Alanine alters carbohydrate metabolism of rainbow trout: Glucose fluxes and cell signaling

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Abstract

In rainbow trout, dietary carbohydrates are poorly metabolized compared to other macronutrients. One prevalent hypothesis suggests that high dietary amino acid levels could contribute to the poor utilization of carbohydrates in trout. In mammals, alanine is considered an important gluconeogenic precursor, but has recently been found to stimulate AMP-activated protein kinase (AMPK) to reduce glucose levels. In trout, the effect of alanine on glucose fluxes is unknown. The goal of this study was to determine the effects of 4h exogenous alanine infusion on glucose metabolism in rainbow trout. Glucose fluxes, glucose appearance (Ra), and disposal (Rd) were measured in vivo. Key glycolytic and gluconeogenic enzyme expression and activity and cell signaling molecules relevant to glucose metabolism were assessed in liver and muscle. Results show that alanine inhibits Ra glucose (from 13.2+/-2.5 to 7.3+/-1.6 micromol / kg min) and Rd glucose (from 13.2+/-2.5 to 7.4+/-1.5 micromol / kg min) and the slight mismatch

between Ra and Rd caused a reduction in glycemia, similar to the effects of insulin in trout. The reduction in Rd glucose can be partially explained by a reduction in glut4b expression in red muscle. In contrast to mammals, alanine-dependent glucose-lowering effects in trout did not involve hepatic AMPK activation, suggesting a different mechanistic basis. Interestingly, protein kinase B (AKT) activation increased only in muscle similar to effects observed in insulin-infused trout. We speculate that alanine-dependent effects were probably mediated through stimulation of insulin secretion which could indirectly promote alanine oxidation to provide the needed energy.

KEY WORDS: Glucose fluxes, Glycolysis, Gluconeogenesis, AMPK signaling, Gene expression, Enzyme activity

Introduction

Glucose is a key metabolic fuel and the preferred substrate of many tissues in mammals (Polakof et al., 2011b). Generally, circulating glucose levels, turnover, and oxidation rate are lower in fish compared to mammals and birds, except possibly for American eel and skipjack tuna (Hemre et al., 2002; Polakof et al., 2011b; Polakof et al., 2012). Even after effective digestion and absorption, dietary carbohydrates only represent a minor energy source compared to proteins and lipids in carnivorous trout (Polakof et al., 2012). However, current evidence suggests that glucose nevertheless constitutes an essential fuel in some tissues, especially in the brain (Polakof et al., 2012). One prevalent hypothesis suggests that high levels of dietary amino acids are responsible for the comparatively poor metabolization of carbohydrates observed in trout (Panserat et al., 2013).

How fish adjust their glucose fluxes to regulate glycemia can be assessed by tracer methods that allow measurement of the rate of steady-state glucose turnover (R_t), or the rates of glucose appearance in (R_a) and disposal from the circulation (R_d) separately, under non-steady-state conditions (Haman and Weber, 1996). Even though some information is already available on the regulation of trout glucose fluxes (Forbes et al., 2019a; Forbes et al., 2019b; Weber et al., 2016), the effects of amino acids on *in vivo* glucose kinetics are unknown. Alanine is a dominant amino acid in all tissues in rainbow trout (Storey, 1991) and has one of the highest disposal rates indicating a high requirement for alanine in this species (Robinson et al., 2011). Alanine is generally considered an important gluconeogenic precursor (Felig, 1973; Jürss and Bastrop, 1995). In mammals, exogenous alanine activates gluconeogenesis, but it fails to affect R_a glucose (Diamond et al., 1988; Jahoor et al., 1990; Wolfe et al., 1987). The extra glucose produced from alanine appears to be predominantly channeled towards hepatic glycogen rather than released in the circulation. A recent study on cats in diabetic remission, which are described as diabetic cats that maintain euglycemia for at least two weeks after insulin therapy, found that alanine was one of the gluconeogenic amino acids that showed a decrease in serum levels (Gottlieb et al., 2020). On the other hand, the authors suggested that an increase in alanine metabolism through the glucose-alanine cycle might be linked to the observed increase in plasma urea in remission cats (Gottlieb et al., 2020). The potential effects of alanine on trout gluconeogenesis are less clear because activation of the pathway has been reported *in vivo* (Cowey et al., 1977) and for isolated hepatocytes (Canals et al., 1992), but some studies show no stimulation of gluconeogenic enzymes (Kirchner et al., 2003) or suggest that alanine is not an

important precursor for glucose (French et al., 1981). Alternately, amino acids can be significant oxidative substrates for carnivorous fish (Ballantyne, 2001) and trout hepatocytes prefer to oxidize alanine rather than to convert it to glucose (French et al., 1981; Pereira et al., 1995). Therefore, it is conceivable that exogenous alanine could partly replace glucose as an oxidative fuel.

Recent evidence shows that alanine also acts as a signaling molecule because it stimulates AMP-activated protein kinase (AMPK) in mammals (Adachi et al., 2018). A high AMP/ATP ratio activates AMPK via phosphorylation, thereby stimulating catabolic pathways (to restore normal ATP levels) and inhibiting anabolic pathways such as protein synthesis via target of rapamycin (TOR)(Hardie, 2018). AMPK activity is regulated by insulin and nutrient availability through inhibition by AKT (also known as protein kinase B)(Jeon, 2016). In mice, alanine activation of AMPK via alanine aminotransferase (ALT) inhibits downstream TOR effectors and promotes glucose utilization (Adachi et al., 2018). Current knowledge of how AMPK (Polakof et al., 2011a) and TOR (Lansard et al., 2010) affect trout metabolism suggests a conserved role for these signals. However, it is not known whether alanine acts on AMPK, AKT and TOR in trout in the same way as it does in mammals (Adachi et al., 2018).

The aim of the present study was to integrate organismal and molecular information to characterize the effects of exogenous alanine on trout glucose metabolism. Our first goal was to measure whether alanine modulates R_t, R_a and/or R_d glucose. Current literature suggests two alternative hypotheses about how glucose fluxes could be affected. If exogenous alanine acts mainly as a gluconeogenic precursor, R_a glucose and glycemia will both increase (hypothesis 1). Alternately, if alanine is predominantly

used as an oxidative fuel, it will replace some glucose and reduce R_a and R_d (hypothesis 2). The second goal of this study was to explore potential mechanisms responsible for the observed changes in glucose fluxes. Therefore, the expression of key genes involved in glucose transport, glycolysis, and gluconeogenesis, as well as the activities of essential enzymes were measured in muscle and liver. Protein levels of AMPK, AKT and two TOR targets, ribosomal protein S6 (S6) and eukaryotic translation initiation factor 4E binding protein 1 (4-EBP1), were also quantified. This allowed to test whether alanine activates AMPK signaling in rainbow trout as recently shown in mammals (Adachi et al., 2018).

Material and methods

Animals

Adult rainbow trout (*Oncorhynchus mykiss*) of both sexes were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). The fish were maintained in 1,200-liter flow-through tanks supplied with dechlorinated Ottawa tap water at 13°C and were exposed to a 12 h:12 h light:dark photoperiod. They were acclimated to these conditions for a minimum of 2 weeks before experiments. Trout were fed Orient LP diet (Skretting, New Brunswick, Canada) 5 days/week. The diet contains 39% protein, 16% fat and 3% fiber. This study consisted of two groups of fish; the first was used for *in vivo* measurements of glucose kinetics by continuous tracer infusion and the second involved measurements at the molecular level by real-time RT-PCR, Western blots and enzyme activity for key indices of glucose metabolism (physical characteristics of the

experimental groups are given in Table 1). All procedures were approved by the Animal Care Committee of the University of Ottawa and complied with the guidelines established by Canadian Council on Animal Care (CCAC).

Cannulations

Fish were anesthetized using ethyl 3-aminobenzoate methanesulfonate (60 mg/l MS-222 buffered with 0.2 g/l sodium bicarbonate) and cannulated with BTPE-50 catheters (Instech Laboratories, Plymouth Meeting, PA, USA) in the dorsal aorta as described previously (Haman and Weber, 1996). Fish used for glucose kinetics were doubly cannulated and those used for the molecular experiments were singly cannulated as labeled glucose was not infused in the latter. Cortland saline containing 50 U/ml heparin (Sigma-Aldrich, St Louis, MO, USA) was used to flush the catheters after being placed in the dorsal aorta and 25 U/ml of heparin in Cortland saline was used during blood sampling to prevent clotting. For the glucose kinetics experiments, alanine or Cortland saline (for the control group) and labeled glucose were infused through one catheter and blood was sampled through the second. For the molecular experiments, blood samples were taken before the infusion and at the end of the experiment and alanine or Cortland saline were infused through one catheter. Fish were kept in a 90-liter swim tunnel respirometer (Loligo Systems, Tjele, Denmark) supplied 13°C aerated and dechlorinated Ottawa tap water to recover overnight before the infusion. All experiments were conducted in resting fish at a water velocity of 0.5 body length/s.

Glucose kinetics experiments

The catheters were made accessible through the swim tunnel lid by channeling them through a water-tight port. Fish (N=9 for the alanine group and 7 for controls) were continuously infused with [6-^{3}H] glucose (2220 GBq/mmol; Perkin Elmer, Boston, MA) to measure R_t, R_a and R_d glucose. The infusate was prepared for each fish immediately before infusion by drying 20 µl of the tracer solution under N_2 and resuspending in 10 ml of Cortland saline. A priming dose of the tracer, equivalent to 90 min of infusion, was injected as a bolus to reach isotopic steady-state in <45 min (Shanghavi and Weber, 1999). Thereafter, the tracer was infused at ~1 ml/h (finely adjusted for each fish to account for differences in body mass) using a calibrated syringe pump (Harvard Apparatus, South Natick, MA). Labeled glucose infusion rate averaged 2149±346 Bq kg^{-1} min^{-1} (N=9) for the alanine-infused fish and 1676±135 Bq kg^{-1} min^{-1} (N=7) for the control fish. These trace amounts of labeled glucose accounted for <0.00001% of the baseline rate of hepatic glucose production in trout (Shanghavi and Weber, 1999). Blood samples (~100 µl each) were taken 50, 55 and 60 min after the start of infusion to establish baseline glucose kinetics and then every 30 min during the alanine (Sigma - Aldrich A7627; at a rate of 443 µg.kg^{-1}.min^{-1}) or Cortland saline infusions that lasted 4 h. The alanine infusion rate was selected on the basis of a previous study (Robinson et al., 2011) and on pilot experiments. The amount of blood drawn from each fish accounted for <10% of total blood volume. Blood samples were collected in tubes containing heparin and aprotinin, and they were immediately centrifuged for 5 min at 12,000 rpm (Eppendorf 5415C, Brinkman, Rexdale, Canada). Plasma was separated and stored at −20°C until analyses.

Plasma sample analyses

Plasma alanine and glucose concentrations were measured spectrophotometrically using a Spectra Max Plus 384 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Plasma alanine concentration was measured using an EnzyChrom L-Alanine Assay Kit (BioAssay Systems, USA). Glucose concentration was determined using an enzymatic assay that measures the absorbance of NADH at 340 nm with 7 U/ml hexokinase (H4502, Sigma) and 0.1 U/ml glucose-6-phosphate dehydrogenase (G5885, Sigma) in a buffer containing 59.84 mM Trizma base, 39.98 mM Tris-HCl, 1.0142 mM $MgSO_4.7H_2O$, 2.215 mM NAD^+ and 1.106 mM ATP. For the glucose kinetics experiments, radioactivity was measured by scintillation counting (Beckman Coulter LS 6500, Fullerton, CA) by drying 20 µl of plasma under nitrogen gas to eliminate tritiated water, resuspending in 1 ml of deionized water and adding 10 ml of Bio-Safe II scintillation cocktail (RPI, Mount Prospect, IL).

Molecular experiments

These experiments were carried out on different fish than those used for the glucose kinetics to avoid handling radioactivity unnecessarily. The same rate of alanine (alanine group, N=7; 443.2 µg alanine.kg^{-1}.min^{-1}) or Cortland saline infusion (control group, N=7) was administered at ~1 ml/h for 4 h. A baseline blood sample was taken at time 0 h before the start of alanine/saline infusion and at 4 h. The fish were then euthanized by a sharp blow on the head and tissues were collected. Whole liver, white

muscle (perpendicular to the dorsal fin) and red muscle (along the lateral line) were collected and stored at –80°C until analyses.

RNA extraction and gene expression

Total RNA from white and red muscle was extracted by homogenizing 50 mg of tissue in 1.5 ml tubes using a sonicator (Fisher Scientific Sonic Dismembrator model 100, San Diego, CA, USA) and Trizol reagent (life technologies). RNA was quantified using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo-Fisher Scientific).

QuantiTect reverse transcriptase kit (Qiagen, Toronto, ON, Canada) was used to generate cDNA using total RNA from white and red muscle tissues following the manufacturer's protocol. A no-reverse transcriptase (RT) negative control, where the RT was replaced with water, and a no-template negative control, where the RNA was replaced with water, were included to check for genomic DNA contamination.

White and red muscle mRNA gene expression of glucose transporter (*glut4a* and *glut4b*) and hexokinase (*hk2*; presented in supplementary data, Fig. S2) were assessed by two-step real-time RT-PCR in each tissue using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Mississauga, ON, Canada) following the manufacturer's protocol and using the *BioRad* CFX96- Real-Time System- C1000 Thermal Cycler. The expression of the reference gene elongation factor 1α (*ef1α*) was stable in both groups regardless of the treatment in the red and white muscle (Fig. S5). Therefore, *ef1α* was used to normalize the mRNA abundance of the white and red muscle genes (*glut4a*, *glut4b* and *hk2*). The reference gene *ef1α* has been previously used in rainbow trout

(Kostyniuk et al., 2019; Polakof et al., 2011a). Liver mRNA gene expression (presented in supplementary data, Fig. S1 and Fig. S2) of key gluconeogenic genes (phosphoenolpyruvate carboxykinase, *pck1*, *pck2a*, *pck2b*, and glucose-6-phosphatase, *g6pca*, *g6pcb1a*, *g6pcb2a*, *g6pcb1b* and *g6pcb2b*) and glycolytic genes (glucokinase, *gka* and *gkb*) were assessed similarly using iTaq Universal SYBER Green SuperMix (Bio-Rad).Transcript abundance of the reference gene *β-actin* was stable in both the control and alanine-infused trout in the liver (Fig. S5). Therefore, *β-actin* was used to normalize transcript abundance of the genes assessed in the liver. The reference gene *β-actin* has been previously used in trout (Moltesen et al., 2016). Standard curves from pooled cDNA serial dilution were generated to optimize/check reaction conditions. Diluted samples were run in duplicate. The total volume of the reaction was 20 µl including 1 µl cDNA, 1 µl specific forward primer, 1 µl specific reverse primer (Table S1), 10 µl Universal SYBR Green Supermix and 7 µl nuclease free water. An initial step at 98°C (2 min) was required for the activation of the enzymes in the mix. Then, two steps were repeated 40 times, the denaturation step at 95°C (20 s) and the annealing combined with the extension step (30 s) at the optimized temperature for each primer pair (Table S1). At the end, a melt step from 65°C to 95°C (0.5°C increment/5 s) was included to check the specificity of the produced amplicon by assessing the melt curve where a single melt peak indicates a specific product. The amplification efficiencies and R^2 are included in Table S1. The abundance of mRNA was calculated relative to the control group.

Western blotting

Total protein was extracted from frozen liver and muscle tissue by homogenizing 100 mg of tissue in 400 ul of buffer on ice using a sonicator (Fisher Scientific Sonic Dismembrator model 100, San Diego, CA, USA). Liver homogenization buffer (pH ~ 8) contained 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, 1 mM EDTA, 100 mM sodium fluoride, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate and a protease inhibitor cocktail (A32953, Thermo Scientific). Muscle tissues were homogenized in a buffer as previously described (Forbes et al., 2019a; Forbes et al., 2019b). Homogenates were centrifuged at 15,000 g for 10 min at 4°C, and the supernatant was taken and stored at -20°C. Protein concentrations were determined using bicinchoninic acid (BCA) assay (B9643, Sigma) with bovine serum albumin (BSA) as a standard. Protein samples were denatured and reduced before being loaded into the gels. Protein samples were diluted in equal amount of the above-described buffer and 2X Laemmli buffer for a total amount of 25 µg protein in 20 µl of loading volume. The samples were denatured at 95°C for 5 min and quickly placed on ice. To load protein samples, gels were casted as a 10% resolving gel consisting of 5 ml ddH_2O, 2.5 ml buffer B (1.5 M Tris base and 0.04% SDS dissolved in dH_2O, pH 8.8), 2.5 ml 40%Acryl/Bis (Bio-Rad), 50 µl 10% ammonium persulfate (APS) (A3678, Sigma) and 20 µl TEMED (Invitrogen); and a 4% stacking gel consisting of 3.25 ml ddH_2O, 1.25 ml buffer C (0.5 M Tris and 0.04% SDS dissolved in dH_2O, pH 6.8), 0.5 ml 40%Acryl/Bis, 25 µl 10% APS and 10 µl TEMED. Gels were placed in 1X Tris glycine SDS (TGS) running buffer consisting of 2.5 mM Tris base, 0.192 M glycine and 0.1% SDS dissolved in dH_2O. Protein samples were loaded with 5 µl of Page Ruler

prestained protein ladder (26616, Thermo Scientific, Lithuania). The proteins were migrated for ~2 h at 100 V using the Mini Protean Tetra Cell (Bio-Rad, China). Immediately after migration, the gels were blotted for ~2 h at 100 V on nitrocellulose membrane (pore size 0.2 µm, Bio-Rad, Germany) in 20% Western transfer buffer consisting of 250 mM Tris base, 1.92 M glycine dissolved in dH_2O. Then, the membranes were incubated in Odyssey blocking buffer (LI-COR) for 1 h at room temperature on an orbital shaker. Membrane were incubated overnight at 4°C in rabbit-raised primary antibodies diluted to 1:1000 in the blocking buffer specific for p-AKT (S473; no. 9271S) and total AMPKα (no. 2532) all from Cell Signaling Technology. AKT (Forbes et al., 2019a) and AMPKα (Gilmour et al., 2017; Polakof et al., 2011a) antibodies were previously used in rainbow trout. The phosphorylated form of AMPKα (T172, no. 2535S), and two TOR targets, S6 (S235/236, no. 4856S) and 4-EBP1 (T37/46, no. 9459S), were also quantified in liver and muscle (see supplementary data). Membranes were then washed 4 times for 5 min with phosphate buffered saline (PBS) + 0.1% Tween 20 (P9416, Sigma) and incubated with IRDye 800 CW goat-anti-rabbit secondary antibody (925-32211, LI-COR) diluted to 1:5000 in the blocking buffer for 1.5 h at room temperature on the shaker protected from light. Membranes were then washed 4 times for 5 min with PBS + 0.1% Tween 20 and 1 time for 5 min with PBS and visualized by infrared fluorescence using the Odyssey Imaging System (LI-COR) and band intensity was quantified by Odyssey Infrared imaging system software (LI-COR). β-tubulin (no. 2146S, Cell Signaling Technology) was visualized and quantified to normalize the measured protein intensities and it has been previously used in rainbow trout to normalize protein levels (Forbes et al., 2019a; Polakof et al., 2011a).

Enzyme activities

Liver tissue (1:4 w/v), red and white muscle tissue (1:16 w/v) were sonicated on ice in cold 50 mM Tris buffer pH 7.5 with protease inhibitor mini tablets (A32953, Thermo Scientific) as previously described (Best et al., 2014; Ings et al., 2011). Whole homogenate was used. Aliquots were prepared by diluting the homogenate in 50% glycerol buffer pH 7.5 (50% glycerol, 21 mM Na_2HPO_4, 0.5 mM EDTA-Na_2 dihydrate, 0.2% BSA, 5 mM β-mercaptoethanol) and stored at -20°C. Enzyme activity was determined kinetically at 22°C by measuring the reduction of NADH at 340 nm using a microplate reader. Enzyme activity was expressed as µmol/min/g tissue. The activity was assayed in triplicate. Control reactions were run for 10 min for each enzyme assay after adding all the reagents except the starter. Background activities were subtracted from the actual read (after adding the starter). The conditions of the individual enzyme assays were as detailed below (final concentration in the well; all reagents dissolved in 50 mM imidazole buffer pH 7.4). Phosphoenolpyruvate carboxykinase (PCK; EC 4.1.1.32) reaction mixture contained 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.5 mM PEP (Na_3), 0.12 mM NADH and 0.2 mM deoxyguanosine diphosphate (dGDP; to start the reaction). Alanine aminotransferase (ALT; EC 2.6.1.2) reaction mixture contained 0.12 mM NADH, 200 mM L-alanine, 0.025 mM pyridoxal 5-phosphate, 12 U/ml LDH (no ammonium sulphate) and 10.5 mM α-ketoglutarate (to start the reaction).

Calculations and statistics

Fulton's condition factor was calculated using the equation $K= (10^5 \times M_b)/L^3$, where M_b is body mass in g and L is length in mm (Blackwell et al., 2000). Steady-state and non-steady-state equations of Steele were used to calculate glucose fluxes in two ways (Steele, 1959). The steady-state equation was used to calculate R_t glucose. R_a and R_d glucose were calculated separately using the non-steady-state equations (Forbes et al., 2019a; Steele, 1959) using glucose specific activities curve-fitted by second-degree polynomial regression for each fish individually (Wolfe, 1992). Statistical analysis for glucose kinetics was performed using one-way repeated-measures analysis of variance (RM-ANOVA) with Dunnett's post hoc test to determine which time point was statistically different from the average baseline value using SigmaPlot v12.5 (Systat Software, San Jose, CA, USA). Two-way RM-ANOVA was used to analyze the non-steady-state data where time and R_a or R_d were the 2 factors. When the assumption of normality or equal variances were not met, data were transformed to their ln or square root. Kruskal-Wallis non-parametric one-way ANOVA on ranks was used when transformation failed to meet the assumptions of parametric tests. Molecular data (gene expression, signaling and enzyme activity) were analyzed by two-tailed unpaired t-tests using GraphPad Prism 8.3.1. Data were checked for normality using the Shapiro-Wilk test. When the normality assumption was not met, data were transformed (ln or sin transformations). The Grubb's test was used on normally distributed data to check for outliers. When variances of the two groups, control and alanine, were not equal, Welch's test was used for correction. Mann-Whitney non-parametric test was applied when the normality assumption was not met after transformation. Glucose and alanine

concentrations were analyzed by two-way RM ANOVA. The mean + s.e.m. (± s.e.m. for glucose kinetics) are shown and P<0.05 was considered significant in all tests.

Results

Plasma alanine, glycemia and glucose kinetics

The effects of exogenous alanine infusion on plasma alanine levels, glucose concentration and specific activity are presented in Fig. 1. The infusion of exogenous alanine caused a significant increase in plasma alanine concentration (*p=0.04*; Fig. 1A), specifically at 0.5, 3 and 4 h compared to baseline. Alanine increased from an average baseline value (0 h) of 0.6±0.1 µmol ml^{-1} to a final value (4 h) of 3.0±0.8 µmol ml^{-1}. Alanine administration caused a decrease in glucose concentration after 2 h (*p<0.001*; Fig. 1B). Glucose levels decreased from a baseline value of 8.7±0.3 µmol ml^{-1} to a final value of 7.3±0.7 µmol ml^{-1}. Glucose specific activity increased after 3 h of exogenous alanine infusion (*p=0.003*; Fig. 1C). Glucose specific activity increased from a baseline value of 197.4±42.9 Bq $µmol^{-1}$ to a final value of 373.0±83.4 Bq $µmol^{-1}$.

The effect of alanine on glucose flux is presented in Fig. 2. R_t glucose decreased over time (*p<0.001*; Fig. 2A) from a baseline value of 14.9±2.7 µmol kg^{-1} min^{-1} to a final value of 6.8±0.9 µmol kg^{-1} min^{-1}. Similarly, hepatic R_a and R_d glucose decreased over the 4 h of alanine infusion (*P<0.01*; Fig. 2B). R_a glucose decreased from a baseline value of 13.2±2.5 µmol kg^{-1} min^{-1} to a final value 7.3±1.6 µmol kg^{-1} min^{-1}. R_d glucose decreased from a baseline value of 13.2±2.5 µmol kg^{-1} min^{-1} to a final value of 7.4±1.5

μmol kg^{-1} min^{-1}. R_d glucose is slightly higher than R_a glucose as it can be seen in Fig. 2B.

Plasma alanine and glucose levels as well as glucose specific activity were measured in the control fish infused with Cortland saline and they are presented in Fig. 3. All the measured parameters did not change from the average baseline (*p>0.05* for all the parameters; Fig. 3A, B and C). R_t glucose did not show significant change over time compared to the average baseline *(p>0.05*; Fig. 4).

Plasma alanine and glucose for molecular experiments

Baseline and final plasma alanine and glucose concentrations in the alanine and control groups are presented in Table 2. Alanine levels increased after 4 h of exogenous alanine administration (*p=0.002*) and compared to the control group (*p=0.0006*). The interaction between the time and treatment was significant (*p=0.002*). Plasma glucose concentration decreased after 4 h of exogenous alanine infusion regardless of the treatment, Cortland saline or alanine (*p=0.03*).

Gene expression

The effect of alanine on red and white muscle *glut4a* and *glut4b* mRNA abundance is shown in Fig. 5. The relative abundance of red muscle *glut4b* decreased in the alanine group (*p=0.01*; Fig. 5), but the mRNA abundance of the other genes was unaffected by the treatment (*p>0.05*; Fig. 5). The mRNA abundance of the paralogues of two gluconeogenic genes, *pck* and *g6pc*, was assessed in the liver as shown in Fig. S1. Alanine had no effect on the transcript abundance of *pck* paralogues (*p>0.05*; Fig

S1A). Among the five paralogues of *g6pc*, only *g6pcb1b* increased in the alanine group (*p=0.04*; Fig. S1B). The transcript abundance of the glycolytic genes *gka* and *gkb* in the liver and *hk2* in red and white muscle is presented in Fig. S2. Alanine had no effect on the mRNA abundance of these genes in the liver, white and red muscle (*p>0.05*; Fig. S2).

Protein signaling

The effect of alanine on the relative level of total AMPKα was assessed by Western blotting in the liver as shown in Fig 6. After 4 h of exogenous alanine administration, total AMPKα increased in the alanine group (*p=0.02*; Fig. 6). The level of phosphorylated AKT was quantified in the liver and red muscle as presented in Fig. 7. Alanine had no effect on liver p-AKT, but there is a trend of a decrease in the alanine group (*p=0.06*; Fig. 7). However, the level of red muscle p-AKT increased in the alanine group after 4 h of exogeneous alanine infusion (*p=0.03*; Fig.7). The relative level of phosphorylated AMPKα and two TOR targets, S6 and 4-EBP1, was assessed in the liver (Fig. S3) and red muscle (Fig. S4). Alanine had no effect on the phosphorylated form of these proteins in the liver (*p>0.05*; Fig. S3) and red muscle (*p>0.05*; Fig. S4).

Enzyme activities

Enzyme activity of ALT and PCK in the liver is shown in Fig 8. Alanine had no significant effect on the activity of the measured enzymes in liver ($p>0.05$; Fig. 8).

Discussion

This study is the first to show that alanine reduces glycemia in rainbow trout. This reduction is linked to decreases in R_a and, to a lesser extent, R_d glucose. At the molecular level, the alanine-dependent decrease in R_d glucose can be partially explained by a reduction in *glut4b* gene expression in red muscle. Together, these findings show that alanine does not stimulate hepatic glucose release but limits peripheral glucose utilization. The reduction in glycemia observed here is consistent with recent findings in mice (Adachi et al., 2018). However, the molecular mechanisms involved appear to be different. In mice, the alanine-dependent reduction in glycemia is linked to the activation of hepatic AMPK, the stimulation of glucose uptake and reduction of its release (Adachi et al., 2018). In trout, alanine failed to stimulate hepatic p-AMPK, although total AMPK increased. However, p-AKT increased in red muscle, but not in liver, mimicking earlier findings after insulin infusion in trout (Forbes et al., 2019a). Together, present results suggest that the glucose-lowering effects of alanine is indirectly mediated by insulin in trout.

Inhibition of glucose appearance

Alanine inhibits R_a glucose in trout (Fig. 2), but had no such effect in humans (Jahoor et al., 1990; Wolfe et al., 1987) or dogs (Diamond et al., 1988). R_a glucose is controlled by gluconeogenesis and glycogen breakdown (glycogenolysis). The key enzymes catalyzing the first step in gluconeogenesis and glycogenolysis are PCK and glycogen phosphorylase (GP), respectively. The last step of both gluconeogenic and glycogenolytic pathways is catalyzed by G6Pc (Enes et al., 2009). In trout, alanine affected neither *pck* expression (Fig. S1A) or PCK activity (Fig. 8), nor *g6pc* expression (except for a single paralogue; Fig. S1B). In juvenile trout fed an alanine-rich diet over several weeks, no effect on cytosolic PCK activity and an inhibition of the expression and activity of G6Pc were found (Kirchner et al., 2003). Despite the different experimental designs, both the current work and the dietary study by Kirchner and colleagues show that alanine does not stimulate hepatic glucose production in trout. In mammalian and avian species, alanine had no effect on PCK and G6Pc activities (Donaldson and Christensen, 1992; Friedrichs and Schoner, 1974).

Given the observed alanine-dependent reduction of R_a glucose in the absence of effects on PCK activity, it is possible that alanine inhibited hepatic glycogenolysis. Glycogen metabolism is principally regulated by phosphorylation, which elicits opposing effects on glycogen phosphorylase (activated by phosphorylation), and glycogen synthase (inhibited by phosphorylation) (Enes et al., 2009). While the hepatic expression and activity of GP and glycogen content were not measured in the current study, findings in fish and mammals suggest a role for alanine in promoting hepatic glycogen synthesis. In sea raven (*Hemitripterus americanus*) hepatocytes, insulin

increased gluconeogenesis from alanine and had a small positive effect on glycogen content (Foster and Moon, 1987). In mammals, hepatic glycogen synthesis from alanine was reported (Shalwitz et al., 1989). Together, our findings refute the hypothesis that alanine increases R_a glucose. However, future studies investigating potential effects of alanine on liver glycogen metabolism are warranted.

Inhibition of glucose utilization

Alanine inhibits R_d glucose in rainbow trout (Fig. 2). The same effect was reported in humans (Jahoor et al., 1990) and a weaker but similar response was found in dogs (Diamond et al., 1988). However, glucose utilization was unaffected by alanine *in vitro* in rat brain (Lütz et al., 2003). Glucose disposal is regulated by glucose uptake, glycolysis and glycogen synthesis. Glucose uptake is mainly mediated through GLUTs (Navale and Paranjape, 2016). The insulin-sensitive GLUT4, found mostly in muscle and adipose tissue, plays a key role in glucose disposal in mammals and trout (Díaz et al., 2007; Huang and Czech, 2007). The first step in the glycolytic pathway is catalyzed by HK in muscle and GK in liver (Enes et al., 2009). Glycogen synthase (GS) is a key enzyme in glycogenesis (Enes et al., 2009).

Alanine decreased *glut4b* in red muscle (Fig. 5) but did not change *hk* expression in red or white muscle (Fig. S2). This reduction in *glut4b* expression may partially explain the decrease in R_d glucose. Alanine did not affect the expression (Fig. S2) of *gk* in the liver. Because the expression of *gk* in trout is a sensitive marker of hepatic glucose concentration (Panserat et al., 2000), this finding suggests that hepatic glucose levels

were not modulated by alanine. Similarly, a 9-week dietary alanine treatment had no effect on hepatic glycolysis in juvenile rainbow trout, as indicated by a lack of changes in expression and activity of GK (Kirchner et al., 2003). In *vitro* incubation of mammalian H4IIE hepatocytes with alanine increased glucose uptake (Adachi et al., 2018), but the amino acid did not affect GK activity *in situ* (Weber et al., 1968). Because studies on the effect of alanine (after feeding, injection or infusion) on hepatic glycolytic enzyme activity in mammals have not been reported to our knowledge, it is currently unclear whether these differences reflect methodological or true species differences.

While indices of glycogen metabolism were not quantified in our study, previous work using fish and mammalian hepatocytes suggests that exogenous alanine is metabolized to glycogen (Baquet et al., 1991; Foster and Moon, 1989; Plomp et al., 1990). It is therefore feasible that alanine may be used to support hepatic glycogen synthesis in rainbow trout. Overall, the decrease in R_d glucose suggests that preference for glucose as an oxidative fuel decreased and that it was partly replaced by alanine, although the amino acid was probably also used for hepatic glycogen synthesis. Decreased glucose import and utilization is further corroborated at the molecular level in muscle, where alanine caused reductions in the expression of glucose transporters.

Increase in total but not activated hepatic AMPK

To our knowledge, this is the first study examining the impact of alanine on AMPK in trout or any fish species. Alanine increased total AMPK in liver (Fig. 6) with no significant effect on p-AMPK (Fig. S3 and S4). AMPK is a heterotrimeric complex

composed of 3 subunits (Jeon, 2016). It is activated when phosphorylated at threonine (Thr) 172 within the α-subunit and by binding of AMP within the γ-subunit (Craig et al., 2018). In mammals, AMPK activation by alanine inhibited TOR targets, S6 kinase and S6, and reduced glycemia *in vivo* (Adachi et al., 2018). In the current study, TOR targets, S6 and 4EBP1, were unaffected by alanine further supporting that AMPK was not activated by alanine (Fig S3 and S4).

A differential response of AMPK activation following alanine administration may be linked to physiological and molecular differences between trout and mammals. At the physiological level, activation of hepatic AMPK in mammals was linked to increased transamination of alanine by ALT and subsequent urea synthesis, whose energetic cost contributed to an increase in AMP levels (Adachi et al., 2018). Conversely, ALT activity was unaffected by alanine in trout liver (Fig. 8), in line with previous studies in trout reporting either no change (French et al., 1981) or only a small increase (Walton, 1986) in ALT activity after changes in dietary protein levels. Nitrogen waste in adult trout is mainly excreted as ammonia, while urea accounts for a small amount (Panserat et al., 2013). Ammonia-based nitrogen detoxification is energetically less costly compared to urea-based strategies (Ip and Chew, 2010). This difference in energetic cost may partially explain the lack of AMPK phosphorylation in trout. This would result in a comparatively smaller increase in AMP levels in rainbow trout, which in turn may not be high enough to trigger AMPK activation. At the molecular level, salmonid-specific whole genome duplication led to the retention of fifteen genes encoding AMPK subunits in rainbow trout, whereas only 7 genes exist in humans (Causey et al., 2019). This complexity and potential divergent regulation of paralogues may also contribute to the

different responses. In contrast to the recent mammalian study (Adachi et al., 2018), our results suggest that the alanine-dependent decrease in glycemia is not mediated by AMPK activation in rainbow trout. The biological consequences of the observed increase in total hepatic AMPK are unknown but may suggest an increased capacity to respond to changes in AMP/ATP ratios.

Activation of AKT in red muscle

Alanine increased p-AKT levels in red muscle but not liver (Fig.7). Interestingly, this finding is consistent with results after exogenous insulin infusion in rainbow trout (Forbes et al., 2019a). In addition to the similar pattern of p-AKT stimulation following alanine and insulin infusion, the reductions in glycemia, as well as R_a and R_d glucose were strikingly similar between the two studies. This suggests that alanine may have stimulated insulin secretion. Indeed, previous studies support the idea that alanine as well as other amino acids stimulate insulin secretion in teleost fishes, including rainbow trout (Andoh, 2007; Plisetskaya et al., 1991). Unfortunately, insulin levels cannot be readily measured in rainbow trout (Moon, 2001). Mammalian studies report different effects of alanine on pancreatic hormone release. For example, alanine increased insulin (Genuth and Castro, 1974) and glucagon (Sann et al., 1978; Wise et al., 1973) levels in humans. Interestingly, alanine can differentially regulate both hormones under different conditions in dogs (Müller et al., 1971). When glucose was available (exogenous load), alanine raised the ratio of insulin to glucagon, whereas it stimulated glucagon secretion when glucose was lacking (Müller et al., 1971). Conversely, other findings in mammals suggest alanine had no effect on both hormones (Adachi et al.,

2018). In the current study, glucagon was not measured because of the limited plasma volume available. However, given that exogenous glucagon increased glycemia and R_a glucose, and decreased R_d glucose in trout (Forbes et al., 2019b), our findings suggest that alanine mediated its effects through insulin, but not glucagon in trout.

Possible fates of alanine and consequences on glucose metabolism

The concurrent reduction in R_a and R_d glucose suggests that glucose was not the preferred fuel under high alanine availability. This indicates that alanine was predominantly used as an oxidative fuel replacing glucose to some extent which supports our second hypothesis. Glucose represents a minor energy source in trout and amino acids (from dietary proteins) can be oxidized to provide energy (Panserat et al., 2013). The lack of expression and activity changes of the key gluconeogenic enzyme PCK in this study suggests that alanine was also possibly used for hepatic glycogen synthesis via gluconeogenesis. Moreover, glycogen breakdown was probably inhibited in the liver given the reduction in R_a glucose. This likely occurred at the level of glycogen phosphorylase because a general lack of change in the expression of *g6pc* paralogues was observed. However, additional studies will be necessary to probe the specific effect of alanine on hepatic glycogen metabolism. After the conversion of alanine to pyruvate, the latter can be oxidized to provide energy (Mommsen et al., 1980). Under our conditions, energy requirements were probably met by alanine oxidation and it would be less energetically favorable to convert alanine to glucose before oxidation. In trout muscle, we speculate that alanine was preferentially oxidized over glucose because consistent reductions in molecular indices of glucose transport

were found. Unfortunately, the fate of alanine or other amino acids in trout muscle remains largely unexplored (Weber et al., 2016).

In hepatocytes from long-term fasted rainbow trout, alanine oxidation, as assessed by CO_2 production, was significantly higher compared to glucose (Pereira et al., 1995). The production of CO_2 from alanine was higher than its conversion to glucose in trout hepatocytes indicating that alanine is a good oxidative substrate (French et al., 1981). In sea raven hepatocytes, insulin stimulated CO_2 production from alanine (Foster and Moon, 1987). The above-mentioned studies were conducted *in vitro*. Therefore, they should be interpreted with caution. In mammals, many reports found no effect of alanine on R_a glucose (Diamond et al., 1988; Jahoor et al., 1990; Wolfe et al., 1987). Thus, three fates were suggested for alanine in mammals. First, hepatic glucose produced from alanine would be converted to glycogen (Wolfe et al., 1987). Second, an excess in alanine would inhibit gluconeogenesis from other precursors; and third, alanine is possibly preferentially used instead of glucose for energy production (Jahoor et al., 1990). The similar effect of both alanine (present study) and insulin (Forbes et al., 2019a) on glucose fluxes suggests that alanine probably stimulated insulin secretion which could indirectly promote alanine oxidation to provide the needed energy.

Conclusions

This is the first study that investigates the effects of alanine on glucose metabolism in trout by integrating glucose fluxes at the whole organism level and molecular indices in liver and muscle tissues. Proteins, and thus amino acids, represent the main nutritional energy source in this species. In addition to providing a better comparative understanding of their impact on glucose metabolism in trout, our findings provide a starting-step toward a better understanding of the effect of individual amino acids on glucose metabolism in the applied context of the salmonid aquaculture sector, for which the replacement of (costly) dietary proteins with carbohydrate protein represents and economical advantage.

The reduction in glucose fluxes suggests that glucose was not the preferred substrate under our conditions (high alanine availability and trout were at rest) and that alanine oxidation was probably sufficient to meet energy requirements. Mechanistically, alanine does not change molecular indices of hepatic glycolysis and gluconeogenesis raising the possibility that the reduction in R_a is linked to altered glycogen metabolism. The reduction of molecular indices related to glucose import in muscle tissue support decreased peripheral utilization of glucose under conditions of high alanine availability. Comparatively, the alanine-dependent reduction in glycemia at the whole animal level is consistent with findings in mammals. However, while reduction in glycemia was mediated through AMPK activation in mammals, our data suggest that the effects of alanine do not involve AMPK activation in trout and might be indirectly mediated through insulin. Thus, different mechanisms underlie alanine's glucose-lowering effects in trout and mammals. Given the striking similarities in regulation and mechanistic

underpinnings of glucose fluxes between alanine and insulin-infused trout, we hypothesize that alanine alters glucose fluxes by acting as an insulin secretagogue in this species. Future studies using insulin receptor antagonist are warranted to investigate this hypothesis.

Acknowledgements

We thank Christine Archer for expert care of the animals and Katie Gilmour for providing AMPK antibodies.

Competing interests

No competing interests declared.

Author Contributions

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

Funding

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to Jean-Michel Weber (NSERC discovery grant 105639-2012) and to Jan Mennigen (NSERC discovery grant 2114456-2017) and Canadian Foundation for Innovation (John Evans Leader's fund 148035).

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Figures

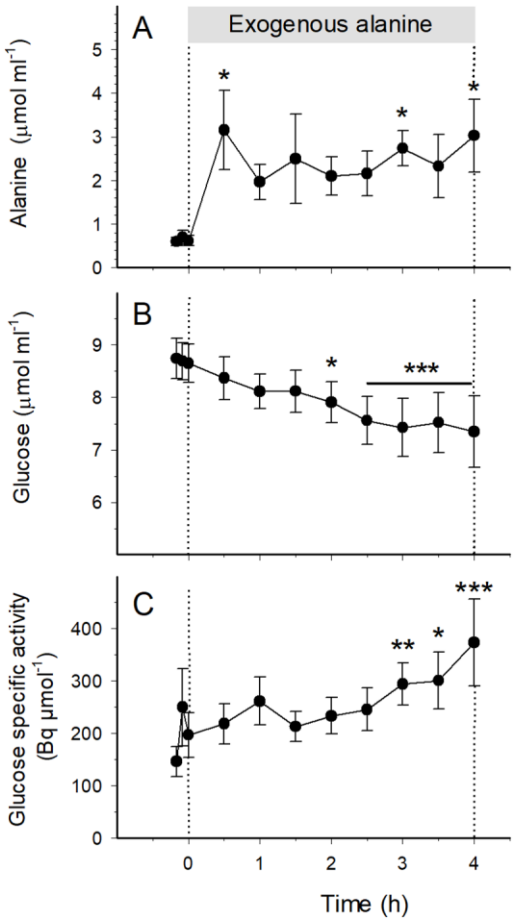

Fig. 1. Effects of exogenous alanine administration on trout plasma metabolites and glucose specific activity during the measurement of glucose kinetics. (A) Alanine concentration. (B) glucose concentration, and (C) glucose specific activity. Values are means ± s.e.m. (N=6-9). Means significantly different from baseline are indicated by asterisks (* P<0.05; ** P<0.01; *** P<0.001.

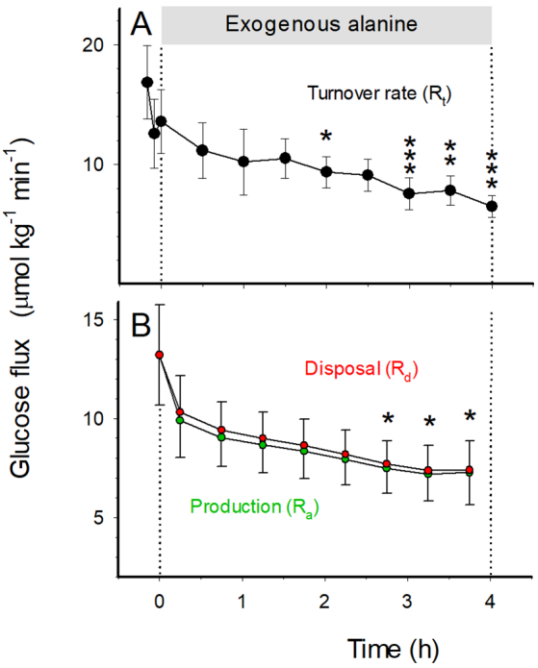

Fig. 2. Effects of exogenous alanine administration on the glucose kinetics of rainbow trout. (A) Glucose turnover rate (R_t) and (B) rate of glucose production (or appearance in the circulation: R_a) and rate of glucose disposal (or disappearance from the circulation: R_d). R_t was calculated with the steady state equation, but R_a and R_d with the non steady-state equations of Steele (Steele, 1959). Values are means ± s.e.m. (N=9). Means significantly different from baseline are indicated by asterisks (* P<0.05; ** P<0.01; *** P<0.001).

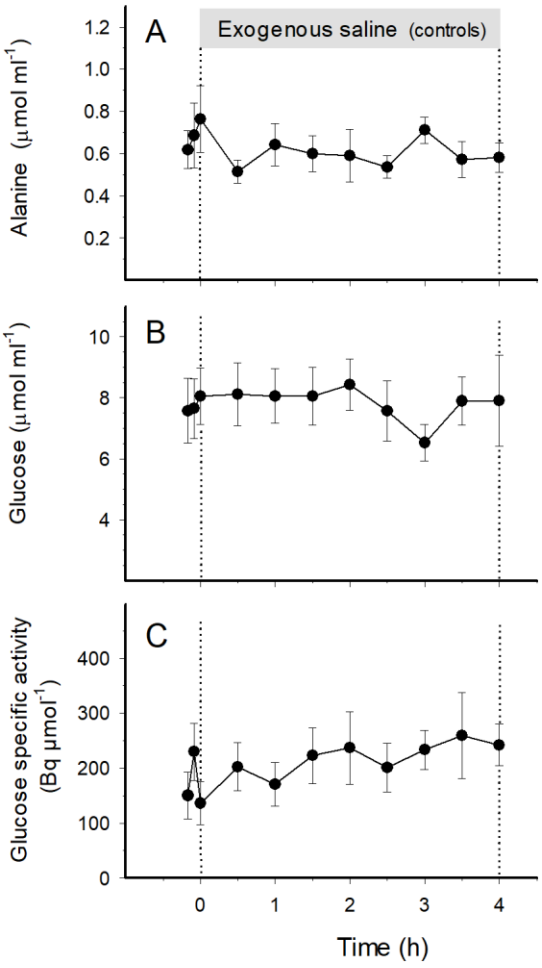

Fig. 3. Effects of exogenous saline administration on plasma metabolites and glucose specific activity of control trout during the measurement of glucose kinetics. (A) Alanine concentration. (B) glucose concentration, and (C) glucose specific activity. Values are means ± s.e.m. (N=7). Saline had no effect on these parameters (P>0.05).

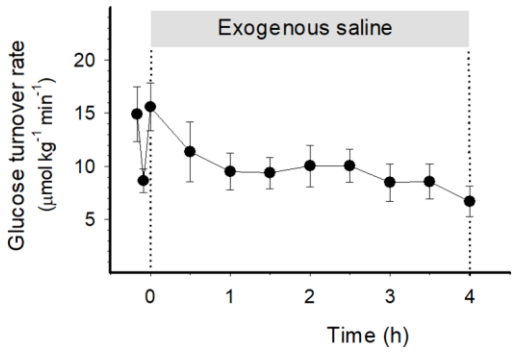

Fig. 4. Effects of exogenous saline administration on glucose turnover rate (R_t) of control rainbow trout. R_t was calculated with the steady state equation of Steele (Steele, 1959). Values are means ± s.e.m. (N=7). Saline had no effect on turnover rate (P=0.237).

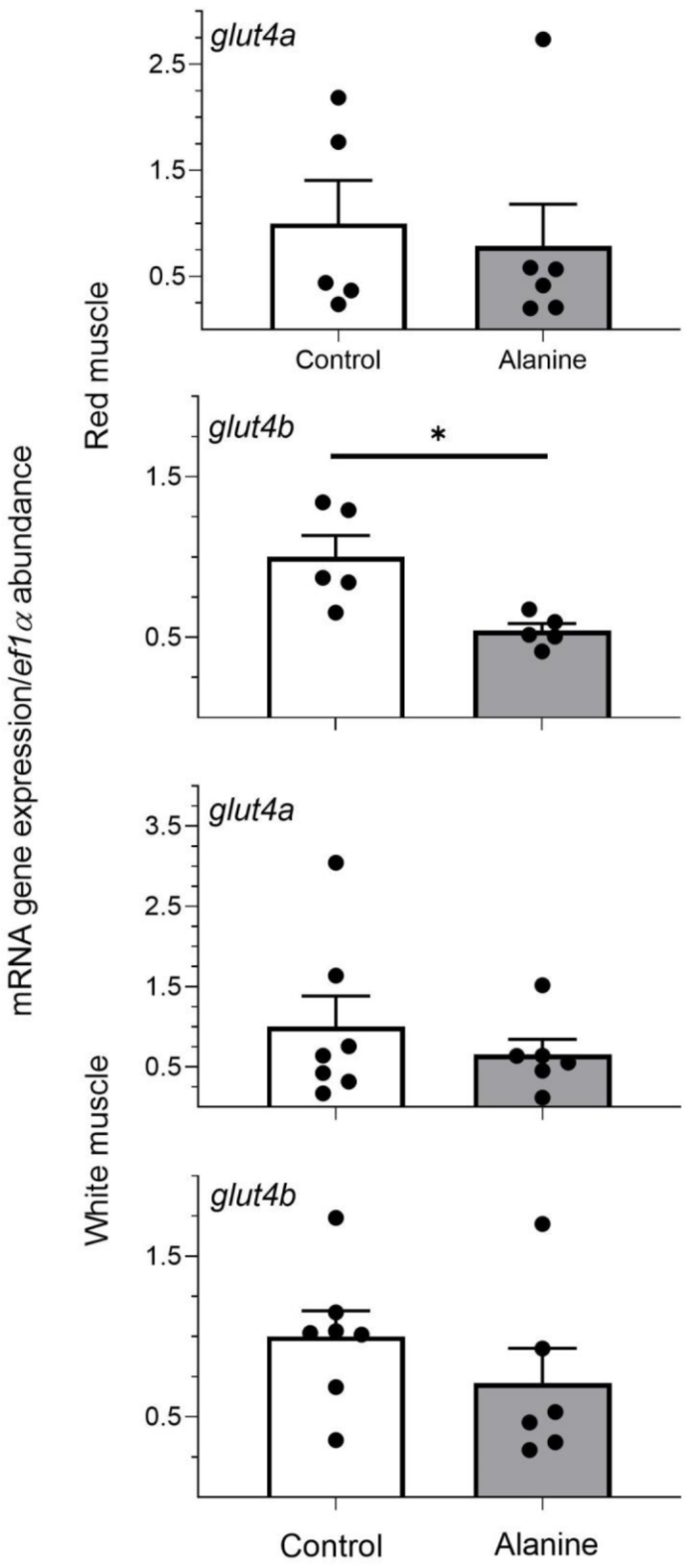

Fig. 5. Red and white muscle relative mRNA abundance of *glucose transporter* 4 (*glut4a* and *glut4b*) in the control and alanine-infused rainbow trout. Data were normalized to the reference gene *ef1α*. The mean + s.e.m. are represented (N=5-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test and means significantly different from control are indicated by an asterisk ($p<0.05$).

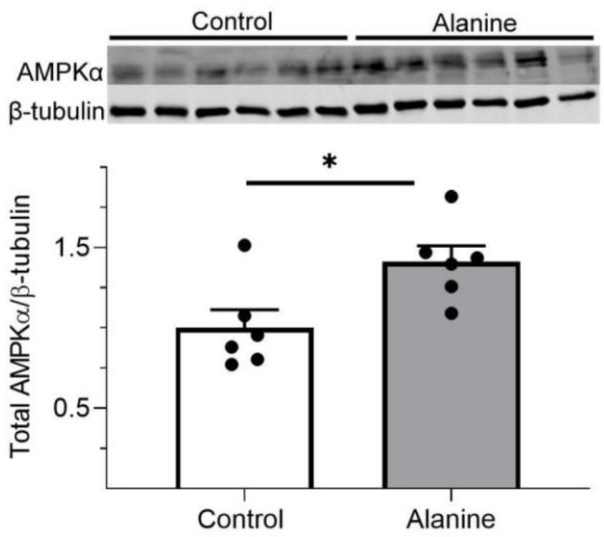

Fig. 6. Relative level of total AMPKα in the liver in the control and the alanine-infused groups. Data were normalized to β-tubulin and are represented as fold changes relative to the control group. The western blot is shown on top of the figure. The mean + s.e.m. are represented (N=6). Filled circles represent individual data points. Data were analyzed using two-tailed t-test and means significantly different from control are indicated by an asterisk ($p<0.05$).

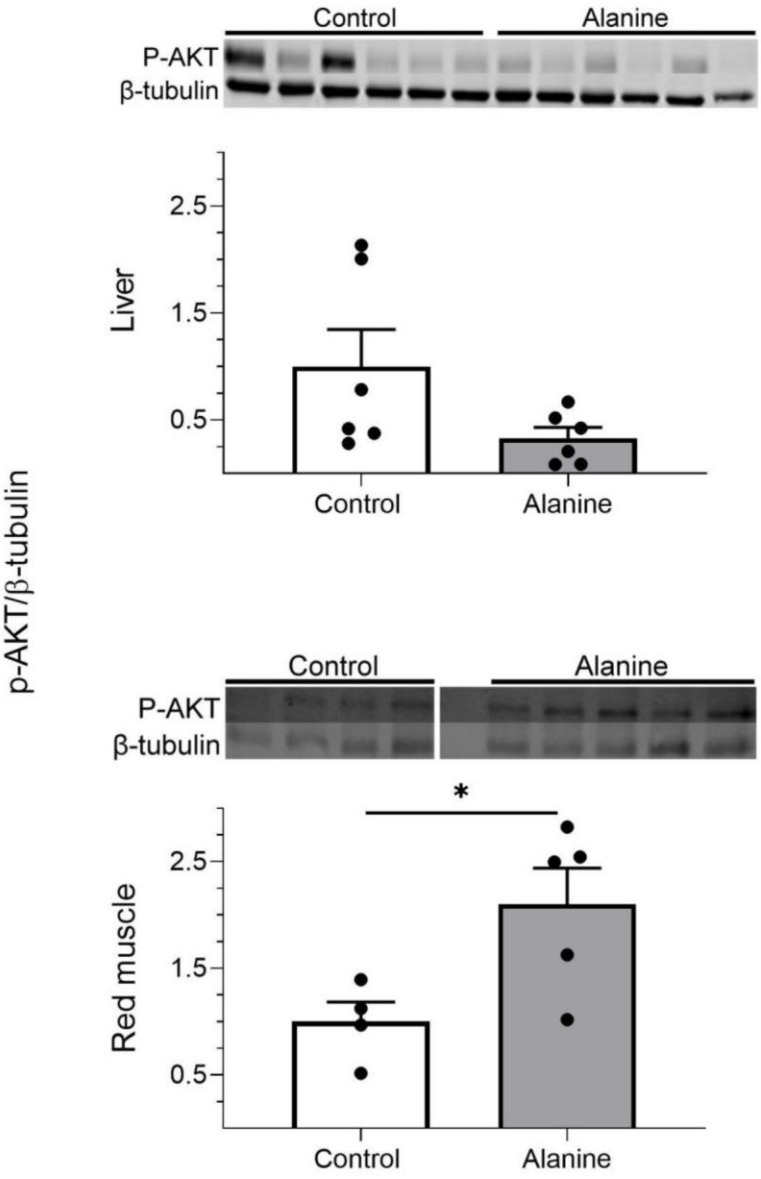

Fig. 7. Relative level of phosphorylated AKT (at S473) in the liver and red muscle in the control and the alanine-infused groups. Data were normalized by β-tubulin and are represented as fold changes relative to the control group. The western blot of each phosphorylated protein is shown on top of its figure. The mean + s.e.m. are represented (N=4-6). Filled circles represent individual data points. Data were analyzed using two-tailed t-test and means significantly different from control are indicated by an asterisk ($p<0.05$). A white space indicates the removal of a lane (outlier).

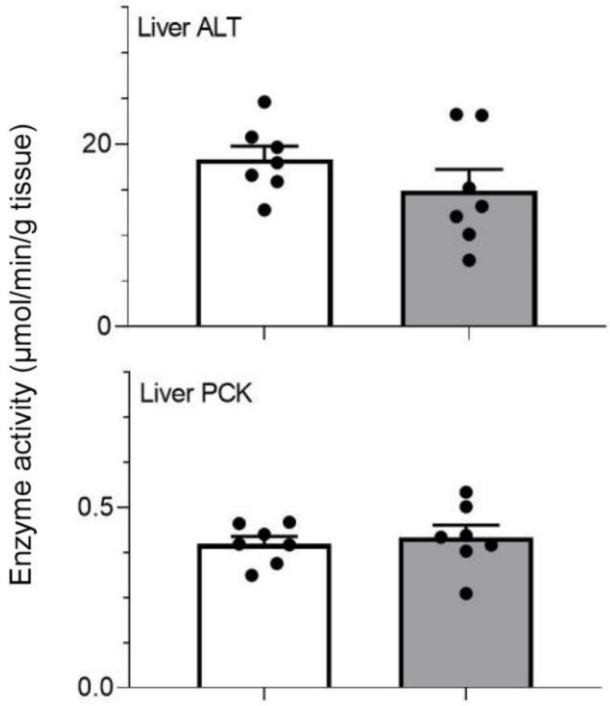

Fig. 8. Liver and muscle enzyme activity (µmol/min/g tissue) for alanine aminotransferase (ALT) and phosphoenolpyruvate carboxykinase (PCK) in the control and the alanine-infused groups. The mean + s.e.m. are represented (N=5-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Means significantly different from control are indicated by asterisks (* P<0.05; ** P<0.01).

Table 1. Physical characteristics and hematocrit of the two groups of rainbow trout.

	Glucose kinetics (N=16)	Molecular experiment (N=14)
Body mass (g)	463.7±16.0	412.4±14.9
Body length (cm)	33.7±0.4	33.2±0.4
Hematocrit (%)	28.7±1.9	19.2±2.1

Trout were used for (i) *in vivo* glucose kinetics measurements or (ii) tissue gene expression, signaling and enzyme activity. Fulton's condition factor *K* for all fish was 1.17±0.02 (N=30). Body mass and length were measured before the surgery. Hematocrit was measured on the second day, after recovery from the surgery to minimize blood loss. Mean values ± s.e.m. are presented.

Table 2. Plasma alanine and glucose concentrations for the molecular experiment.

Metabolite	Group	Baseline (0h)	Final (4h)	Time effect	Treatment effect	Time-treatment interaction
Plasma alanine ($\mu mol\ ml^{-1}$)	Control Alanine	0.5±0.1 0.8±0.1	0.4±0.1 7.4±1.7	**	***	**
Plasma glucose ($\mu mol\ ml^{-1}$)	Control Alanine	7.7±0.9 6.6±0.7	5.9±0.8 5.7±0.7	*	ns	ns

Values are means ± s.e.m. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns indicates no significant difference ($p>0.05$).

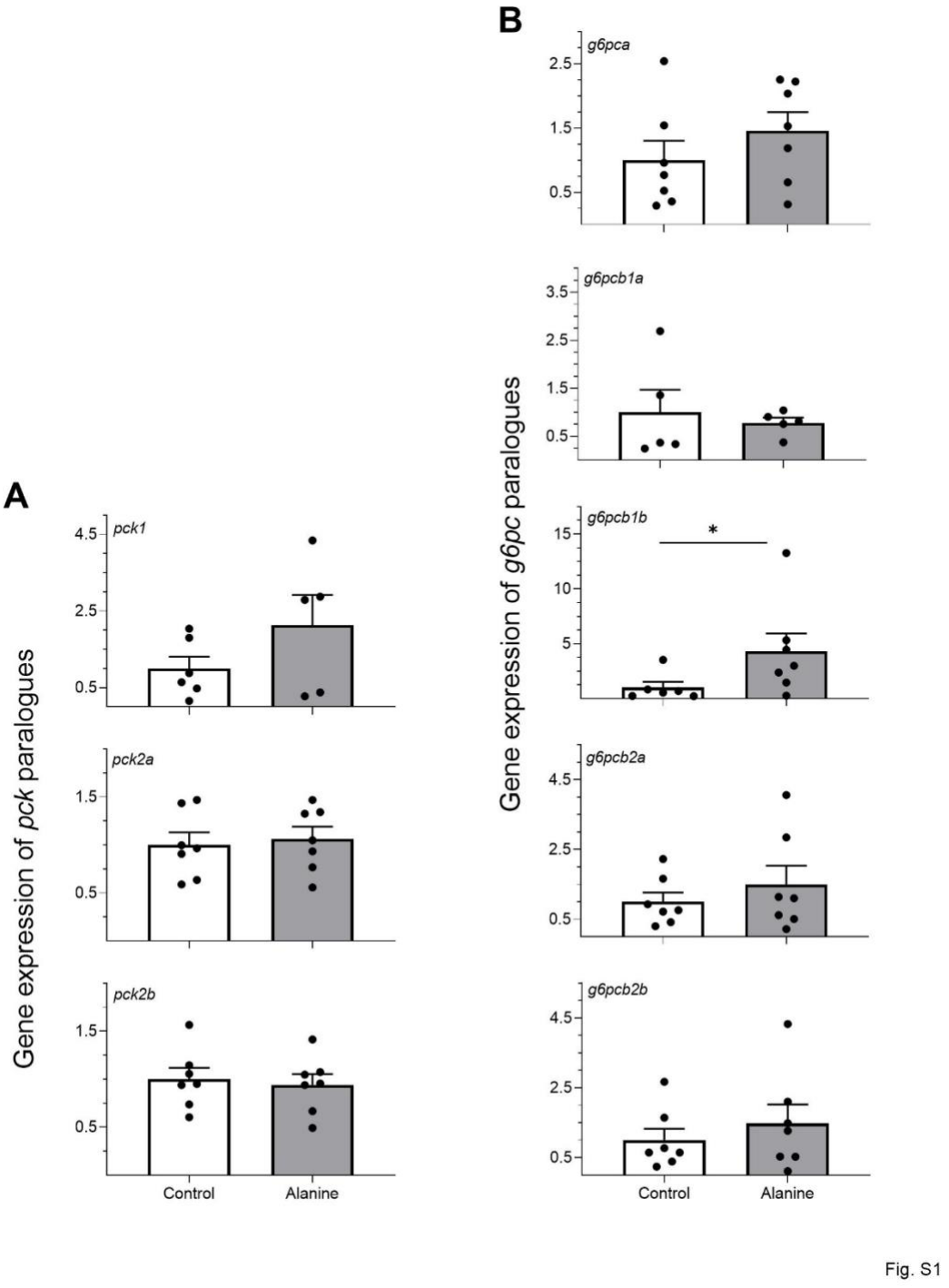

Fig. S1. Liver relative mRNA abundance of (A) phosphoenolpyruvate carboxykinase (*pck*) and (B) glucose-6-phosphatase (*g6pc*) paralogues in the control and alanine-infused rainbow trout. Data were normalized by the reference gene *β-actin*. The mean + s.e.m. are represented (N=5-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test and means significantly different from control are indicated by an asterisk ($p<0.05$).

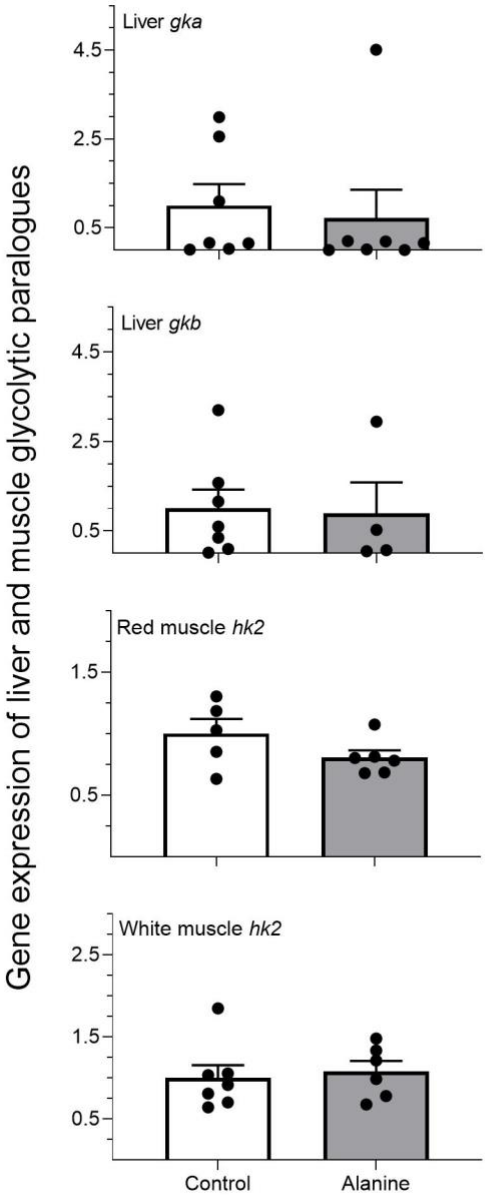

Fig. S2

Fig. S2. Relative mRNA abundance of glucokinase (*gk*) in the liver and hexokinase 2 (*hk2*) in the red and white muscle in the control and alanine-infused rainbow trout. Data were normalized by the reference gene *β-actin* for the liver and *ef1α* for red and white muscle. The mean + s.e.m. are represented (N=4-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Alanine had no effect on the measured glycolytic mRNA transcript abundance ($p>0.05$).

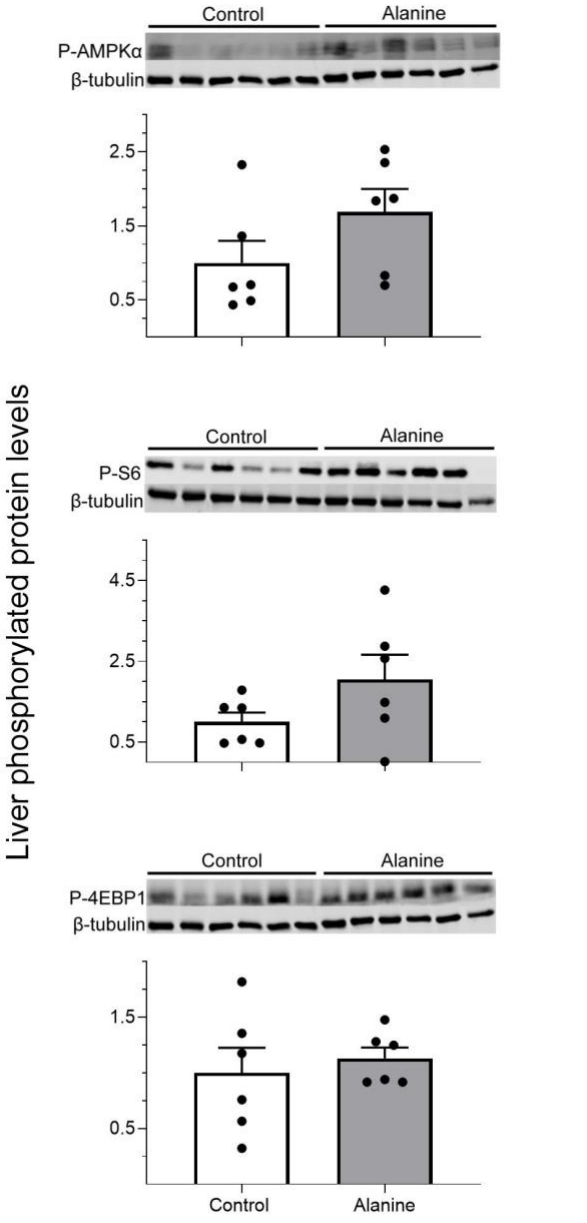

Fig. S3

Fig. S3. Liver relative abundance of phosphorylated AMPKα (at T172), ribosomal protein S6 (S6; at S235/236) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1; at T37/46) in the control and the alanine-infused groups. Data were normalized by β-tubulin and are represented as fold changes relative to the control group. The western blot of each phosphorylated protein is shown on top of its figure. The mean + s.e.m. are represented (N=6). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Alanine had no effect on the phosphorylated level of these proteins in the liver ($p>0.05$).

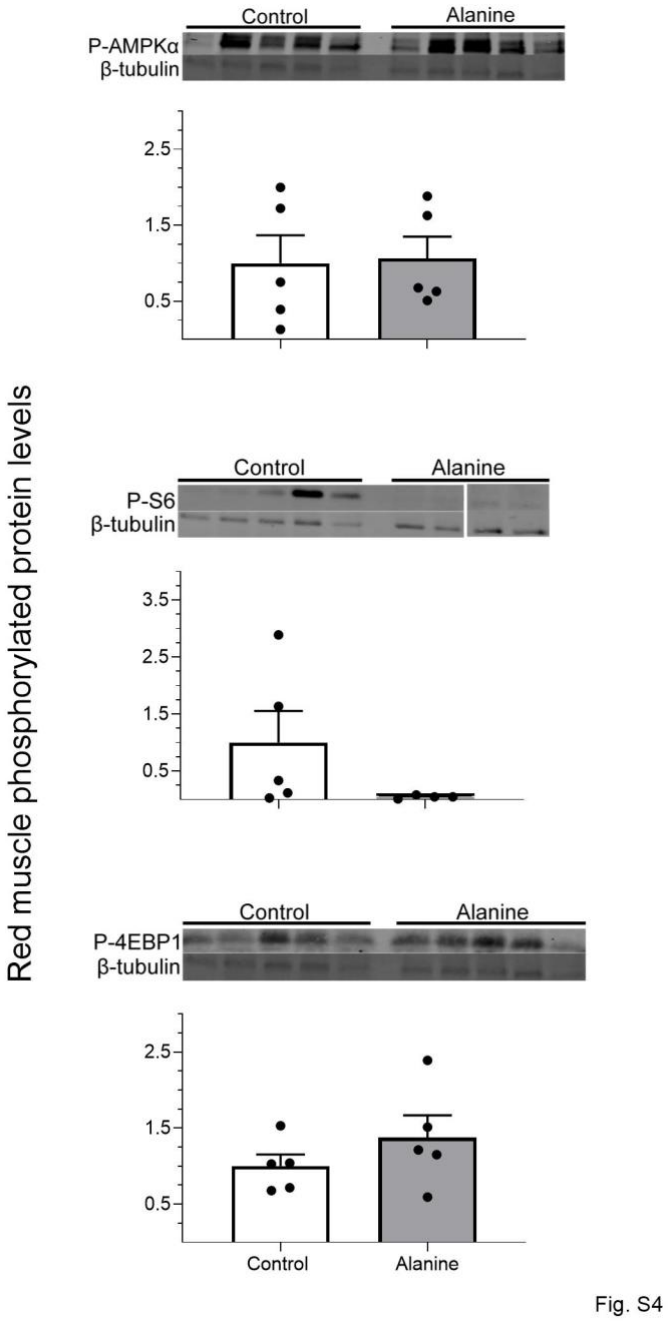

Fig. S4. Red muscle relative levels of phosphorylated AMPKα (at T172), S6 (at S235/236) and 4EBP1 (at T37/46) in the control and the alanine-infused groups. Data were normalized by β-tubulin and are represented as fold changes relative to the control group. The western blot of each phosphorylated protein is shown on top of its figure. The mean + s.e.m. are represented (N=4-5). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Alanine had no effect on the phosphorylated level of these proteins in the red muscle ($p>0.05$). A white space indicates the removal of a lane (outlier).

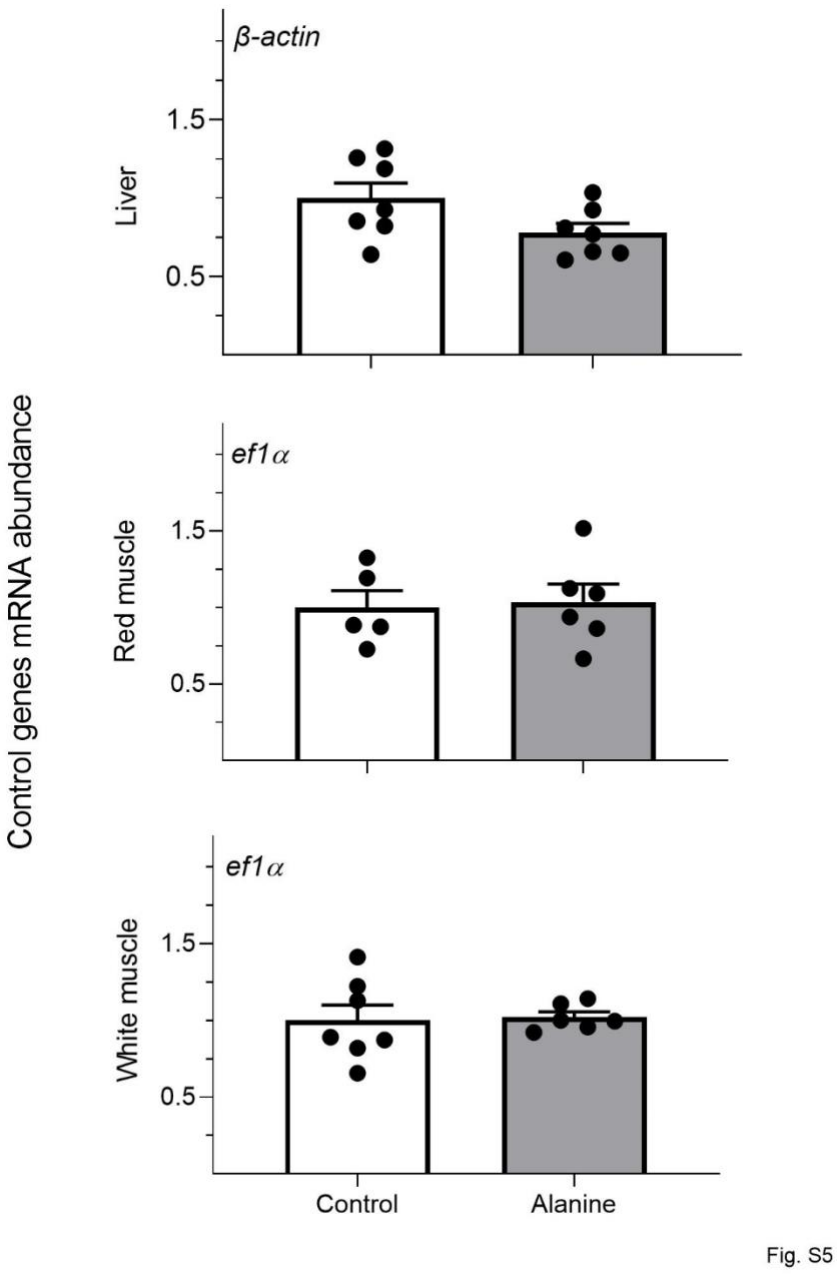

Fig. S5. Relative mRNA abundance of the control genes *β-actin* in the liver and elongation factor 1α (*ef1α*) in the muscle of the control and alanine-infused rainbow trout. Data points are relative to the control group for each gene. The mean + s.e.m. are represented (N=5-7). Filled circles represent individual data points. Data were analyzed using two-tailed t-test. Alanine had no effect on the measured mRNA transcript abundance of the control genes ($p>0.05$).

Table S1. Primer pair conditions used for mRNA quantification by real-time RT PCR.

mRNA	Primer sequence (5' to 3')	Annealing temperature °C	Efficiency %, R^2	Reference
pck1	F: ACAGGGTGAGGCAGATGTAGG R: CTAGTCTGTGGAGGTCTAAGGGC	55	91.9, 0.995	Marandel et al., 2015
pck2a	F: ACAATGAGATGATGTGACTGCA R: TGCTCCATCACCTACAACCT	55	90.4, 0.997	Marandel et al., 2015
pck2b	F: AGTAGGAGCAGGGACAGGAT R: CCGTTCAGCAAAGGTTAGGC	55	102.8, 0.989	Marandel et al., 2019
g6pca	F: GATGGCTTGACGTTCTCCT R: AGATCCAGGAGAGTCCTCC	55	91.9, 0.995	Marandel et al., 2015
g6pcb1a	F: GCAAGGTCCAAAGATCAGGC R: GCCAATGTGAGATGTGATGGG	59	105.9, 0.975	Marandel et al., 2015
g6pcb1b	F: GCTACAGTGCTCTCCTTCTG R: TCACCCCATAGCCCTGAAA	55	91.6, 0.997	Marandel et al., 2015
g6pcb2a	F: ATCGGACAATACACACAGAACT R: CAACTGATCTATAGCTGCTGCCT	54	91.3, 0.994	Marandel et al., 2015
g6pcb2b	F: CCTCTGCTCTTCTGACGTAG R: TGTCCATGGCTGCTCTCTAG	55	92.3, 0.985	Marandel et al., 2015
gka	F: CTGCCCACCTACGTCTGT R: GTCATGGCGTCCTCAGAGAT	54	96.3, 0.993	Marandel et al., 2015
gkb	F: TCTGTGCTAGAGACAGCCC R: CATTTTGACGCTGGACTCCT	57	90.9, 0.993	Marandel et al., 2015
hk2	F: TGAAAAGGGACATGCAGAGA R: GGCCCTAAAAGCAAGGAAA	58	92.3-96.1,0.992-0.988	Designed
glut4a	F: CATCTTTGCAGTGCTCCTTG R: CAGCTCTGTACTCTGCTTGC	56	106.1-101.1, 0.997-0.982	Liu et al., 2017
glut4b	F: TCGGCTTTGGCTTCCAATATG R: GTTTGCTGAAGGTGTTGGAG	56	101.4-106.2, 0.995-0.996	Liu et al., 2017
β-actin	F: AGAGCTACGAGCTGCCTGAC R: GTGTTGGCGTACAGGTCCTT	60	90.4, 0.996	Moltesen et al., 2016
ef1α	F: CACATCGCCTGCAAGTTT R: GAAGCTCTCCACACACATGG	58	106.6-110.1, 0.985-0.988	Designed

F and R represent forward and reverse primer sequences, respectively. Primer sequences for *hk2* and ***ef1α*** was designed using Primer 3 algorithm. The efficiency and R^2 values are presented for both the red and white muscle *glut4a*, *glut4b*, *hk2* and *ef1α*. The reference genes for the liver and muscle are ***β-actin*** and ***ef1α,*** respectively.