions alone (Andrews et al., 2002), requiring an additional stress to increase (Kirschbaum et al., 1997).

To assess the hypothesis that glucose levels modulate cortisol release in rainbow trout, in a first experiment we evaluated changes in cortisol release (in the absence or presence of ACTH) when head kidneys were superfused with media mimicking conditions of hypoglycaemic, normoglycaemic and hyperglycaemic conditions (2, 4 and 8 mmol l<sup>-1</sup> glucose, respectively) as previously described (Polakof et al., 2007b). As changes were noted, we carried out a second experiment in which cortisol release from head kidney slices incubated *in vitro* was evaluated in parallel with changes in parameters related to cortisol synthesis (mRNA abundance of StAR, P450<sub>scc</sub>, 3βHSD and 11βH) and to the glucosensor characterized in rainbow trout (Polakof et al., 2011), including levels of glycogen and glucose, activities of GK, glycogen synthase (GSase) and pyruvate kinase (PK), and mRNA levels of GK, GLUT-2, inward rectifier K<sup>+</sup> channel pore type 6-like (Kir6.x-like) and sulfonylurea receptor-like (SUR-like). In addition, we evaluated in the same experiment the presence of components of other putative glucose sensor mechanisms, similar to those described in mammals. These include (1) the electrogenic sensor (González et al., 2009), evaluated by the mRNA abundance of sodium–glucose-linked transporter type 1 (SGLT-1); (2) the nuclear receptor sensor (Mitro et al., 2007), evaluated by the mRNA abundance of liver X receptor (LXR); and (3) the taste receptor sensor (Nakagawa et al., 2009), evaluated by the mRNA abundance of gustducin (Gnat3). In a third series of experiments, we evaluated changes in the same parameters described in experiment 2 but in the presence of two well-known inhibitors of glucose transport, *viz.* cytochalasin B (GLUT inhibitor) and phlorizin (SGLT inhibitor). Finally, we carried out histochemical studies to substantiate and localize several of the main components of the glucosensing in head kidney tissue.

## MATERIALS AND METHODS

### Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from local fish farms either in The Netherlands (Forellenkwekerij De Keijzersberg, Blitterswijck, experiment 1) or Spain (A Estrada, experiments 2 and 3, and immunohistochemical studies). Fish were maintained for 15 days under laboratory conditions and a 12 h:12 h light:dark photoperiod in dechlorinated tap water at 17°C. Fish mass was 149±4 g. Fish were fed once daily (10:00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg, Segovia, Spain; proximate food analysis: 48% crude protein, 14% carbohydrates, 25% crude fat and 11.5% ash; 20.2 MJ kg<sup>-1</sup> of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/EU), and of the Governments of The Netherlands (code 2011139.a, Lelystad) and Spain (RD1201/2005) for the use of animals in research. Animal protocols were approved by the Animal Care Committees at the Universities of Nijmegen and Vigo.

### Experimental design

We used head kidney tissue (acknowledging the heterogeneity of the tissue) rather than isolated inter-renal cells to avoid possible dedifferentiation, which is usually associated with cell isolation. In addition to the convenience of collecting head kidneys as a source

of cortisol-producing cells, the specificities of our assays and histology allowed us to define our findings unequivocally.

### Experiment 1: *in vitro* superfusion of head kidneys with different glucose concentrations in the absence/presence of ACTH

Fish were fasted for 24 h before treatment to ensure basal hormone levels were achieved. After anaesthesia with 2-phenoxyethanol (0.5% v/v), fish were killed by decapitation, and head kidneys (left and right) were removed carefully and placed on a cheese-cloth filter in a superfusion chamber gassed with a 0.5% CO<sub>2</sub>/99.5% O<sub>2</sub> mixture. Tissues (~100 mg) were superfused with modified Hanks' medium (92.56 mmol l<sup>-1</sup> NaCl, 3.63 mmol l<sup>-1</sup> KCl, 2.81 mmol l<sup>-1</sup> NaHCO<sub>3</sub>, 0.85 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 0.55 mmol l<sup>-1</sup> MgSO<sub>4</sub>, 0.4 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.23 mmol l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 7.5 mmol l<sup>-1</sup> Hepes, 0.03% (w/v) BSA, pH 7.15) at three different concentrations of D-glucose: 2, 4 and 8 mmol l<sup>-1</sup> (indicative of hypoglycaemic, normoglycaemic and hyperglycaemic conditions in rainbow trout, respectively), which was pumped through the chambers at 30 μl min<sup>-1</sup> by a multichannel peristaltic pump (Watson-Marlow, Wilmington, MA, USA). All glucose treatments were evaluated in each replicate of head kidneys used. After 150 min, when cortisol release had reached a steady state, the medium was supplemented for 20 min with human ACTH (Sigma, St Louis, MO, USA) at a concentration of 3.3×10<sup>-7</sup> mol l<sup>-1</sup> determined in a previous assay, in agreement with previous studies (Brodeur et al., 1998), except in control chambers, where no ACTH was supplied. This procedure was repeated eight times for each glucose concentration (2, 4 and 8 mmol l<sup>-1</sup>). Fractions (5, 10 or 30 min) were collected over 4 h and stored at -80°C for cortisol assay. The area under the curve (AUC) was calculated following the trapezoidal rule using SigmaPlot software.

**Experiment 2: *in vitro* incubation of head kidney tissue with different glucose concentrations in the absence/presence of ACTH**  
The superfusion experiments described above are not suitable for evaluating the mechanisms involved in the relationship between cortisol synthesis and release and glucose levels; thus, static incubations were used to explore the underlying mechanisms.

Every morning of an experiment, fish that had been fasted for 24 h (to ensure basal levels of metabolic hormones were achieved) were dip-netted, anaesthetized with MS-222 (50 mg l<sup>-1</sup>) buffered to pH 7.4 with sodium bicarbonate, weighed and killed by decapitation. Head kidneys were removed and rinsed by immersion in modified Hanks' medium. In order to have enough mass, tissues were pooled from 3–4 fish. Tissues from each pool were finely sliced in a chilled Petri dish and mixed, and then placed in other Petri dishes containing 100 ml of modified Hanks' medium per g tissue (gassed with a 0.5% CO<sub>2</sub>/99.5% O<sub>2</sub> mixture) at three different concentrations of D-glucose: 2, 4 and 8 mmol l<sup>-1</sup> for 1.5 h to ensure basal levels of cortisol release. After this time, tissue was placed in 48-well culture plates (25 mg of tissue in 250 μl of modified Hanks' medium per well) with 2, 4 and 8 mmol l<sup>-1</sup> glucose alone or in the presence of 3.3×10<sup>-7</sup> mol l<sup>-1</sup> (3.3×10<sup>5</sup> IU) ACTH to assess differences in response to glucose under constitutive (basal) and regulated (ACTH-stimulated) conditions. All glucose treatments were assessed at the same time in each replicate of head kidneys used. After 10, 30 or 60 min incubation, tissues were quickly removed, snap-frozen in liquid nitrogen and stored at -80°C until assayed; the medium was also taken for cortisol assessment. The sampling times used were selected based on the dynamics of cortisol release observed in experiment 1 after addition of ACTH.

For each experiment, two different sets of tissue pools (2 treatments × 3 glucose concentrations × 3 sampling times) were

used. The first set was used to assess enzyme activities (GK, GSase and PK) and metabolite levels (glucose and glycogen) whereas the second set was used for quantification of mRNA abundance. Cortisol levels in the medium were evaluated in all samples from the two sets used. The same procedure was performed in four independent replicates per set ( $N=4$ ).

Experiment 3: *in vitro* incubation of head kidney tissue with  $8\text{ mmol l}^{-1}$  glucose with inhibitors of glucose transport in the presence of ACTH

Samples were obtained and processed as described in experiment 2. Tissue was placed in 48-well culture plates (25 mg of tissue in  $250\ \mu\text{l}$  of modified Hanks' medium per well) with  $8\text{ mmol l}^{-1}$  glucose alone (control) or in the presence of  $3.3\times 10^{-7}\text{ mol l}^{-1}$  ACTH, or  $3.3\times 10^{-7}\text{ mol l}^{-1}$  ACTH and  $10\ \mu\text{mol l}^{-1}$  cytochalasin B, or  $3.3\times 10^{-7}\text{ mol l}^{-1}$  ACTH and  $1\text{ mmol l}^{-1}$  phlorizin. The concentrations of inhibitors were selected based on previous studies on glucose transport in fish (Soengas and Moon, 1995; Soengas and Moon, 1998). After 10, 30 or 60 min incubation, tissues were quickly removed, snap-frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until assayed; the medium was also taken for cortisol assessment.

In each experiment, two different sets of tissue pools were used. The first set was used to assess enzyme activities (GK, GSase and PK) and metabolite levels (glucose and glycogen) whereas the second set was used for quantification of mRNA abundance. Cortisol levels in the medium were evaluated in all samples from the two sets used. The same procedure was performed in four independent replicates per set ( $N=4$ ).

### Analytical procedures

#### Cortisol measurement

In experiment 1, cortisol was measured by radioimmunoassay (RIA). A 96-well micro-assay plate was incubated overnight at  $4^\circ\text{C}$  with  $100\ \mu\text{l}$  of cortisol antibody (Abcam, Cambridge, UK) diluted 1:2000 in coating buffer ( $50\text{ mmol l}^{-1}\ \text{NaHCO}_3$ ,  $50\text{ mmol l}^{-1}\ \text{Na}_2\text{CO}_3$  and  $0.02\% \text{ NaN}_3$ ). The plate was washed with wash buffer ( $100\text{ mmol l}^{-1}$  Tris,  $0.9\% \text{ NaCl}$  and  $0.02\% \text{ NaN}_3$ ) then incubated for 1 h at  $37^\circ\text{C}$  with  $100\ \mu\text{l}$  of blocking buffer (normal calf serum at  $0.25\%$  in wash buffer);  $10\ \mu\text{l}$  of standards, plasma or superfusion fractions was incubated for 4 h with  $^3\text{H}$ -cortisol tracer (Perkin Elmer, Waltham, MA, USA) diluted in assay buffer ( $100\text{ mmol l}^{-1}$  Tris,  $0.9\% \text{ NaCl}$ ,  $0.02\% \text{ NaN}_3$  and  $0.1\% \text{ ANS}$ , pH 7.4 with HCl). This RIA had been validated before for the measurement of cortisol in culture medium (Gorissen et al., 2012). In experiments 2 and 3, cortisol levels were

measured by ELISA using a commercially available kit (Cayman, Ann Arbor, MI, USA).

#### Assessment of metabolite levels and enzyme activities

Samples used ( $\sim 20\text{ mg}$ ) to assess metabolite levels were homogenized immediately by ultrasonic disruption in  $7.5\text{ vol. ice-cold } 6\% \text{ perchloric acid}$ , and neutralized (using  $1\text{ mol l}^{-1}$  potassium bicarbonate). The homogenate was centrifuged, and the supernatant used to assay tissue metabolites. Tissue glycogen levels were assessed following a previously described method (Keppler and Decker, 1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit (Biomérieux, Grenoble, France). Samples for enzyme activities ( $\sim 25\text{ mg}$ ) were homogenized by ultrasonic disruption with  $9\text{ vol. ice-cold buffer}$  consisting of  $50\text{ mmol l}^{-1}$  Tris (pH 7.6),  $5\text{ mmol l}^{-1}$  EDTA,  $2\text{ mmol l}^{-1}$  1,4-dithiothreitol and protease inhibitor cocktail (Sigma). The homogenate was centrifuged and the supernatant used immediately for enzyme assays. Enzyme activities were determined using a microplate reader INFINITE 200 Pro (Tecan, Männedorf, Switzerland) and microplates. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at  $340\text{ nm}$ . The reactions were started by the addition of supernatant ( $15\ \mu\text{l}$ ) at a pre-established protein concentration, omitting the substrate in control wells (final volume  $265\text{--}295\ \mu\text{l}$ ), and allowing the reactions to proceed at  $20^\circ\text{C}$  for pre-established times ( $3\text{--}10\text{ min}$ ). Enzyme activities are expressed relative to protein content of the sample (activity per mg protein). Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with BSA (Sigma) as standard. GK (EC 2.7.1.2), GSase (EC 2.4.1.11) and PK (EC 2.7.1.40) activities were determined as described previously (Polakof et al., 2007a; Polakof et al., 2008a; Polakof et al., 2008b; Polakof et al., 2008c). Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations.

#### mRNA abundance analysis by real-time quantitative RT-PCR

Total RNA was extracted from tissues ( $\sim 20\text{ mg}$ ) using Trizol reagent (Life Technologies, Grand Island, NY, USA) and treated with RQ1-DNase (Promega, Madison, WI, USA). A  $2\ \mu\text{g}$  sample of total RNA was reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies) and random hexaprimers (Life Technologies). Gene expression levels were determined by real-time quantitative RT-PCR (qPCR) using the iCycler iQ (Bio-Rad, Hercules, CA, USA). Analyses were performed on  $1\ \mu\text{l}$  cDNA

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT-PCR (qPCR)

	Forward primer	Reverse primer	Annealing temperature ( $^\circ\text{C}$ )
EF1 $\alpha$	GGGCAAGGGCTCTTTCAAGT	CGCAATCAGCCTGAGAGGT	60
GK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCCTTGGTCCAG	60
GLUT-2	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	59
Gnat3	GCAAGACGTGCTGAGGACCA	ATGGCGGTGACTCCCTCAA	60
3 $\beta$ HSD	TCACAGGGTCAACGTCAAAG	CCTCCTTCTTGGTCTTGCTG	56
11 $\beta$ H	ATTTGCCCTGTACGAGTTGG	GGATGATGATGTCTCTGACTG	56
Kir6.x-like	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACCTGGA	60
LXR	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	62
P450scc	ATGCGTCAGGACACTAACAC	CAGCGGTATCATCTTCAGCA	56
SGLT-1	GGCTGAAACATCTACCTTGCT	CTCATAACCTCCCACCTCATTG	59
StAR	CTTCTACAGACATGAGGAAC	GCCTCCTCCTCCCTGCTTAC	56
SUR-like	CGAGGACTGGCCCCAGCA	GACTTCCACTTCTGTGCGTCC	62

EF1 $\alpha$ , elongation factor 1 $\alpha$ ; GK, glucokinase; GLUT-2, glucose facilitative transporter type 2; Gnat3, gustducin; 3 $\beta$ HSD, 3 $\beta$ -hydroxysteroid dehydrogenase; 11 $\beta$ H, 11 $\beta$ -hydroxylase; Kir6.x-like, inward rectifier  $\text{K}^+$  channel pore type 6-like; LXR, liver X receptor; P450scc, cytochrome P450 cholesterol side-chain cleavage; SGLT-1, sodium-glucose-linked transporter type 1; StAR, steroidogenic acute regulatory protein; SUR-like, sulfonylurea receptor-like.



using the MAXIMA SYBR Green qPCR Mastermix (Fermentas, Vilnius, Lithuania), in a total PCR reaction volume of 15  $\mu$ l, containing 50–500  $\text{nmol l}^{-1}$  of each primer. The abundance of GK, GLUT-2, Kir6.x-like and SUR-like mRNAs was determined as previously described (Polakof et al., 2008a; Polakof et al., 2008c); mRNA abundance of  $3\beta$ HSD,  $11\beta$ H, LXR, P450<sub>scc</sub>, SGLT-1 and StAR was determined as previously described in the same species (Geslin and Auperin, 2004; Geurden et al., 2007; Cruz-Garcia et al., 2009) and mRNA abundance of Gnat3 was determined using specific primers developed for rainbow trout based on available sequences (CU073912, Sigenae database, INRA; courtesy of Dr S. Polakof, INRA Clermont/Theix, France). Relative quantification of the target gene transcripts was done using elongation factor 1 $\alpha$  (EF1 $\alpha$ ) gene expression as a reference, which was stably expressed in this experiment. Sequences of the forward and reverse primers used for each gene expression are shown in Table 1.

Thermal cycling was initiated with incubation at 95°C for 15 min using hot-start iTaq DNA polymerase activation; 40 steps of PCR were performed, each consisting of denaturation at 95°C for 15 s, annealing at specific temperatures (Table 1) for 30 s, and extension at 72°C for 30 s. Following the final PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C s<sup>-1</sup> from 55 to 95°C) to ensure that only one fragment was amplified. Each sample was analysed in triplicate. All the replicates of each sample for each gene were located on the same plate to allow comparisons. In all plates, we included the standard curve (in triplicate), and no-template and no-reverse transcriptase controls (in duplicate). Only efficiency values between 85% and 100% were accepted (the  $R^2$  for all genes assessed was always higher than 0.985). Relative quantification of the target gene transcript with the EF1 $\alpha$  reference gene transcript was made following a previous method (Pfaffl, 2001).

#### Immunohistochemistry

Fish were anaesthetized with MS-222, their head kidneys were excised, and small pieces were immersion-fixed in Bouin's fluid or in 4% paraformaldehyde in 0.1  $\text{mol l}^{-1}$  phosphate-buffered saline (PBS) at pH 7.4 for 24 h at 4°C. Pieces were then paraffin embedded and 6–12  $\mu$ m thick sections were stained using haematoxylin and eosin to study the overall structure. Other head kidney pieces were cryo-protected in 30% sucrose and were embedded in Tissue-Tec OCT compound (Sakura, Torrance, CA, USA). Horizontal and transverse sections (20  $\mu$ m) were made on a cryostat. The primary antibodies used in this study were polyclonal rabbit anti-GK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-SGLT-1 (Millipore, Billerica, MA, USA) and monoclonal mouse anti-TH (tyrosine hydroxylase, Millipore). The specificity of GK and SGLT-1 antibodies was previously tested by western blotting (Polakof et al., 2010).

Sections were processed as follows: (1) blocking endogenous peroxidase activity with 3% H<sub>2</sub>O<sub>2</sub> for 30 min; (2) pre-incubation with 0.1% BSA for 1 h, to inhibit non-specific reactivity; (3) incubation with anti-GK (1:100), anti-SGLT-1 (1:100) and anti-TH (1:200) antibodies overnight at room temperature in a humid chamber; (4) incubation with biotinylated goat anti-rabbit IgG (GAR 1:100; Vector, Burlingame, CA, USA) for GK and SGLT-1 and biotinylated goat anti-mouse IgG (GAM; Sigma, 1:100) for TH; and (5) rinsing in PBS followed by incubation with ABC-complex (1:100; Vector). The reaction was developed by incubation with 3,3'-diaminobenzidine (DAB; Sigma, 0.003%) and H<sub>2</sub>O<sub>2</sub> (0.01%). All dilutions were made in PBS. Finally, some sections were counterstained with Mayer's haematoxylin solution, dehydrated and

coverslipped with DEPEX mounting medium. Negative controls were run by omitting primary antisera during incubation of tissues.

To study co-localization of GK, SGLT-1 and TH, selected sections were processed for double immunofluorescence following steps 1–2 above. Then, sections were incubated in a cocktail of SGLT-1/TH or GK/TH antibodies (dilution already described) and then with a cocktail of secondary specific antibodies (Alexa Fluor 488 GAR-conjugated and Alexa Fluor 594 GAM-conjugated, Life Technologies) diluted 1:400 in PBS. After incubation, sections were washed in PBS and coverslipped using Prolong Gold with DAPI (Life Technologies) to delay fluorescence fading. For co-localization of SGLT-1/GK, sections were incubated sequentially with anti-SGLT1 antibody and secondary antibody (Alexa Fluor 488 GAR-conjugated), observed and photographed. The same sections were then incubated with anti-GK followed by secondary specific antibody (Alexa Fluor 594 GAR-conjugated).

Slides were observed and photographed with an Olympus photomicroscope (BX51) equipped with a digital camera (Olympus DP71). Confocal images were acquired with a spectral laser confocal microscope Leica SP5X.

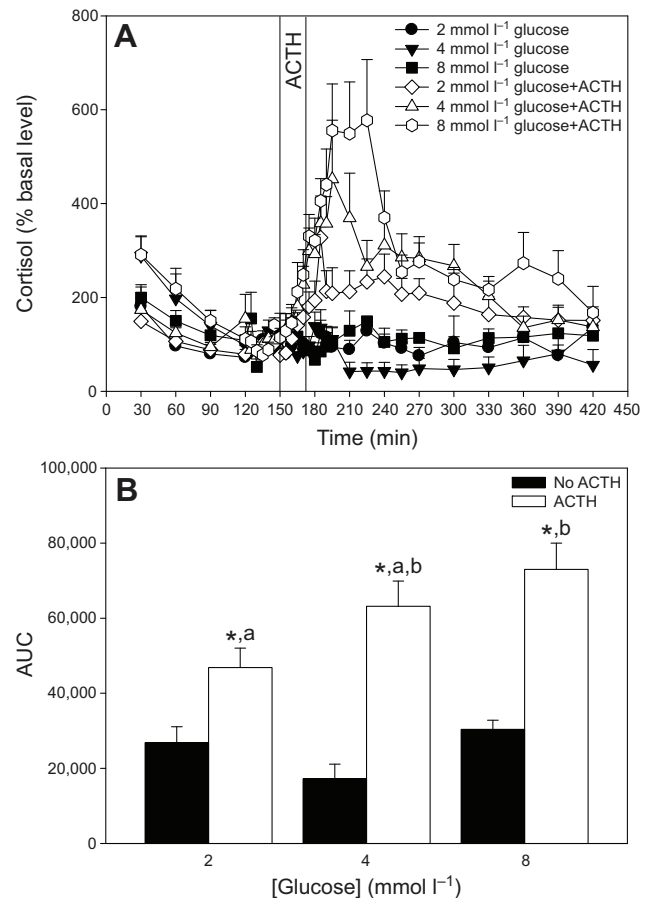


Fig. 1. Time course (A) and area under curve (AUC) of the time course (B) of cortisol levels released to the incubation medium by head kidneys of rainbow trout superfused at 15°C in modified Hanks' medium containing 2, 4 or 8  $\text{mmol l}^{-1}$  D-glucose alone or in the presence (after 150 min preincubation) of  $3.3 \times 10^{-7}$   $\text{mol l}^{-1}$  ACTH for 20 min. Each value is the mean + s.e.m. of eight independent experiments. Percentage basal level is the average cortisol level measured in the incubation medium once the superfusion became stabilized (in the interval between 120 and 150 min). In B, different letters indicate significant differences ( $P < 0.05$ ) among glucose concentrations within each treatment. \*Significantly different from group without ACTH at the same glucose concentration.

Table 2. *P*-values obtained after three-way ANOVA of parameters assessed in head kidneys of rainbow trout in experiment 2

Parameter	Time	ACTH	Glucose	Glucose × time	Glucose × ACTH	Time × ACTH	Glucose × ACTH × time
<b>Cortisol metabolism</b>							
Cortisol levels	<0.001	0.028	0.045	<0.001	–	0.051	0.037
StAR mRNA	0.015	0.039	0.046	–	–	0.017	–
P450scc mRNA	0.005	0.047	0.031	–	0.038	0.021	–
3βHSD mRNA	0.036	0.033	0.037	–	–	0.067	–
11βH mRNA	0.012	0.042	0.022	–	0.045	<0.001	–
<b>Glucosensing</b>							
Glucose	–	0.050	<0.001	–	–	0.002	<0.001
Glycogen	–	–	0.043	–	–	–	–
GK activity	<0.001	–	0.014	0.001	–	0.012	–
GSase activity	0.046	–	<0.001	0.012	–	–	–
PK activity	0.011	–	–	–	–	–	–
GK mRNA	0.016	–	0.041	–	0.033	–	–
GLUT-2 mRNA	<0.001	–	–	–	–	–	–
Kir6.x-like mRNA	<0.001	0.043	0.021	–	0.002	0.046	0.006
SUR-like mRNA	0.008	0.039	0.006	–	–	0.041	–
SGLT-1 mRNA	<0.001	0.047	0.039	–	0.003	–	–
LXR mRNA	0.010	–	–	–	–	–	–
Gnat3 mRNA	<0.001	–	–	–	–	–	–

Time (10, 30 and 60 min), adrenocorticotrophic hormone (ACTH; presence and absence) and glucose concentration (2, 4 and 8 mmol l<sup>-1</sup>) were the main factors. Glucose × time, glucose × ACTH, and time × ACTH are first-order interactions. Glucose × ACTH × time is a second-order interaction. All values are significantly different unless noted by a dash.

### Statistics

Comparisons among groups were carried out using three-way ANOVA with glucose concentration, presence/absence of ACTH and time as main factors. Only in those cases where a significant effect was noted were *post hoc* comparisons carried out by a Student–Newman–Keuls test, and differences were considered statistically significant at *P*<0.05. When necessary, data were log transformed to fulfil the conditions of the ANOVA.

## RESULTS

### Experiment 1

Significant time, ACTH and glucose effects (*P*<0.001) were noted using a three-way ANOVA. The time course and the AUC of the time course of cortisol release from head kidney tissue displayed no major changes with the increase in glucose concentration in the medium when ACTH was not present; however, in the presence of ACTH, cortisol release increased in parallel with the increase in glucose (Fig. 1).

### Experiment 2

Cortisol released into the medium displayed significant time, ACTH and glucose effects as well as significant interactions (Table 2). Levels were higher after 60 min of incubation with 2 mmol l<sup>-1</sup> glucose either in the absence or presence of ACTH, and after 30 min with 8 mmol l<sup>-1</sup> glucose in the presence of ACTH (Fig. 2); in the presence of ACTH, higher cortisol levels were noted at 8 mmol l<sup>-1</sup> glucose after 30 min and at 2 mmol l<sup>-1</sup> glucose after 60 min (Fig. 2).

mRNA levels of genes related to cortisol synthesis (StAR, P450scc, 3βHSD and 11βH) displayed glucose, ACTH and time effects (Table 2). With 8 mmol l<sup>-1</sup> glucose in the absence of ACTH, the four parameters displayed higher values at 10 min than at 30 or 60 min of incubation, and with 2 mmol l<sup>-1</sup> glucose, lower values were noted after 60 min incubation for StAR and P450scc mRNA levels (Fig. 3). In the presence of ACTH, higher values were noted for the four parameters after 10 min of incubation with 4 and 8 mmol l<sup>-1</sup> glucose (Fig. 3). Levels with 8 mmol l<sup>-1</sup> glucose were higher than

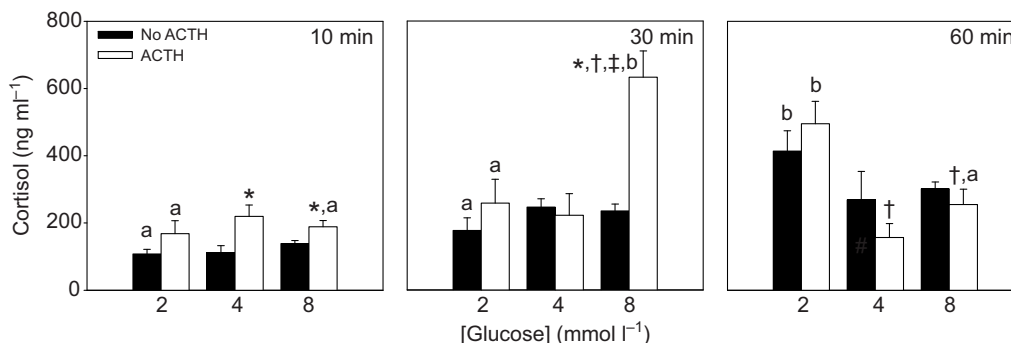


Fig. 2. Time course of cortisol levels released into the incubation medium by head kidney slices of rainbow trout incubated at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l<sup>-1</sup> D-glucose alone (No ACTH) or in the presence of 3.3 × 10<sup>-7</sup> mol l<sup>-1</sup> ACTH. Each value is the mean + s.e.m. of four independent experiments. Comparisons among groups were carried out using three-way ANOVA with glucose concentration, presence/absence of ACTH, and time as main factors. Only in those cases where a significant effect was noted were *post hoc* comparisons carried out by a Student–Newman–Keuls test. \*Significantly different from group incubated without ACTH at the same time and glucose concentration. Different letters indicate significant differences (*P*<0.05) among sampling times within each glucose concentration. †Significantly different from group incubated with 2 mmol l<sup>-1</sup> glucose at the same sampling time (*P*<0.05). ‡Significantly different from group incubated with 4 mmol l<sup>-1</sup> glucose at the same sampling time (*P*<0.05).

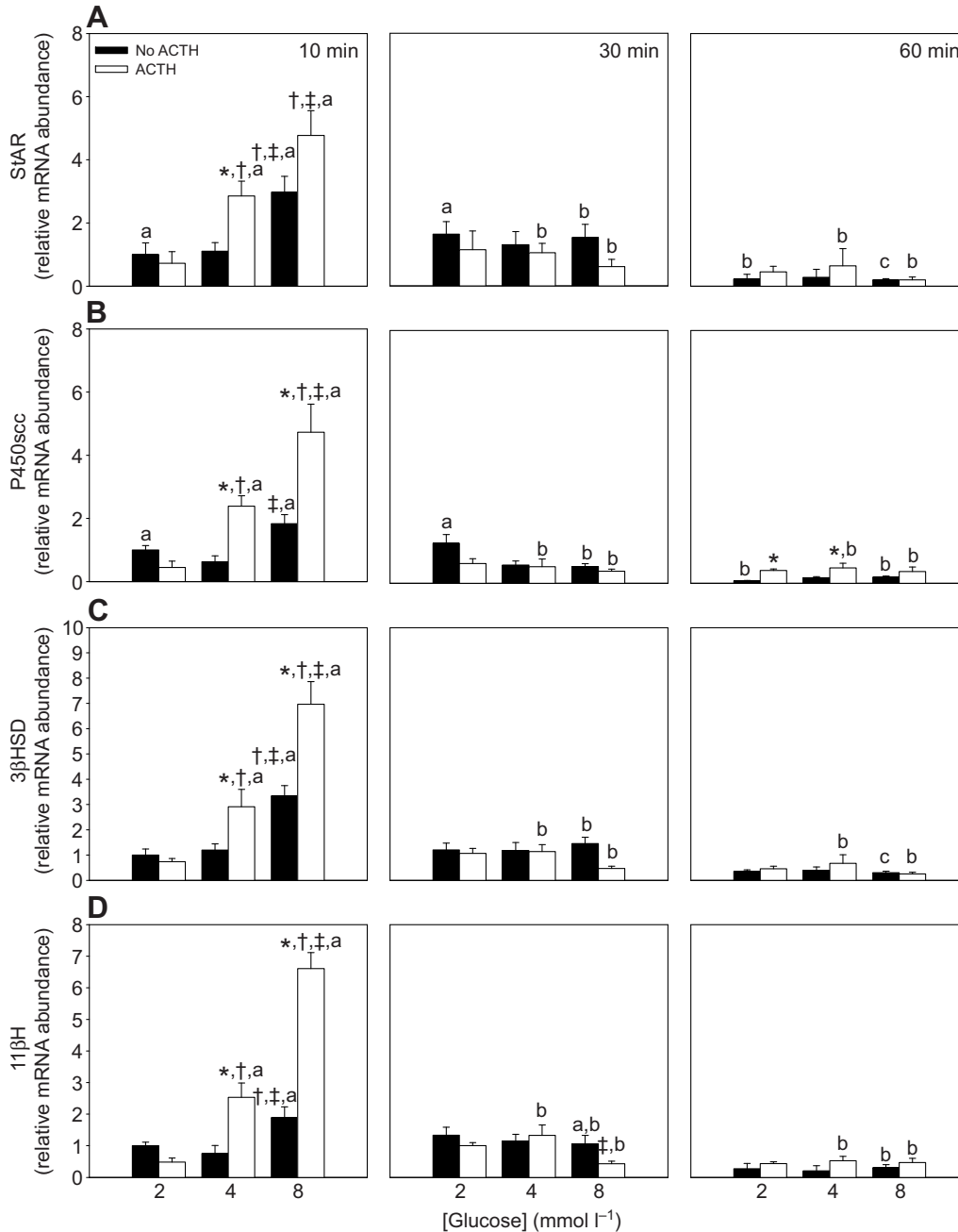


Fig. 3. Time course of mRNA abundance of StAR (steroidogenic acute regulatory protein) (A), P450scc (cytochrome P450 cholesterol side-chain cleavage) (B), 3βHSD (3β-hydroxysteroid dehydrogenase) (C) and 11βH (11β-hydroxylase) (D) in head kidney slices of rainbow trout incubated at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l<sup>-1</sup> D-glucose alone (No ACTH) or in the presence of 3.3×10<sup>-7</sup> mol l<sup>-1</sup> ACTH. Each value is the mean + s.e.m. of four independent experiments. Differences in mRNA levels between treatments are presented as an x-fold-change with respect to 2 mmol l<sup>-1</sup> glucose control (no ACTH) at 10 min. Expression results were normalized to EF1α (elongation factor 1α) mRNA levels (mRNA levels – no variation). For further details, see Fig. 2 legend.

those with 2 and 4 mmol l<sup>-1</sup> glucose after 10 min of incubation in the absence of ACTH, whereas in the presence of ACTH a dose-dependent increase was noted in the four parameters assessed (Fig. 3).

Glucose levels displayed ACTH and glucose effects, and glycogen levels displayed glucose effects (Table 2). Glucose levels (Fig. 4A) were higher with 8 mmol l<sup>-1</sup> glucose than with 2 and 4 mmol l<sup>-1</sup> glucose in the absence of ACTH, whereas a dose-dependent increase was noted in the presence of ACTH. Glycogen levels (Fig. 4B) were lower with 2 mmol l<sup>-1</sup> glucose than with 4 and 8 mmol l<sup>-1</sup> glucose after 10 min incubation in the absence of ACTH, whereas levels were higher with 8 mmol l<sup>-1</sup> glucose than with 2 and 4 mmol l<sup>-1</sup> glucose after 60 min incubation in the presence of ACTH.

The activities of GK and GSase were affected by time, glucose and the glucose × time interaction, whereas PK activity displayed

only time effects (Table 2). GK activity (Fig. 5A) in the absence of ACTH increased after 60 min with 4 mmol l<sup>-1</sup> glucose compared with that at 30 min, and after 30 min with 8 mmol l<sup>-1</sup> glucose compared with that at 10 min, whereas in the presence of ACTH the activity was higher after 60 min with 2 and 8 mmol l<sup>-1</sup> glucose and lower after 30 min with 4 mmol l<sup>-1</sup> glucose; in the absence of ACTH the activity was lower with 2 mmol l<sup>-1</sup> glucose than with 4 mmol l<sup>-1</sup> glucose after 60 min, and also lower with 2 than with 4 mmol l<sup>-1</sup> glucose after 60 min, whereas in the presence of ACTH the activity was lower with 2 mmol l<sup>-1</sup> glucose than with 4 and 8 mmol l<sup>-1</sup> glucose after 10 and 60 min of incubation. GSase activity (Fig. 5B) increased with time with 2 mmol l<sup>-1</sup> glucose in the absence of ACTH, whereas activity decreased with time with 8 mmol l<sup>-1</sup> glucose both in the absence and in the presence of ACTH; the activity with 4 and 8 mmol l<sup>-1</sup> glucose was lower than that with 2 mmol l<sup>-1</sup> glucose after 30 and 60 min incubation in both the absence and the presence of

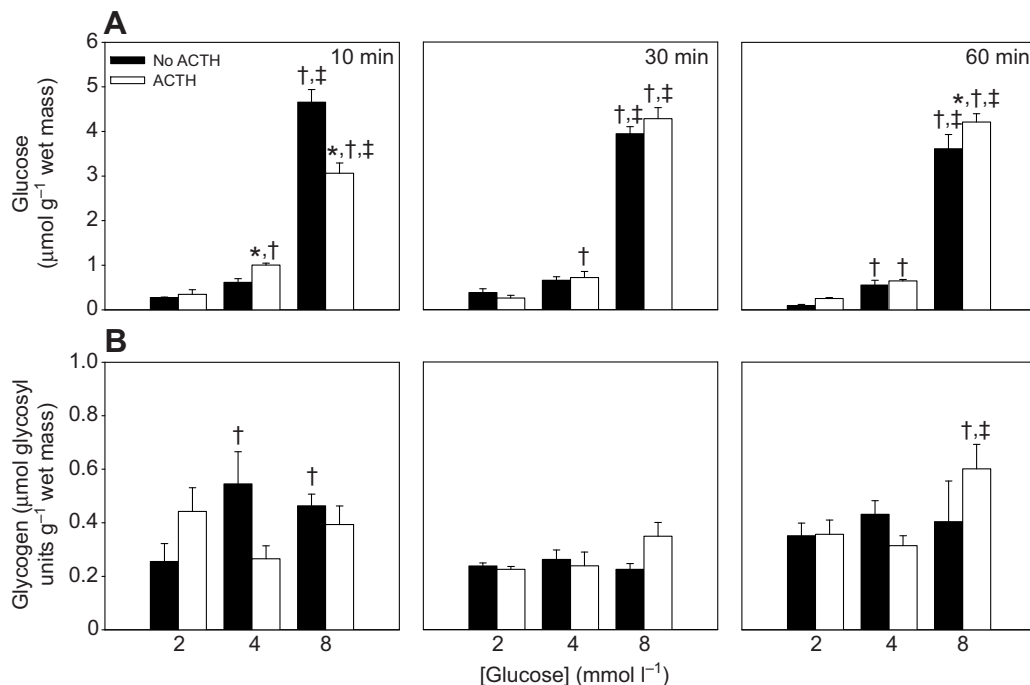


Fig. 4. Time course of levels of glucose (A) and glycogen (B) in head kidney slices of rainbow trout incubated at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l<sup>-1</sup> D-glucose alone (No ACTH) or in the presence of 3.3 × 10<sup>-7</sup> mol l<sup>-1</sup> ACTH. Each value is the mean + s.e.m. of four independent experiments. For further details, see Fig. 2 legend.

ACTH. PK activity (Fig. 5C) was higher after 30 min of incubation with 4 mmol l<sup>-1</sup> glucose in the absence of ACTH or with 2 mmol l<sup>-1</sup> glucose in the presence of ACTH.

mRNA levels of Kir6.x-like and SUR-like were affected by ACTH, time and glucose, whereas mRNA levels of GK were affected by time and glucose, and mRNA for GLUT-2 was affected only by time, as displayed in Table 2. GK mRNA levels (Fig. 6A) were lower after 60 min of incubation with 8 mmol l<sup>-1</sup> glucose in the absence of ACTH, whereas in the presence of ACTH the levels were higher after 30 and 60 min with 4 mmol l<sup>-1</sup> glucose and after 30 min with 8 mmol l<sup>-1</sup> glucose; levels with 8 mmol l<sup>-1</sup> glucose were higher than those with 2 and 4 mmol l<sup>-1</sup> glucose after 15 and 30 min of incubation, lower after 60 min of incubation in the absence of ACTH and higher after 30 min of incubation in the presence of ACTH; moreover, levels were higher with 4 mmol l<sup>-1</sup> glucose than with 2 mmol l<sup>-1</sup> glucose after 10 min of incubation in the absence of ACTH and after 30 min of incubation in the presence of ACTH. GLUT-2 mRNA levels (Fig. 6B) increased after 30 and 60 min of incubation with 2 and 4 mmol l<sup>-1</sup> glucose both in the absence and in the presence of ACTH, whereas with 8 mmol l<sup>-1</sup> glucose mRNA levels were higher after 30 min incubation in the absence of ACTH. Kir6.x-like mRNA levels (Fig. 6C) and SUR-like mRNA levels (Fig. 6D) increased after 30 and 60 min of incubation with 4 and 8 mmol l<sup>-1</sup> glucose in the absence of ACTH and with 2 and 4 mmol l<sup>-1</sup> glucose in the presence of ACTH; mRNA levels of both genes were higher with 2 mmol l<sup>-1</sup> glucose than with 4 and 8 mmol l<sup>-1</sup> glucose after 30 and 60 min of incubation in the presence of ACTH.

mRNA levels of LXR and Gnat-3 displayed no significant effects of glucose treatment though they were affected by time, whereas SGLT-1 mRNA was affected by ACTH, time and glucose (Table 2). SGLT-1 mRNA levels (Fig. 7A) increased after 60 min of incubation with 2 and 8 mmol l<sup>-1</sup> glucose in the absence of ACTH and with 2 and 4 mmol l<sup>-1</sup> glucose in the presence of ACTH; levels at 8 mmol l<sup>-1</sup> glucose were higher than those at 2 and 4 mmol l<sup>-1</sup> glucose in the absence of ACTH, whereas levels at 2 mmol l<sup>-1</sup> glucose were higher than those at 4 and 8 mmol l<sup>-1</sup> glucose after 10 and 60 min of incubation in the presence of ACTH. LXR mRNA levels (Fig. 7B)

were higher after 30 and 60 min of incubation with 4 mmol l<sup>-1</sup> glucose and after 30 min of incubation with 8 mmol l<sup>-1</sup> glucose in the absence of ACTH. Gnat3 mRNA levels (Fig. 7C) increased after 60 min of incubation with 2 and 4 mmol l<sup>-1</sup> glucose in the absence or presence of ACTH.

### Experiment 3

The presence of phlorizin did not induce significant changes in any of the parameters assessed (data not shown). The effects of the presence of cytochalasin B on the parameters assessed in head kidney at 8 mmol l<sup>-1</sup> glucose are shown in Table 3. Levels of cortisol and glycogen, and mRNA abundance of P450<sub>scc</sub>, 11βH and GLUT-2, which increased in the presence of ACTH, returned to values similar to those of controls when cytochalasin B was present. GSase activity and GK mRNA abundance decreased in tissues treated with ACTH and cytochalasin B compared with controls and tissues treated with ACTH alone.

### Immunohistochemical studies

In sections stained with haematoxylin and eosin, haematopoietic, inter-renal and chromaffin cells were easily recognizable (Fig. 8). In the areas surrounding vessels, we observed inter-renal (polygonal with rounded nuclei and dense cytoplasm, forming cords or follicles) and chromaffin (irregular or elongated in shape with light cytoplasm, arranged in groups or cords) cells, which can be contiguous but are never mingled. GK immunoreactivity was present in inter-renal cells but not in chromaffin cells. In contrast, SGLT-1 immunoreactivity was present in most (but not all) chromaffin cells.

In the colocalization studies (Fig. 9), we observed that TH co-localized with SGLT-1 in most cells but never co-localized with GK. In the cells where GK was present, we observed a co-localization with SGLT-1.

### DISCUSSION

A well-established paradigm in stress physiology is that corticosteroids stimulate glucose production to fuel metabolic processes for re-establishing homeostasis (Wendelaar Bonga, 1997).

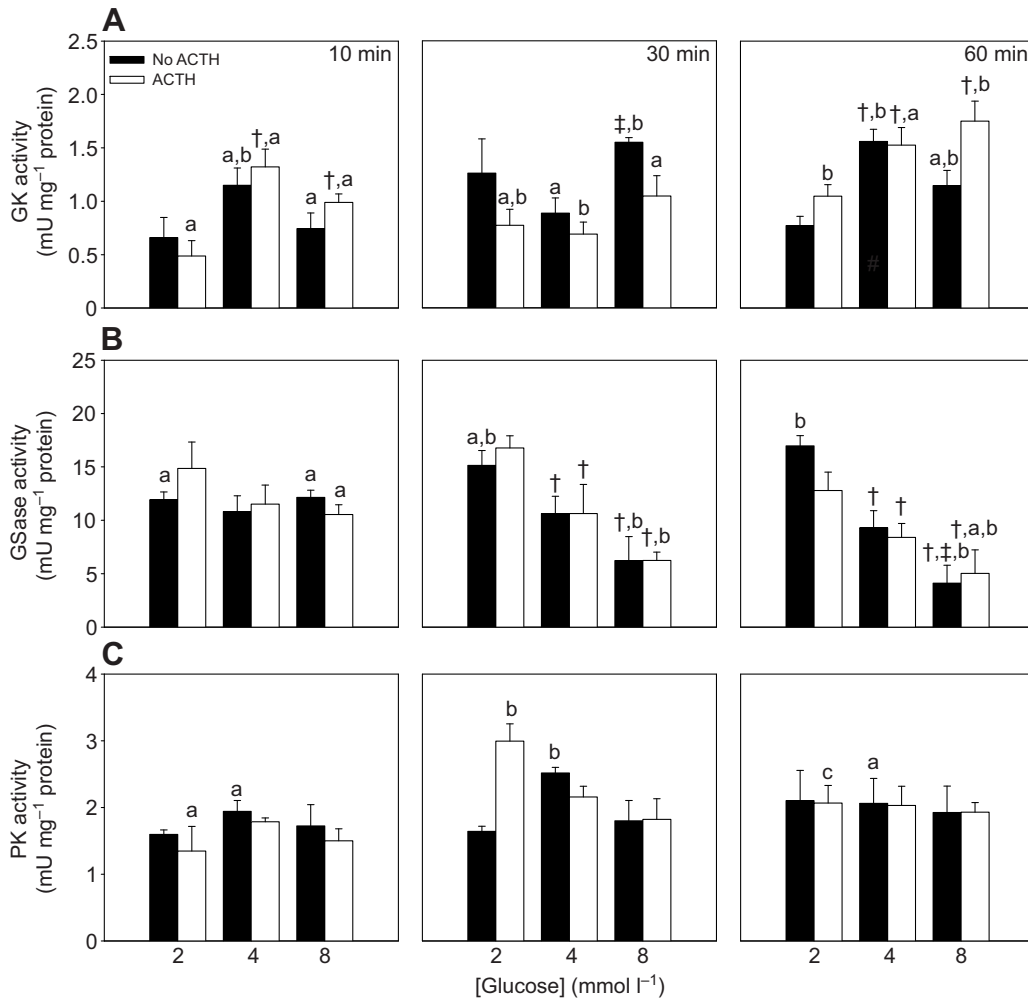


Fig. 5. Time course of the activities of GK (glucokinase) (A), GSase (glycogen synthase) (B) and PK (pyruvate kinase) (C) in head kidney slices of rainbow trout incubated at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l<sup>-1</sup> D-glucose alone (No ACTH) or in the presence of 3.3 × 10<sup>-7</sup> mol l<sup>-1</sup> ACTH. Each value is the mean + s.e.m. of four independent experiments. For further details, see Fig. 2 legend.

However, an allostatic control of cortisol levels requires adjustment of set points for cortisol production and release. Levels of circulating metabolites like glucose could be involved in such mechanisms through either inhibitory or stimulatory effects. According to this model, ACTH-stimulated cortisol release from head kidney of rainbow trout was clearly modulated by glucose concentration as increasing glucose levels elicited increased cortisol release (more clear in superfusion experiments), whereas ACTH-stimulated cortisol release was inhibited by the presence of cytochalasin B (an inhibitor of the GLUT glucose carrier). These results apparently disagree with those obtained in zebrafish, where cortisol levels were dependent on glucose in the absence of ACTH (Powers et al., 2010), though the study in zebrafish evaluated changes in whole-body content, not in medium levels as studied here, and used a much higher glucose concentration than those used in the present study. As ACTH levels increase under stress conditions in fish (Wendelaar Bonga, 1997), our results may indicate that the release of cortisol in response to increased glucose concentration occurs only under stress conditions. This situation would be comparable to that already reported in mammals where an additional stress is needed to increase cortisol release in response to an increase in glucose levels (Kirschbaum et al., 1997). However, in previous studies we observed that stress induced by high stocking density in rainbow trout did not result in a parallel response of cortisol levels in plasma to increased glucose levels (Conde-Sieira et al., 2010a; Conde-Sieira et al., 2010b), which could be related to the fact that cortisol levels

in plasma are the net result of release and breakdown as well as clearance by excretion, and the balance determines plasma levels. Moreover, the response of cortisol levels to changes in glucose concentration could be dependent upon the type of stress and/or exposure to ACTH.

The increased release of cortisol from head kidney could be related to an enhanced production, and therefore we evaluated the mRNA abundance of several genes involved in cortisol synthesis such as those encoding StAR, P450scc, 3βHSD and 11βH, the mRNA levels of which are known to mirror the level of cortisol release in rainbow trout (Aluru and Vijayan, 2006; Hagen et al., 2006). The presence of ACTH enhanced mRNA levels of P450scc, 11βH and StAR under normoglycaemic conditions (4 mmol l<sup>-1</sup> glucose), which is in agreement with results previously obtained in rainbow trout (Geslin and Auperin, 2004; Aluru and Vijayan, 2006). Furthermore, mRNA levels of genes related to cortisol synthesis increased in general in parallel with the increase in glucose concentration in the medium in the presence of ACTH, thus supporting glucose modulation of ACTH-stimulated cortisol synthesis. In the absence of ACTH, only 8 mmol l<sup>-1</sup> glucose increased the levels of these transcripts, suggesting that basal cortisol can modulate transcript levels but only under hyperglycaemic conditions, which is also in agreement with the increase noted in cortisol levels under the same condition. It is also interesting to note that the modulation of glucose levels was clearly observed following short-duration incubation (10 min), as from that time onwards



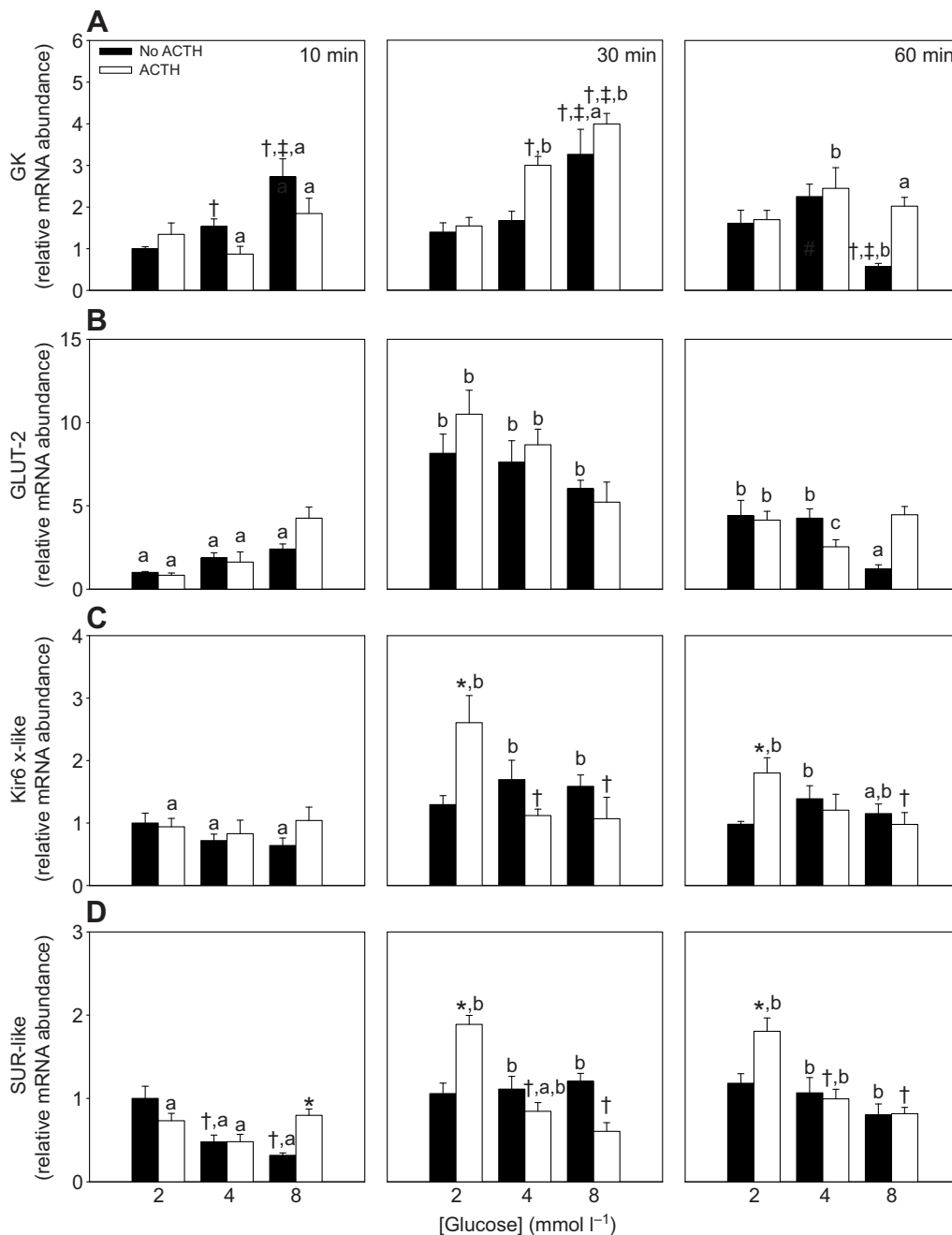


Fig. 6. Time course of mRNA abundance of GK (A), GLUT-2 (glucose facilitative transporter type 2) (B), Kir6.x-like (inward rectifier K<sup>+</sup> channel pore type 6.x-like) (C) and SUR-like (sulfonylurea receptor-like) (D) in head kidney slices of rainbow trout incubated at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l<sup>-1</sup> D-glucose alone (No ACTH) or in the presence of 3.3 × 10<sup>-7</sup> mol l<sup>-1</sup> ACTH. Each value is the mean + s.e.m. of four independent experiments. Differences in mRNA levels between treatments are presented as an x-fold-change with respect to 2 mmol l<sup>-1</sup> glucose control (no ACTH) at 10 min. Expression results were normalized to EF1α mRNA levels (mRNA levels – no variation). For further details, see Fig. 2 legend.

mRNA levels tended to be similar. This probably suggests a fast regulation of gene expression, which is later followed by changes in the level of cortisol itself, which showed higher levels thereafter (30 min). The effect of ACTH on genes involved in cortisol synthesis was also maximal in those short time periods. Further support for the relationship between glucose levels and cortisol synthesis comes from results obtained in the presence of cytochalasin B, which counteracted the increase induced by ACTH in the mRNA abundance of P450<sub>scc</sub> and 11βH.

Could glucosensors have a role in the connection between increased glucose levels and increased synthesis and release of cortisol? The best known glucosensor mechanism in mammals, the GK-mediated glucosensor (Marty et al., 2007), requires glucose uptake through the low-affinity glucose transporter GLUT-2, glucose phosphorylation by GK, and subsequent metabolism of glucose through glycolysis to increase the intracellular ATP/ADP ratio. This

leads to the closure of K<sub>ATP</sub> channels, membrane depolarization and the entry of Ca<sup>2+</sup>, which triggers increased neuronal activity and neurotransmitter secretion in brain regions and insulin release in pancreatic β-cells. We have characterized in hypothalamus, hindbrain and Brockmann bodies (main accumulation site of pancreatic endocrine tissue) of rainbow trout the presence of a similar GK-mediated glucosensor system (for review, see Polakof et al., 2011).

The possibility of a GK-mediated glucosensor system present in inter-renal cells that could relate glucose detection to cortisol release is supported by our immunohistochemical studies in head kidney. In the areas surrounding blood vessels we observed two different cell types, which can be identified as inter-renal and chromaffin cells in agreement with previous studies in rainbow trout (Gallo and Civinini, 2001). In chromaffin cells (also identified by TH immunoreactivity), SGLT-1 was present in 60–70% and GK was

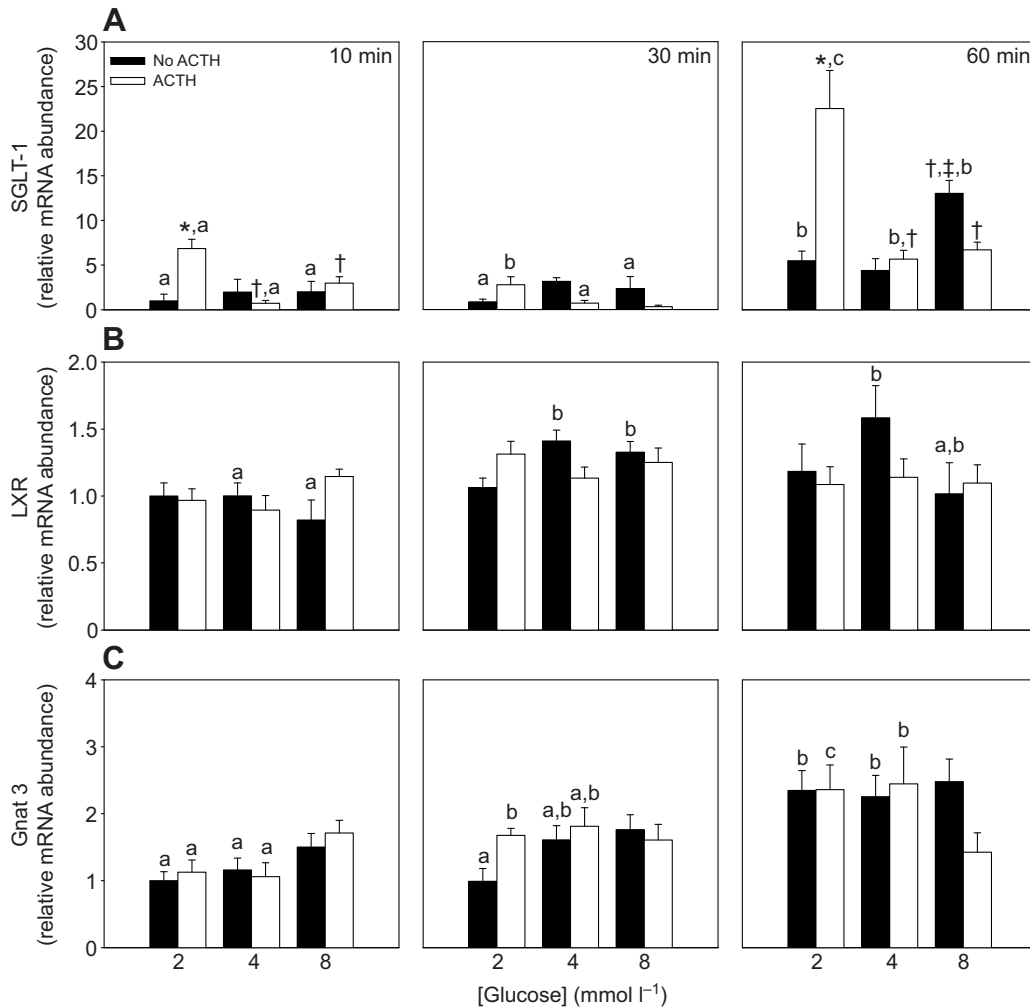


Fig. 7. Time course of mRNA abundance of SGLT-1 (sodium–glucose-linked transporter type 1) (A), LXR (liver X receptor) (B) and Gnat3 (gustducin) (C) in head kidney slices of rainbow trout incubated at 15°C in modified Hanks' medium containing 2, 4 or 8 mmol l<sup>-1</sup> D-glucose alone (No ACTH) or in the presence of 3.3×10<sup>-7</sup> mol l<sup>-1</sup> ACTH. Each value is the mean + s.e.m. of four independent experiments. Differences in mRNA levels between treatments are presented as an x-fold-change with respect to 2 mmol l<sup>-1</sup> glucose control (no ACTH) at 10 min. Expression results were normalized to EF1α mRNA levels (mRNA levels – no variation). For further details, see Fig. 2 legend.

not detected. In inter-renal cells, GK and SGLT-1 were co-localized. The presence of GK in inter-renal cells provides direct evidence for a relationship between cortisol biosynthesis and glucosensing

through a glucosensor GK-related mechanism. Therefore, we assessed several of the parameters related to GK-mediated glucosensing in response to increased glucose concentration in head

Table 3. Effects of cytochalasin B on the response to ACTH in cortisol levels and parameters related to cortisol metabolism and glucosensing

Parameter	Control	ACTH	ACTH + cytochalasin B
Cortisol level	100.0±8.89 <sup>a</sup>	180.3±13.9 <sup>b</sup>	137.5±7.73 <sup>a</sup>
Cortisol metabolism			
STAR mRNA	100.0±13.9	101.5±15.2	96.5±12.9
P450 <sub>scc</sub> mRNA	100.0±6.56 <sup>a</sup>	181.1±14.1 <sup>b</sup>	96.7±8.60 <sup>a</sup>
3βHSD mRNA	100.0±8.2	114.7±9.7	83.3±10.6
11βH mRNA	100.0±9.78 <sup>a</sup>	167.5±7.78 <sup>b</sup>	89.7±15.01 <sup>a</sup>
Glucosensing			
Glucose levels	100.0±3.36	107.1±3.25	114.4±3.19
Glycogen levels	100.0±6.62 <sup>a</sup>	142.1±10.2 <sup>b</sup>	81.7±5.55 <sup>a</sup>
GK activity	100.0±4.24	78.5±8.95	102.6±5.99
GSase activity	100.0±8.91 <sup>a</sup>	99.4±10.4 <sup>a</sup>	62.7±3.16 <sup>b</sup>
PK activity	100.0±10.6	98.2±7.43	119.9±6.79
GK mRNA	100.0±3.36 <sup>a</sup>	126.7±5.95 <sup>a</sup>	60.6±12.1 <sup>b</sup>
GLUT-2 mRNA	100.0±5.4 <sup>a</sup>	138.6±8.72 <sup>b</sup>	72.7±11.9 <sup>a</sup>
Kir6.x-like mRNA	100.0±7.31	81.8±12.4	142.2±12.3
SUR-like mRNA	100.0±9.91	77.9±8.48	146.7±11.4

Mean (±s.e.m.) cortisol level released to the incubation medium, parameters related to cortisol metabolism, and parameters related to glucosensing mediated by GK in head kidneys of rainbow trout incubated for 10, 30 and 60 min at 15°C in modified Hanks' medium containing 8 mmol l<sup>-1</sup> D-glucose alone (Control) or in the presence of 3.3×10<sup>-7</sup> mol l<sup>-1</sup> ACTH or 3.3×10<sup>-7</sup> mol l<sup>-1</sup> ACTH and 10 μmol l<sup>-1</sup> cytochalasin B.

Results are shown as a percentage of control values (control 100%). Each value is the mean of eight (cortisol) or four (remaining parameters) independent experiments carried out with pools of head kidneys from 3–4 different fish. Different letters indicate significant differences (P < 0.05) among treatments.

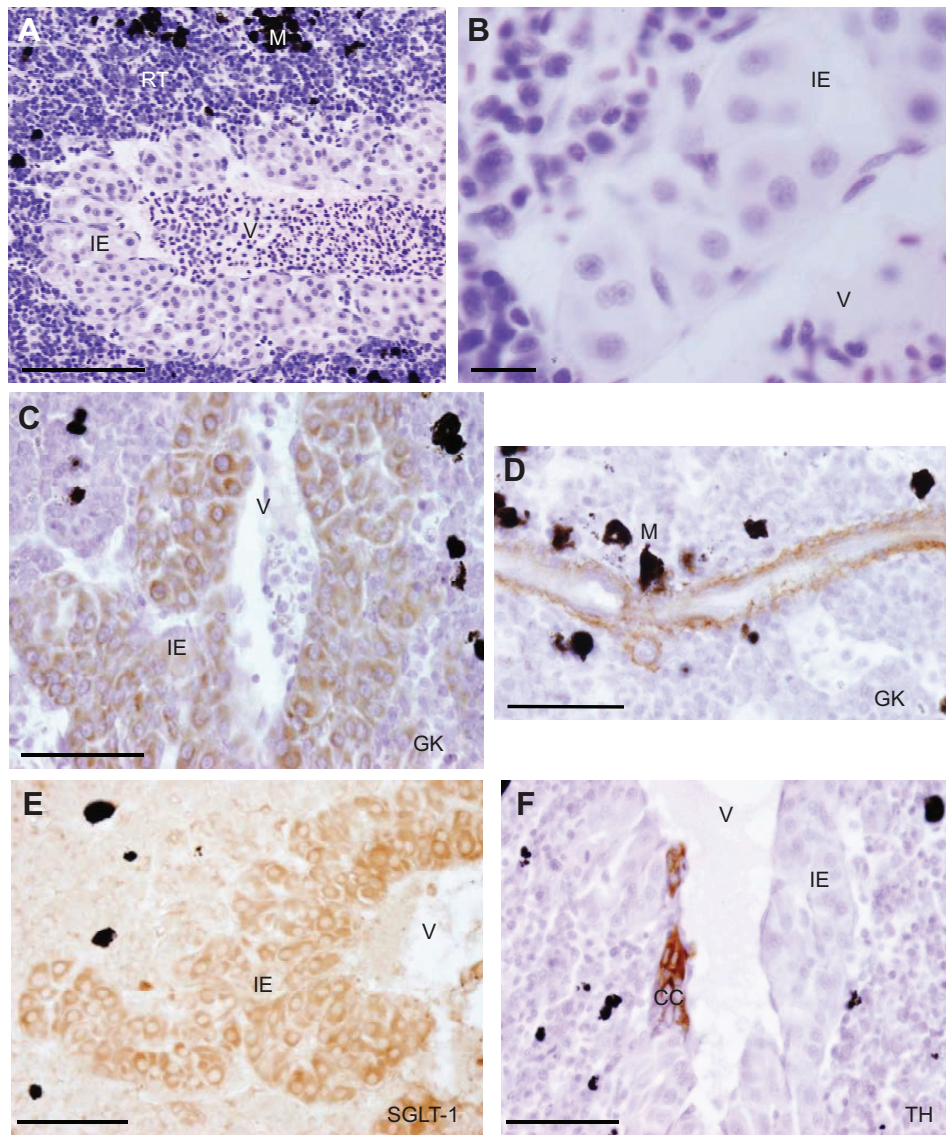


Fig. 8. Histological sections of rainbow trout head kidney stained with haematoxylin and eosin. The inter-renal tissue is easily recognizable, being arranged in groups or cords (A,B). The inter-renal cells are positive for GK (C,D) and SGLT-1 (E). The GK-immunoreactive cells (ir) line the wall of the blood vessel (E). The chromaffin cells are identified by the positive reaction for TH (tyrosine hydroxylase) (F). Dispersed in the head kidney are small ganglia and nerve fibres, as well as TH-ir (not shown). CC, chromaffin cells; IE, inter-renal tissue; M, melano-macrophages; RT, renal tissue; V, blood vessel. Scale bars: 100  $\mu$ m (A), 25  $\mu$ m (B), 50  $\mu$ m (C–F).

kidney slices. Changes observed in several of these parameters are in agreement with the functioning described in other glucosensing tissues in rainbow trout (Polakof et al., 2007a; Polakof et al., 2007b; Polakof et al., 2008a; Polakof et al., 2008b; Polakof et al., 2008c), such as the increase in glucose and glycogen levels, the increase of GK mRNA levels or the decrease in mRNA abundance of the components of the  $K_{ATP}$  channel (Kir6.x-like and SUR-like) in response to increased glucose levels.

In contrast to those parameters, no changes were noted in other parameters such as GK activity or mRNA abundance of GLUT-2, which usually increase in response to raised glucose concentration in other glucosensing tissues in rainbow trout (Polakof et al., 2008a; Polakof et al., 2008b; Polakof et al., 2008c; Conde-Sieira et al., 2010a; Conde-Sieira et al., 2010b; Conde-Sieira et al., 2011). More surprising was the finding that under increased glucose concentration, GSase activity, which normally increases in glucosensor tissues of mammals (Marty et al., 2007) and fish (Polakof et al., 2008a; Polakof et al., 2008b; Polakof et al., 2008c; Conde-Sieira et al., 2010a; Conde-Sieira et al., 2011), actually decreased in head kidney.

Further support for the presence of glucosensor mechanisms based on GK come from the results obtained when head kidney slices

were incubated in the presence of the GLUT-2 antagonist cytochalasin B. Cytochalasin B treatment in the presence of ACTH reversed the response of several parameters (glycogen levels and mRNA abundance of GK and GLUT-2) to ACTH though no changes were noted in others (even in the case of GSase activity an agonistic effect was noted). In general, it seems that several of the responses of parameters related to glucosensing are blocked by the inhibition of glucose transport through GLUT-2, one of the compulsory steps of glucosensing.

Therefore, not all of the components of a putative GK-mediated glucosensor system seem to be operational as predicted from a mammalian model in head kidney but several of them actually responded to increased glucose levels in a way similar to that previously addressed in other glucosensing tissues in rainbow trout. The lack of response in several parameters could be related to the fact that head kidney is a heterogeneous tissue composed of lymphoid cells, inter-renal cells, melanomacrophages and chromaffin cells (Wendelaar Bonga, 1997; Mommsen et al., 1999). In fact, Hontela and colleagues (Hontela et al., 2008) found that only 0.01% of cells in rainbow trout head kidneys were inter-renal cells. The fact that only a few of the cells (inter-renal) may possess glucosensing mechanisms related to GK would help to



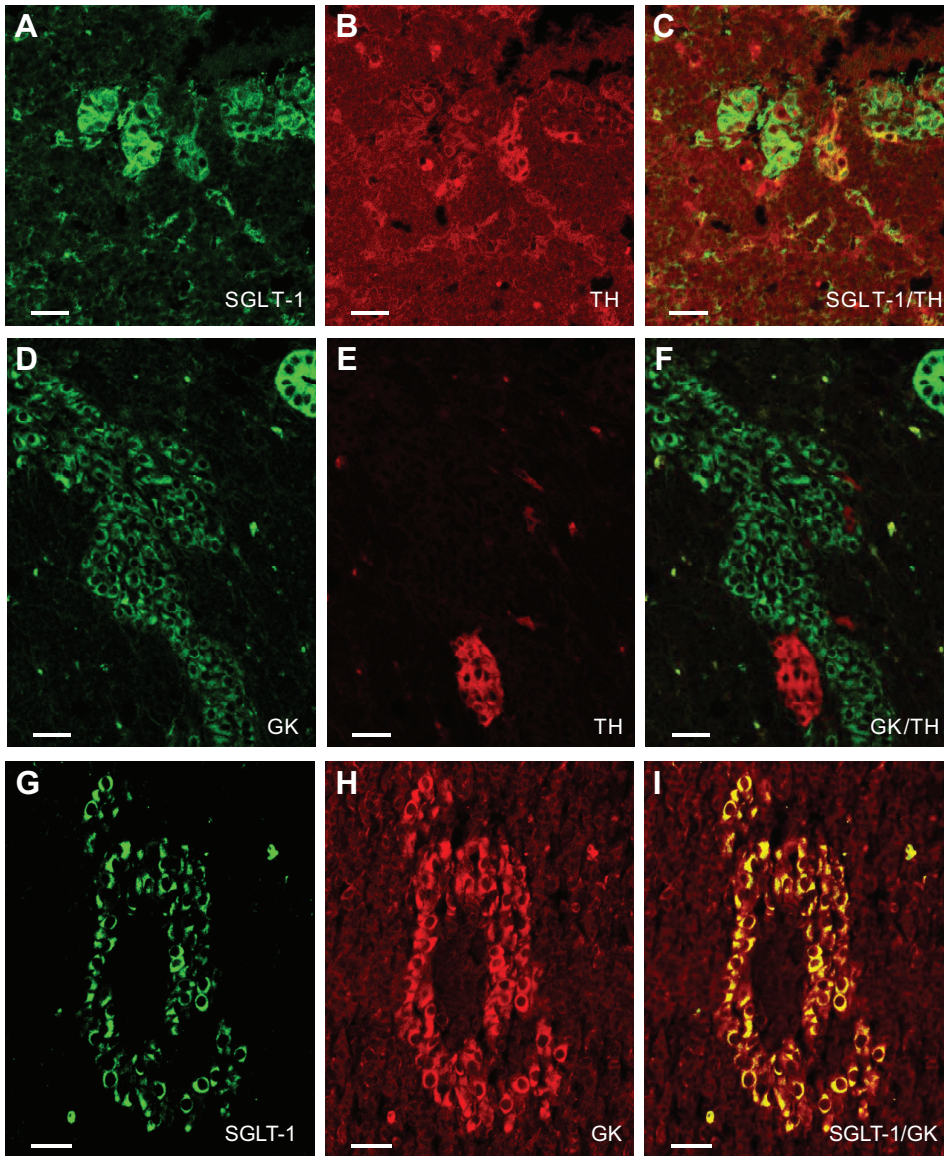


Fig. 9. Confocal photomicrographs of selected double-labelled sections of rainbow trout head kidney showing the distribution/colocalization of SGLT1/TH (A–C), GK/TH (D–F) and SGLT-1/GK (G–I) immunoreactivity. Cells showing colocalization SGLT-1/TH and SGLT-1/GK appear yellow. Note the intense GK-ir cells in the renal tubule (green); these cells are also SGLT-1-ir (results not shown). Scale bars: 25  $\mu$ m.

explain why less clear changes were noted in several parameters as values obtained in this study are the result of the dilution effect of different cell types available in the tissue assessed. The differential expression patterns observed in the immunohistochemical studies would also support such contention. Moreover, GK is also present in other cell types in head kidney (such as in tubular cells of nephrons as observed in our immunohistochemical studies) whose response to glucose may be different from that occurring in inter-renal cells.

We have no explanation regarding the connection between activation of a putative GK-mediated glucosensor mechanism in inter-renal cells and increased cortisol release. In pancreatic endocrine tissue, the activation of a glucosensor system based on GK induces the entry of calcium through L-type calcium channels, resulting in subsequent insulin release. A comparable mechanism (though certainly operating through other effectors) may be present in inter-renal cells of rainbow trout.

Other glucosensor systems described in mammalian brain regions and endocrine pancreas are the electrogenic sensor based on SGLT-1 (González et al., 2009), the nuclear receptor sensor based on LXR (Mitro et al., 2007) and the taste receptor sensor

based on gustducin (Nakagawa et al., 2009). In contrast to the GK-mediated sensor, there is no clear evidence in fish regarding the presence of any of these sensors yet, with only preliminary evidence describing SGLT-1 in rainbow trout intestine (Polakof et al., 2010). We have evaluated the response of several parameters related to these putative glucosensor systems in head kidneys of rainbow trout maintained under different glucose concentrations. The nuclear receptor and the taste receptor sensors seem to be present but not operational under the conditions used in the present studies as no changes were noted for mRNA abundance. As for the electrogenic sensor, it could be operational in the absence of ACTH as increased mRNA abundance of SGLT-1 was noted in response to increased levels of glucose. Moreover, in our immunohistochemical studies we observed that SGLT-1 co-localized with GK in inter-renal cells. However, comparing the response of SGLT-1 mRNA with that of cortisol levels and cortisol-related genes, which mainly responded to glucose in the presence of ACTH, it seems that it is not operational as a sensor involved in cortisol release as in the presence of ACTH, SGLT-1 mRNA abundance decreased. These results are also supported by the lack of effect of the SGLT inhibitor phlorizin on cortisol



release and mRNA levels of genes expressing proteins involved in cortisol metabolism. Therefore, a differential involvement of these sensing mechanisms in constitutive and regulated pathways could be proposed.

In summary, we have demonstrated that ACTH-induced release of cortisol by trout head kidney is modulated by glucose levels such that increased release occurs under high glucose levels and decreased release occurs when glucose transport is inhibited by cytochalasin B. The release of cortisol may be associated with increased synthesis as enhanced mRNA abundance of genes related to cortisol synthesis was also noted under conditions of increased glucose. These results indicate that a glucosensor may exist in inter-renal cells of rainbow trout. Accordingly, we obtained evidence regarding the presence of a glucosensor mediated by GK in head kidney, presumably in inter-renal cells, as immunoreactivity for GK was always present in inter-renal cells but absent in chromaffin cells, and increased glucose levels resulted in changes in several parameters related to GK-mediated glucosensing similar to those already reported in glucosensor tissues of the same species. In contrast, no changes compatible with those of glucose levels and cortisol release/synthesis in the presence of ACTH were noted for any other putative glucosensor mechanisms based on LXR, SGLT-1 or Gnat3. In this way, mammalian studies have shown that glucose can modulate chromaffin cell capacity for catecholamine synthesis (Piskuric et al., 2008). However, because of the heterogeneity of the tissue used, specific studies were not carried out in isolated inter-renal cells; therefore, we caution that the effects observed may be indirect, not reflecting a direct relationship between glucose levels and steroidogenic capacity.

The results combined are the first evidence for a mechanism in fish linking the synthesis and release of a non-pancreatic hormone like cortisol with circulating glucose levels, in a way comparable to that occurring in pancreatic  $\beta$ -cells responding to increased glucose levels with raised insulin release. The relationship was evident for the regulated (ACTH-dependent) pathway and this suggests that under acute stress conditions glucose is important for the regulation of cortisol synthesis and release. A coupling of ACTH-stimulated cortisol release to circulating glucose seems logical as a hyperglycaemic state supports the cortisol surges (and their impact on glucose utilization) under acute stress conditions.

#### LIST OF ABBREVIATIONS

11 $\beta$ H	11 $\beta$ -hydroxylase
3 $\beta$ HSD	3 $\beta$ -hydroxysteroid dehydrogenase
ACTH	adrenocorticotrophic hormone
CRF	corticotropin releasing factor
EF1 $\alpha$	elongation factor 1 $\alpha$
GK	hexokinase IV or glucokinase (EC 2.7.1.2)
GLUT-2	glucose facilitative transporter type 2
Gnat3	gustducin
GSase	glycogen synthase (EC 2.4.1.11)
K <sub>ATP</sub>	ATP-dependent inward rectifier potassium channel
Kir6.x-like	inward rectifier K <sup>+</sup> channel pore type 6.x-like
LXR	liver X receptor
P450 <sub>sc</sub>	cytochrome P450 cholesterol side-chain cleavage
PK	pyruvate kinase (EC 2.7.1.40)
SGLT-1	sodium–glucose-linked transporter type 1
StAR	steroidogenic acute regulatory protein
SUR-like	sulfonylurea receptor-like
TH	tyrosine hydroxylase

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