Effects of food-deprivation and refeeding on the regulation and sources of blood glucose appearance in European seabass (*Dicentrarchus labrax* L.)

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ABSTRACT

Sources of blood glucose in European seabass (initial weight 218.0 ± 43.0 g; mean $\pm$ S.D., n = 18) were quantified by supplementing seawater with deuterated water (5%-^2H_2O) for 72 h and analyzing blood glucose ^2H-enrichments by ^2H NMR. Three different nutritional states were studied: continuously fed, 21-day of fast and 21-day fast followed by 3 days of refeeding. Plasma glucose levels (mM) were 10.7 ± 6.3 (fed), 4.8 ± 1.2 (fasted), and 9.3 ± 1.4 (refed) (means $\pm$ S.D., n = 6), showing poor glycemic control. For all conditions, ^2H-enrichment of glucose position 5 was equivalent to that of position 2 indicating that blood glucose appearance from endogenous glucose 6-phosphate (G6P) was derived by gluconeogenesis. G6P-derived glucose accounted for $65 \pm 7\%$ and $44 \pm 10\%$ of blood glucose appearance in fed and refed fish, respectively, with the unlabeled fraction assumed to be derived from dietary carbohydrate ($35 \pm 7\%$ and $56 \pm 10\%$, respectively). For 21-day fasted fish, blood glucose appearance also had significant contributions from unlabeled glucose ($52 \pm 16\%$) despite the unavailability of dietary carbohydrates. To assess the role of hepatic enzymes in glycemic control, activity and mRNA levels of hepatic glucokinase (GK) and glucose 6-phosphatase (G6Pase) were assessed. Both G6Pase activity and expression declined with fasting indicating the absence of a classical counter-regulatory stimulation of hepatic glucose production in response to declining glucose levels. GK activities were basal during fed and fasted conditions, but were strongly stimulated by refeeding. Overall, hepatic G6Pase and GK showed limited capacity in regulating glucose levels between feeding and fasting states.

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1. Introduction

Glucose is an essential energy substrate for many cells and as principal regulator of its supply, the liver controls the requirements for endogenous glucose production (EGP) in order to maintain homeostasis. Despite large variation in dietary carbohydrate (CHO) availability and whole body glucose demands between species, the biochemical pathways and control mechanisms of glucose metabolism are highly conserved (Polakof et al., 2011). Therefore, the plasticity of this system determines how well a particular organism adapts to dietary CHO. For farmed carnivorous fish species such as the European seabass *Dicentrarchus labrax* L., there is current interest from both economic and ecological perspectives about their capacity to

adapt from their natural high protein/low CHO diet to feedstock where a significant portion of protein is replaced by CHO (Fernández et al., 2007; Enes et al., 2009).

It is widely accepted that seabass and other carnivorous fish ineffectively utilize dietary CHO, instead they are highly dependent on gluconeogenesis from amino acids for sustaining endogenous glucose demands (Moon, 2001; Stone, 2003; Enes et al., 2009; Polakof et al., 2012). In addition, glycemia is poorly controlled in these fish compared to mammals (Polakof et al., 2011). This may in part reflect a weak coupling between plasma glucose levels and regulation of glucose production and disposal pathways. For example, in seabass the activities of hepatic gluconeogenic enzymes were not suppressed by chronic high-CHO feeding thereby contributing to postprandial hyperglycemia (Enes et al., 2006, 2008b). Meanwhile, the transition from feeding to fasting is characterized by a steep drop in plasma glucose concentrations (Gutiérrez et al., 1991; Echevarría et al., 1997; Pérez-Jiménez et al., 2007; Viegas et al., 2011; Pérez-Jiménez et al., 2012) among other tissue-dependent effects (Blasco et al.,

2001). Other fish species can tolerate extended periods of starvation without apparently suffering from hypoglycemia like *Brycon cephalus* (Figueiredo-Garutti et al., 2002), gilthead seabream *Sparus aurata* (Sangiao-Alvarellos et al., 2005; Polakof et al., 2006) or Senegalese sole *Solea senegalensis* (Costas et al., 2011). To date, our knowledge of fish CHO metabolism is largely informed by relating measurements of activities and gene expression levels of key enzymes in the regulation of glucose fluxes with changes in plasma glucose levels. While this approach has provided important insights into fish glucose metabolism, it does not directly unravel the metabolic processes that contribute to the appearance of blood glucose. Hence, the contribution of dietary CHO absorption vs. EGP to blood remains incompletely defined.

Deuterated water (^2H_2O) reveals the fraction of blood glucose derived from glucose 6-phosphate (G6P) via glucose 6-phosphatase (Basu et al., 2008; Nunes and Jones, 2009). In addition, the sources of G6P can be resolved into gluconeogenic and glycogenolytic contributions by measuring the ratio of enrichment in positions 5 and 2 (H5/H2) of blood glucose (Landau et al., 1996) (Fig. 1). This approach was recently applied in seabass (Viegas et al., 2011) revealing that gluconeogenesis was the main source of hepatic G6P production regardless of nutritional state. However, for fasted fish, where blood glucose appearance was expected to be completely dependent on G6P hydrolysis, the fraction of blood glucose derived from G6P was unexpectedly low, accounting for only ~70% of the total. Therefore, to confirm feeding to fasting transitions and metabolic rebound by refeeding, we aimed at assessing the available endogenous sources of hepatic G6P. For this purpose we also measured hepatic glycogen concentration as well as the activities and mRNA levels of the hepatic catalytic subunit of glucose 6-phosphatase (G6Pase, EC 3.1.3.9) and glucokinase (hexokinase IV; GK, EC 2.7.1.2). To our knowledge, mRNA levels for these enzymes have never been assessed in any studies using seabass.

2. Material and methods

2.1. Fish handling and sampling

A total of 18 fish provided by a local farm (initial mean length of 28.0 ± 1.7 cm and initial mean body weight of 218.0 ± 43.0 g) were transported to the laboratory and randomly assigned to 3 different tanks (n = 6). These consisted of ~200 L polyethylene circular tanks supplied with well-aerated filtered seawater from a recirculation system set to 18 ± 1 °C temperature, 30 ± 1‰ salinity and O_2 levels above 80% saturation under natural photoperiod. These conditions were kept throughout the experiment. One of the tanks served as control and was provided with food once a day, 5 d per week, with ration of 2% mean body weight d^{-1} (Table 1) for 21 d. Fish from the remaining two tanks were fasted for 21 d. Following this period, each group was transferred to a separate 5% ^2H-enriched seawater tank for a period of 72 h. This consisted of a ~200 L tank of similar characteristics to the ones used in the previous phase. In order to insure similar water parameters, this tank was maintained with an independent closed filtering system. During this 72 h period in 5% ^2H-enriched seawater, the fed fish were kept in the same feeding protocol and one group of fasted fish was kept in the fasting protocol. The remaining fasted group started a refeeding protocol with the same ration size and diet than the fed fish. Fed and refed fish were provided with last meal 24 h before sacrifice. At this stage, fish were anesthetized in 5% ^2H-enriched seawater containing 0.1 g L^{-1} of MS-222, measured, weighed and sampled for blood from the caudal vein with heparinized syringes. Fish were sacrificed by cervical section; liver was excised, weighed, freeze-clamped in liquid N_2, ground and stored at -80 °C.

2.2. Sample treatment

From the total blood withdrawn, an aliquot was separated and kept on ice until centrifugation (3000 *g* for 10 min) to separate plasma. This aliquot was used to quantify plasma glucose (Amplex® Red Glucose Assay Kit, Invitrogen, Spain) and assess plasma ^2H-enrichment
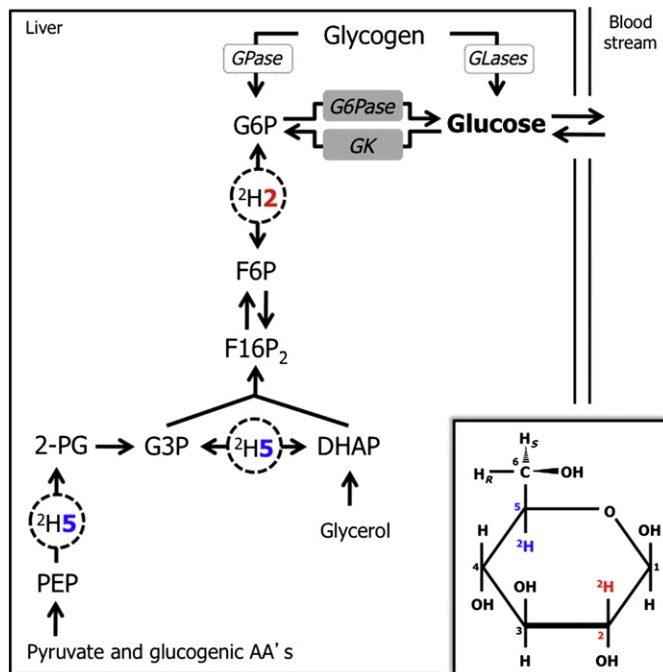
Fig. 1. Metabolic model representing gluconeogenesis and glycogenolysis in the liver. Gluconeogenic precursors are represented by pyruvate and gluconeogenic amino acids (metabolized via the anaplerotic pathways of the Krebs cycle) as well as glycerol from lipolysis. The sites for glucose enrichment in position 5 ($^2H5$) and position 2 ($^2H2$) from ^2H_2O are indicated. Note that for $^2H5$, the incorporation mechanisms include the addition of water hydrogen via enolase and formation of glyceraldehyde 3-phosphate from dihydroxyacetone phosphate via triose phosphate isomerase hence enrichment at this site is obligatory for glucose derived from gluconeogenic precursors. Glucose molecule with incorporation of ^2H in positions 2 and 5 is presented in detail. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P = glucose 6-phosphate; F6P = fructose 6-phosphate; F16P$_2$ = fructose 1,6-bisphosphate; DHAP = dihydroxyacetone phosphate; G3P = glyceraldehyde 3-phosphate; 2-PG = 2-phosphoglycerate; PEP = phosphoenolpyruvate; AA's = amino acids; GK = glucokinase; G6Pase = glucose 6-phosphatase; GPase = glycogen phosphorylase (EC 2.4.1.1); GLases = glycosidases (E.C. 3.2.1) which include glucosidases, amylases and glycogen debranching enzymes that do not hydrolyze glycogen into G6P.

Table 1
Ingredients and proximate composition of the diet provided to *D. labrax* (5 mm standard pellet).

Ingredients (%)	Diet
Fishmeal	37.0
Soybean meal	21.0
Corn gluten	13.5
Wheat gluten	0.3
Fish oil	13.5
Rapeseed oil	5.0
Wheat	4.5
Pea starch	2.5
Premix	0.2
Co-product of formation of L-glutamate from fermentation	2.5
Proximate composition (% dry weight)	
Crude protein	44.1
Crude fat	18.0
Starch	2.2
Ash	9.2
Phosphorous	1.3
Gross energy (kJ g^{-1} dry mass)	20

(see ^{2}H NMR analysis below). The remainder of the blood was used to isolate glucose and analyze its positional [2]H-enrichments. For this purpose whole blood was mixed with 0.3 N $ZnSO_4$ and 0.3 N $Ba(OH)_2$ (1.5 mL of each solution per mL of blood) and protein was removed by centrifugation (3500 g for 15 min). The supernatant was desalted by passage through sequential columns containing Dowex® 50WX8 (hydrogen form, Sigma-Aldrich) and Amberlite® IRA-67 (free base, Fluka, Sigma-Aldrich), then lyophilized and stored at −20 °C. In order to optimize the signal resolution in the NMR spectra, glucose was converted into its derivative, monoacetone glucose (MAG), as previously described (Viegas et al., 2011). Hepatic glycogen was extracted by precipitation with ethanol after alkaline tissue hydrolysis and glycogen was broken into its glycosyl units with amyloglucosidase from *Aspergillus niger* (glucose-free preparation, Sigma-Aldrich) as previously described (Viegas et al., 2012). After digestion free glucose was quantified using Amplex® Red Glucose Assay Kit (Invitrogen, Spain).

2.3. ^{2}H NMR analysis

Plasma water [2]H-enrichments were determined from 10 μL plasma volumes by ^{2}H NMR as previously indicated (Jones et al., 2001). Proton-decoupled ^{2}H NMR spectra of MAG samples were obtained at 50 °C with a 14.1 T Varian spectrometer (Varian, Palo Alto, CA, USA) equipped with a 3 mm broadband probe with the observe coil tuned to ^{2}H as previously described (Viegas et al., 2011). Approximately 12,000 to 17,000 scans were acquired per sample (corresponding to 6–8 h of collection time) and field-frequency drift was compensated by the Scout Scan software (Varian). All spectra were analyzed with the NUTS PC-based NMR spectral analysis software (Acorn NMR Inc., USA).

2.4. Quantification of blood glucose sources

Sources of blood glucose were resolved by positional [2]H-enrichment analysis relative to that of plasma water (PW) using the intramolecular ^{2}H methyl signals (Jones et al., 2006). Contributions from G6P were quantified by enrichment in position 2 relative to PW. For fasted fish, glucose that was not enriched in position 2 was assumed to be derived from non-G6P sources. For fed and refed fish, the contribution of non-G6P sources also includes that of absorbed dietary glucose (Fig. 1).

$$\text{Blood glucose from G6P } (\%) = 100 \times \text{H2/PW} \qquad (1)$$

$$\text{Blood glucose from non-G6P sources} (\%) = 100 - \text{G6P sources} \qquad (2)$$

The contributions of glycogenolytic and gluconeogenic fluxes to G6P were estimated from the ratio of position 5 to position 2 enrichments (H5/H2) as follows:

$$\text{G6P from gluconeogenesis} (\%) = 100 \times \text{H5/H2} \qquad (3)$$

$$\text{G6P from glycogenolysis} (\%) = 100 - \text{G6P from gluconeogenesis}. \qquad (4)$$

2.5. Enzymatic activities

GK activity assays were carried out as previously described by Caseras et al. (2000). Briefly, crude extracts were obtained by homogenization of the powdered frozen liver in a buffer (1/5, w/v) containing 50 mM Tris–HCl pH 7.5, 4 mM EDTA, 50 mM NaF, 0.5 mM PMSF, 1 mM 1,4-dithiothreitol (DTT), 250 mM sucrose and 100 mM KCl, using a PTA-7 Polytron mixer (Kinematica GmbH, Switzerland; position 3, for 30 s) followed by centrifugation at 15,800 g for 40 min at 4 °C. Crude extracts were filtered through 1 mL Sephadex G 25 column and 5 μL was assayed in a final volume of 200 μL containing

100 mM Tris–HCl pH 7.75, 7.5 mM $MgCl_2$, 100 mM KCl, 1 mM NADP, 2.5 mM 2-mercaptoethanol, 100 mM glucose and 1 mU mL^{-1} glucose 6-phosphate dehydrogenase (G6PDH). The reaction was triggered with the addition of 6 mM ATP and 100 mM or 0.5 mM glucose. GK activity was calculated as the rate of NADPH formation at 100 mM glucose minus the rate at 0.5 mM glucose (hexokinase activity), after correction for glucose dehydrogenase activity, determined as GK activity in the presence of 1 mM NADP and absence of both ATP and G6PDH.

G6Pase was assayed as previously described by Caseras et al. (2002) with minor modifications. Briefly, microsomes were isolated from powdered frozen liver homogenized in buffer A (50 mM Imidazole-HCl pH 6.5, 250 mM sucrose and 0.1% Triton X-100) (1/5, w/v) using a PTA-7 Polytron mixer (Kinematica GmbH, Switzerland; position 3, for 30 s) followed by centrifugation at 10,000 g for 15 min at 4 °C. The supernatant was recovered and centrifuged at 100,000 g for 90 min at 4 °C using a Beckman Coulter Optima™ MAX-XP ultracentrifuge. Microsomal fractions were resuspended at a protein concentration of 10–20 mg mL^{-1} in buffer A and immediately used for G6Pase assays. The reaction mixture consisted of buffer B (100 mM imidazole-HCl pH 6.5, 40 mM G6P) and 50 mg mL^{-1} of protein from the microsomal suspension for a final volume of 20 μL. Incubations, carried out for 0, 8 and 15 min at 30 °C, were stopped by the addition of 80 μL of cold mixture of trichloroacetic acid (100 g L^{-1}) and ascorbic acid (20 g L^{-1}). G6Pase activity was determined by the increment in glucose production and glucose was determined at 560 nm by a colorimetric assay kit (Glucose MR, Cromatest, Linear Chemicals, Spain). Total protein was determined at 600 nm by the Bradford method (Bio-Rad, Spain) using bovine serum albumin as a standard. All the assays were performed at 30 °C. All the assays, except for G6Pase, were adapted for automated measurement using a Cobas Mira S spectrophotometric analyzer (Hoffman-La Roche, Switzerland). Enzyme activities were expressed per mg of soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme necessary to transform 1 μmol of substrate per min.

2.6. Total RNA extraction and reverse transcription (RT)

Total mRNA was isolated from frozen liver samples using the Speedtools Total RNA Extraction Kit (Biotools, Spain). The RNA obtained served as the template for RT-PCR. RNA was spectrophotometrically quantified using a NanoDrop ND-1000 (Thermo Scientific) and quality was determined by using the ratio of absorbance at 260 and 280 nm. Single strand cDNA templates for PCR amplification were synthesized from 1 μg of total RNA by incubation with M-MLV RT (Promega, Spain) at 37 °C for 1 h, according to supplier's instructions.

2.7. RT-PCR analysis

Amplification by RT-PCR was carried out using the pairs of primers presented in Table 2. The primers were designed from highly conserved regions of nucleotide sequences published in GenBank for both GK and G6Pase, using whenever possible fish species such as *Oncorhynchus mykiss*, *Platichthys flesus*, *Cyprinus carpio*, *Rhabdosargus sarba* and *S. aurata* (partial or complete sequences). The sequences were aligned for comparing homologies using EMBL-EBI ClustalW2— Multiple Sequence Alignment Tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and primers were designed with Oligo Explorer 1.2 Software (Gene Link). RT-PCR mixture (30 μL total volume) consisted of 3 μL of single-strand cDNA product, 3 μL of 10× PCR buffer, 200 μM dNTPs, 0.5 μM of primers (forward and reverse), and 1 U of Expand High Fidelity PCR System (Roche). Amplification was conducted through 39 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and DNA synthesis at 72 °C for 2 min, followed by a final extension step of 5 min. The PCR products obtained were separated electrophoretically on 1% agarose gel alongside 1 kbp ladder (Biotools). Bands of

Table 2
Primer pairs used for the GK and G6Pase partial cDNA cloning by RT-PCR and primers used to assay GK and G6Pase mRNA levels by quantitative real-time RT-PCR analysis.

			Primer sequence (5′–3′)	Band extension (in base pairs)
RT-PCR	GK	Forward	AGAGACGCTATCAAGAGACG	411
		Reverse	GCTCAGACGCTTCACCATTA	
	G6Pase	Forward	TCTGGTTCCACCTGCGGAGGGACAC	652
		Reverse	CCCATGTTACGCAGGAGGCTGGC	
qRT-PCR	18S[a]	Forward	ACGGACGAAAGCGAAAGCA	91
		Reverse	GGAACTACGACGGTATCTGATC	
	GK[b]	Forward	GAATGACACAGTAGCCACCA	115
		Reverse	CACAGTCCTCATCTCCTC	
	G6Pase[c]	Forward	CCTGTGGATGCTAATGGGTC	83
		Reverse	TGACCTGATGTGGGAAGTGG	

[a] GenBank accession no. AM419038.
[b] GenBank accession no. JX073704.
[c] GenBank accession no. JX073707.

appropriate size were excised, purified using High Pure PCR Cleanup Micro Kit (Roche), ligated into pGEM-T Easy plasmid (Promega), transformed by thermic shock in DH5α competent cells and selected colonies were allowed to grow overnight in LB/ampicillin broth at 37 °C. Recombinant plasmid DNA was isolated using GenElute™ Plasmid Miniprep Kit (Sigma) and sequenced with ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The identities of insert sequences were verified using BLASTx against the National Center for Biotechnology Information (NCBI) public databases and aligned among different cDNA clones. Sequences were introduced in GenBank (accession numbers for GK and G6Pase are JX073704 and JX073707, respectively).

2.8. Real time quantitative RT-PCR analysis

Specific primers for real time quantitative RT-PCR (qRT-PCR) were designed with Oligo Explorer 1.2 (Gene Link) (Table 2). Linearity and efficiency of amplification on qRT-PCR for the chosen pair of primers were tested by generating standard curves with consecutive dilutions of a cDNA test sample. PCR product from a non-diluted cDNA sample and a blank (with milliQ water instead of cDNA) were separated electrophoretically on 2% agarose gel alongside GeneRuler™ 100 bp DNA ladder (Fermentas) for length confirmation and discard the presence of secondary bands. Extraction of mRNA and subsequent synthesis of single strand cDNA templates were performed as mentioned above from frozen liver samples. The reaction product was diluted $10\times$ in milliQ water and qRT-PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems®) using 0.4 μM of each primer, 10 μL of Power SYBR® Green (Applied Biosystems®) and 1.6 μL of diluted cDNA. The temperature cycle protocol for amplification was: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s and 62 °C for 1 min. Ribosomal subunit 18 (18S) from *D. labrax* was chosen as the reference gene to normalize expression levels of targets between different samples. Primers were selected from a partial sequence (GenBank accession no. AM419038). Variations in gene expression and n-fold changes were calculated relative to fed fish using the standard ΔΔCt method including the efficiencies for both the experimental gene and 18S (house-keeping gene).

2.9. Statistical analysis

Values are presented as mean ± standard deviation (S.D.). Analysis of variance (ANOVA) was used to test the statistical differences between nutritional statuses. A posteriori Tukey's test was performed when significant differences were found. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Physiological parameters and hepatic enzyme activities

The 21 d fasting period resulted in a significant decrease in glycemia to about half of observed in fed fish while hepatic glycogen levels were nearly depleted (Table 3). The activity and mRNA levels of hepatic GK and G6Pase are shown in Figs. 2 and 3, respectively. Significant decline was observed in both G6Pase activity and mRNA levels after food deprivation while GK activity and mRNA levels showed no significant differences compared to fed fish. To determine whether hepatic metabolism of the 21 d fasted fish was still responsive to dietary cues, a group of 21 d fasted fish were subsequently refed over 3 d. For these fish, glycemia and glycogen levels were restored compared to the fed fish (Table 3) and this was accompanied by a significant increase in GK activity and expression. At this stage, an increase of G6Pase activity to the levels found in fed fish occurred but without observable changes in mRNA levels which were retained in the same levels found in the fasted fish.

3.2. ²H-Enrichments of plasma water and glucose

Plasma water ^{2}H-enrichment was equivalent to that of the tank water (4.7 ± 0.3% PW vs. 5.0 ± 0.1% tank water) confirming equilibrium from constant ^{2}H$_2$O precursor. Blood glucose enrichment from ^{2}H$_2$O was quantified by ^{2}H NMR for all positions of the MAG molecule (Fig. 4). The blood spectra from fasted and fed fish were highly consistent with earlier studies and allowed to precisely measure the glucose positional ^{2}H-enrichments, namely in positions 2 and 5 as shown in Table 4. The enrichment level of position 2 in relation to PW enrichment indicated the amount of blood glucose derived from G6P (65% for fed fish; 44% for refed fish; 48% for fasted fish). Enrichments of positions 5 and 2 were similar (student's t-test, $P = 0.28$, $P = 0.93$ and $P = 0.34$ for fed, fasted and refed fish, respectively), indicating that gluconeogenesis was the principal contributor to hepatic G6P synthesis (Table 5). The blood glucose that was not enriched with ^{2}H (35% for fed fish and 56% for refed fish) was assumed to be derived from dietary CHO absorption. For the 21 d fasted fish, an unexpectedly high fraction of unlabeled glucose (~52%) was estimated despite the absence of dietary CHO contributing for blood glucose via absorption (Table 5).

4. Discussion

4.1. Nutritional regulation of G6Pase and GK

The liver plays a key role in fish glucose homeostasis in part by the reciprocal regulation of glucose phosphorylation to G6P via GK and through the hydrolysis of G6P to glucose via G6Pase (Fig. 1). Transition of hepatic metabolism from fasting to feeding has provided insight into the functioning and responsiveness of fish species to dietary cues (Metón et al., 2003; Metón et al., 2004; Soengas et al., 2006; Pérez-Jiménez et al., 2007, 2012). Under a low CHO diet as the one used in the present study, GK activity was very low in fed seabass as reported previously (Enes et al., 2008b; Moreira et al.,

Table 3
Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on body weight, plasma glucose concentration and hepatic and muscle glycogen concentration.

	Feeding condition		
	Fed	Fasted	Refed
Final body mass, in g	247.3 ± 54.3[b]	173.3 ± 38.1[a]	211.5 ± 27.0[ab]
Plasma glucose, in mM	10.7 ± 6.3[b]	4.8 ± 1.2[a]	9.3 ± 1.4[b]
Liver glycogen[1]	167.7 ± 52.5[b]	36.3 ± 24.7[a]	191.8 ± 23.9[b]

Mean values ± S.D. are presented. Significant differences between feeding conditions are indicated by different letters (one-way ANOVA followed by Tukey's test).
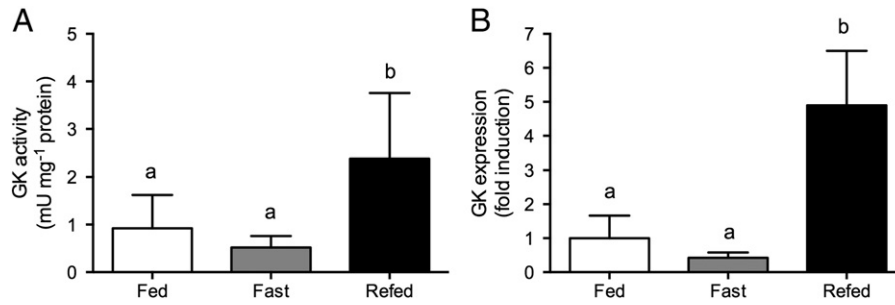[1] In μmol glycosyl units g^{-1} tissue.

Fig. 2. Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) (A) and relative abundance of mRNA (in arbitrary units) (B) of glucokinase in liver. Mean values ± S.D. (n = 6) are presented. Significant differences are indicated by different letters (one-way ANOVA followed by Tukey's test).

2008) as well as in other species (Enes et al., 2009). Moreover, no noticeable differences in regard to fasted fish were observed suggesting a limited role of GK in glycemic control in this setting. A more effective nutritional control exerted by GK seems to be confined to the postprandial period (Caseras et al., 2000; Panserat et al., 2000; Kirchner et al., 2003, 2005; Soengas et al., 2006). In contrast to the lack of significant GK activity excursions during regular daily feeding, a refeeding protocol (with the same diet) following a prolonged fasting period resulted in high GK activity, a characteristic also observed by others. After 3 d refeeding GK activity is enhanced in seabass (2.6-fold induction, present study; 2.8-fold induction, Pérez-Jiménez et al., 2007) but can last up to 7 d in rainbow trout (2.7-fold induction, Soengas et al., 2006). Interestingly, despite normoglycemic levels coupled with elevated GK activity, the direct pathway is not a significant contributor to hepatic glycogen synthesis during the refeeding phase (Viegas et al., 2012). Thus dietary glucose that is phosphorylated to G6P via GK is mobilized to glycogen predominantly via the indirect pathway after being metabolized to 3-carbon intermediates. Besides, differences in feed intake after fasting could also in part explain this enhanced activity and mRNA levels for GK in comparison to fed fish. Food ration was not corrected during the trial; nevertheless, these discrepancies alone could hardly explain such acute differences (Metón et al., 1999; Eroldogan et al., 2004).

G6Pase on the other hand, seems to be sensitive to nutritional status as in rainbow trout (Panserat et al., 2000), seabream (Caseras et al., 2002; Enes et al., 2008a), common carp (Panserat et al., 2002b) and seabass (Enes et al., 2006) but does not necessarily correlate with efficient glycemic control, particularly for carnivorous fish. In this study, fasting provoked a significant decrease in G6Pase activity and mRNA levels, which contradict others' work that demonstrated either, increased G6Pase activity for seabream (Caseras et al., 2002; Metón et al., 2004; Sangiao-Alvarellos et al., 2005), or unchanged activity for rainbow trout (Panserat et al., 2001, 2002a; Kirchner et al., 2008). It is worth noting that in these studies, plasma glucose levels did not change significantly between fed and fasted states therefore suggesting a better co-ordination of EGP with whole body glucose demand compared to the fish from this study. The decline in both

activity and mRNA levels of G6Pase was therefore consistent with the observed hypoglycemia. The rapid recovery of hepatic glycogen stores after the 3 d refeeding protocol restored the liver's capacity to export glucose into the blood stream. This was verified by the rebound in G6Pase activity probably boosted by abundance of substrate to dephosphorylate even if G6Pase mRNA levels did not accompany such recovery. Administration of glucose (in vivo) and glucose plus insulin (in vitro) has shown to significantly inhibit transcription of this enzyme in seabream liver (Salgado et al., 2004). Post-translational regulation (Egea et al., 2008) and other mechanisms of non-transcriptional regulation such as the effects of recovering normoglycemic levels on EGP (Guignot and Mithieux, 1999) cannot be discarded.

4.2. Sources of blood glucose during prolonged fasting and consequent refeeding

Our results demonstrate that for 21 d fasted fish, approximately half of the blood glucose was not enriched with ^{2}H from ^{2}H$_2$O. Under the conditions of our study, this unenriched glucose could not have been derived from G6P since exchange between G6P and fructose 6-phosphate (F6P) results in enrichment of position 2 from ^{2}H$_2$O regardless of the G6P source (Landau et al., 1996). The possibility of the unlabeled glucose fraction reflecting incomplete turnover of the blood glucose pool (i.e. pre-existing glucose before ^{2}H$_2$O administration) is unlikely, based on the known glucose transit times—equivalent to the half-life of blood glucose residence. The glucose transit time using the single injection tracer method for 2–9 d fasted seabass was estimated to be ~3 h (Garin et al., 1987) and for 20 d fasted kelp bass (*Paralabrax* sp.), a closely related species, ~3.5 h (Bever et al., 1977). Given that after 7 half-lives (a period of 21–25 h based on the reported transit times), greater than 99% of blood glucose will have turned over. Of the known alternative glucose-producing pathways that could generate unlabeled glucose in the presence of ^{2}H$_2$O, glycogen conversion to glucose via glycosidases (E.C. 3.2.1; includes glucosidases, amylases and glycogen-debranching enzymes) is the most plausible, since these do not involve exchange of glucose and water hydrogens. In fish, there is widespread α-glucosidase (Castilla et al., 1980) and γ-amylase (Sharma and
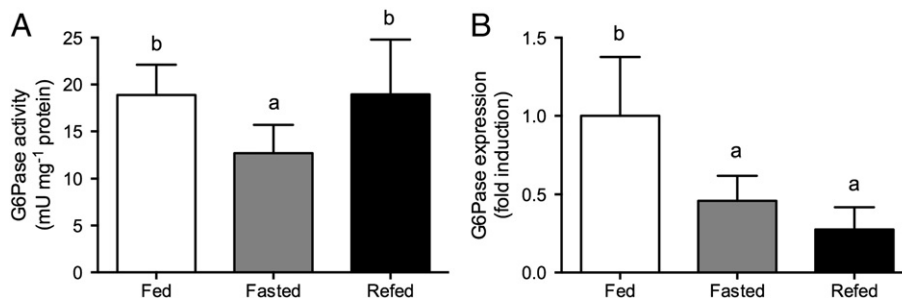

Fig. 3. Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) (A) and relative abundance of mRNA (in arbitrary units) (B) of glucose 6-phosphatase in liver. Mean values ± S.D. (n = 6) are presented. Significant differences are indicated by different letters (one-way ANOVA followed by Tukey's test).
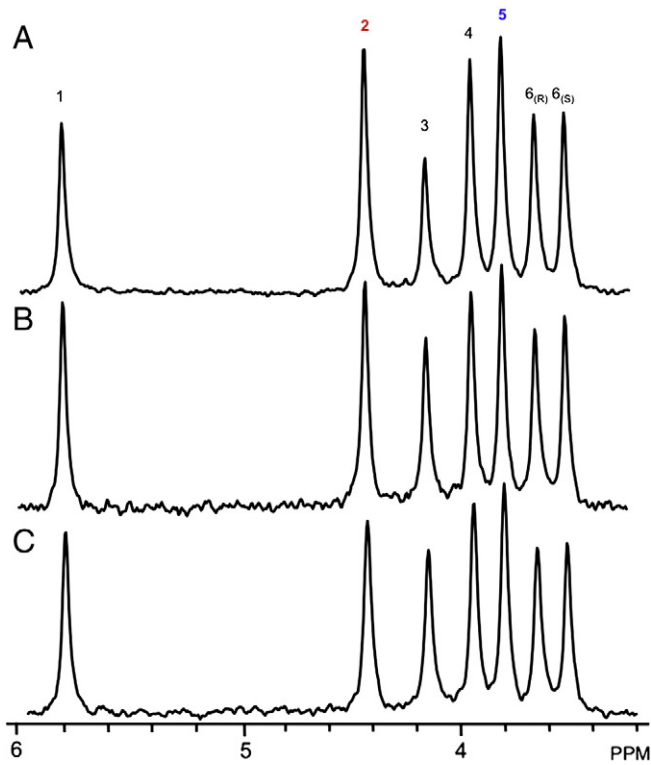
Fig. 4. ^{2}H NMR representative spectra of monoacetone glucose (MAG) samples derived from blood glucose of fed (A), fasted (B) and refed (C) European seabass (*D. labrax*) sampled after 72 h residence in tank with 5% ^{2}H-enriched water. The numbers above each signal (each of the 7 aliphatic hydrogen) represent each hydrogen within the original glucose molecule.

Table 5
Plasma glucose ^{2}H-enrichments for positions 2 and 5 as quantified by ^{2}H NMR, hepatic glucose sources and synthesis fluxes for fed, fasted and refed placed 72 h in 5%-enriched deuterated water.

Fractional contributions ± %	Fed	Fasted	Refed
G6P sources[1]	65 ± 7[b]	48 ± 16[ab]	44 ± 10[a]
Gluconeogenesis[2]	93 ± 6[a]	98 ± 3[b]	113 ± 3[b]
Glycogenolysis[3]	7 ± 6[b]	2 ± 3[b]	− 13 ± 3[a]
Non G6P sources[4]	35 ± 7[a]	52 ± 16[ab]	56 ± 10[b]

Mean values (S.D.) are presented. Significant differences between feeding conditions are indicated by different letters (one-way ANOVA followed by Tukey's test).
[1] Following Eq. (1).
[2] Following Eq. (3).
[3] Following Eq. (4).
[4] Following Eq. (2) where in the case of fasted fish corresponds only to EGP and in the case of fed and refed fish corresponds to EGP + absorbed dietary carbohydrates.

After refeeding, and despite replenishment of hepatic glycogen levels to fed fish levels as expected (Metón et al., 1999; Pérez-Jiménez et al., 2007), the glycogenolytic potential was not restored. Yet, these by-difference calculations as defined by the equations presented in this work rely on the assumption that G6P fully exchanges with body water such that the enrichment of hepatic G6P position 2 is equivalent to that of body water (Viegas et al., 2011). To the extent that this exchange is incomplete, the fractional synthetic rate of G6P from non-gluconeogenic precursors is underestimated. In contrast, position 5 enrichment of glucose or glycogen derived from gluconeogenesis is obligatory and is not known to be influenced by isotope effects (Landau et al., 1996). Therefore, with incomplete G6P–F6P exchange, the ^{2}H5/^{2}H2 ratio will tend to overestimate the fractional gluconeogenic contribution to glucose and glycogen synthesis.

5. Conclusions

In seabass fed with conventional commercial diet, plasma glucose levels show considerable fluctuations between feeding and fasting. Gluconeogenesis appears to be the principal source of EGP and is a significant contributor to blood glucose levels during both feeding and fasting. The observed decrease in G6Pase activity and mRNA levels during fasting suggested that glycemic control, if present, may be exerted elsewhere within the metabolic network. Finally, indications for significant contribution of hepatic glycosidases and participation of extrahepatic glucose production in the maintenance of glycemia during starvation were unveiled.

Sengupta, 1993) activity in liver, kidney, muscle and brain. Their responsiveness to glycogenolytic stimuli such as exhaustive exercise (Mehrani and Storey, 1993) and fasting (Murat, 1976; Sharma and Sengupta, 1993) has also been demonstrated. Moreover, for extrahepatic glucose production, the kidney must be taken as putative contributor for glycemic regulation under starvation. In rainbow trout at rest the kidney can be responsible for approximately 10% of total glucose production (Haman et al., 1997) and in fact, starvation does not alter renal G6Pase activity in seabream (Kirchner et al., 2005; Sangiao-Alvarellos et al., 2005). The energy demand and functionality of this important osmoregulatory organ are maintained under starvation as confirmed also by unaltered glycogen levels (Sangiao-Alvarellos et al., 2005). The ubiquitous presence of ^{2}H in the body means that any tissue that can generate glucose via G6P and export it into blood can potentially contribute to the observed plasma glucose ^{2}H-enrichment pattern.

For most fish species, including the seabass, hepatic glycogen stores are depleted during fasting (Metón et al., 2003; Soengas et al., 2006; Pérez-Jiménez et al., 2007, 2012) being therefore unable to sustain significant rates of EGP via glycogenolysis as observed. During refeeding with fishmeal-based diet there is an increased dependence on gluconeogenesis for EGP and for the repletion of hepatic glycogen (Viegas et al., 2012), however the decreased contribution of G6P sources to blood glucose during refeeding compared to the fed state may suggest better utilization of dietary CHO.

Table 4
Plasma glucose positional ^{2}H-enrichments (%) and plasma water (PW) enrichment (% as quantified by ^{2}H NMR).

	^{2}H-positional enrichment							PW
	1	2	3	4	5	6_S	6_R	
Fed	2.1 ± 0.5	3.1 ± 0.3[b]	2.1 ± 0.7	2.8 ± 0.3	2.8 ± 0.3	2.0 ± 0.4	2.0 ± 0.3	
Fasted	2.0 ± 0.2	2.4 ± 0.7[ab]	1.8 ± 0.3	2.4 ± 0.6	2.4 ± 0.7	1.7 ± 0.3	1.7 ± 0.3	4.7 ± 0.3
Refed	1.9 ± 0.4	2.1 ± 0.5[a]	1.8 ± 0.5	2.2 ± 0.5	2.3 ± 0.6	1.7 ± 0.5	1.7 ± 0.5	

Mean values (± S.D.) are presented. Significant differences between feeding conditions are indicated by different letters (one-way ANOVA followed by Tukey's test).

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