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Advances and Applications of Tracer Measurements of Carbohydrate Metabolism in Fish

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1. Introduction

1.1. Relevance of carbohydrate metabolism studies in fish

The zebrafish *Danio rerio* has long been used as an animal model for developmental studies due to a number of desirable characteristics for lab study including its short generation time, large number of offspring, transparent embryos, and *ex utero* development of the embryo. This setting proved to be particularly useful for research on vertebrate development [1] and modeling of human disease [2], namely the hematopoietic [3, 4] and the cardiac systems [5, 6]. However, the use of fish for scientific studies has evolved substantially in the last 30 years. The direction of metabolic fish studies changed drastically with the advent of modern aquaculture and a high demand for models to study nutrition, physiology and metabolism. Capture of fish products in the end of the 1990's represented almost 75% of total production but since 2001 that has leveled at around 90 million tons. Meanwhile, aquaculture production has been increasing at an average annual growth rate of 6.2% from 38.9 million tons in 2003 to 52.5 million tons in 2008 [7]. Aquaculture in 2009 already accounted for 38% of the 145 million tones of total fish products, 81% of which were for human consumption [8]. It is estimated that by 2030, half of the production for human consumption will be derived from aquaculture while the harvest of wild fish will not show any significant growth [9]. 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* [10]. These artisanal methods, with little or no active manipulation of the animals

or diets, 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 in farming methodologies. Aquaculture is highly dependent on capture fisheries to provide fishmeal and fish oil required to produce feeds [11, 12]. Thus, the development of well-suited and cost-effective substitute feeds based on carbohydrates has become a matter of extreme importance to the sustainability and profitability of the sector. Along with it, comes the need to improve our understanding of fish carbohydrate nutrition, its physiology, biochemistry and metabolism.

1.2. Carbohydrate metabolism in carnivorous fish

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 [13]. Carbohydrates (CHO) are a basic nutritional source of energy and carbon, but carnivorous fish have a limited capability to digest and metabolize them since they are adapted to a diet high in protein [14]. CHO influence growth, feed utilization and deposition of nutrients according to species, quantity, origin and treatment of dietary CHO used [15, 16, 17]. Fish are considered to be glucose intolerant on the basis of sluggish clearance of a glucose load and their metabolism of glucose has often been compared to that of mammals with insulin-dependent diabetes *mellitus* (IDDM) [18]. 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 [19].

Figure 1 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. As for mammals, many tissues such as skeletal muscle can oxidize alternative substrates to glucose for energy such as triglycerides and can therefore function with variable levels of blood glucose [20] but the brain is highly dependent on glucose as an energy substrate therefore a threshold level of plasma glucose is required for maintaining its function. Not surprisingly, the brain is regarded as key component of endogenous glucose sensing mechanisms that have recently been described in fish models [21]. The plasma glucose concentration also has considerable influence on the selection of myocardial substrates for oxidation in resting and active fish [22] and this adaptation is representative of the changes that occur in whole-body glucose utilization in response to changes in glucose availability [23]. During hypoxia, tissues such as the heart and brain upregulate anaerobic metabolic pathways and become more reliant on glycolysis for ATP production [24]. However under normoxic conditions, glucose uptake by muscle is surpassed by that of amino acids and lipids for growth and energy [25]; this also happens during the recovery of muscle glycogen following exhaustive exercise. Thus in general, the direct contribution of blood glucose to muscle energy metabolism seems to be minor [20, 26, 27, 28, 29]. The generally low reliance of fish muscle on glucose as an energy substrate is compatible with the limited availability of carbohydrate in fish diets [18].

1.3. 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 [19]. 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 [30]. Recently, the presence of GLUT-2 transporter was confirmed at both biochemical and molecular levels in species like rainbow trout *Oncorhynchus mykiss* [31, 32] and its expression in seabass *D. labrax* hepatocytes seems to be regulated by hypoxia.

Blood glucose concentration is the net result of the difference between rates of glucose appearance and disposal. Following uptake into the hepatocyte, the ATP-dependent conversion of glucose to glucose 6-phosphate (G6P) is catalyzed by hexokinase IV or glucokinase (GK). GK is the major hexokinase expressed in liver and due to its relatively high K_m, its role in glucose homeostasis, is to increase the hepatic capacity for glucose metabolism during hyperglycemia. Uniquely, GK is not inhibited by its G6P product so it is able to maintain high rates of glucose phosphorylation while the activities of other hexokinases become limited by increased G6P levels. Moreover, GK activity is hormonally controlled via the glucokinase regulatory protein (GKRP), such that its activity is increased by the high insulin levels of the fed state, but rapidly suppressed during the onset of fasting when both portal vein glucose and insulin levels are waning. As a result, when the liver becomes a net producer of glucose during the fasting state, GK is prevented from converting this back to G6P.

The G6P product can be utilized by the pentose phosphate pathway, be recruited for glycogen synthesis (glycogenesis) or be metabolized by the glycolytic pathway to pyruvate. Pyruvate in turn may be oxidized to acetyl coenzyme A (acetyl-CoA) or carboxylated to

oxaloacetate or malate – a process known as anaplerosis. Acetyl-CoA may be oxidized to CO_2 by the tricarboxylic acid cycle (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). In fish, it has been shown that G6Pase is poorly regulated by dietary CHO in comparison to GK [33]. When G6Pase and GK are both active at the same time, glucose and G6P are interconverted in a "futile cycle" that results in the consumption of ATP but no net conversion of glucose to products. This activity has important implications for interpreting the metabolism of certain glucose tracers such as [2-^{2}H]- or [2-^{3}H]glucose since it results in disappearance of the label without any net glucose consumption.
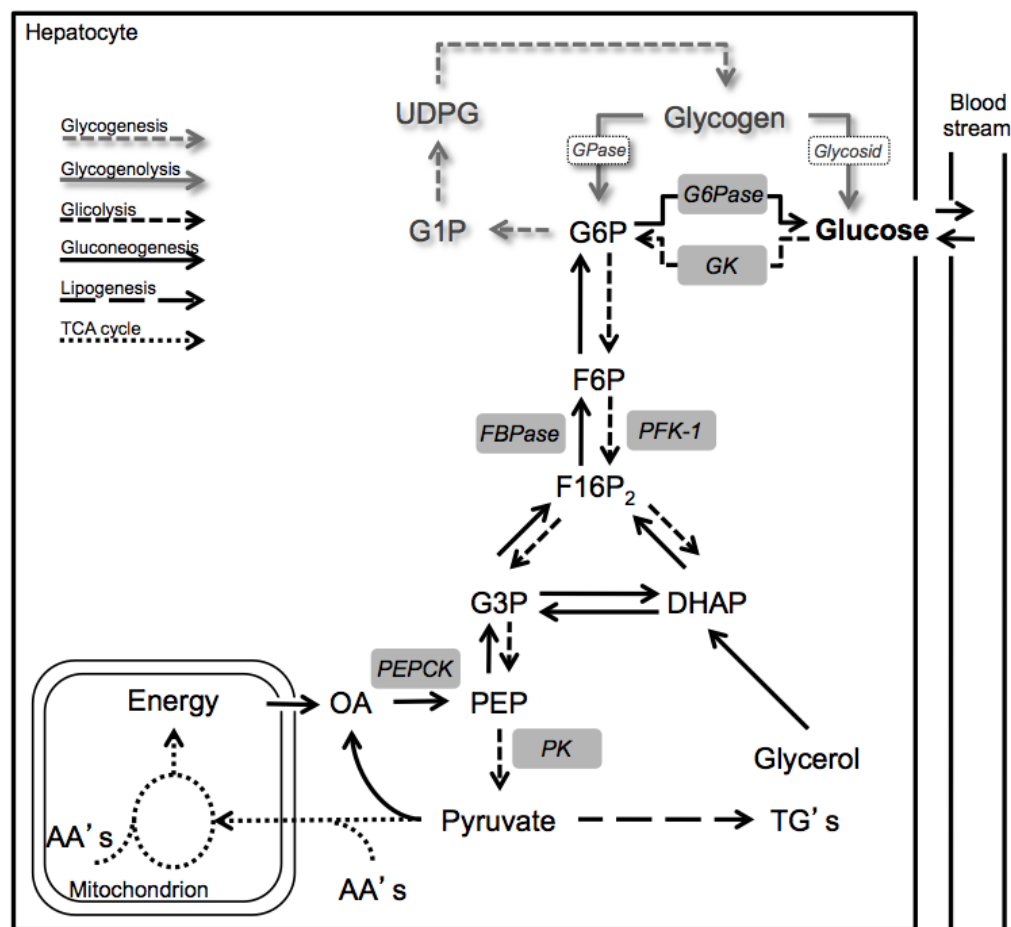
Figure 1. Metabolic model representing main pathways in the liver involving catabolism and anabolism of glucose. Gluconeogenic precursors are represented by pyruvate (and gluconeogenic amino acids, metabolized via the anaplerotic pathways of TCA cycle) as well as glycerol from lipolysis. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P - glucose 6-phosphate; G1P - glucose 1-phosphate; UDPG - uridine diphosphoglucose; F6P - fructose 6-phosphate; F16P2 - 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 bisphosphatase; PFK-1 - 6-phosphofructo-1-kinase; PEPCK - phosphoenolpyruvate carboxykinase; PK - pyruvate kinase; GPase - glycogen phosphatase; Glycosid - glycosidic enzymes; TCA cycle - tricarboxylic acid cycle.

1.4. Glycogen synthesis and hydrolysis

The 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 [34].

GSase 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 glycosidic linkages. Hepatic glycogen can be synthesized by the incorporation of glucose into glycogen via a sequence of reactions that was initially characterized in muscle. In this so-called "direct pathway", the glucosyl moiety is transferred into glycogen as an intact entity. It was subsequently discovered that in the liver, glycogen can also be synthesized independently from glucose via gluconeogenic precursors [19]. These contributions are collectively referred to as the "indirect pathway. Since the indirect pathway bypasses GK, and uses precursors that are independent of blood glucose, its contribution to glycogen synthesis may respond to nutritional and hormonal states in a different way to that of the direct pathway. Quantifying the direct and indirect pathway flux components of hepatic glycogen synthesis can therefore further inform adaptations of hepatic carbohydrate metabolism to different diets and hormonal states.

Under fasting conditions, the liver becomes a net producer of glucose and the hydrolysis of glycogen is an important source of glucose production at least in the early stages of fasting. To avoid futile cycling between G6P and glycogen, the activities of GSase and GPase are regulated in a reciprocal manner such that only one or the other is active at a given time. Glycogen may be also hydrolyzed to glucose by the action of glucosidases enzymes that hydrolyze the non-reducing end of polysaccharides directly to glucose. There is evidence of glucosidase activity alongside that of GPase in living fish [35, 36, 37], but the extent of its contribution to glucose synthesis from glycogen and modulation of activity by feeding and fasting are little known.

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 [38] and the liver plays an important role in the endogenous control of fuel storage and mobilization [39, 40, 41]. 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 results 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 refeeding [39, 40, 42, 43]. The general consensus is that the liver of fish plays a role in glycemic control through the regulation of hepatic glucose storage and mobilization [33] but its actions are sluggish in comparison to mammalian liver.

1.5. Glycolysis and gluconeogenesis

Glycolysis involves the metabolism of glucose or glycogen to pyruvate. From glucose, the first step in the glycolytic pathway is the phosphorylation of glucose by GK into G6P, as described. From glycogen, G6P is generated following GPase 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-phospho-fructo 1-kinase (PFK-1). These reactions commit the hexose 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, contributes to control fluxes through PFK-1 and PK. The free energy released in this process is coupled to substrate-level ATP and NADH formation. Pyruvate is the final product of glycolysis and can undergo further oxidation to acetyl-CoA via pyruvate dehydrogenase (PDH), carboxylation via pyruvate carboxylase (PC) or NADP-malic enzyme or reduction to lactate. In the liver, the relative activities of PDH and PC determine 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 unfavorable free energy change of pyruvate to PEP conversion. The conversion of $F16P_2$ to F6P and G6P to glucose are also mediated by different enzymes to their glycolytic counter-parts (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.

1.6. 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 [44, 45], salinity and osmoregulation [46, 47], feeding status [39, 41, 48, 49], rearing densities [50], diets [51, 52, 53, 54, 55, 56, 57] and to study the effects of hormones [58, 59, 60, 61].

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. The mammalian paradigm, to which the hormonal regulation in fish is usually compared, has shown to be poorly representative of fish metabolism in a number of cases [62]. Glucose is weakly effective at stimulating insulin secretion in fish compared to mammalian species, although, as expected, an improved response is observed in CHO-tolerant species [63]. 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 carps 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 important component of glucose homeostasis. However, it is unclear in fish to what extent the regulatory and counterregulatory processes are coupled [62]. 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 [64]. 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 [65].

2. Tracer studies of carbohydrate metabolism in fish

2.1. Overview

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 from the tracee. In order that the tracee pool of interest be not altered by administration of the tracer (thereby potentially perturbing the metabolic activity under observation), its chemical concentration should be insignificant in comparison to that of the tracee. At the same time, this amount of material must possess a high abundance of label relative to the tracee since metabolic flux parameters are typically derived by administering a high density of label and monitoring its dilution by unlabeled tracee molecules. With radioisotopes, the label concentration is measured in terms of specific activity, defined as the number of decays per minute measured per mol of glucose. With stable isotopes, tracer concentration is defined in terms of percent excess enrichment per mol of glucose. Since most stable isotopes are typically

present at background levels (for example, 1.1% of carbon in nature is ^{13}C), this must be accounted for when attributing metabolite enrichment to inflow from a tracer.

Since blood glucose and other carbohydrates can be synthesized endogenously as well as absorbed from the diet, several tracer strategies may be applied to inform carbohydrate metabolism. The first and most easily understood is to incorporate the tracer into dietary carbohydrate and monitor its rate of appearance in endogenous carbohydrate pools (i.e. plasma glucose and liver glycogen) as well as metabolic endproducts (i.e. respired CO_2). This approach provides a direct measure of how a specific dietary carbohydrate source is utilized and disposed into endogenous oxidative and nonoxidative pathways. Oral administration of labeled food [66], either by pellets with ^{13}C-labeled starch and ^{15}N-labeled protein [67] or ^{13}C-labeled rotifers [68], and measurement of isotope appearance in endogenous carbohydrate and protein have also been used to measure substrate utilization in fish. In the case of carbohydrate studies, while the dilution of ^{13}C-enrichment between the feed precursor and sampled product metabolite may qualitatively inform the extent of endogenous glucose production (i.e. high dilution equates to high rates of endogenous glucose production relative to absorption of the ^{13}C-enriched carbohydrate), quantitative data rely on knowing the rate of entry of the ^{13}C-enriched dietary carbohydrate into blood glucose. Since this is a function of absorption and cannot be directly measured, this approach provides only a limited insight into endogenous glucose production. In the second type of measurement, a glucose tracer is directly administered into the blood at a known rate or quantity, and by measuring its dilution by unlabeled endogenous glucose, the rate of appearance (R_a) of blood glucose may be quantitatively defined. Since both absorbed and endogenously-produced glucose contribute to the dilution of the labeled glucose, this measurement does not resolve the contributions of dietary and endogenous sources. Under fasting conditions where there is no input of dietary glucose, glucose R_a equates to endogenous glucose production. In mammals and humans, dietary and endogenous components of glucose R_a may be resolved by combining a feed tracer with a second glucose tracer administered intravenously [69]. The detection method must have the capability of resolving the labeling contribution of blood glucose from each tracer. For example, radioactivity from infused [3-^{3}H]glucose and ingested [U-^{14}C]glucose are resolved by scintillation counters on the basis of their different emission energies. Finally, as previously discussed, endogenous glucose can be derived from multiple sources including gluconeogenesis from a diverse range of precursors as well as glycogen hydrolysis. Neither the labeled dietary carbohydrate nor glucose isotope dilution measurements inform the sources of endogenous glucose production. This represents a significant limitation for understanding carbohydrate metabolism of carnivorous fish since their diet is low in carbohydrate therefore the plasma glucose rate of appearance (R_a) is likely to dominated by synthesis from endogenous precursors. Moreover in aquaculture, weaning of carnivorous fish from pure fishmeal to diets supplemented with carbohydrates is an important objective for improved sustainability and reduced environmental impact [18]. Hence, there is continuing interest in understanding to what extent their metabolic phenotype can adapt to increased dietary carbohydrate availability and if gluconeogenic utilization of amino acids is spared under these conditions [16]. To effectively address such questions and to im-

prove 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.

2.2. Radioactive versus stable isotope carbohydrate tracers

Historically, the use of radioactive isotopes such as ^{14}C and ^{3}H has 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 many 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. For this same reason, radioactive isotopes have been particularly useful in the study of larval nutrition. A supply of good quality fish fry is essential for a successful production of juveniles however knowledge of their nutritional requirements is often qualitative rather than quantitative [70]. In this context the innovative methodologies of incorporation of ^{14}C-labeled amino acids [71] or fatty acids [72] in larvae live prey *Artemia* sp. nauplii has improved our understanding of larval nutrition. However, for conventional nutritional studies involving larger fish and tanks, containment of radioactive tracers is often not practically possible therefore in these settings, their stable isotope counterparts (^{13}C and ^{2}H) are typically deployed. Methods for obtaining glucose rates of appearance (R_a) that were originally developed using [3]H- and ^{14}C-labeled glucose [23, 73, 74, 75, 76] can be easily adapted for stable isotope studies. Moreover, stable isotope tracers can potentially yield much richer metabolic information than their radioactive counterparts due to advances in positional and isotopomer analysis of metabolite enrichment from ^{13}C and ^{2}H that go above and beyond those which are possible for radiolabeled tracers.

2.3. Tracer administration and measurements in fish compared to mammals

Tracer measurements of whole-body carbohydrate metabolism were originally developed in humans and experimental animal models such as rodents, dogs and primates. Translating these methodologies to fish presents some additional challenges, the most important being the following: firstly, most mammals have accessible peripheral veins that can be catheterized for tracer administration and blood sampling. Catheters may be installed temporarily under anesthesia for the duration of the tracer study, or as indwelling entities for longitudinal studies of conscious free-moving animals. In fish, the dorsal aorta proximal to the gill arches is the main accessible vessel for tracer studies. It has been successfully cannulated for tracer infusion and blood sampling in undisturbed, non-anaesthetized rainbow trout of 800-1200 grams in weight [77, 78, 79]. However, it is unclear if this method can be easily applied to smaller sized fish, or to fish with a different gill-body configuration to rainbow trout, for example flatfish. The second major impediment for fish carbohydrate tracer studies is the fact that glucose appearance and turnover rates are much slower in comparison to mammals, necessitating far longer infusion periods for achieving isotopic steady-state con-

ditions – a critical requirement for investigating precursor-product relationships of biosynthetic pathways. This is particularly problematic for quantitative studies of gluconeogenesis based on the delivery of a labeled precursor substrate such as ^{14}C-lactate or alanine, since both the tracee precursor and glucose product pools must be at isotopic equilibrium during the sampling period.

2.4. Measurement of glucose R_a by bolus injection and by primed infusion

There are two approaches for quantifying plasma glucose R_a by isotope dilution. The first involves an intravenous injection of a tracer bolus and monitoring the decrease in specific activity or enrichment of plasma glucose as the tracer is being diluted by unlabeled glucose [75]. The dilution kinetics are best represented by more than one exponential decay function indicating the presence of separate pools of glucose in the body with different clearance characteristics. Because of the relative simplicity of a single injection tracer delivery, this method has been applied in many fish species including kelpbass *Paralabrax clathratus* [73], seabass *D. labrax* [74], *Hoplias malabaricus* [76], common carp *C. carpio* [23]. However, this approach requires frequent blood sampling over a sustained period to adequately describe the complex tracer clearance kinetics. Limited by the number of blood samples that could be drawn from the fish, the study described in [73] extrapolated the clearance kinetics from a smaller set of initial measurements, but the uncertainties of this approach were acknowledged. The alternative primed-infusion method establishes a constant level of tracer in the bloodstream following an appropriate priming dose. Under these conditions, the dilution of the tracer, measured from the ratio of specific activity or enrichment of infused label to that of blood glucose, is equal to the ratio of endogenous glucose appearance and tracer infusion rates. If glucose R_a is variable (for example during meal ingestion) then the rate of infusion needs to be adjusted accordingly in order to maintain a constant ratio of infused glucose to blood glucose specific activity or enrichment. Compared to the bolus injection method, primed infusion measurements require fewer blood samplings; in fact the sole rationale for multiple blood samplings is to verify a constant ratio of infused to blood glucose specific activity or enrichment. Calculation of glucose R_a from steady-state isotope data is more robust compared to single injection since it is independent of the complex and often poorly defined clearance kinetics. However, a primed infusion requires catheterization of a vein to deliver the tracer over an extended period and is technically more difficult than a single bolus injection. While these procedures now allowed glucose R_a to be well determined, and by combination with dietary tracers can determine the contributions of absorbed and endogenous glucose production to glucose R_a, they do not inform the sources of endogenous glucose production. Novel methodologies of resolving the sources of glucose R_a using deuterated water (^2H_2O) have been developed and can be integrated with primed-infusion glucose R_a measurements [80, 81, 82]. ^2H_2O is ideally suited for fish metabolic studies since it can be incorporated into aquarium water for an indefinite period and is rapidly incorporated into the fish body water such that the ^2H-enrichment level of the fish tissue water is also fixed for the duration in the ^2H-enriched tank water. As a result of enzyme-catalyzed exchange reactions between bulk water and metabolite hydro-

gens, many important precursor metabolites of glucose and glycogen biosynthesis, such as lactate, pyruvate and alanine also become rapidly enriched to the same level as body water for the study duration. Thus, a constant precursor enrichment that can be indefinitely maintained is possible. ^2H_2O has recently been used to study protein synthesis kinetics in the catfish *Ictalarus punctatus* [83] and gilthead seabream *S. aurata* [84] as well as blood glucose and hepatic glycogen turnover in seabass *D. labrax* [43, 85]. Because of its enormous potential for informing endogenous carbohydrate metabolism in fish, we will discuss its use in more detail.

3. Deuterated water as a tracer of endogenous glucose and glycogen synthesis

3.1. Overview

Fish and mammals share common pathways for glucose production and consumption [86] hence the underlying principles of plasma glucose ^2H-enrichment from ^2H_2O that are well described and validated for mammals [80, 87, 88] can be applied to fish. 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 administration of classical carbon tracers.

3.2. Basic principles

Deuterium (^2H) is a stable isotope of hydrogen with a nucleus containing one proton and one neutron (the ^1H nucleus contains no neutron). In the NMR experiment, ^2H resonates at a different frequency compared to its ^1H counterpart, allowing tracer levels of ^2H to be observed in the presence of the tracee ^1H. The inherent sensitivity of ^2H (at constant field and with an equivalent number of nuclei) is about 0.9% that of ^1H. Metabolism of ^2H is not exactly equivalent to that of ^1H 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-^2H bond is stronger than a C-^1H for any compound. Since metabolite transformation is governed in part by breaking and formation of C-H bonds, the presence of ^2H makes the bonds harder to break thereby potentially slowing the rate of C-^2H *vs.* C-^1H transformation. This can discriminate the transformation of ^2H-enriched metabolites compared to their tracees resulting in apparently slower rates of transformation. Moreover, with bulk levels of ^2H tracers, notably

^2H_2O, the aggregate isotope effects are toxic and indeed lethal to most living organisms. With tracer studies that utilize ^2H_2O, toxicity from isotope effects is minimized by substituting a relatively low proportion of ^1H by ^2H (<10%). Furthermore, discrimination against ^2H incorporation into metabolites via enzymatic reactions is minimized when the reaction that transfers ^2H 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 ^2H incorporation via kinetic isotope effects is not significant. Indeed, when ^2H-discrimination is observed, it informs the unidirectionality of a particular enzymatic step.

These caveats notwithstanding, ^2H_2O is an inexpensive tracer that is easily delivered into body water by immersion of fish in ^2H-enriched water. It has been successfully used in humans [89, 90, 91] and other mammals [82, 92, 93], for study of hepatic carbohydrate metabolism in physiological and pathophysiological conditions. The methodology was pioneered in humans [80, 87, 94] and was rapidly adopted by others in tissues [81, 88, 95]. As previously discussed, G6P is a common precursor to both glycogen and glucose and in the presence of ^2H_2O, G6P is labeled with ^2H 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 ^2H-enrichment level can be maintained indefinitely [96]. In fish, incorporation of ^2H from a 5%-enriched saltwater tank into plasma water is rapid, reaching more than 1% in 15 minutes, half of the enrichment of the tank water within 1 hour and approaching that of the tank water after 6h [85].

For studies of carbohydrate metabolism, the ^2H-enrichment distribution of plasma glucose from ^2H_2O is established according to origin of the G6P precursor (Figure 2.).

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. In mammals, G6P-F6P exchange is extensive and essentially complete hence the hepatic G6P pool is quantitatively 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 [93, 97]. There is some evidence that in fish, hepatic G6P-isomerase activity is sensitive to the nutritional state [43]. In common carp *C. carpio* hepatic G6P-isomerase activity decreased in direct relation to feeding rates [98, 99] 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 or its utilization by the pentose phosphate pathway.
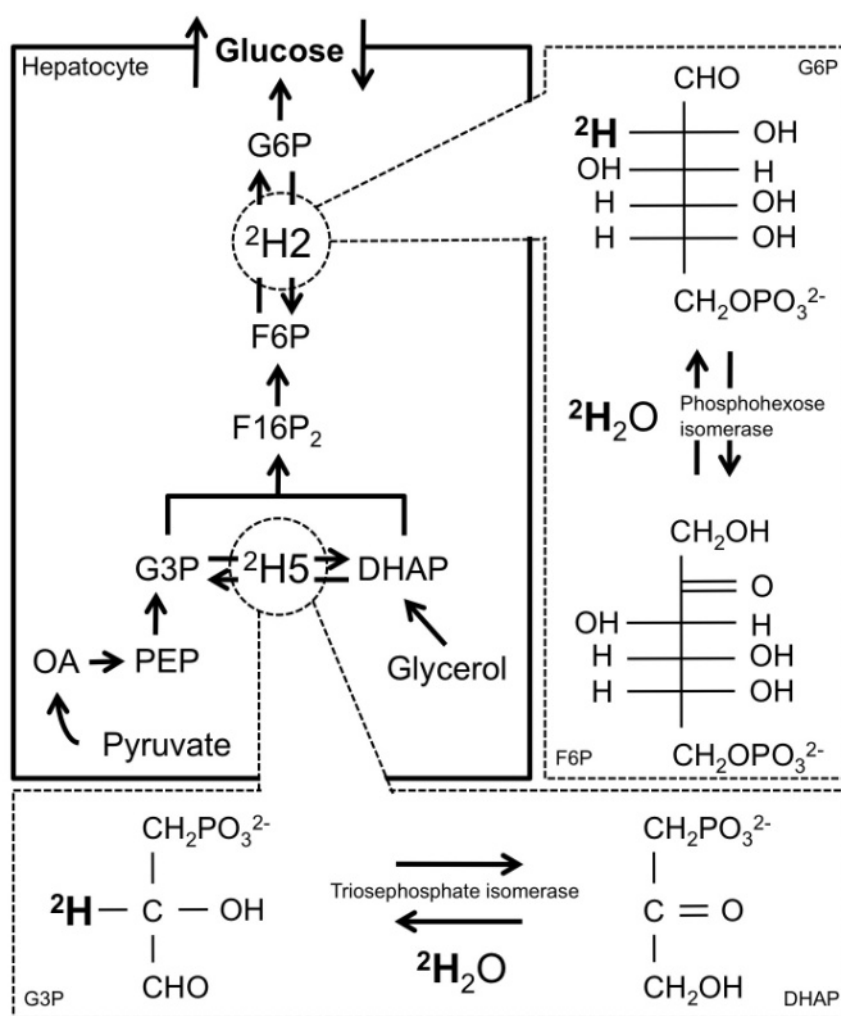
Figure 2. Metabolic model representing gluconeogenesis in the liver with special detail to the labeling with deuterium in position 2 (^{2}H2) and 5 (^{2}H5) of the glucose molecule. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P - glucose 6-phosphate; F6P - fructose 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.

Besides, ^{2}H-Enrichment in position 5 of G6P occurs at the level of triose phosphates as shown in Figure 2. Therefore, enrichment of plasma glucose or hepatic glycogen in position 5 reflects the contribution of gluconeogenic fluxes to endogenous glucose production or indirect pathway contributions to hepatic glycogen synthesis.

3.3. NMR and MS methods for ^{2}H enrichment analysis

Many improvements on the measurement of stable isotope tracer enrichment and analysis of positional labeling information have been largely driven by the development of nuclear magnetic resonance (NMR) and mass spectrometry (MS) technologies. 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 techni-

ques quantify 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 [87]. 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.

Following a simple method based on a LC-MS/MS procedure for quantifying plasma [6,6-^{2}H$_2$]glucose enrichment [100] which does not require glucose derivatization, analysis of glucose 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 [85]. 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 [85].

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$_2$O 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 [101]. 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 and 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 [102, 103, 104]. 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).

4. Conclusions

There is a compelling need to better understand the metabolism of carbohydrates by fish in general and aquaculture species in particular. Stable-isotope tracer methodologies have evolved such that safe, inexpensive and practical measurements of carbohydrate metabolism may be directly performed on naturally feeding fish in the aquaculture setting. These studies have great potential for informing the efficacy of novel dietary supplements in sparing the conversion of feed protein to carbohydrate as well as improving our general understanding of fish nutritional physiology.

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