Glucagon regulation of carbohydrate metabolism in rainbow trout: *in vivo* glucose fluxes and gene expression

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Summary statement

Glucagon causes hyperglycemia in rainbow trout by increasing hepatic glucose production via activation of gluconeogenesis, and by decreasing glucose disposal via inhibition of glycogen synthesis and glycolysis.

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Abstract

Glucagon increases fish glycemia, but how it affects glucose fluxes *in vivo* has never been characterized. The goal of this study was to test the hypothesis that glucagon stimulates hepatic glucose production (R_a) and inhibits disposal (R_d) of rainbow trout. Changes in the mRNA abundance of key proteins involved in glycolysis, gluconeogenesis, and glycogen breakdown were also monitored. Results show that glucagon increases glycemia (+38%) by causing a temporary mismatch between R_a and R_d before both fluxes converge below baseline (-17%). A novel aspect of the regulation of trout gluconeogenesis is also demonstrated: the completely different effects of glucagon on the expression of three Pepck isoforms (stimulation of *pck1*, inhibition of *pck2a*, and no response of *pck2b*). Glycogen phosphorylase was modulated differently among tissues, and muscle upregulated *pygb* and downregulated *pygm*. Glucagon failed to activate the cAMP-dependent protein kinase or FoxO1 signalling cascades. We conclude that trout hyperglycemia results from the combination of two responses: (i) an increase in R_a glucose induced by the stimulation of gluconeogenesis through transcriptional activation of *pck1* (and possibly glycogen phosphorylase), and (ii) a decrease in R_d glucose via inhibition of glycogen synthase and glycolysis. The observed decrease in glucose fluxes after 4 h of glucagon administration may be caused by a counterregulatory response of insulin, potentially linked to the decrease in *pygm* transcript abundance. Overall, however, these integrated effects of glucagon only lead to modest changes in glucose fluxes that partly explain why trout seem to be unable to control glycemia very tightly.

Key words: fish glucoregulation, glucose production and disposal, gluconeogenesis, glycogen, glucagon signalling.

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Introduction

The regulation of circulating glucose levels is necessary for adequate fuel supply to the brain and working muscles (Shrayyef and Gerich, 2010; Wasserman et al., 2011), but fish do not generally control glycemia as tightly as birds or mammals (Enes et al., 2009; Polakof et al., 2012). Blood glucose concentration depends on changing rates of hepatic production (R_a glucose) and peripheral disposal (R_d glucose)(Weber et al., 2016). Although thoroughly characterized in mammals (Wasserman, 2009), the hormonal regulation of glucose fluxes remains poorly explored in fish. Only two previous studies have addressed this issue by examining how epinephrine (Weber and Shanghavi, 2000) and insulin (Forbes et al., 2019) regulate fish glucose kinetics. The catabolic hormone glucagon is another important endocrine signal that controls glucose fluxes in mammals, and possibly also in fish (Polakof et al., 2012; Shrayyef and Gerich, 2010). Multiple metabolic responses to glucagon have been demonstrated in mammals where it stimulates glucose production, and weakly inhibits (Lins et al., 1983), or has no effect on glucose disposal (Hinshaw et al., 2015). Glucagon increases glycemia in fish (Polakof et al., 2012), but this response could be mediated through an increase in R_a, a decrease in R_d, or a combination of both. Current information suggests that fish R_a glucose is probably stimulated by glucagon because gluconeogenic and glycogenolytic enzymes are upregulated in isolated hepatocytes (Brighenti et al., 1991; Foster and Moon, 1990; Puviani et al., 1990; Sugita et al., 2001). Several studies also support the idea that fish R_d glucose could be inhibited because glucagon decreases liver activities of glycogen synthase (Murat and Plisetskaya, 1977), phosphofructokinase (Foster et al., 1989) and pyruvate kinase (Petersen et al., 1987). However, *in vivo*

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measurements of glucose kinetics are necessary to establish how glucagon-induced changes in R_a and R_d glucose cause an increase in fish glycemia.

The effects of glucagon on glucose metabolism are mediated by two pathways: cyclic AMP (cAMP) and calcium signalling. Glucagon binds to the G-protein-coupled receptor that raises GTP, causing increases in cAMP and inositol trisphosphate (IP_3) (Moon et al., 1997). Then, the cAMP and calcium signalling pathways are mobilized to activate cAMP-dependent protein kinase (PKA) which, in turn, regulates several downstream targets (PKA substrates)(Plisetskaya and Mommsen, 1996), and possibly also FoxO1 as in mammals (Eijkelenboom and Burgering, 2013). Phosphorylated forms of PKA substrates and FoxO1 are associated with the regulation of metabolic processes that include gluconeogenesis and glycogen breakdown (Habegger et al., 2010; Moon, 1998), but whether glucagon causes phosphorylation of these targets has not been determined for fish. In particular, It would be useful to know if liver and muscle are affected because these two tissues play essential roles in glucose metabolism.

The main goal of this study was to test the hypothesis that glucagon-induced fish hyperglycemia is caused by stimulating glucose production and inhibiting glucose disposal as it does in mammals. We anticipated that glucagon would have a weaker effect on fish glucose production because fish generally have a lower capacity for glucoregulation. Other goals were: (i) to measure levels of phosphorylated PKA substrates and FoxO1 in muscle and liver to determine whether these signalling cascades are activated, and (ii) to monitor potential changes in transcript levels of key proteins involved in glycolysis, gluconeogenesis, glycogen mobilization, and transmembrane glucose transport. Because salmonids have experienced several whole genome duplications in their evolutionary history

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(Berthelot et al., 2014), we have quantified the mRNA expression of multiple paralogs of phosphoenolpyruvate carboxykinase (Pepck) and glycogen phosphorylase (Gp) that could show different responses to glucagon.

Methods

Animals

Adult rainbow trout (*Oncorhynchus mykiss*) of both sexes were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). All results presented are pooled male and female values because no significant sex differences were observed for the measured parameters of this study. Two groups of fish were used: (i) for *in vivo* measurements of glucose kinetics, and (ii) for measurements of gene expression of mRNA (qPCR) and glucagon signalling proteins (Western blots). The physical characteristics of each experimental group are given in Table 1. The fish were held in a 1,200 liter flow-through tank supplied with dechloraminated Ottawa tap water at 13°C, on a 12 h:12 h light:dark photoperiod and they were fed Profishnet floating fish pellets (Martin Mills, Elmira, ON, Canada) 5 days a week. They were acclimated to these conditions for a minimum of 2 weeks before experiments. All the procedures were approved by the Animal Care Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care.

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Catheterizations and respirometry

Fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222 at a concentration of 60 mg l^{-1} buffered with 0.2 g l^{-1} sodium bicarbonate) and surgically fitted with 2 catheters (for glucose kinetics experiments) or a single catheter (for signalling protein and gene expression experiments)(BTPE-50 catheters, Instech Laboratories, Plymouth Meeting, PA, USA) in the dorsal aorta (Haman and Weber, 1996). The catheters were kept patent by flushing with Cortland saline containing 50 U ml^{-1} heparin (Sigma-Aldrich, St Louis, MO, USA). After surgery, the fish were left to recover overnight in a 90 liter swim-tunnel respirometer (Loligo Systems, Tjele, Denmark) where all the *in vivo* measurements were carried out in resting animals at a water velocity of 0.5 body length per second (BL s^{-1}). This weak current reduces stress and enhances the flow of water over the gills, but it does not require swimming to maintain body position because the animals are sitting at the bottom of the respirometer chamber (Choi and Weber, 2015). The respirometer was supplied with the same quality water as the holding tank and was kept at 13°C.

Glucose kinetics experiments

The catheters were made accessible through the respirometer lid by channeling them through a water-tight port. The rates of glucose turnover (R_t), hepatic glucose production (R_a) and glucose disposal (R_d) were measured by continuous infusion of [6-^{3}H]glucose (Perkin Elmer, Boston, MA, USA; specific activity 222 GBq $mmol^{-1}$) in a group of 8 fish (see Table 1). This tracer method has been validated to quantify glucose kinetics in fish (Haman et al., 1997a) and

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thoroughly tested in rainbow trout under a variety of physiological stresses (Choi and Weber, 2016; Haman et al., 1997b; Shanghavi and Weber, 1999; Weber et al., 2016). The infusate was freshly prepared immediately before each experiment by drying an aliquot of the solution obtained from the supplier under N_2 and resuspending in Cortland saline. A priming dose of tracer equivalent to 3 h of infusion was injected as a bolus at the start of each infusion to reach isotopic steady state in <45 min. The infusate was then administered continuously at ~1 ml/h (exact infusion rates were determined individually for each fish to correct for differences in body mass) using a calibrated syringe pump (Harvard Apparatus, South Natick, MA). Infusion rates for labeled glucose averaged 2007 ± 181 Bq kg^{-1} min^{-1} (N=8) and these trace amounts of glucose (labeled and unlabeled) had no effect on glucose metabolism because they only accounted for 0.00001% of the baseline endogenous rate of hepatic glucose production. Blood samples (~100 µl each) were drawn 50, 55 and 60 min after starting the tracer infusion to determine baseline glucose kinetics, and every 20 min thereafter during glucagon administration (8.3 µg bovine glucagon kg^{-1} min^{-1} for 4 h at 1 ml/h). Sham infusion of saline has no effect on glucose kinetics (see Fig. 2 in Weber and Shanghavi, 2000). Values measured before the start of glucagon infusion were therefore used as baseline. Bovine glucagon has been commonly used to investigate the effects of teleost glucagon (Foster and Moon, 1990; Foster et al., 1989; Plisetskaya and Mommsen, 1996). The rate of glucagon infusion selected was based on a previous study where the hormone was also administered *in vivo* to rainbow trout and where it elicited significant changes in glucose metabolism (de la Higuera and Cardenas, 1986). The total amount of blood sampled from each fish accounted for <10% of total blood volume. Samples were collected in tubes containing heparin and aprotinin (500 KIU ml^{-1} to stabilize

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glucagon). They were centrifuged to separate plasma (5 min; 12,000 RPM) that was stored at -20ºC until analyses.

Gene expression and signalling protein experiments

To avoid making the measurements in radioactive tissues, these experiments were carried out on different fish than those used for glucose kinetics, but they received the same infusions: saline (control group) or glucagon (treatment group; 8.3 $\mu g\ kg^{-1}\ min^{-1}$) that were administered at 1 ml/h through the catheter for 4 h. Fourteen fish were used for these experiments (see Table 1). The animals were then euthanized by a sharp blow on the head before collecting the liver and ~4 g of white muscle (dorsal region, anteriorly to the dorsal fin). The tissue samples were stored at -80ºC until analyses.

Sample analyses

Glucose kinetics: Plasma glucagon and glucose concentrations were measured spectrophotometrically using a Spectra Max Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Glucagon was measured using a commercial ELISA kit (Crystal Chem, Downers Grove, IL). This kit uses a particular COOH-terminal anti-glucagon fragment that has been previously validated for measuring fish glucagon (Navarro et al., 1995). Unfortunately, fish insulin cannot be measured accurately at the present time (Moon, 2001). A radioimmunoassay was developed decades ago (Plisetskaya, 1998), but it also measures pro-insulins and, therefore, overestimates true insulin concentration. Glucose activity was quantified by drying plasma under N_2 to eliminate tritiated water and by resuspending in distilled water. Radioactivity was then measured by

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scintillation counting (Perkin-Elmer Tricarb 2910TR, Perkin-Elmer, Inc., Waltham, MA, USA) in Bio-Safe II scintillation fluid (RPI Corp., Mount Prospect, IL, USA).

mRNA transcript abundance: Total RNA was extracted from 20 to 100 mg of individual liver or muscle samples (N=7 per group) using TRIzol (Invitrogen, Burlington, ON, Canada) following the manufacturer's protocol. Tissues (control N=7; glucagon N=7) were homogenized using a sonicator Model 100 (Thermo-Fisher Scientific, Ottawa, ON, Canada) on ice until tissue fragments were no longer visible. Extracted RNA was quantified using a NanoDrop® 2000c UV-Vis Spectrophotometer (Thermo-Fisher Scientific). Next cDNA was generated from the total RNA using a QuantiTech Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's protocol. Two step quantitative real-time RT-PCR assays were performed on a Bio-Rad CFX96 instrument (Bio-Rad, Mississauga, ON, Canada) to quantify fold changes in relative liver and muscle mRNA abundances of key transcripts involved in gluconeogenesis (*pck1, pck2a, pck2b, fbpase*), glycogenolysis (glycogen phosphorylase brain-associated *pygb,* glycogen phosphorylase muscle-associated *pygm, g6pase*), glycolysis (*pfk*) and glucose transport (*glut1, glut2, glut4*). A standard curve consisting of serial dilutions of pooled cDNA, a negative no-RT control consisting of cDNA generated in a reaction that did not include reverse transcriptase, a negative no-template control generated in a reaction that substituted water for RNA and individual diluted samples were run in duplicate. For each individual reaction, the total volume was 20 µl, that consisted of 4 µl diluted cDNA template, 0.5 µl 10 µM specific forward and 0.5 µl 10 µM specific reverse primer (Table 2), 10 µl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 5 µl nuclease free H_2O. For each assay, cycling parameters were a 2 min activation step at 98ºC followed by 40 cycles consisting of a 20 sec denaturation step at 95ºC and a

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combined 30 sec annealing and extension step at primer specific temperatures (Table 2). Following each run, melting curves were produced (65ºC – 95ºC at 0.5ºC every 5 seconds) by gradually increasing temperature and the final curves were monitored for single peaks to confirm the specificity of the reaction and the absence of primer dimers. In cases where primers were newly designed, pooled samples were sent for sequencing (Ottawa Hospital Research Institute, Ottawa, ON, Canada), followed by BLAST search (NCBI), to confirm amplicon specificity. The acceptable range for amplification efficiency calculated from serially diluted standard curves was 90-110% with R^2 values >0.97. Assays were subsequently normalized using the NORMA-Gene approach as described by Heckmann et al. (Heckmann et al., 2011). Finally, mRNA fold-changes were calculated relative to the control group.

Glucagon signalling proteins: Frozen liver and muscle samples (control: N=7; glucagon: N=7; ~200 mg) were homogenized on ice with a sonicator (Fisher Scientific Sonic Dismembrator Model 100, San Diego, CA, USA) in 400 µl of per 100 mg of tissue. During homogenization, samples were kept in a buffer containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA (pH 7.4), 100 mmol l^{-1} sodium fluoride, 4 mmol l^{-1} sodium pyrophosphate, 2 mmol l^{-1} sodium orthovanadate, 1% (v/v) Triton X-100, 0.5% (v/v) NP40-IGEPAL and a protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenates were centrifuged at 15,000 g for 5 min at 4ºC, the resulting supernatants were recovered and stored at -80ºC. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (200 µg of total protein for liver and 50 µg for muscle) were diluted in the previously described buffer and 2× Laemmli. The prepared samples were denatured at 95°C for 2 min and quick chilled on ice before being subjected to SDS-PAGE and western blotting

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using the appropriate antibodies (monoclonal rabbit anti-p-PKA substrates (RRXS*/T*) (9624; Cell Signaling Technology, Whitby, Canada) validated by (Dindia et al., 2012), and polyclonal rabbit anti-p-FOX01 (9464; Cell Signaling Technology, Whitby, Canada) validated by (Cleveland and Weber, 2010; Seiliez et al., 2010), and were normalized using REVERT Total Protein Stain (LI-COR Biosciences, Lincoln, NE, USA). Gels were cast as 10% resolving gel consisting of 5 ml ddH20, 2.5 ml buffer B pH 8.8 (1.5 M Tris base, 0.04% SDS; both BioShop, Burlington, ON Canada) dissolved in dH2O, 2.5 ml 40% acryl/Bis (Bio-Rad, Mississauga, ON, Canada) and polymerized with 50 µl 10% APS (Sigma-Aldrich Oakville, ON, Canada) and 20 µl TEMED (Life Technologies Burlington, ON, Canada), and a 4% stacking gel [consisting of 3.25 ml ddH2O, 1.25 ml buffer C pH 6.8 (0.5 M Tris, 0.04% SDS dissolved (BioShop) in dH2O], 0.5 ml 40% acryl/bis polymerized with 25 µl 10% APS, and 10 µl TEMED. Gels were immersed in 1× Tris glycine SDS (TGS) running buffer, consisting of Tris base 2.5 mM, glycine 0.192 M, and 0.1% SDS (all BioShop Canada) dissolved in dH2O, and samples were loaded with 5 µl of Page Ruler prestained protein ladder (Thermo Fisher, Ottawa, ON, Canada). Proteins were migrated in the gel at 100 V. After migration, they were transferred onto nitrocellulose 0.45-mm pore size membrane paper (Millipore, Etobicoke, ON, Canada) by wet transfer using the Mini TransBlot system (Bio-Rad) with transfer buffer (250 mM Tris base, 1,920 mM glycine; all BioShop Canada) dissolved in dH2O, by applying 100 V for 2 h. Membranes were incubated with Odyssey blocking buffer (LI-COR Biosciences Lincoln, NE) for 1 h at room temperature using an orbital shaker was used to prevent nonspecific binding and then incubated in primary antibody at a concentration of 1:10,000 on an orbital shaker at 4°C overnight. Membranes were washed four times for 5 min with PBS + 0.1% TWEEN20 then

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incubated with an IRDye Infrared secondary goat anti-rabbit IgG antibody (LIC-925-68071; LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (v.3.0, LI-COR Biosciences).

Calculations and statistics

Fulton's condition factor was computed as $K = (10^5 \times M_b) / L^3$; where M_b = body mass in g and L = total length in mm (Blackwell et al., 2000). Glucose fluxes were calculated 2 ways, using either the steady-state or the non-steady-state equations of Steele (Forbes et al., 2019; Steele, 1959). Glucose turnover (R_t) was calculated using the steady-state equation. Then, the non-steady-state equations were used to calculate R_a and R_d glucose separately after changes in specific activity over time were curve-fitted by 2^{nd}-degree polynomial regression for each animal (see Wolfe, 1992) as described in more detail previously (Forbes et al., 2019). Statistical comparisons were performed using one-way repeated-measures analysis of variance (RM-ANOVA) with the Dunnett's post hoc test to determine which means were significantly different from baseline (SigmaPlot v12, Systat Software, San Jose, CA, USA). When the assumptions of normality or equality of variances were not met and if data transformations failed to normalize data distribution, Friedman's non-parametric RM-ANOVA on ranks was used. Gene expression and signalling protein data were analyzed using Mann-Whitney rank sum test. Values are presented as means ± s.e.m. and a level of significance of $P<0.05$ was used in all tests.

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Results

Glucagon, glycemia, and glucose kinetics

The administration of exogenous glucagon caused a steady increase in plasma glucagon concentration that became significantly higher than baseline after 2 h ($P<0.05$). Glucagon increased from a baseline value of 18.6±2.0 pg ml^{-1} (N=8) to a final value of 124.8±42.0 ng ml^{-1} (supplementary Fig. S1) after 4 h (N=8; $P<0.001$). Fig. 1 shows the changes in plasma glucose concentration and glucose specific activity before and during glucagon administration. Glucose concentration increased progressively for the first 3 h before reaching a plateau during the last hour of the experiment ($P<0.05$; Fig. 1A). Glucose specific activity showed no significant change from baseline ($P>0.05$; Fig. 1B). The effects of glucagon on glucose turnover rate (R_t), hepatic glucose production (R_a), glucose disposal (R_d), and the capacity to increase glycemia [R_a glucose – R_d glucose] are presented in Fig. 2. R_t glucose showed no significant response to the hormone ($P>0.05$; Fig. 2A). Both R_a and R_d glucose remained constant for the first 2 h of glucagon infusion before decreasing progressively to reach a common final value lower than baseline ($P<0.05$; Fig. 2B). The capacity to increase glycemia [R_a glucose – R_d glucose] was stimulated to a maximum of 1.5 µmol kg^{-1} min^{-1} during the first few minutes of glucagon infusion ($P<0.05$; Fig. 2C) before decreasing steadily to zero over the course of the experiment. Table 3 summarizes mean initial (baseline) and final values (after 4 h of glucagon infusion) for glucose concentration and fluxes.

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Gene expression

The effects of glucagon on muscle and liver mRNA abundance for key proteins of glucose metabolism are shown in Figs. 3 to 5. In muscle, glucagon caused an increase in the transcript abundance for glycogen phosphorylase, brain-associated (*pygb*) ($P<0.01$), but a decrease for glycogen phosphorylase, muscle-associated (*pygm*) ($P<0.05$; Fig. 3). In this tissue, no significant changes were observed for phosphofructokinase-1 (*pfk*), and glucose transporters 1 or 4 (*glut1, glut4*) ($P>0.05$; Fig. 3). In liver, the 3 isoforms of phosphoenolpyruvate carboxykinase responded differently to glucagon (Fig. 4). Phosphoenolpyruvate carboxykinase 1 (*pck1*) increased by 42-fold ($P<0.01$), phosphoenolpyruvate carboxykinase 2a (*pck2a*) decreased ($P<0.05$), and phosphoenolpyruvate carboxykinase 2b (*pck2b*) was not affected by the hormone ($P>0.05$; Fig. 4). The liver expressions for the other gluconeogenic enzymes fructose-bis-phosphatase (*fbpase*) and glucose-6-phosphatase (*g6pase*) as well as for glucose transporter 2 (*glut2*) and glycogen phosphorylase, brain associated (*pygb*) were not affected by glucagon ($P>0.05$; Fig. 5).

Glucagon signalling

The effects of exogenous glucagon on the active (phosphorylated) form of PKA substrates and FoxO1 in muscle and liver are shown in Fig. 6. Glucagon did not significantly activate PKA substrates in muscle ($P=0.93$) or liver ($P=0.13$). Similarly, the hormone did not cause the activation of FoxO1 in either tissue (muscle: $P=0.21$; liver: $P=0.29$).

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Discussion

This study is the first to characterize the effects of glucagon on fish glucose fluxes *in vivo*. It shows that the hormone elicits hyperglycemia by causing a temporary mismatch between hepatic glucose production and peripheral glucose utilization that slowly disappears over 4 h. Both R_a and R_d glucose eventually decrease below baseline during the last 2 h of glucagon administration. Results also show a novel aspect of the regulation of trout gluconeogenesis: the completely different effects of glucagon on the expression of the three isoforms of Pepck: stimulation of *pck1,* inhibition of *pck2a*, and no response of *pck2b*. Furthermore, the transcript abundance of glycogen phosphorylase is modulated differently among tissues, or even within muscle that responds by upregulating *pygb* and downregulating *pygm*.

Initial effects of glucagon on glucose fluxes

Increasing circulating glucagon causes an initial divergence between R_a and R_d that raises blood glucose concentration by 38%. These immediate responses (stimulation of R_a and inhibition of R_d) fail to reach statistical significance (Fig. 2B), but the divergence clearly occurs because hyperglycemia ensues (Fig. 1A), and the difference between R_a and R_d jumps significantly to 1.5 μmol kg^{-1} min^{-1} immediately upon glucagon administration (Fig. 2C). Upregulating hepatic glucose production can be achieved by activating gluconeogenesis, glycogen breakdown or both. Previous fish studies show that glucagon stimulates gluconeogenesis by increasing Pepck activity (Foster and Moon, 1990) and by stimulating FBPase and G6Pase in isolated hepatocytes (Sugita et al., 2001). Similar responses have been documented for

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mammals that increase the expression and activity of these same enzymes (Band and Jones, 1980; Beale et al., 1984; Christ et al., 1988; Pilkis et al., 1982; Striffler et al., 1984). Glucagon also stimulates glycogen breakdown by increasing glycogen phosphorylase activity by ~50% in trout (Puviani et al., 1990), and ~160% in mammals (Malbon et al., 1978). Observed changes in mRNA transcript abundance suggest that glucagon increases trout R_a glucose by upregulating gluconeogenesis as well as glycogen breakdown. However, mRNA abundance, protein abundance, and protein activity often show differential responses and direct measurements of protein activity will be needed to confirm this conclusion. The potential mechanisms involved in decreasing glucose disposal are not clear, but the inhibition of glycogen synthesis and hepatic glycolysis could be at play. Experiments on fish liver have shown that glucagon reduces the activities of glycogen synthase (*Gs*)(Murat and Plisetskaya, 1977), pyruvate kinase (*Pk*)(Petersen et al., 1987) and phosphofructokinase (*Pfk*)(Foster et al., 1989), similarly to what occurs in mammals (Jiang and Zhang, 2003). Overall, therefore, the glucagon-driven hyperglycemia is probably caused by a series of integrated, mostly hepatic responses including the stimulation of gluconeogenesis and glycogen phosphorylase, and the inhibition of glycolysis and glycogen synthase.

Longer term effects of glucagon

After 2 hours of glucagon infusion, blood glucose concentration stabilizes at ~12 μmol ml^{-1} (Fig. 1A), rates of glucose production and disposal decrease progressively below baseline to converge to the same lower value (Fig. 2B), and the capacity to raise glycemia (measured as [R_a - R_d]) is eventually reduced to zero (Fig.

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2C). These longer-term changes suggest that exogenous glucagon triggers a counterregulatory response by insulin that prevents a further increase in circulating glucose concentration, and ultimately tries to restore normoglycemia. When fish experience hyperglycemia, glucosensing neurons and beta cells of the Brockmann bodies are known to stimulate insulin secretion (Blasco et al., 2001; de Celis et al., 2004; Furuichi and Yone, 1981; Ince, 1979). Raising insulin levels inhibits glucose production more strongly than glucose disposal (Forbes et al., 2019), and this could explain why [R_a – R_d] returns to zero during the last hour of our experiments (Fig. 2C). Whether a counterregulatory response actually takes place cannot presently be tested because measuring fish insulin accurately is not possible (Moon, 2001). A radioimmunoassay was developed more than 2 decades ago (Plisetskaya, 1998), but it also measures pro-insulin, and, therefore, overestimates true insulin concentration to some unknown and varying extent. Nevertheless, insulin is the most likely signal that could cause the decrease in glucose fluxes observed after 4 h of glucagon administration.

Regulation of phosphoenolpyruvate carboxykinase (Pepck)

This study shows that the 3 isoforms of trout hepatic Pepck respond very differently to glucagon (Fig. 5). The best characterized Pepck isoform (trout *Pck1* and mammalian *PEPCK-C*) is strongly stimulated by glucagon in fish (a more than 40-fold increase; top Fig. 4) as well as in mammals (Iynedjian and Salavert, 1984). This cytosolic form of the enzyme is tightly associated with the regulation of gluconeogenesis in both groups of animals (Méndez-Lucas et al., 2013; Mommsen, 1986; Suarez and Mommsen, 1987). Such a large transcriptional induction of *pck*1 is likely to play an important role in stimulating hepatic glucose production in trout. Less

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is known about the regulation of the mitochondrial form of Pepck in fish (*pck2*) or mammals (*PEPCK-M*). Salmonids have 2 mitochondrial Pepck isoforms, but glucagon only affects *pck2a* (downregulation) whereas *pck2b* does not respond to the hormone (Fig. 4). In mammals, *PEPCK-M* potentiates the *PEPCK-C*-driven gluconeogenesis (Méndez-Lucas et al., 2013), but it is not sensitive to glucoregulatory hormones like glucagon and insulin (Stark and Kibbey, 2014). The functional relevance of differential glucagon regulation in these duplicated mitochondrial *pck2* paralogues of rainbow trout is not known and warrants further investigation. Overall, this study reveals that strong transcriptional regulation of cytosolic *pck1* by glucagon greatly contributes to increased hepatic *de novo* gluconeogenesis, as PEPCK-C does in mammals (Iynedjian and Salavert, 1984).

Regulation of glycogen phosphorylase

In trout muscle, glucagon regulates the expression of 2 isoforms of glycogen phosphorylase in opposite ways: it inhibits *pygm* but stimulates *pygb* (Fig. 3). While these results reveal that key enzymes involved in muscle glycogen metabolism are affected at the transcript level, the exact functional roles of these trout isoforms have not been elucidated. Transcript regulation suggests a predominant limitation of glycogenolysis by glucagon, but the measurement of enzyme activities will be needed to test this possibility. In mammalian muscle, insulin strongly decreases glycogen phosphorylase activity (Dimitriadis et al., 2011), and reduces its transcript level (Reynet et al., 1996). This study shows transcript regulation of *pygm* in rainbow trout, and the observed decrease seems to support the idea that insulin mounts a counterregulatory response during the final 2 hours of our experiment. However, insulin infusion failed to alter muscle glycogen phosphorylase activity in a previous

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study (Polakof et al., 2010). Overall, this suggests that the measured changes in *pygm* transcript abundance, while indicating some effect on glycogen metabolism, should be interpreted with caution in terms of function.

Glucagon signalling

Glucagon did not activate PKA substrates and FoxO1 in muscle or liver (Fig. 6). A previous study had shown that the hormone has a weak glycogenolytic effect in muscle and mainly acts on the liver (Polakof et al., 2012). Here, it is unclear why the glucagon signalling pathways were not activated in either tissue. However, several different factors may be responsible including the timing of tissue sampling, post-transcriptional mechanisms, and downregulation of the glucagon receptors. The most likely explanation is that glucagon signalling occurred before the tissues were collected (e.g. sampling 4 h after the onset of glucagon administration may have been too late to catch the effects of glucagon on FoxO1 or PKA substrates, and could have provided enough time for insulin to counter the initial response to glucagon). The changes in glucose fluxes observed within just a few hours may also have been elicited by post-transcriptional activation/inhibition of key enzymes in gluconeogenesis and glycogen metabolism (Brighenti et al., 1991; Mommsen and Moon, 1990; Murat and Plisetskaya, 1977; Puviani et al., 1990). Finally, the lack of effects on glucagon signalling may be associated with an overabundance of circulating glucagon. Several *in vivo* studies in rats, report that high levels of glucagon cause downregulation of glucagon receptors (Dighe et al., 1984; Soman and Felig, 1978; Srikant et al., 1977). The hormone might also regulate glucagon receptor concentration in trout hepatocytes (Navarro and Moon, 1994), as it does in mammals (Horwitz and Gurd, 1988; Santos and Blazquez, 1982).

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Conclusions

This study provides the first *in vivo* measurements of fish glucose fluxes during glucagon administration. It shows that glucagon increases blood glucose concentration by causing a rapid mismatch between R_a and R_d glucose that gradually disappears over the duration of the experiment (Figs. 1A and 2C). This hyperglycemia results from: (i) an increase in R_a glucose induced by the stimulation of gluconeogenesis (possibly through transcriptional activation of *pck1* and glycogen phosphorylase, as well as (ii) a decrease in R_d glucose via inhibition of glycogen synthase and glycolysis. After 4 h of glucagon administration, both R_a and R_d glucose decrease below baseline and converge to the same lower level. This late reduction of glucose fluxes may be caused by a counterregulatory response of insulin, potentially linked to a decrease in *pygm* transcript abundance. Results also show that the three known Pepck isoforms of trout are regulated differently, pointing to a conserved role of transcriptionally regulated cytoplasmic Pepck by glucagon between fish and mammals (Fig. 4). This similarity with mammals does not apply to the mitochondrial form of Pepck because the two trout *pck2* transcripts are differentially affected by glucagon, whereas the equivalent mammalian enzyme (PEPCK-M) does not respond to glucoregulatory hormones. Glycogen phosphorylase isoform transcripts are regulated differently between tissues, but also within muscle. Overall, however, the integrated, multi-organ response to glucagon characterized here only leads to modest changes in glucose fluxes and this is another reason why rainbow trout seem unable to exert tight control over glycemia.

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Competing interests

No competing interests declared.

Author Contributions

Author contributions: J.F. and J.-M.W. conception and design of research; J.F. performed experiments; J.F. analyzed data; J.F. and J.-M.W. interpreted results of experiments; J.F. and J.-M.W prepared figures; J.F. drafted manuscript; J.F., J.-M.W., D.J.K and J.A.M. edited and revised manuscript; J.F., J.-M.W., D.J.K. and J.A.M. approved final version of manuscript.

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Table 1: Mean physical characteristics and hematocrit of the 2 groups of catheterized rainbow trout used (i) for *in vivo* measurements of glucose kinetics or (ii) for tissue measurements of glucagon signalling proteins and gene expression of enzymes. Fulton's condition factor K for all fish was 1.09±0.02 (N=22). Measurements were made before surgery (for body mass and length) or after recovery from surgery (for hematocrit) to make sure that minimal blood loss occurred during and after surgery. Values are means ± s.e.m.

	Glucose kinetics experiments (N=8)	Protein and gene expression experiments (N=14)
Body mass (g)	350 ± 16	344.4 ± 15
Body length (cm)	31.4 ± 0.4	31.7 ± 0.4
Hematocrit (%)	19.5 ± 0.3	19.5 ± 0.4

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Table 2: Primer sequences and annealing temperatures used for mRNA quantification by real-time RT-PCR. Primers for new assays were designed using NCBI-derived trout mRNA sequences in the Primer 3 algorithm (http://bioinfo.ut.ee/primer3-0.4.0/) using standard parameters with the following modifications: Amplicon size: 100-300 bp; Primer size: 19-21 nt; Primer T_m 55-65 ºC; Primer GC% 45-55; Maximal self-complementarity: 4; Maximal 3' self-complementarity: 4.

Target	Forward primer sequence (5'3')	Reverse primer sequence (3'5')	Annealing temperature [ºC]	Reference
pck1	ACAGGGTGAGGCAGATGTAGG	CTAGTCTGTGGAGGTCTAAGGGC	55	(Marandel et al., 2015)
pck2a	ACAATGAGATGATGTGACTGCA	TGCTCCATCACCTACAACCT	56	(Marandel et al., 2015)
pck2b	AGTAGGAGCAGGGACAGGAT	CCGTTCAGCAAAGGTTAGGC	59	Designed
g6pase	TAGCCATCATGCTGACCAAG	CAGAAGAACGCCCACAGAGT	55	(Panserat et al., 2009)

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fbpase	GCTGGACCCTTCCATCGG	CGACATAACGCCCACCATAGG	60	(Panserat et al., 2009)
glut4	GGCGATCGTCACAGGGATTC	AGCCTCCCAAGCCGCTCT T	57	(Panserat et al., 2009)
glut2	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	60	Designed
pygm	CCCGGCTACAGGAACAACAT	ACAGCCTGAATGTAGCCACC	55	Designed
pygb	GTGATCCCTGCAGCTGACTT	TCCTCTACCCTCATGCCGAA	59	Designed
glut1bb	GTGATCCCTGCAGCTGACTT	AGGACATCCATGGCAGCTTG	57	(Liu et al., 2017)
pfk	CGTAGGCATGGTGGGTTCTA	AGCCACAGTGTCTACCCATC	59	Designed

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Table 3: Initial (baseline) and final values after 4 h of glucagon administration for various parameters of glucose metabolism in rainbow trout. Values are means ± s.e.m (N=8). Glucose turnover rate (R_t) was obtained with the steady state equation, whereas the rates of appearance (R_a) and disposal (R_d) were obtained with the non-steady state equations of Steele (Steele, 1959). The effects of glucagon are indicated as * P<0.01 or ** P<0.001 (paired t-test).

	Baseline	Final (after 4 h of glucagon infusion)
Glucose (μmol ml^{-1})	8.5 ± 0.4	11.7 ± 0.4 **
R_t Glucose (μmol kg^{-1} min^{-1})	11.5 ± 1.2	10.2 ± 0.9
R_a Glucose (μmol kg^{-1} min^{-1})	10.6 ± 1.4	8.8 ± 1.3
R_d Glucose (μmol kg^{-1} min^{-1})	10.6 ± 1.4	8.8 ± 1.2

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Figures

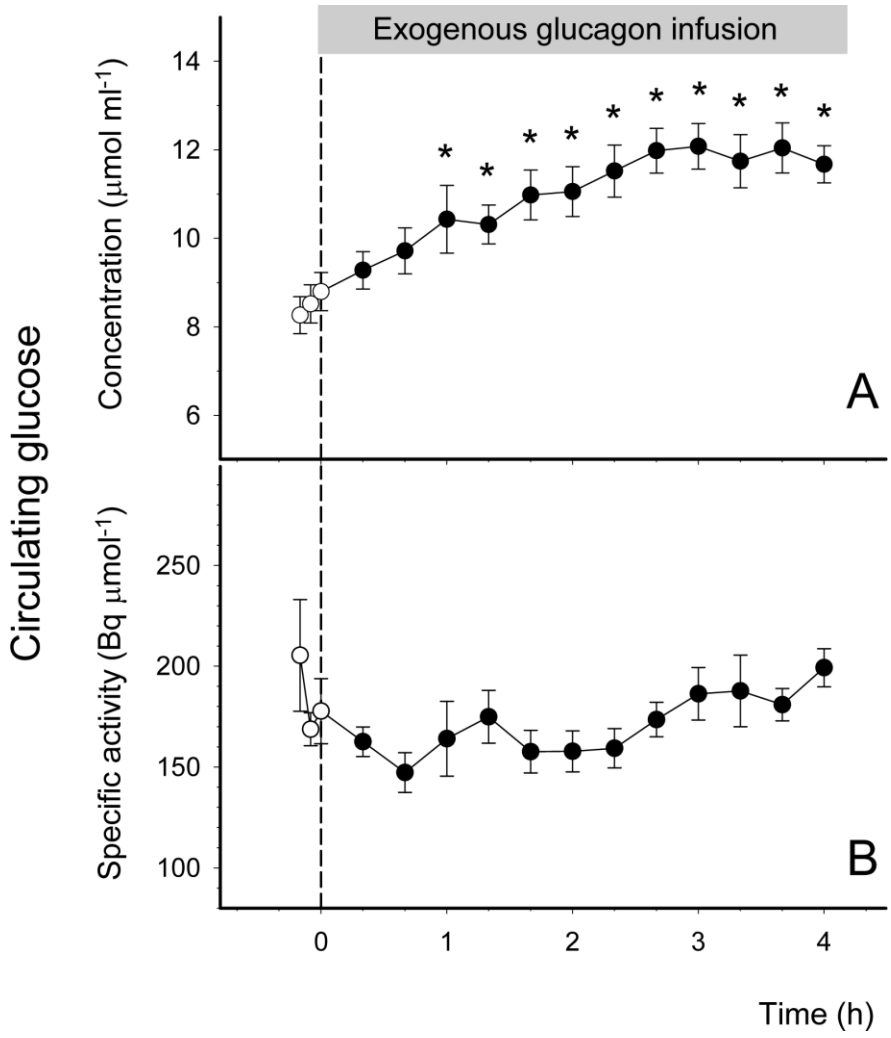

Fig. 1. Effects of exogenous glucagon on (A) plasma glucose concentration and (B) glucose specific activity. Values are means ± s.e.m (N=8). Open symbols are for baseline values before starting glucagon administration. Means significantly different from baseline are indicated by * (P<0.05).

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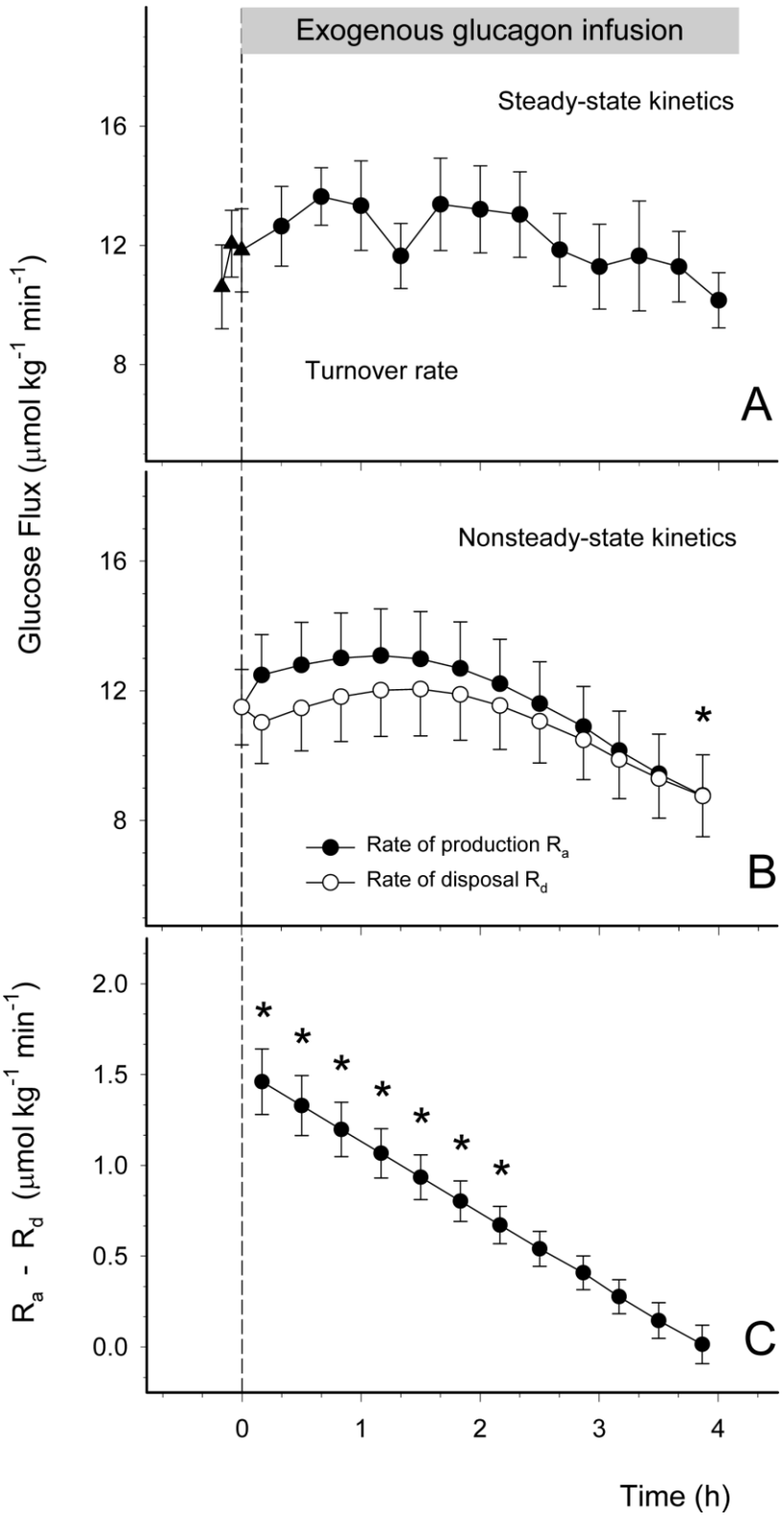

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Fig. 2. Effects of glucagon on the glucose fluxes of rainbow trout. Fluxes were either calculated with the steady-state equation [turnover rate (R_t), panel A where triangles are for baseline values] or the non-steady-state equations of Steele [glucose disposal (R_d) and hepatic glucose production (R_a), panel B] (Steele, 1959). Panel C shows the effects of glucagon on the capacity to increase glycemia [R_a glucose – R_d glucose]. Values are means ± s.e.m. (N=8). Means significantly different from baseline (panels A and B) or significantly different from 0 (panel C) are indicated by * (P<0.05).

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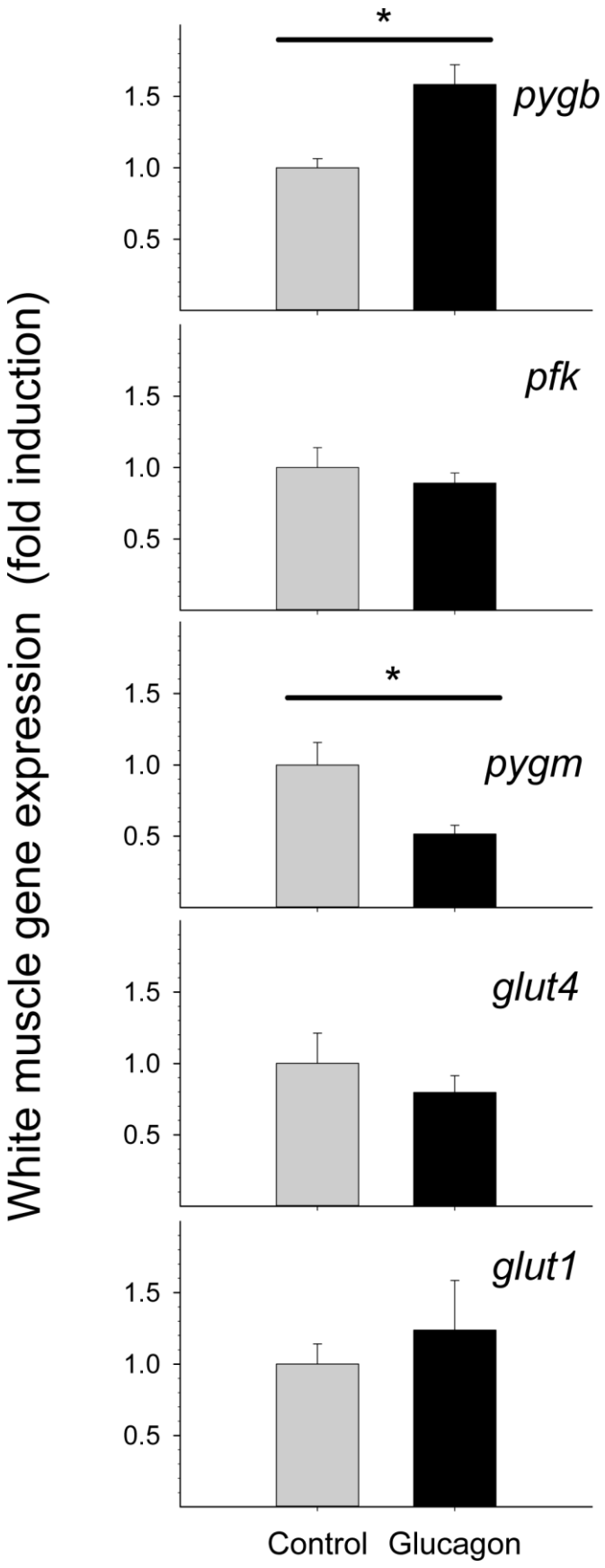

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Fig. 3. Relative effects of glucagon on white muscle mRNA transcript abundance of glycogen phosphorylase brain-associated b (*pygb*), phosphofructokinase (*pfk*), glycogen phosphorylase muscle-associated (*pygm*), glucose transporter 4 (*glut4*), and glucose transporter 1 (*glut1*). Values are means ± s.e.m. (N=7 for each group). Means significantly different from control are indicated by * (P<0.05).

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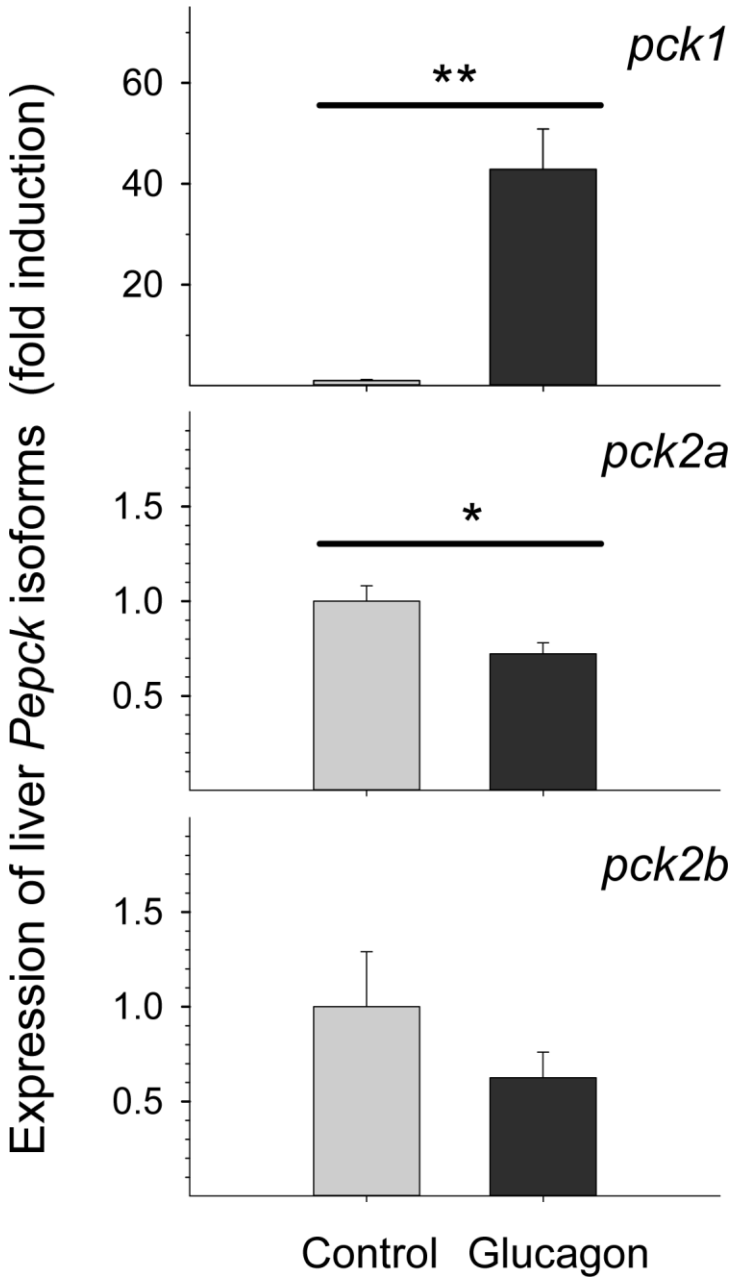

Fig. 4. Relative effects of glucagon on liver mRNA transcript abundance of 3 isoforms of phosphoenolpyruvate carboxykinase: *pck1, pck2a, and pck2b.* Values are means ± s.e.m. (N=7 for each group). Means significantly different from control are indicated by * (*P*<0.05) or ** (*P*<0.001).

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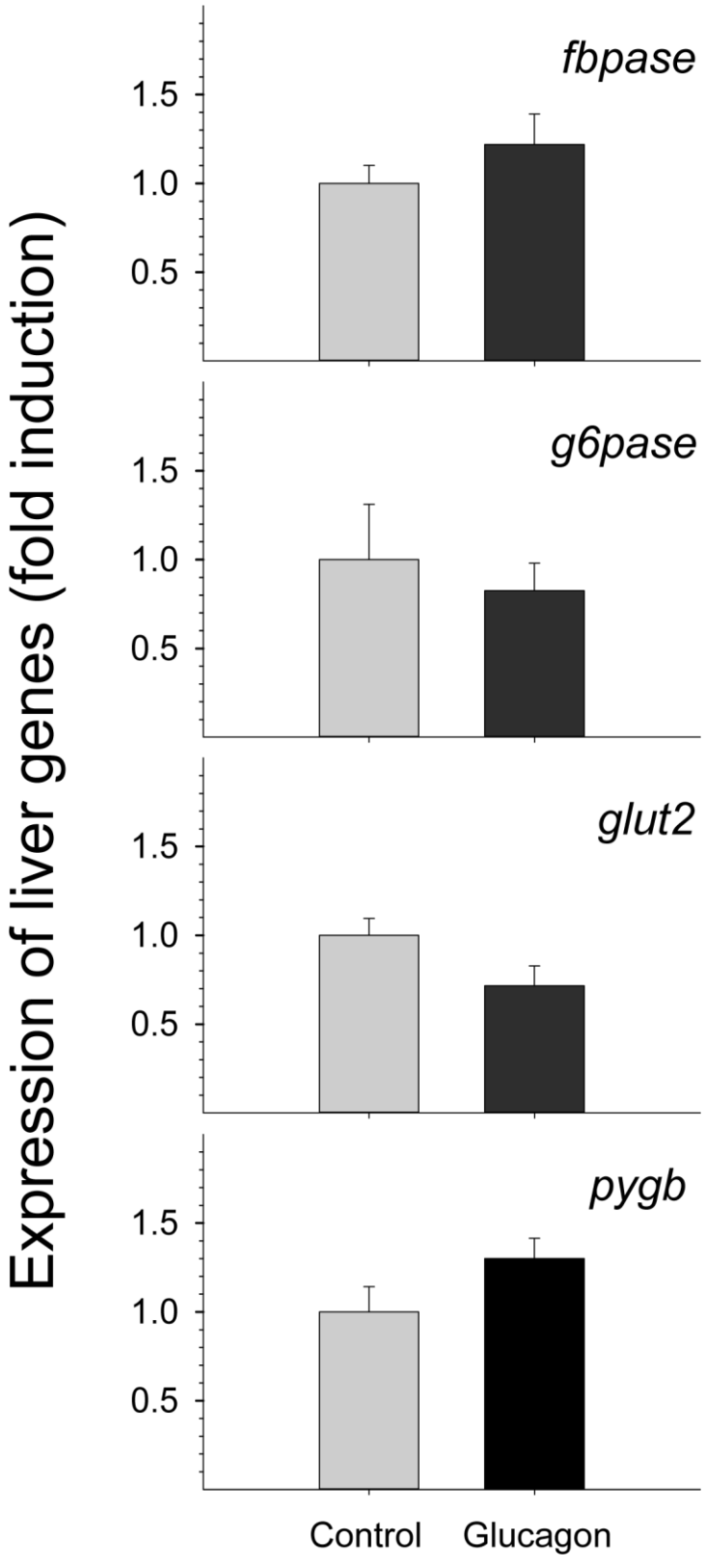

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Fig. 5. Relative effects of glucagon on liver mRNA transcript abundance of fructose 1,6-bisphosphatase (*fbpase*), glucose-6-phosphatase (*g6pase*), glucose transporter 2 (*glut2*) and glycogen phosphorylase brain-associated (*pygb*). Values are means ± s.e.m. (N=7 for each group). Glucagon had no effect (P>0.05).

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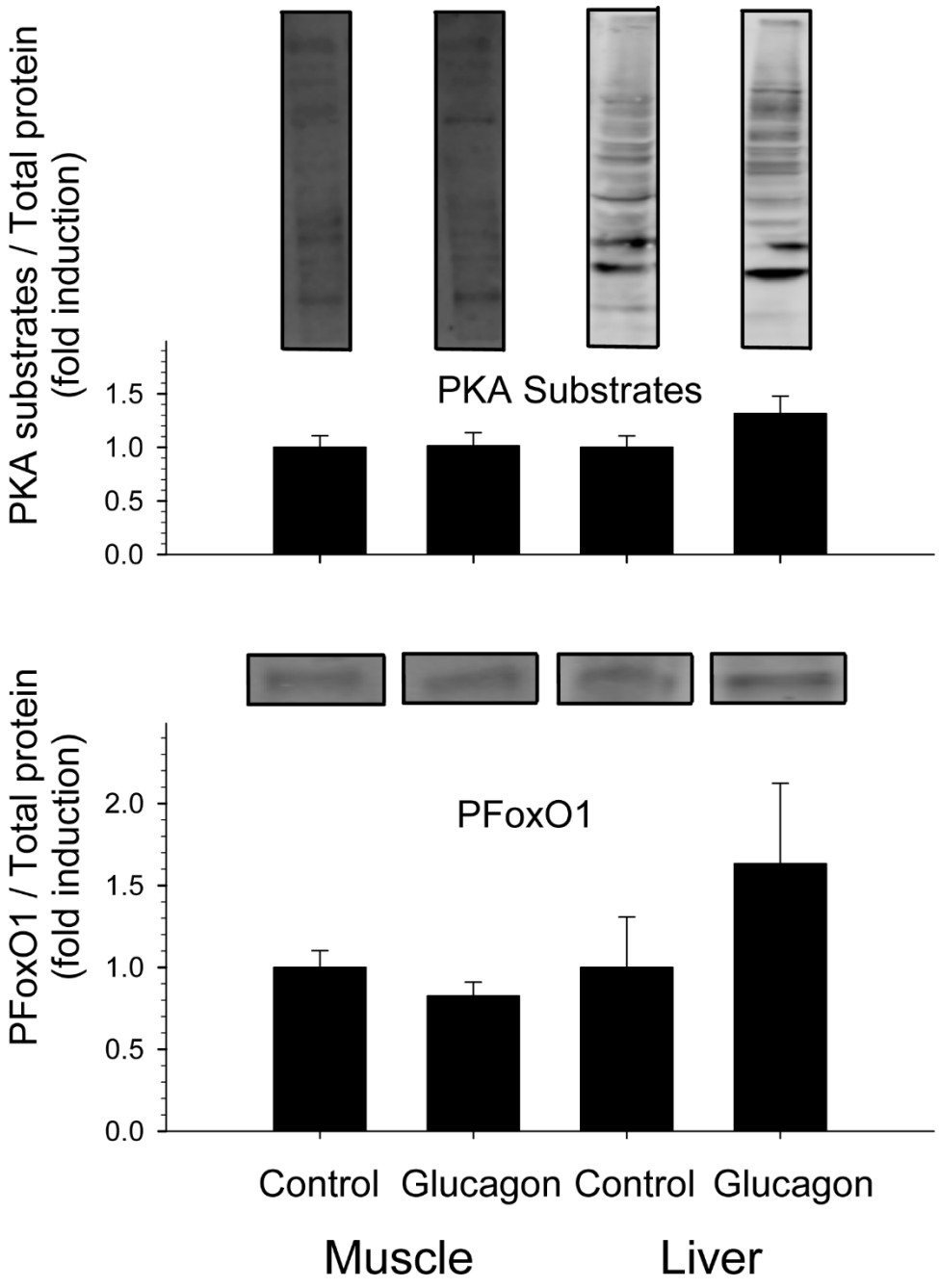

Fig. 6. Relative effects of glucagon on the levels of the key signalling proteins, PKA substrates and PFoxO1 in white muscle and liver. For each mean, western blots are given for the phosphorylated protein(s). Values are means ± s.e.m. (N=6 for each group). Glucagon had no effect on these proteins (P>0.05).

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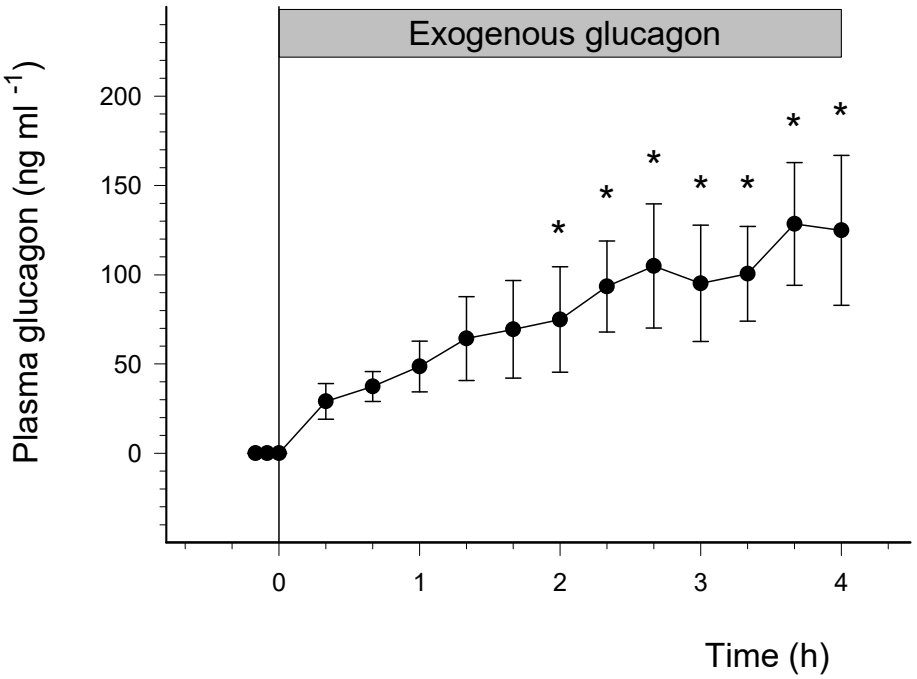

Fig. S1. Changes in plasma glucagon concentration caused by the administration of exogenous glucagon in adult rainbow trout during measurements of glucose kinetics. Values are means ± s.e.m (N=8). Means significantly higher than baseline are indicated by * (*P<0.05*).

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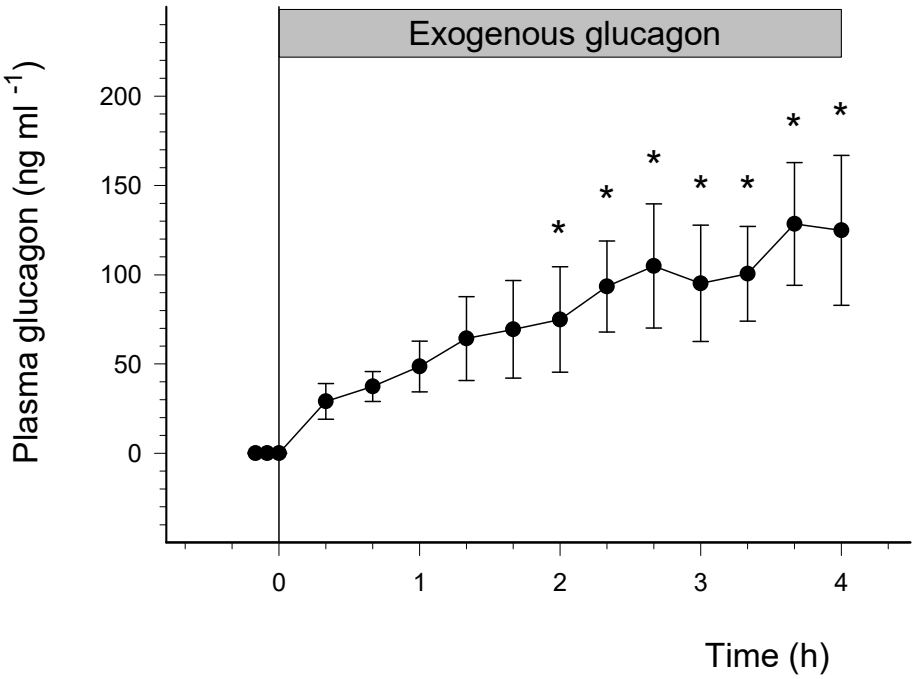

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