



Disposition of [U-²H₇]glucose into hepatic glycogen in rat and in seabass



Fátima O. Martins^{a,b}, João Rito^{a,c}, Ivana Jarak^a, Ivan Viegas^{a,c}, Miguel A. Pardal^c, M. Paula Macedo^{d,e}, John G. Jones^{a,b,e,*}

^a CNC—Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal

^b Institute for Interdisciplinary Research, University of Coimbra (IIIUC), Coimbra, Portugal

^c CFE—Center for Functional Ecology, University of Coimbra, Apartado 3046, 3001-401 Coimbra, Portugal

^d CEDOC, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisbon, Portugal

^e APDP—Portuguese Diabetes Association, Lisbon, Portugal

ARTICLE INFO

Article history:

Received 3 April 2013

Received in revised form 17 June 2013

Accepted 1 July 2013

Available online 7 July 2013

Keywords:

Direct pathway

Transaldolase

Glycogenesis

Gluconeogenesis

Seabass

Rat

ABSTRACT

The stimulation of hepatic glycogenesis is a ubiquitous response to a glucose challenge and quantifying its contribution to glucose uptake informs its role in restoring euglycemia. Glycogenesis can be quantified with labeled water provided that exchange of glucose-6-phosphate hydrogen 2 (G6P-H2) and body water via glucose-6-phosphate isomerase, and exchange of positions 4, 5 and 6 hydrogens (G6P-H456) via transaldolase, are known. These exchanges were quantified in 24-h fasted rats (*Rattus norvegicus*; n = 6) and 21-day fasted seabass (*Dicentrarchus labrax*; n = 8) by administration of a glucose load (2000 mg·kg⁻¹) enriched with [U-²H₇]glucose and by quantifying hepatic glycogen ²H-enrichments after 2 h (rats) and 48 h (seabass). Direct pathway contributions of the glucose load to glycogenesis were also estimated. G6P-H2 and body water exchange was 61 ± 1% for rat and 47 ± 3% for seabass. Transaldolase-mediated exchange of G6P-H456 was 5 ± 1% for rat and 10 ± 1% for seabass. Conversion of the glucose load to hepatic glycogen was significant in seabass (249 ± 54 mg·kg⁻¹) but negligible in rats (12 ± 1 mg·kg⁻¹). Preload plasma glucose levels were similar for seabass and rats (3.3 ± 0.7 and 4.4 ± 0.1 mmol·L⁻¹, respectively) but post-load plasma glucose was significantly higher in seabass compared to rats (14.6 ± 1.8 versus 5.8 ± 0.3 mmol·L⁻¹, p < 0.01). In conclusion, G6P-H2 and body water exchange is incomplete for both species and has to be accounted for in estimating hepatic glycogen synthesis and direct pathway activities with labeled water tracers. Transaldolase-mediated exchange is insignificant. Hepatic direct pathway glycogenesis plays a prominent role in seabass glucose load disposal, but a negligible role in the rat.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

The clearance of a glucose load is used to assess the capacity of an organism to control plasma glucose levels and measurements of hepatic glycogen synthesis under these conditions inform the role of hepatic glycogenesis in glucose homeostasis. Alterations in net hepatic glycogen synthesis rates can reveal hormonal or cell signaling defects in glycemic control, for example defective insulin secretion in Type 1 Diabetes (Bischof et al., 2001) or impaired insulin signaling in Type 2 Diabetes (Torres et al., 2011). These measurements also provide insight into the importance of hepatic glycogenesis relative to other mechanisms of glucose disposal for dissipating postprandial hyperglycemia. Compared to mammals, carnivorous fish such as

seabass (*Dicentrarchus labrax* L.) have poor control of plasma glucose levels and are slow to clear a glucose load (Peres et al., 1999; Enes et al., 2011b). However, it is unclear if this reflects a limited capacity for hepatic glycogen synthesis from glucose, a limited uptake of glucose by other tissues, or both.

Hepatic glycogen can be synthesized from glucose via the so called “direct pathway” or from gluconeogenic precursors by the indirect pathway (Newgard et al., 1983), as depicted in Fig. 1. Indirect pathway precursors can be derived from glucose metabolism (i.e. lactate) or from other sources, for example glycerol from triglyceride hydrolysis or dietary amino acids. Carnivorous fish such as seabass that are fed with a high protein/low carbohydrate diet synthesize the bulk of liver glycogen via the indirect pathway (Viegas et al., 2012). In contrast, humans and rodents typically synthesize the majority of glycogen via the direct pathway reflecting a higher proportion of dietary carbohydrate (Jones et al., 2006b; Delgado et al., 2009; Soares et al., 2009, 2012). Alterations in direct and indirect pathway activities can reflect changes in hepatic insulin actions, such as in insulin-dependent diabetes (Bischof et al.,

* Corresponding author at: Center for Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal. Tel.: +351 239820190.

E-mail address: john.griffith.jones@gmail.com (J.G. Jones).

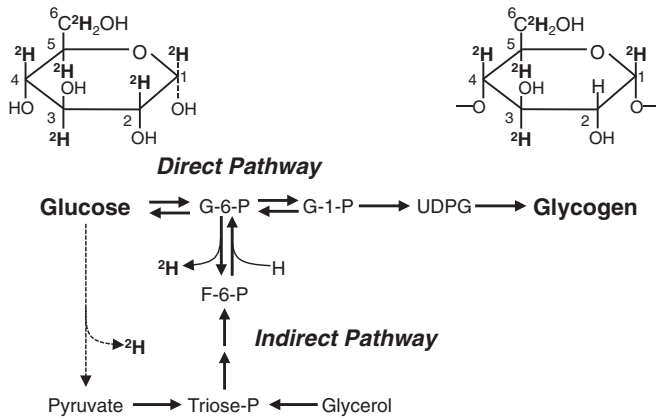


Fig. 1. Depiction of direct and indirect pathways and deuterium/hydrogen exchanges during conversion of [$^2\text{H}_7$]glucose to glycogen. As a result of interconversion of glucose-6-phosphate and fructose-6-phosphate, the deuterium (^2H) of G6P position 2 is exchanged with a proton (H) from water. Some metabolic intermediates are omitted for clarity. F6P = fructose-6-phosphate, G6P = glucose-6-phosphate, G1P = glucose-1-phosphate, Triose-P = triose phosphates, UDPG = uridine diphosphoglucose.

2002) as well as adaptation to different dietary regimes (Obeid et al., 2005, 2006).

Deuterated water ($^2\text{H}_2\text{O}$) is an inexpensive and versatile tracer for the study of hepatic glycogen synthesis under both natural and artificial feeding conditions (Delgado et al., 2009; Soares et al., 2009, 2012; Barosa et al., 2012). Analysis of glycogen positional ^2H -enrichments provides estimates of the fractional synthetic rate as well as direct and indirect pathway contributions to glycogen synthesis (Soares et al., 2009, 2010). These estimates rely on two key assumptions concerning exchange of metabolite and water hydrogens. First, the position 2 hydrogen of glucose-6-phosphate (G6P) is fully exchanged with body water such that the enrichment of hepatic G6P position 2 is equivalent to that of body water. When this assumption holds, the fractional synthetic rate of glycogen from G6P can be calculated from the ratio of glycogen position 2 to body water ^2H -enrichments (Soares et al., 2009). To the extent that exchange between position 2 and water is incomplete, the fractional synthetic rate is underestimated (Soares et al., 2010). Second, the position 5 hydrogen of G6P is assumed to be enriched by indirect pathway activity. When this assumption holds, the fractional contribution of the indirect pathway can be calculated from the ratio of glycogen position 5 to body water enrichment. To the extent that G6P position 5 is enriched by processes other than indirect pathway flux, such as transaldolase exchange (Delgado et al., 2009), the indirect pathway contribution is overestimated and that of the direct pathway is correspondingly underevaluated.

These assumptions have been tested in humans given an oral glucose tolerance test (OGTT) as well as a regular breakfast meal by monitoring the fate of the deuterium labels of [$^2\text{H}_7$]glucose following its conversion to UDP-glucose, sampled as urinary glucuronide. Analysis of UDP-glucose ^2H -enrichment revealed that a), exchange of G6P position 2 with body water was essentially complete (Delgado et al., 2009; Barosa et al., 2012) and b), ~15–20% of G6P derived from the direct pathway underwent transaldolase exchange activity, resulting in a modest overestimation of indirect pathway and underestimation of direct pathway contributions (Delgado et al., 2009; Barosa et al., 2012). The goal of this study was to apply the same methodology to quantify hepatic glycogenic G6P–fructose-6-phosphate (F6P) and transaldolase exchanges in rats and seabass following a glucose challenge. Aside from the interest of interspecies comparison of these parameters, these studies are also important for the following reasons. The rat is widely used as a small animal model of diabetes and liver carbohydrate metabolism, hence there is widespread interest in studying hepatic glycogen fluxes in this animal, (e.g. Soares et al., 2012). The seabass is a widely cultivated carnivorous saltwater fish and there is currently

high interest in studying its capacity to utilize carbohydrate-based substrates as a cheaper and more sustainable alternative to fishmeal-based diets. The disposal of glucose into hepatic glycogen is a widely used parameter for evaluating carbohydrate utilization in this and other carnivorous fish species (Enes et al., 2011a).

2. Material and methods

2.1. Fish handling and sampling

For the present study, 8 European seabass (*D. labrax* L.) provided by a local farm were transported to the lab and acclimated at 20 °C and 30‰ salinity in a 200 L tank supplied with well aerated filtered seawater from a recirculation system equipped with a central filtering unit and UV unit. Tank water temperature, salinity, pH, and dissolved oxygen were continuously monitored and NH_4^+ , NO_3^- and NO_2^- were assessed every 7 days and maintained within optimal ranges. During acclimation, fish were provided with food until satiated once a day (Dourasoja Ultra 5, SORGAL, S.A.; 44% protein, 18% fat, 2.2% carbohydrates, 9.2% ash, 5 mm standard pellet). Following acclimation, the fish were fasted for 21 days. At the end of this period, fish were anesthetized in a 30 L tank of saltwater containing 0.1 g L^{-1} of MS-222 for approximately 2 min. Fish were measured (21 ± 0 cm), weighed (88 ± 7 g) and sampled for blood from the caudal vein with heparinized syringes. Blood was kept on ice, centrifuged (3000 g for 10 min) to separate plasma and stored at -20 °C for posterior assessment of ^2H -enrichment of body water and glucose quantification with a commercial assay kit (Invitrogen, Spain). Fish were then injected intraperitoneally (10 mL kg^{-1}) with saline solution with 200 mg/mL of glucose enriched to 49% with [$^2\text{H}_7$]glucose for a glucose load of 2000 mg \cdot kg^{-1} . Fish were allowed to recover in the tank for 48 h after which were once again anesthetized, measured, weighed and sampled for blood as previously described. This time, the maximum possible volume of blood was withdrawn. Fish were then sacrificed by cervical section; livers were excised, weighed, freeze-clamped in liquid N_2 and stored at -80 °C until further analysis. Experimental procedures complied with the Guidelines of the European Union Council (86/609/EU).

2.2. Rat studies

Six male Wistar rats (*Rattus norvegicus*) at 8 weeks of age were studied. Animal handling followed the ethical proceedings for scientific experiments of Institutional Animal Care and Use Committee. Animals were maintained in a 12 h dark–light cycle (19:00/07:00) with ad libitum access to food and water. After 24 h of fasting, awake animals were submitted to an intraperitoneal glucose tolerance test (2000 mg \cdot kg^{-1}), using 98% [$^2\text{H}_7$]glucose. Glucose was monitored from tail tip samples 15 min before the load and at 120 min after the load, using a One-Touch Vita glucometer (LifeScan). At this point animals were sacrificed by cervical dislocation and the abdominal cavity was opened. Blood was collected from the *vena cava* and immediately centrifuged (1000 g for 5 min) to obtain plasma samples, which were stored at -20 °C for later assessment of ^2H -enrichment of body water. Livers were excised, weighed, freeze-clamped in liquid N_2 and stored at -80 °C until further analysis.

2.3. Blood glucose and liver glycogen extraction

Plasma was mixed with 0.3 N ZnSO_4 and 0.3 N $\text{Ba}(\text{OH})_2$ (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-50- H^+ and Amberlite, then lyophilized and stored at -20 °C.

Glycogen was extracted by alcoholic precipitation after alkaline tissue hydrolysis as previously described (Soares et al., 2009). Briefly,

frozen liver powder was treated with 30% KOH (2 mL per gram of liver) at 70 °C for 30 min. After vigorous vortex, the mixture was treated with 6% Na₂SO₄ (1 mL per g of liver) and 99.9% ethanol (to a final concentration of 70%, 7 mL per gram of liver) and left overnight at 4 °C to precipitate glycogen. After centrifugation, the upper liquid phase was discarded and the solid residue was dried. The residue was resuspended in 5 mL acetate buffer (0.05 M pH = 4.5) and 20 µL of an aqueous solution containing 16 U of amyloglucosidase from *Aspergillus niger* (glucose-free preparation, Sigma-Aldrich, Germany) was added to hydrolyze glycogen to its glucosyl units. Samples were incubated overnight at 55 °C and centrifuged. The supernatant was collected and a 100 µL aliquot was stored separately so glucose derived from glycogen enzymatic hydrolysis could be quantified with standard assay kit (Invitrogen, Spain). The remainder was lyophilized.

2.4. Derivatization of glucose to monoacetone glucose (MAG)

Each lyophilized extract was vigorously mixed with 5 mL acetone containing 4% sulphuric acid (v/v), both enriched with deuterium to 2%. Anhydrous ²H-enriched acetone was prepared as previously described (Jones et al., 2006a; Nunes and Jones, 2009). The mixture was stirred overnight at room temperature to yield diacetone glucose. The acetonation reaction was quenched by adding 5 mL of water (also enriched with deuterium to 2%) and the pH was adjusted to 8 with 1 M NaHCO₃ followed by acidification of the solution to pH 2.0 with 1 M HCl. The newly formed diacetone glucose was hydrolyzed to MAG by incubation at 40 °C for 5 h. The solution pH was then increased again to 8.0 with 1 M NaHCO₃ and the samples were dried by rotary evaporation under vacuum. MAG in the residue was extracted with 4 mL of boiling ethyl acetate. Following evaporation of ethyl acetate, the residue was dissolved in 0.6 mL mixture of 1:10 water/acetonitrile (v/v) buffered with NaHCO₃ (9 mg·mL⁻¹) for ²H NMR analysis.

2.5. ²H NMR analysis

Proton-decoupled ²H NMR spectra were acquired at a temperature of 50 °C with a Varian VNMRs 600 MHz NMR (Agilent, Santa Clara, CA, USA) spectrometer equipped with a 3 mm broadband probe. Acquisition parameters included a 90° hard pulse angle, a sweep width of 10 ppm, 1.6 s acquisition time, 0.1 s pulse delay and continuous WALTZ-16 broadband ¹H decoupling. Up to 2000 scans were collected per sample, corresponding to ~1 h of collection time. The summed free induction decays were processed with 0.5–1.0 Hz line-broadening before Fourier transform. MAG ²H-enrichments were quantified from the ²H NMR spectra by measuring the intensity of each signal of the MAG hexose moiety relative to the mean intensity of the two intramolecular methyl reference signals at 1.28 and 1.40 ppm (Nunes and Jones, 2009). Spectra were analyzed with the NUTS PC-based NMR spectral analysis software (Acorn NMR Inc., USA).

2.6. Quantification of deuterium–hydrogen exchanges and glycogen synthesis pathway contributions

2.6.1. Quantifying G6P–F6P exchange with [U-²H₇]glucose

When [U-²H₇]glucose is metabolized to glycogen via the direct pathway, the position 3 deuterium (H3) is fully retained, while the position 2 deuterium (H2) is exchanged for a solvent proton during exchange of G6P and F6P. Hence, the ratio of glycogen H2 to H3 informs the fraction of direct pathway G6P that exchanged with F6P as follows:

$$\text{Percent of direct pathway G6P exchanged with F6P} = 100 \times (1 - \text{H2}/\text{H3}). \quad (1)$$

2.6.2. Quantification of transaldolase exchange activity from analysis of glycogen and glucose ²H-enrichments

Transaldolase (TA; EC 2.2.1.2) exchange catalyzes the exchange of the 4, 5 and 6 carbon fragments of F6P and glyceraldehyde-3-phosphate (G3P) independently of oxidative pentose phosphate pathway flux (see Fig. 1). Thus, TA exchange activity results in a loss of ²H-enrichment in positions 4, 5 and 6 relative to position 3 hence the mean enrichment of these hydrogens relative to that of position 3 (H456^m/H3) reflects the fraction of G6P that participated in TA exchange.

$$\text{Percent of G6P participating in TA exchange} = 100 \times (1 - \text{H456}_m/\text{H3}). \quad (2)$$

2.6.3. Quantification of glucose load contribution to hepatic glycogen synthesis via the direct pathway

When [U-²H₇]glucose is metabolized through the indirect pathway, the ²H-label in position 3 is assumed to be quantitatively removed following conversion of [U-²H₇]glucose-6-phosphate to triose phosphates and pyruvate. Hence, glycogen that is subsequently synthesized via indirect pathway is not enriched with ²H in this position. Meanwhile, H3 is retained during direct pathway metabolism of [U-²H₇]glucose to glycogen. Therefore, the direct pathway contribution of the load to hepatic glycogen synthesis can be estimated as the ratio of glycogen H3 to load glucose H3 (H3_{GLY}/H3_{GLC}):

$$\text{Percent of glycogen derived from the load via direct pathway} = 100 \times \text{H3}_{\text{GLY}}/\text{H3}_{\text{GLC}}. \quad (3)$$

2.6.4. Estimation of whole-body glucose pool size

For rats, the glucose pool size before and after the load was estimated from the product of the plasma glucose levels, (expressed as mg/100 g) and 0.25 times the body mass, where 0.25 is the reported glucose space for rats (Smadja et al., 1988). For seabass, the same calculation was performed but with a glucose space of 0.13 (Garin et al., 1987).

2.6.5. Load glucose contribution to hepatic glycogen synthesis

The amount of hepatic glycogen derived from the glucose load via the direct pathway was estimated as the product of the hepatic glycogen level and fractional direct pathway contribution to glycogen synthesis. Values are expressed as mg·kg⁻¹ body mass. The fraction of the glucose load that was converted to hepatic glycogen via the direct pathway was estimated as the ratio of hepatic glycogen synthesized from the load via the direct pathway to the quantity of the glucose load.

2.7. Statistical analysis

Values are presented as mean ± standard error of the mean (S.E.M.). Analysis of variance (ANOVA) was used to test the existence of significant differences between basal and post-load states for various parameters. Student's two-tailed unpaired *t*-test was used to compare means between rat and seabass parameters. Differences were considered statistically significant at *p* < 0.05.

3. Results

For rats, the glucose load resulted in a transient doubling of plasma glucose levels by 30 min (data not shown) followed by a return towards basal values by the sampling period of 120 min, as shown in Table 1. For the seabass, plasma glucose levels remained significantly elevated at 48 h after the injection and are characteristic of the inferior glucose tolerance of carnivorous marine fish compared

Table 1

Characteristics of glucose dosage and plasma glucose excursion in fasted rats and seabass following an intraperitoneal glucose load.

Parameter	Rat	Seabass
Mass	358 ± 5 g	88 ± 7 g
Fasting interval	24 h	21 days
Glucose load	2000 mg·kg ⁻¹	2000 mg·kg ⁻¹
Basal plasma glucose	4.4 ± 0.1 mmol·L ⁻¹	3.3 ± 0.7 mmol·L ^{-1ns}
Endpoint plasma glucose	5.8 ± 0.3 mmol·L ⁻¹	14.6 ± 1.8 mmol·L ^{-1**}
Basal plasma glucose pool size	198 ± 5 mg·kg ⁻¹	79 ± 15 mg·kg ^{-1***}
Endpoint plasma glucose pool size	262 ± 12 mg·kg ⁻¹	342 ± 41 mg·kg ^{-1ns}
Endpoint liver glycogen pool size	108 ± 18 mg·kg ⁻¹	659 ± 56 mg·kg ^{-1***}
Load disposed into liver glycogen via direct pathway	12 ± 1 mg·kg ⁻¹	249 ± 54 mg·kg ^{-1***}

Mean values ± S.E.M. are presented. Differences from correspondent value in rats are indicated by asterisks (*t*-test, ^{ns}*p* > 0.05, **p* < 0.05, ***p* < 0.01 and ****p* < 0.001).

to mammals. Blood glucose ²H-enrichments at the endpoints are shown in Table 2.

For rats, the enrichment of glucose position 3 was ~20% that of the load, indicating that approximately one-fifth of the blood glucose at 120 min was derived from the load. The remaining fraction was accounted for by endogenous production and/or pre-existing unlabeled glucose. The difference between basal and post-load whole body glucose pool was ~100 mg·kg⁻¹, equivalent to ~5% of the glucose load. For the seabass, blood glucose position 3 enrichment was significantly higher and indicated that about one-half of the blood glucose was derived from the load at 48 h after administration (see Table 3). The change in whole body glucose pool size between pre- and post-load was ~260 mg·kg⁻¹, equivalent to ~13% of the glucose load. For both rat and seabass, the position 2 enrichment of blood glucose was significantly depleted compared to all other sites (Fig. 2). This indicates futile cycling activity between blood glucose and hepatic G6P, where the deuterium in position 2 is exchanged with unlabeled water hydrogen as a result of G6P–F6P interconversion, while the other deuterium labels remain bound to the hexose skeleton. There was no significant depletion of position 4, 5 and 6 enrichments relative to position 3 indicating that the blood glucose pool had not undergone any significant transaldolase exchange activity.

Hepatic glycogen enrichments were systematically lower than that of blood glucose for both rat and fish (Table 2). This is consistent with the dilution of the ²H-enriched glucosyl units by a residual amount of pre-existing unlabeled glycogen and by unlabeled glycogen that was synthesized via the indirect pathway. In previous studies, the amount of pre-existing hepatic glycogen in 21-day fasted seabass was ~70 mg·kg⁻¹ body mass (Viegas et al., 2012) and ~9 mg·kg⁻¹ for 24-hour fasted rats (Soares et al., 2010). These levels represent ~10% of the endpoint values shown in Table 1. As for blood glucose, hepatic glycogen showed a significant depletion of position 2 enrichment relative to all other sites, indicative of exchange between this hydrogen and that of body water by glucose-6-phosphate isomerase (EC 5.3.1.9). Compared to humans, where ~90% of the position 2 deuterium is exchanged at the level of hepatic G6P (Delgado et al.,

Table 2

Blood glucose and liver glycogen ²H-enrichments, in percentage, following administration of a glucose load enriched with [U-²H₇]glucose to a group of rats and a group of seabass. Metabolite enrichments are normalized for differences in [U-²H₇]glucose load enrichments between the different groups.

Metabolite	Species	Glucose and glycogen positional ² H-enrichments						
		1	2	3	4	5	6 _R	6 _S
Blood glucose	Rat (n = 6)	16.8 ± 0.5 ^{a,c}	11.9 ± 0.5 ^b	18.5 ± 0.4 ^c	18.1 ± 0.2 ^{a,c}	16.8 ± 0.3 ^a	18.9 ± 0.3 ^{a,c}	17.5 ± 0.6 ^{a,c}
	Seabass (n = 8)	41.9 ± 2.7 ^{a,***}	22.3 ± 2.2 ^{b,**}	50.1 ± 2.8 ^{a,***}	42.1 ± 2.5 ^{a,***}	40.7 ± 2.7 ^{a,***}	47.9 ± 2.7 ^{a,***}	45.7 ± 2.6 ^{a,***}
Liver glycogen	Rat (n = 6)	10.2 ± 1.1 ^a	4.4 ± 0.5 ^b	11.5 ± 1.2 ^a	10.6 ± 1.1 ^a	10.5 ± 1.1 ^a	11.4 ± 1.1 ^a	11.2 ± 1.2 ^a
	Seabass (n = 8)	30.4 ± 5.0 ^{a,**}	18.3 ± 3.5 ^{b,***}	33.7 ± 5.2 ^{a,**}	29.4 ± 4.8 ^{a,**}	29.0 ± 4.8 ^{a,**}	32.2 ± 4.8 ^{a,**}	31.2 ± 4.9 ^{a,**}

Mean values ± S.E.M. are presented. Within each set of blood glucose and liver glycogen enrichments, values not sharing a common superscript letter are significantly different from each other (one-way ANOVA followed by Tukey test, *p* < 0.0001 for seabass blood glucose; *p* < 0.05 for rat and seabass liver glycogen). Differences from correspondent value in rats are indicated by asterisks (*t*-test, **p* < 0.05, ***p* < 0.01 and ****p* < 0.0001).

Table 3

Contribution of the glucose load, in percentage, to blood glucose and hepatic glycogen levels at the endpoint and the fraction of G6P hydrogen 2 that was removed by exchange with F6P via G6P-isomerase and the fraction of G6P hydrogens 4, 5 and 6 that were removed via a combination of G6P-isomerase and transaldolase exchanges.

Species	Load contributions		Glucose-6-phosphate exchanges	
	Blood glucose	Hepatic glycogen via direct pathway	G6P-isomerase	Transaldolase
Rat (n = 6)	19 ± 1	12 ± 1	61 ± 1	5 ± 1
Seabass (n = 8)	51 ± 3 ^{**}	34 ± 5 [*]	47 ± 3 [*]	10 ± 1 [*]

Mean values ± S.E.M. are presented. Differences from correspondent value in rats are indicated by asterisks (*t*-test, **p* < 0.01 and ***p* < 0.0001).

2009), this fraction was considerably lower for the rat (~61%) and even more so for seabass (~47%), as shown in Table 3. For the rat, enrichment of positions 4, 5 and 6 was ~95% that of position 3 indicating that transaldolase-mediated exchange of glucogenic G6P was negligible. For the seabass, while there was no significant difference between enrichment of positions 4, 5 and 6 to that of position 3, the ratio of position 4, 5 and 6 mean enrichment to that of position 3 was systematically less than 1.0, consistent with a minor amount of transaldolase exchange activity (see Table 3).

In seabass, hepatic glycogenesis via the direct pathway played a significant role in sequestering the glucose load, accounting for about one-eighth of the load at the time of sampling. In contrast, the direct pathway accounted for an insignificant fraction of the glucose load for the rat (see Table 1). This indicates that essentially all of the glucose load must have been disposed into peripheral tissues such as the skeletal muscle and adipocytes.

4. Discussion

The disposal of a deuterated glucose load into hepatic glycogen was examined in fasted rats and seabass. The initial fasting conditions (24 h for rats and 21 days for seabass) were chosen as to deplete hepatic glycogen to residual levels so that the opportunity for net hepatic glycogen deposition from the glucose load was maximized for both species. The relatively slow rates of glucose turnover and hepatic glycogenolysis in seabass compared to rat necessitates a longer fasting period to achieve similar degrees of hepatic glycogen depletion (i.e. ≤10% of postprandial levels). Likewise, the longer post-load interval chosen for hepatic glycogen sampling in seabass (48 h) compared to the rat (2 h) reflects the inherently slower disposal and metabolism of a glucose load in seabass compared to rat. Given that seabass intermediary metabolic fluxes are fundamentally governed by ambient water temperature, it is almost certain that glucose disposal rates will be accelerated at higher temperatures. Our measurements were performed at 18 °C, representing the median of the temperature range for cultivating seabass, but they have maximum feed utilization and growth rates at 24 °C and 26 °C, respectively (Person-Le Ruyet et al., 2004).

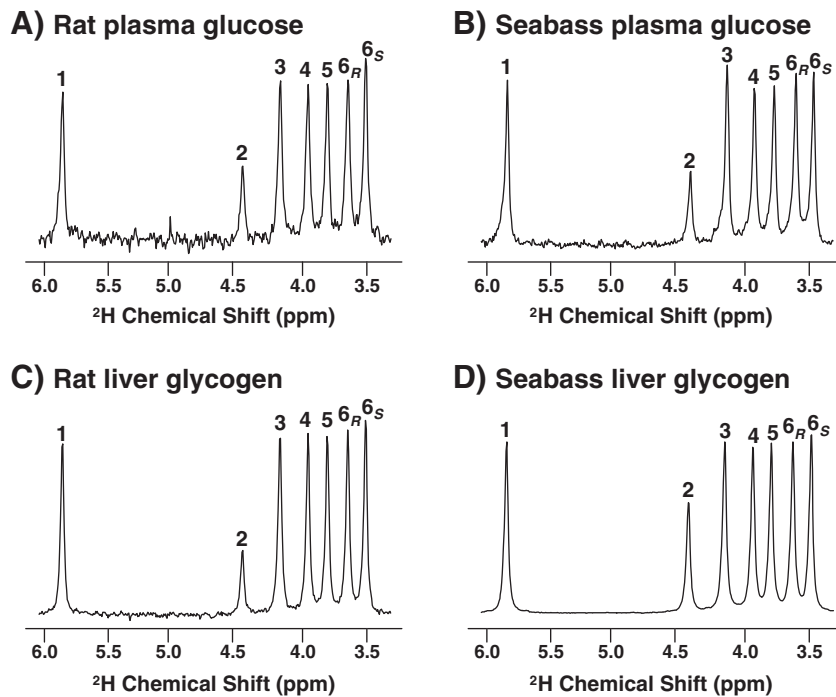


Fig. 2. ^2H NMR spectra of monoacetone glucose samples derived from blood glucose of rat (A) and seabass (B) and liver glycogen of rat (C) and seabass (D) following administration of a glucose load enriched with $[\text{U-}^2\text{H}_7]\text{glucose}$. The number above each signal indicates its glucosyl positional origin.

Our study provided insights on hepatic disposal of glucose into glycogen following a glucose load, as well as exchanges between the hydrogens of glucose and body water during this process. Our studies indicate that for rats, hepatic glycogen synthesis via the direct pathway does not have a significant role in disposal of a glucose load in the setting of a glucose tolerance test. We have previously shown that in rats subjected to the same protocol, direct pathway synthesis of hepatic glycogen accounted for only ~1% of the glucose load while gluconeogenesis from endogenous substrates accounted for the large majority of glycogen that was synthesized (Soares et al., 2010). This suggests that for rats, the restoration of euglycemia following a glucose load is almost entirely mediated by clearance into peripheral tissues such as the skeletal muscle and adipocytes. For seabass, hyperglycemia persisted at 48 h post-load while a significantly higher fraction of hepatic glycogen was synthesized via the direct pathway. This suggests that hepatic glycogenesis may have a more prominent role for glucose disposal in fish compared to rats. It also demonstrates that the seabass has a significant capacity for upregulation of direct pathway fluxes in response to a glucose load. In comparison, when fasted seabass are refed with standard fishmeal, the direct pathway contribution is insignificant (Viegas et al., 2012). Despite this, the clearance of the glucose load is much slower in comparison to the rat suggesting that glucose uptake by peripheral tissues has a much more limited role for clearing a glucose load in seabass compared to the rat. The fraction of the glucose load disposed into urine is likely to be negligible for non-diabetic rats, but it is possible that in the seabass, a portion of the load was cleared in this manner. In rainbow trout, the plasma glucose threshold for glucosuria is about 22 mM or 400 mg/dL (Bucking and Wood, 2005). Above these levels about 0.5–2% of renal glucose flux is lost to the urine. Assuming that seabass have a similar threshold and given that the endpoint glucose was around 340 mg/dL, it is likely that earlier glucose values would have exceeded the 400 mg/dL threshold. Under these conditions, it is possible that a portion of the glucose load was cleared via urine.

The analysis of hepatic glycogen synthesis with labeled water is a versatile approach that provides information about the fractional

replacement of glycogen during feeding as well as informing the substrates that contribute to glycogen synthesis (Postle and Bloxham, 1980; Jones et al., 2006b; Soares et al., 2012; Viegas et al., 2012; Delgado et al., 2013). The analysis relies on certain assumptions concerning exchange of hydrogens between sugar phosphates and body water. These include the complete exchange of G6P hydrogen 2 and body water via G6P-isomerase so that G6P that is synthesized from unlabeled glucose becomes enriched in position 2 to the same extent as body water while G6P derived via the indirect pathway is also quantitatively enriched in this position. Thus, the fraction of hepatic glycogen derived from all G6P sources is equivalent to the ratio of position 2 enrichment to body water (Jones et al., 2006b; Soares et al., 2009; Barosa et al., 2012). In humans, exchange of G6P hydrogen 2 and body water is >90% complete (Jones et al., 2006b; Delgado et al., 2009; Barosa et al., 2012). In comparison, the data of this report indicate that this exchange is considerably less extensive in both rat and seabass. There are two mechanisms that can contribute to incomplete exchange of G6P hydrogen 2 and body water. First, if G6P derived from glucose is recruited for glycogenesis before it has the opportunity to be converted to F6P and back by G6P-isomerase, then it will not become enriched in position 2. The second mechanism is isotopic discrimination against ^2H incorporation by G6P-isomerase during conversion of F6P to G6P. This latter process becomes significant when the rate of F6P and G6P exchange is relatively slow compared to G6P uptake by glycogenesis and results in a reduced enrichment of position 2 relative to body water regardless of the G6P source (i.e. either direct or indirect pathways). Conversely, when G6P–F6P exchange is fast compared to glycolytic G6P utilization, this isotopic discrimination is abolished.

For rats, there is evidence suggesting that isotopic discrimination of G6P position 2 enrichment is not significant. Under extended fasting conditions where essentially all G6P is derived via gluconeogenesis, the enrichment of G6P position 2 approaches that of body water and is also equivalent to position 5, a site that is also enriched during gluconeogenesis but is not subject to isotopic discrimination. However, under postprandial conditions there is evidence for limited G6P–F6P exchange during the conversion of glucose to glycogen via

the direct pathway due to the channeling of glycogenic G6P into the glycogen synthesis pathway (Agius et al., 1996, 2002). If this is the mechanism that accounts for the retention of position 2 enrichment following direct pathway conversion of [U-²H₇]glucose to glycogen, then our data suggest that ~40% of the G6P intermediate did not undergo G6P–F6P exchange (Table 3). Therefore, in the ²H₂O experiment, glycogen units derived via the direct pathway would be enriched in position 2 to only ~60% of body water while the remaining 40% would be unlabeled. Thus for rats, the direct pathway contribution measured from the enrichment of glycogen from ²H₂O (Soares et al., 2009) is an underestimate. Based on the present data, these estimates need to be corrected with a factor of 1.64 (i.e. 1 + 39/61). Meanwhile, enrichment of glycogen position 5 from ²H₂O via the indirect pathway is not limited by exchange or isotope effects hence the indirect pathway contribution as read by the enrichment of position 5 relative to body water does not need any correction.

For seabass, the retention of position 2 enrichment following metabolism of [U-²H₇]glucose to glycogen was even greater than that of the rat, with less than one-half of the position 2 deuterium removed via F6P–G6P exchange. On this basis, the calculated direct pathway contribution based on the enrichment of glycogen from ²H₂O needs to be corrected by a factor of 2.11 (1 + 52.7/47.3). For the seabass, the limited ²H–¹H exchange at position 2 of G6P is at least partially contributed by a kinetic isotope effect mediated by G6P-isomerase, as described in plants (Schleucher et al., 1999). Under conditions of low dietary carbohydrate and residual direct pathway activity, hepatic glycogen synthesis is almost entirely mediated by the indirect pathway (Viegas et al., 2012) and enrichment of glycogen positions 2 and 5 from ²H₂O via this pathway is theoretically equivalent. We observed a significantly lower enrichment of glycogen position 2 compared to position 5 that is consistent with the isotopic discrimination of ²H-incorporation into position 2 of G6P via G6P-isomerase (Viegas et al., 2012).

Hydrogens 4, 5 and 6 of G6P can also exchange by a combination of G6P-isomerase and transaldolase activities (Bock et al., 2008; Basu et al., 2009, 2010). In overnight-fasted humans, this exchange accounts for ~20% of hepatic G6P following a glucose tolerance test (Delgado et al., 2009) or a mixed meal (Barosa et al., 2012). Transaldolase exchange results in the incorporation of ²H₂O or other gluconeogenic tracers into G6P and glycogen without any net indirect pathway flux and therefore results in an overestimation of the indirect pathway contribution by these methods. Accordingly, if direct and indirect pathway fluxes are measured with a glucose tracer labeled in positions 4, 5 or 6, such as [5-³H]glucose, (Stingl et al., 2006) transaldolase activity will remove this label at the level of G6P. This results in an apparently higher dilution of the label by indirect pathway flux and an underestimation of the direct pathway contribution (Stingl et al., 2006). Our data indicate that transaldolase-mediated exchange of hepatic G6P is insignificant in rat and is of minor importance in seabass, at least under the conditions of a glucose load. Therefore, estimations of indirect pathway contributions from the enrichment of glycogen position 5 from ²H₂O do not need to be corrected for this process.

Finally, our study has highlighted some key differences between a carnivorous fish and an omnivorous rodent in the role of hepatic glycogenesis during clearance of a glucose load. Paradoxically, for a species that is considered to be glucose intolerant, the seabass liver exhibited “textbook behavior” in that it sequestered a significant fraction of the load as glycogen via the direct pathway. In contrast, this mechanism played a very minor role in glucose disposal by the rat, in agreement with previous studies (Soares et al., 2010). Instead, the vast majority of the load was likely disposed into insulin-sensitive peripheral tissues including the skeletal muscle and adipocytes, and this process was able to counteract hyperglycemia within a relatively short interval (~1–2 h). In contrast, the seabass remained hyperglycemic 48 h after the load despite the higher amount of load sequestered into hepatic

glycogen. This indicates that glucose uptake by peripheral tissues (principally muscle) plays a much less prominent role in restoring euglycemia following a glucose load in seabass compared to rat.

Acknowledgments

The authors acknowledge the valuable assistance of all colleagues who participated in any of the stages of this work. The authors acknowledge the financial support from Fundação para a Ciência e a Tecnologia (FCT) in the form of a Ph.D. Fellowship to F.O.M.: SFRH/BD/51194/2010, and Research Grants to J.G.J.: PTDC/EBB-BIO/098111/2008 & PTDC/SAU-MET/111398/2009. The NMR spectrometer is part of the National NMR Network and was purchased in the framework of the National Programme for Scientific re-equipment, contract REDE/1517/RMN/2005, with funds from POCI 2010 (FEDER) and FCT.

References

- Agius, L., Peak, M., Newgard, C.B., Gomez-Foix, A.M., Guinovart, J.J., 1996. Evidence for a role of glucose-induced translocation of glucokinase in the control of hepatic glycogen synthesis. *J. Biol. Chem.* 271, 30479–30486.
- Agius, L., Centelles, J., Cascante, M., 2002. Multiple glucose 6-phosphate pools or channelling of flux in diverse pathways? *Biochem. Soc. Trans.* 30, 38–43.
- Barosa, C., Silva, C., Fagulha, A., Barros, L., Caldeira, M.M., Carvalho, M., Jones, J.G., 2012. Sources of hepatic glycogen synthesis following a milk-containing breakfast meal in healthy subjects. *Metabolism* 61, 250–254.
- Basu, R., Chandramouli, V., Schumann, W., Basu, A., Landau, B.R., Rizza, R.A., 2009. Additional evidence that transaldolase exchange, isotope discrimination during the triose-isomerase reaction, or both occur in humans: effects of Type 2 Diabetes. *Diabetes* 58, 1539–1543.
- Basu, R., Barosa, C., Basu, A., Pattan, V., Saad, A., Jones, J., Rizza, R.A., 2010. Transaldolase exchange and its effects on measurements of gluconeogenesis in humans. *Am. J. Physiol. Endocrinol. Metab.* 300, E296–E303.
- Bischof, M.G., Krssak, M., Krebs, M., Bernroider, E., Stingl, H., Waldhäusl, W., Roden, M., 2001. Effects of short-term improvement of insulin treatment and glycemia on hepatic glycogen metabolism in Type 1 Diabetes. *Diabetes* 50, 392–398.
- Bischof, M.G., Bernroider, E., Krssak, M., Krebs, M., Stingl, H., Nowotny, P., Yu, C., Shulman, G.I., Waldhäusl, W., Roden, M., 2002. Hepatic glycogen metabolism in Type 1 Diabetes after long-term near normoglycemia. *Diabetes* 51, 49–54.
- Bock, G., Schumann, W.C., Basu, R., Burgess, S.C., Yan, Z., Chandramouli, V., Rizza, R.A., Landau, B.R., 2008. Evidence that processes other than gluconeogenesis may influence the ratio of deuterium on the fifth and third carbons of glucose: implications for the use of ²H₂O to measure gluconeogenesis in humans. *Diabetes* 57, 50–55.
- Bucking, C., Wood, C.M., 2005. Renal regulation of plasma glucose in the freshwater rainbow trout. *J. Exp. Biol.* 208, 2731–2739.
- Delgado, T.C., Silva, C., Fernandes, I., Caldeira, M., Bastos, M., Baptista, C., Carvalho, M., Geraldes, C.F.G.C., Jones, J.G., 2009. Sources of hepatic glycogen synthesis during an oral glucose tolerance test: effect of transaldolase exchange on flux estimates. *Magn. Reson. Med.* 62, 1120–1128.
- Delgado, T.C., Martins, F.O., Carvalho, F., Gonçalves, A., Scott, D.K., O’Doherty, R., Macedo, M.P., Jones, J.G., 2013. ²H enrichment distribution of hepatic glycogen from ²H₂O reveals the contribution of dietary fructose to glycogen synthesis. *Am. J. Physiol. Endocrinol. Metab.* 304, E384–E391.
- Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2011a. Dietary carbohydrate utilization by European sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.) juveniles. *Rev. Fish. Sci.* 19, 201–215.
- Enes, P., Peres, H., Sanchez-Gurmaches, J., Navarro, I., Gutiérrez, J., Oliva-Teles, A., 2011b. Insulin and IGF-I response to a glucose load in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* 315, 321–326.
- Garin, D., Rombaut, A., Fréminet, A., 1987. Determination of glucose turnover in sea bass *Dicentrarchus labrax*. Comparative aspects of glucose utilization. *Comp. Biochem. Physiol. B* 87, 981–988.
- Jones, J.G., Barosa, C., Gomes, F., Carina Mendes, A., Delgado, T.C., Diogo, L., Garcia, P., Bastos, M., Barros, L., Fagulha, A., Baptista, C., Carvalho, M., Madalena Caldeira, M., 2006a. NMR derivatives for quantification of ²H and ¹³C-enrichment of human glucuronide from metabolic tracers. *J. Carbohydr. Chem.* 25, 203–217.
- Jones, J.G., Fagulha, A., Barosa, C., Bastos, M., Barros, L., Baptista, C., Caldeira, M.M., Carvalho, M., 2006b. Noninvasive analysis of hepatic glycogen kinetics before and after breakfast with deuterated water and acetaminophen. *Diabetes* 55, 2294–2300.
- Newgard, C.B., Hirsch, L.J., Foster, D.W., McGarry, J.D., 1983. Studies on the mechanism by which exogenous glucose is converted into liver glycogen in the rat. A direct or an indirect pathway? *J. Biol. Chem.* 258, 8046–8052.
- Nunes, P.M., Jones, J.G., 2009. Quantifying endogenous glucose production and contributing source fluxes from a single ²H NMR spectrum. *Magn. Reson. Med.* 62, 802–807.
- Obeid, O.A., Bittar, S.T., Hwalla, N., Emery, P.W., 2005. Effect of diet supplementation with glutamine, dihydroxyacetone, and leucine on food intake, weight gain, and postprandial glycogen metabolism of rats. *Nutrition* 21, 224–229.

- Obeid, O.A., Boukarim, L.K., Al Awar, R.M., Hwalla, N., 2006. Postprandial glycogen and lipid synthesis in prednisolone-treated rats maintained on high-protein diets with varied carbohydrate levels. *Nutrition* 22, 288–294.
- Peres, H., Goncalves, P., Oliva-Teles, A., 1999. Glucose tolerance in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). *Aquaculture* 179, 415–423.
- Person-Le Ruyet, J., Mahe, K., Le Bayon, N., Le Delliou, H., 2004. Effects of temperature on growth and metabolism in a Mediterranean population of European sea bass, *Dicentrarchus labrax*. *Aquaculture* 237, 269–280.
- Postle, A.D., Bloxham, D.P., 1980. The use of tritiated water to measure absolute rates of hepatic glycogen synthesis. *Biochem. J.* 192, 65–73.
- Schleucher, J., Vanderveer, P., Markley, J.L., Sharkey, T.D., 1999. Intramolecular deuterium distributions reveal disequilibrium of chloroplast phosphoglucose isomerase. *Plant Cell Environ.* 22, 525–533.
- Smadja, C., Morin, J., Ferre, P., Girard, J., 1988. Metabolic fate of a gastric glucose load in unrestrained rats bearing a portal vein catheter. *Am. J. Physiol. Endocrinol. Metab.* 254, E407–E413.
- Soares, A.F., Viegas, F.J., Carvalho, R.A., Jones, J.G., 2009. Quantifying hepatic glycogen synthesis by direct and indirect pathways in rats under normal ad libitum feeding conditions. *Magn. Reson. Med.* 61, 1–5.
- Soares, A.F., Carvalho, R.A., Veiga, F.J., Jones, J.G., 2010. Effects of galactose on direct and indirect pathway estimates of hepatic glycogen synthesis. *Metab. Eng.* 12, 552–560.
- Soares, A., Carvalho, R., Veiga, F., Alves, M., Viegas, I., González, J., Metón, I., Baanante, I., Jones, J., 2012. Restoration of direct pathway glycogen synthesis flux in the STZ-diabetes rat model by insulin administration. *Am. J. Physiol. Endocrinol. Metab.* 303, E875–E885.
- Stingl, H., Chandramouli, V., Schumann, W.C., Brehm, A., Nowotny, P., Waldhäusl, W., Landau, B.R., Roden, M., 2006. Changes in hepatic glycogen cycling during a glucose load in healthy humans. *Diabetologia* 49, 360–368.
- Torres, T.P., Fujimoto, Y., Donahue, E.P., Printz, R.L., Houseknecht, K.L., Treadway, J.L., Shiota, M., 2011. Defective glycogenesis contributes toward the inability to suppress hepatic glucose production in response to hyperglycemia and hyperinsulinemia in Zucker Diabetic Fatty rats. *Diabetes* 60, 2225–2233.
- Viegas, I., Rito, J., Jarak, I., Leston, S., Carvalho, R.A., Metón, I., Pardal, M.A., Baanante, I.V., Jones, J.G., 2012. Hepatic glycogen synthesis in farmed European seabass (*Dicentrarchus labrax* L.) is dominated by indirect pathway fluxes. *Comp. Biochem. Physiol. A* 163, 22–29.