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Sources of blood glucose and liver glycogen in the seabass (*Dicentrarchus labrax* L.):

implications to carbohydrate metabolism in fish

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Universidade de Coimbra
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Sources of blood glucose and liver glycogen in the seabass (*Dicentrarchus labrax* L.): implications to carbohydrate metabolism in fish

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"*Un mot d'esprit ne prouve rien*"

Voltaire (1694-1778)

Symbols, abbreviations and expressions

6PGDH	6-phosphogluconate dehydrogenase
AA	Amino acids
AMP/ ADP/ATP	Adenosine monophosphate/diphosphate/triphosphate
ANOVA	Analysis of variance
$^{13}C/^{14}C$	Carbon-13/Carbon-14
cDNA	Complementary deoxyribonucleic acid
CHO	Carbohydrates
d	Day
DHAP	Dihydroxyacetonephosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e.g.	*Exempli gratia,* Latin expression meaning “for the sake of example”
EGP	Endogenous glucose production
et al.	*Et alii,* Latin expression meaning “and others”
EU	European Union
$F16P_2$	Fructose 1,6-bispohsphate
F6P	Fructose 6-phosphate
FAO	Food and Agriculture Organization of the United Nations
FBPase	Fructose 1,6-bisphosphatase
FBW	Final body weight
G1P	Glucose 1-phosphate
G3P	Glyceraldehyde 3-phosphate
Gly3P	Glycerol 3-phosphate
G6P	Glucose 6-phosphate
G6Pase	Glucose 6-phosphatase
G6PDH	Glucose 6-phosphate dehydrogenase
GNG	Gluconeogenesis
GK	Glucokinase
GPase	Glycogen phosphorylase
GSase	Glycogen synthase
h	Hour

^{1}H / ^{2}H / ^{3}H	Proton / Deuterium / Tritium
$^{2}H_2O$	Deuterated water
H2/H5	Hidrogen in position 2/position 5 of glucose molecule
i.e.	*Id est*, Latin expression meaning "that is"
In situ	Latin expression meaning "in site" referring to the original location
In vitro	Latin expression meaning "within glass" - occurring outside a living organism
In vivo	Latin expression meaning "within the living" - occurring in a living organism
LC-MS/MS	Liquid Chromatography-Mass Spectrometry
min	Minute
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP/ NADPH	Nicotinamide adenine dinucleotide phosphate (oxydized form/reduced form)
NMR	Nuclear magnetic resonance
OA	Oxaloacetate
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
per capita	Latin expression meaning "for each head" - per individual or per person
PFK-1	6-phosphofructo 1-kinase
PK	Pyruvate kinase
PW	Plasma water
ppm/PPM	Parts per million
RT	Reverse transcriptase
SEM	Standard error of the mean
SD	Standard deviation
TCA	Tricarboxylic acid
TG	Triglyceride
Tris	Tris(hydroxymethyl)aminomethane
USD	Dollars (United States currency)
UN	United Nations
vs.	*Versus*, latin expression meaning "against"

List of publications

Part of the scientific work presented in this thesis resulted in the publication/submission in peer-reviewed international scientific journals of the following manuscripts:

Viegas I, Mendes VM, Leston S, Jarak I, Carvalho RA, Pardal MÂ, Manadas B, Jones JG (2011) Analysis of glucose metabolism in farmed European seabass (*Dicentrarchus labrax* L.) using deuterated water. Comp Biochem Physiol A 160, 341-347

Viegas I, Rito J, Jarak I, Leston S, Carvalho RA, Metón I, Pardal MA, Baanante IV, Jones JG (2012) Hepatic glycogen synthesis in farmed European seabass (*Dicentrarchus labrax* L.) is dominated by indirect pathway fluxes. Comp Biochem Physiol A 163, 22-29

Viegas I, Rito J, González JD, Jarak I, Carvalho RA, Metón I, Pardal MA, Baanante IV, Jones JG (2012) Effects of food-deprivation and refeeding on the regulation and sources of plasma glucose appearance in European seabass (*Dicentrarchus labrax* L.). Submitted to Marine Biotechnology

During the course of this PhD thesis, active participation in the development of colleagues' work resulted in the publication/submission in peer-reviewed international scientific journals of the following manuscripts:

González JD, Caballero A, Viegas I, Metón I, Jones JG, Barra J, Fernández F, Baanante IV (2012) Effects of alanine aminotransferase inhibition on the intermediary metabolism in *Sparus aurata* through dietary amino-oxyacetate supplementation. Brit J Nutr 107, 1747-1756

Soares A, Carvalho RA, Veiga F, Alves M, Viegas I, González J, Metón I, Baanante IV, Jones JG (2012) Restoration of Direct Pathway Glycogen Synthesis Flux in the STZ-diabetes Rat Model by Insulin Administration. Submitted to Am J Physiol Endocrinol Metab

Mendes VM, Viegas I, Jarak I, Jones JG, Manadas B (2012) Quantification of plasma glucose ^{2}H-enrichment from deuterated water by tandem mass spectrometry. In preparation.

Abstract

The European seabass (*Dicentrarchus labrax* L.) is an euryhalin and eurythermic species occurring in marine, estuarine and coastal lagoon habitats along the European Atlantic coast and in the Mediterranean Sea. Is one of the most economically important fish species farmed in temperate areas however, carnivorous fish like the seabass present limited capacity for digesting carbohydrate (CHO) since they are adapted to a diet high in protein and low in carbohydrate. The liver is an organ that can produce, utilize and store CHO, and is a key component of glucose homeostasis in higher organisms. In recent years, the study of fish hepatic carbohydrate metabolism has been applied to improve nutrient utilization in aquaculture and to serve as model for diabetes. The develpment of tracer methods for the study of hepatic fish metabolism has been an important component of these studies.

Deuterated water (^2H_2O) is a relatively inexpensive non-radioactive tracer that can be incorporated in drinking water, or in the case of fish studies, in the tank water. It has been successfully used in humans and other mammals for the study of hepatic intermediary metabolism in both normal and pathological conditions. It rapidly equilibrates with total body water and is distributed evenly into all tissues. It is a practical tracer for both short and long-term metabolic studies. ^2H_2O is ideally suited for studying fish metabolism since it can be added to the tank water for an indefinite period, during which time it is incorporated into hepatic metabolites such as glycogen and glucose by specific enzymatic reactions in their biosynthetic pathways, as previously described for mammals. Applying these principles to free-swimming fish provides an authentic metabolic profile that is unadulterated by anesthesia or infusion procedures that characterize the adminstration of classical carbon tracers. With the principal purpose of developing and applying the ^2H_2O method for characterizing fish hepatic CHO metabolism, this thesis was pursued and the results were presented as six chapters.

In **Chapter I**, a general introduction was presented, providing a general scope for this work and introducing the basic concepts and methods to be used. In order to understand the

purpose of studying fish metabolism and using the European seabass as model of study, this work was framed in the context of aquaculture. Due to consistent differences in CHO metabolism in comparison to mammals, a review of hepatic CHO metabolism in fish was also presented, namely the main pathways in which glucose is involved (glycogen synthesis and hydrolysis, glycolysis and gluconeogenesis) and its enzymatic and hormonal regulation. This review also included the state of the art of knowledge and methodologies for the study of carbohydrate metabolism in seabass.

In **Chapter II**, blood glucose metabolism in free-swimming fasted and fed seabass was studied using $^{2}H_2O$ with the purpose of validating the methodology for detection and quantification of glucose ^{2}H-enrichment from the tracer. Metabolite enrichment from $^{2}H_2O$ is a function of two processes; an intitial rapid equilibration of fish body water with the external ^{2}H-enriched tank water resulting in the ^{2}H-enrichment of body water equivalent to that of tank water and enrichment of glucose from the ^{2}H-enriched body water via metabolic activity. This latter process is limited by the rate of glucose turnover and is typically slow compared to the initial enrichment of body water. Preliminary studies were therefore aimed at following the evolution of plasma water ^{2}H-enrichment following transfer of fish to ^{2}H-enriched tank water in order to establish the minimum interval for plasma water (the effective precursor of endogenous ^{2}H-enriched glucose) to reach isotopic steady-state. Once this interval was characterized, fish were transferred to seawater enriched with 5% $^{2}H_2O$ for 6h and 72h where the effects of glucose turnover on the ^{2}H-enrichment patterns could be evaluated. Positional and mole percent enrichment of plasma glucose and water were quantified by nuclear magnetic resonance (^{2}H-NMR) and liquid chromatography-mass spectrometry (LC-MS/MS).

In **Chapter III**, hepatic glycogen synthesis fluxes from direct and indirect pathways were quantified in seabass by ^{2}H-NMR analysis of plasma water and glycogen glucosyl ^{2}H-enrichments from ^{2}H-enriched seawater. Eighteen fish (28.0±1.7 cm total length and 218.0±43.0 g total weight) were divided into three groups of six and studied over 24d with transfer to 5% ^{2}H-seawater after day 21. Over this period, one group was fed daily with fishmeal, a second group

was fasted, and a third group was fasted for 21d followed by 3d refeeding. Glycogen turnover and sources were determined from the ratio of glucosyl position 5 enrichment to that of plasma water.

In **Chapter IV** the sources of plasma glucose in seabass were quantified in the same fish used in Chapter III under the same three different nutritional states: continuously fed, 21d fasted and 21d fasted followed by 3d of refeeding. For all conditions, glucose derived from glucose 6-phosphate and contributions from unlabeled glucose were evaluated, while ^{2}H-enrichment of glucose position 5 in relation to that of position 2 indicated plasma glucose appearance from endogenous sources. To assess the role of hepatic enzymes in glycemic control, activities and mRNA levels of hepatic glucokinase (GK) and glucose 6-phosphatase (G6Pase) were assessed.

Chapter V addressed the effects of food deprivation and refeeding in other enzymes directly or indirectly involved in the hepatic glucose metabolism in seabass. Besides GK and G6Pase, already presented in Chapter IV of this thesis, activities and mRNA levels (after sequencing and development of specific primers) were quantified several other enzymes. Amongst them, 6-phosphofructo 1-kinase (PFK-1; EC 2.7.1.11) and pyruvate kinase (PK; EC 2.7.1.40) from the glycolytic pathway and fructose 1,6-biphosphatase (FBPase; EC 3.1.3.11) a gluconeogenic enzyme, were assayed for activity only. The transaminases, alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) were evaluated for both cytosolic and mitochondrial isoforms. The pentose phosphate pathway is an alternative route to glycolysis so glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.43) were assayed for activity and expression.

Finally, in **Chapter VI** the major findings of this thesis were summarized and general conclusions were drawn. The manner in which the initial objectives were fulfilled, the main limitions that were encountered, and future perspective for lines of research emerging from this thesis were also addressed.

Resumo

O robalo (*Dicentrarchus labrax* L.) é uma espécie eurihalina e euritérmica presente em ambientes marinhos, estuarinos e lagoas costeiras ao largo da costa Atlântica Europeia e Mar Mediterrâneo. É uma das espécies de peixe economicamente mais importantes ao nível da aquacultura em zonas temperadas, no entanto, espécies carnívoras como o robalo apresentam uma capacidade limitada de digestão de hidratos de carbono (HC) dependendo de um metabolismo adaptado para lidar com altos níveis de proteína na dieta. O fígado é um órgão que utiliza, produz e armazena glucose e é considerado um glucostato em organismos vertebrados. Nos últimos anos, quer no contexto da aquacultura como para mimetizar modelos de mamíferos insulino-dependentes, o estudo do metabolismo hepático nos peixes tem sofrido avanços consideráveis, nomeadamente a nível dos estudos com traçadores.

Água deuterada (^2H_2O) é um traçador relativamente barato, de manejável toxicidade e de administração fácil. Distribui-se facilmente pelos tecidos e equilibra-se com a água corporal tornando-se um práctico traçador para estudos de curta e longa duração. A ^2H_2O pareceu perfeitamente ajustada para estudos metabólicos em peixes já que poderia ser adicionada à água dos tanques por tempo indefinido e incorporada no glicogénio hepático e glucose sanguínea, como descrito para mamíferos. A aplicação destes princípios a peixes poderia portanto fornecer um verdadeiro perfil metabólico. Foi com o objectivo de desenvolver esta premissa inicial que esta tese foi realizada e que é apresentada neste documento dividido em seis capítulos.

No **Capítulo I**, é apresentada uma introdução geral, providenciando um âmbito geral para este trabalho e os conceitos básicos e metodologias a usar. De modo a perceber o propósito de estudar metabolismo em peixes e de usar o robalo como modelo de estudo, este trabalho foi enquadrado no contexto da aquacultura. Devido às constantes diferenças em relação ao observado em mamíferos, o metabolismo hepático de HC em peixes foi sujeito a uma breve revisão, nomeadamente no que concerne às principais vias metabólicas onde a glucose é

interveniente (síntese e hidrólise de glicogénio, glicólise e gluconeogénese) e respectiva regulação enzimática e hormonal. O estado da arte nesta temática para o robalo foi também apresentado.

No **Capítulo II**, o metabolismo da glucose sanguínea em robalos alimentados e em jejum foi estudado com recurso à $^{2}H_2O$, com o objectivo de validar a metodologia e os métodos de detecção do traçador. Estudos preliminares foram dedicados a seguir a evolução do enriquecimento em deutério (^{2}H) da água corporal em diferentes tempos de residência de modo a verificar quando o enriquecimento chegava a estado estacionário isotópico. Uma vez estabelecidos tempos de residência de referência os peixes foram transferidos para tanques de água salgada enriquecida a 5% de $^{2}H_2O$ por 6h e 72h. O enriquecimento posicional foi quantificado por ressonância magnética nuclear (^{2}H-RMN) e cromatografia líquida acoplada à espectrometria de massas (LC-MS/MS).

No **Capitulo III**, os fluxos de síntese de glicogénio hepático por via directa ou indirecta foram quantificados em robalos por análise de ^{2}H-RMN. Dezoito peixes (28.0±1.7 cm comprimento total e 218.0±43.0 g peso total) foram divididos em três grupos de 6 e estudados por um período de 24 dias com transferência para tanques de água salgada com 5% de $^{2}H_2O$ após o 21º dia. Durante este período um grupo foi alimentado diariamente com dieta comercial, outro grupo foi jejuado por 21 dias e o terceiro grupo foi igualmente jejuado por 21 dias mas depois realimentado por 3 dias. O *turnover* e do glicogénio e a sua proveniência foram determinados pelo rácio do enriquecimento da posição 5 da unidade glicosil em relação ao enriquecimento da água corporal.

No **Capítulo IV**, a proveniência da glucose sanguínea foi quantificada nos mesmos robalos descritos no capítulo III sob as mesmas condições experimentais: alimentados, jejuados por 21 dias ou jejuados por 21 dias mas posteriormente realimentados por 3 dias. Para todas as condições a glucose derivada da glucose 6-fosfato e as contribuições para glucose não enriquecida foram quantificadas, enquanto que o enriquecimento em ^{2}H da posição 5 em relação à posição 2 indicaram a proveniência endógena da glucose. De modo a avaliar o papel de

enzimas hepáticas no controlo da glicemia quantificaram-se as actividades e níveis de RNA mensageiro (mRNA) da glucoquinase (GK, EC 2.7.1.2) e da glucose 6-fosfatase (G6Pase, EC 3.1.3.9).

O **Capítulo V**, foi dedicado a analisar os efeitos do jejum e da realimentação em outras enzimas envolvidas directa ou indirectamente no metabolismo hepático de glucose em robalo. Para além da GK e da G6Pase já apresentadas no capítulo IV desta tese, quantificaram-se as actividades e níveis de mRNA (após sequenciação e desenho de oligonucleótidos específicos) para outras enzimas. Entre elas , 6-fosfofruto 1-quinase (PFK-1; EC 2.7.1.11) e a piruvato quinase (PK; EC 2.7.1.40) da via glucolítica e a frutose 1,6-bifosfatase (FBPase; EC 3.1.3.11) da via gluconeogénica, foram analisadas apenas a nível da actividade. As transaminases, alanina aminotransferase (ALT; EC 2.6.1.2) e aspartato aminotransferase (AST; EC 2.6.1.1) foram avaliadas para as suas isoformas citosólica e mitocondrial. A via das pentoses-fostato é uma via alternativa à glicólise, logo a glucose 6-fosfato desidrogenase (G6PDH; EC 1.1.1.49), e a 6-fosfogluconato desidrogenase (6PGDH; EC 1.1.1.43) foram avaliadas em termos de actividade e níveis de mRNA.

Finalmente, no **Capítulo VI**, foram resumidos os resultados mais relevantes e tiradas as principais conclusões desta tese. É também realçado de que forma os objectivos iniciais foram atingidos bem como as principais limitações e perspectivas futuras da linha de investigação que emergiu desta tese.

CHAPTER I

General introduction

The state of Aquaculture

Overview

According to the 2010 revision of the official United Nations population estimates, the world population was projected to reach 7 billion in late 2011 (UN 2011) and at least one billion rely on fish as their main source of animal proteins (FAO 1997). The food supply (in kg *per capita*) has almost doubled since the 1960's and increased demand has been accompanied by a steady growth in production of fish species (finfish, shellfish and cuttlefish) worldwide (Fig. 1).

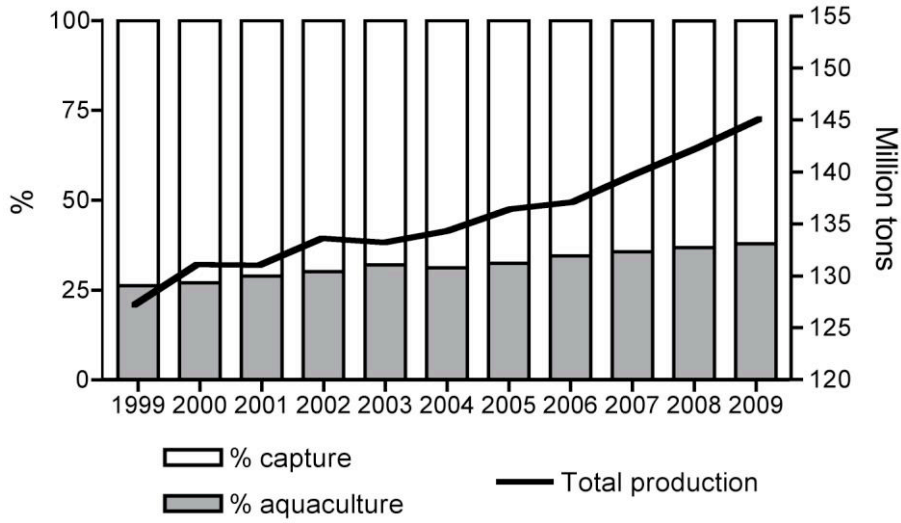

Fig. 1 World fish total production and percentage of origin (capture and aquaculture) from 1999 to 2009. Data from the reports on "The state of world fisheries and aquaculture" published by FAO (2002; 2004; 2007; 2009; 2010).

Production from capture has leveled at around 90 million tons since 2001 and in the end of the 1990's represented almost 75% of total production. Aquaculture production has been gaining more importance and increasing at an average annual growth rate of 6.2% from 38.9 million tons in 2003 to 52.5 million tons in 2008 (FAO 2010). With market value estimated at 98.4 billion USD, aquaculture was in 2009, responsible for producing 38% of the 145 million tones of

total fish products, 81% of which were for human consumption (FAO 2011). It is estimated that by 2030 half of the production for human consumption can be derived from aquaculture while wild fish capture by European vessels will face zero growth in production up until then (Failler 2007).

According to the Food and Agriculture Organization (FAO 1997a), aquaculture is the farming of aquatic organisms: fish, mollusks, crustaceans, and aquatic plants. Aquaculture is markedly different from capture for two main reasons: i) some form of intervention in the rearing process to enhance production (regular stocking, feeding, protection from predators, etc.) and ii) individual or corporate ownership of the stock being cultivated. Farming of aquatic species has existed for many thousands of years: in ancient Asia, carp were left to grow in ponds and rice paddies and later harvested. Similar practices were thought to take place in ancient Egypt with tilapia and in southern Europe in a polyculture regime, including mullet *Mugil* spp, sole *Solea* spp, seabass *Dicentrarchus labrax* and gilthead seabream *Sparus aurata* (Pillay & Cutty 2005). These artisanal methods, with little or no active manipulation of the animals, are still practiced in some parts of the globe. However, as it has evolved into a highly competitive and commercialized business, the technique of aquaculture has also significantly evolved, both in targeted species and farming methodologies. Growth in aquaculture production is in part due to the stagnation of fishing efforts worldwide directed to reduce fishing pressure in exploited, and many times over-exploited, natural stocks. However, aquaculture does not hold the solution for the sustainability of ocean fisheries because some types of farming interfere greatly with ocean and coastal resources. Habitat destruction, waste disposal, exotic species and pathogen invasions are some of the consequences of irresponsible farming practices (Naylor et al. 2000). Besides, aquaculture is highly dependent on capture fisheries to provide fishmeal and fish oil required to produce feeds (Kristofersson & Anderson 2006; Tacon & Metian 2008). Thus, the development of well-suited and cost-effective substitute feeds has become a matter of extreme importance to the sustainability and profitability of the sector. Along with it, came the need to improve our understanding of how fish metabolize different diets.

In Portugal

Consumption of seafood in Portugal is amongst the highest worldwide and by far the highest within the EU with 60 kg/year/*per capita* consumed compared to the EU average of 22 kg/year/*per capita* (Failler 2007). Portugal is the world's 18th biggest buyer of fish products, importing in 2009 more than 1.5 billion USD of fish products (FAO 2011).

Artisanal farming practices have been reported in salt farms in Portugal for many decades where juvenile fishes of various species were trapped in the reserve tanks and left to grow until the following drying season. Nevertheless Portuguese aquaculture has come a long way even without a strategic solution to the increasing demand. In 2009, from the 206,000 tons of total fish products, 97% still came from capture, while aquaculture was responsible for only the remaining 3%. Freshwater cultures are generally of lesser importance than marine cultures and amongst the latter, mollusk bottom cultures are the most representative (Fig. 2).

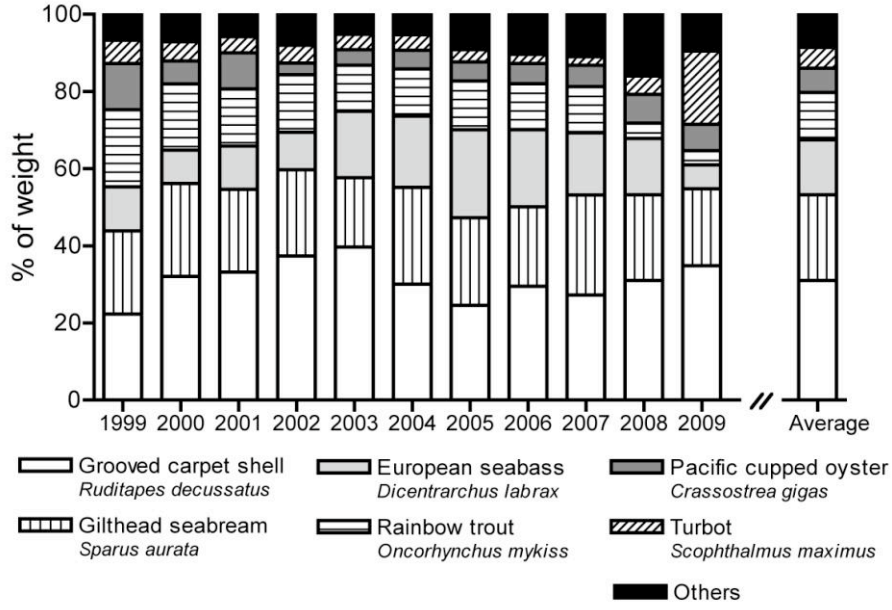

Fig. 2 Portuguese aquaculture production in percentage of weight per species from 1999 to 2009 and average. Data from FAO's database (FAO 2012).

Data from the last years (average from 1999 to 2009) confirms the trend: cultures of grooved carpet shell *Ruditapes decussatus* represents an average ~30% of total production (42% of revenue), gilthead seabream *Sparus aurata* 22%, seabass *Dicentrarchus labrax* 14% and rainbow trout *Oncorhynchus mykiss* 12% (FAO 2012a). It is interesting to note that between 2006 and 2009, important shifts were observed in Portuguese aquaculture with turbot *Scophthalmus maximus* production increasing 3-fold due to the recent activation of a high-production plant. Meanwhile, production of seabream and seabass was reduced by half as consequence of the closing of four growing farms and one nursery (INE 2011). This may change the Portuguese aquaculture *status quo* for the years to come.

European seabass *Dicentrarchus labrax* L.

The European seabass (*Dicentrarchus labrax* L.) is an euryhalin and eurythermic species occurring in marine, estuarine and coastal lagoon habitats along the European Atlantic coasts and in the Mediterranean Sea being one of the most economically important fish species farmed in temperate areas (Pickett & Pawson 1994).

This species is well adapted to farming due to a combination of different biological and physiological features. Interactions between salinity, temperature and feeding rate have been established (Eroldogan et al. 2004) so its capability to grow within a wide range of temperatures and salinities is of particular importance in coastal lagoons and estuarine areas (Conides et al. 2006), precisely where most semi-intensive farms are found in Portugal. Good food conversion ratios (Peres & Oliva-Teles 2005) and increasing knowledge of its nutritional requirements (Oliva-Teles 2000) also favoured this species as a target for aquaculture. In addition, due to its organoleptic characteristics (flavor, aroma, consistency, taste), its fillet is of high demand by the consumers thus having good market value. The main handicap is the lack of dependable source of fry and fingerlings and the reliance on wild fry is normally highly inconsistent and inadequate (Pillay & Cutty 2005). Aquaculture of this species has been growing steadily in terms of weight

and market value since the 1990's and since 2007 surpasses the annual production of 100,000 tons representing nowadays a market of almost 700,000 USD (FAO 2012a) (Fig. 3).

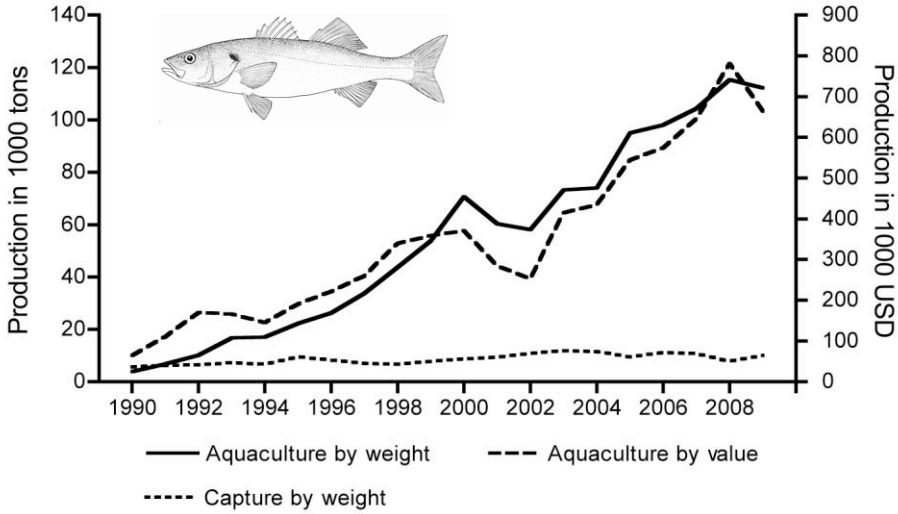

Fig. 3 World production of European seabass by weight and value from 1999 to 2009. Data from FAO's database (FAO 2012a; 2012b) and image of European seabass *D. labrax* from FAO's *D. labrax* fact sheet at http://www.fao.org/figis/servlet/ServerFileServlet?f=figis/species/images/Dicentrarchus/dic_lab_2291_0.gif

Worldwide production of European seabass is an industry dominated by Mediterranean countries, that profiting from favorable water temperatures, mild meteorological and sea conditions, farm in sea-cage systems. Amongst them, Greece and Turkey together are responsible for approximately 70% of total production (Fig. 4). Portugal, encounters several limitations to such large-scale farming being thus responsible for only 1.3% of total production (average 1999-2009, by weight; FAO 2012a). The rough conditions of the Atlantic Ocean do not allow intensive and open-cage farming so production is confined to inland plants, pond systems in marine/brackish protected estuaries and coastal lagoons. In Portugal, ~15% of the seabass is originated from capture (FAO 2012b), meaning that the reminding 85% originates a 7,824,000€ revenue per year (average 1999-2009, by weight; FAO 2012a).

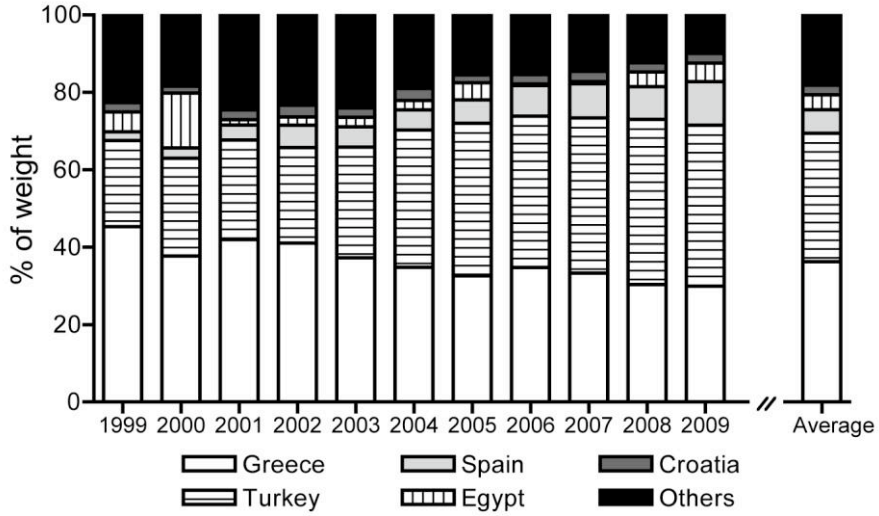

Fig. 4 World production of European seabass by country in percentage, from 1999 to 2009 and average. Data from FAO's database (FAO 2012a).

Nutritional requirements of the seabass

In its natural environment the seabass display predatory behavior with carnivorous habits and their diet includes small fish, prawns, crabs and cuttlefish (Pickett & Pawson 1994). In the aquaculture setting, its highly carnivorous nature is reflected in terms of the high dietary protein level for optimum growth (Oliva-Teles 2012). Maintenance energy requirements for the seabass have been estimated to be 40-50 kJ DE/$kg^{0.8}$ BW/day at 25°C (DE: digestible energy; BW: body weight) (Peres & Oliva-Teles 2005; Lupatsch et al. 2001) and food intake seems to be adjusted to the dietary digestible energy content (Boujard et al. 2004).

The optimum protein to energy ratio of the diets for seabass should include 45-50% protein (Oliva-Teles 2000) however best overall growth performance, combined with low nitrogenous losses could be achieved at a crude protein level of 43% (Dias et al. 1998). Until the recent years, fishmeal was the major protein source and the most expensive ingredient in the feeds for carnivorous species, however, responding to the concerns of the industry, feed

formulations have been redesigned and optimized. For carnivorous finfish, a partial or total replacement of dietary fishmeal protein by plant protein source has been shown to be achievable (Adamidou et al. 2009b), but overall growth performance is impaired (Dias et al. 2003). A combination of low levels of fishmeal, appropriate supplementation of essential amino acids and inorganic phosphorus may improve growth performance and nitrogen utilization (Kaushik et al. 2004).

The main lipid source in fish feeds comes from fish oil, due to its traditionally high availability and high content of polyunsaturated fatty acids which supply essential fatty acids for marine fish species (Izquierdo et al. 2003). Dietary lipid levels can reach up to 30% with no observable depression in growth rate, but protein and energy retention efficiencies can be significantly reduced (Peres et al. 1999). Such high dietary lipids does lead to increased fat deposition in the visceral and hepatic tissues, but on the other hand, it does not promote excessive muscle fat deposition and exerts a positive effect on nitrogen balance by increasing retention and reducing losses (Boujard et al. 2004). However, a level of 9–12% lipids has been established since no significant overall beneficial effects have been identified above this range (Oliva-Teles 2000). As for protein sources, partial replacement of fish oils by vegetable oils has been used to offset the increasing cost and decreasing availability of fish oil (Tacon & Metian 2008). A partial substitution of 60% of fish oil with vegetable oils of various sources provoked no negative effects on fish growth performance and flesh quality. Nevertheless, to guarantee the quality of the final product, special care must be taken regarding both the inclusion level and source of the dietary lipids (Parpoura & Alexis 2001; Izquierdo et al. 2003). Despite its limited availability in the world market, fish oil is an excellent source of both phosphorous and high quality lipids (polyunsaturated fatty acids). These fatty acids are essential for marine species including the seabass (Person-Le Ruyet et al. 2004).

Carnivorous fish like the seabass have a limited capability to digest and metabolize carbohydrates (CHO). Carbohydrate digestibility is affected by the complexity of the molecule, concentration in the diets, feed intake and technological treatments applied. In general, seabass

perform better with starch than with glucose (Enes et al. 2008a) and this can be related to the slower absorption and more efficient metabolic utilization of the linked glucose found in starch (Enes et al. 2011a). A decrease in starch digestibility is observed with increasing dietary inclusion levels (Enes et al. 2006b; Moreira et al. 2008). So, in order to ensure high digestibility, growth and feed utilization, diets for seabass should include around 20% digestible CHO (Enes et al. 2011a). Digestibility of native starch is high (above 70%), but starch gelatinization or extrusion improves CHO digestibility (above 90%) (Dias et al. 1998; Enes et al. 2006b; Moreira et al. 2008).

Vitamin and mineral requirement data are only available for a limited number of fish species and for a limited number of vitamins so information is still needed on the requirements for seabass (Oliva-Teles 2000). Practical diets are formulated based on recommendations for other species and regarding water-soluble vitamins differences are negligible within fish species (Oliva-Teles 2012). Nevertheless, vitamin and mineral deficiencies are easy to avoid and if necessary corrected by dietary supplementation (Halver 2003; Lall 2003).

Carbohydrate metabolism in fish

Overview

The central organization of metabolic pathways is highly conserved amongst vertebrates. While the metabolic machinery of fish is much the same as that of mammals, the main differences lie in the nutritional and endocrine control of the pathways via various feedback mechanisms. As a water-living organism, fish have specific adaptations in relation to their terrestrial vertebrate counter-parts. As consequence of living in water, their constant thermal equilibrium with the environment is a principal determinant of overall metabolic rates while excess dietary nitrogen can be cleared as ammonia, a highly toxic but rapidly diffusible molecule that is efficiently transferred from blood to the outside water via the gills (Ip & Chew 2010).

CHO are a basic nutritional source of energy and carbon, but as stated previously, carnivorous fish like the seabass have a limited capability to digest and metabolize them. CHO influence growth, feed utilization and deposition of nutrients according to species, quantity,

origin and treatment of dietary CHO used (Hemre et al. 2002; Stone 2003; Enes et al. 2011a). Fish are considered to be glucose intolerant as seen by their poor ability to clear a glucose load and their metabolism of glucose has been compared with that of mammals with insulin-dependent diabetes *mellitus* (IDDM) (Moon 2001). However, unlike IDDM, the causes of this intolerance cannot be attributed to a simple deficiency of insulin but is instead a reflection of different enzymatic and hormonal control regimes for glucose regulation in fish compared to mammals. The liver utilizes, produces and stores CHO and is an important component of systemic glycemic control in vertebrates. The rate of nutrient absorption coupled with continuous monitoring of blood glucose levels by various organs including the brain and pancreas mediates a combination of nutritional, endocrine and nerve-mediated regulation of hepatic glucose metabolism. This results in net hepatic glucose uptake when CHO is abundant, such as after feeding and net hepatic glucose output when dietary CHO is unavailable, such as during starvation. Glucose is the major energy source for the central nervous system and the only energy source for erythrocytes and the functions of both are dependent on a threshold concentration of blood glucose. Blood glucose levels are maintained through a balance of several factors, including the rate of consumption and intestinal absorption of dietary CHO, the rate of utilization of glucose by peripheral tissues and the loss of glucose through the kidney and finally the rate of removal or release of glucose by the liver (Nordlie & Foster 1999).

Figure 5 depicts the pathways by which the liver disposes glucose into the bloodstream during fasting and storage and utilization of imported glucose during feeding. The main enzymes involved in these pathways are also represented as well as possible final fates of glucose anabolism. At the physiologic level, glucose utilization rate in fish is tissue-specific (Blasco et al. 2001) but some tissues like the brain depend on a steady supply of glucose for energy metabolism. Fish brain can utilize other fuels besides glucose, including ketone bodies, lactate, fatty acids or amino acids (Soengas & Aldegunde 2002), since they are often subjected to long periods of starvation.

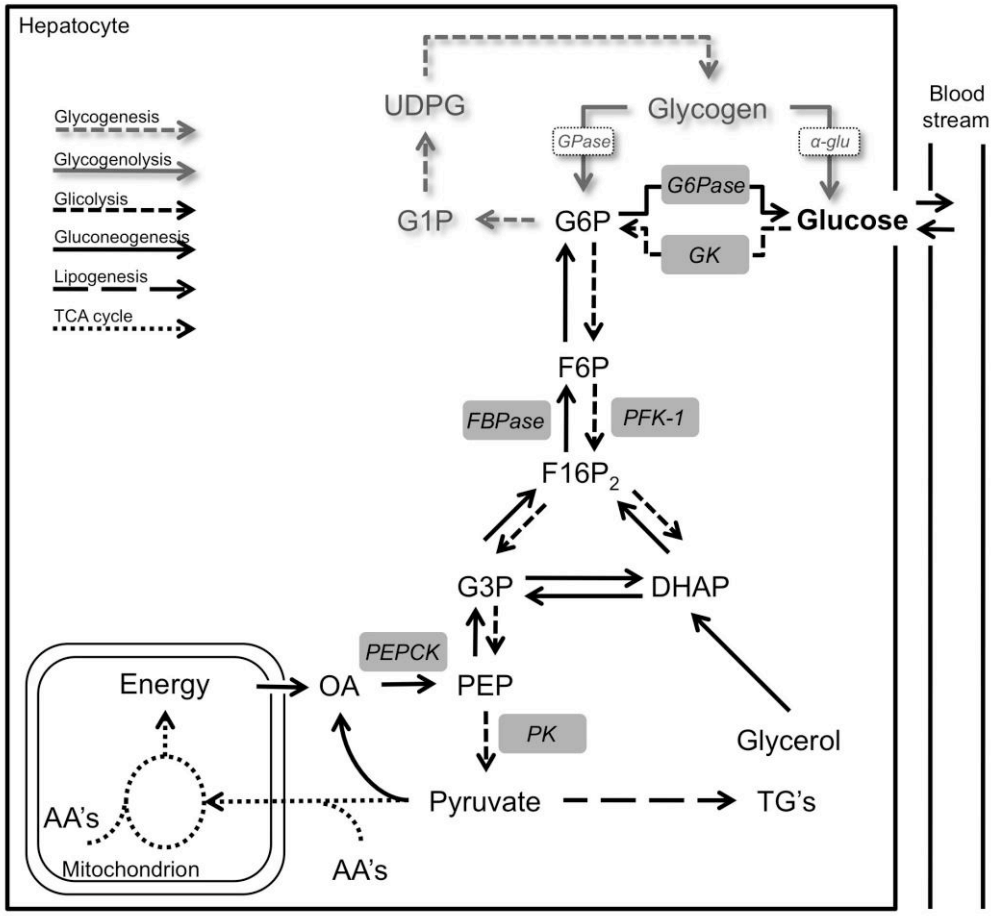

Fig. 5 Metabolic model representing main pathways in the liver involving catabolism and anabolism of glucose. Gluconeogenic precursors are represented by pyruvate (and gluconeogenic amino acids, metabolized via the anaplerotic pathways of TCA cycle) as well as glycerol from lipolysis. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P = glucose 6-phosphate; G1P = glucose 1-phosphate; UDPG = uridine diphosphoglucose; F6P = fructose 6-phosphate; $F16P_2$ = fructose 1,6-bisphosphate; G3P = glyceraldehyde 3-phosphate; DHAP = dihydroxyacetone phosphate; PEP = phosphoenolpyruvate; TG's = triglycerides; OA = oxaloacetate; AA's = amino acids; GK = glucokinase; G6Pase = glucose 6-phosphatase; FBPase = fructose biphosphatase; PFK-1 = 6-phosphofructo 1-kinase; PEPCK = phosphoenolpyruvate carboxykinase; PK = pyruvate kinase; GPase = glycogen phosphatase;α-glu = α-glucosidases; TCA cycle = tricarboxylic acid cicle.

Indeed, not only the capacity of brain to use exogenous glucose decreases during starvation (Soengas et al. 1996), as well as induced hyperglycemia does not alter glucose uptake into brain (Blasco et al. 1996). This suggests that fish brain could be metabolically independent on the blood-borne glucose supply relying on endogenous glycogen. Even so, the brain is regarded as key part of glucosensing mechanisms as described in recent years in fish models (Polakof et al. 2011). The blood-borne glucose availability has also considerable influence on the pattern of myocardial substrate oxidation in resting and active fish (West et al. 1994b) and in the whole responsiveness towards utilization of glucose (West et al. 1994a). During hypoxia, tissues such as the heart and brain upregulate anaerobic metabolic pathways and become reliant on glycolysis for ATP production (MacCormack & Driedzic 2007). However under normoxic conditions, glucose uptake by muscle is surpassed by that of amino acids for exercise and growth (Thillard 1986) even during the recovery of muscle glycogen following exhaustive exercise. Thus, the direct contribution of blood glucose to muscle energy metabolism seems to be minor (Milligan 1996; Pagnotta & Milligan 1991; West et al. 1993; Blasco et al. 1996; Blasco et al. 2001). The generally low reliance of fish tissues on glucose as an energy substrate is compatible with the limited availability of carbohydrates in fish diets (Moon 2001).

Hepatic glucose storage and disposal

In mammals, glucose enters the hepatocyte through the GLUT-2 transporter, which is primarily expressed in the liver but is also present in kidney, intestine and β-cells of pancreatic islets (Nordlie & Foster 1999). Due to its low affinity and high capacity, GLUT-2 transports hexoses in a large range of physiological concentrations necessary for glucose homeostasis, displaying bidirectional fluxes in and out the cells, namely in hepatocytes (Leturque et al. 2005). Recently, the presence of GLUT-2 transporter was confirmed at both biochemical and molecular levels in rainbow trout (Polakof et al. 2007a, 2007b) and its expression in seabass hepatocytes seems to be regulated by hypoxia (Terova et al. 2009).

Blood glucose concentration depends on a wide variety of factors and its level at any time is the net result of the difference between rates of glucose appearance and disposal. Once glucose is inside the hepatocyte, the ATP-dependent conversion of glucose to glucose 6-phosphate (G6P) occurs and is catalyzed by hexokinase IV or glucokinase (GK). GK is the major hexokinase expressed in liver and due to its relatively high K_m, it is thought to play a major role in hepatic glucose homeostasis, namely under mildly hyperglycemic conditions. During postprandial uptake of glucose by the liver, GK metabolically traps glucose as G6P for the liver cell to metabolize. It is not inhibited by G6P so as a consequence GK is able to maintain high rates of glucose phosphorylation while other hexokinases rapidly become saturated. But when the liver is producing glucose, it is essential that newly formed glucose does not become a substrate for GK, thus its low affinity. Full-length cDNA coding for GK in the liver of rainbow trout, common carp and gilthead seabream were accomplished, as well as the GK mRNA expression in different postprandial times and tissues (Caseras et al. 2000; Panserat et al. 2000a). The G6P product can be partially oxidized by the pentose phosphate pathway, be recruited for glycogen synthesis (glycogenesis) or be metabolized by the glycolytic pathway into two pyruvate molecules. Pyruvate in turn may be oxidized to acetyl-CoA or carboxylated to oxaloacetate or malate – a process known as anaplerosis. Acetyl-CoA may be fully oxidized to CO_2 by the TCA cycle or utilized for lipid production (lipogenesis) while anaplerotic products can be utilized for gluconeogenesis. G6P can also be hydrolyzed back to glucose, a reaction catalyzed by glucose 6-phosphatase (G6Pase). Isolated cDNA encoding full-length G6Pase catalytic subunit from gilthead seabream has been described, as well as partial cloning of G6Pase gene was also reported in rainbow trout, common carp and gilthead seabream livers (Panserat et al. 2000b, 2002c). Evidence seem to show that G6Pase is poorly regulated by dietary CHO in comparison to GK (Enes et al. 2009). When G6Pase and GK are both active at the same time glucose and G6P are interconverted forming a "futile cycle" that results in the consumption of ATP but no net conversion of glucose to products.

Glycogen synthesis and hydrolysis

Liver is the main storage site for glycogen, but tissues such as gills, kidney and brain also sustain relatively high rates of glycogen synthesis from glucose. Net glycogen synthesis results from the glycogen synthase (GSase) and glycogen phosphorylase (GPase) activities that are reciprocally regulated both allosterically and by phosphorylation (Ferrer et al. 2003).

GS exists in a phosphorylated inactive form in the cytosol and it is activated by binding to G6P and this causes a conformational change that makes it a better substrate for protein phosphatases, which then convert the enzyme to the active dephosphorylated isoform. In glycogenesis, G6P is first converted to glucose 1-phosphate (G1P) by phosphoglucomutase and subsequently to uridine diphosphate glucose (UDPG) by UDPG pyrophosphorylase. Glycogen synthase then adds glucose residues from the UDPG donor to the growing glycogen molecule via α-1,4 bonds. The hepatic glycogen synthesis is based both on the incorporation of blood-borne glucose into glycogen and on gluconeogenesis from lactate and amino acids. So, depending on the source of G6P that will fuel glycogenesis, two pathways need to be distinguished. In the direct pathway, glucose is converted to glycogen via GK and GS coordinated activities. In the indirect pathway glucose is first metabolized to 3-carbon intermediates that will be used to generate G6P for glycogen synthesis. This pathway also allows non-hexose precursors such as glycerol or gluconeogenic amino acids to be converted to glycogen (Nordlie & Foster 1999).

The activity of GPase, which catalyzes the breakdown of glycogen to G1P molecules, is also regulated by phosphorylation in a coordinated fashion with GS, with the dephosphorylated isoform being less active than the phosphorylated one (Ferrer et al. 2003). However, glycogenolysis besides occurring via GPase, producing G6P, can also occur via the action of α-glucosidases (α-glu) that hydrolyze the non-reducing end of polysaccharides directly to glucose.

Most fish species need to periodically cope with starvation as a result of seasonal food limitation or in some cases as natural consequence of their life cycle (Navarro & Gutiérrez 1995) and the liver plays an important role in the endogenous control of fuel storage and mobilization (Metón et al. 2003; Soengas et al. 2006; Pérez-Jiménez et al. 2007). Glycogen represents an

important source of glucose to be released in the fasted state or when responding to acute stressors. The transition from fasting to feeding states result in a comprehensive realignment of hepatic carbohydrate metabolic fluxes from minimal maintenance of glycemia and peripheral glucose demands by endogenous glucose production during the fasting phase to a high nutrient inflow and replenishment of liver glycogen stores during feeding. The general concensus is that the liver of fish plays a role in glycemic control through the regulation of hepatic glucose storage and mobilization (Enes et al. 2009) but its actions are sluggish in comparison to mammalian liver.

Glicolysis and gluconeogenesis

Glycolysis involves the metabolism of glucose or glycogen to pyruvate. The first step in the glycolytic pathway is the phosphorylation of glucose by GK into G6P, as described. G6P is also the entry point for glycogen following glycogen phosphorylase and phosphoglucomutase activities. G6P is isomerized to F6P via G6P isomerase and this is followed by another phosphorylation of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate ($F16P_2$) catalyzed by 6-phosphofructo 1-kinase (PFK-1). These reactions serve primarily to increase the equilibrium constant of the glycolytic pathway and to commit the carbon skeletons to pyruvate production. Both phosphorylations are highly regulated and along with conversion of phosphoenolpyruvate (PEP) to pyruvate catalyzed by pyruvate kinase (PK) constitute the irreversible steps of glycolysis. The energy charge of the cell, as well as allosteric and transcriptional processes, contribute to control the flux through PFK-1 and PK. The free energy released in this process is used to form energy-rich molecules ATP and NADH. Pyruvate is the final product of glycolysis and can undergo further oxidation to acetyl-CoA via pyruvate dehydrogenase (PDH) or undergo carboxylation via pyruvate carboxylase (PC) or NADP-malic enzyme. In the liver, the relative activities of PDH and PC detemine whether pyruvate is oxidized or utilized by anaplerotic pathways such as gluconeogensis hence the activities of these enzymes are highly regulated depending on the nutritional state. Gluconeogenesis is the principal metabolic pathway that generates glucose in fish. Its main precursors include lactate (converted into pyruvate by lactate dehydrogenase) and

gluconeogenic amino acids (those that are metabolized to pyruvate or C4 and C5 TCA cycle intermediates), with minor contributions from glycerol, derived by lipolysis of triglycerides. While conversion of pyruvate to glucose occurs via the same intermediates as glycolysis, different enzymes are used to overcome the unfavourable free energy change of pyruvate to PEP conversion. The conversion of fructose 1,6-bisphosphate to fructose 6-phosphate and glucose 6-phosphate to glucose are also mediated by different enzymes to their glycolytic counterparts (fructose 1,6-bisphosphatase and glucose 6-phosphatase, respectively). Pyruvate is converted to PEP at the expense of two ATP equivalents. First, pyruvate carboxylase generates oxaloacetate (OA) from pyruvate and second, PEP carboxykinase (PEPCK) converts OA to PEP. The free energy change of pyruvate conversion to glucose is positive, hence pyruvate gluconeogenesis requires energy, in the form of ATP and reducing equivalents. This is not the case for all gluconeogenic precursors, notably glutamate and glutamine. These are metabolized to α-ketoglutarate, which during its oxidation to OA via the TCA cycle, generates ATP and reducing equivalents in excess of those consumed by PEPCK and glyceraldehyde 3-phosphate dehydrogenase. It is important to note that these amino acids are among the most abundant in dietary protein, therefore from a thermodynamic viewpoint, they facilitate gluconeogenesis from protein.

Enzymatic and hormonal regulation

Measurement of enzymatic activities involved directly or indirectly in CHO metabolism has been performed in various species and tissues and has proved to be of crucial importance in evaluating adaptation to changes in temperature (Couto et al. 2008; Enes et al. 2008c), salinity and osmoregulation (Laiz-Carión et al. 2005; Sangiao-Alvarellos et al. 2003), feeding status (Soengas et al. 1996, 2006; Caseras et al. 2000; Metón et al. 2003), rearing densities (Sangiao-Alvarellos et al. 2005) and diets (Melo et al. 2008; Metón et al. 1999b; Panserat et al. 2000, 2001a, 2001b, 2001c; Fernández et al. 2007; Pérez-Jiménez et al. 2009, 2012). In the case of the seabass, enzymatic assays have been used to address some of these issues but main focus was attributed to the relation between temperature and diets with different levels and sources of

CHO (Dias et al. 1998; Enes et al. 2006a, 2006b 2008c; Moreira et al. 2008), starvation and refeeding regimes (Pérez-Jiménez et al. 2007) and more recently to study the effects of hormones (Enes et al. 2010, 2011b).

Hormones in vertebrate organisms are a diverse but well conserved group of signaling molecules that regulate and modulate metabolic fluxes. The role of hormones in the regulation of CHO metabolism of fish are far from being completely known due to the variety of responses observed - many of which are dependent on whether studies are performed on intact fish, isolated organs or primary cell cultures. *In vitro* studies in hepatocyte cultures advance the knowledge in this field despite some limitations as for example, the reported negative glycogen imbalance (Mommsen et al. 1988; Weber & Shanghavi 2000; Moon 2004). The response of individual cells may not represent the response of the intact organ but in recent years a combination *in vitro* and *in vivo* experiments has provided a more realistic description of the responsiveness of enzyme activities and metabolic fluxes to hormones (Weber et al. 2000; Plagnes-Juan et al. 2008; Polakof & Soengas 2008). The mammalian paradigm, to which the hormonal regulation in fish is usually compared, has shown to be unrepresentative of fish metabolism in a number of cases (Moon 2004). Glucose is poorly effective at stimulating insulin secretion in fish compared to mammalian species, although, as expected, an improved response is observed in CHO-tolerant species (Navarro et al. 2002). On the other hand, amino acids are potent stimulators of insulin secretion even though the intensity of response varies greatly between salmonids and other fish like carp and seabream. This bears some resemblance to mammals, where arginine is also a powerful insulin secretagogue.

In mammals, the actions of insulin are directly opposed by glucagon and adrenal hormones such as adrenaline and these counterregulatory actions are an imporant component of glucose homeostasis. However, it is unclear in fish to what extent the regulatory and counterregulatory processes are coupled (Moon 2004). For example, in response to stress or hypoglycemia, the adrenaline-induced hyperglycemic response of rainbow trout is caused by the stimulation of hepatic glucose production that happens in a dose-dependent fashion (Weber et

al. 2000). However, the accompanying suppression of glucose clearance described in mammals is not observed in fish. Cortisol is a major regulator of intermediary metabolism and promotes hepatic glucose production and hyperglycemia, primarily as a result of increased hepatic gluconeogenesis fuelled in part by amino acid products of peripheral proteolysis.

State of the art for the seabass

Due to its carnivorous nature, the seabass has a limited capacity for digesting CHO relying instead on metabolism of dietary proteins for generating endogenous glucose. High digestible carbohydrate intake results in prolonged postprandial hyperglycemia lasting between 6 and 10h depending on the type of CHO (Enes et al. 2009, 2010). During a glucose tolerance test (GTT), a glucose load is administered, normally by bolus injection, and glucose clearance is assessed by periodic measurements of plasma glucose levels after the load (Peres et al. 1999; Enes et al. 2011b). Intra-peritoneal injections of 1 g glucose kg^{-1} FBW immediately raises glycemia by 3-fold within 1h and 4-fold by 3h with return to basal levels occurring only at 12h post-injection. This process coincides with a significant increase in hepatic glycogen, protein and plasma triacylglycerides suggesting that these components are linked to the clearance of glucose and may also have a role in glucose regulation (Peres et al. 1999). As reflected by the sluggish glucose excursions, the secretion and subsequent clearance of plasma insulin and insulin-like growth factor-I levels after GTT are also slow compared to mammal. Peak levels are reached only after 4 hours, compared to 15-30 minutes for rodents, while control levels are reestablished after 9-12h (Enes et al. 2011b), compared to <2h for rodents. As previously mentioned, this suggessts that glucose *per se* is less effective in stimulating insulin secretion in fish species than in mammals and the role of insulin in the regulation of fish CHO metabolism is still incompletely understood.

Glucose turnover in fish has been reported to be lower than in mammals and birds as estimated by bolus injection with [U-^{14}C]glucose. The reported rates of ~0.3 µmol min^{-1} kg^{-1} for 200 g fish are more than 2 orders of magnitude less than that of rats of similar weight (50-100 µmol min^{-1} kg^{-1}). As for mammals, glucose turnover measured with [2-^{3}H]glucose was higher

than that for [U-^{14}C]glucose indicating the presence of futile glucose-G6P recycling (Garin et al. 1986).

Use of stable isotopes to trace carbohydrate metabolism

Overview

The hepatic intermediary metabolic network encompasses the entry of precursor substrates such as gluconeogenic amino acids and the exit of end-products such as glucose and glycogen. Branch points at the level of glucose 6-phosphate, pyruvate, oxaloacetate and citrate commit metabolite carbons to oxidation for energy and/or reducing equivalents or utlilization for biosynthesis of nucleotides, carbohydrates, amino acids and lipids. The liver is able to utilize a wide range of substrastes both for energy provision and as precursors for biosynthetic pathways. In many cases, this is dictated by diet, the prime example being the extensive utilization of amino acids derived from for glucose and glycogen synthesis in carnivorous fish whose natural diet is high in protein and low in CHO. Therefore, identifying the nutrient precursors of hepatic biosynthetic pathways such as gluconeogenesis and glycogenesis is at least as important as measuring the overall rates of glucose and glycogen synthesis. A metabolic tracer is, by definition, a substance used to follow the biological transformation of an endogenous substrate (tracee). The tracer must have a unique property that allows its detection but at the same time be chemically identical to the tracee. In most cases, tracers consist of synthetic substrate molecules where one or more atoms in the molecule are substituted for an atom of the same chemical element, but of a different isotope. Isotopic tracers may be identified by radioactivity, or differences in mass and/or nuclear spin.

The use of radioactive isotopes (such as ^{14}C and ^{3}H) provided valuable information characterizing the main pathways of hepatic carbohydrate metabolism. They are easily detected by scintillation counting and their background radioactivity is very low in comparison to metabolite specific activity. Due to the fact that they are weak α- and β-ray emitters, and are therefore easily contained and have relatively high maximum permissible dosages, these tracers

are still used in some human and animal studies. They are more widely used in cell cultures for substrate uptake studies and isotope-dilution measurements where the small scale of the experiments can be accommodated by reasonable radiation containment measures. The use of stable isotopes such as ^{13}C and ^{2}H has expanded on the previous knowledge obtained from radioactive isotopes, principally by simplifying the analysis of positional labeling information. Improving and simplifying the analysis of positional labeling information has been largely driven by the developent of nuclear magnetic resonance (NMR) and mass spectrometry (MS) technologies.

Deuterated water as a tracer to glucose and glycogen metabolism

Deuterium (^{2}H) is a stable isotope of hydrogen with a nucleus containing one proton and one neutron (the ^{1}H nucleus contains no neutron). In the NMR experiment, ^{2}H resonates at a different frequency compared to its ^{1}H counterpart, allowing tracer levels of ^{2}H to be observed in the presence of the tracee ^{1}H. The inherent sensitivity of ^{2}H (at constant field and with an equivalent number of nuclei) is about 0.9% that of ^{1}H. Metabolism of ^{2}H is not exactly equivalent to that of ^{1}H because of kinetic isotope effects. The strength of a chemical bond between two atoms is dependent in part on their relative masses, hence a C-^{2}H bond is stronger than a C-^{1}H for any compound. Since metabolite transformation is governed in part by breaking and formation of C-H bonds, the presence of ^{2}H makes the bonds harder to break thereby potentially slowing the rate of C-^{2}H *vs.* C-^{1}H transformation. This can discriminate the transformation of ^{2}H-enriched metabolites compared to their tracees resulting in apparently slower rates of transformation. Moreover, with bulk levels of ^{2}H tracers, notably deuterated water ($^{2}H_2O$) the aggregate isotope effects are toxic and indeed lethal to most living organisms. With tracer studies that utilize $^{2}H_2O$, toxicity from isotope effects is minimized by substituting a relatively low proportion of ^{1}H by ^{2}H (<10%). Furthermore, discrimination against ^{2}H incorporation into metabolites via enzymatic reactions is minimized when the reaction that transfers ^{2}H from water to the metabolite hydrogen is reversible. Most of the enzymatic steps of intermediary

metabolism are reversible with extensive exchange of precursor and product, and under these conditions, discrimination of ^{2}H incorpration via kinetic isotope effects is not significant. Indeed, when ^{2}H-discrimination is observed, it informs the unidirectionality of a particular enzymatic step. For example, exchange of G6P and F6P is catalyzed by glucose 6-phosphate isomerase and results in the transfer of ^{2}H from water to position 2 of G6P (Landau et al. 1996). This exchange is typically extensive hence isotopic discrimination is normally not observed for ^{2}H incorporation into G6P from deuterated water ($^{2}H_2O$), despite a significant kinetic isotope effect for the hydrogen transfer step via glucose 6-phosphate isomerase. However, as described in Chapter III, G6P position 2 enrichment from $^{2}H_2O$ is significantly lower than expected, consistent with discrimination of ^{2}H incorporation during the conversion of F6P to G6P and informing that exchange between G6P and F6P is limited.

These caveats notwithstanding, $^{2}H_2O$ is an inexpensive tracer that is easy delivered into body water by drinking, or in the case of fish by immersion in ^{2}H-enriched water. It has been successfully used in humans (Chacko et al. 2008; Jones et al. 2009; Barosa et al. 2011) and other mammals (Sena et al. 2007; Nunes & Jones, 2009; Soares et al. 2009), for study of hepatic carbohydrate metabolism in physiological and pathophysiological conditions. The methodology was pioneered in humans by Landau´s group (Landau et al. 1996; Chandramouli et al. 1997; Landau 2001) and was rapidly adpoted by others (Saadatian et al. 2000; Jones et al. 2001; Gastaldelli et al. 2000). As previously discussed, G6P is a common precursor to both glycogen and glucose (Fig. 6) and in the presence of $^{2}H_2O$, G6P is labeled with ^{2}H in several positions due to the incorporation of that isotope via exchange with body water. It rapidly equilibrates with total body water and distributes homogeneously within tissues and the body water ^{2}H-enrichment level can be maintained indefinitely (Jones 2007). It has been also used in inveterbrates like earthworms (Qiu et al. 2000) as well as perfused mammalian organs and cells (Moldes et al. 1994; Rodrigues et al. 2005).

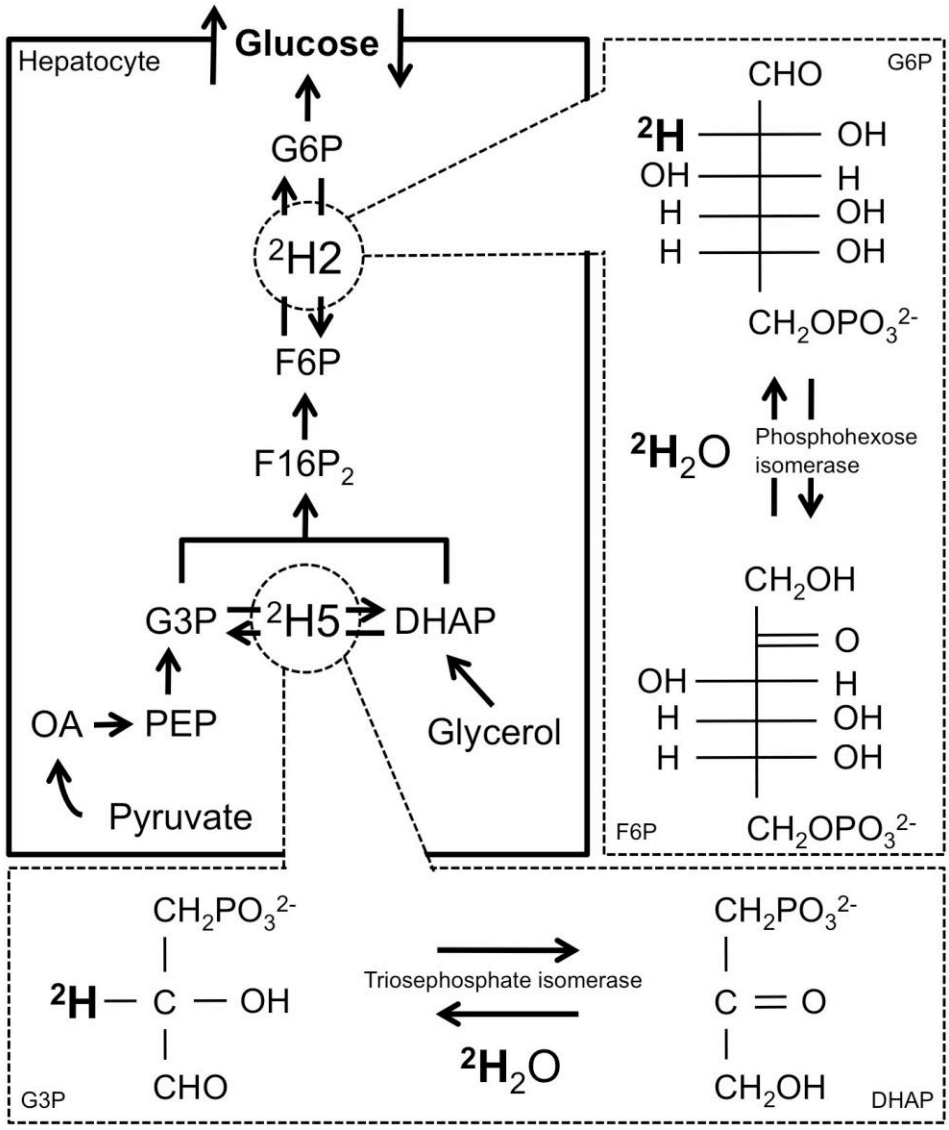

Fig. 6 Metabolic model representing gluconeogenesis in the liver with special detail to the labeling with deuterium in position 2 (^{2}H2) and 5 (^{2}H5) of the glucose molecule. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P = glucose 6-phosphate; F6P = fructose 6-phosphate; F16P$_2$ = fructose 1,6-bisphosphate; G3P = glyceraldehyde 3-phosphate; DHAP = dihydroxyacetone phosphate; PEP = phosphoenolpyruvate; OA = oxaloacetate; ^{2}H$_2$O = deuterated water.

At the onset of this thesis work, to the best of our knowledge, its use in fish had not been reported. During the course of these studies Gasier et al. (2009) reported the use of $^{2}H_2O$ to assess protein synthesis in channel catfish *Ictalurus punctatus*. For studies of carbohydrate metabolism, the ^{2}H-enrichment distrubution of plasma glucose from $^{2}H_2O$ is established according to origin of the G6P precursor. If produced from gluconeogenic substrates (gluconeogenic amino acids, pyruvate or glycerol) enrichment in position 2 (H2) is obligatory since conversion of F6P to G6P (facilitated by G6P-isomerase) is part of the gluconeogenic pathway. Besides, ^{2}H-enrichment in position 5 (H5) also occurs at the level of the triose phosphates isomerization due to exchange glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (via triosephosphate isomerase). If produced from glycolysis exchange of protons with ^{2}H from the $^{2}H_2O$ in H2 is conditional depending on the keto-enolic on the equilibrium between G6P and F6P (Jones et al. 2006b).

In mammals, G6P-F6P exchange is extensive and essentially complete hence hepatic G6P pool is quantitiatively enriched in H2 regardless of its origin. This means that newly-synthesized glycogen from G6P is also enriched in this position, regardless of whether the G6P was derived via the direct or indirect pathway (Soares et al. 2009; Jones et al. 2006). Moreover, incorporation of ^{2}H in H5 of glycogen reflects G6P derived via gluconeogenic intermediates and is therefore a specific marker of the indirect pathway (Soares et al. 2009; Jones et al. 2006).

NMR and MS spectroscopy in metabolism

Measurement of stable isotope tracer enrichment is normally performed by MS or NMR. Choosing between these methods depends on the kind of enrichment information that is required from the experiment, the available sample size and access to instrumentation. MS techniques quantifiy metabolite enrichment by resolving heavier labeled molecules from lighter unlabeled ones. For most MS instruments, the presence of two isotopes with similar increase in the molecular mass, (i.e. ^{2}H and ^{13}C) cannot be resolved, placing limitations on multiple isotope studies. Positional enrichment can be inferred from fragmentation and analysis of the mass of

the daughter fragments (MS-MS). Nevertheless, as fragmentation is dependent on the molecule's chemical structure, the label of interest may or may not be isolated. Chemical derivatization of metabolites is often used to facilitate fragmentation and positional enrichment analysis (Landau et al. 1996). MS is highly sensitive and can quantify enrichments from submicromole to picomole amounts of analyte and with appropriate signal calibration and sample purification safeguards, it can be configured for high throughput measurements.

Analysis of ^{2}H enrichment by ^{2}H NMR spectroscopy is a method with much lower sensitivity compared to MS, requiring 5-50 μmol of analyte in the typical experimental setting for $^{2}H_2O$ studies (0.5-5.0 % body water enrichment). However, in addition to being nondestructive to the sample, NMR provides a much higher level of positional enrichment information, allows enrichment from multiple stable isotope tracers to be selectively observed, and can provide a global analysis of metabolite enrichments from a complex mixture of metabolites, such as cellular extracts, biological fluids, and intact tissues (Wu et al. 2008). This technique relies on the ability of atomic nuclei with odd mass and/or atomic number to align if subjected to an external magnetic field. When irradiated with a certain frequency signal the nuclei in a molecule can change their alignment and the energy frequency at which this occurs can be measured and displayed as an NMR spectrum. Common biologically relevant nuclei that are present at ~100% natural abundance that are observed by NMR include ^{1}H and ^{31}P and ^{23}Na. Isotopes that are more rare in nature such as ^{2}H (0.015% of hydrogen) and ^{13}C (1.11% of carbon) can also be observed at natural abundance levels, but molecules that are enriched to higher levels from ^{2}H- or ^{13}C-enriched precursors can be measured against this background. Since isotopes resonate at a specific frequency, its signals can be uniquely isolated from any other isotope that may be present. Derivatization of the target molecule can be used to provide a more heterogeneous chemical environment therefore improving signal dispersion (Schleucher et al. 1999; Jones et al. 2000; Kunert et al. 2003). This is particularly important for analysis of carbohydrate ^{2}H enrichment, which feature highly crowded hydrogen signals that are poorly resolved by the inherently small dispersion of ^{2}H signals (~15% of ^{1}H signals).

NMR assessments can also be performed *in vivo*, which represents a major breakthrough in metabolic studies of intact organs such as the brain and liver (Gruetter et al. 1994) and in general, NMR techniques have contributed significantly to our current unserstanding of glucose and glycogen metabolism in humans (Roden et al. 2001). In fish, NMR has been utilized to monitor *postmortem* changes during storage of fish (Aursand et al. 1995), to followin metabolic recovery (Borger et al. 1998) and substrate uptake (Pereira et al. 1995b). It has also being used to estimate the bioavailability of amino acids in different diets (Conceição et al. 2003). In recent years major interest has been focused on ^{1}H NMR metabolomics and development of metabolic profiling in the context of stress factors (Karakach et al. 2009), industrial processing of final fish product (Savorani et al. 2010) and fish nutrition (Kullgren et al. 2010).

CHAPTER II

Analysis of glucose metabolism in farmed European seabass (*Dicentrarchus labrax* L.) using deuterated water

Introduction

Fish, like any other vertebrate, rely on the effective coupling of nutrient uptake with biological demands (growth, reproduction, migration, exercise) and environmental challenges (hypoxia, temperature and salinity oscillations). To this end, fish rely on the coordination of substrate and metabolite production rates, turnover, inter-tissue fluxes and oxidation for energy (Weber & Zwingelstein 1995). Metabolic control of glucose and its storage equivalent, glycogen, are key components since glucose is an obligatory energy substrate for erythrocytes (Walsh et al. 1990; Wood et al. 1990) and central nervous system tissues (Soengas & Aldegunde 2002) and is also a key component of the protective mucin coating that fish continuously produce (Shephard 1994). In terms of whole-body energy metabolism, carbohydrates are not the principal oxidizable fuels (Thillart & Van Raaij 1995) however their utilization may be elevated in situations of burst exercise (Wells & Baldwin 2006) or hypoxia (Haman et al. 1997). The diet of carnivorous fish is low in carbohydrate therefore the plasma glucose rate of appearance (R_a), representing the availability of glucose for whole-body consumption, is expected to be dominated by its synthesis from endogenous precursors rather than through dietary absorption.

In aquaculture, the weaning of carnivorous fish from pure fishmeal to diets supplemented with carbohydrate is an important objective for improved sustainability and reduced environmental impact as put in perspective by Moon (2001). Hence, there is continuing interest in understanding to what extent their metabolic phenotype can adapt to increased dietary carbohydrate availability and if the utilization of amino acids for glucose synthesis is spared under these conditions (Hemre et al. 2002). To effectively address such questions and to improve our overall understanding of piscine glycemic control, endogenous and exogenous contributions to glucose R_a need to be better defined than they are at present. These include the endogenous glucose production pathways of glycogenolysis and gluconeogenesis as well as the appearance of absorbed glucose from dietary carbohydrate.

Methods for obtaining glucose R_a and pool sizes based on modeling the clearance of a radiolabeled glucose tracer bolus (Katz et al. 1974) have been applied in many fish species

including kelpbass *Paralabrax clathratus* (Bever et al. 1977), seabass *Dicentrarchus labrax* (Garin et al. 1987), *Hoplias malabaricus* (Machado et al. 1989) and carp *Cyprinus carpio* (West et al. 1994b). However, this approach requires frequent blood sampling over a sustained period to adequately describe the tracer clearance kinetics. Limited by the number of blood samples that could be drawn from the fish, Bever et al. (1977) extrapolated the clearance kinetics from a set of initial measurements, but the uncertainties of this approach were acknowledged. The dorsal aorta cannulation developed by Soivio et al. (1975) facilitates multiple blood samplings in undisturbed, non-anaesthetized fish but cannot accommodate simultaneous tracer administration and blood sampling. This was solved by the double dorsal aorta catheterization developed by Haman & Weber (1996) allowing glucose R_a in rainbow trout *Oncorhynchus mykiss* to be measured by the primed-constant infusion tracer dilution method – a widely used and robust method for estimating glucose appearance rates in other animals including humans. While these procedures now allow glucose R_a to be well determined, they poorly inform the endogenous and exogenous glucose contributions to this flux.

Novel methodologies of resolving the sources of glucose R_a using deuterated water (^2H_2O) have developed and can be integrated with primed-infusion glucose R_a measurements (Chandramouli et al. 1997; Jones et al. 2001; Nunes & Jones 2009). ^2H_2O is ideally suited for fish metabolic studies since it incorporates into the aquarium water for an indefinite period and has recently been used to study protein synthesis kinetics in the catfish (Gasier et al. 2009). Fish and mammals share common pathways for glucose production and consumption (Cowey & Walton 1989) hence the underlying principles of plasma glucose ^2H-enrichment from ^2H_2O that are well described and validated for mammals (Landau et al. 1996; Chandramouli et al. 1997; Saadatian et al. 2000) can be applied to fish.

The principal metabolite transformation and exchanges involved in glucose metabolism are shown in Figure 7. During fasting, the liver synthesizes glucose by hydrolysis of glucose 6-phosphate (G6P). Due to the rapid interchange of hepatic G6P and fructose 6-phosphate (F6P) via G6P-isomerase, the hydrogen at position 2 of glucose (H2) and that of bulk water (BW) are

extensively exchanged. Thus, when body water is enriched with deuterium, glucose produced from the liver will be equivalently enriched in H2. Gluconeogenesis generates molecules enriched in multiple sites including position 5 (H5). Therefore, the fraction of endogenous glucose output derived directly from gluconeogenesis *vs.* other sources can be estimated by analysis of ^{2}H-enrichment in position 5 relative to position 2 (H5/H2) of blood glucose. The H5/H2 analysis does require steady-state levels of body water enrichment (Chandramouli et al. 1997), but instead only requires sufficient ^{2}H-enrichment in the glucose sites to be within the limits of quantification by NMR.

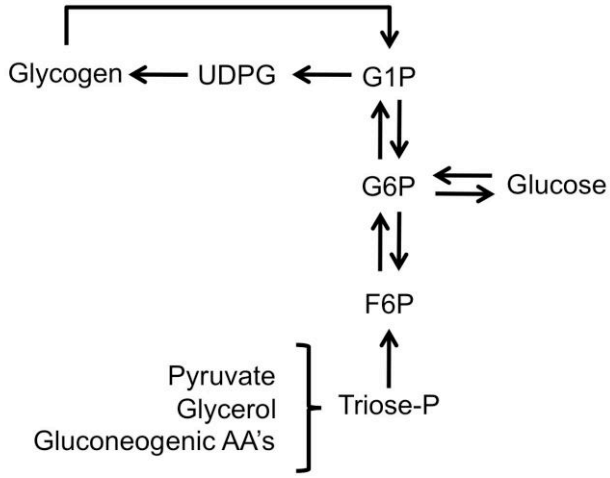

Fig. 7 Metabolic model representing the principal sources of endogenous glucose production and exchanges between glucose and various hexose phosphates. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: F6P = fructose 6-phosphate; G6P = glucose 6-phosphate; G1P = glucose 1-phosphate; Triose-P = triose phosphates; UDPG = uridine diphosphate glucose.

Under feeding conditions, dietary glucose can be directly absorbed into the splanchnic circulation thereby contributing to the rate of appearance of plasma glucose. Initially, this fraction is not enriched with ^{2}H in any position but it has the possibility of becoming enriched in position 2 as a

result of glucose-G6P cycling. At isotopic steady-state (i.e. when the plasma glucose pool has completely turned over in the presence of $^{2}H_2O$), the fraction of plasma glucose molecules derived from gluconeogenesis is estimated as the ratio of position 5 enrichment to that of plasma water (H5/PW) with the balance representing the contribution from non-gluconeogenic sources.

In this report, we demonstrate the application of $^{2}H_2O$ to identify the sources of glucose R_a in the seabass *D. labrax* under feeding and fasted conditions. We also demonstrate a sensitive, high-throughput procedure for obtaining glucose ^{2}H-enrichment information by liquid chromatography-mass spectrometry (LC-MS/MS). This method was validated by direct comparison with the established ^{2}H-NMR method for determining plasma glucose ^{2}H-enrichment levels (Jones et al. 2006). The $^{2}H_2O$ measurement procedure and analysis can be easily integrated with isotope-dilution glucose R_a measurements.

Methods

Fish handling and plasma sampling

For the present study, two groups of fish provided by a local farm were transported to the lab. The first group, hereafter designated as small fish, had a mean length of 9.1±0.1 cm and a mean body weight of 9.1±0.1 g. The second group, hereafter referred to as large fish, had a mean length of 28.0±3.8 cm and mean body weight of 231.0±80.3 g. Fish were acclimated for 2 weeks at 18°C and 30‰ in 200 L tanks with aerated, recirculated seawater passed through a mechanical filter, a biological filter, activated carbon and a UV unit. Tank water temperature, salinity, pH, and dissolved oxygen were continuously monitored and NH_4^+, NO_3^- and NO_2^- were assessed every 7d. After the acclimation period, a subset of fish from each group were fed with a commercial diet (small: Aquagold 4, SORGAL, S.A; 52% protein, 18% fat, 1% carbohydrates, 11% ash, 1-2 mm standard pellet; large: Dourasoja Ultra 5, SORGAL, S.A; 44% protein, 18% fat, 2.2% carbohydrates, 9.2% ash, 5 mm standard pellet) while the remaining fish were fasted (small: for 5d; large: for 21d). Another 100 L tank equipped with the same filters and monitors was filled

with seawater enriched with ^2H_2O to 5%. This was prepared by mixing 95 L of seawater with 5 L of 99%-enriched ^2H_2O (Eurisotop, France). The seawater salinity level was maintained by addition of commercial mineral salt suited for fish rearing. Fish were transferred to the ^2H_2O-enriched seawater tank and then sampled for blood at various times afterwards, ranging from 10min to 72h. For blood sampling, fish were anaesthetized by placing in a 30 L tank of ^2H_2O-enriched seawater containing 0.1 g L^{-1} MS-222 for 2-3min. For a subset of fish (control group), this procedure was performed in the absence of ^2H-enrichment. Fish were sampled for blood from the caudal vein (0.1 mL in 50 mg EDTA) with syringes. Blood was kept on ice and centrifuged (3000g for 10min) to separate plasma which was stored at -20ºC for successive glucose quantification with a commercial assay kit (Invitrogen, Spain).

Blood sampling and blood glucose derivatization

The remaining blood was collected with heparinized syringes from the caudal vein only in fish placed 6h and 3d in the 5%-^2H_2O enriched tank and was processed in two different ways: i) a small drop of blood was retained for LC-MS/MS analysis by spotting on a filter paper strip followed by drying and storage at room temperature in a container with silica-gel desiccant; ii) plasma 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 15min). 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 -20ºC.

For derivatization of glucose to monoacetone glucose (MAG), the dried extract was stirred for 4h in a 3 mL solution of acetone and 4% sulphuric acid enriched to 2% with ^2H. MAG that is synthesized in this way has the two isopropylidene methyl groups enriched to 2% which serve as intramolecular ^2H-enrichment standards for quantifying ^2H-enrichment of the glucosidic hydrogens (Jones et al. 2006; Soares et al. 2009). The reaction was quenched by the addition of an equal volume of water (2% with ^2H) and the pH was adjusted to 2.0 by the addition of solid sodium carbonate. This solution was stirred for 24h before the pH was adjusted to 8.0 by the

addition of more sodium carbonate. The sample was then freeze-dried and MAG was extracted with 5 ml boiling ethyl acetate. The ethyl acetate was evaporated and MAG was dissolved in 90% acetonitrile 10% water for ^{2}H-NMR analysis.

^{2}H-NMR analysis

Plasma water $^{2}H_2O$ enrichments were determined from 10 µL plasma volumes by ^{2}H-NMR as described Jones et al. (2001). Proton-decoupled ^{2}H-NMR spectra of MAG samples were obtained at 50ºC with a 14.1 T Varian 600 system (Varian, Palo Alto, CA) equipped with a 3-mm broadband probe with the observe coil tuned to ^{2}H. Fully-relaxed spectra were acquired with a 90º pulse and 1.7s of recycling time (1.6s of acquisition time and 0.1s pulse delay). The number of scans ranged from 12.000 to 17.000 (corresponding to 6-8h of collection time). Field-frequency drift was compensated by the Scout Scan software (Varian, Palo Alto, CA) as previously described (Nunes & Jones 2009).

LC-MS/MS analysis

A disk (6.0 mm in diameter) was punched out of the bloodspots, transferred to an Eppendorff microtube and wetted with 40 µl of water followed by 400 µl of ethanol. The tubes were shaken for 45min at room temperature, then transferred into eppendorf tubes fitted with a Polyethersulfone membrane and thin channel filtration chamber (Sartorius Vivaspin 500) and centrifuged for 1h. The filtrate was dried under nitrogen gas. Samples were resuspended in 60 µL of Milli-Q water, vortexed and spun. Fifty microliters of the supernatant were combined with 20 µL of internal standard ([U- ^{13}C]glucose 9.3 ng/µL in 80% CH_3CN, final concentration 1.86 ng/µL), and 30 µL of CH_3CN. Samples were analyzed by flow injection analysis (FIA) on an Ultimate™3000 LC system (LC Packings, Dionex) coupled to an ESI Turbo V ion source and an hybrid triple quadrupole/linear ion-trap 4000 QTrap mass spectrometer operated by Analyst 1.4 (Applied Biosystems/MDS Sciex). Samples were injected at 60 µL/min using 80% of acetonitrile in water as mobile phase. The ionization source operated in negative mode set to an ion spray voltage of -

4800 V, the nebulizer gas 1 (GS1) was 35 psi and the temperature 150°C. Glucose enrichment was quantified using Multiple Reaction Monitoring (MRM) triple quadrupole scan mode. The parameters of the mass spectrometer were the same for all MRM transitions: curtain gas (CUR) 50 psi, collision energy (CE) -13 eV, entrance potential (EP) -4 eV, collision cell exit potential (CXP) -8 eV and the collision gas (CAD) was set to high. The MRM transition used for monitoring unlabeled glucose was 179 → 89, for labeled glucose ([^{2}H]glucose and [1-^{13}C] glucose) 180 → 89 and 180 → 90, and for [U- ^{13}C]glucose 185 → 92. Peak areas were integrated using MultiQuant v1.2 (Applied Biosystems).

Unlabeled glucose was quantified using a calibration curve with five different known concentrations of standard glucose (0.45 – 45 ng/µL). Each standard was spiked with 20 µL of internal standard (9.3 ng/µL [U-^{13}C]glucose in 80% ACN, final concentration 1.86 ng/µL). The quantification of [^{2}H]glucose in blood fish samples was performed using [1-^{13}C]glucose as standard for the calibration curve. The injection volume was 1 µL.

Quantification of the M+1 signal component representing excess enrichment from $^{2}H_2O$ was corrected for ^{13}C and ^{2}H natural abundance by performing an empirical quantification of the background M+1 signal. Briefly, a calibration curve representing the M+1 was obtained from non-enriched glucose, such that for a given sample the background M+1 could be calculated. The M+1 signal derived from the fish samples (representing both excess ^{2}H-enrichment from $^{2}H_2O$ and the background ^{13}C and ^{2}H abundance) was then corrected by subtracting the calculated background fraction.

Calculating glucose enrichment from $^{2}H_2O$ by ^{2}H-NMR and by LC-MS/MS

From the ^{2}H-NMR spectrum of MAG, the ^{2}H-enrichment at a given hexose position, *i*, was determined from the ratio of the positional ^{2}H hexose signal (H_i) to that of the two methyl (CH_3) intramolecular standard signals (Jones et al. 2001; Nunes & Jones 2009; Soares et al. 2009). With the LC-MS/MS approach, glucose m+1 enrichment was thus calculated as the ratio of 179.0→89

transition, representing unenriched glucose molecules, to the sum of 180→89 and 180→90 transitions - representing glucose molecules labeled with a single heavy isotope.

Absolute amounts of unlabeled (m+0) and labeled (m+1) glucose in both samples and standards were calculated by comparison with the 185→92 transition signal intensity from a fixed amount of [U-^{13}C]glucose internal standard. The internal standard was also used to match the amounts of sample m+0 and m+1 glucose to the range of the glucose calibration curves.

Statistics

Data are presented as means ± SD (except when stated otherwise). Student's two-tailed unpaired t-test was used to compare means of the experimental data. Whenever necessary one-way ANOVA was used and significant differences among groups were determined by the Tukey's multiple range test. The probability level of 0.05 was used for rejection of the null hypothesis.

Results

Plasma water ^{2}H-enrichment kinetics and plasma glucose levels

Tank water ^{2}H-enrichment was measured after all experiments and was found to have minimal variance between different batches of studies (4.9±0.2%). For the smaller fish, plasma water ^{2}H-enrichment was rapid, reaching more than 1% in 15min, half of the enrichment of the tank water within 1h and approaching that of the tank water after 6h (see Figure 8). Between 6h and 72h, the plasma water enrichment remained constant. Larger fish showed similar plasma water enrichment kinetics to their smaller counterparts as no statistical differences were found between both at each of the sampling times. The importance of maintaining the tank water ^{2}H-enrichment during the brief anesthetization procedure is underlined by our finding that plasma water ^{2}H-enrichments were found to be significantly less than those of tank water (3.3±0.2% *vs.* 4.9±0.2%, *P*=0.0004) for a group of fish that were anesthetized in non-deuterated water after 72h in the deuterated water tank.

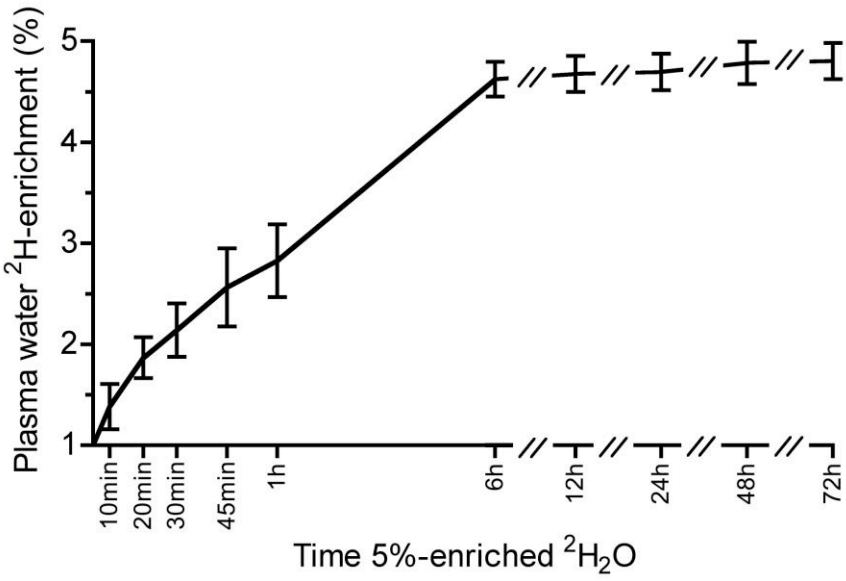

Fig. 8 Evolution of plasma water ^{2}H-enrichment with the residence time in aquarium water enriched with 4.9% ^{2}H. Each time point represents the mean ± SD (n=6).

Table 1 shows the blood glucose levels that were recorded following transfer of the fish from the 100 L tank enriched with ^2H_2O where the fish had been kept for 6 or 72h to a smaller 30 L tank containing anesthetic. Significant differences between small and large fish within the same time and condition were always found. Amongst small fishes only the ones sampled after 6h in the fed condition presented higher levels of glycemia. The same trend was observed for large fish (Table 1).

Table 1 Plasma glucose levels (in mM)

	Fed		**Fasted**	
	6h	**72h**	**6h**	**72h**
Small	8.05±1.14^{a}	3.43±0.54^{b}	3.55±0.45^{b}	2.95±0.18^{b}
Large	18.24±1.95^{a*}	10.61±2.53^{b*}	13.19±1.08^{b*}	10.24±0.86^{b*}

Mean values ± standard error are presented. Significant differences within small or large fish (one-way ANOVA) are indicated by different letters (Tukey test, $P<0.05$). Significant differences between small and large fish within the same time and condition are indicated by asterisks (*t*-test, $*P<0.05$).

Plasma glucose ^{2}H-enrichment

For the larger fish, plasma glucose enrichment from $^{2}H_2O$ in all positions was well-characterized by ^{2}H-NMR, as shown by Figure 9 and Table 2. For fish that had been administered $^{2}H_2O$ for 6h, plasma glucose H2 enrichment was significantly lower compared to that observed at 72h, amounting to ~20-40% of plasma water enrichment (see Table 2).

Table 2 Plasma glucose positional ^{2}H-enrichments as quantified by ^{2}H-NMR

		^{2}H-positional enrichment						
		1	**2**	**3**	**4**	**5**	**6_S**	**6_R**
6h	**Fasted**	1.0±0.1	1.1±0.1	0.7±0.1	1.0±0.2	1.1±0.1	0.9±0.0	1.0±0.1
	Fed	1.4 ± 0.2^{ab}	$2.0\pm0.1^{a*}$	1.1 ± 0.2^{b}	$1.5\pm0.1^{ab*}$	$1.6\pm0.2^{ab*}$	$1.5\pm0.2^{ab*}$	1.3 ± 0.2^{ab}
72h	**Fasted**	3.5 ± 0.1^{ab}	3.8 ± 0.2^{a}	2.9 ± 0.2^{ab}	3.0 ± 0.3^{ab}	3.6 ± 0.2^{ab}	2.9 ± 0.1^{b}	3.1 ± 0.2^{ab}
	Fed	$2.8\pm0.3^{abc*}$	3.3 ± 0.1^{a}	2.3 ± 0.2^{bc}	2.8 ± 0.2^{abc}	3.3 ± 0.1^{ab}	$2.3\pm0.2^{c*}$	$2.1\pm0.3^{abc*}$

Mean values ± SEM are presented. Significant differences between positional enrichment (one-way ANOVA) are indicated by different letters (Tukey test, $P<0.05$). Significant differences between fed and fasted fish within the same time are indicated by asterisks (*t*-test, $*P<0.05$).

These lower enrichments reflect the wash-in period of $^{2}H_2O$ into body water pool in addition to the limited turnover of the plasma glucose pool. The H5/H2 ratio approached unity (0.94±0.03) for the fasted fish indicating that the source of enriched glucose was essentially all gluconeogenic with no input from glycogenolysis or glucose-G6P cycling. A significantly lower H5/H2 ratio was measured for the fed fish (0.79±0.05). Statistical differences were found between fed and fasted fish in positions 2, 4, 5 and 6_S. For fish that had been administered $^{2}H_2O$ for 72h, plasma glucose ^{2}H-enrichment of H2 was ~65-75% that of body water for fed and fasted fish respectively indicating that the majority of plasma glucose had been metabolically derived. The H5/H2 ratio was 0.98±0.01 for both fed and fasted fish (as for the 6h sampled fasted fish) indicating that the metabolically derived glucose originated almost entirely from gluconeogenesis. Enrichment of the remaining positions (1, 3, 4, and $6_{R,S}$) tended to be less than that of H5 reflecting the fact that ^{2}H-enrichment of these hydrogens is conditional on exchange reactions between bulk water and gluconeogenic precursors.

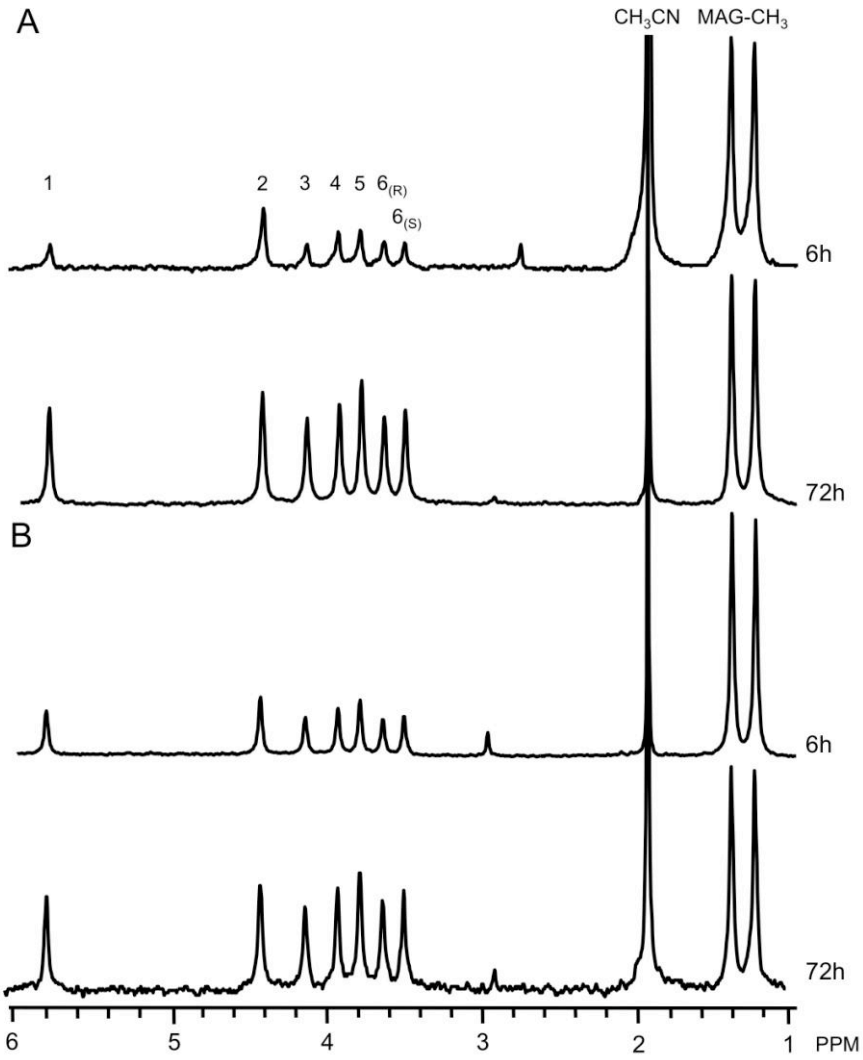

Fig. 9 ^{2}H-NMR spectra of monoacetone glucose samples from plasma glucose of fasted (A) and fed (B) fish sampled following 6 hours and 72 hours residence time in aquarium water enriched with 4.9% ^{2}H. The numbers above each signal represents its position within the original glucose molecule. Other signals include the pair of ^{2}H-enriched methyl signals used as internal ^{2}H enrichment references (MAG-CH_3) and the signal from the acetonitrile solvent (CH_3CN). The signal at 2.9 ppm is a residual ^{2}H-signal from water.

Quantifying plasma glucose ^{2}H-enrichment and gluconeogenic fraction by LC-MS/MS

As shown in Table 3, MPE values derived by LC-MS/MS are in agreement with the summed positional enrichments from the ^{2}H-NMR analyses.

Table 3 Mole percent glucose ^{2}H-enrichments (MPE) as quantified by both ^{2}H-NMR and LC-MS

		MPE	
		NMR	**LC-MS/MS**
6h	**Fasted**	6.8±0.6 †	10.4±1.1*
	Fed	10.3±1.2	11.4±1.4
72h	**Fasted**	23.1±1.2 †	20.3±0.5*
	Fed	18.8±1.0	19.0±0.6

Mean values ± SEM are presented. Significant differences between methods of detection within the same time are indicated by asterisks (*t*-test, **P*<0.05). Significant differences between fed and fasted fish within the same time and method are indicated by crosses (*t*-test, †*P*<0.05).

No statistical differences between both methods were found for fed fish, contrary to observed in fasted fish (P=0.027 and P=0.037; for 6h and 72h respectively). Besides, NMR was able to perceive differences between fed and fasted fish (P=0.040 and P=0.032, for 6h and 72h respectively), while LC-MS/MS was not. Thus, the increase in glucose ^{2}H-enrichment after 6 to 72h of $^{2}H_2O$ administration can be followed by LC-MS/MS as well as by ^{2}H-NMR. However, the LC-MS/MS analysis does not provide positional ^{2}H-enrichment information hence the sources of metabolically-derived glucose are unresolved by this method.

Discussion

In this study we show that plasma water ^{2}H-enrichment from tank water in both small and large individuals was rapid demonstrating a facile exchange of internal and external water molecules. A rapid equilibration of ^{2}H- between fish plasma water and tank water was observed allowing stable precursor ^{2}H-enrichment levels to be established by 6h. However, even a brief hiatus from the ^{2}H-enriched tank water resulted in a rapid washout of $^{2}H_2O$ from the tissue water, as demonstrated by the anesthesia tank studies. The rapid washout of ^{2}H-plasma water

under these conditions is likely enhanced by hyperventilation of the fish in reaction to the transfer and anesthetization procedure.

Handling of most fish species, including seabass generates a stress response that is characterized by an acute development of hyperglycemia that persists for several hours (Chavin & Young 1970; Bever et al. 1977). This is difficult or impossible to avoid for tracer infusion protocols (Bever et al. 1977; Garin et al. 1987) hence it is uncertain to what extent these measurements of glucose kinetics are influenced by stress hyperglycemia. This was later circumvented by other methods like the double-cannulation presented by Haman & Weber (1996). It has been shown that netting and handling procedures of short duration do not evoke the hyperglycemic stress response in captive seabass (Marino et al. 2001). On this basis, the transfer of fish from the deuterated water tank to a separate anesthesia tank for blood sampling without evoking the stress response should be feasible. For the small fish, the feeding and fasting plasma glucose levels measured after transfer to the anesthesia tank were in good agreement with the study of Gutiérrez et al. (1991) where glucose levels during feeding were ~8.3 mM, falling to ~3.9 mM at 4d of fasting for 40-60 g seabass. Moreira et al. (2008), working with 30 g fish and Pérez-Jimenéz et al. (2007) working with 90 g fish reported similar excursions in plasma glucose levels during the fed to fasted transition. We therefore conclude that stress hyperglycemia was avoided during sampling of the small fish and the observed differences in blood glucose levels were attributable to different feeding-fasting states. In contrast, fasting plasma glucose levels for the larger fish were well above the normal range suggesting that they had incurred stress hyperglycemia – possibly due to the relative confinement of the smaller anesthesia tank, a procedure that can be easily adapted in future experiments. Echevarría et al. (1997) working with ~315 g seabass reported fasting blood glucose concentrations of 5.2 mM - less than half of that observed for the larger fish of our study. Glucose concentrations during early absorption were significantly higher than those measured at late absorption and fasting suggesting that the effects of stress hyperglycemia and nutritional state on plasma glucose levels were additive.

The ^{2}H-NMR method allowed enrichment of all seven aliphatic glucose hydrogens to be precisely quantified. Hydrogen 2 is biochemically the most labile since it can be exchanged with body water by the equilibration of G6P and F6P catalyzed by G6P-isomerase – an ubiquitous enzyme of glycolysis and gluconeogenesis. Enrichment of plasma glucose hydrogen 2 in relation to the tank water therefore reflects the contribution of metabolically-derived glucose to overall plasma glucose levels, thus a simple and direct reference for turnover studies in fish species. This includes net synthetic fluxes from gluconeogenesis and glycogenolysis (Landau et al. 1996) as well as futile cycling of plasma glucose and G6P via glucokinase and glucose 6-phosphatase (Dunn et al. 1976). The remainder of plasma glucose that was unlabeled in this position most likely represents a residual pool of endogenous unlabeled glucose molecules that were present before $^{2}H_2O$ administration, reflecting the slow turnover of the whole body glucose pool under the fasting conditions (Garin et al. 1987). Hydrogen 5 enrichment is considered to be the most precise marker of gluconeogenic output since the conversion of all gluconeogenic precursor molecules (i.e. glycerol or phosphoenolpyruvate) to G6P involves the obligatory addition of water hydrogen that is destined to become the position 5 hydrogen of glucose (Landau et al. 1996). Enrichment of the remaining positions (1, 3, 4, and $6_{R,S}$) tended to be less than that of position 5 reflecting the fact that ^{2}H-enrichment of these hydrogens is conditional on exchange reactions between bulk water and gluconeogenic precursors, and in the case of positions 1 and 6 hydrogens, may be diluted by the inflow of triose phosphate units with unlabeled methylene hydrogens derived from glycerol. To the extent that the exchange reactions are incomplete, and/or glycerol is contributing to gluconeogenesis, enrichment of positions 1, 3, 4 and $6_{R,S}$ will be less than theoretical values. In contrast, incorporation of the water hydrogen destined for position 5, either via enolase or via triose phosphate isomerase, is obligatory and is not subject to significant isotope effects. Therefore, enrichment at this site is considered to quantitatively represent the contribution from all gluconeogenic presursors (i.e. glycerol plus anaplerotic substrates) (Landau et al. 1996).

The process of digestion and absorption is much slower in fish compared to mammals lasting for, in the case of the seabass, up to 50h (Adamidou et al. 2009b). Hence, our 72h sampling interval immediately following feeding was deemed sufficient for the fish to achieve complete evacuation and the ^{2}H-enrichment distribution measured during this period is a reflection of glucose metabolism during absorptive and initial post-absorptive stages. Under these conditions, plasma glucose was substantially enriched with ^{2}H, with enrichment levels approaching those measured in the fasted fish. These data indicate that the majority (~70%) of plasma glucose was derived metabolically while absorption of unlabeled glucose units from dietary carbohydrate contributed at most only 30% of plasma glucose appearance. The low carbohydrate content of the feed (1-2%) coupled with the poor ability of carnivorous fish such as seabass to digest and absorb dietary carbohydrate (Enes et al. 2006; Moreira et al. 2008) is consistent with this observation.

The H5/H2 ratio indicated that the metabolically derived glucose originated almost entirely from gluconeogenesis with no contributions from G6P precursor molecules that were unlabeled in H5 (either derived from glycogenolysis of unlabeled glucosyl units or from futile cycling of unlabeled glucose). Shanghavi & Weber (1999) attempted to calculate glycogenolysis by estimating glycogen depletion in the liver and kidney during exercise (gluconeogenesis would account for the remainder), however no significant change could be demonstrated due to high variability among individuals. The H5/H2 ratio of 0.98±0.01 indicates that gluconeogenesis contributed to virtually all of the metabolically derived glucose with no significant input from glycogenolysis.

Analysis of blood glucose from fed fish after 6h of $^{2}H_2O$ administration revealed a low enrichment of glucose H2 relative to that of plasma water (BW) indicating a partial replacement of the unlabeled glucose pool by endogenously produced glucose. However, unlike the fasted fish, the glucose ^{2}H-enrichment pattern was not uniform with position 2 enrichment being substantially higher than the other sites - including position 5 - resulting in a significantly lower H5/H2 ratio (0.79±0.05) compared to either fasted fish or fed fish kept for 72h in $^{2}H_2O$. These

observations are consistent with the generation of glucose molecules enriched in H2 only ([2-^{2}H]glucose) in addition to the synthesis of glucose enriched in all positions via gluconeogenesis. As discussed previously, the source of [2-^{2}H]glucose may include the hydrolysis of unlabeled hepatic glycogen to glucose via G6P or futile cycling of unlabeled plasma glucose. The fact that detectable levels of [2-^{2}H]glucose were only observed during brief $^{2}H_2O$ administration, when plasma glucose enrichment had not reached isotopic steady-state, suggests that it was formed by futile glucose-G6P cycling (see Figure 7). Under these conditions, the fraction of unlabeled plasma glucose is large relative to that of ^{2}H-enriched glucose molecules hence the probability of forming [2-^{2}H]glucose from unlabeled glucose via futile cycling is high. When isotopic steady-state conditions are approached following the longer period of $^{2}H_2O$ administration, plasma glucose enrichment approaches that of the uniformly-enriched hepatic G6P pool. Under these conditions, glucose-G6P cycling has no significant effect on the ^{2}H-enrichment distribution or the H5/H2 ratio of plasma glucose. Significant glucose-G6P cycling activity was reported for seabass under similar conditions (48h after feeding) using the [2-^{3}H]glucose/[U-^{14}C]glucose dual tracer method (Garin et al. 1987).

The reduced H5/H2 ratio observed at 6h post-feeding could also reflect net hepatic glycogenolytic activity that was contributing to endogenous glucose production at this time (but not at 72h post-feeding). Glucosyl units released from hepatic glycogen are not enriched in position 5, but become extensively enriched in H2 through G6P/F6P isomerization (Landau et al. 1996; Jones et al. 2001; Nunes & Jones 2009). We concur that hepatic glycogenolysis is unlikely to be a significant contributor to the reduced H5/H2 ratio under these conditions since 1); high absorptive glucose and insulin levels are expected to favor hepatic glycogen synthesis over glycogenolysis and 2); hepatic glycogen levels at 6 and 72h post-feeding were relatively abundant and constant and were not indicative of significant glycogen depletion (data not shown).

The ^{2}H-NMR method is informative but impractical for sampling plasma glucose enrichments from <1 ml whole blood because of poor sensitivity. Therefore, this approach is limited to single end-point studies of relatively large fish (>200 g). The ^{2}H-enrichment of glucose

in positions 2, 5 and 6 can be measured by gas-chromatography-mass spectrometry (GC-MS) (Landau et al. 1996; Chandramouli et al. 1997; Schumann et al. 2001). While this approach is sufficiently sensitive for analyzing much smaller quantities of plasma glucose than the ^{2}H-NMR method, the derivatization procedure is complex and laborious. We explored a simpler method based on a LC-MS/MS procedure developed by Ullah et al. (2009) for quantifying plasma [6,6-$^{2}H_2$]glucose enrichment. This approach does not require glucose derivatization and it can be performed on a few microliters of blood, either whole or as a dried spot on filter paper. This means it can be applied to any size fish and can also be used for repeated sampling of the same fish. This LC-MS/MS measurement provides the mole percent enrichment (MPE) of the glucose molecule, equivalent to the sum of all seven positional enrichments. The principal uncertainties of utilizing plasma MPE levels as a marker of gluconeogenic contribution include the incomplete incorporation of ^{2}H into sites other than position 5, as seen by the tendency for lower enrichments in positions 1, 3, 4, 6_R and 6_S compared to position 5 by ^{2}H-NMR analysis. Provided that this enrichment pattern does not vary significantly between different experimental conditions, it should be feasible to derive a correction factor that accounts for incomplete enrichment into each of these positions - analogous to the *N* value of *de-novo* lipogenesis measurements that accounts for the average number of deuterium atoms incorporated into each newly-synthesized lipid molecule (Diraison et al. 1996). The principal limitation of this approach is the assumption that enrichment of different glucose sites derived via gluconeogenic activity (i.e. positions 1, 3, 4, 5 6_R and 6_S) is uniform, whereas in reality there may be significant variation between positional enrichments due in part to gluconeogenic substrate selection (Burgess et al. 2008). Additional uncertainties are caused by ^{2}H-incorporation by processes that are unrelated to gluconeogenesis – principally the selective enrichment of H2 via glucose-G6P cycling or glycogenolysis. Position 2 enrichment contributions may be potentially avoided by LC-MS/MS analysis of selected glucose fragments. This approach was applied to GC-MS analysis of glucose ^{2}H-enrichment from the pentaacetate derivative where a fragment containing all hydrogens except hydrogen 2 was identified and analyzed for ^{2}H-enrichment (Chacko et al. 2008).

CHAPTER III

Hepatic glycogen synthesis in farmed European seabass (*Dicentrarchus labrax* L.) is dominated by indirect pathway fluxes

Introduction

The liver plays a key role in the control of endogenous carbohydrate storage and glucose mobilization in fish (Metón et al. 2003; Soengas et al. 2006; Pérez-Jiménez et al. 2007). Glucose is an essential substrate for the brain, kidney and gills, and these organs account for about two-thirds of whole-body fish glucose disposal (Blasco et al. 2001). Glucose is also a key precursor for mucin (Shephard 1994), a glycoprotein that is continuously synthesized and secreted. Thus, during starvation, a baseline level of plasma glucose is continuously maintained by hepatic glucose production. Under these conditions, hepatic glycogen is depleted (Navarro and Gutiérrez 1995) and glucose production is sustained by gluconeogenesis. When fasted fish are refed, both plasma glucose and hepatic glycogen levels quickly rebound and may even exceed those of fish that are fed on a regular basis (Metón et al. 1999b; Soengas et al. 2006; Pérez-Jiménez et al. 2007). It is unclear if nutrient utilization is more efficient under these conditions compared to regular feeding, hence there have been numerous studies of nutrient uptake and assimilation during compensated growth (Eroldogan et al. 2006; Huang et al. 2008; Peres et al. 2011). The status of carbohydrate metabolism during refeeding is of particular interest since there is a sharp transition from fasting to feeding states resulting in a rapid reversal of hepatic carbohydrate metabolic fluxes (Metón et al. 2003; Polakof et al. 2006; Furné et al. 2012), from net glucose export to carbohydrate inflow and replenishment of glycogen stores. While endogenous glucose synthesis in carnivorous fish is highly dependent on gluconeogenesis from amino acids, hepatic glycogen synthesis is considered to be at least partly derived from dietary carbohydrate (Enes et al. 2009). However, given the fact that the liver can synthesize glycogen from gluconeogenic precursors via the indirect pathway it is likely that during refeeding with a standard fishmeal formulation that is low in carbohydrate content, a significant portion of hepatic glycogen repletion is sustained by gluconeogenic metabolism of amino acids. However, to date, this has not been established for seabass or indeed any fish species.

As shown in Fig. 10, hepatic glycogen can be synthesized from glucose via direct pathway or generated from gluconeogenic precursors via the indirect pathway (Newgard et al. 1983).

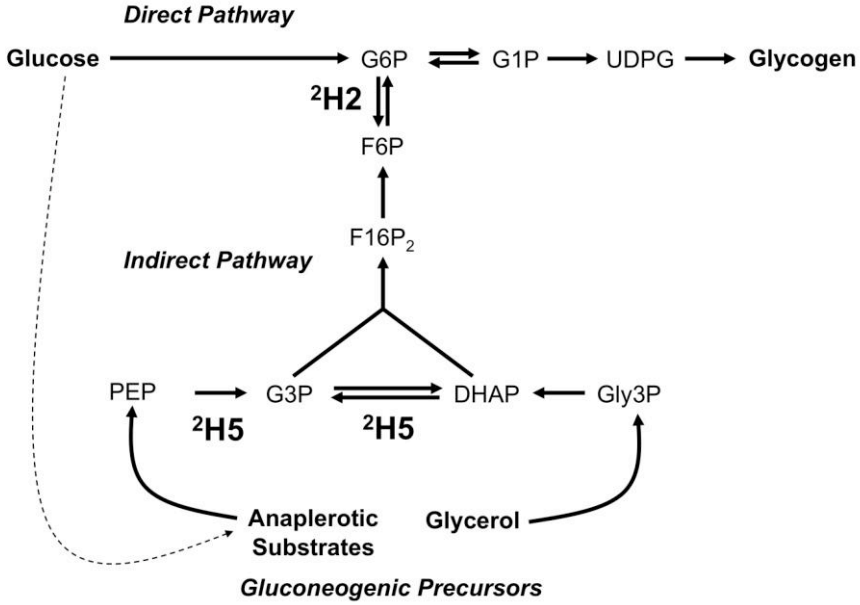

Fig. 10 Metabolic model representing the direct and indirect pathways of hepatic glycogen synthesis. Indirect pathway sources include gluconeogenic precursors that are metabolized via the anaplerotic pathways of TCA cycle (i.e. pyruvate and gluconeogenic amino acids) as well as glycerol from lipolysis. Indirect pathway sources also formally include pyruvate derived from glucose, as indicated by the dashed line. The metabolic pathway sites for enrichment of glycogen position 5 (^{2}H5) from ^2H_2O, namely enolase and triose phosphate isomerase, and enrichment of position 2 (^{2}H2) via glucose 6-phosphate isomerase are also indicated. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P = glucose 6-phosphate; G1P = glucose 1-phosphate; UDPG = uridine diphosphate glucose; F6P = fructose 6-phosphate; $F16P_2$ = fructose 1,6-bisphosphate; G3P = glyceraldehyde 3-phosphate; DHAP = dihydroxyacetone phosphate; Gly3P = glycerol-3-phosphate; PEP = phosphoenolpyruvate.

This method is readily adaptable to the study of fish glycogen kinetics since ^2H_2O can be added to the bulk aquarium water where it rapidly equilibrates with the fish's body water (Viegas et al. 2011). The tracer is incorporated into hepatic glycogen under natural free-swimming conditions thereby providing a true metabolic profile of feeding and fasting fish. Hepatic

glycogen that is synthesized during the period in $^{2}H_2O$ is enriched with ^{2}H, and the ^{2}H-enrichment distribution within its glucosyl units informs the fractional synthetic or turnover rate of the hepatic glycogen pool and also the direct and indirect pathway contributions to this flux (Soares et al. 2009). The glucosyl ^{2}H-enrichment distribution can be quantified by deuterium nuclear magnetic resonance (^{2}H-NMR) analysis of the monoacetone glucose (MAG) derivative (Schleucher et al. 1999; Jones et al. 2001b; Jones et al. 2006a). With this approach, we demonstrate that for captive seabass 21d fasted fish, refed on a commercial fishmeal diet, essentially all postprandial hepatic glycogen was synthesized from gluconeogenic precursors via the indirect pathway. Moreover, the observed ^{2}H-enrichment distribution suggests a high control of glycogen synthesis under these conditions by G6P-isomerase.

Material and Methods

Fish handling and sampling

For the present study, a total of 18 European seabass (*D. labrax*) provided by a local farm were transported to the lab and randomly assigned to 3 different tanks (n=6 per tank; initial mean length of 28.0±1.7 cm and initial mean body weight of 218.0±43.0 g). Each group of fish was acclimated at 18°C and 30‰ salinity in 200 L tanks 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 7d and maintained within optimal ranges. Fish in one of the tanks were provided with food once a day (2% mean body weight d^{-1}; Dourasoja Ultra 5, SORGAL, S.A; 44% protein, 18% fat, 2.2% carbohydrates, 9.2% ash, 5 mm standard pellet). Fish in the remaining two tanks were fasted for 21d. Following this period, each group was transferred to a $^{2}H_2O$-enriched seawater tank for 3d. This 200 L tank was maintained with an independent closed filtering system but had similar characteristics as the other tanks used during the rearing experiment in terms of size, opacity, filtering material and water parameters. Seawater was enriched with $^{2}H_2O$ to 5% with the addition of 99%-enriched $^{2}H_2O$ (Eurisotop, France) as

previously described by Viegas et al. (2011). Feeding of the previously fed group was maintained following transfer to the ^{2}H-enriched seawater. For the two fasted groups, one was kept fasted while the other was refed once a day over the 3d interval in ^{2}H-enriched seawater. Fed and refed fish were provided with last meal 24h before sacrifice (after 48h in 5% $^{2}H_2O$-enriched seawater). For metabolite sampling, fish were anesthetized in a 30 L tank of 5% ^{2}H-enriched saltwater containing 0.1 g L^{-1} of MS-222 for approximately 2min. Fish were measured, weighed and sampled for blood from the caudal vein with heparinized syringes. Blood was kept on ice and centrifuged (3000 g for 10min) to separate plasma which was stored at -20ºC for later assessment of ^{2}H-enrichment of plasma water (PW) and glucose quantification with a commercial assay kit (Invitrogen, Spain). Fish were sacrificed by cervical section; livers were excised, weighed, freeze-clamped in liquid N_2 and stored at -80ºC until further analysis. Experimental procedures complied with the Guidelines of the European Union Council (86/609/EU).

Hepatic glycogen extraction

Glycogen was extracted by alcoholic precipitation after alkaline tissue hydrolysis following Good et al. (1933). Briefly, frozen liver powder was treated with 30% KOH (2 mL per gram of liver) at 70ºC for 30min After vigorous vortex, the mixture was treated with 6% Na_2SO_4 (1 mL per gram of liver) and 99.9% ethanol (to a final concentration of 70%, 7 mL per gram of liver) and left overnight at 4ºC to precipitate glycogen. After centrifugation, the upper liquid phase was discarded and the solid residue 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 hydrolyse glycogen to its glucosyl units. Samples were incubated overnight at 55ºC 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.

Derivatization of glucose to monoacetone glucose (MAG)

In order to optimize the signal resolution in the NMR spectra, glucose obtained from glycogen isolation and hydrolysis was derivatized to MAG. Briefly, the glucose preparation was lyophilized and vigorously mixed with 5 mL acetone containing 4% sulphuric acid (v/v), both enriched with deuterium to 2%. The ^{2}H-enriched acetone was prepared and dried as previously described by Nunes & 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 1M $NaHCO_3$ followed by reacidification of the solution to pH 2.0 with 1M HCl. The newly formed diacetone glucose was hydrolyzed to MAG by incubation at 40ºC for 5h. The solution pH was then increased again to 8 with 1M $NaHCO_3$ 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 $NaHCO_3$ (9 mg/mL) for ^{2}H-NMR analysis.

^{2}H-NMR analysis

Plasma body water ^{2}H-enrichments were determined from 10 µL of plasma by ^{2}H-NMR as described by Jones et al. (2001a). Proton-decoupled ^{2}H-NMR spectra of MAG samples were obtained at 50ºC with a 11.75 T with a Varian Unity 500 system equipped with a 5-mm broadband probe (Varian, Palo Alto, CA) with the observe coil tuned to ^{2}H. Fully relaxed spectra were acquired with a 90º pulse and 1.7s of recycling time (1.6s of acquisition time and 0.1s pulse delay). Up to 12,000 scans were collected per sample, corresponding to a maximum of 6h collection time. The summed free induction decays were processed with 0.5–1.0 Hz line-broadening before Fourier transform. MAG ^{2}H-enrichments were quantified from the ^{2}H-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. Each reference signal is derived from three equivalent hydrogens enriched to 2.5% with ^{2}H such that

each signal represents an enrichment level of 7.5%. Spectra were analyzed with the NUTS PC-based NMR spectral analysis software (Acorn NMR Inc., USA).

Quantification of hepatic glycogen synthesis rates and sources

Fractional synthetic or turnover rates for hepatic glycogen synthesis during the period in $^{2}H_2O$-enriched water were estimated from the fractional enrichment of glycogen glucosyl units relative to that of plasma water (PW). Direct and indirect pathway contributions to glycogen synthesis were resolved by positional ^{2}H-enrichment analysis. For the refeeding study, net glycogen synthesis rates were estimated from the mean difference in glycogen concentrations between fasted and refed groups. The indirect pathway contribution to net glycogen synthesis was estimated from the ratio of glucosyl position 5 enrichment (H5) of glycogen and that of PW as follows:

$$\text{Indirect pathway contribution (\%)} = 100 \times H5/PW \quad (1)$$

The fraction of liver glycogen that was not enriched in H5 consists of glycogen units that were synthesized via the direct pathway and endogenous unlabeled glycogen that was present before administration of $^{2}H_2O$, represented as follows:

$$\text{Pre-existing glycogen + direct pathway contribution (\%)} = 100 - \text{Indirect pathway contribution} \quad (2)$$

The fraction of pre-existing glycogen was estimated from the ratio of glycogen levels measured in the group of fasted fish according to the following equation:

$$\text{Pre-existing glycogen fraction (\%)} = 100 \times \text{fasting glycogen/refeeding glycogen} \quad (3)$$

The fraction of glycogen derived from the direct pathway was calculated from the difference between total unlabeled glycogen and pre-existing glycogen fractions.

For fish that were either continually fed or continually fasted, hepatic glycogen levels were assumed to be constant over the 72h $^{2}H_2O$ administration period. Therefore, enrichment of hepatic glycogen under these conditions was assumed to reflect glycogen turnover, i.e. the simultaneous breakdown and synthesis of glycogen. The synthesis phase results in the labeling of glycogen from $^{2}H_2O$. Since there is no net glycogen synthesis under these conditions, estimation of direct pathway synthesis by equations (2) and (3) are not applicable. Instead, the direct pathway contribution was inferred from the ratio of H5 to position 2 (H2) glycogen enrichment (H5/H2) on the basis that the indirect pathway results in enrichment of both H5 and H2, while the direct pathway results in enrichment of H2 only (Jones et al. 2006b; Soares et al. 2009).

Glycogen fractional turnover via indirect pathway (%) = 100 × H5/PW (4)

Glycogen fractional turnover via direct pathway (%) = 100 × (1-H5/H2)/PW (5)

Statistical analysis

Values are presented as mean ± standard deviation (SD). Analysis of Variance (ANOVA) was used to test the existence of significant differences between nutritional statuses for various parameters. *A posteriori* Tukey's multiple comparisons test was performed when significant differences were found. Student's two-tailed unpaired t-test was used to compare means between fasted and refed fish. Differences were considered statistically significant at $P<0.05$.

Results

The 21d fasting interval resulted in a 30% lower final body weight (FBW) and 10% smaller final body length (FBL) compared to daily-fed controls. Fish that were fasted for 21d followed by refeeding once a day for 3d showed intermediate values of weights and lengths that were not

significantly different from either continuous fed or fasted fish. The physiologic indices, HSI and PFSI, did not differ between the three groups reflecting no gross alterations in energy reserves between fasted and fed fish. Meanwhile, plasma glucose and hepatic glycogen levels were highly sensitive to nutritional status, with fasting glucose levels being about one-half and hepatic glycogen levels being approximately 1/6th those of daily-fed fish. After 3d of refeeding, both plasma glucose and hepatic glycogen levels were fully restored to those of daily-fed fish (Table 4).

Table 4 Effects of different feeding status (fed, fasted and refed) on total length, body weight, hepatosomatic index, perivisceral fat somatic index, plasma glucose concentration and hepatic glycogen concentration of European seabass (*D. labrax*)

	Feeding condition		
	Fed	**Fasted**	**Refed**
Final total length, in cm	29.2±1.6^{a}	26.3±1.9^{b}	27.6±1.3^{ab}
Final body weight, in g	247.3±54.3^{a}	173.3±38.1^{b}	211.5±27.0^{ab}
HSI[1]	1.9±0.2	1.7±0.4	1.9±0.3
PFSI[2]	3.9±0.8	4.3±1.8	4.3±1.3
Plasma glucose, in mM	10.7±6.3^{a}	4.8±1.2^{b}	9.3±1.4^{a}
Glycogen concentration			
in µmol glycosyl units g^{-1} liver	167.7±52.5^{a}	36.3±24.7^{b}	191.8±23.9^{a}
in µmol glycosyl units liver^{-1}	665.4±345.2^{a}	77.2±59.54^{b}	584.6±140.4^{a}

Values are means ± SD. Significant differences between different feeding conditions are indicated by different letters (one-way ANOVA followed by Tukey test, $P<0.05$).
[1]Hepatosomatic index = 100 x (liver weight / body weight).
[2]Perivisceral fat index = 100 x (perivisceral fat weight / body weight).

After 72h in $^{2}H_2O$-enriched tank water, plasma water ^{2}H-enrichment measured in selected fed fish was equivalent to that of the tank water (4.7±0.3% plasma body water *vs.* 5.0±0.1% tank water; not significant, $P=0.19$) thereby verifying that the body water precursor enrichment was equivalent to that of the tank water. As we recently demonstrated, equilibration of plasma and tank water enrichments occurs within 6h (Viegas et al. 2011).

The quantity of glycogen isolated from fed fish (~600 mmol) provided sufficient sample mass to precisely quantify ^{2}H-enrichment levels of ≥0.1% (the limit of quantification of the NMR

signal being defined by a signal to noise ratio of ≥10:1) and ≥0.5% for the smaller amount of glycogen (~60 mmol) recovered from fasted fish. These limits of quantification correspond to fractional synthetic rates over the 72h period in ^{2}H-enriched seawater of 0.1/4.7, or ~2% and 0.5/4.7, or ~10% for fed and fasted fish, respectively. In other words, the methodology was able to reliably quantify newly-synthesized glycogen fractions as low as 2% for fed fish and as low as 10% for fasted fish. ^{2}H-NMR spectra for MAG derived from hepatic glycogen are shown in Fig. 11. The ^{2}H-enrichment levels of hepatic glycogen, represented by the ^{2}H-NMR signals in the 3.0-6.0 ppm region, show distinctive differences between the three groups.

For the fed fish (Fig. 11A), there was no quantifiable excess ^{2}H-enrichment of glycogen from $^{2}H_2O$ in any of the hexose positions, including H5, meaning that the fractional synthetic rate was less than the 2% quantification limit of our measurement. This indicates a minimal turnover of hepatic glycogen under these conditions (Fig. 12). In contrast, spectra from fasted fish (Fig. 11B) showed strong signals from all seven carbon-bound glucosyl hydrogens indicating that glycogen had been synthesized during the 72h period in deuterated water. The glycogen ^{2}H-enrichment levels were about one half that of body water indicating that about one-half of the glycogen pool had been replaced over this period (Fig. 12).

The synthesized glycogen had equivalent enrichments of H5 and H2 hydrogens, showing that it was entirely derived via the indirect pathway (Soares et al. 2009); also see equations (4) and (5). This indirect pathway flux was likely to have had a significant contribution from *de novo* gluconeogenesis given the lower plasma glucose levels and fasting state (Table 4). For refed fish, hepatic glycogen ^{2}H-enrichment was even more extensive than for the fasted group (Fig. 11C) with H5 enrichment approaching that of plasma water. This indicates that the net increase in glycogen that was observed between fasting and re-feeding during the 72h period in $^{2}H_2O$ was entirely supported by indirect pathway flux despite the more favorable nutritional conditions for direct pathway activity.

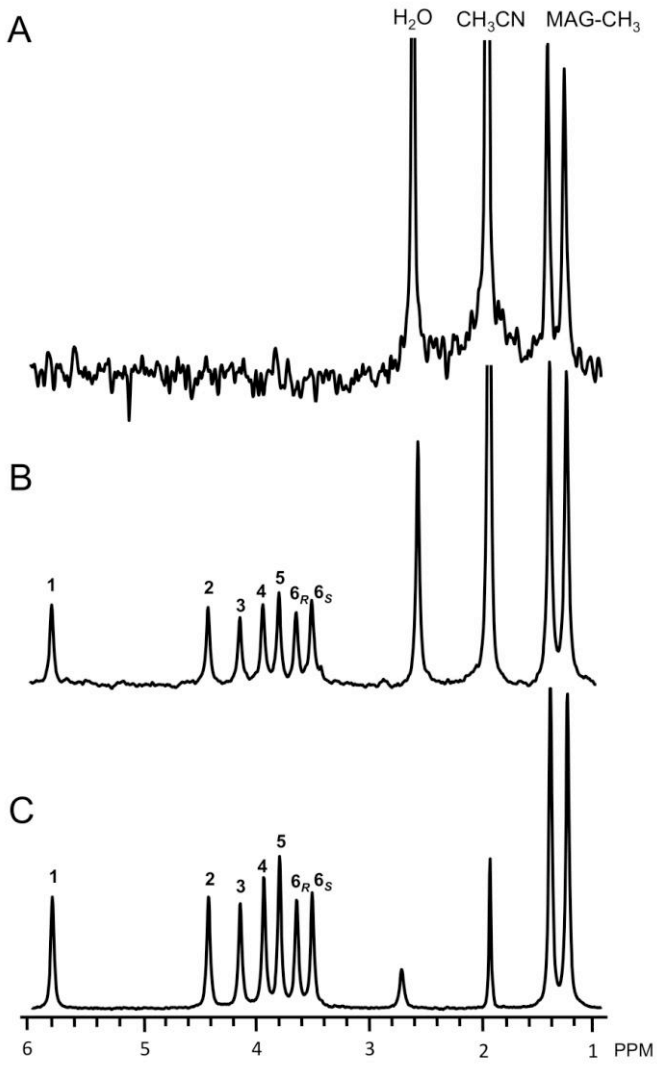

Fig. 11 ^{2}H-NMR representative spectra of monoacetone glucose samples derived from liver glycogen of fed (A) fasted (B) and refed (C) European seabass (*D. labrax*) sampled after 72 hours residence in tank with 5% ^{2}H-enriched water. The numbers above each signal represents its position within the original glucose molecule. Other signals include the pair of ^{2}H-enriched methyl signals used as internal ^{2}H enrichment references (MAG-CH_3) and the signal from the acetonitrile solvent (CH_3CN). The signal at 2.5 ppm is a residual ^{2}H-signal from water.

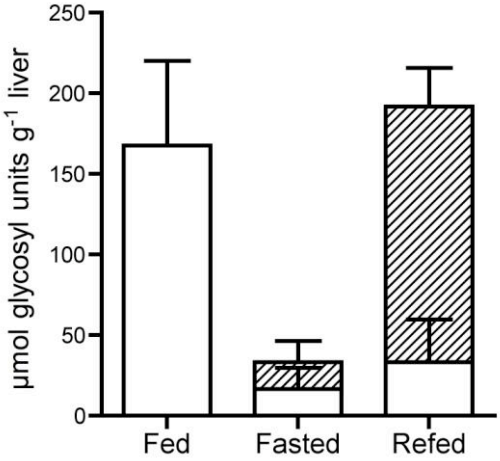

Fig. 12 Hepatic glycogen sources in European seabass (*D. labrax*) over 3 days following 21 days of feeding once per day (fed), 21 days of fasting (fasted) and 21 days of fasting followed by 3 days of feeding, once per day (refed). For each condition, the preexisting glycogen fraction is represented in white and the fraction of newly synthesized glycogen is represented as shaded.Values are means ± SD.

There was also a significantly higher level of enrichment in H5 compared to the other sites, including that of position 2. The lower enrichment levels of positions 1, 3, 4, 6_R and 6_S compared to H5 reflect the fact that enrichment of these positions is conditional on exchange between body water and metabolite hydrogens, whereas H5 enrichment is obligatory (Landau et al. 1996) (Table 5).

Table 5 Hepatic glycogen positional ^{2}H-enrichments (%) and plasma water (PW) enrichment (%) as quantified by ^{2}H-NMR

	^{2}H-positional enrichment							
	1	**2**	**3**	**4**	**5**	**6_S**	**6_R**	**PW**
Fed	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Fasted	2.1±0.2^{cde}	2.2±0.1^{bc}	1.7±0.1^{e}	2.3±0.2^{abc}	2.5±0.0^{a}	1.9±0.2^{d}	2.2±0.1^{ac}	4.7±0.3
Refed	3.5±0.2^{a§}	3.4±0.2^{a§}	2.7±0.2^{b§}	3.4±0.4^{a§}	4.5±0.4^{c§}	3.3±0.4^{a§}	3.2±0.5^{a†}	

Values are means ± SD; n.d. not determined. Significant differences between positional enrichments (one-way ANOVA) are indicated by different letters (Tukey test, $P<0.05$). Significant differences in enrichments of the same position between fasted and refed fish are indicated by symbols (*t*-test, $^{*}P<0.05$, $^{†}P<0.01$ and $^{§}P<0.001$).

Moreover, certain metabolite hydrogens, such as the methylenes of glycerol, are incorporated into glycogen without undergoing exchange with body water. Glycerol utilization thus dilutes the enrichments of positions 1 and $6_{R,S}$ relative to H5 (Landau et al. 1996; Jones 2007). The higher enrichment of glucose or glycogen H5 relative to positions 1, 3, 4, 6_R and 6_S from ^2H_2O is generally observed in humans (Jones et al. 2001b; Weis et al. 2004; Chevalier et al. 2006; Browning et al. 2008), other mammals (Jin et al. 2004; Burgess et al. 2005; Soares et al. 2009; Soares et al. 2010) and for plasma glucose of seabass (Viegas et al. 2011). H2 enrichment is an important exception, since theoretically, it cannot be less than that of H5 based on the known exchanges between metabolite precursor hydrogens and body water. For refed fish, our observation of significantly lower ^2H-enrichment of glycogen H2 relative to H5 is inconsistent with this premise and indicates that additional factors besides metabolite-water hydrogen exchanges may be involved in the glycogen ^2H-enrichment distribution.

Discussion

General considerations

Hepatic glycogen synthesis and turnover in seabass were studied under defined nutritional conditions using a novel ^2H_2O tracer method combined with biochemical assay of hepatic glycogen levels. The observed sensitivity of hepatic glycogen levels to fed and fasted states and the rapid repletion of hepatic glycogen during refeeding have been well described in seabass and other fish species (Soengas et al. 1996; Metón et al. 1999b; Pérez-Jiménez et al. 2007). The novel insights that were gained with the ^2H_2O tracer include observation of glycogen synthesis and turnover fluxes under different nutritional states and defining the direct and indirect pathway contributions to these fluxes in free-swimming fish. Overall, while our results are in agreement with the general consensus of limited dietary glucose utilization by carnivorous fish they nevertheless provide new insights on the relationship between dietary carbohydrate utilization and hepatic glycogen metabolism.

Postprandial hepatic glycogen synthesis in carnivorous fish

In humans and other omnivorous mammals, about 20% of dietary carbohydrate is utilized as hepatic glycogen and the direct pathway accounts for the majority of this conversion. For carnivorous fish, dietary carbohydrate levels are much lower, and their fractional conversion to hepatic glycogen is also substantially less (Blasco et al. 2001). Nevertheless, glycogenesis from dietary carbohydrate may be highly influenced by different diets and nutritional states.

In studies with ^{14}C-glucose, Blasco et al. (2001) reported that 0.9-1.3% of plasma glucose was incorporated into hepatic glycogen in 3d or 18d fasted brown trout (*Salmo trutta*). Following intra-aortic administration of an exogenous glucose load (500 mg/kg) to mimic refeeding, this fraction rose substantially to ~7.4% of total glucose utilization - still well below that of humans and mammals. Felip et al. (2012) studied rainbow trout (*Oncorhynchus mykiss*) that was fed once a day with a diet containing 30% raw or gelatinized starch, and measured after a single meal the incorporation of 3%-enriched ^{13}C-starch into hepatic glycogen. Under these conditions, hepatic glycogen synthesis accounted for only 0.4-0.6% of ingested isotopic starch over a 24h period. In our study, fish received ~4.9 g per fish per day of food with the carbohydrate portion accounting for 0.1 g per fish per day, or 0.3 g over the 72h refeeding period. Assuming similar fractional carbohydrate utilization rates for hepatic glycogen synthesis to those reported by Felip et al. (2012) (~0.5%), this means that 1.6 mg or 9.1 µmol of carbohydrate were converted to liver glycogen over the 72h period. This amounts to only ~2% of the ~500 µmol of hepatic glycogen per fish that was newly synthesized over this period. Our observations of negligible hepatic glycogen turnover for daily-fed fish are concordant with these estimates. Moreover, they indicate that turnover of hepatic glycogen by either direct or indirect pathways was negligible for daily-fed fish under our study conditions.

As expected, hepatic glycogen kinetics were more dynamic during refeeding, with a high rate of net synthesis over 72h. While the fishmeal diet was low in carbohydrate, there was nevertheless sufficient available over the 3d feeding period (1540 µmol) to account for all net hepatic glycogen synthesis (~500 µmol) - had it been efficiently utilized for this purpose. If the

fraction of dietary carbohydrate utilized for hepatic glycogen synthesis was equivalent to the 7.4% reported for the glucose refeeding studies of Blasco et al. (2001), this would have contributed ~115 µmol of glucosyl equivalents and would have accounted for ~20% of net hepatic glycogen synthesis. If this had been metabolized via the direct pathway, it would have been detected as a decrease in the ratio of glycogen H5 enrichment to PW. On the other hand, if the carbohydrate fraction had been metabolized to glycogen via the indirect pathway, it would have contributed to the observed H5 enrichment and would not be distinguishable from *de novo* gluconeogenic contributions. The 7.4% utilization rate of meal carbohydrate for hepatic glycogen synthesis might represent an upper limit since a pure glucose load is more effective at promoting insulin secretion and activating hepatic glycogen synthesis compared to standard fishmeal (Blasco et al. 2001; Enes et al. 2011b). Accordingly, the 20% dietary carbohydrate contribution to glycogen repletion based on the utilization rates of Blasco et al. (2001) is also a high-end estimate and we conclude that the large majority of hepatic glycogen was synthesized *de novo* from dietary amino acids.

Comparing our results from seabass with the data of Blasco et al. (2001) from trout, it appears that saltwater and freshwater carnivorous fish share similar characteristics of sluggish postprandial hepatic glycogen synthesis from glucose when fed with a diet low in carbohydrate. However, there appear to be adaptations in hepatic glycogen utilization that are linked to osmoregulation. Freshwater fish such as the rainbow and brown trout generally up-regulate glucose metabolism and supply of systemic glucose to fuel glycolysis in gills for ion-regulation mechanisms may come from mobilization of liver glycogen reserves (Tseng & Hwang 2008). Accordingly, changes in hepatic glycogenolysis have been attributed as part of the overall metabolic adaptation to salinity changes in rainbow trout (Sangiao-Alvarellos et al. 2003; Chang et al. 2007). It is not known to what extent hepatic glycogen synthesis rates and sources are altered in these settings.

Previous evidence for indirect pathway synthesis of hepatic glycogen in fish

In mammalian and human liver, glycogen synthesis via the indirect pathway is supported by a substantial and consistent body of experimental data. For fish, the data are fewer and less consistent. This is due in part to methodological constraints, both for *in situ* liver and isolated hepatocytes. However, the evolving definition of the indirect pathway has also played an important factor. Initially, the indirect pathway was formally defined as a glucose-metabolizing pathway (i.e. glucose→ G6P→ C3 intermediates→ G6P→ glycogen) and the first studies of direct and indirect pathway fluxes were performed with glucose tracers. Subsequently, with the realization that the "C3-intermediates" included pyruvate generated via PEP-carboxykinase (Newgard et al. 1984) it became clear that true gluconeogenic precursors such as glycerol, alanine and other gluconeogenic amino acids could also contribute to indirect pathway flux. Hence nowadays, the indirect pathway is assumed to include *de novo* gluconeogenic contributions as well as that from glucose. Therefore in principle, indirect pathway activity may be revealed with either labeled glucose or labeled gluconeogenic precursors. With labeled glucose, indirect pathway activity results in a dilution of the label at the level of UDP-glucose or glycogen. If no other substrates are being metabolized by the liver (a standard assumption for isolated hepatocytes in minimal medium with glucose), labeled glucose that is metabolized via the indirect pathway undergoes randomization of the label via the TCA cycle resulting in the appearance of new [2-^{13}C], [5-^{13}C] and [6-^{13}C]glycogen isotopomers. Thus, the amount of [6-^{13}C] relative to [1-^{13}C]glycogen was used to estimate direct and indirect pathway activities in rainbow trout hepatocytes incubated with 10 mM [1-^{13}C]glucose (Pereira et al. 1995). Excess enrichment of [1-^{13}C]glycogen, but not [6-^{13}C]glycogen was reported, and it was concluded that indirect pathway activity was insignificant. The major limitation of this analysis is that even if glucose is the only substrate utilized by the liver, there is a substantial but undefined dilution of the indirect pathway ^{13}C-isotopomers due to TCA cycle exchange. Metabolism of unlabeled exogenous or endogenous gluconeogenic substrates further adds to this dilution. Thus, the inability to detect [6-^{13}C]glycogen does not necessarily translate to the absence of indirect pathway activity.

In vitro studies of fish hepatocyte glycogen labeling from gluconeogenic precursors such as ^{14}C-alanine have also been inconclusive or negative. When Canals et al. incubated both postabsorptive and fasted brown trout hepatocytes with [U-^{14}C]alanine, radioactivity was recovered in glucose but not in glycogen (Canals et al. 1992). French et al. (1981) incubated rainbow trout hepatocytes from both fed and fasted fish with a mixture of ^{14}C-labeled substrates that included amino acids and lactate. They found no significant incorporation of ^{14}C-label into glycogen for either group. Such *in vitro* studies of glycogen synthesis may be hampered by the effects of stress-induced catecholamine release triggered by the isolation procedure that promotes glycogenolysis. Mommsen et al. (1988) demonstrated that without prior β-adrenergic blockade, isolated hepatocytes continuously hydrolyzed their endogenous glycogen regardless of the media nutrient composition. Under these conditions, indirect pathway activity would be difficult or impossible to detect with any certainty. We are unaware of any previous measurements of direct and indirect pathway fluxes that have been described for *in situ* fish liver (Table 6).

Table 6 Hepatic glycogen sources and synthesis fluxes in refed European seabass (*D. labrax*) placed in deuterated water for 72 hours. Estimates based on the ratio of glycogen position 5 enrichment to plasma water (H5/PW) are shown

	Hepatic Glycogen Sources			
	Pre-existing	Newly Synthesized		Total Glycogen
		Indirect pathway	Direct pathway	
Absolute Contributions (μmol g^{-1} refed liver)	33±26†	171±15	-13±15	192±24
Fractional Contributions (%)	17±13	90±8	-7±10	100
Mean synthetic rate (μmol g^{-1} refed liver hr^{-1})	-	2.38±0.21	-0.18±0.21	-

Values are means ± SD. †From the mean liver glycogen concentrations observed in 24-day fasted fish.

The methodologies of Felip et al. (2012) and Blasco et al. (2001) discussed earlier do not distinguish between direct and indirect pathway incorporation of the starch or glucose precursor

into glycogen since positional labeling information of glycogen was not recovered by the described IRMS or radioisotope analyses.

Methodological considerations for *in situ* measurements of direct and indirect pathway fluxes

Compared to classical carbon tracer methods, $^{2}H_2O$ has certain advantages and disadvantages for studying hepatic glycogen synthesis. The $^{2}H_2O$ method is versatile in that 1); it can be applied to any diet and feeding regime 2); it captures the metabolic profile of naturally feeding fish, and 3); the tracer cost per fish studied is low since the ^{2}H-enriched water tank may be repeatedly used. Its principal disadvantage is that direct pathway precursors are not uniquely labeled like those of the indirect pathway (i.e. enrichment of hepatic G6P position 2 is common to both direct and indirect pathway activities). Moreover, as shown by our data, there appears to be discrimination of H2 enrichment from $^{2}H_2O$ relative to H5 as a result of a kinetic isotope effect - a phenomenon that is not observed with ^{13}C tracers. These results underestimate direct pathway contributions as calculated by the ratio of glycogen H5 to H2 ^{2}H-enrichments. Because of this, the direct pathway contribution is calculated from the ratio of glycogen H5 to plasma water enrichment after correction for pre-existing glycogen measured from a fasted cohort. As a result, the methodology does not provide precise estimates for very low fractional direct pathway contributions, i.e. below ~10% of newly synthesized glycogen. On the other hand, the $^{2}H_2O$ method does provide a direct readout of indirect pathway contributions and provides definitive proof of this pathway's activity in refeeding fish. With glucose tracers, direct and indirect pathway contributions are inferred from the isotopic dilution of the tracer following its uptake from the plasma glucose pool into hepatic glycogen (Taylor et al. 1996; O'Doherty et al. 2000; Soares et al. 2010). Therefore, this approach also requires correction for label dilution by pre-existing glycogen. Moreover, direct and indirect pathway estimates obtained from glucose tracers assume a constant level of plasma glucose precursor enrichment during the glycogen synthesis interval. This condition may be difficult to establish during feeding due to postprandial

fluctuations in plasma glucose concentrations and rates of appearance. In any case, frequent sampling of plasma glucose over the feeding interval is required for quantifying precursor enrichment levels. With $^{2}H_2O$, constant precursor enrichment levels are established and maintained throughout the feeding protocol.

Depleted ^{2}H-enrichment of glycogen position 2 from $^{2}H_2O$

Our experimental data show a significantly lower H2 enrichment relative to H5 for refed fish which contradicts the assumption that for glycogen derived via the indirect pathway, the positions 2 and 5 hydrogens are both enriched to the same level as body water. The possibility that this could be an artifact of the NMR analysis or sample preparation can be dismissed since 1); ^{1}H-NMR analysis of the samples shows no other metabolites that could contribute ^{2}H signals in the spectral region of interest, and 2); test spectra of MAG prepared from [U-$^{2}H_7$]glucose show uniform signal intensities (data not shown). The remaining possibility is the selective discrimination of position 2 enrichment from $^{2}H_2O$ via G6P-isomerase as a result of a kinetic isotope effect. To our knowledge, this has never been observed in mammals and humans, but it has been reported for the synthesis of starch in plant chloroplasts (Schleucher et al. 1999) using the same ^{2}H-NMR methodology as described here. In these studies, ^{2}H-enrichment in position 2 of chloroplastic starch was found to be depleted by ~40% in comparison to other positions, including H5. This is identical to the relative depletion of H2 to H5 enrichments observed for glycogen after refeeding (2.7% *vs.* 4.5%). The depleted enrichment of position 2 implies that the rate of F6P to G6P conversion via G6P-isomerase is comparable to the rate of G6P disposal into polysaccharide synthesis (Schleucher et al. 1999) since isotope discrimination is abolished by fast exchange of F6P and G6P relative to G6P disposal into glycogen synthesis. These data suggest that G6P-isomerase may exert significant control over indirect pathway flux into hepatic glycogen under the experimental refeeding conditions. In contrast, glycogen that was enriched during fasting did not show a significant depletion of H2 enrichment suggesting that under these conditions, G6P-F6P exchange mediated by G6P-isomerase was relatively fast compared to

glycogenic G6P utilization and was likely not a rate limiting step for glycogen synthesis. In humans, G6P-F6P exchange has been shown to be fast relative to glycogenic G6P flux accounting for 1); near complete exchange of G6P position 2 hydrogen and that of body water and 2); no discernible isotopic discrimination of glucose or UDP-glucose H2 enrichment from $^{2}H_2O$ (Delgado et al. 2009; Barosa et al. 2012). There is some evidence that in fish, hepatic G6P-isomerase activity is sensitive to the nutritional state. In common carp (*Cyprinus carpio*) hepatic G6P-isomerase activity decreased in direct relation to feeding rates (Shimeno & Shikata 1993; Shimeno et al. 1997) but there is no information on how G6P-isomerase activity is modified during the fasting to feeding transition. One possible explanation is that induction of G6P-isomerase activity by feeding is slow compared to activation of glycogen synthesis fluxes. Under these conditions, G6P-isomerase activity could be a rate-liming step for indirect pathway synthesis of glycogen, at least in the initial stages of refeeding. In principle, sub-maximal G6P-isomerase activity could limit the glycolytic metabolism of G6P derived from glucose and favor its conversion to glycogen via the direct pathway (although this did not appear to be significant in our case) or its utilization by the pentose phosphate pathway.

CHAPTER IV

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

Introduction

For all higher organisms there is an absolute requirement for endogenous glucose provision since it is an essential energy substrate for the central nervous system and erythrocytes. Despite a large variation in dietary carbohydrate availability and whole body glucose demands between different 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 a change in dietary carbohydrate. For farmed carnivorous fish species such as the European seabass *D. labrax* L., there is current interest from both economic and ecological perspectives about their capacity to adapt from their natural high protein/low carbohydrate diet to feedstock where a significant portion of protein is replaced by carbohydrate (Fernández et al. 2007; Enes et al. 2009).

It is widely accepted that dietary carbohydrates are poorly utilized by seabass and other carnivorous fish: instead they are highly dependent on gluconeogenesis from amino acids for sustaining endogenous glucose demands (Enes et al. 2009; Moon 2001; Polakof et al. 2012; Stone 2003). In addition, plasma glucose levels are much less 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 and expression of hepatic gluconeogenic enzymes was not suppressed by chronic high-carbohydrate feeding thereby contributing to excessive postprandial levels of plasma glucose (Enes et al. 2006b, 2008b) and resembling the insulin resistant state of mammals. Meanwhile, the transition from feeding to fasting has been characterized by a steep drop in plasma glucose concentrations (Echevarría et al. 1997; Gutiérrez et al. 1991; Pérez-Jiménez et al. 2007; Viegas et al. 2011). Paradoxically, other fish species can tolerate extended periods of starvation without apparently suffering from complications of hypoglycemia like *Brycon cephalus* (Figueiredo-Garutti et al. 2002), gilthead seabream *Sparus aurata* (Polakof et al. 2006; Sangiao-Alvarellos et al. 2005) or Senegalese sole *Solea senegalensis* (Costas et al. 2011). To date, our knowledge of fish carbohydrate 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 insight into fish glucose metabolism, it does not directly unravel the metabolic and/or absorptive processes that contribute to the appearance of plasma glucose. Hence, the contribution of dietary carbohydrate absorption *vs.* endogenous synthesis to plasma glucose levels remains incompletely defined.

Deuterated water (^2H_2O) reveals the fraction of plasma glucose derived from glucose 6-phosphate (G6P) via glucose 6-phosphatase (Basu et al. 2008; Nunes & 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 plasma glucose (Landau et al. 1996) (Fig. 13). This approach was recently applied to determine sources of glucose synthesis in seabass (Viegas et al. 2011). These studies revealed that for both fed and fasted fish, plasma glucose rapidly became enriched with ^2H with a H5/H2 of ~1.0, indicating that gluconeogenesis was the main source of hepatic G6P production regardless of nutritional state. However, for fasted fish, where plasma glucose appearance was expected to be completely dependent on G6P hydrolysis, the fraction of plasma glucose derived from G6P, as determined by the ratio of H2 enrichment to that of plasma water (PW), was unexpectedly low, accounting for only 60-70% of the total. This implies that a significant fraction (30-40%) of plasma glucose was unlabeled with ^2H and therefore could not have been derived via G6P. Furthermore, the fish were sampled for plasma glucose enrichment after 72h in ^2H_2O to ensure that the plasma glucose pool had completely turned over in the presence of the tracer. This ensured that the presence of unlabeled plasma glucose could not be explained by pre-existing unlabeled glucose. 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 (GK, EC 2.7.1.2). To our knowledge, mRNA levels for both of the enzymes have never been addressed in the seabass in any condition.

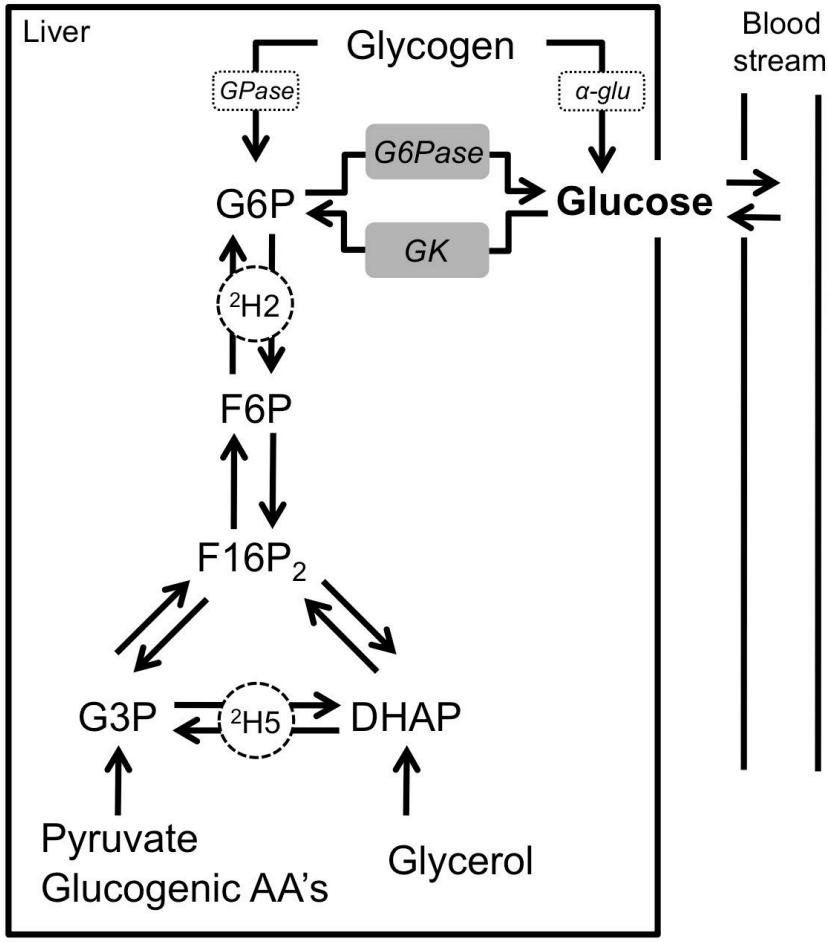

Fig. 13 Metabolic model representing gluconeogenis and glycogenolysis in the liver. Gluconeogenic precursors are represented by pyruvate and gluconeogenic amino acids (metabolized via the anaplerotic pathways of TCA cycle) as well as glycerol from lipolysis. The sites for glucose enrichment in position 5 (^{2}H5) (via triose phosphate isomerase) and enrichment in position 2 (^{2}H2) via glucose 6-phosphate isomerase from ^2H_2O are also indicated. 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; G3P = glyceraldehyde 3-phosphate; DHAP = dihydroxyacetone phosphate; AA's = amino acids; GK = glucokinase; G6Pase = glucose 6-phosphatase; GPase = glycogen phosphatase; α-glu = α-glucosidase

Methods

Fish handling and sampling

Fish handling and rearing was performed as described in the Methods Section of Chapter III. For sampling, fish were sacrificed by cervical section; liver and muscle were excised, weighed, freeze-clamped in liquid N_2, grinded and stored at -80ºC until further analysis. Glycogen was extracted by alcoholic precipitation after alkaline tissue hydrolysis and glycogen was broken into its glucosyl units with amyloglucosidase from *Aspergillus niger* (Glucose-free preparation, Sigma-Aldrich, Germany) as described in Viegas et al. (2012). After digestion free glucose was quantified using a commercial assay kit (Invitrogen, Spain).

Blood sampling and blood glucose derivatization

Blood sampling and blood glucose derivatization was performed as described in the Methods Section of Chapter II.

^{2}H-NMR analysis

^{2}H-NMR analysis was performed as described in the Methods Section of Chapter II.

Quantification of plasma glucose sources

For fasting fish, sources of plasma glucose were resolved by positional ^{2}H-enrichment analysis relative to that of plasma water (PW) as described earlier (Viegas et al. 2011). For fasted fish, glucose that was not enriched in position 2 was assumed to be derived from unlabeled glycogen via α-glucosidase or glycogen debranching enzymes (non-G6P sources), processes that do not incorporate ^{2}H into this (or any other) position.

$$\text{Plasma glucose from G6P (\%)} = 100 \times H2/PW \quad (6)$$

$$\text{Plasma glucose from non-G6P sources (\%)} = 100 - \text{G6P sources} \quad (7)$$

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:

$$\text{G6P from gluconeogenesis (\%)} = 100 \times H5/H2 \quad (8)$$

$$\text{G6P from glycogenolysis (\%)} = 100 - \text{G6P from gluconeogenesis} \quad (9)$$

For fed and refed fish, the contribution of non-G6P sources also includes that of absorbed dietary glucose (Fig. 13).

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 acid, 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 (Kinematica GmbH, Switzerland) mixer (position 3, 30s) followed by centrifugation at 15,800 g for 40min at 4ºC. Crude extracts were filtered through 1 mL Sephadex G 25 column and 5 µL were 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 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 (Kinematica GmbH, Switzerland) mixer (position 3, 30s) followed centrifugation at

10,000 g for 15min at 4ºC. The supernatant was recovered and centrifuged at 100,000 g for 90min at 4ºC 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 15min at 30ºC, were stopped by the addition of 80 µL cold buffer C (100 g L^{-1} trichloroacetic acid, 20 g L^{-1} ascorbic acid) and G6Pase activity was determined by the increment in glucose production. Glucose was determined at 500 nm by a colorimetric assay kit (Glucose MR, Cromatest, Linear Chemicals, Spain) and total protein was determined at 600 nm by the Bradford method (Bio-Rad, Spain) using bovine serum albumin as a standard. Both of the above mentioned assays were performed at 30ºC and adapted for automated measurement using a Cobas Mira S spectrophotometric analyzer (Hoffman-La Roche, Switzerland).

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 37ºC for 1h, according to supplier's instructions.

RT-PCR analysis

Amplification by RT-PCR was carried out using the pairs of primers presented in Table 7. These 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 *Sparus aurata* (partial or

complete sequences). The sequences were aligned using EMBL-EBI ClustalW2 - Multiple Sequence Alignment Tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) for comparing homologies 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 10x PCR buffer, 200 µM dNTPs, 0.5 µM of primers (forward and reverse), and 1 U of Expand High Fidelity PCR System (Roche).

Table 7 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**[1]	**Forward**	ACGGACGAAAGCGAAAGCA	91
		Reverse	GGAACTACGACGGTATCTGATC	
	GK[2]	**Forward**	GAATGACACAGTAGCCACCA	115
		Reverse	CACAGTCCTCATCTCCTC	
	G6Pase[3]	**Forward**	CCTGTGGATGCTAATGGGTC	83
		Reverse	TGACCTGATGTGGGAAGTGG	

[1]GenBank accession no. AM419038; [2]GenBank accession no. JX073704; [3]GenBank accession no. JX073707.

Amplification was conducted through 39 cycles of denaturation at 94ºC for 30s, annealing at 55ºC for 30s and DNA synthesis at 72ºC for 2min, followed by a final extension step of 5min. The PCR products obtained were separated electrophoretically on 1% agarose gel alongside 1 Kbp ladder (Biotools). Bands of 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 37ºC. 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 amongst different cDNA clones. Sequences were introduced in GenBank (accession numbers for GK and G6Pase are JX073704 and JX073707, respectively).

Quantitative real-time RT-PCR analysis

Specific primers for quantitative real time RT-PCR (qRT-PCR) were designed with Oligo Explorer 1.2 (Gene Link) (Table 7). 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 miliQ water instead of cDNA) were separated electrophoretically on 2% agarose gel alongside GeneRuler™ 100 bp DNA ladder (Fermentas) for length confirmation and discard presence of secondary bands. Extraction of mRNA and subsequent synthesis of single strand cDNA templates was performed as mentioned above from frozen liver samples. The reaction product was diluted 10x in miliQ water and real-time quantitative PCR was performed in an ABI Prism 7000 Sequence Detection 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: 50ºC for 2min, 95ºC for 10min, followed by 40 cycles with 95ºC for 15s and 62ºC for 1min.

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 n.º 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).

Statistical analysis

Values are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to test the statistical differences between nutritional statuses. *A posteriori* Dunnett's test was performed when significant differences were found in order to assess which conditions were significantly different from the control (fed fish). Differences were considered statistically significant at $P<0.05$.

Results

Physiological parameters and hepatic enzyme activities

The 21d fasting period resulted in significant decrease of fish weight thereby suggesting its dependence on endogenous sources for the endogenous glucose production (EGP). Plasma glucose concentrations were about one-half of fed fish while hepatic glycogen levels were highly depleted (Table 8).

Table 8 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on total length, body weight, plasma glucose and hepatic and muscle glycogen concentration

	Feeding condition		
	Fed	**Fasted**	**Refed**
Final total length, in cm	29.2±1.6	26.3±1.9*	27.6±1.3^{ns}
Final body weight, in g	247.3±54.3	173.3±38.1**	211.5±27.0^{ns}
Plasma glucose, in mM	10.7±6.3	4.8±1.2*	9.3±1.4^{ns}
Liver glycogen[1]	167.7±52.5	36.3±24.7**	191.8±23.9^{ns}
Muscle glycogen[1]	25.1±5.1	10.4±2.7**	14.4±2.7**

Mean values ± SD are presented. Significant differences in relation to fed fish are indicated by asterisks (one-way ANOVA followed by Dunnett's test, $^{*}P<0.05$, $^{**}P<0.01$ and ns not significant).
[1]in µmol glycosyl units g^{-1} tissue.

The activities and mRNA levels of hepatic GK and G6Pase for fed and for 21d fasted fish are shown in Figures 14 and 15, respectively. Both G6Pase activity and expression showed significant declines, suggesting a decreased capacity for EGP from hepatic G6P after prolonged fasting.

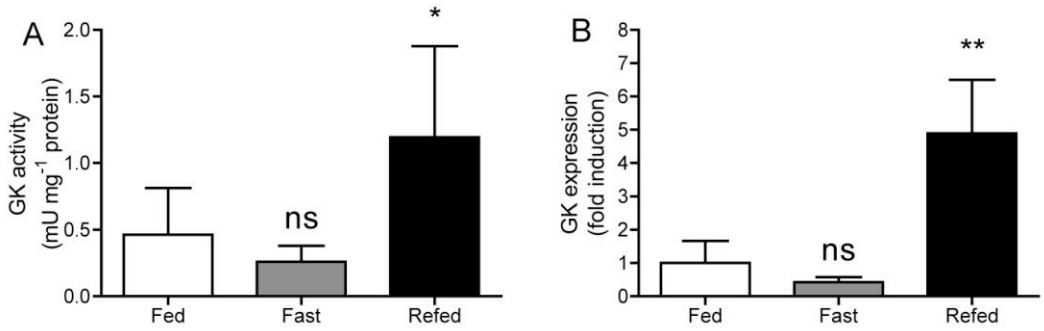

Fig. 14 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) (A) and relative abundance of mRNA (in arbitrary units) (B) of glucokinase. Mean values ± SD are presented. Significant differences in relation to fed fish are indicated by asterisks (one-way ANOVA followed by Dunnett's test, $^{*}P<0.05$, $^{**}P<0.01$ and ns not significant).

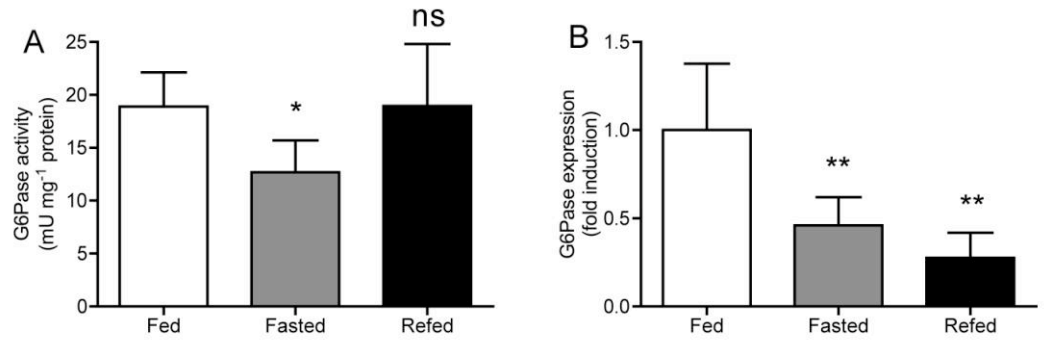

Fig. 15 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) (A) and relative abundance of mRNA (in arbitrary units) (B) of glucose 6-phosphatase. Mean values ± SD are presented. Significant differences in relation tofed fish are indicated by asterisks (one-way ANOVA followed by Dunnett's test, $^{*}P<0.05$, $^{**}P<0.01$ and ns not significant).

This was also consistent with the observed hypoglycemia. GK activity and its mRNA expression showed slight tendency to decrease in fasted fish compared to fed fish.

To determine whether hepatic metabolism of the 21d fasted fish was still responsive to dietary cues, a group of 21d fasted fish were subsequently refed over 3d. For these fish, glycogen levels

were restored to those of fed fish and this was accompanied by a significant increase in GK activity and expression while decrease in G6Pase expression was observed. Thus, the characteristic transition of hepatic metabolic activity from fasting to feeding state was functional after 21d of fasting for this group of fish.

^{2}H-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) verifying that the body water precursor enrichment was at isotopic steady-state. Plasma glucose enrichment from $^{2}H_2O$ was quantified by ^{2}H-NMR for all positions of the MAG molecule (Fig. 16). The plasma spectra from fasted and fed fish are highly consistent with our earlier studies and allowed us to precisely measure the glucose positional ^{2}H-enrichments, namely in positions 2 and 5 as shown in Table 9, using the intramolecular ^{2}H methyl signals (Jones et al. 2006b).

Table 9 Plasma glucose positional ^{2}H-enrichments (%) and plasma water (PW) enrichment (%) as quantified by ^{2}H-NMR

	^{2}H-positional enrichment							
	1	**2**	**3**	**4**	**5**	**6_S**	**6_R**	**PW**
Fed	2.1±0.5	3.1±0.3	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	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**	1.8±0.5	2.2±0.5	2.3±0.6	1.7±0.5	1.7±0.5	

Mean values ± SD are presented. Significant differences in relation to fed fish are indicated by asterisks (one-way ANOVA followed by Dunnett's test, *P<0.05, **P<0.01).

For fed fish, plasma glucose was relatively uniformly enriched, with equivalent enrichments of positions 5 and 2. The enrichment level of position 2 was 65% that of PW indicating that 65% of plasma glucose was derived from G6P with gluconeogenesis being the principal contributor (93%) to hepatic G6P synthesis. The remaining 35% of plasma glucose that was not enriched with ^{2}H was assumed to be derived from dietary carbohydrate absorption. For fish that were refed after the fasting period, G6P contributions to plasma glucose levels tended to be lower (44%),

compared to fed fish, possibly reflecting the reduced G6Pase activity as well as the inhibitory effects of high plasma glucose levels *per se* on EGP (Ader et al. 1985). As for the fed fish, the unlabeled fraction that accounted for ~56% of plasma glucose in the refed fish was presumed to be derived from dietary carbohydrate.

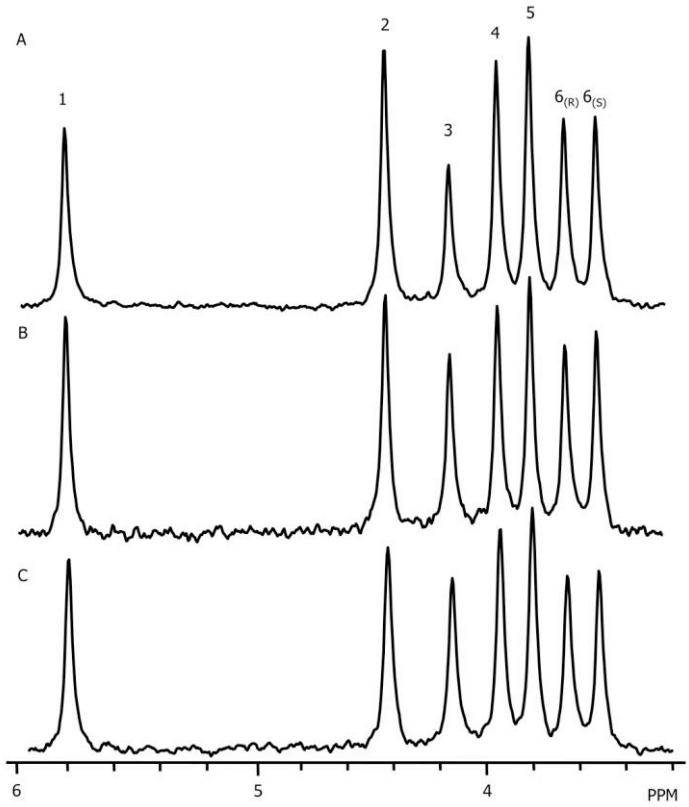

Fig. 16 ^{2}H-NMR representative spectra of monoacetone glucose (MAG) samples derived from blood glucose of fed (A), fasted (B) and refed (C) European seabass (*D. labrax*) sampled after 72h residence in tank with 5% ^{2}H-enriched water. The numbers above each signal (each of the 7 aliphatic hydrogen) represent each hydrogen within the original glucose molecule.

For the 21d fasted fish, analysis of plasma glucose ^{2}H-enrichment revealed an unexpectedly high fraction of unlabeled glucose (~52%) despite the absence of dietary carbohydrate sources for contributing plasma glucose via absorption. Enrichments of positions 5 and 2 were equal (student's t-test, *P*=0.28, *P*=0.93 and *P*=0.34 for fed, fasted and refed fish, respectively), indicating that hepatic G6P had been almost exclusively derived *via* gluconeogenesis (Table 10).

Table 10 Plasma glucose ^{2}H-enrichments for position 2 and 5 as quantified by ^{2}H-NMR, hepatic glucose sources and synthesis fluxes for fed, fasted and refed placed 72h in 5%-enriched deuterated water

Fractional contributions (%)	Fed	Fasted	Refed
G6P sources[a]	65±7	51±15^{ns}	44±10**
Gluconeogenesis[b]	93±6	98±3^{ns}	113±3**
Glycogenolysis[c]	7±6	2±3^{ns}	-13±3**
Non G6P sources[d]	35±7	48±15^{ns}	56±10**

Mean values ± SD are presented. Significant differences in relation to fed fish are indicated by asterisks (one-way ANOVA followed by Dunnett's test, $^{*}P<0.05$, $^{**}P<0.01$ and ns not significant).
[a]following equation (6); [b]following equation (8); [c]following equation (9); [d]following equation (7) where in the case of fasted fish corresponds only to EGP and in the case of fed and refed fish corresponds to EPG + absorbed dietary CHO.

Discussion

Nutritional regulation of G6Pase and GK

The liver plays a key role in fish glucose homeostasis during feeding and fasting in part by the reciprocal regulation of glucose phosphorylation to G6P via GK and through the hydrolysis of G6P to glucose via G6Pase (Fig. 13). Hepatic GK expression in fish is stimulated following feeding with moderate to high carbohydrate meals but this process takes several hours and is therefore ineffective at counter-acting the initial hours of postprandial hyperglycemia (Caseras et al. 2000; Panserat et al. 2001a). With low carbohydrate diets, hepatic GK activity and expression were maintained at basal levels suggesting a limited role of GK in glycemic control in this setting. During the onset of fasting, hepatic G6Pase activity and gene expression is increased above its postprandial level (Caseras et al. 2002; Kirchner et al. 2003a; Metón et al. 2004; Sangiao-Alvarellos et al. 2005) but plasma glucose levels fall towards the hypoglycemic range. In the early

postprandial stages when hepatic glycogen levels are maximal, G6P may be derived from both glycogenolysis and gluconeogenesis (Viegas et al. 2011). For most fish species, including the European seabass, hepatic glycogen stores are depleted during fasting, albeit at a slower rate compared to mammals (Metón et al. 1999, 2003; Pérez-Jiménez et al. 2007; Soengas et al. 2006). After an extended period of fasting, the residual levels of hepatic glycogen are unable to sustain significant rates of glucose production via glycogenolysis, hence there is an increased dependence on gluconeogenesis for EGP. This same pathway is also largely responsible for the repletion of hepatic glycogen during refeeding with standard fishmeal (Viegas et al. 2012).

G6Pase expression and activity seem to be insensitive to nutritional status in the rainbow trout (Panserat et al. 2000c), gilthead seabream (Caseras et al. 2002; Enes et al. 2008c), common carp (Panserat et al. 2002b) and seabass (Enes et al. 2006b) and this characteristic may in part explain the inefficient hepatic carbohydrate utilization by carnivorous fish. In this study, fasting provoked a significant decrease in G6Pase activity and mRNA levels, which contradicts other work that demonstrated either increased G6Pase activity in gilthead seabream (Caseras et al. 2002; Metón et al. 2004; Sangiao-Alvarellos et al. 2005), or no change in rainbow trout (Kirchner et al. 2008; Panserat et al. 2002a) in response to fasting. 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 hepatic glucose production with whole body glucose demand compared to the fish from our study.

Under a low carbohydrate diet as the one used in the present study, GK activity was very low in fed seabass as reported previously (Enes et al. 2008c; Moreira et al. 2008) as well as in others species (Enes et al. 2009), with no notable differences in regard to fasted fish (Pérez-Jiménez et al. 2007). Compared to the sluggish hormonal responses to feeding (Enes et al. 2010), postprandial GK activity is relatively dynamic with maximal activities reached after 6-8h post-feeding in rainbow trout (Kirchner et al. 2003a, 2005; Soengas et al. 2006; Panserat et al. 2000c) and gilthead seabream (Caseras et al. 2000) with a return to basal levels at 10h (diets with 6-17% starch). In contrast to the lack of significant GK activity excursions during regular daily feeding,

refeeding of fish with the same diet following a prolonged fasting period resulted in a high and sustained GK activity over several days, a characteristic also observed by others. After 3d 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 7d in rainbow trout (2.7-fold induction; Soengas et al. 2006). The return to control levels can be observed before the 12th day (Pérez-Jiménez et al. 2007). Interestingly, despite the high plasma glucose 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.

Sources of plasma glucose during prolonged fasting

Our results demonstrate that for 21d fasted fish, approximately half of the plasma glucose was not enriched with ^{2}H from $^{2}H_2O$. 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 $^{2}H_2O$ regardless of the G6P source (Landau et al. 1996). The possibility that the unlabeled glucose fraction reflects incomplete turnover of the plasma glucose pool (i.e. glucose that was present before $^{2}H_2O$ administration), is unlikely based on the known glucose transit times - equivalent to the half-life of plasma glucose residence. For 2-9d fasted seabass, the glucose transit time was estimated to be ~3h using the single injection tracer method (Garin et al. 1987) and for 20d fasted kelp bass (*Paralabrax* sp.), a closely related species, transit times of ~3.5h were reported using the same method (Bever et al. 1977). Given that after 7 half-lives (a period of 21-25h based on the reported transit times), greater than 99% of plasma glucose will have turned over. In this light, the 72h period in ^{2}H-enriched tank water administration was more than sufficient for complete turnover of the plasma glucose pool with constant ^{2}H-water precursor enrichment. It is worth noting that Haman and Weber (1996) measured glucose turnover in rainbow trout by primed-constant infusion - considered to be the

gold standard method - and found significantly higher glucose appearance rates compared to previous measurements based on tracer single-injection. If the glucose appearance rates of seabass were also underestimated by single-injection, then the true plasma glucose transit time may be even shorter than the aforementioned published estimates.

Of the known alternative glucose-producing pathways that could generate unlabeled glucose in the presence of $^{2}H_2O$, glycogen conversion to glucose via α-glucosidase and/or glycogen-debranching enzymes is the most plausible, since these do not involve exchange of glucose and water hydrogens. If the reported EGP rates of ~1.4 mmol kg^{-1} min^{-1} for 25-42d fasted kelp bass (Garin et al. 1987) apply to the fish in our study, the unlabeled plasma glucose fraction would require a rate of appearance of ~0.6 mmol kg^{-1} min^{-1} of unlabeled glucose, or ~3,600 mmol of glucosyl units over the 21d fasting period. The difference in hepatic glycogen levels between fed and 21d fasted fish is ~600 mmol (Viegas et al. 2012) therefore even if all hepatic glycogen was hydrolyzed via glucosidic pathways, it would only account for a minority of unlabeled glucose appearance. There is a possibility that glucosidic hydrolysis of muscle glycogen stores could contribute to the formation of unlabeled plasma glucose. Muscle glycogen stores fell by 15 mmol g^{-1} wet weight between postprandial and 21d fasted states (see Table 8). Given the large muscle mass of fish (~45-65% of total body weight), this change in glycogen levels could contribute a significant fraction of the unlabeled glucose were it hydrolyzed to glucose via glucosidic enzymes. For fish of ~220 g (initial body weight), 55% of muscle accounts for 120 g. A decrease of 15 mmol glycogen g^{-1} muscle corresponds to a total of 1800 mmol glucosyl units.

There is evidence that under certain conditions, muscle glycogen may be preferentially hydrolyzed by glucosidase in both amphibians and fish. In frog muscle, glucosidic pathways of glycogen conversion to glucose were shown to make a significant contribution to whole body plasma glucose (Fournier & Guderley 1993; Fournier et al. 1992). In fish, there is widespread α-glucosidase activity in liver, kidney, brain and muscle (Castilla et al. 1980) and there is also evidence that it may be responsive to glycogenolytic stimuli (Mehrani & Storey 1993). Moreover, α-glucosidase activity in liver is increased relative to glycogen phosphorylase activity during

prolonged fasting. In 8d fasted carp, hepatic glycogen phosphorylase activity was undetectable, so α-glucosidase was proposed to be the principal glycogenolytic enzyme under these conditions (Murat 1976). In catfish *Heteropneustes fossilis*, a 20d starvation provoked a 3-fold increase in hepatic α-glucosidase activity (Sharma and Sengupta 1993) as well as in kidney and brain. In masu salmon (*Oncorhynchus masou*) muscle, acid α-glucosidase activity was much higher compared to that of glycogen phosphorylase suggesting that the lysosomal hydrolytic pathway of glycogenolysis is relatively active (Konishi et al. 1991).

CHAPTER V

Effects of food-deprivation and refeeding on key enzyme activities and mRNA levels in the liver of European seabass (*Dicentrarchus labrax* L.)

Introduction

The European seabass (*Dicentrarchus labrax* L.) is nowadays one of the most important marine fish species farmed in Southern Europe. As a carnivorous fish, its metabolism is adapted to high levels of dietary protein therefore its capacity to utilize dietary CHO is considered to be poor (Hemre et al. 2002). Assaying enzymatic activities involved directly or indirectly in CHO metabolism has been performed in various species and tissues and has proved to be of crucial importance to evaluate metabolic changes in different contexts such as rearing temperature (Couto et al. 2008; Enes et al. 2008c), salinity changes and osmotic adaptation (Sangiao-Alvarellos et al. 2003; Laiz-Carrión et al. 2005), feeding status (Soengas et al. 1996, 2006; Caseras et al. 2000; Metón et al. 2003), rearing densities (Sangiao-Alvarellos et al. 2005) and novel diet formulations (Metón et al. 1999b; Panserat et al. 2000, 2001a; Fernández et al. 2007; Pérez-Jiménez et al. 2007; Melo et al. 2008). In the case of the seabass, enzymatic assays have been used to address some of these issues but the main focus was has been in determining relationships between temperature and diets with different levels and sources of carbohydrate (Dias et al. 1998; Enes et al. 2006b; Moreira et al. 2008), starvation and refeeding regimes (Pérez-Jiménez et al. 2007) and more recently to study the effects of hormones on carbohydrate metabolism (Enes et al. 2010).

Besides GK and G6Pase, already presented in Chapter III of this thesis, we sought to assay activities and developing specific primers for quantifying mRNA levels of other enzymes involved in carbohydrate metabolism. A key-regulatory step in the glycolytic pathway is catalyzed by 6-phosphofructo 1-kinase (PFK-1; EC 2.7.1.11), which phosphorylates fructose 6-phosphate. Another important glycolytic enzyme the pyruvate kinase (PK; EC 2.7.1.40), catalyzes the transfer of a phosphate group from PEP to ADP, yielding pyruvate, the final product of glycolysis. The conversion of glucose to pyruvate via glycolysis is opposed by the conversion of pyruvate and other C3 precursors to glucose via gluconeogenesis. Fructose 1,6-biphosphatase (FBPase; EC 3.1.3.11) is a key control enzyme of gluconeogenesis and therefore influences the direction of carbon flow between G6P and pyruvate.

In the scope of aquaculture, feed formulation aims to balance efficient utilization of dietary CHO with conversion of feed protein to muscle. This would be economically and environmentally beneficial, since high-cost fishmeal could be substituted with low-cost plant-derived material while dietary carbohydrate utilization would spare the conversion of protein to glucose and the resulting generation of nitrogenous waste. So along with the activities directly involved in CHO metabolism, activities of amino acid-degrading enzymes such as transaminases are also studied to determine the correlation between amino acid catabolism and gluconeogenic activity. Amongst the transaminases, alanine aminotransferase (ALT; EC 2.6.1.2) is more responsive than aspartate aminotransferase (AST; EC 2.6.1.1) and its activity has been shown to significantly increase with rising protein content in the diet in some cases (Fynn-Aikins et al. 1995; Metón et al. 1999b; Gaye-Siessegger et al. 2006; Enes et al. 2008a; Pérez-Jiménez et al. 2007). However, other studies including those on the seabass (Enes et al. 2006a) and rainbow trout (Kirchner et al. 2003) did not show this relationship.

The Pentose phosphate pathway (PPP) is an alternative route for G6P consumption, generating 5-carbon sugars that can be utilized for nucleotide biosynthesis as well as being a principal source of NADPH for the synthesis of lipids in fish (Dias et al. 1998). Metabolism of G6P through this pathway comprises an oxidative and a nonoxidative phase and within the former, glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), a rate-controlling enzyme, and 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.43) are often analyzed along with other enzymes directly involved in CHO metabolism. Starvation seems to negatively affect the activity of G6PDH and 6PGDH (Metón et al. 2003; Pérez-Jiménez et al. 2007), but response to dietary starch levels is variable (Dias et al. 1998; Enes et al. 2006b).

Despite the valuable information produced using the European seabass as animal model, the underlying alterations in carbohydrate metabolic fluxes in fish have lagged far behind that of enzyme activity (Viegas et al. 2011) and the development of specific molecular probes to assess transcription and genetic expression of these enzymes also, to our knowledge, remains to be addressed. We therefore aimed at sequencing key-enzymes involved CHO metabolism in seabass

from various pathways (gluconeogenesis, glycolysis, pentose phosphate pathway and amino acid-degrading enzymes) in order to design valid primers to measure the expression patterns and to compare mRNA levels. Finally we aimed at comparing enzymatic activities with the correspondent mRNA levels and analyzing the obtained results in three different nutritional statuses in seabass: fed, fasted and refed.

Material and Methods

Fish handling and sampling

Fish handling and sampling was as described in the Methods Section of Chapter III.

Enzyme activities

Enzyme activity assays were carried out as previously described (Metón et al. 1999b; Caseras et al. 2000), with minor modifications. Crude extracts for assaying enzyme activities were obtained by homogenization of the powdered frozen liver (1/5, w/v) 50 mM Tris-HCl pH 7.5, 4 mM ethylenediaminetetracetic acid, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM 1,4-dithiothreitol and 250 mM sucrose using a PTA-7 Polytron mixer (Kinematica GmbH, Switzerland; position 3, 30 seconds), and centrifugation at 15,800 g for 40 min at 4ºC. PFK-1 activity was assayed in a final volume of 200 µL containing 100 mM Tris HCl pH 8.25, 5 mM $MgCl_2$, 50 mM KCl, 0.15 mM NADH, 4 mM ammonium sulfate, 12 mM 2-mercaptoethanol, 10 mM F6P, 30 mM G6P, 0.675 U/mL fructose bisphosphate aldolase, 5 U/mL triose-phosphate isomerase, 2 U/mL glycerol-3-phosphate dehydrogenase and 4 µL crude extract. The reaction was triggered after addition of 10 mM ATP. Specific assay conditions for PK activity were as follows: 250 µL of final volume containing 70 mM glycylglycine pH 7.4, 10 mM $MgCl_2$, 100 mM KCl, 0.15 mM NADH, 2.8 mM phosphoenolpyruvate, 21 U/mL lactate dehydrogenase and 4 µL of sample. ADP (2.5 mM) was added to monitor the PK reaction. G6PDH activity was measured using 4 µL sample in a final volume of 200 µL containing 77.5 mM imidazole-HCl pH 7.7, 5 mM $MgCl_2$, 1 mM NADP and 1 mM G6P. The assay conditions for 6PGDH activity were: 82.7 mM

imidazole-HCl pH 7.7, 3 mM $MgCl_2$, 0.5 mM NADP, 2 mM 6-phosphogluconate and 4 µL crude extract in a final volume of 200 µL.

Mitochondrial fractions were prepared as described by Metón et al. (2004) at 4 °C, from liver homogenized (1/5, w/v) using a Dounce homogenizer in buffer A (50 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 50 mM NaF, 0.5 mM PMSF, 1 mM DTT, 200 mM mannitol and 70 mM sucrose). To remove nuclear and cell debris, the homogenate was centrifuged at 500 g for 10 min. The mitochondrial fraction was pelleted by centrifugation at 9000 g for 20 min. The resulting supernatant contained the cytosolic fraction. The pellet was yet washed twice with buffer A followed by centrifugation at 9000 g for 20 min. Mitochondria were resuspended and disrupted in buffer A without mannitol and sucrose. ALT and AST activity in cytosolic and mitochondrial fractions was determined using commercial kits (Cromatest, Linear Chemicals, Spain). All enzyme assays were carried out at 30ºC and followed at 340 nm. The total protein content was determined by the Bradford method (Bio-Rad, Spain) at 30ºC in liver crude extracts using bovine serum albumin as a standard and followed at 600 nm. All assays for enzyme activities and total protein were adapted for automated measurement using a Cobas Mira S spectrophotometric analyzer (Hoffman-La Roche, Switzerland).

Total RNA extraction and reverse transcription (RT)

Total RNA extraction and reverse transcription was performed as described in the Methods Section of Chapter IV.

RT-PCR analysis and primer design for qPCR

The pairs of primers, annealing temperatures and length of bands generated in the amplification by RT-PCR for the different enzymes are presented in Table 11. These primers were designed from highly conserved regions of nucleotide sequences published in GenBank for each of the enzymes, using whenever possible fish species such as *Danio rerio, Oncorhynchus mykiss, Platichthys flesus, Cyprinus carpio*, *Rhabdosargus sarba* and *Sparus aurata* (partial or complete

sequences). RT-PCR analysis and primer design for qPCR were performed as described in the Methods Section of Chapter IV. Specific details for each enzyme such as primers used, length of bands generated and GenBank accession numbers are listed in Table 12.

qRT-PCR analysis

qRT-PCR was performed as described in the Methods Section of Chapter IV.

Results

Data on the physiological condition (TL and W), indexes (HIS and PFSI), glycogen concentration and glycaemia were already presented and discussed in Chapters III and IV of this thesis. The activity for the glycolytic enzymes PK and PFK-1 (Fig. 17A and 17B) revealed higher values for fed fish in comparison to the fasted ones, however, refeeding after a 21d fast generated a different profile to that of regular feeding. While activities of PFK-1 rebounded to values close to those found in the fed fish, those of PK were similar to fish kept in the fasted state. Meanwhile, the gluconeogenic enzyme FBPase also presented higher values for fed fish in comparison to the fasted ones (Fig. 17C).

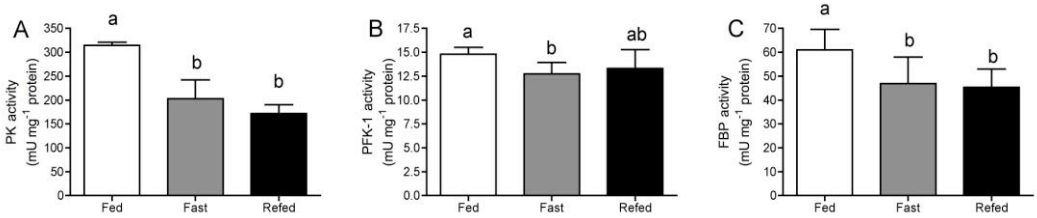

Fig. 17 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) of PK (A), PFK-1 (B) and FBPase (C). Mean values ± SD are presented. Significant differences between conditions are indicated by different letters (one-way ANOVA followed by Tukey's test, $P<0.05$).

Despite all efforts these results could not be corroborated with mRNA levels because at present sequences for these enzymes for *D. labrax* are not available and cloning was not successful. A sequence for PK was obtained but informed about the muscular isoform.

Activities for ALT were successfully assayed for both cytosolic (cALT; Fig. 18) and mitochondrial (mALT; Fig. 19) isoforms.

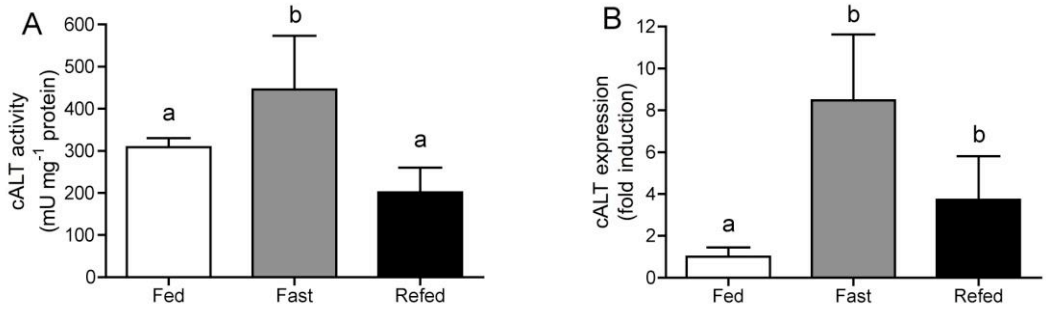

Fig. 18 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) (A) and relative abundance of mRNA (in arbitrary units) (B) of cALT. Mean values ± SD are presented. Significant differences between conditions are indicated by different letters (one-way ANOVA followed by Tukey's test, $P<0.05$).

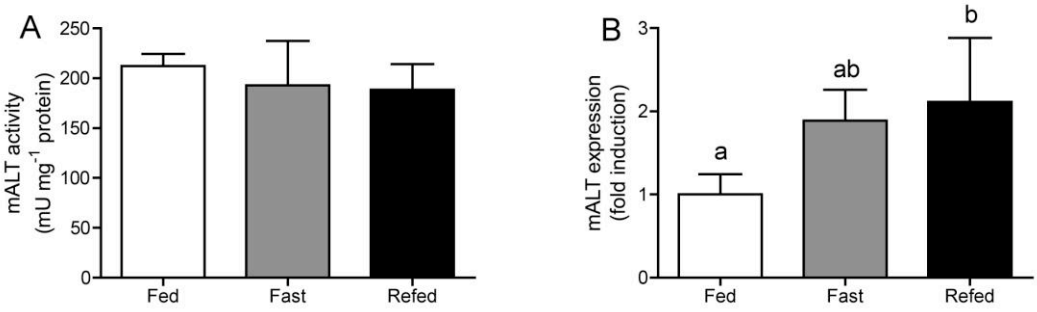

Fig. 19 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) (A) and relative abundance of mRNA (in arbitrary units) (B) of mALT. Mean values ± SD are presented. Significant differences between conditions are indicated by different letters (one-way ANOVA followed by Tukey's test, $P<0.05$).

Fasting provoked an increase in cALT and the 3d refeeding period resulted in a reestablishment of the values observed in the fed state. Expression of mRNA levels corroborated these observations. While mALT isoform activity showed no differences among the different nutritional conditions its mRNA levels were significantly enhanced in the refed state. This agrees with the observation that in terms of total activity, the mitochondrial isoform gains a small but significant increase in contribution during refeeding (cALT: 90±3% vs mALT: 10±3%) compared to the fed and fasted states, where the cytosolic isoform accounts for essentially all activity (95±3% vs: 5±3% for mALT for fed fish; 94±4% vs 6±4% for mALT in fasted fish). Tissue distribution of cALT and mALT are presented in Figure 20.

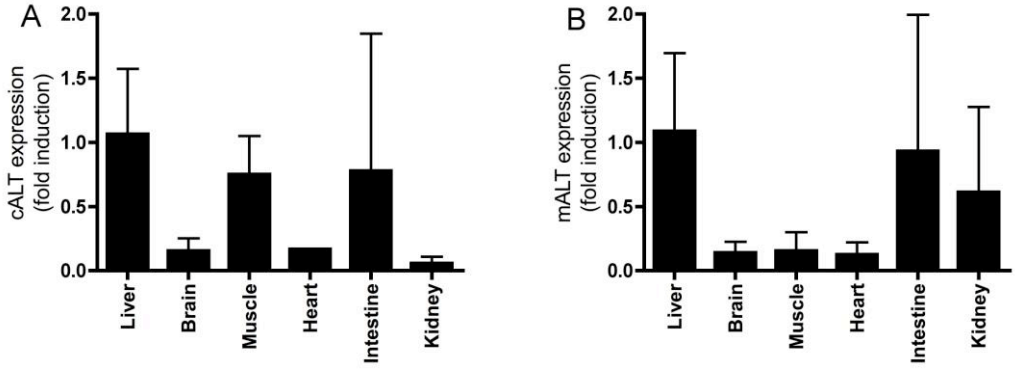

Fig. 20 Tissue distribution for cALT (A) and mALT (B) as quantified by qPCR. Mean values ± SD are presented (n=2).

Both were highly expressed in liver and intestine, but while the cytosolic isoform was also found in muscle the mitochondrial isoform was largely localized in kidney. Activity for AST was also successfully assayed for both cytosolic (cAST) and mitochondrial (mAST) isoforms (Fig. 21) however design of specific primers to perform qPCR was not pursued. As was observed for cALT in the fasted fish, cAST activity was enhanced and after refeeding activities returned to fed state levels. Once again, refeeding stimulated the contribution of the mitochondrial isoform to total activity (15±6% of total AST activity compared to 5±1% for fed fish and 5±3% for fasted fish).

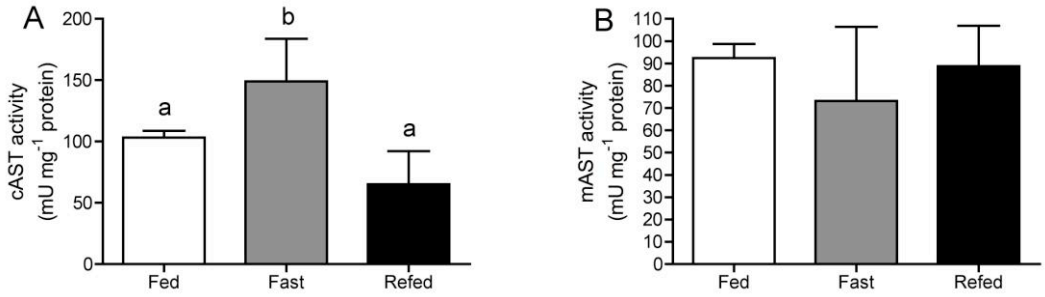

Fig. 21 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding)on the activity (in mU mg^{-1} protein) of cAST (A) and mAST. Mean values ± SD are presented. Significant differences between conditions are indicated by different letters (one-way ANOVA followed by Tukey's test, $P<0.05$).

The dehydrogenases of the PPP responded similarly to nutritional changes in terms of enzymatic activity. Both G6PDH (Fig. 22) and 6PGDH (Fig. 23) activities were significantly higher in the fed state compared to the fasted state.

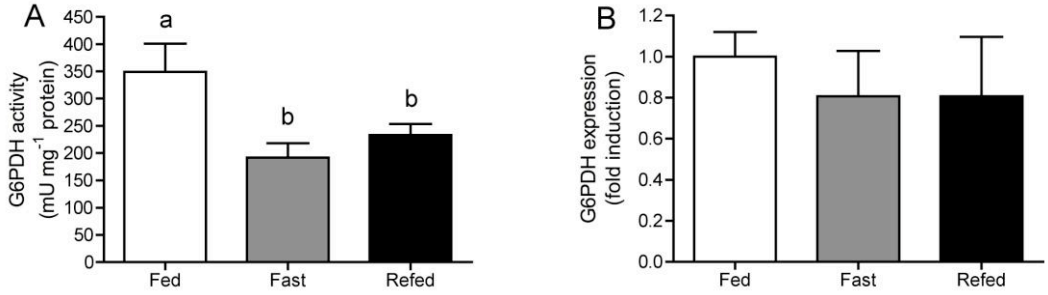

Fig. 22 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) (A) and relative abundance of mRNA (in arbitrary units) (B) of G6PDH. Mean values ± SD are presented. Significant differences between conditions are indicated by different letters (one-way ANOVA followed by Tukey's test, $P<0.05$).

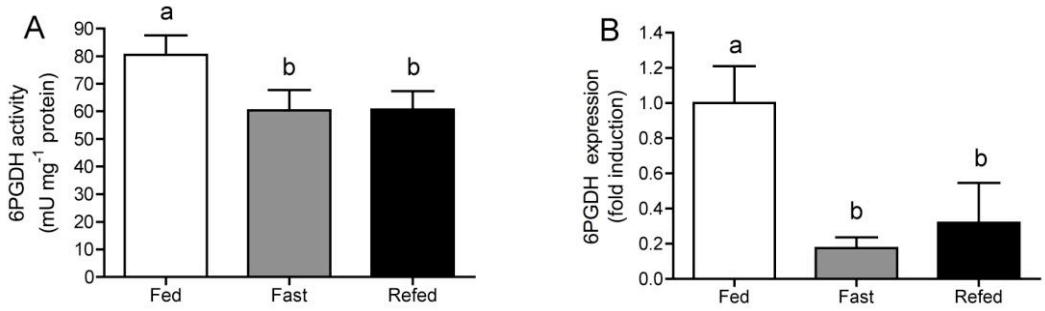

Fig. 23 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasted, 3 days refeeding) on the activity (in mU mg^{-1} protein) (A) and relative abundance of mRNA (in arbitrary units) (B) of 6PGDH. Mean values ± SD are presented. Significant differences between conditions are indicated by different letters (one-way ANOVA followed by Tukey's test, $P<0.05$)

After the 3d refeeding period, activities did not rebound and fasted state values were maintained. For 6PGDH, mRNA levels reflected the pattern observed for enzymatic activities. Expression in refed fish had a weak tendency to be higher compared to either fed or fasted fish. For G6PDH mRNA levels, no distinguishable patterns were observed between the different nutritional conditions. Tissue distributions for both of these enzymes is presented in Figure 24.

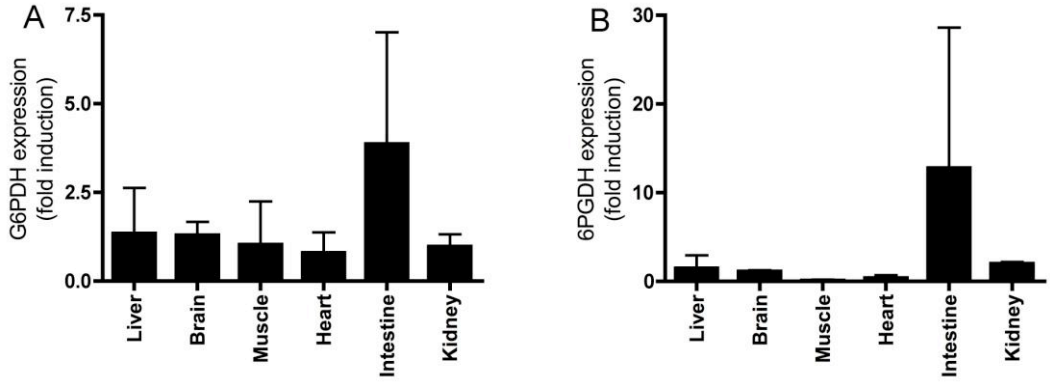

Fig. 24 Tissue distribution for G6PDH (A) and 6PGDH (B) as quantified by qPCR. Mean values ± SD are presented (n=2).

Discussion

The existence of efficient enzymatic mechanisms for regulating blood glucose in carnivorous fish such as seabass - that appear to be poorly adapted for carbohydrate metabolism - remains controversial. Observation of the response in expression and activity of key regulatory enzymes involved in glucose production and disposal to nutritional challenges may inform the presence of such mechanisms. Under conditions of fasting where there is a requirement for increased endogenous glucose production to maintain glycemia, these can be generally defined as increased glycogenolysis and gluconeogenesis and an enhanced capacity of liver for exporting glucose (Caseras et al. 2002; Metón et al. 2004; Sangiao-Alvarellos et al. 2005). At the same time expression and activities of enzymes involved in glucose consumption are generally downregulated.

A glycolytic enzyme like PK, seems to be strongly related to glucose concentration and other intermediary metabolite of glycolysis (Gómez-Milán et al. 2007) so as would be expected, PK activity significantly fell after 21d of fasting. This has also been observed in other species like rainbow trout (Furné et al. 2012), common dentex *Dentex dentex* (Pérez-Jiménez et al. 2012) and gilthead seabream (Bonamusa et al. 1992; Metón et al. 1999a, 2003). However this decrease not always is clear or significant as the activities also reported for rainbow trout (Soengas et al. 2006) which adds to the fact that no difference in PK mRNA levels between food-deprived and fed fish were observed in this species (Panserat et al. 2001c; Kirchner et al. 2003b). Changes in PK activity during shorter periods of fasting is less well defined as confirmed by studies with both gilthead seabream (Sangiao-Alvarellos et al. 2005) and sturgeon *Acipenser naccarii* (Furné et al. 2012). Indeed for the latter species, PK activity increased significantly at 5 days of food deprivation (Furné et al. 2012). Response to refeeding seems to be dependent of the time of recovery and specific variability. PK denotes lack of effectiveness on the response to refeeding as short-term refeeding was not sufficient to produce significant changes on hepatic PK relative to fasted fish as observed in the present study. In comparison, the restoration of hepatic glycogen reserves seems to be a more robust response during the initial stages of refeeding (Viegas et al. 2012).

Restoration of PK activity levels is attained only after 8d of refeeding (Metón et al. 2003; Soengas et al. 2006, Furné et al. 2012; Pérez-Jiménez et al. 2012) which suggests a long-term stimulation by food intake (Enes et al. 2009). PFK-1, the other glycolytic enzyme analyzed in the present study also revealed lower activities in food deprived fish compared with fed fish (Soengas et al. 1996). In contrast to that of PK, the induction of PFK-1 activity by refeeding was much more robust. This may be explained by increased levels of its allosteric activator, fructose 2,6-bisphosphate, whose synthesis and degradation is catalyzed by the bifunctional enzyme 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase (Metón et al. 1999a; 2003).

FBPase is also considered to be an important control enzyme of gluconeogensis. As such, its activity is expected to be sensitive to the transition between feeding and fasting states, to show a high degree of correlation with activities and expression of other gluconeogenic enzymes, and to show a reciprocal relationship with activities of glycolytic control enzymes. However, data from fasting and refeeding studies do not provide consistent evidence for this. In the present study, a decrease in FBPase activity was observed in fasted fish while other studies reported either no change between feeding and fasting like in common dentex (Pérez-Jiménez et al. 2012) and rainbow trout (Kirchner et al. 2005)) or an increase in activity following transition from feeding to fasting as observed in gilthead seabream (Metón et al. 2003; Sangiao-Alvarellos et al. 2005), Atlantic salmon *Salmo salar* (Soengas et al. 1996) and rainbow trout (Soengas et al. 2006)). These alterations were also observed for another gluconeogenic enzyme (G6Pase in Chapter IV). It is worth noting that in the cases where FBPase and G6Pase activities were upregulated by fasting, fasting plasma glucose levels did not fall by a large amount. In contrast, plasma glucose levels of fish from the current study showed a much larger fall in concentration following the fed to fasted transition, which corroborates with the poor induction of FBPase and G6Pase activities. The apparently low sensitivity of FBPase activity to fasting plasma glucose levels in seabass, as well as in gene expression in the liver of rainbow trout for example (Panserat et al. 2001c), brings into question the importance of this enzyme in control of plasma glucose levels during prolonged food deprivation. Besides, FBPase activity and/or mRNA levels of this

enzyme appear to be more sensitive to dietary protein rather than carbohydrates (Kirchner et al. 2003b, 2005; Enes et al. 2006a; Pérez-Jiménez et al. 2007; Enes et al. 2008a). Such insensitivity to plasma glucose levels has also been observed previously for two other key hepatic enzymes, PEPCK and G6Pase (Panserat et al. 2000a, 2001b, 2002a), suggesting that hepatic glucose production is poorly responsive to plasma glucose levels (Panserat et al. 2001c).

ALT and AST enzyme activities are quantitatively the most important aminotransferases in the teleost fish liver (Cowey & Walton 1989). Liver is the main site for transamination, a process by which energy can be obtained either by direct oxidation of the carbon skeleton of the amino acids or from glucose produced after the conversion of the carbon skeleton through from gluconeogenesis. Both ALT and AST responded in a coherent fashion to fasting with an increase in activity, however higher activity reported for ALT seemed to indicate its greater importance in the scope of protein mobilization. Besides, mRNA levels for cALT, which as responsible for most of total activity corroborated this pattern. Response to starvation does not follow a clear pattern throughout the literature between both transaminases and between different fish species. The common denominator seems to be the proportional response in relation to dietary protein content (Fernández et al. 2007; Pérez-Jiménez et al. 2007, 2012; Furné et al. 2011). Refeeding induced the recovery of initial activities as found in other studies (Metón et al. 1999; Pérez-Jiménez et al. 2007, 2012; Furné et al. 2011).

Within the PPP, despite being one of the possible pathways of channeling glucose into catabolism, G6PDH activity in the seabass does not seems to be influenced by dietary starch levels (Dias et al. 1998; Enes et al. 2006a). In Atlantic salmon hepatocytes, glucose and alanine alone were able to modulate the oxidative part of the PPP, but no interaction between the two could be found (Sanden et al. 2003). Contributions of this pathway on achieving an improved use of CHO with fish diets seem to be correlated with interaction between protein but also strongly related to dietary lipid intake (Dias et al. 1998, 2004). Starvation decreased the activities of both G6PDH, 6PGDH, as already described (Metón et al. 2003; Morales et al. 2004; Pérez-Jiménez et al. 2007, 2012), nevertheless other studies report little or no alterations (Sangiao-Alvarellos et al.

2005; Furné et al. 2011). Recovery time from fasting by refeeding may differ between species however in the seabass Pérez-Jiménez et al. (2007) reported full recovery of G6PDH activity in after 1d of refeeding. This was not observed in this study after 3d despite slight increase. Different levels of protein in the diet may explain such difference, however mRNA levels found in the present study seemed to confirm the obtained activities for both G6PDH, 6PGDH.

CHAPTER VI

General conclusions and future perspectives

General conclusions

In recent years, both in the scope of aquaculture or to mimic insulin-dependent diabetic mammalian models, the study of fish metabolism has suffered serious advances, namely in terms of tracer studies. Isolated hepatocytes have been an important system to probe hepatic physiology and biochemistry (Moon 2004) however many limitations emerge when extrapolating to *in vivo* models. So, other methods had to overcome mandatory adaptations to effectively deliver tracers to an intact aquatic organism. Bolus injection using ^{3}H- and ^{14}C-labeled substrates has been widely used in various species like kelpbass *Paralabrax clathratus* (Bever et al. 1977), seabass *Dicentrarchus labrax* (Garin et al. 1987), wolf fish *Hoplias malabaricus* (Machado et al. 1989) and carp *Cyprinus carpio* (West et al. 1994b). This approach required frequent blood sampling over a sustained period to adequately describe the tracer clearance kinetics but is limited by the number of blood samples that could be drawn from the fish. The dorsal aorta cannulation developed by Soivio et al. (1975) facilitated multiple blood samplings in undisturbed, non-anaesthetized fish but cannot accommodate simultaneous tracer administration and blood sampling. This was solved by the double dorsal aorta catheterization developed by Haman & Weber (1996) allowing R_a in rainbow trout *Oncorhynchus mykiss* to be measured by the primed-constant infusion tracer dilution method. While these procedures now allow glucose R_a to be well determined, they poorly inform the endogenous and exogenous glucose contributions to this flux. Oral administration of labeled food (Hemre & Storebakken 2000), either by pellets with ^{13}C-labeled starch and ^{15}N-labeled protein (Felip et al. 2012) or ^{13}C-labeled rotifers (Conceição et al. 2003), also offer interesting solutions to better understand glucose utilization in fish.

NMR methods and tracer-based studies have used $^{2}H_2O$ in humans and other mammals concerning various types of hepatic-metabolism related disorders such as insulin-dependent diabetes *mellitus* or glycogen storage disease. This was due to well-established methods and equations for labeling patterns of G6P (Landau et al. 1996). $^{2}H_2O$ seemed ideally suited for metabolic studies in fish since it could be added into the tank water for an indefinite period and the tracer would be incorporated into hepatic glycogen. From this starting point we aimed at

developing a novel noninvasive analysis of hepatic glucose and glycogen production using $^{2}H_2O$ and NMR in free-swimming seabass. This premise was summarized and postulated in the original project as the following objectives:

1) Contribute to the understanding of glucose utilization and dynamics in fish using NMR by estimation of the ratio of deuterium enrichment reported in its monoacetone glucose MAG derivative spectrum:

1.1) measurement of hepatic glucose output and identification of sources of blood glucose: derived from glycogen, tricarboxylic acid cycle and glycerol;

1.2) measurement of hepatic glycogen and identification of sources of the glucose used to form the liver glycogen pool;

1.3) clarify causes to shifts in the utilization of sources of glucose and follow its evolution through consecutive sampling in different rearing scenarios, diet composition and starvation/refeeding regimes, which have proven to significantly influence the metabolic responses.

2) In order to complement and possibly corroborate data provided by the NMR spectra, key enzymes activity in CHO metabolism will be quantified to assess the response to nutritional and environmental challenges drawn in the experiments:

2.1) enzymes involved in gluconeogenesis, glycolysis and glycogen-glucose reaction.

Objective 1.1 was complied in **Chapter II** since endogenous glucose synthesis in fish could be measured following a period of time in ^{2}H-enriched water. It was demonstrated that this non-invasive methodology was well suited for fish and allowed the quantification of the total rate of glucose production as well as the contribution of gluconeogenesis to this flux. With small fish, this procedure did not induce stress hyperglycemia – an unavoidable complication of earlier and more invasive tracer studies of fish glucose kinetics. From the positional enrichment analysis of plasma glucose enrichment by ^{2}H-NMR, it was demonstrated that with a standard fishmeal diet, plasma glucose appearance is almost entirely derived from gluconeogenesis regardless of

nutritional status. While this has long been hypothesized for carnivorous fish, this data represented the first direct proof of this key aspect of fish metabolism. Finally, we demonstrated that the appearance of plasma glucose ^{2}H-enrichment from $^{2}H_2O$ could also be determined by a rapid and sensitive LC-MS/MS measurement of plasma glucose ^{2}H-enrichment levels thereby increasing the scope and versatility of the method. Main findings were:

- plasma body water ^{2}H-enrichment reached that of seawater within 6h;
- in both fasted and fed fish, plasma glucose mole percent enrichment increased asymptotically attaining ~55% of plasma water enrichment by 72h;
- for both fed and fasted fish, gluconeogenesis accounted for 98±1% of the glucose that was produced during the 72h $^{2}H_2O$ administration period;
- for fasted fish, gluconeogenic contributions measured after 6h were identical to 72h values (94±3%);
- for fed fish, the apparent gluconeogenic contribution at 6h was significantly lower compared to 72h (79±5% *vs.* 98±1%, $P<0.05$) this reflected a brief augmentation of gluconeogenic flux by glycogenolysis after feeding and/or selective enrichment of plasma glucose position 2 via futile glucose-G6P cycling.

In **Chapter III**, using the method based on $^{2}H_2O$ detailed information was provided on hepatic glycogen kinetics in free-swimming seabass as postulated in **Objective 1.2**. There was slow fractional turnover of glycogen in fasted fish, but negligible turnover in continually fed fish. For refed fish, the rapid repletion of hepatic glycogen was completely sustained by *de novo* gluconeogenesis, presumably from dietary amino acids. There was significant discrimination of ^{2}H-enrichment of position 2 via G6P-isomerase suggesting that conversion of F6P to G6P via G6P-isomerase may be a rate-limiting step for glycogen synthesis under certain conditions. While this characteristic was reported for starch synthesis in plants it has not been observed for hepatic glycogen synthesis in mammals. Main findings were:

- glycogen levels of fed fish were significantly higher than fasted (665.4±345.2 mmol g^{-1} liver *vs.* 77.2±59.5 mmol g^{-1} liver, $P<0.05$) while refed fish had comparable levels to fed fish (584.6±140.4 mmol g^{-1} liver);
- glycogen enrichment of fed fish was undetectable indicating negligible turnover over 3d;
- for fasted fish, H5/PW was ~50% indicating that half of the glycogen had turned over via indirect pathway flux;
- for refed fish, H5/PW was ~100% indicating that the indirect pathway accounted for all net glycogen synthesis;
- direct pathway conversion of dietary CHO to glycogen was not detected in any of the groups.

In **Chapter IV**, **objective 1.1** was reinforced and along with Chapter II and III, **objective 1.3** was achieved. In seabass fed with conventional fishmeal, plasma glucose levels showed considerable fluctuations between feeding and fasting. Gluconeogenesis was the principal source of endogenous glucose production and is a significant contributor to plasma glucose levels during both feeding and fasting. Evidence for significant participation of extrahepatic glucose production in the maintenance of glycemia during starvation suggests that the liver is not the sole source of endogenous glucose production. With the exception of refeeding, where GK was strongly stimulated, activities of G6Pase and GK showed relatively little variation in expression and activities between fed and fasted states. This suggested that glycemic control, if present, may be exerted elsewhere within the metabolic network. This set of data already fit into **objective 2/2.1**. Main findings were:

- plasma glucose levels (mM) were 10.7±6.3 (fed), 4.8±1.2 (fasted), and 9.3±1.4 (refed) indicating poor glycemic control between feeding and fasting states;
- for all conditions, ^{2}H-enrichment of glucose position 5 was equivalent to that of position 2 indicating that plasma glucose appearance from endogenous G6P was derived by gluconeogenesis;

- G6P-derived glucose accounted for 65±7% and 44±10% of plasma glucose appearance in fed and refed fish, respectively, with the unlabeled fraction assumed to be derived from dietary CHO (37±7% and 56±10%, respectively);
- for 21d fasted fish, plasma glucose appearance also had significant contributions from unlabeled glucose (51±15%) despite the unavailability of dietary glucose;
- under these conditions, unlabeled glucose was postulated to be derived from hydrolysis of endogenous glycogen to glucose via glucosidase;
- G6Pase expression and activity declined with fasting, consistent with low glucose levels and increased contributions from glucosidase activity;
- GK activities were basal during fed and fasted conditions, but were strongly stimulated by refeeding.

Finally, in **Chapter V**, **objective 2/2.1** was completed with the analysis of two glycolytic enzymes (PK and PFK-1), one gluconeogenic enzyme (FBPase), two transaminases (ALT and AST) and two enzymes of the penthose phosphate pathway. Along with GK and G6Pase presented in Chapter IV, this set of results provided a reliable profile of how the hepatic enzymatic machinery responds to the three different feeding statuses tested. The obtained results not only casted light to the functioning of CHO metabolism as also revealed its interaction with other pathways (protein catabolism and penthose-phosphate pathway). Main findings were:

- liver of seabass was unable to properly counterbalance hypoglycemia under fasting by enhancing gluconeogenesis (FBPase and G6Pase);
- 3d refeeding period was on the one hand enough to reestablish moderate transaminase activities and on the other hand too short to rebound PPP enzymes to control (fed) levels;
- novel information on the genetics of the seabass and the sequences introduced in GenBank will assist greatly in studies to come.

Future perspectives

Other species, diets and rearing conditions

The constant demand of the aquaculture sector for feeds that optimize nutrient utilization at a low cost (normally by substitution of fishmeal for vegetable equivalents) without compromising growth rates and quality of the final product has enhanced the importance of understanding fish metabolism through enzymatic regulation of CHO. Thus it seems adjusted to pursue this line of research namely in terms of developing new methods of profiling CHO utilization in fish species targeted in aquaculture as well as in other species whose potential to intensive farming is still to be assessed. Now that baseline values have been established for standard setups in fish experiments (fed, fasted and refed), more complex scenarios can be tested, including different formulations of diets/supplements and rearing conditions.

^{13}C tracers

While $^{2}H_2O$ informs of the main pathways from which glucose has been derived, it does not specifically point out its provenance. For example, while $^{2}H_2O$ method can clearly distinguish the gluconeogenic origin of glucose it is unable to discriminate individually which TCA cycle metabolite contributed to this flux. So using ^{13}C-labeled amino acids and other labeled substrates, metabolic fluxes could be derived also by ^{13}C-isotopomer analysis of hepatic metabolites. ^{2}H measurement is not compromised by the presence of ^{13}C tracers thus ^{2}H- and ^{13}C-NMR in conjugation may be practical a method to provide reliable information. Proper tuning of the NMR apparatus would allow, with one sole sample, analysis of both isotopomers. The main challenge would be calibrating the concentration of tracer to be administered and adjusting its delivery to ensure proper distribution. This can be achieved either by forced-feeding of tailor-made pellets or by intra-peritoneal injection but both protocols would have to be carefully adjusted to the size of fish.

Unlabeled blood glucose on fasted fish

Paradoxically, one of the most intriguing results from the tracer-based studies was the fraction of unlabeled glucose found in fasted fish blood. For the 21d fasted fish, analysis of plasma glucose ^{2}H-enrichment revealed an unexpectedly high fraction of unlabeled glucose (~52%) despite the absence of dietary carbohydrate sources for contributing plasma glucose. Of the known alternative glucose-producing pathways that could generate unlabeled glucose in the presence of $^{2}H_2O$, glycogen conversion to glucose via α-glucosidase and/or glycogen-debranching enzymes is the most plausible. So evaluating the contribution of these enzymes, in comparison with GPase, could provide some insight into how glycogen hydrolysis takes place and possible regulation between them. Insight on contribution of extrahepatic tissues to glucose production could add valuable information to whole body glucose kinetics.

Isotope effect from G6P-isomerase

Another interesting point of the presented experimental data was the significantly lower H2 enrichment relative to H5 for refed fish hepatic glycogen. This contradicts the assumption that for glycogen derived via the indirect pathway, the positions 2 and 5 hydrogens are both enriched to the same level as plasma water. The possibility that this could be an artifact of the NMR analysis or sample preparation was dismissed. The selective discrimination of position 2 enrichment from $^{2}H_2O$ via G6P-isomerase as a result of a kinetic isotope effect was pointed out as most plausible cause. Investigate if G6P-isomerase exerts significant control over indirect pathway flux into hepatic glycogen under the experimental refeeding conditions would help clarify this matter. Under these conditions, G6P-isomerase activity could be a rate-liming step for indirect pathway synthesis of glycogen, at least in the initial stages of refeeding.

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