UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

Early nutritional programming in fish: tailoring the metabolic use of dietary carbohydrates

Filipa Soares Rocha

Tese

Doutoramento em Ciências da Vida, do Mar, da Terra e do Ambiente
Ramo de Aquacultura
(Especialidade em Nutrição)

Trabalho efetuado sob a orientação de:

Prof. Dr. Maria Teresa Dinis, Professor Emérito da Universidade do Algarve, Portugal.

Dr. Jorge Dias, Diretor Geral da SPAROS Lda., Portugal.

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Declaração de autoria do trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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SUMMARY

Sustainable feeding practices in aquaculture require a higher use of vegetable feedstuffs, which are naturally rich in carbohydrates. This can raise some constrains to fish species with carnivorous feeding habits, due to their poor ability to use dietary carbohydrates. This thesis aimed to explore the potential of nutritional programming as a new strategy to better understand the mechanisms underlying the impaired utilization of dietary carbohydrates in fish. The experimental work relied on multiple approaches: a) rearing trials with larvae and juvenile fish for assessment of zootechnical criteria; b) analysis with radiolabeled tracers to follow the metabolic flux of nutrients; c) and genomic expression of key metabolic-genes. In **Chapters 2 and 3**, we evaluated how the supplementation of egg-yolk with glucose, through microinjection, could act as a stimulus and permanently alter some metabolic pathways in zebrafish. Microinjection was proven an efficient technique to alter the nutritional composition of embryo's yolk. We saw that the embryonic window for stimulus delivery is crucial in determining future outcomes. The late embryo stage was found more suitable to exert a glucidic stimulus compared with incipient stages of embryogenesis, based on molecular and metabolic analyses that suggested an improved capacity for glucose utilization. **Chapters 4 and 5** aimed the nutritional programming of the carbohydrate-related metabolic pathways in gilthead seabream. Recurrent hyperglucidic stimuli were delivered at early larval development using live preys and a glucose-rich diet, demonstrating that nutritional stimuli can be performed in a marine fish species during sensitive stages of development, without compromising survival and growth. We found some short-term effects after stimulus delivery, on gene expression pattern and metabolic utilization of glucose of post-larvae. In contrast, juvenile fish exposed to the early stimuli showed only few changes on glucose utilization, namely a higher absorption of dietary starch. This thesis has generated new knowledge on the triggering effect of early glucidic events upon the regulation of key metabolic processes, contributing to a better comprehension over the concept of nutritional programming in fish.

Keywords: Fish nutrition; Nutritional programming; Glucose metabolism; Nutrigenomics; Nutrient flux;

RESUMO

A crescente procura por práticas de alimentação sustentáveis no sector da aquacultura tem conduzido a uma maior utilização de ingredientes de origem vegetal, o que acarreta um aumento do teor de hidratos de carbono nas dietas de peixe, quer pela incorporação direta de amidos ou indiretamente através de subprodutos das proteínas vegetais. No entanto, a aplicação desta prática alimentar não é bem aceite por espécies de peixes com hábitos alimentares preferencialmente carnívoros, devido à sua fraca capacidade metabólica de utilizar hidratos de carbono. Esta Tese visa explorar o potencial da programação nutricional como uma nova estratégia nutricional para peixes, de forma a compreender as limitações inerentes à utilização metabólica de alguns nutrientes, nomeadamente os hidratos de carbono. Como principal objetivo, foi colocada a hipótese de que um estímulo hiper-glucídico, exercido durante as fases iniciais de desenvolvimento dos peixes, podia causar alterações permanentes sobre as vias metabólicas relacionadas com os hidratos de carbono. A componente experimental realizada nesta Tese contou com uma abordagem multidisciplinar, com foco em: a) ensaios de cultivo larval e de juvenis, para a avaliação de critérios zootécnicos; b) ensaios com marcadores radioativos, para analisar o fluxo metabólico de vários nutrientes; e c) análises da expressão de genes-chave envolvidos em relevantes vias metabólicas. Nos **Capítulos 2 e 3**, avaliamos se uma suplementação de glucose nos ovos de peixe-zebra (*Danio rerio*), por meio da microinjeção, poderia funcionar como um estímulo nutricional e afetar de forma permanente algumas vias metabólicas dos peixes juvenis. Pela primeira vez foi demonstrada a eficiência da técnica de microinjecção para alterar a composição nutricional das reservas presentes nos ovos de peixe. Verificou-se ainda que a "janela" embrionária selecionada para a entrega do estímulo é crucial na determinação dos efeitos a longo prazo. Os resultados indicam uma ação mais eficiente e promissora dos estímulos quando aplicados durante uma fase embrionária tardia (organogénese), comparativamente com períodos mais precoces do desenvolvimento embrionário (gastrulação); com base em análises moleculares e metabólicas verificou-se que os peixes inicialmente submetidos aos estímulos glucídicos demonstravam uma melhor capacidade de utilização metabólica da glucose assim como uma menor produção de glucose endógena. Os **Capítulos 4 e 5** foram dirigidos à programação nutricional das vias metabólicas de hidratos de carbono da dourada (*Sparus aurata*). A abordagem selecionada foi a entrega de repetidos estímulos híper-glucídicos, por meio da bioencapsulação do alimento vivo (rotíferos e artémia), a partir do início da alimentação exógena das larvas. O presente trabalho testou, pela

primeira vez, este procedimento numa espécie de peixes marinhos. Deste modo demonstrou-se que os estímulos nutricionais podem ser realizados durante períodos sensíveis do desenvolvimento inicial dos peixes, sem comprometer a sobrevivência e o crescimento larval. Alguns efeitos a curto prazo foram detetados imediatamente após a entrega dos estímulos às larvas de dourada, no padrão de expressão genética e utilização metabólica da glucose das pós-larvas. Em contraste, os juvenis de dourada expostos aos estímulos iniciais não sofreram alterações a nível da regulação molecular, apenas sofreram algumas mudanças no modo de utilização da glucose, refletidas por uma maior absorção de glucose proveniente do amido alimentar. Esta Tese permitiu gerar novo conhecimento sobre os potenciais efeitos da nutrição precoce de peixes, exercida em estadios críticos da embriogénese ou de larvas recém-eclodidas, sobre a regulação de importantes processos metabólicos, contribuindo assim para uma melhor compreensão sobre o conceito de programação nutricional em peixes.

Palavras-chave: Nutrição de peixe; Programação nutricional; Metabolismo da glucose; Nutrigenómica; Fluxo de nutrientes;

T**ABLE OF CONTENTS**

General Introduction

Chapter I

1.1. Sustainable production in aquaculture

Aquaculture is the fastest growing animal producing sector in the world and is expected to play an important role in global food supply (FAO, 2014). The expansion of this sector is dictated not only by the choices and needs of the consumers but also by the current trends of sustainable practices for nature exploitation, increasingly demanded by today's society. The path towards a sustainable development of aquaculture embraces numerous challenges at a social, economic and ecological level, that should be addressed in a dynamic and extensive manner (Klinger and Naylor, 2012). Efforts to develop a) novel culture systems (to reduce land occupation, intensify the production and reduce water pollution) b) alternative feed strategies (less expensive formulations that maintain efficient growth at lower cost per unit gain) and c) species selection (to perform a selective breeding, genetic traits) are some examples of the various factors that can be considered for aquaculture growth (Klinger and Naylor, 2012). Above all, the future of fish production is highly dependent on the production of high quality and healthy fry (larvae) and juveniles, so the question now is how to maintain high production standards and lower its costs without choosing unsustainable practices? Providing the adequate nutrition and fulfilling all the nutritional requirements are one of the key-factors to a successful growth, development and survival of fish larvae and juvenile (Holt, 2011). Furthermore, there is a permanent pressure around aquaculture industry to reduce its dependence on marine-based ingredients, namely fish meal and fish oil (Gatlin et al., 2007). The replacement of these high priced and limited ingredients has been done mainly through the use of plant ingredients, being soybean one of the most used. The high availability, global production levels and the wide range of products makes plant feedstuffs strong candidates for a sustainable aquafeed production (Hardy, 2010). However, several disadvantages of using plant-based ingredients in fish diets were reported comparatively to fish-based ingredients, such as low crude protein content, amino acids unbalances, low palatability, anti-nutritional factors and high amount of carbohydrates (Gatlin et al., 2007). Counting with all recent advances in the field of fish nutrition, several improvements were made through dietary manipulations, supplementation with additives and processing technologies of raw vegetable material to enhance growth and feed efficiency of farmed fish raised on plant feeds, but despite great efforts it is inevitable the increased intake of carbohydrates associated with either a protein or a starch vegetable sources (Klinger and Naylor, 2012).

Most marine teleosts, in particular those with carnivorous feeding habits, demonstrate a limited capacity to use dietary carbohydrates as energy-yielding substrate, mainly characterized by a persistent postprandial hyperglycaemia (Hemre et al., 2002; Moon, 2001; Polakof et al., 2012). Therefore, in the near future is expected some major changes in the field of fish nutrition, towards a higher use of plant ingredients in aquafeeds for marine species (particularly to carnivorous fish) and suitable to different life stages, from early larval stages until the final phase of production. A better understanding of the physiological mechanisms of fish, from an embryo to adulthood, underlying the use of dietary carbohydrates may allow exploitation of other feeding strategies, less conventional for fish.

1.2. Feeding marine fish with dietary carbohydrates

Nowadays, the major farmed species in Europe are the Atlantic salmon (*Salmo salar*), in cold waters and gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*), in the Mediterranean, holding 84.7% of the total production in 2013 (FEAP, 2014). Thus, European fish production relies mainly in carnivorous species, whose natural diet is characterized by a high-protein and low-carbohydrate contents. The natural feeding habit of a species is crucial to determine their ability to use dietary carbohydrates as energy substrate. Indeed, omnivorous fish, such as common carp (*Cyprinus carpio*), present a relatively high tolerance and effective use of dietary carbohydrates comparatively with carnivorous fish like salmon, trout and seabream (Polakof et al., 2012). This is mainly due to the marked differences among species regarding the morphology of the digestive tract, the efficiency of digestive processes, nutrient uptake and how all mechanisms are regulated at different levels. Additionally, throughout development the nutritional capacity and physiology of a species suffers major alterations, as most marine larvae start to feed before the digestive system has developed into its adult form (Rønnestad et al., 2013). Thus, to achieve a successful feeding and nutrient utilization by fish, it should be considered the nutritional composition of the diet and its suitability to the feeding behaviour of the species, as well as to the developmental stage in which is provided (Rust, 2003).

The digestive process involves a series of coordinated events beginning with food intake, followed by a chain of mechanical and enzymatic processes for reducing complex molecules into simple absorbable units, which are transported across the intestinal epithelium to the

blood stream for cellular metabolism (Figure 1.1) (Rust, 2003). Dietary inclusion levels and bioavailability of plant-based ingredients have a great impact on diet acceptance and utilization by fish. For several species of commercial interest, as salmon, rainbow trout (*Onchorhynchus mykiss*), seabream and seabass, the replacement of fish meal by plant proteins was successfully achieved, reaching a reduction of 25 to 50% since the beginning of their production (Hardy, 2010). In addition, carbohydrates have been used in diets primarily as energy sources and for their binding properties, however the possibility of sparing protein utilization has not yet been clearly demonstrated in fish (Hemre et al., 2002). Based on several studies, it was recommended that the inclusion level of dietary carbohydrates should not exceed 20% for carnivorous and marine species, whereas for omnivorous and warm water species the levels could be higher and up to 40% (Stone, 2003; Wilson, 1994). In agreement with these values, Enes et al. (2011) suggested that diets for gilthead seabream juveniles could include up to 20% of digestible carbohydrates without compromising growth performance and feed utilization.

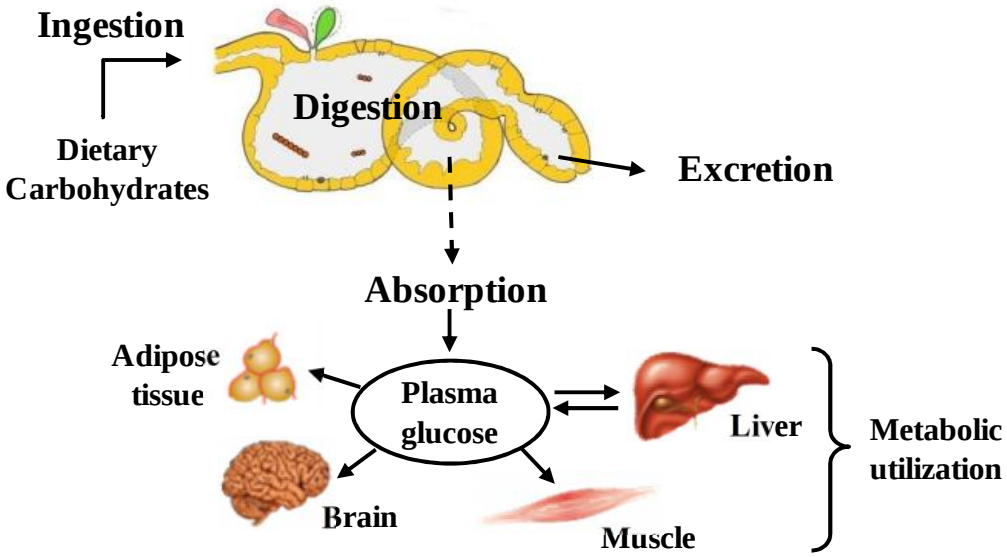

Figure 1.1 – Digestive processes during dietary carbohydrate flux, from the intake to metabolic utilization. Adapted from Rønnestad et al. (2013).

But a negative correlation between carbohydrates digestibility and increasing dietary levels was reported for several fish species, including gilthead seabream (Enes et al., 2008; Hemre et al., 1989; Krogdahl et al., 2004; Moreira et al., 2008). Also, a comparative study between the

omnivorous common carp and the carnivorous rainbow trout, both fed the same diet containing 28% of starch, showed a higher starch digestibility in the carp (90%) compared to with the trout (78%) (Yamamoto et al., 2001). These data suggest a limited capacity to digest starch, reflecting anatomical and functional differences of the gastrointestinal tract and digestive system of fish. In all teleost fish investigated to date, it was reported the presence and activity of digestive enzyme (carbohydrases), including the main enzyme involved in starch hydrolysis, the α-amylase (Enes et al., 2011; Krogdahl et al., 2005; Stone, 2003). The reduction in starch digestibility may be related to fish feeding habits and consequent variations on carbohydrases activity, since higher activities of these enzymes were reported in the intestinal tract of herbivorous fish in relation to carnivorous fish (Stone, 2003). This event might also be explained by the inhibition of some carbohydrases due to the saturation stage attained with an overload of the enzyme substrate, as it was described for amylase - starch reaction (Dona et al., 2010; NCR, 2011).

The digestion and subsequent metabolic use of dietary carbohydrates by fish can be affected by other factors related to the botanical origin of the starch, its molecular complexity and physical state and to abiotic factors like water temperature or handling-induced stress (Hemre et al., 2002; Stone, 2003; Wilson, 1994). For instance, it was demonstrated that the effect of raising water temperature improves the apparent digestibility of dietary starch of seabass and rainbow trout (Brauge et al., 1995; Moreira et al., 2008), while for gilthead seabream, the digestibility showed to be unaffected by the temperature (Couto et al., 2008). Concerning the botanical origin of the starch, it was found that rainbow trout and gilthead seabream presented higher apparent digestibility for wheat starch (58%, 91%, respectively) than for potato (5%) or maize starch (72%) respectively (Bergot, 1993; Venou et al., 2003); whereas seabass showed that, with a dietary level of 10%, maize starch was better digested (97%) compared to wheat starch (79%) (Dias et al., 1998; Enes et al., 2006). These differences may be attributed to the structural properties of the starch grain, namely the granule size and the ratio between amylose/ amylopectin, the major components of starch (Bergot, 1993; Dona et al., 2010). Starches that present large granules and a high content of amylose are often poorly digested (Dona et al., 2010). The use of processing techniques, like extrusion and gelatinization, to alter the physical state of native starches granules, allowed to improve its digestibility in several species (Enes et al., 2011; Krogdahl et al., 2005; Rust, 2003).

After carbohydrate digestion the main final product is glucose, which is then absorbed along the intestinal tract and transported to the major metabolic organs – liver, muscle and adipose tissue – via the circulatory system (Figure 1.1). The uptake of glucose in fish intestinal tract is mediated by active glucose-transporters present in the brush border membrane which is followed by a simple diffusion across the basolateral membrane, in a similar way to that found in mammals (Krogdahl et al., 2005). As so, the glucose transport can occur passively and Na^+ independent, through the GLUT carries of the facilitated glucose transporters family and by electrogenic, Na^+ dependent transport by the action of the SGLT cotransporters family (Bakke et al., 2010). To present date, several members of glucose transporters were identified, cloned and analised at a molecular level, in different fish species, namely the GLUT1 (Balmaceda-Aguilera et al., 2012; Hrytsenko et al., 2010), GLUT2 (Castillo et al., 2009; Terova et al., 2009), GLUT3 (Hall et al., 2005), GLUT4 (Planas et al., 2000) as well as SGLT1 (Geurden et al., 2007; Polakof et al., 2010) types. Glucose transporters seem to be fairly conserved within the vertebrates group, based on the degree of similarity found between the mRNA of fish and mammals (Krogdahl et al., 2005). Nevertheless, when compared to mammals, fish exhibit a lower capacity for glucose uptake associated with variations of transporters towards glucose affinity along the intestinal tract (Collie and Ferraris, 1995; Krogdahl et al., 2005). Two hypotheses were placed to explain the reduced rate of glucose absorption in fish intestine, comparatively to mammalian intestine: a) a lower densities of transporters or b) a lower amount of absorptive tissue (Collie and Ferraris, 1995). Intestinal transporters of glucose play major roles in the intermediary metabolism, regulation of hormones release (as glucose sensors) and maintenance of glucose homeostasis (Enes et al., 2009; Polakof et al., 2010).

Whenever the intake of dietary carbohydrates leads to a high blood glucose level that exceeds the renal threshold of fish, the excess glucose can be excreted through urine (glycosuria) or through the gills (Bucking and Wood, 2005; Deng et al., 2001; Hemre and Kahrs, 1997). Summarizing, each step of carbohydrates utilization by fish, from the intake to absorption and transport, present several factors that can narrow the use of plant-based diets, naturally rich in carbohydrates. However, the full potential and efficiency of using carbohydrates as energy yielding-substrates in diets is not only limited to its digestibility or absorption, the metabolic utilization of glucose and mechanisms underlying its regulation are equally important and crucial.

1.3. Metabolic network for glucose utilization

Regardless of the efficiency level of intestinal absorption, fish usually exhibit a poor metabolic use of dietary glucose, especially those species of a high trophic level (carnivorous) whose natural feed is rich in protein and poor in carbohydrates (Moon, 2001; Wilson, 1994). Following the intake of a carbohydrate-rich diet, fish tend to react with a persistent postprandial hyperglycaemia along with a lack of control on glucose homeostasis, which can later be reflected in a decrease of growth and development of a "fatty" liver (Figure 1.2) (Enes et al., 2011; Hemre et al., 2002; Polakof et al., 2012). Even with a significant research effort devoted to this issue, the physiological mechanisms underlying the apparent "glucose intolerance" in fish remain unclear. Until now, several hypothesis have been proposed to explain this inefficient use such as: lack of inhibition of endogenous glucose production (Panserat et al., 2000b; Panserat et al., 2001a), weak induction of hepatic lipogenesis from glucose (Ekmann et al., 2013; Polakof et al., 2012), inadequate response towards the control of blood glucose levels in particular after a high glucose load (Figure 1.2) (Legate et al., 2001; Peres et al., 1999) as well as the lower potency of glucose over amino acids as insulin secretagogues (Andoh, 2007; Mommsen and Plisetskaya, 1991) and the relatively low number of muscle insulin receptors (Navarro et al., 1999).

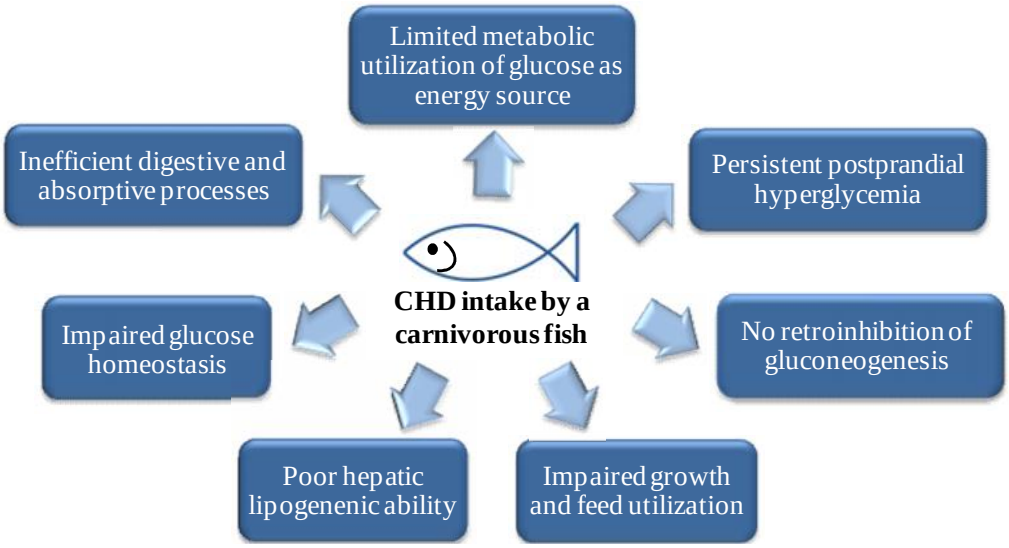

Figure 1.2 – Relevant negative effects related to dietary carbohydrate (CHD) intake and glucose metabolism by a carnivorous fish species.

Surprisingly, fish have the whole enzymatic machinery for carbohydrates metabolism fully functional, and the metabolic pathways are active similarly to mammals, however their regulation by nutritional and hormonal factors has shown to be different (Figure 1.3) (Dabrowski and Guderley, 2003; Enes et al., 2009). The activity and gene expression of the major enzymes involved in starch digestion, glucose phosphorylation (glycolysis), glucose production (gluconeogenesis), glycogen synthesis (glycogenesis) and breakdown (glycogenolysis) and the presence of glucose transporters have been confirmed and characterized in several fish species (Dabrowski and Guderley, 2003; Enes et al., 2009; 2011; Hemre et al., 2002; Krasnov et al., 2001; Krogdahl et al., 2005; Panserat et al., 2000a; Planas et al., 2000). Furthermore, the enzymes involved in lipogenesis and in the pentose phosphate pathway have also been described in fish livers (Dabrowski and Guderley, 2003; Figueiredo-Silva et al., 2012; Kamalam et al., 2012; Polakof et al., 2011; Richard et al., 2006). Although not directly linked to glucose metabolism, these two metabolic pathways are important for the maintenance of glucose homeostasis, since the glucose in excess can be converted into fatty acids through lipogenesis, which is dependent of the reducing potential of NADPH, that is mainly originated through the pentose pathway (Towle et al., 1997). The induction or repression of the activity of these metabolic enzymes by the intake of carbohydrate-rich diets can be observed at different levels through: a) regulation of gene expression; b) post-translational modifications of the proteins (e.g. phosphorylation, acetylation) and c) allosteric regulation of the proteins (Enes et al., 2009; Pilkis and Granner, 1992). In fish, as in mammals, glucose oxidation by the citric acid cycle and the respiratory chain results in ATP production (Dabrowski and Guderley, 2003). The citric acid cycle is a key metabolic pathway that unifies the glucose, fatty acid and amino acid metabolisms through a series of anaplerotic reactions (input of intermediates) and cataplerotic reactions (deplete of intermediates). Concerning cataplerosis, the carbons produced from glycolysis that enter the cycle can be used for nonessential amino acid synthesis as well for lipogenesis or gluconeogenesis (Bequette et al., 2006). Therefore, the proportion and availability of dietary carbohydrates affects not only the main pathways of glucose metabolism, but also the parallel metabolic fluxes that are interconnected to it, which indicates a dynamic and complex regulation of the whole metabolic network underlying the inefficient use of glucose by fish. Figure 1.3 summarizes the main metabolic pathways that occur in the hepatocytes focused in the catabolism and production of glucose.

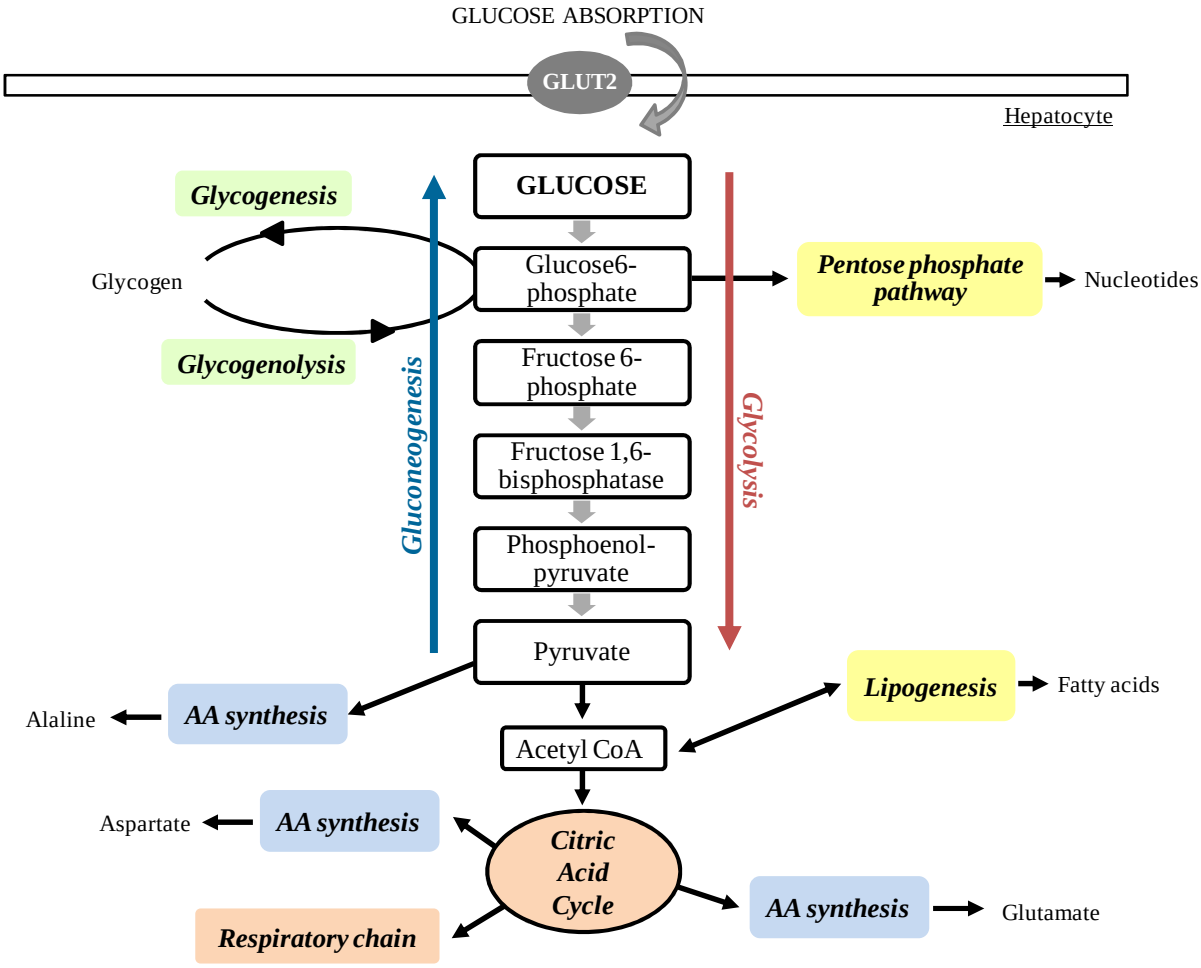

Figure 1.3 – Representation of the hepatic intermediary metabolism: the pathways for glucose phosphorylation (glycolysis) and glucose production (gluconeogenesis) and their connections with other metabolic pathways involved in the storage of excess glucose (glycogenesis and lipogenesis), energy metabolism (pentose phosphate pathway, citric acid cycle and respiratory chain) and amino acid biosynthesis. The main end-products of each pathway are, as well, presented.

The main pathways for glucose metabolism are the glycolysis, where occurs the phosphorylation of glucose to pyruvate and its opposite pathway, the gluconeogenesis, where glucose is synthesised *de novo* from non-carbohydrate substrates (Pilkis and Granner, 1992). The glycolytic pathway is the major route for glucose utilization in all living organisms, and its flux is regulated at three limiting steps catalysed by the hexokinase (HK), 6-phosphofructo-1-kinase (6PFK) and pyruvate kinase (PK) (Figure 1.4.) (Oosterveer and Schoonjans, 2014; Pilkis and Granner, 1992). When glucose is present in excess, the hepatic-specific hexokinase VI, named as glucokinase (GK), acts firstly to induce the storage of glucose as glycogen, instead of promoting the glycolytic path. In mammals the activity of this

enzyme is under a strong nutritional and hormonal control (Iynedjian, 1993). A similar induction was found for the piscine form of GK, which has been already characterized at a biochemical and molecular levels in several fish species (Panserat et al., 2014). The increase of GK activity in rainbow trout fed with high dietary carbohydrates was often associated with a significant induction of GK gene expression (Panserat et al., 2001b; Seiliez et al., 2011; Soengas et al., 2006), which strongly suggests the capacity of carnivorous species to adapt to carbohydrate intake and, therefore, to future use of plant-based diets.

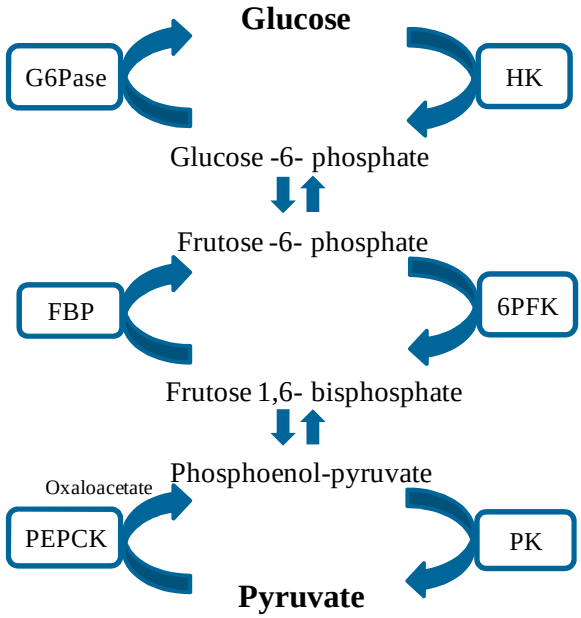

Figure 1.4 – Enzyme / Substrate cycles in the glycolytic and gluconeogenic pathways. Adapted from (Pilkis and Granner, 1992).

The regulation of the gluconeogenic pathway is dependent on three key-enzymes, the phosphoenolpyruvate carboxykinase (PEPCK), the fructose-1,6-bisphosphatase (FBPase) and the glucose-6-phosphatase (G6Pase), each corresponding to a rate-limiting step of glucose endogenous production (Figure 1.4.) (Nordlie et al., 1999; Oosterveer and Schoonjans, 2014; Pilkis and Granner, 1992). Unlike glycolysis which is a non-tissue specific pathway, gluconeogenesis takes place mainly in the liver and, in less extent, in the kidney and intestine (Kirchner et al., 2008; Knox et al., 1980). Also, this pathway was found to be sensitive to a nutritional and hormonal regulation in omnivorous fish, such as common carp (Shimeno et al., 1995; Sugita et al., 2001), in a similar way to that observed in higher vertebrates. Thus, the

induction of the gluconeogenic enzymes occurs during glucagon release and starvation periods, whereas its repression occurs with insulin release, refeeding and high intake of dietary carbohydrates (Pilkis and Granner, 1992). On the contrary, carnivorous fish such as rainbow trout, gilthead seabream and European seabass, fed with high carbohydrate levels showed poor regulation of hepatic gluconeogenesis, reflected by a lack of inhibition of the major involved enzymes (Caseras et al., 2002; Enes et al., 2006; Panserat et al., 2000b; Panserat et al., 2001a). As previously mentioned, the absence of inhibition of endogenous glucose production, regardless the carbohydrate content of the diet, is one of the possible reasons for the poor use of dietary carbohydrates and "glucose intolerance" of carnivorous fish. But this hypothesis needs to be deeply investigated, since it was shown in seabream a down-regulation of some gluconeogenic genes after the intake of high carbohydrates (Panserat et al., 2002).

Liver plays a key role in regulating the intermediary metabolism and glucose homeostasis in fish. However, it was found that the white muscle has a relatively lower glycolytic capacity compared to the liver, and that is not regulated by dietary carbohydrates, at least at a molecular level, in several fish species (Hemre et al., 2002; Kamalam et al., 2012; Seiliez et al., 2011; Seiliez et al., 2013). The presence of an insulin sensitive glucose transporter (GLUT4) was reported in the muscle of rainbow trout (Diaz et al., 2009), however even with a functional glucose flux into the muscle, the glycolytic pathways remained unaffected after the intake of carbohydrates or injection with glucose (Legate et al., 2001; Panserat et al., 2009). Therefore, it seems that muscle does not have a major role in the regulation of glucose metabolism, despite being the largest tissue in fish, by representing almost 50% of the body weight of a fish (Moon, 2001).

Fish possess the two major pancreatic endocrine hormones, insulin and glucagon, responsible for the regulation of glucose homeostasis and all the metabolic pathways underlying carbohydrate metabolism (Navarro et al., 2002). Piscine insulins forms are structurally close to other vertebrates insulins while the glucagon hormone was found to be well conserved across most vertebrates (Navarro et al., 2002). The mechanisms underlying hormones secretion are also similar among vertebrates. After feeding (regardless the carbohydrate content) or a glucose load, insulin is released in the blood and induces glycolysis at the same time that inhibits gluconeogenesis and glycogen synthesis in the liver, muscle and adipose tissues (Caruso and Sheridan, 2011; Mommsen and Plisetskaya, 1991). However, in many

carnivorous species, dietary amino acids appear to play a more important role in simulating insulin secretion than glucose (Andoh, 2007; Mommsen and Plisetskaya, 1991). Several *in vivo* and *in vitro* studies have shown the high insulinotropic potential of some amino acids, such as glycine, arginine, alanine and lysine, in different fish species (Andersen et al., 2014; Navarro et al., 2002; Rojas et al., 2009). Contrary to this, under a situation of food deprivation, the secretion of glucagon increases and glucose *de novo* synthesis is stimulated by the breakdown of glycogen and hepatic gluconeogenesis (Dabrowski and Guderley, 2003; Moon, 1998; Navarro et al., 1999). It is now accepted that both pancreatic hormones, insulin and glucagon, are fully functional in fish and thereby are not directly related to the poor metabolic use of carbohydrates, nevertheless their regulation by nutritional factors is still scarcely studied in fish (Polakof et al., 2012).

1.4. Specificities on carbohydrate utilization at embryonic and larval stages

Once spawned and fertilized the oviparous fish eggs operate as semi-closed system and only respiratory gases, heat and negligible amounts of solutes and water are exchanged freely (Kamler, 2008). The low permeability to external factors results from the hardening of the egg surface membrane, after being exposed to water (Heming and Buddington, 1988). The newly spawned eggs are therefore totally dependent on its endogenous yolk reserves to supply all the energetic substrates needed during growth and development. In turn, the nutritional composition of the egg/embryo yolk reserves was shown to be strictly dependent on the nutritional status and feeding habits of the broodstock (Fernández-Palacios et al., 2011). Therefore, the source, availability and pattern of depletion of the metabolic fuels during embryogenesis and the yolk-sac larval stage can vary between fish species and is associated to specific nutritional state of broodstock (Rønnestad et al., 1999). Even not being considered as a definite sequence for nutrient depletion, it is generally accepted that fish ontogeny begins with a short period of glucose utilization, soon switched to free amino acids (FAA). Recent studies have shown that marine fish eggs have a significant pool of FAA, which was found to play an important role in energy metabolism and protein synthesis during early development (Rønnestad et al., 1999), and in species that possess pelagic eggs (with oil globules), such as seabream, the FAA pool is mainly consumed prior to hatching (Rønnestad et al., 1994). Then, after hatching, lipids were found to be mostly mobilized (in triacylglycerols and phospholipids forms) in response of increased energy demand from the newly hatched larvae.

Finally, a second peak of amino acid catabolism occurs after the exhaustion of the yolk reserves, when body protein-bound amino acids are mobilized (Kamler, 2008). Several studies in freshwater and marine fish have demonstrated that carbohydrates, derived from maternal glycogen reserves or glycoproteins, are used as primary energy source for catabolism during a very short period of early embryogenesis, mostly prior to gastrulation (Heming and Buddington, 1988; Kamler, 2008). Although fish eggs contain relatively low levels of carbohydrates compared to the amount on protein and lipids, in the common carp, an omnivorous species, carbohydrates represent 1.5- 6.2% of the egg dry mass, while in the carnivorous rainbow trout is found only in trace amounts (0.6%) (Heming and Buddington, 1988), thus the availability and use of carbohydrates in catabolic processes is species-specific. By analyzing the profile of glycogen and glucose levels during zebrafish embryogenesis, becomes clear the importance of these substrates as fuels prior to hatching, since the decrease of glycogen endogenous reserves is followed by an increase of free glucose levels (Soanes et al., 2011). Also, throughout zebrafish embryogenesis occurs a relatively steady increase of amino acids levels, which might explain, in part, the small pool of FAA found in some freshwater species eggs (Rønnestad et al., 1999; Soanes et al., 2011).

It is well known that yolk-sac stage represents an important developmental period for all fish larvae. At this stage, larvae undergo major morphological, anatomical and functional changes before the onset of exogenous feeding. Marine and small-sized eggs freshwater fish species have similar basic mechanisms involved in the differentiation of the gastrointestinal tract, although differences among species can be found in the relative timing of the ontogeny (Lazo et al., 2011). Fish larvae present an immature and undifferentiated digestive tract at hatching time, namely the two fish species studied in this thesis, seabream and zebrafish. The development of the main metabolic organs occurs between the transitional period of endogenous to exogenous feeding; commonly the liver, pancreas and intestine are formed and partially functional by the time of mouth opening and the ingestion of external food, whereas the morphogenesis of the stomach is species dependent (Zambonino-Infante et al., 2008). Also, the enzymes responsible for digestion of proteins, lipids, and carbohydrates are present at first-feeding (Lazo et al., 2011). In particular, the pancreatic enzyme α-amylase, a key enzyme for the digestion of complex carbohydrates, has been detected in early larvae of several fish species, including seabream (Naz, 2009; Zambonino-Infante et al., 2008) and zebrafish (Yee et al., 2005). In general, α-amylase activity is higher at the onset of exogenous feeding, then according to the feeding habits, the pattern of activity gradually declines, in

carnivorous species, or progressively increases, in omnivorous species, along fish development (Zambonino-Infante et al., 2008; Zouiten et al., 2008). Concerning the molecular regulation of α-amylase, high mRNA levels of amylase have been observed during the initial stages of larval development, followed by a progressive decrease at later stages, which suggests that the activity and expression of amylase might be genetically programmed and regulated at a transcriptional level, during the development of marine larvae (Peres et al., 1996; Zambonino-Infante et al., 2008). Although the increase of activity of this digestive enzyme may be related to the onset of exogenous feeding, the common live preys of marine larvae are not naturally rich in starches, therefore the physiological function of this enzyme during the early life of marine fish are not completely understood. Nevertheless, the particular enzymatic action of amylase may suggest a natural predisposition of young fish larvae to use dietary carbohydrates (Krogdahl et al., 2005).

1.5. Nutritional programming: emerging tool for fish nutrition

As firstly mentioned, it would be of great interest, economically and environmentally, to improve the ability of fish to use dietary carbohydrates as energy substrates that allowed a higher incorporation of plant feedstuffs in their diets. From our brief review, it is clear that fish possess the whole enzymatic machinery required for the digestion, absorption and metabolism of carbohydrates however some species, particularly those with carnivorous feeding habits, have a limited capacity to use dietary carbohydrates, mainly reflected in the poor regulation of glucose metabolism. Despite the significant research work developed over the last years, the physiological basis underlying such metabolic deficiency remains unclear. The development of alternative nutritional strategies may contribute to generate novel knowledge, essential for overcoming the bottlenecks related to the poor utilization of carbohydrates as energy sources. Under this scope, the approach by means of nutritional programming appears to be as interesting as challenging.

1.5.1. Nutritional programming in mammals

The term “programming” was introduced into scientific literature by Lucas (1991) to describe the process by which exposure to specific stimulus or insult, during critical windows of development, can result in permanent changes in somatic structures, physiological systems or

metabolic status of the organism. The extension of this concept to the field of early nutrition is known as ”nutritional programming” and has been largely studied in mammalian models to understand the consequences in adulthood of an altered nutrition during the intrauterine or post-natal periods, (Burdge and Lillycrop, 2010; Lucas, 1998; Metges et al., 2014; Ozanne and Hales, 1999; Patel and Srinivasan, 2002; Patel et al., 2009; Srinivasan et al., 2003). The time frame in which the programming event can occur is often confined to sensitive periods of high developmental plasticity. The majority of tissues retain their maximum plasticity during the periods of embryonic and fetal development, however the period immediately after birth, during lactation, is also considered of high susceptibility (Gluckman et al., 2011; Patel and Srinivasan, 2002). In human health, considerable efforts have been devoted to knowing how nutritional stimulus during pregnancy can induce the risks for the onset of chronic diseases, such as the metabolic syndrome, obesity and diabetes (Buckley et al., 2005; Burdge and Lillycrop, 2010; Langley-Evans, 2015; Patel and Srinivasan, 2002; Vieau, 2011). As a result there are clear evidences of the persistence of the programmed phenotype, present in the mother, as well as the possibility of its transmission to the offspring (generational effect) (Lim and Brunet, 2013; Vickers, 2014).

The mechanisms involved in metabolic regulation by early nutritional events are still not fully understood. Based on strong evidences that the genome can be “imprinted” to store genetic memory of the resulting nutritional event, mechanisms related to epigenetic modifications, such as DNA methylation, histone acetylation and non-coding RNAs have been suggested as potential sources for reprogramming the gene expression pattern (Anderson et al., 2012; Badeaux and Shi, 2013; Jaenisch and Bird, 2003; Lillycrop and Burdge, 2012). Although not encoded in the genes, epigenetic changes are known to be transmitted hereditarily, which largely supports the evidences of vertical transmission of metabolic-related diseases, from mothers to their progeny (Jaenisch and Bird, 2003; Youngson and Whitelaw, 2008). Moreover, new data on the role of nutrients, and their availability levels, as modulators of epigenetic mechanisms shows an increasing list of dietary components involved in chromatin remodeling (Badeaux and Shi, 2013; Milagro et al., 2013). Other possible mechanisms responsible for the programming outcomes were suggested, such as tissue remodelling and differential cellular proliferation, impaired mitochondrial function and nutrient-sensitive signalling pathways. Early events exerted during the phases of cellular proliferation and differentiation can caused tissue remodelling and compromise its function, which automatically causes a major impact upon the organ structure (Langley-Evans, 2015). Several

studies have shown that the pancreas is particularly sensitive to early nutritional manipulations, as was the case of a study performed in rats submitted to a low- protein regimen during pregnancy that resulted in a decrease of islet size and cell proliferation of the offspring (Snoeck et al., 1990). An impaired mitochondrial function can result from long-term adaptations to early diets, being reflected in an imbalance of energy homeostasis. For instance, the increase of the reactive oxidative species and oxidative stress together with the decrease of the antioxidative defense capacity, was reported in pancreatic islets of a model of intrauterine growth retardation (rat), as being the result of the reprogramming of mitochondrial function (Simmons et al., 2005). For last, if the metabolic pathways of energy are affected it can alter the production of metabolites and interfere with their role as cofactors in signaling pathways, which may or may not alter DNA transcription by means of epigenetic mechanisms (Zawia et al., 2009).

The long-term effects of early nutritional stimulus on the glucose metabolism are well documented by numerous studies in adult animals, mostly on mammals. Also, recent studies have suggested that glucose may interact, as a cofactor, in signaling pathways involved in the regulation of the epigenome (Badeaux and Shi, 2013). An acute exposure to an increasing range of insulin levels during the gestational period induced glucose intolerance in the progeny of rats (Harder et al., 1998) while a restricted intake of proteins during the intrauterine period had a significant effect on the offspring tolerance to glucose, which revealed to be age-dependent, so in adulthood the offspring develop some diabetic symptoms (Ozanne and Hales, 2002). Changes in the immediate neonatal nutrition (lactation) were also found to have life-long consequences on carbohydrate uptake and metabolism. It is the case of the well study high-carbohydrate (HC) rat model. The use of an artificially high-carbohydrate milk formula during the lactation of rats pups before weaning resulted in an increased activity of the hepatic GK (Patel and Hiremagalur, 1992) and in altered mRNA transcription levels of genes involved in energy metabolism and appetite control, that persisted into adulthood (Srinivasan et al., 2008). Together these evidences prove the potential of glucose as a programming nutrient as well as demonstrate the vulnerability of carbohydrate metabolic pathways to nutritional regulation.

Programming by non-nutritional factors during early life is also documented in mammals. Extensive literature on the hazardous effects of the consumption of coffee, tobacco, alcohol and drugs during pregnancy clearly demonstrate the development of several chronic diseases,

as respiratory failure, hepatic failure, abnormal endocrine function, behavior problems and also of some disorders as stress, depression and anxiety, in adulthood (Hellemans et al., 2010; Huizink and Mulder, 2006; Loomans et al., 2012; Maritz and Harding, 2011). Also it was shown that the exposure to stressful conditions during early neonatal period, involving an excessive maternal care (licking and grooming) alters the DNA methylation pattern of the offspring (Meaney and Szyf, 2005).

1.5.2. Nutritional programming in fish

In fish development, the windows of highest sensitivity and metabolic plasticity are restricted to the periods of embryogenesis and early larval development, similar to what is observed in mammals. When the concept of nutritional programming, based on previous studies performed in higher vertebrates is applied to fish, caution should be taken in the interpretation and extrapolation of possible results, since mammals and fish exhibit a distinct embryonic development. Therefore, while the foetus is directly linked to maternal nutritional experiences, possibly in a continuous state, through the umbilical cord and placenta, the fish embryo rely exclusively on his endogenous yolk reserves, acting as a semi-closed system unresponsive to external factors. Thus, the possibility of delivering a nutritional stimulus with success becomes more difficult to accomplish during the early development of fish.

Despite the mechanisms involved in metabolic regulation by nutritional factors are not fully understood, there are strong evidences that the genome can be “imprinted” to store genetic memory of the resulting event. These mechanisms, described in more detail in the previous section, can be (partly) related to epigenetic regulation, through covalent modifications of DNA and histone proteins, that include methylation, acetylation, phosphorylation, among others. Although not targeting nutritional factors, recent studies have demonstrated that specific abiotic factors like temperature can induce epigenetic alterations in fish through changes on the DNA methylation pattern, as observed in terrestrial vertebrates. A study performed in Senegalese sole showed that muscle growth of larvae undergoing metamorphosis was affected by the early rearing temperature of newly-hatched larvae, by means of methylation of the myogenin promoter. The lowest rearing temperature (15ºC) induced the methylation of myogenin promoter, which subsequently affected the expression of myogenin gene in the muscle and reduced the growth rate and muscle cellularity (Campos et al., 2013). In the same environmental frame, a research in European seabass upon the shift

of sex-ratio in response to temperature showed that exposure to high temperatures (21ºC) increased the methylation level of the gonadal aromatase (cyp19a) promoter in both males and females. As result, the expression of aromatase gene was repressed and through the silencing of this gene, the development of males during sex differentiation was promoted. Thus, it was presented strong evidences that temperature-dependent sex determination in European seabass might be controlled by epigenetic mechanisms of DNA methylation (Navarro-Martín et al., 2011). Several other studies were performed in different fish species to enlighten the role of environmental factors as modulators of epigenetic mechanisms during the differentiation and determination of sexual phenotypes (Piferrer et al., 2012). A recent study in zebrafish showed that gene expression during early stages of embryogenesis can be markedly regulated by DNA methylation mechanisms, indicating this species as susceptible to early genomic imprinting (Andersen et al., 2012). Indeed, Olsen et al. (2012) showed that the phenomenon of diabetic metabolic memory might been transmitted to new generations of hyperglycemic zebrafish, through mechanisms related to DNA demethylation. It was also demonstrated how an exposure to nutrients excess, such as to glucose, can permanently increase the number of β-cells in zebrafish pancreas, showing that other mechanisms besides DNA methylation can be involved in the epigenetic regulation of the genome (Maddison and Chen, 2012). Current studies in mammalian model systems have shown that microRNA constitute important regulators of the intermediary metabolism (Dumortier et al., 2013). These findings were further extended to the effects of early nutritional events on programming the expression of microRNA. In mammals, a maternal fat-rich diet during gestation was found to alter the expression of key metabolic microRNAs of the offspring, possibly associated with altered DNA methylation (Zhang et al., 2009). These evidences of microRNAs involvement in nutritional programming of mammals could be potentially applied to fish. In fact, it was shown that, in rainbow trout alevins, the nutritional shift from endogenous to exogenous feeding changed the expression of specific microRNAs involved in the regulation of metabolism, being the new pattern consistent with the storage of external feed (Mennigen et al., 2013). Despite being fragmented and little related to nutritional factors, these evidences strongly support the hypothesis that nutritional events can potentially trigger permanent changes in the genome in fish.

Critical windows of development are highly restricted in fish. Thus the periods to exert a nutritional stimulus during stages of high developmental plasticity are narrowed to: a) maternal nutrition, through nutrient transfer to the yolk reserves and b) the onset of exogenous

feeding, when larval nutrition can be manipulated by live prey enrichments or inert diets. In gilthead seabream, the positive effects of feeding the broodstock with a high quality diet, enriched with essential fatty acids, were reflected in a better egg quality and larval development (Fernández-Palacios et al., 1995; Fernández-Palacios et al., 1997). Other dietary components such as vitamin E, vitamin C, carotenoids and phospholipids were also considered as important nutrients to be fed during reproduction, since it was found an improvement of egg, sperm and larval quality (Izquierdo et al., 2001). Just recently, a study in gilthead seabream revealed that early nutritional programming could be achieved through parental feeding, with positive long-term metabolic changes of the offspring. When the broodstock was fed with a linseed-oil rich diet (over 60% of fish oil replacement) the resulting progeny proved to be better adapted to use diets with high vegetable oils /vegetable proteins, by showing improved growth rate and feed utilization. However, even not relevant to the nutritional programming aim, some short-term negative effects were found when fish-oil was replaced 100% by linseed-oil in the broodstock diets, the fecundity, spawn quality, growth and Δ6 desaturase gene expression of 45DAH post-larvae were significantly affected (Izquierdo et al., 2015). Currently, research on nutritional programming in fish is still scarce, but the promising results shown at different studies are raising a growing interest in this subject.

The concept of nutritional programming was applied for the first time to fish by Geurden et al. (2007). The authors have demonstrated that an acute hyperglucidic stimulus exerted at the onset of exogenous feeding, induced the expression of carbohydrate digestive enzymes (maltase and amylase) in rainbow trout juveniles, suggesting some long-term physiological changes. Although the absence of phenotypic differences in growth and postprandial glycaemia has been observed, data showed persistent molecular adaptations to the early nutritional event, which may be related to epigenetic mechanisms, and thus, in line with the mammalian concept of nutritional programming. Since then, only few studies were performed under this topic. Two of these studies reported an enhancement of the expression of delta 6-desaturase gene in European seabass juveniles, which had been previously submitted, from first-feeding, to a dietary deficiency in long-chain 3-n polyunsaturated fatty acids (Vagner et al., 2007; Vagner et al., 2009). The remaining studies were focused on the effects of early exposure to high levels of dietary carbohydrates, during the first-feeding period, on the modulation of carbohydrate metabolic pathway. Therefore, these studies demonstrated some persistent adaptations on molecular markers related to the carbohydrate metabolism in

rainbow trout (Geurden et al., 2014), zebrafish (Fang et al., 2014) and Siberian sturgeon (Gong et al., 2015).

1.6. Objectives

The aim of this thesis is to generate novel knowledge on the concept of nutritional programming in fish, during the early life stages of development, which is still scarcely investigated in the field of fish nutrition. This thesis intends to unravel how the glucidic stimuli, operating as nutritional events during sensitive periods of fish development, affect the regulation of key-metabolic pathways, in particular those involved in glucose homeostasis, and if these effects can persist in the adult life of the fish. Also, a novel technique for *in ovo* supplementation of nutrients is presented as possible accurate tool to alter the nutritional content of fish embryos. For this purpose, the following goals were proposed:

» Establishment of the technical and biological basis for the supplementation of zebrafish eggs with glucose: tolerance of embryos to a range of high doses of glucose and molecular characterization of metabolic pathways during the embryonic development of zebrafish (**Chapter 2**).

» Deliver a single hyper-glucidic stimulus directly into the yolk-sac of zebrafish embryos, at two different stages of embryogenesis, and assess the effects on survival, growth performance, glucose metabolism and gene expression of target metabolic pathways, in juvenile fish fed with a glucose-rich diet during a confined period (**Chapters 2 and 3**).

» Deliver three recurrent glucidic stimuli to seabream larvae during critical stages of development and evaluate the short- and long-term effects on growth performance, survival, nutrients metabolism and gene expression of seabream post- larvae (short-term) and juveniles (long-term), both fed with a high glucose diet (**Chapters 4 and 5**).

A schematic view of the plan work, detailing the four experimental trails developed under this thesis is provided in Figure 1.5. The resulting knowledge from this thesis can potentially pave the way towards a better use of carbohydrates as energy substrates for fish, and therefore allow a higher incorporation of plant-ingredients in fish diets, necessary for a sustainable

production. It will, therefore, contribute to reinforce the scientific bases of the nutritional programming concept in the field of fish nutrition.

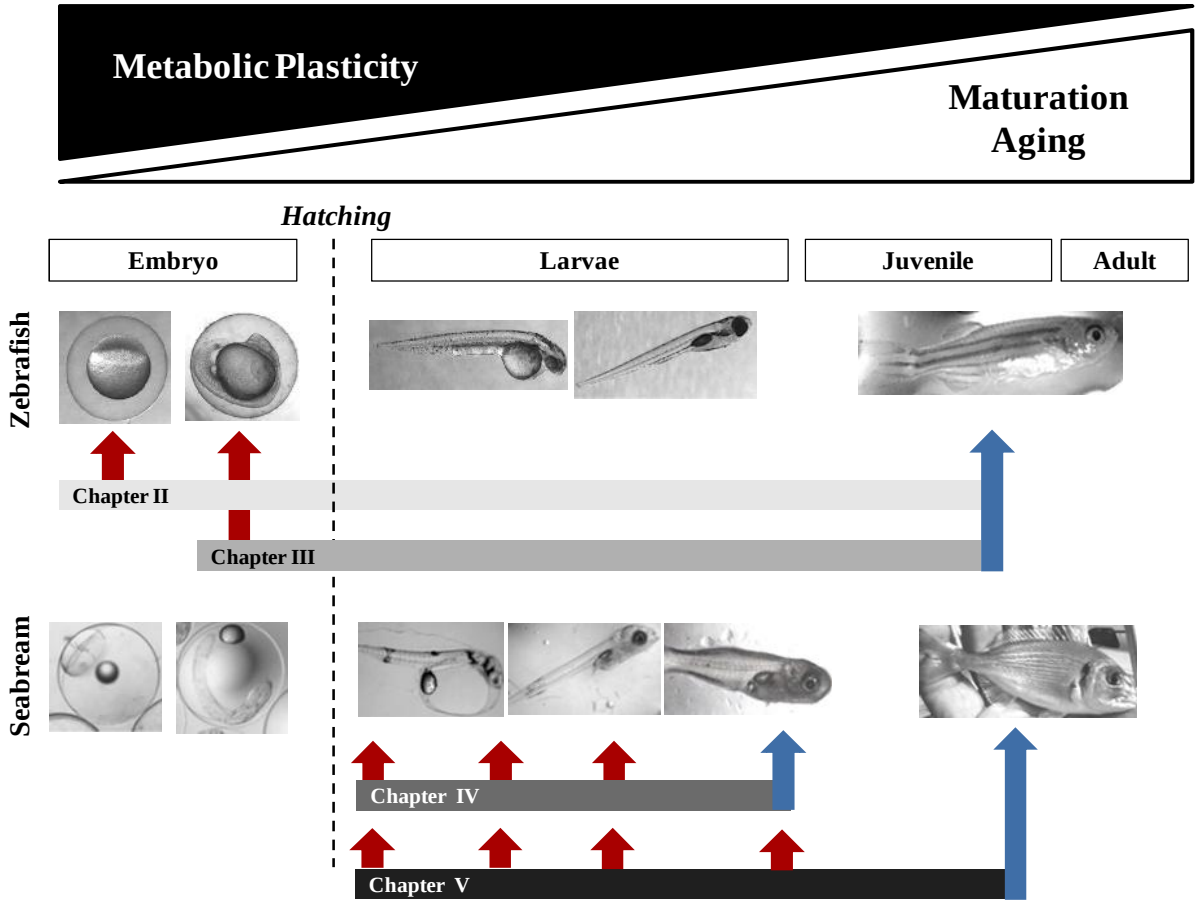

Figure 1.5 – Schematic plan of the four experiments developed with zebrafish and seabream focusing the early nutritional programming with glucose. Red arrows indicate the time period for glucidic stimulus delivery; Blue arrow indicates the periods of dietary challenge for the analysis of the long-term effects of early stimulus; Chapter II to V are indicated to each corresponding experiment.

1.7. References

Andersen, I., Reiner, A., Aanes, H., Alestrom, P., Collas, P., 2012. Developmental features of DNA methylation during activation of the embryonic zebrafish genome. Genome Biology. 13, R65.

Andersen, S.M., Taylor, R., Holen, E., Aksnes, A., Espe, M., 2014. Arginine supplementation and exposure time affects polyamine and glucose metabolism in primary liver cells isolated from Atlantic salmon. Amino Acids.

Anderson, O.S., Sant, K.E., Dolinoy, D.C., 2012. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J Nutr Biochem. 23, 853-859.

Andoh, T., 2007. Amino acids are more important insulinotropins than glucose in a teleost fish, barfin flounder (Verasper moseri). Gen Comp Endocrinol. 151, 308-317.

Badeaux, A.I., Shi, Y., 2013. Emerging roles for chromatin as a signal integration and storage platform. Nat Rev Mol Cell Biol. 14, 211-224.

Bakke, A.M., Glover, C., Krogdahl, A., 2010. Feeding, digestion and absorption of nutrients. in: Grosell, M., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology: The Multifunctional Gut of Fish. Academic Press, USA, pp. 57- 110.

Balmaceda-Aguilera, C., Martos-Sitcha, J.A., Mancera, J.M., Martínez-Rodríguez, G., 2012. Cloning and expression pattern of facilitative glucose transporter 1 (GLUT1) in gilthead sea bream Sparus aurata in response to salinity acclimation. Comp Biochem Physiol A Mol Integr Physiol. 163, 38-46.

Bequette, B.J., Sunny, N.E., El-Kadi, S.W., Owens, S.L., 2006. Application of stable isotopes and mass isotopomer distribution analysis to the study of intermediary metabolism of nutrients. J Anim Sci. 84 Suppl, E50-59.

Bergot, F., 1993. Digestibility of native starches of various botanical origins by rainbow trout (*Oncorhynchus mykiss*). in: Kaushik, S.J., P., L. (Eds.), Fish Nutrition in Practice. INRA, Paris, France, pp. 857-865.

Brauge, C., Corraze, G., Medale, F., 1995. Effect of dietary levels of lipid and carbohydrate on growth performance, body composition, nitrogen excretion and plasma glucose levels in rainbow trout reared at 8 or 18 degrees C. Reprod Nutr Dev. 35, 277-290.

Bucking, C., Wood, C.M., 2005. Renal regulation of plasma glucose in the freshwater rainbow trout. J Exp Biol. 208, 2731-2739.

Buckley, A.J., Jaquiery, A.L., Harding, J.E., 2005. Nutritional programming of adult disease. Cell Tissue Res. 322, 73-79.

Burdge, G.C., Lillycrop, K.A., 2010. Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr. 30, 315-339.

Campos, C., Valente, L., Conceicao, L., Engrola, S., Fernandes, J., 2013. Temperature affects methylation of the myogenin putative promoter, its expression and muscle cellularity in Senegalese sole larvae. Epigenetics. 8.

Caruso, M.A., Sheridan, M.A., 2011. New insights into the signaling system and function of insulin in fish. Gen Comp Endocrinol. 173, 227-247.

Caseras, A., Meton, I., Vives, C., Egea, M., Fernandez, F., Baanante, I.V., 2002. Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (Sparus aurata). Br J Nutr. 88, 607-614.

Castillo, J., Crespo, D., Capilla, E., Diaz, M., Chauvigne, F., Cerda, J., Planas, J.V., 2009. Evolutionary structural and functional conservation of an ortholog of the GLUT2 glucose transporter gene (SLC2A2) in zebrafish. Am J Physiol Regul Integr Comp Physiol. 297, 1570-1581.

Collie, N.L., Ferraris, R.P., 1995. Nutrient fluxes and regulation in fish intestine. in: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes. Elsevier Science, Amsterdam, The Netherland, pp. 221-239.

Couto, A., Enes, P., Peres, H., Oliva-Teles, A., 2008. Effect of water temperature and dietary starch on growth and metabolic utilization of diets in gilthead sea bream (Sparus aurata) juveniles. Comp Biochem Physiol A Mol Integr Physiol. 151, 45-50.

Dabrowski, K., Guderley, H., 2003. Intermediary Metabolism. in: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition (Third Edition). Academic Press, San Diego, pp. 309-365.

Deng, D.F., Refstie, S., Hung, S.S.O., 2001. Glycemic and glycosuric responses in white sturgeon (*Acipenser transmontanus*) after oral administration of simple and complex carbohydrates. Aquaculture. 199, 107-117.

Dias, J., Alvarez, M.J., Diez, A., Arzel, J., Corraze, G., Bautista, J.M., Kaushik, S.J., 1998. Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (Dicentrarchus labrax). Aquaculture. 161, 169-186.

Diaz, M., Vraskou, Y., Gutierrez, J., Planas, J.V., 2009. Expression of rainbow trout glucose transporters GLUT1 and GLUT4 during in vitro muscle cell differentiation and regulation by insulin and IGF-I. Am J Physiol Regul Integr Comp Physiol. 296, R794-800.

Dona, A.C., Pages, G., Gilbert, R.G., Kuchel, P.W., 2010. Digestion of starch: In vivo and in vitro kinetic models used to characterise oligosaccharide or glucose release. Carbohydrate Polymers. 80, 599-617.

Dumortier, O., Hinault, C., Van Obberghen, E., 2013. MicroRNAs and metabolism crosstalk in energy homeostasis. Cell Metab. 18, 312-324.

Ekmann, K.S., Dalsgaard, J., Holm, J., Campbell, P.J., Skov, P.V., 2013. Glycogenesis and de novo lipid synthesis from dietary starch in juvenile gilthead sea bream (Sparus aurata) quantified with stable isotopes. Br J Nutr. 109, 2135-2146.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2006. Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (Dicentrarchus labrax) juveniles. Comp Biochem Physiol A Mol Integr Physiol. 143, 89-96.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008. Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (Sparus aurata) juveniles. Aquaculture. 274, 101-108.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2009. Nutritional regulation of hepatic glucose metabolism in fish. Fish Physiol Biochem. 35, 519-539.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2011. Dietary carbohydrate utilization by European sea bass (Dicentrarchus labrax L.) and gilthead sea bream (Sparus aurata L.) juveniles. Reviews in Fisheries Science. 19, 201-215.

Fang, L., Liang, X.F., Zhou, Y., Guo, X.Z., He, Y., Yi, T.L., Liu, L.W., Yuan, X.C., Tao, Y.X., 2014 Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, Danio rerio. Br J Nutr. 111, 808-818.

FAO, 2014. The State of World Fisheries and Aquaculture FAO, Rome.

FEAP, 2014. Annual Report 2014. Federation of European Aquaculture Producers, Belgium.

Fernández-Palacios, H., Izquierdo, M.S., Robaina, L., Valencia, A., Salhi, M., Vergara, J., 1995. Effect of n − 3 HUFA level in broodstock diets on egg quality of gilthead sea bream (Sparus aurata L.). Aquaculture. 132, 325-337.

Fernández-Palacios, H., Izquierdo, M., Robaina, L., Valencia, A., Salhi, M., Montero, D., 1997. The effect of dietary protein and lipid from squid and fish meals on egg quality of broodstock for gilthead seabream (Sparus aurata). Aquaculture. 148, 233-246.

Fernández-Palacios, H., Norberg, B., Izquierdo, M., Hamre, K., 2011. Effects of Broodstock Diet on Eggs and Larvae. in: Holt, G.J. (Ed.), Larval Fish Nutrition. Wiley-Blackwell, Oxford, pp. 151-181.

Figueiredo-Silva, A.C., Panserat, S., Kaushik, S., Geurden, I., Polakof, S., 2012. High levels of dietary fat impair glucose homeostasis in rainbow trout. J Exp Biol. 215, 169-178.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., J Souza, E., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac Res. 38, 551-579.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (Oncorhynchus mykiss) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am J Physiol Regul Integr Comp Physiol. 292, 2275-2283.

Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D., Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S., Panserat, S., 2014. High or low dietary

carbohydrate:protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout. J Exp Biol. 217, 3396-3406.

Gluckman, P.D., Hanson, M.A., Low, F.M., 2011. The role of developmental plasticity and epigenetics in human health. Birth Defects Res C Embryo Today. 93, 12-18.

Gong, G., Xue, M., Wang, J., Wu, X.-f., Zheng, Y.-h., Han, F., Liang, X.-f., Su, X.-o., 2015. The regulation of gluconeogenesis in the Siberian sturgeon (Acipenser baerii) affected later in life by a short-term high-glucose programming during early life. Aquaculture. 436, 127-136.

Hall, J.R., Richards, R.C., MacCormack, T.J., Ewart, K.V., Driedzic, W.R., 2005. Cloning of GLUT3 cDNA from Atlantic cod (Gadus morhua) and expression of GLUT1 and GLUT3 in response to hypoxia. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression. 1730, 245-252.

Harder, T., Plagemann, A., Rohde, W., Dorner, G., 1998. Syndrome X-like alterations in adult female rats due to neonatal insulin treatment. Metabolism. 47, 855-862.

Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquaculture Research. 41, 770-776.

Hellemans, K.G., Sliwowska, J.H., Verma, P., Weinberg, J., 2010. Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders. Neurosci Biobehav Rev. 34, 791-807.

Heming, T.A., Buddington, R.K., 1988. Yolk Absorption in Embryonic and Larval Fishes. in: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, The Physiology of Developing Fish - Eggs and Larvae. Academic Press, San Diego, California, pp. 407-446.

Hemre, G.I., Lie, Ø., Lied, E., Lambertsen, G., 1989. Starch as an energy source in feed for cod (Gadus morhua): Digestibility and retention. Aquaculture. 80, 261-270.

Hemre, G.I., Kahrs, F., 1997. 14C-glucose injection in Atlantic cod, Gadus morhua, metabolic responses and excretion via the gill membrane. Aquaculture Nutrition. 3, 3-8.

Hemre, G.I., Mommsen, T.P., Krogdahl, A., 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquacult Nutr. 8, 175-194.

Holt, G.J., 2011. Larval Fish Nutrition. Wiley-Blackwell, John Wiley & Sons.

Hrytsenko, O., Pohajdak, B., Xu, B.Y., Morrison, C., van Tol, B., Wright, J.R., Jr., 2010. Cloning and molecular characterization of the glucose transporter 1 in tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol. 165, 293-303.

Huizink, A.C., Mulder, E.J., 2006. Maternal smoking, drinking or cannabis use during pregnancy and neurobehavioral and cognitive functioning in human offspring. Neurosci Biobehav Rev. 30, 24-41.

Iynedjian, P.B., 1993. Mammalian glucokinase and its gene. Biochemical Journal. 293, 1-13.

Izquierdo, M.S., Fernández-Palacios, H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture. 197, 25-42.

Izquierdo, M.S., Turkmen, S., Montero, D., Zamorano, M.J., Afonso, J.M., Karalazos, V., Fernández-Palacios, H., 2015. Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream. Aquaculture.

Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 33 Suppl, 245-254.

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S., 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J Exp Biol. 215, 2567-2578.

Kamler, E., 2008. Resource allocation in yolk-feeding fish. Rev Fish Biol Fisheries. 18, 143-200.

Kirchner, S., Panserat, S., Lim, P.L., Kaushik, S., Ferraris, R.P., 2008. The role of hepatic, renal and intestinal gluconeogenic enzymes in glucose homeostasis of juvenile rainbow trout. J Comp Physiol B. 178, 429-438.

Klinger, D., Naylor, R., 2012. Searching for Solutions in Aquaculture: Charting a Sustainable Course. Annual Review of Environment and Resources. 37, 247-276.

Knox, D., Walton, M.J., Cowey, C.B., 1980. Distribution of enzymes of glycolysis and gluconeogenesis in fish tissues. Marine Biology. 56, 7-10.

Krasnov, A., Teerijoki, H., Molsa, H., 2001. Rainbow trout (Onchorhynchus mykiss) hepatic glucose transporter. Biochim Biophys Acta. 1520, 174-178.

Krogdahl, A., Sundby, A., Olli, J.J., 2004. Atlantic salmon (*Salmo salar)* and rainbow trout (*Oncorhynchus mykiss*) digest and metabolize nutrients differently. Effects of water salinity and dietary starch level. Aquaculture. 229, 335-360.

Krogdahl, A., Hemre, G.I., Mommsen, T.P., 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquacult Nutr. 11, 103-122.

Langley-Evans, S.C., 2015. Nutrition in early life and the programming of adult disease: a review. J Hum Nutr Diet. 28 Suppl 1, 1-14.

Lazo, J.P., Darias, M.J., Gisbert, E., 2011. Ontogeny of the Digestive Tract. in: Holt, G.J. (Ed.), Larval Fish Nutrition. Wiley-Blackwell, John Wiley & Sons, pp. 3-46.

Legate, N.J., Bonen, A., Moon, T.W., 2001. Glucose tolerance and peripheral glucose utilization in rainbow trout (Oncorhynchus mykiss), American eel (Anguilla rostrata), and black bullhead catfish (Ameiurus melas). Gen Comp Endocrinol. 122, 48-59.

Lillycrop, K.A., Burdge, G.C., 2012. Epigenetic mechanisms linking early nutrition to long term health. Best Pract Res Clin Endocrinol Metab 26, 667-676.

Lim, J.P., Brunet, A., 2013. Bridging the transgenerational gap with epigenetic memory. Trends Genet. 29, 176-186.

Loomans, E.M., Hofland, L., van der Stelt, O., van der Wal, M.F., Koot, H.M., Van den Bergh, B.R.H., Vrijkotte, T.G.M., 2012. Caffeine Intake During Pregnancy and Risk of Problem Behavior in 5- to 6-Year-Old Children. Pediatrics. 130, e305-e313.

Lucas, A., 1991. Programming by early nutrition in man. Ciba Found Symp. 156, 38-50; discussion 50-35.

Lucas, A., 1998. Programming by early nutrition: an experimental approach. J Nutr 128, 401-406.

Maddison, L.A., Chen, W., 2012. Nutrient Excess Stimulates β-Cell Neogenesis in Zebrafish. Diabetes.

Maritz, G.S., Harding, R., 2011. Life-long Programming Implications of Exposure to Tobacco Smoking and Nicotine Before and Soon After Birth: Evidence for Altered Lung Development. International Journal of Environmental Research and Public Health. 8, 875-898.

Meaney, M.J., Szyf, M., 2005. Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. Dialogues Clin Neurosci. 7, 103-123.

Mennigen, J.A., Skiba-Cassy, S., Panserat, S., 2013. Ontogenetic expression of metabolic genes and microRNAs in rainbow trout alevins during the transition from the endogenous to the exogenous feeding period. The Journal of Experimental Biology. 216, 1597-1608.

Metges, C.C., Gors, S., Lang, I.S., Hammon, H.M., Brussow, K.P., Weitzel, J.M., Nurnberg, G., Rehfeldt, C., Otten, W., 2014. Low and high dietary protein:carbohydrate ratios during pregnancy affect materno-fetal glucose metabolism in pigs. J Nutr. 144, 155-163.

Milagro, F.I., Mansego, M.L., De Miguel, C., Martinez, J.A., 2013. Dietary factors, epigenetic modifications and obesity outcomes: progresses and perspectives. Mol Aspects Med. 34, 782-812.

Mommsen, T.P., Plisetskaya, E.M., 1991. Insulin in fishes and agnathans: history structure and metabolic regulation. Rev Aquat Sci 4, 225-260.

Moon, T.W., 1998. Glucagon: from hepatic binding to metabolism in teleost fish. Comp Biochem Physiol B Biochem Mol Biol. 121, 27-34.

Moon, T.W., 2001. Glucose intolerance in teleost fish: fact or fiction? Comp Biochem Physiol B Biochem Mol Biol. 129, 243-249.

Moreira, I.S., Peres, H., Couto, A., Enes, P., Oliva-Teles, A., 2008. Temperature and dietary carbohydrate level effects on performance and metabolic utilisation of diets in European sea bass (Dicentrarchus labrax) juveniles. Aquaculture. 274, 153-160.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., Piferrer, F., 2011. DNA Methylation of the Gonadal Aromatase (cyp19a) Promoter Is Involved in Temperature-Dependent Sex Ratio Shifts in the European Sea Bass. PLoS Genet. 7, e1002447.

Navarro, I., Leibush, B., Moon, T.W., Plisetskaya, E.M., Baños, N., Méndez, E., Planas, J.V., Gutiérrez, J., 1999. Insulin, insulin-like growth factor-I (IGF-I) and glucagon: the evolution of their receptors. Comp Biochem Physiol B Biochem Mol Biol 122, 137-153.

Navarro, I., Rojas, P., Capilla, E., Albalat, A., Castillo, J., Montserrat, N., Codina, M., Gutiérrez, J., 2002. Insights into Insulin and Glucagon Responses in Fish. Fish Physiol Biochem. 27, 205-216.

Naz, M., 2009. Ontogeny of Biochemical Phases of Fertilized Eggs and Yolk Sac Larvae of Gilthead Seabream (Sparus aurata L.). Turk J Fish Aquat Sci. 9, 77-83.

NCR, 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washington, DC.

Nordlie, R.C., Foster, J.D., Lange, A.J., 1999. Regulation of glucose production by the liver. Annu Rev Nutr. 19, 379-406.

Olsen, A.S., Sarras, M.P., Jr., Leontovich, A., Intine, R.V., 2012. Heritable transmission of diabetic metabolic memory in zebrafish correlates with DNA hypomethylation and aberrant gene expression. Diabetes. 61, 485-491.

Oosterveer, M., Schoonjans, K., 2014. Hepatic glucose sensing and integrative pathways in the liver. Cell. Mol. Life Sci. 71, 1453-1467.

Ozanne, S.E., Hales, C.N., 1999. The long-term consequences of intra-uterine protein malnutrition for glucose metabolism. Proc Nutr Soc. 58, 615-619.

Ozanne, S.E., Hales, C.N., 2002. Early programming of glucose–insulin metabolism. Trends in Endocrinology & Metabolism. 13, 368-373.

Panserat, S., Medale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R., Kaushik, S., 2000a. Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. Am J Physiol Regul Integr Comp Physiol. 278, 1164-1170.

Panserat, S., Medale, F., Breque, J., Plagnes-Juan, E., Kaushik, S., 2000b. Lack of significant long-term effect of dietary carbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout (Oncorhynchus mykiss). J Nutr Biochem. 11, 22-29.

Panserat, S., Plagnes-Juan, E., Breque, J., Kaushik, S., 2001a. Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol. 204, 359-365.

Panserat, S., Plagnes-Juan, E., Kaushik, S., 2001b. Nutritional regulation and tissue specificity of gene expression for proteins involved in hepatic glucose metabolism in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol. 204, 2351-2360.

Panserat, S., Plagnes-Juan, E., Kaushik, S., 2002. Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). Biochim Biophys Acta. 1579, 35-42.

Panserat, S., Skiba-Cassy, S., Seiliez, I., Lansard, M., Plagnes-Juan, E., Vachot, C., Aguirre, P., Larroquet, L., Chavernac, G., Medale, F., Corraze, G., Kaushik, S., Moon, T.W., 2009. Metformin improves postprandial glucose homeostasis in rainbow trout fed dietary carbohydrates: a link with the induction of hepatic lipogenic capacities? Am J Physiol Regul Integr Comp Physiol. 297, R707-R715.

Panserat, S., Rideau, N., Polakof, S., 2014. Nutritional regulation of glucokinase: a cross-species story. Nutr Res Rev. 27, 21-47.

Patel, M.S., Hiremagalur, B.K., 1992. Artificial-rearing technique: its usefulness in nutrition research. J Nutr. 122, 412-419.

Patel, M.S., Srinivasan, M., 2002. Metabolic programming: causes and consequences. J Biol Chem. 277, 1629-1632.

Patel, M.S., Srinivasan, M., Laychock, S.G., 2009. Metabolic programming: Role of nutrition in the immediate postnatal life. J Inherit Metab Dis. 32, 218-228.

Peres, A., Cahu, C.L., Zambonino Infante, J.L., Le Gall, M.M., Quazuguel, P., 1996. Amylase and trypsin responses to intake of dietary carbohydrate and protein depend on the developmental stage in sea bass (Dicentrarchus labrax) larvae. Fish Physiol Biochem. 15, 237-242.

Peres, H., Gonçalves, P., Oliva-Teles, A., 1999. Glucose tolerance in gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax). Aquaculture. 179, 415-423.

Piferrer, F., Ribas, L., Diaz, N., 2012. Genomic approaches to study genetic and environmental influences on fish sex determination and differentiation. Mar Biotechnol (NY). 14, 591-604.

Pilkis, S.J., Granner, D.K., 1992. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. Annu Rev Physiol. 54, 885-909.

Planas, J.V., Capilla, E., Gutiérrez, J., 2000. Molecular identification of a glucose transporter from fish muscle. FEBS Letters. 481, 266-270.

Polakof, S., Alvarez, R., Soengas, J.L., 2010. Gut glucose metabolism in rainbow trout: implications in glucose homeostasis and glucosensing capacity. Am J Physiol Regul Integr Comp Physiol. 299, R19-32.

Polakof, S., Medale, F., Larroquet, L., Vachot, C., Corraze, G., Panserat, S., 2011. Insulin stimulates lipogenesis and attenuates Beta-oxidation in white adipose tissue of fed rainbow trout. Lipids. 46, 189-199.

Polakof, S., Panserat, S., Soengas, J.L., Moon, T.W., 2012. Glucose metabolism in fish: a review. J Comp Physiol B.

Richard, N., Kaushik, S., Larroquet, L., Panserat, S., Corraze, G., 2006. Replacing dietary fish oil by vegetable oils has little effect on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (Oncorhynchus mykiss). Br J Nutr. 96, 299-309.

Rojas, P., Albalat, A., Santigosa, E., PÉRez-SÁNchez, J., Kaushik, S.J., GutiÉRrez, J., Navarro, I., 2009. Dietary effects on insulin and glucagon plasma levels in rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata). Aquaculture Nutrition. 15, 166-176.

Rønnestad, I., Koven, W.M., Tandler, A., Harel, M., Fyhn, H.J., 1994. Energy metabolism during development of eggs and larvae of gilthead sea bream (Sparus aurata). Marine Biology. 120, 187-196.

Rønnestad, I., Thorsen, A., Finn, R.N., 1999. Fish larval nutrition: a review of recent advances in the roles of amino acids. Aquaculture 177, 201-216.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev Aquaculture. 5, S59-S98.

Rust, M.B., 2003. Nutritional Physiology. in: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition Academic Press, San Diego, pp. 367-452.

Seiliez, I., Panserat, S., Lansard, M., Polakof, S., Plagnes-Juan, E., Surget, A., Dias, K., Larquier, M., Kaushik, S., Skiba-Cassy, S., 2011. Dietary carbohydrate-to-protein ratio affects TOR signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. Am J Physiol Regul Integr Comp Physiol. 300, R733-R743.

Seiliez, I., Medale, F., Aguirre, P., Larquier, M., Lanneretonne, L., Alami-Durante, H., Panserat, S., Skiba-Cassy, S., 2013. Postprandial regulation of growth- and metabolism-related factors in zebrafish. Zebrafish. 10, 237-248.

Shimeno, S., Kheyyali, D., Shikata, T., 1995. Metabolic Response to Dietary Carbohydrate to Protein Rations in Carp. Fish Sci. 61, 277-281.

Simmons, R.A., Suponitsky-Kroyter, I., Selak, M.A., 2005. Progressive Accumulation of Mitochondrial DNA Mutations and Decline in Mitochondrial Function Lead to β-Cell Failure. Journal of Biological Chemistry. 280, 28785-28791.

Snoeck, A., Remacle, C., Reusens, B., Hoet, J.J., 1990. Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate. 57, 107-118.

Soanes, K.H., Achenbach, J.C., Burton, I.W., Hui, J.P., Penny, S.L., Karakach, T.K., 2011. Molecular characterization of zebrafish embryogenesis via DNA microarrays and multiplatform time course metabolomics studies. J Proteome Res. 10, 5102-5117.

Soengas, J.L., Polakof, S., Chen, X., Sangiao-Alvarellos, S., Moon, T.W., 2006. Glucokinase and hexokinase expression and activities in rainbow trout tissues: changes with food deprivation and refeeding. Am J Physiol Regul Integr Comp Physiol. 291, R810-821.

Srinivasan, M., Laychock, S.G., Hill, D.J., Patel, M.S., 2003. Neonatal nutrition: metabolic programming of pancreatic islets and obesity. Exp Biol Med (Maywood). 228, 15-23.

Srinivasan, M., Mitrani, P., Sadhanandan, G., Dodds, C., Shbeir-ElDika, S., Thamotharan, S., Ghanim, H., Dandona, P., Devaskar, S.U., Patel, M.S., 2008. A high-carbohydrate diet in the immediate postnatal life of rats induces adaptations predisposing to adult-onset obesity. Journal of Endocrinology. 197, 565–574.

Stone, D.A.J., 2003. Dietary Carbohydrate Utilization by Fish. Reviews in Fisheries Science. 11, 337-369.

Sugita, T., Shimeno, S., Ohkubo, Y., Hosokawa, H., Masumoto, T., 2001. Response of enzyme activities and metabolic intermediate concentrations to glucagon administration in hepatopancreas and muscle of carp. Fish. Sci. 67, 157-162.

Terova, G., Rimoldi, S., Brambilla, F., Gornati, R., Bernardini, G., Saroglia, M., 2009. In vivo regulation of GLUT2 mRNA in sea bass (Dicentrarchus labrax) in response to acute and chronic hypoxia. Comp Biochem Physiol B Biochem Mol Biol. 152, 306-316.

Towle, H.C., Kaytor, E.N., Shih, H.M., 1997. Regulation of the expression of lipogenic enzyme genes by carbohydrate. Annu Rev Nutr. 17, 405-433.

Vagner, M., Zambonino-Infante, J.L., Robin, J.H., Person, J., 2007. Is it possible to influence European sea bass (Dicentrarchus labrax) juvenile metabolism by a nutritional conditioning during larval stage? Aquaculture. 267, 165-174.

Vagner, M., Robin, J.H., Zambonino-Infante, J.L., Tocher, D.R., Person-Le Ruyet, J., 2009. Ontogenic effects of early feeding of sea bass (Dicentrarchus labrax) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. Br J Nutr. 101, 1452-1462.

Venou, B., Alexis, M.N., Fountoulaki, E., Nengas, I., Apostolopoulou, M., Castritsi-Catharíou, I., 2003. Effect of extrusion of wheat and corn on gilthead sea bream (Sparus aurata) growth, nutrient utilization efficiency, rates of gastric evacuation and digestive enzyme activities. Aquaculture. 225, 207-223.

Vickers, M.H., 2014. Early life nutrition, epigenetics and programming of later life disease. Nutrients. 6, 2165-2178.

Vieau, D., 2011. Perinatal nutritional programming of health and metabolic adult disease. World J Diabetes. 2, 133-136.

Wilson, R.P., 1994. Utilization of dietary carbohydrate by fish. Aquaculture. 124, 67-80.

Yamamoto, T., Shima, T., Furuita, H., Suzuki, N., Shiraishi, M., 2001. Nutrient digestibility values of a test diet determined by manual feeding and self-feeding in rainbow trout and common carp. Fisheries Science. 67, 355-357.

Yee, N.S., Lorent, K., Pack, M., 2005. Exocrine pancreas development in zebrafish. Dev Biol. 284, 84-101.

Youngson, N.A., Whitelaw, E., 2008. Transgenerational epigenetic effects. Annu Rev Genomics Hum Genet. 9, 233-257.

Zambonino-Infante, J.L., Gisbert, E., Sarasquete, C., Navarro, I., Gutiérrez, J., Cahu, C.L., 2008. Ontogeny and Physiology of the Digestive System of Marine Fish Larvae. in: Cyrino, J.E.P., Bureau, D., Kapoor, B.G. (Eds.), Feeding and Digestive Functions in Fishes. CRC Press, pp. 281-348.

Zawia, N.H., Lahiri, D.K., Cardozo–Pelaez, F., 2009. Epigenetics, oxidative stress and Alzheimer's Disease. Free radical biology & medicine. 46, 1241-1249.

Zhang, J., Zhang, F., Didelot, X., Bruce, K., Cagampang, F., Vatish, M., Hanson, M., Lehnert, H., Ceriello, A., Byrne, C., 2009. Maternal high fat diet during pregnancy and lactation alters hepatic expression of insulin like growth factor-2 and key microRNAs in the adult offspring. BMC Genomics. 10, 1-12.

Zouiten, D., Khemis, I.B., Besbes, R., Cahu, C., 2008. Ontogeny of the digestive tract of thick lipped grey mullet (Chelon labrosus) larvae reared in "mesocosms". Aquaculture. 279, 166-172.

Nutritional programming at embryonic stages of fish

Chapter II

Glucose overload in yolk has little effects on the long term modulation of carbohydrate metabolic genes in zebrafish (*Danio rerio*)

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Glucose overload in yolk has little effects on the long term modulation of carbohydrate metabolic genes in zebrafish (*Danio rerio*)

Abstract

Some fish show a low metabolic ability to use dietary carbohydrates. The use of early nutritional stimuli to program metabolic pathways in fish is ill defined. Therefore, studies were undertaken with zebrafish to assess the effect of high glucose levels during the embryonic stage as a lifelong modulator of genes involved in carbohydrate metabolism. Genes related to carbohydrate metabolism were expressed at low levels at 0.2 and 1 days post-fertilization (dpf). However, from 4 dpf onwards there was a significant increase on expression of all genes, suggesting that all analysed pathways were active. By microinjection, we successfully enriched zebrafish egg yolk with glucose (a 43-fold increase of basal levels). Acute effects of glucose injection on gene expression were assessed in larvae up to 10 dpf, and the programming concept was evaluated in juveniles (41 dpf) challenged with a hyperglucidic diet. At 4 dpf, larvae from glucose-enriched eggs showed a down-regulation of several genes related to glycolysis, glycogenolysis, lipogenesis and carbohydrate digestion in comparison with control (saline-injected) embryos. This inhibitory regulation was suppressed after 10 dpf. At the juvenile stage and upon switching from a low to a high digestible carbohydrate diet, early glucose enrichment had no significant effect on most analysed genes. However, these same fish showed altered expression of the genes for cytosolic phosphoenolpyruvate carboxykinase, sodium-dependent glucose cotransporter 1 and glycogen synthase, suggesting changes to the glucose storage capacity in muscle and glucose production and transport in viscera. Overall, supplementation of egg yolk supplementation with high glucose levels had little effects on the long term modulation of carbohydrate metabolic genes in zebrafish.

Keywords: Glucose metabolism; Nutritional programming, Gene expression; Zebrafish.

2.1. Introduction

Mammalian models show that prenatal or early neonatal events exerted at critical developmental windows may result in lifelong contributions to postnatal growth potential and health status (Burdge and Lillycrop, 2010; Lucas, 1998; Patel and Srinivasan, 2002; Patel et al., 2009). Critical periods are likely to occur when the developing organism has high genetic plasticity such as cell proliferation, organs and tissues formation or in a later stage, when immature regulatory mechanisms that still are not functional (Srinivasan and Patel, 2008). Possible biological mechanisms for "imprinting" the nutritional event until adulthood comprise adaptive changes on: gene expression pattern or cellular phenotype (epigenetic phenomenon), nutrient-sensitive signalling pathways and adaptive clonal selection, which could be transmitted to future offspring (Lucas, 1998; Symonds et al., 2009; Waterland and Jirtle, 2004). Knowledge on the broad concept of nutritional programming in fish is extremely scarce. Despite not targeting nutrients, there are now several studies indicating that, as in terrestrial vertebrates, environmental factors (e.g. temperature) can lead to genomic imprinting in fish, namely through changes in DNA methylation (Campos et al., 2013; Navarro-Martín et al., 2011). Moreover, important elements associated with epigenetic mechanisms have been recently reported in zebrafish (Andersen et al., 2012).

Some teleost fish exhibit a poor utilization of dietary carbohydrates, especially those with carnivorous feeding habits (Panserat and Kaushik, 2010; Wilson, 1994). Important research work was undertaken to establish the role of carbohydrates at a metabolic and physiological level in fish (Panserat and Kaushik, 2010). Such studies focused mainly on the role of dietary factors as modulators of glucose utilization and expression of key enzymes of the intermediary metabolism (Enes et al., 2011; Panserat et al., 2002; Seiliez et al., 2013). However, the mechanisms underlying the relatively poor ability of fish to utilize dietary carbohydrates as a major energy yielding substrate remain to be elucidated. Numerous studies in mammals have shown that increasing carbohydrate intake through maternal nutrition (prenatal) or by newborn during suckling period (neonatal) can cause long-term modifications on glucose metabolism and later diseases related to metabolic syndromes and diabetes (Burdge and Lillycrop, 2010; Olsen et al., 2012; Waterland and Jirtle, 2004). The opportunities to exert a nutritional stimulus during a stage of high metabolic plasticity, such as fish embryogenesis or early larval development, are limited to e.g. maternal nutrient transfer (Fernández-Palacios et al., 1995; Fernández-Palacios et al., 1997) and the onset of

exogenous feeding (Geurden et al., 2007; Vagner et al., 2007). A previous study by Geurden et al. (2007) has shown that a short hyperglucidic stimulus exerted at the onset of feeding, up-regulated carbohydrate digestive enzymes in rainbow trout at a later juvenile stage, suggesting some long-term physiological changes.

Once spawned and fertilized, fish eggs operate as semi-closed systems given the extremely low permeability of the egg surface membrane (Babin et al., 2007; Kamler, 2008). This period of rapid embryonic development, prior to hatching, when fish rely exclusively on yolk nutrients appears of specific interest for testing the nutritional programming concept in fish. At this stage, target delivery of specific nutrient loads during early embryonic development may be performed through microinjection directly in the yolk. Zebrafish (*Danio rerio,* F. Hamilton 1822) is now firmly established as an important and informative model system for studying vertebrate embryogenesis and organogenesis, as well for the analysis of developmentally regulated genes in aspects related to human disease modelling (Ali et al., 2011; Kudoh et al., 2001). In addition to the wide variety of molecular tools and resources available for genomic analysis in zebrafish, the ease of breeding, the large number of offspring (embryos), the ex-utero development of embryos and its optical transparency during early embryogenesis (Ulloa et al., 2011) make it a powerful model for studying early nutritional programming in fish through modification of embryo nutritional status. Fish eggs contain relatively low levels of carbohydrates in the vitellus and are generally rich in free amino acids and fatty acids (Hoar and Randall, 1988; Kamler, 2008). Whether a targeted supplementation of yolk reserves with glucose during embryogenesis could act as a nutritional stimulus to program life-long metabolic pathways in fish is unknown.

The main objective of our work was to assess the short and long-term effects of supplementing zebrafish embryos with high levels of glucose on the gene regulation of several pathways of the intermediary metabolism.

2.2. Materials and methods

2.2.1. Rearing conditions

For each trial, zebrafish eggs were obtained from natural spawning of wild-type fish maintained at the Centre of Marine Sciences (University of Algarve, Faro, Portugal). Eggs were incubated and larvae were raised under identical and standardized conditions as described previously (Westerfield, 2000). In all trials, experimental treatments were conducted in triplicate and larvae were conditioned to an initial stocking density of 100 individuals L^{-1}. At 5 dpf, larvae were fed with *Artemia* nauplii, three times per day in excess. In the long-term trial, a gradual replacement of *Artemia* nauplii with an inert diet occurred from 10 to 15 dpf, after which the larvae were fed exclusively with an inert diet. A schematic view of the feeding plan adopted for the various trials is presented in Figure 2.1. Animal care and experiments were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals.

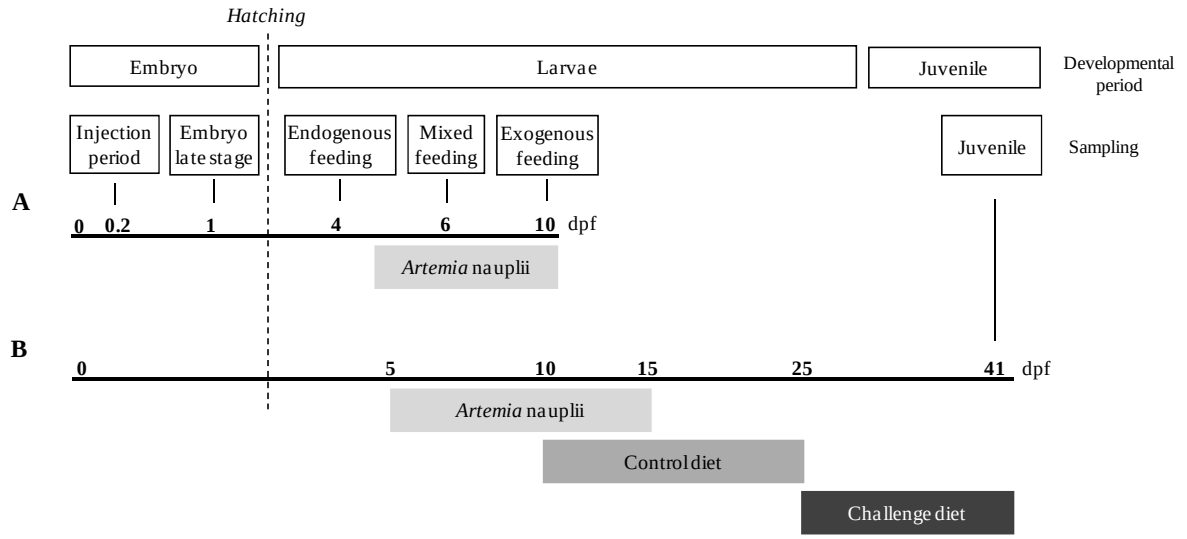

Figure 2.1- Feeding plan and samplings regimes during the various experimental trials: (A) Gene expression profile and short-term trial; (B) long-term trial. Age is given in days post-fertilization (dpf) at 28ºC.

2.2.2. Microinjection of zebrafish eggs with glucose

Embryos were microinjected using a nanoliter 2000 injector (World Precision Instruments, Sarasota, FL, USA) linked to a stereoscopic microscope. Needles for microinjection were made in a puller (Narishige PN-30, Setagaya, Tokyo, Japan) using glass capillary with an internal filament (1.14 OD; 0.5 mm ID). For injection, embryos were lined up on an agar holder (1.5%), as described by Westerfield (2000). Eggs were injected at 0.2 dpf (approximately at 30% epiboly stage, according to Kimmel et al. (1995)) into the yolk with 4.6 nL of either saline solution (Danieau: 58 mmol l^{-1} NaCl, 0.7 mmol l^{-1} KCl, 0.4 mmol l^{-1} $MgSO_4$, 0.6 mmol l^{-1} $Ca(NO_3)_2$, 5 mmol l^{-1} Hepes, pH 7.6) or glucose solutions. Glucose solutions were prepared with D-glucose (Sigma-Aldrich, St Louis. MO. USA) dissolved in saline solution. To assess the tolerance of zebrafish embryos to yolk supplementation with glucose by microinjection, preliminary experiments were carried out with graded concentrations of glucose (0.2, 1.4 and 2.2 mol l^{-1}). The highest dose was estimated based on the maximum solubility of glucose in water. Additional treatments consisted of no injection and saline injection. Criteria used to assess glucose supplementation tolerance of zebrafish were egg osmolality, embryonic development (total length and yolk volume; n=15 larvae), growth (dry mass; n=3 of pooled samples) and mortality.

2.2.3. Experimental design and sampling

Three experimental trials were conducted separately, each using a pool of eggs from several zebrafish breeders.

2.2.3.1. Expression of target metabolic genes at relevant embryonic and larval stages

To assess the expression levels of target metabolic genes, triplicate groups of zebrafish (n=300) were reared up to 10 dpf according to standardized procedures mentioned above. Glucose levels in eggs, embryos and larvae (25 individuals per replicate) were determined from fertilization time (0 dpf) to 4 dpf. Based on critical periods for larval nutrition (exclusive reliance on endogenous yolk nutrients, transition to exogenous feeding and exclusive reliance on exogenous feeding), samples for gene expression analysis (n=3 per developmental stage) were collected at 0.2, 1, 4, 6, 10 dpf (Figure 2.1). Embryos and larvae were randomly sampled, snap-frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.2.3.2. Short-term effect of early glucose injection in zebrafish larvae

This trial evaluated the effect of yolk injection with glucose on the gene expression of target metabolic pathways in zebrafish larvae. The concentration of glucose solution was set at 2 mol l^{-1} in order to achieve a high supplementation dose. Fertilized eggs were injected either with glucose or with saline solution. Triplicate groups (n= 200) of each treatment were reared up to 10 dpf and larvae were fed with *Artemia* nauplii (Figure 2.1-A). An additional group of non-injected eggs was maintained as a biological control of egg quality. Mortality was recorded daily. Immediately after injection, eggs (n=30) we collected to confirm glucose supplementation. Larvae (whole body) of each injection treatment as well as the non-manipulated group were randomly sampled at 4, 6 and 10 dpf (n=6, per treatment), frozen in liquid nitrogen and stored at -80ºC for molecular analyses. Exogenous feeding larvae (6 and 10 dpf) were sampled 4 hours after the last meal to prevent the presence of feed in the gastric tract.

2.2.3.3. Long-term effect of early glucose injection in zebrafish juveniles

This trial assessed the long-term effects of yolk fortification with glucose on growth and gene expression of target metabolic pathways of zebrafish juveniles subjected to a dietary challenge with a high carbohydrate diet. Triplicate groups of eggs (n= 100) were injected with either glucose (2 mol l^{-1}) or saline solution. Each treatment was reared under standardized conditions until 41 dpf (juvenile stage). A group of non injected eggs was included as egg quality control. After injection, samples (n=30 eggs) were taken from both treatments for glucose quantification. Larvae from both injection treatments were initially fed with *Artemia* nauplii for 10 days (5 -15 dpf). During this period, a gradual transition to inert diet was conducted by co-feeding. From 15 to 25 dfp fish were fed exclusively with Control diet (low in digestible carbohydrates). From 25 to 41 dpf, both treatments received the Challenge diet with high level of digestible carbohydrates. The feeding plan is detailed in Figure 2.1-B. Both the Control and Challenge diets were isoproteic (52% crude protein) and isolipidic (14.5% crude lipid), but contained very distinct levels of digestible carbohydrates. Changes on the digestible carbohydrate content of the diets were achieved by the use of raw starch and cellulose as carbohydrate sources in the Control diet and their total replacement by a pre-gelatinized starch and D-glucose in the Challenge diet. Diets were manufactured by low-shear extrusion (SPAROS Lda., Olhão, Portugal). Formulation and proximate composition of diets is presented in Table 2.1. Diets were sieved into three size classes of 50-100 µm; 100-200 µm

and 200-400 µm and delivered according to larval size. Feed was distributed by hand, three to four times a day to visual satiety, and a good acceptance of both diets was recorded. At the end of the trial (41dpf), individual samples were randomly collected from each treatment for: dry weight and fork length determination (n=30 per treatment); gene expression analysis (n= 8 per treatment) where each individual was dissected for separate collection of viscera (all abdominal content) and muscle (remaining body parts). Samples were then snap-frozen in liquid nitrogen and kept at -80ºC until molecular analysis.

Table 2.1 – Formulation and composition of diets used for zebrafish larvae and juveniles

Ingredients (%)	**Control**	**Challenge**
Fishmeal	45.0	45.0
Fish protein concentrate	5.0	5.0
Wheat gluten	10.0	10.0
Raw peas starch	10.0	–
Gelatinized pea starch	–	10.0
Cellulose	10.0	–
Fish oil	5.0	5.0
Krill phospholipids	10.0	10.0
Vitamin & Mineral Premix*	2.0	2.0
D-glucose	–	10.0
Binder (guar gum)	3.0	3.0
Proximate composition		
Dry matter (DM) (%)	95.6 ± 0.21	95.3 ± 0.17
Crude protein (% DM)	52.1 ± 0.92	52.2 ± 0.56
Crude fat (% DM)	14.6 ± 0.43	14.6 ± 0.32
Ash (% DM)	9.2 ± 0.07	9.2 ± 0.02
Gross Energy (KJ g^{-1})	19.3 ± 0.05	19.3 ± 0.03

* Commercial premix for freshwater fish. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; B12, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt sulphate, 2.5mg; copper sulphate, 1.1mg; ferric citrate, 0.2g; potassium iodide, 5mg; manganese sulphate, 15mg; sodium selenite, 0.2mg; zinc sulphate, 40mg; magnesium hydroxide, 0.6g; potassium chloride 1.1g; sodium chloride, 0.5 g; calcium carbonate, 4g.

2.2.4. Analytical methods

Diets were analysed for proximate composition according to the following procedures: dry matter after drying at 105°C for 24 h; ash content by incineration in a muffle furnace at 500ºC for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428, St. Joseph, MI, USA); fat by dichloromethane extraction (Soxhlet); gross energy in an adiabatic bomb calorimeter (IKA C2000, Germany). Glucose levels of eggs and embryos were determined by fluorescent spectroscopy using a commercial kit (Amplit Glucose Quantitation Kit, AAT Bioquest, USA). Fluorescence readings were performed in triplicate using a Synergy^{TM} 4 Multi-Mode Microplate Reader controlled by Gen5^{TM} software (BioTek Instruments, USA).
The osmolality from non-injected and injected eggs as well as of all injected solutions was measured using the OSMOMAT 030 system (Gonotec, Germany). For dry weigh determination samples were freeze-dried for 24 h and weighed in a balance with 0.01 mg precision (Denver Instruments, USA). Total length, fork length and yolk dimensions were measured from digital photographs using AxioVision 4.8.2 image analysis software (CARL ZEISS LTD., UK). Yolk volume was calculated by the formula $V= (\pi/6)*LH^2$, where L is the length and H is the height of the yolk sac (Bagarinao, 1986).

2.2.5. Analysis of metabolic gene expressions by qRT-PCR

The molecular analysis was focused on the expression of target genes involved in intermediary metabolism such as glucose metabolism (GK; HK1; PK-L, PK-M; PEPCKc; G6Pase) and transport (SGLT1), lipogenesis (FAS; ACCα; G6PDH; MEc), glycogen metabolism (GS; GP) and carbohydrate digestion (AMY). Genes related to lipogenesis were included in this study since most of the lipogenic enzymes are up-regulated by the increase of dietary carbohydrates which stimulates glucose metabolism (Towle et al., 1997). Total RNA was extracted from a pool of embryos and larvae (whole body) and from individual viscera and muscle of juveniles using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and following the manufacturer's recommendations (1 mL for all homogenizations). RNA samples were quantified spectrophotometrically and integrity was assessed by electrophoresis through a 1% agarose gel containing ethidium bromide. Total RNA (1 µg) was reverse transcribed into cDNA using the SuperScript^{TM} III Reverse Transcriptase kit (Invitrogen) and random primers (Promega, Charbonniéres, France). mRNA levels were determined by quantitative real-time RT-PCR using the MYiQ^{TM} iCycler (BIO-RAD**,** Hercules, CA, USA).

Analyses were performed on 5 µl of the diluted cDNA using the iQ™ SYBR® Green supermix in a total PCR reaction volume of 15 µl containing 200 nM of each primer. Specific primers for zebrafish were used according to Seiliez et al. (2013) and for some cases primers were designed using Primer3 software (Table 2.2). Thermal cycling was initiated with the incubation at 95ºC for 90s for Taq DNA polymerase activation. Thirty-five steps of q-PCR were performed, each one consisting of heating at 95ºC for 20s for denaturing and at 55ºC for 30s for annealing and extension. After the final cycle of the PCR, melting curves were systematically monitored (55ºC temperature gradient at 0.5ºC/s from 55 to 94ºC). For some pair of primers the annealing temperature was 62ºC, as indicated in Table 2.2. Each q-PCR run included duplicates of samples (reverse transcription) and negative controls (wells without reverse transcriptase, mRNA and cDNA). *Elongation factor-1alpha* (EF1α) was employed as a reference gene and it was found to be stably expressed in this study, except during early embryo stages of development were it was replaced by *18S* rRNA gene (18S) (McCurley and Callard, 2008). Relative quantification of target gene expression was performed using the mathematical model described by Pfaffl (2001).

Table 2.2 – Primers used for mRNA quantification by qRT-PCR in zebrafish

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Accession	T_a (ºC)
GK	GCTGTGAAGTCGGCATGATA	CTTCAACCAGCTCCACCTTAC	BC122359.1[a]	55
HK1	ACTTTGGGTGCAATCCTGAC	AGACGACGCACTGTTTTGTG	BC067330.1 [a]	55
PK-L	TCCTGGAGCATCTGTGTCTG	GTCTGGCGATGTTCATTCCT	BC152219.1 [a]	55
PK-M	TGGGCTTATTAAGGGCAGTG	TGCACCACCTTTGTGATGTT	BC165710.1[a]	55
PEPCKc	ATCACGCATCGCTAAAGAGG	CCGCTGCGAAATACTTCTTC	NM_214751.1[a]	55
G6Pase	TCACAGCGTTGCTTTCAATC	AACCCAGAAACATCCACAGC	BC164161.1[a]	55
GS	GCAGCTCAGTGTGACGAACC	GGTCCCCTGCTTCCTTATCC	NM_201180.1[b]	62
GP	AGAAGCCGGAGAGGAAAACC	TCTCAGGCTGTTTCGGTGAA	BC085616.2[b]	62
SGLT1	GGATTGACCTGGAGGCAGAC	GCGTTGACCACATTTCTCCA	BC067621.1[b]	62
FAS	GAGGGAAATCCGACAGTTGA	GACTCCAACAGAGCCTGAGC	XM_001923608.3[a]	55
ACCα	CACGATGCTCAGTTGTGTCC	CCATGACAGTGGACTTGACG	XM_001919780.3[a]	55
G6PDH	CGTCTTTTGTGGCAGTCAGA	TGATGGGTGGTGTTTTCTCA	XM_694076.5[a]	55
MEc	TCAAGGCTATGGCATCCTTC	ATATCCCCCTTCCCTCAGTG	BC152078[b]	55
AMY	AGACCAGCCTCCAGGGTACA	AAGCGACCAATAGGCTGGAA	XM_001919100.3[b]	62
18S	GAACGCCACTTGTCCCTCTA	GTTGGTGGAGCGATTTGTCT	FJ915075.1[a]	55
EF1α	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC	NM_131263[b]	55

[a] From Seiliez et al. (2013).
[b] Accession number from http://www.ncbi.nlm.nih.gov
T_a, primer annealing temperature.

2.2.6. Statistical analysis

The results are presented as means ± standard deviation (s.d.). For survival, dry weight, yolk volume and total length of the samples from the preliminary experiment (tolerance to high glucose levels) a one-way ANOVA was performed to assess differences between the saline injection and the several concentrations of glucose injection. Tukey test (HSD) was used to identify differences between the means and in cases of unequal variances the Games-Howell test was used. Differences on gene expression levels during embryogenesis were analysed by the non-parametric Kruskal-Wallis test followed by Dunnett test, due to the reduced sample amount (n=3) analysed in each time point. The control group for gene expression analysis during embryogenesis was 0.2 dpf. To analyse the results between glucose and saline injection on gene expression an unpaired two-tailed Student's t-test was performed. Control group for the injection trials was the saline treatment. Results from growth performance of juveniles were analysed with an unpaired two-tailed Student's t-test. All analyses were performed using software package SPSS® 16.0 for Windows® and the level of significance was set at $P<0.05$.

2.3. Results

2.3.1. Expression of target metabolic genes at relevant embryonic and larval stages

In this first trial, the survival rate was high (88%) for zebrafish fed with *Artemia* nauplii until 10 days post-fertilization (dpf) (Table 2.3). Glucose is scarcely available during zebrafish embryonic development, mainly during the first hours of development. At 0.2 dpf (relevant stage for injection) total glucose level was 0.06 mmol l^{-1}, the lowest concentration recorded. Afterwards, an increase up to 0.4 mmol l^{-1} was recorded at 1 dpf followed by a slight decrease to 0.3 mmol l^{-1} at 4 dpf (Figure 2.2). Figure 2.3 shows data on the transcripts levels during zebrafish embryogenesis of genes related to glycolysis [glucokinase (GK); hexokinase 1 (HK1); pyruvate kinase (PK-L, PK-M, isoforms from liver and muscle, respectively)], gluconeogenesis [cytosolic phosphoenolpyruvate carboxykinase ($PEPCK_c$); glucose-6-phosphatase (G6Pase)], lipogenesis [fatty acid synthase (FAS); acetyl-CoA carboxylase isoform alpha (ACCα); glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme cytosolic (MEc)]; glycogen metabolism [glycogen synthase (GS); glycogen phosphorylase(GP)], glucose transport [sodium-dependent glucose cotransporter 1 (SGLT1)]

and carbohydrate digestion [amylase (AMY)]. These results clearly show that at 0.2 dpf, the expression levels from all genes were significantly lower (P<0.05) than at later stages. Towards the end of the exclusively endogenous feeding stage (4 dpf), the mRNA kevels of all analysed genes related to glucose, glycogen and lipid metabolism as well as to glucose transport and carbohydrate digestion exhibited a considerable increase on mRNA levels. During the transition to exogenous feeding conditions (6 and 10 dpf) all genes continued to be expressed, with a significant (P<0.05) increase for SGLT1, AMY, PEPCKc, G6Pase and PK-L; a significant reduction for GK, PK-M, and unchanged for all other studied genes.

Table 2.3 – Survival of zebrafish larvae and juveniles at 4, 10 and 41 dpf during the trials

		Survival (%)		
Experiment	Condition	4 dpf	10 dpf	41 dpf
Expression profile	Non injected	98 ± 7.3	88 ± 3.1	n.d
Short-term trial	Non injected	97	92	n.d.
	Saline	86 ± 9.1	82 ± 7.0	n.d
	Glucose (2 mol l^{-1})	79 ± 8.0	72 ± 4.6	n.d.
Long-term trial	Non injected	99	84	40
	Saline	87 ± 6.6	73 ± 7.1	42 ± 7.68
	Glucose (2 mol l^{-1})	86 ± 3.1	76 ± 7.8	43 ± 5.24

Values are means ± s.d. (n=3) for saline and glucose injection treatments in the short and long-term trials. Non injected group was reared without replicates. Values were tested with Student's t-test. Absence of superscript letter indicates no statistical differences (P > 0.05). n.d, not determined.

2.3.2. Tolerance of zebrafish embryos to high glucose levels in yolk

Three highly concentrated solutions of D-glucose were injected into yolks and the glucose concentration in zebrafish embryos (0.2 dpf; 30% epiboly) examined (Figure 2.2). Results show that in comparison to saline-injected eggs (controls), it was possible to achieve an increase of 4-, 15- and 43-fold of glucose concentration in eggs injected with 0.2, 1.4 and 2.2 mol l^{-1} doses, respectively. Embryos injected with saline solution had low levels of glucose (0.03 mmol l^{-1}) that were similar to the levels found in non-injected embryos. In contrast, embryos injected with the highest dose (2.2 mol l^{-1}) reach a level of 1.5 mmol l^{-1} of glucose, right after the injection. It is worth mentioning that quantified glucose levels found in zebrafish eggs after injection were considerably lower than estimated injected doses. No

leaking of glucose was registered in the eggs immediately after the injection. However, we cannot exclude the possibility that injected embryos quickly released excess glucose as a mechanism of cellular homeostasis to cope with osmolality changes. Despite the high osmolality value of concentrated of glucose solutions, values measured in eggs were not affected by glucose injection, ranging from 80 mOsm kg^{-1} in glucose-injected eggs to 70 mOsm kg^{-1} in saline-injected and non-injected eggs (data not shown). No differences (P>0.05) in larval weight, embryonic development (length and yolk volume) and survival were found between treatments, at the end of the experiment (Tables 2.3, 2.4). Larvae injected with 1.4 mol l^{-1} glucose were shorter at 4 dpf compared with those injected with 0.2 mol l^{-1} (P=0.002) and 2.2 mol l^{-1} (P=0.01), but not different from the saline-injected embryos. At 10 dpf, survival was high for eggs injected with saline and glucose (Table 2.3). Overall, zebrafish larvae seemed to tolerate well the enrichment of yolk reserves with high concentrations of glucose up to 2.2 mol l^{-1}, without compromising growth and survival

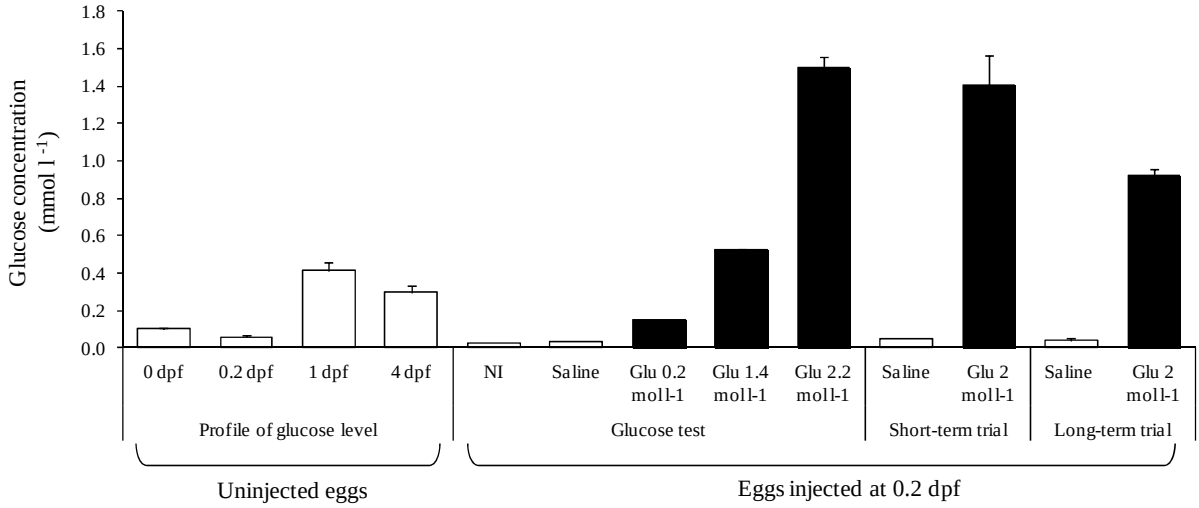

Figure 2.2 – Glucose levels in zebrafish embryos and larvae. Natural changes from zygote to endogenous feeding larvae (up to 4 dpf) in non-injected eggs and changes after injection into the yolk (at 0.2 dpf) of graded glucose doses in comparison with basal levels in saline-injected (saline) and non-injected eggs (NI); Values are means + s.d. of n=3 groups per treatment and time.

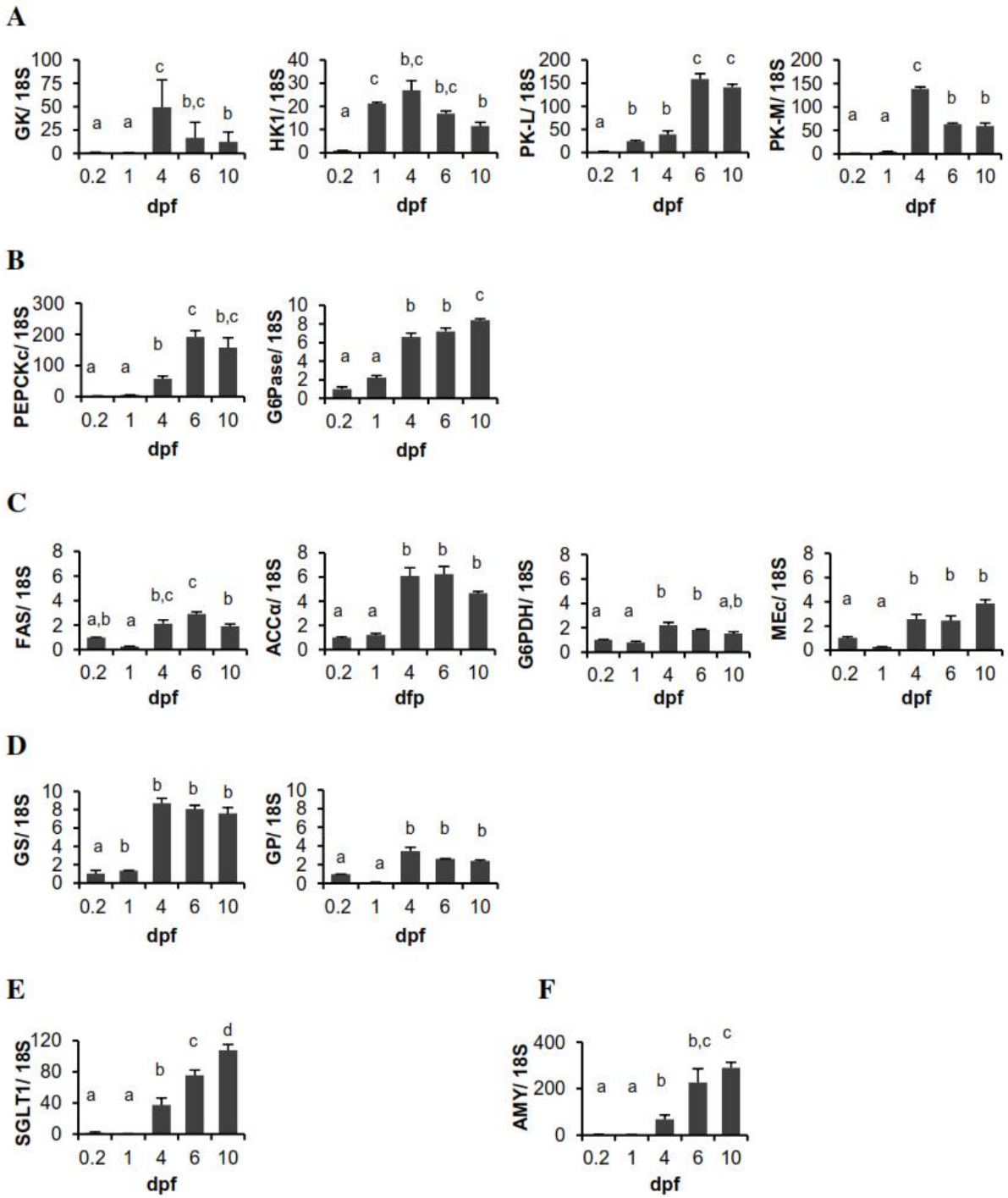

Figure 2.3 – Relative expression profiles (fold variation) of genes from zebrafish larvae up to 10 dpf in comparison to the levels observed at 0.2 dpf (30% epiboly stage). The genes analysed were those involved in (A) glycolysis: GK, HK1, PK-L, PK-M; (B) gluconeogenesis: PEPCKc, G6Pase; (C) lipogenesis: FAS, ACCα, G6PDH, MEc; (D) glycogen metabolism: GS, GP; (E) glucose transport: SGLT1 and (F) carbohydrate digestion: AMY. Total RNA was extracted from whole embryo or larvae (n=3). Relative expression levels were measured using real-time quantitative RT-PCR and normalized with 18S ribosomal RNA gene (18S). Values are means + s.d.; significant differences among developmental stages were analysed using non-parametric test Kruskal-Wallis one-way analysis of variance followed by Dunnett test ($P < 0.05$; different letters above the bars denote a significant difference).

2.3.3. Effect of glucose injection on short-term and long-term metabolic gene expression

Glucose levels were measured in embryos injected (0.2 dpf) with either saline or glucose (2 mol l^{-1}) solution (Figure 2.2). Immediately after microinjection, a 28- and 23-fold increase of basal glucose levels was achieved in the short-term 84, 6, 10 dpf) and long-term (41 dpf) trials, respectively. In addition, saline injected eggs showed similar levels of glucose in both trials (approx. 0.05 mmol l^{-1}). These results confirm that an effective enrichment of yolk glucose reserves of zebrafish embryos at 0.2 dpf was achieved. In both trials, the survival rate at 10 dpf was not significantly different (P>0.05) between the glucose and saline treatments (Table 2.3). Considering the long-term trial, no significant differences (P>0.05) were found for growth (dry mass and fork length) and development of juvenile fish between glucose and saline treatments (Table 2.4).

Table 2.4 – Growth parameters of zebrafish larvae and juveniles injected at 0.2 dpf with different concentrations of glucose.

		Control	**Glucose solution**		
	dpf	Saline	0.2 mol l^{-1}	1.4 mol l^{-1}	2.2 mol l^{-1}
Larvae					
Dry mass (mg.ind^{-1})	4	0.06 ± 0.00	0.06 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
	10	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.03	0.05 ± 0.01
Total length (mm.ind^{-1})	4	4.02 ± 0.07^{ab}	4.22 ± 0.12^{b}	3.92 ± 0.23^{a}	4.17 ± 0.22^{b}
	10	4.75 ± 0.67	4.96 ± 0.97	4.74 ± 0.91	4.45 ± 0.81
Yolk volume (mm^3)	1	0.16 ± 0.04	0.17 ± 0.03	0.15 ± 0.03	0.14 ± 0.04
	4	0.01± 0.00	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01
Juvenile					*
Dry mass (mg.ind^{-1})	41	6.0 ± 4.01	–	–	6.9 ± 4.15
Fork length (mm.ind^{-1})	41	13.3 ± 2.43	–	–	13.9 ± 2.45

* For the long-term trial the injected glucose solution was 2 mol l^{-1}. Values are means ± s.d.; different superscript letters represent significant differences (P < 0.05, one-way ANOVA followed by Tukey post-hoc test or Games-Howell test, when equal variances were not assumed). dpf, days post-fertilization.

For the short-term trial, gene expression was firstly analised between non injected, saline injected and glucose injected embryos. No significant differences were found between non injected eggs and saline injected eggs, meaning that the injection procedure itself (puncturing and injecting) had no effect on gene expression (data not shown). The effects of glucose injection on the expression of metabolic genes in the short-term and long-term trials are illustrated in Figure 2.4. At 4 dpf, a period during which larvae rely exclusively of

endogenous reserves, glucose injected larvae showed a down-regulation of several genes related to glucose metabolism, lipid metabolism, glucose transporter and carbohydrate digestion (Figure 2.4-A, B, C, E) in comparison with the saline injected group. At 6 dpf, when actively feeding, the inhibitory effect of glucose injection on gene expression started to fade. Of all genes that were down-regulated at 4 dpf only those for SGLT1 and GP remained at lower levels of expression, in glucose injected larvae (Figure 2.4-D, E). At 10 dpf the early down-regulation of genes expression associated with glucose injection was suppressed and no significant differences (P>0.05) were found between treatments. Exceptionally, the GS gene expression was up-regulated on the glucose-injected larvae (Figure 2.4-D). In brief, the inhibitory effect of glucose injection on gene expression observed at 4 dpf was no longer present at 10 dpf, which means that effects were reversible. In viscera and muscle of juvenile fish (41 dpf), injected with glucose at early embryo stages and later submitted to a feeding challenge with a high digestible carbohydrate diet, it was possible to detect differences on gene regulation. In viscera, the genes for HK1, PEPCKc, MEc, GS and SGLT1 genes were significantly down-regulated on the glucose injected fish. The decrease in mRNA of visceral tissue of juvenile fish was identical to the short-term effect found at 4 dpf, with the exception of HK1 gene (Figure 2.4). In contrast, in muscle the GS gene was up regulated in the glucose injected treatment (Figure 2.4-D), reflecting once more the pattern found during acute effect (10 dpf) on gene expression. However, for this gene, at 41 dpf, the opposite was found. Therefore the effect of glucose treatment was different depending on the tissue in which the gene was expressed: in viscera the expression was downregulated whereas in muscle it was induced.

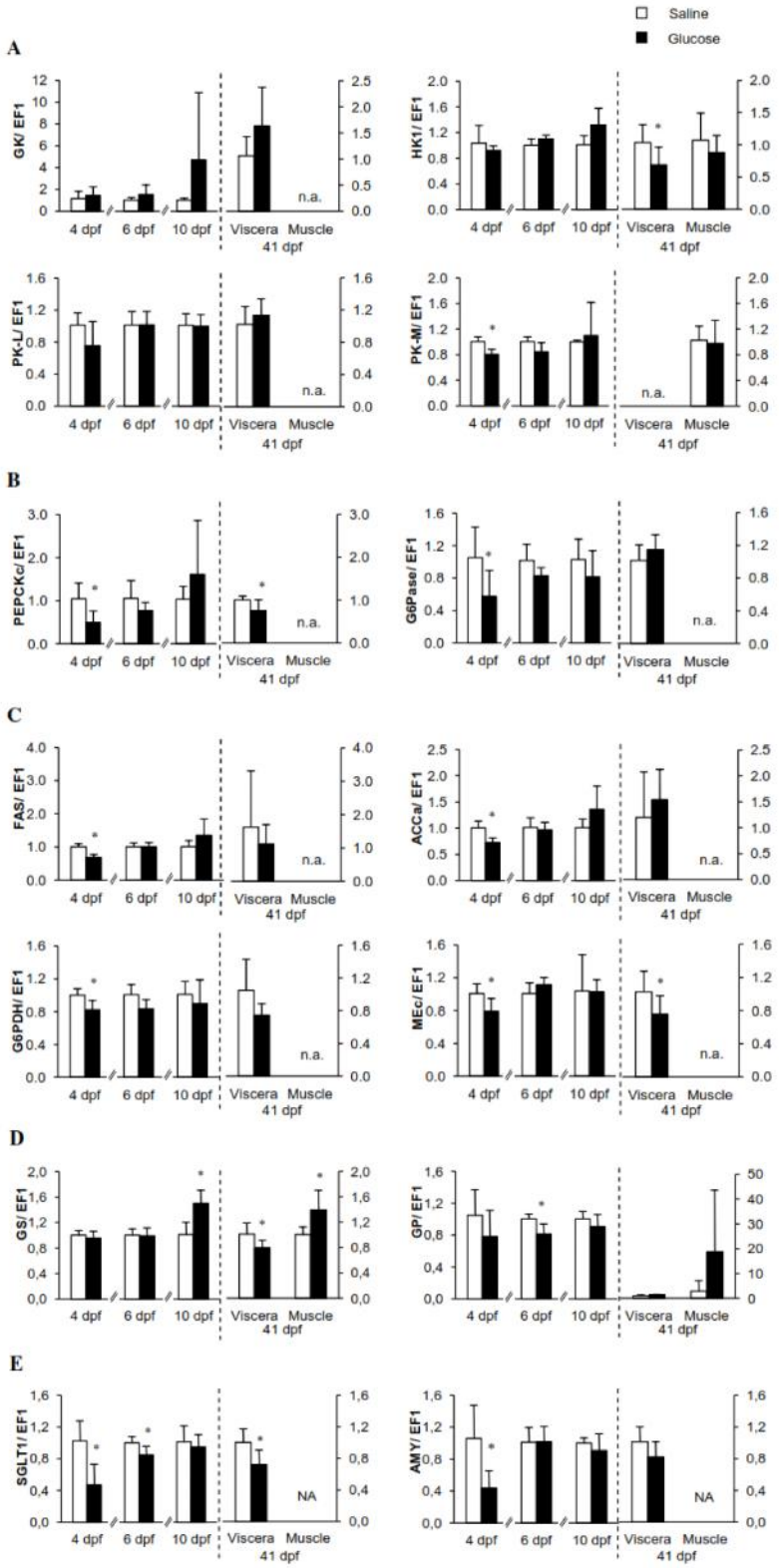

Figure 2.4 - Short-term and long-term effects of glucose injection on gene expression in larvae at 4, 6 and 10 dpf and juveniles at 41 dpf. The genes were those involved in (A) glycolysis: GK, HK1, PK–L, PK–M; (B) gluconeogenesis: PEPCKc, G6Pase; (C) lipogenesis: FAS, ACCα, G6PDH, MEc; (D) glycogen metabolism: GS, GP; (E) glucose transport and carbohydrate digestion: SGLT1 and AMY, respectively. Control group was injected with saline solution. Total RNA was extracted from whole larvae (n=6, up to 10 dpf) or viscera and muscle (n=8, juveniles). Relative expression levels were measured using real-time quantitative RT-PCR and normalized with elongation factor 1 (EF1α) gene. Values are means + s.d. **P*<0.05, significantly different (Student's *t-test*) among injection treatment; n.a., not applicable.

2.4. Discussion

Several studies on metabolic programming in mammals showed that nutrients supply at crucial development stages early in life may have long-term consequences on genetic and physiological consequences during adulthood (Lucas, 1998; Patel and Srinivasan, 2002; Patel et al., 2009). Geurden et al. (2007) tested the concept of nutritional programming in fish, by feeding rainbow trout larvae with a high dextrin diet. This short-term hyperglucidic stimulus resulted in molecular adaptations of carbohydrate digestive enzymes (AMY and Maltase) at a later juvenile stage. However, no persistent effect was recorded for enzymes involved in glucose metabolism or transport. In our study, using microinjection technique, we attempted to modify the glucose content of zebrafish yolk reserves at early stages of embryogenesis in order to assess the short-term and possible long-term modifications on gene regulation. The panel of genes selected for qPCR analysis in this study has been previously validated as good molecular markers for nutritional regulation of glucose metabolism in zebrafish (Seiliez et al., 2013).

2.4.1. Glucose supplementation of yolk has no detrimental effects on growth and development of larvae

In zebrafish, microinjection of RNA, DNA, proteins, antisense oligonucleotides and other small molecules into the developing embryo allows a quick and robust approach for exploring gene function *in vivo* (Xu, 1999). The time frame selected for performing microinjection can vary between the incipient stages of 1- to 2-cell and later gastrula stages, depending on the study requirements (Culp et al., 1991; Janik et al., 2000). In this study, the microinjection of glucose into the yolk of zebrafish embryos was performed at the 30% epiboly stage (0.2 dpf). The choice of this particular developmental stage took into consideration the facts that: (1) the yolk syncytial layer (YSL) was formed; (2) the blastoderm cells were beginning to spread over the yolk; (3) the percentage of yolk coverage by the blastoderm was reduced (30% covered) (Kimmel et al., 1995). The YSL has an important function in transporting nutrients from the yolk to the embryonic cells and later to larval tissues and is also responsible for the degradation of some nutrients (Carvalho and Heisenberg, 2010; Holtta-Vuori et al., 2010). It has been demonstrated before that performing injection into the yolk during the epiboly stage leads to successful diffusion of the injected material within the yolk without later outward flux (Hagedorn et al., 1997; Janik et al., 2000; Robles et al., 2006). Furthermore, injecting the

yolk at 30% epiboly stage can be advantageous for later embryo recovery because the blastoderm is expected to cover the puncture hole during its rapid spreading process. Thus, the injection period as well as the volume (4.6 nL) seemed appropriate for the zebrafish embryos since no negative effects on survival or development were recorded. Using Danieau solution, all injection trials yielded good survival rate up to 4 dpf (86 – 92%), comparable with the results obtained by Janik et al. (2000) who injected with embryo medium. Moreover, the low permeability of YSL and its role in regulating yolk consumption (Carvalho and Heisenberg, 2010), could have protected the developing embryo from immediate exposure to the high glucose load in the yolk reserve. This possibly explains why survival rate was not affected by the injection of such high concentrations of glucose. In natural conditions, a cyprinid egg contains low amount of carbohydrates (1.5 – 6.2% dry mass) of which glycogen represents the major constituent (up to 3 mg per egg) (Hoar and Randall, 1988; Linhart et al., 1995). This is confirmed by the relatively low level of glucose in non-injected eggs at 0.2 dpf ($\approx$ 0.03 mmol l^{-1}), which then slightly increased up to 0.4 mmol l^{-1} prior to hatching. A similar profile of glucose utilization and production has been described for zebrafish by Soanes et al. (2011). Consequently, all injected glucose doses led to a substantial increase of the glucose concentration in eggs, up to 43-fold, showing that yolk reserves can be enriched with this nutrient and that microinjection is an effective way to do it.

2.4.2. Expression of metabolic genes in embryos and larvae

The expression of the selected metabolic genes was analysed during different stages of zebrafish development to better understand the ability to use endogenous glucose at nutritionally relevant stages of development. Genes related to glucose, lipid and glycogen metabolism as well as to glucose transport and carbohydrate digestion where all expressed at low levels at 0.2 dpf, compared to later stages of development. This could be expected because the onset of gene expression that occurs before 0.2 dpf, during the mid-blastula transition stage, is mostly related with early processes of cell cycle and transcriptional regulation (Kane and Kimmel, 1993; Mathavan et al., 2005). This supports the hypothesis that 0.2 dpf could be a relevant period for genomic imprinting in zebrafish. At 4 dpf, larvae still fully rely on yolk-sac endogenous reserves as the only source of nutrients for growth and development (Holt, 2011). During this period the majority of genes analysed showed a significant increase in expression with the exception of HK1 gene that showed increased expression earlier, at 1 dpf. This enhancement should be considered as a peak level during

larval development and not as the onset of expression at that precise time. The high expression level indicates that important metabolic pathways were fully active at 4 dpf. In fact Mathavan et al. described that from segmentation period (0.4 dpf) to later larval stages, a set of genes related to organogenesis begin to be expressed (Mathavan et al., 2005). During the transitional period from endogenous to exogenous feeding (6 – 10 dpf), larvae undergo a remarkable metamorphosis in preparation for ingesting feed (Holt, 2011). PK-L, PEPCKc and AMY genes showed a higher induction of expression at 6 dpf compared to 4 dpf, probably because during this period, the formation and differentiation of two main organs involved in glucose homeostasis (liver and pancreas) is still ongoing (Chu and Sadler, 2009). A similar expression pattern to the one found in this study for the SGLT1 gene was described for zebrafish orthologue of GLUT2, which strongly increased at 3 dpf (Castillo et al., 2009). Together these results tend to demonstrate that, at a molecular level, the metabolic pathways associated with glucose homeostasis are fully functional in zebrafish larvae.

2.4.3. Yolk glucose enrichment transiently modifies metabolic gene expression in larvae

The low transcript levels at 0.2 dpf of the target genes renders this period susceptible for gene modulation. We therefore injected the yolk of embryos with very high levels of glucose, in order to determine if this early glucose stimulus could durably modify the genomic expression at later larval stages. Organogenesis begins early in zebrafish and before the onset of exogenous feeding (5 – 6 dpf), all major organ systems are formed and partially functional: eyes, gut, liver, pancreas among others (Holmberg et al., 2004; Tao and Peng, 2009; Tehrani and Lin, 2011; Wallace and Pack, 2003). However, attention should be given to determining the delicate balance between the establishment of complex metabolic pathways controlling organ formation and the ability to achieve a genomic imprint of the nutritional stimulus exerted at early embryo stage. An acute effect of glucose injection was observed in larvae at 4 dpf, showing that most of the genes related to glucose metabolism and transport, lipid metabolism and carbohydrate digestion were down-regulated compared with those in larvae from saline-injected eggs. Similar results to those found in this study (4 dpf) of the downregulation of PEPCKc and G6Pase, genes involved in gluconeogenesis were described for juveniles of seabream and common carp after feeding on a high carbohydrate diet (Panserat et al., 2002). Also, it was shown that PEPCK expression responded to insulin self-production in zebrafish, suggesting that this pathway could be regulated in a similar manner to that in mammals (Elo et al., 2007). However, some of our results were unexpected, because

it has been demonstrated in different fish species that dietary carbohydrates can induce enzymes involved on glycolysis, lipogenesis and carbohydrate digestion, as occurs in mammals (Caseras et al., 2000; Geurden et al., 2007; Kersten, 2001; Panserat et al., 2000; Panserat et al., 2001a; Panserat et al., 2001b; Polakof et al., 2011; Seiliez et al., 2013).

Several scenarios can be proposed to explain the overall reduction of gene expression in the glucose injected group (4 dpf), as a result of glucose overloading, but further studies are needed to confirm each suggested hypothesis: (1) intensive cellular oxidative stress as a response to excessive nutrient (glucose) uptake (Wellen and Thompson, 2010); (2) susceptibility to modulation of mRNA transcription, as demonstrated for gene phospholipase A2 gene in ß-cells of rat having excess glucose availability (Metz et al., 1991); (3) repression of master genes that control key regulatory systems (Corkey and Shirihai, 2012). Whether such inhibition of gene expression in the glucose injected group at 4 dpf would persist at lower yolk glucose levels remains to be elucidated. Still, cyprinid eggs (data from common carp) contain 68% water, 26% proteins, 2% fat, 1% ash and 2% carbohydrates (Hadjinikolova, 2008). Thus to achieve a marked increase of the relatively small carbohydrate fraction a high supplementation dose is required. A lower dose could mask the stimulus effect by not being enough to alter the nutrients ratio the total biochemical constituents of zebrafish eggs. Throughout larval development, the inhibitory effect on gene regulation was less pronounced, as confirmed at 6 dpf larvae where only glycogen phosphorylase (GP) and glucose transporter (SGLT1) were down-regulated. Later still, at 10 dpf, the inhibition caused by the early glucose supplementation was no longer observed in all metabolic pathways. It seems thus that the early gene expression pattern faded ways after larvae had totally consumed the yolk reserve (in which glucose was injected) suggesting a transient effect on gene regulation. Maddison and Chen (2012) showed that the number of zebrafish ß-cells was rapidly increased after a persistent, but not intermittent, exposure to glucose excess. In our study, from 5 dpf onwards, larvae were only fed with *Artemia* nauplii, which have a low carbohydrate content (aprox. 11% dry mass) (Lavens and Sorgeloos, 1996), making this dietary regime inappropriate for enhancing the expression of glucose related genes. A combination of genetic, metabolic and physiological factors could be associated with the regulation of target genes at 10 dpf. Nevertheless, 10 dpf zebrafish larvae relying exclusively on exogenous food from the glucose-injected eggs showed an up-regulation of the GS gene, suggesting a possible enhancement of glycogen storage. The induction of glycogenesis by carbohydrates intake has been demonstrated for other species such as seabream, cod, Atlantic

salmon and common carp (Ekmann et al., 2013; Hemre et al., 2002; Panserat et al., 2000; Shimeno et al., 1995)

2.4.4. Yolk glucose enrichment has little effect on metabolic genes in juveniles fed high levels of digestible carbohydrates

Mammalian programmed cells are able to quickly react to an external stimulus (e.g. nutrient) and "remember" the response once the stimulus is removed and reintroduced at later stages (Bird, 2002). Recently, Olsen et al. (2012) showed that diabetic metabolic memory could be heritable to new generations of hyperglycemic zebrafish, through mechanisms related to DNA demethylation. After establishing that acute effects of the early glucose injection on the expression of several genes associated with intermediary metabolism were transient, we investigated if such stimulus could have a long-term effect, by challenging juvenile fish with a high digestible carbohydrates regime. A previous study applying a hyperglucidic stimulus at the onset of exogenous feeding of rainbow trout failed to demonstrate persistent molecular adaptations of genes involved in glucose transport or metabolism (Geurden et al., 2007). Similarly in our work, the expression of most studied genes associated with glucose metabolism was not affected by the supplementation of glucose to the embryo and subsequent feeding of juvenile fish with a diet rich in digestible carbohydrates. Only a few genes showed signs of a long-term regulation, similar to the one observed in the acute effect (4 dpf): the PEPCKc and SGLT1 genes were down-regulated in viscera, whereas the GS gene was up-regulated in muscle (but a different effect was registered, with GS inhibition occurring in viscera). These results suggest that the few molecular adaptations achieved on juvenile fish (with the exception of HK1) could be already present at larval stages as a result of the early glucose supplementation. The lower level of SGLT1 gene expression found in juveniles subjected to early glucose stimulus may indicate a potential decrease of postprandial glycaemia by lowering the intestinal transport of glucose. The reason why HK1 gene was down-regulated in visceral tissue remains unclear, because unlike GK, this enzyme does not seem to be regulated by nutritional factors, both at either the molecular expression or activity levels (Enes et al., 2009; Gonzalez-Alvarez et al., 2009; Soengas et al., 2006). Despite being rather weak effects and based only on molecular data, juveniles conditioned by an early glucose stimulus tend to show an enhanced capacity for glucose storage in muscle, lower glucose production in liver and lower glucose transport in intestinal lumen. However, to

substantiate such regulation of metabolic pathways further studies are needed to assess its physiological relevance at enzymatic and metabolic level.

In our study, we have explored the concept of nutritional programming in fish, a process that has mainly been studied in mammals, whereby maternal nutrition can induce alterations on offspring gene regulation. Mammals and fish are physiological different in terms of embryonic development, and therefore embryos are exposed to external stimuli in distinct ways*:* in the uterus the fetus is directly linked to maternal nutritional experiences, whereas in fish eggs the embryos rely exclusively on their endogenous yolk reserves. But despite this difference, it has been shown that fish share a high genetic similarity with mammals and thus some mechanisms for gene regulation, including nutritional regulation, can be conserved (Hemre et al., 2002; Ulloa et al., 2011). The present study showed that glucose supplementation in the early embryo stage had no marked benefits (at a molecular level) on the ability of zebrafish larvae and juvenile to cope with high glucose as a metabolic substrate. A possible impairing effect of glucose overload at such an early embryonic stage should be taken into consideration while assessing the few molecular changes observed in juveniles. Consequently, the nutritional programming concept was not fully established for zebrafish.

2.5. Conclusions

Little is known about the mechanisms underlying nutrient supplementation during fish embryogenesis and the ability to achieve a genomic imprinting at early embryo stages. Given the marked differences existing between mammals and teleost fish regarding the exposure of embryos to nutritional stimuli (continuous in uterus maternal transfer vs. exclusive endogenous egg yolk reserves) comparisons between mammals and fish should be more focused on molecular aspects then physiological aspects. Our study presented a novel approach on how to manipulate nutritional reserves of fish embryos in order to study its effects on the long-term molecular regulation of metabolic pathways. However, our data showed that glucose supplementation in early embryo stage had no marked effect, at a molecular level, on the ability of zebrafish juveniles to cope with high carbohydrate intake. But despite this lack of effects, we cannot conclude that nutritional programming is not viable in fish. Based on our results, future studies on the use of glucose as a trigger for nutritional programming, would benefit from: (1) targeting a highly carnivorous fish species with low

tolerance to dietary carbohydrates; (2) using embryos at a later development stage with fully functional metabolic pathways; (3) defining an adequate supplemental dose of glucose to avoid a potential overload status and consequently cellular damage; (4) exploring the effect of intermittent versus persistent nutritional stimulus and the use of a high carbohydrate/ low protein diet to validate the effects at the juvenile stage.

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2.7. References

Ali, S., Champagne, D.L., Spaink, H.P., Richardson, M.K., 2011. Zebrafish embryos and larvae: A new generation of disease models and drug screens. Birth Defects Research Part C: Embryo Today: Reviews. 93, 115-133.

Andersen, I., Reiner, A., Aanes, H., Alestrom, P., Collas, P., 2012. Developmental features of DNA methylation during activation of the embryonic zebrafish genome. Genome Biology. 13, R65.

Babin, P.J., Cerdà, J., Lubzens, E., 2007. The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer.

Bagarinao, T., 1986. Yolk resorption, onset of feeding and survival potential of larvae of three tropical marine fish species reared in the hatchery. Marine Biology. 91, 449-459.

Bird, A., 2002. DNA methylation patterns and epigenetic memory. Genes Dev. 16, 6-21.

Burdge, G.C., Lillycrop, K.A., 2010. Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr. 30, 315-339.

Campos, C., Valente, L., Conceicao, L., Engrola, S., Fernandes, J., 2013. Temperature affects methylation of the myogenin putative promoter, its expression and muscle cellularity in Senegalese sole larvae. Epigenetics. 8.

Carvalho, L., Heisenberg, C.P., 2010. The yolk syncytial layer in early zebrafish development. Trends in Cell Biology. 20, 586-592.

Caseras, A., Meton, I., Fernandez, F., Baanante, I.V., 2000. Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (Sparus aurata). Biochim Biophys Acta. 1493, 135-141.

Castillo, J., Crespo, D., Capilla, E., Diaz, M., Chauvigne, F., Cerda, J., Planas, J.V., 2009. Evolutionary structural and functional conservation of an ortholog of the GLUT2 glucose transporter gene (SLC2A2) in zebrafish. Am J Physiol Regul Integr Comp Physiol. 297, 1570-1581.

Chu, J., Sadler, K.C., 2009. New school in liver development: lessons from zebrafish. Hepatology. 50, 1656-1663.

Corkey, B.E., Shirihai, O., 2012. Metabolic master regulators: sharing information among multiple systems. Trends Endocrinol Metab. 23, 594-601.

Culp, P., Nusslein-Volhard, C., Hopkins, N., 1991. High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. Proc Natl Acad Sci U S A. 88, 7953-7957.

Ekmann, K.S., Dalsgaard, J., Holm, J., Campbell, P.J., Skov, P.V., 2013. Glycogenesis and de novo lipid synthesis from dietary starch in juvenile gilthead sea bream (Sparus aurata) quantified with stable isotopes. Br J Nutr. 109, 2135-2146.

Elo, B., Villano, C.M., Govorko, D., White, L.A., 2007. Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpyruvate carboxykinase as a marker for exposure to anti-diabetic compounds. J Mol Endocrinol. 38, 433-440.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2009. Nutritional regulation of hepatic glucose metabolism in fish. Fish Physiol Biochem. 35, 519-539.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2011. Dietary carbohydrate utilization by European sea bass (Dicentrarchus labrax L.) and gilthead sea bream (Sparus aurata L.) juveniles. Reviews in Fisheries Science. 19, 201-215.

Fernández-Palacios, H., Izquierdo, M.S., Robaina, L., Valencia, A., Salhi, M., Vergara, J., 1995. Effect of n − 3 HUFA level in broodstock diets on egg quality of gilthead sea bream (Sparus aurata L.). Aquaculture. 132, 325-337.

Fernández-Palacios, H., Izquierdo, M., Robaina, L., Valencia, A., Salhi, M., Montero, D., 1997. The effect of dietary protein and lipid from squid and fish meals on egg quality of broodstock for gilthead seabream (Sparus aurata). Aquaculture. 148, 233-246.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (Oncorhynchus mykiss) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am J Physiol Regul Integr Comp Physiol. 292, 2275-2283.

Gonzalez-Alvarez, R., Ortega-Cuellar, D., Hernandez-Mendoza, A., Moreno-Arriola, E., Villasenor-Mendoza, K., Galvez-Mariscal, A., Perez-Cruz, M.E., Morales-Salas, I., Velazquez-Arellano, A., 2009. The hexokinase gene family in the zebrafish: structure, expression, functional and phylogenetic analysis. Comp Biochem Physiol B Biochem Mol Biol. 152, 189-195.

Hadjinikolova, L., 2008. Investigations on the chemical composition of carp (Cyprinus carpio L.), bighead carp (Aristichthys nobilis Rich.) and pike (Esox lusius L.) during different stages of individual growth. . Bulg. J. Agric. Sci. 14, 121-126.

Hagedorn, M., Hsu, E., Kleinhans, F.W., Wildt, D.E., 1997. New Approaches for Studying the Permeability of Fish Embryos: Toward Successful Cryopreservation. Cryobiology. 34, 335-347.

Hemre, G.I., Mommsen, T.P., Krogdahl, A., 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquacult Nutr. 8, 175-194.

Hoar, W.S., Randall, D.J., 1988. Fish Physiology, The Physiology of Developing Fish - Eggs and Larvae. Academic Press, San Diego, California.

Holmberg, A., Schwerte, T., Pelster, B., Holmgren, S., 2004. Ontogeny of the gut motility control system in zebrafish Danio rerio embryos and larvae. J Exp Biol. 207, 4085-4094.

Holt, G.J., 2011. Larval Fish Nutrition. Wiley-Blackwell, John Wiley & Sons.

Holtta-Vuori, M., Salo, V.T., Nyberg, L., Brackmann, C., Enejder, A., Panula, P., Ikonen, E., 2010. Zebrafish: gaining popularity in lipid research. Biochem J. 429, 235-242.

Janik, M., Kleinhans, F.W., Hagedorn, M., 2000. Overcoming a permeability barrier by microinjecting cryoprotectants into zebrafish embryos (Brachydanio rerio). Cryobiology. 41, 25-34.

Kamler, E., 2008. Resource allocation in yolk-feeding fish. Rev Fish Biol Fisheries. 18, 143-200.

Kane, D.A., Kimmel, C.B., 1993. The zebrafish midblastula transition. Development. 119, 447-456.

Kersten, S., 2001. Mechanisms of nutritional and hormonal regulation of lipogenesis. EMBO Rep. 2, 282-286.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev Dyn. 203, 253-310.

Kudoh, T., Tsang, M., Hukriede, N.A., Chen, X., Dedekian, M., Clarke, C.J., Kiang, A., Schultz, S., Epstein, J.A., Toyama, R., Dawid, I.B., 2001. A gene expression screen in zebrafish embryogenesis. Genome Res. 11, 1979-1987.

Lavens, P., Sorgeloos, P., 1996. Manual on the production and use of live food for aquaculture. Food and Agriculture Organization of the United Nations.

Linhart, O., Kudo, S., Billard, R., Slechta, V., Mikodina, E.V., 1995. Morphology, composition and fertilization of carp eggs: a review. Aquaculture. 129, 75-93.

Lucas, A., 1998. Programming by early nutrition: an experimental approach. J Nutr 128, 401-406.

Maddison, L.A., Chen, W., 2012. Nutrient Excess Stimulates β-Cell Neogenesis in Zebrafish. Diabetes.

Mathavan, S., Lee, S.G.P., Mak, A., Miller, L.D., Murthy, K.R.K., Govindarajan, K.R., Tong, Y., Wu, Y.L., Lam, S.H., Yang, H., Ruan, Y., Korzh, V., Gong, Z., Liu, E.T., Lufkin, T., 2005. Transcriptome Analysis of Zebrafish Embryogenesis Using Microarrays. PLoS Genet. 1, e29.

McCurley, A.T., Callard, G.V., 2008. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. BMC Mol Biol. 9, 102.

Metz, S., Holmes, D., Robertson, R.P., Leitner, W., Draznin, B., 1991. Gene expression of type I phospholipase A2 in pancreatic beta cells: Regulation of mRNA levels by starvation or glucose excess. FEBS Letters. 295, 110-112.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., Piferrer, F., 2011. DNA Methylation of the Gonadal Aromatase (cyp19a) Promoter Is Involved in Temperature-Dependent Sex Ratio Shifts in the European Sea Bass. PLoS Genet. 7, e1002447.

Olsen, A.S., Sarras, M.P., Jr., Leontovich, A., Intine, R.V., 2012. Heritable transmission of diabetic metabolic memory in zebrafish correlates with DNA hypomethylation and aberrant gene expression. Diabetes. 61, 485-491.

Panserat, S., Medale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R., Kaushik, S., 2000. Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. Am J Physiol Regul Integr Comp Physiol. 278, 1164-1170.

Panserat, S., Capilla, E., Gutierrez, J., Frappart, P.O., Vachot, C., Plagnes-Juan, E., Aguirre, P., Breque, J., Kaushik, S., 2001a. Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (Oncorhynchus mykiss) by a single meal with glucose. Comp Biochem Physiol B Biochem Mol Biol. 128, 275-283.

Panserat, S., Plagnes-Juan, E., Kaushik, S., 2001b. Nutritional regulation and tissue specificity of gene expression for proteins involved in hepatic glucose metabolism in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol. 204, 2351-2360.

Panserat, S., Plagnes-Juan, E., Kaushik, S., 2002. Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). Biochim Biophys Acta. 1579, 35-42.

Panserat, S., Kaushik, S., 2010. Regulation of gene expression by nutritional factors in fish. Aquac Res. 41, 751-762.

Patel, M.S., Srinivasan, M., 2002. Metabolic programming: causes and consequences. J Biol Chem. 277, 1629-1632.

Patel, M.S., Srinivasan, M., Laychock, S.G., 2009. Metabolic programming: Role of nutrition in the immediate postnatal life. J Inherit Metab Dis. 32, 218-228.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Polakof, S., Medale, F., Larroquet, L., Vachot, C., Corraze, G., Panserat, S., 2011. Regulation of de novo hepatic lipogenesis by insulin infusion in rainbow trout fed a high-carbohydrate diet. J Anim Sci. 89, 3079-3088.

Robles, V., Cabrita, E., Anel, L., Herráez, M.P., 2006. Microinjection of the antifreeze protein type III (AFPIII) in turbot (Scophthalmus maximus) embryos: Toxicity and protein distribution. Aquaculture. 261, 1299-1306.

Seiliez, I., Medale, F., Aguirre, P., Larquier, M., Lanneretonne, L., Alami-Durante, H., Panserat, S., Skiba-Cassy, S., 2013. Postprandial regulation of growth- and metabolism-related factors in zebrafish. Zebrafish. 10, 237-248.

Shimeno, S., Kheyyali, D., Shikata, T., 1995. Metabolic Response to Dietary Carbohydrate to Protein Rations in Carp. Fish Sci. 61, 277-281.

Soanes, K.H., Achenbach, J.C., Burton, I.W., Hui, J.P., Penny, S.L., Karakach, T.K., 2011. Molecular characterization of zebrafish embryogenesis via DNA microarrays and multiplatform time course metabolomics studies. J Proteome Res. 10, 5102-5117.

Soengas, J.L., Polakof, S., Chen, X., Sangiao-Alvarellos, S., Moon, T.W., 2006. Glucokinase and hexokinase expression and activities in rainbow trout tissues: changes with food deprivation and refeeding. Am J Physiol Regul Integr Comp Physiol. 291, R810-821.

Srinivasan, M., Patel, M.S., 2008. Metabolic programming in the immediate postnatal period. Trends Endocrinol Metab. 19, 146-152.

Symonds, M.E., Sebert, S.P., Hyatt, M.A., Budge, H., 2009. Nutritional programming of the metabolic syndrome. Nat Rev Endocrinol. 5, 604-610.

Tao, T., Peng, J., 2009. Liver development in zebrafish (Danio rerio). J Genet Genomics. 36, 325-334.

Tehrani, Z., Lin, S., 2011. Endocrine pancreas development in zebrafish. Cell Cycle. 10, 3466-3472.

Towle, H.C., Kaytor, E.N., Shih, H.M., 1997. Regulation of the expression of lipogenic enzyme genes by carbohydrate. Annu Rev Nutr. 17, 405-433.

Ulloa, P.E., Iturra, P., Neira, R., Araneda, C., 2011. Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes. Rev Fish Biol Fisheries 21, 646-666.

Vagner, M., Zambonino-Infante, J.L., Robin, J.H., Person, J., 2007. Is it possible to influence European sea bass (Dicentrarchus labrax) juvenile metabolism by a nutritional conditioning during larval stage? Aquaculture. 267, 165-174.

Wallace, K.N., Pack, M., 2003. Unique and conserved aspects of gut development in zebrafish. Dev Biol. 255, 12-29.

Waterland, R.A., Jirtle, R.L., 2004. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 20, 63-68.

Wellen, K.E., Thompson, C.B., 2010. Cellular metabolic stress: considering how cells respond to nutrient excess. Mol Cell. 40, 323-332.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 4th ed. University of Oregon Press, Eugene.

Wilson, R.P., 1994. Utilization of dietary carbohydrate by fish. Aquaculture. 124, 67-80.

Xu, Q., 1999. Microinjection into zebrafish embryos. in: Guille, M. (Ed.), Molecular Methods in Developmental Biology. Humana Press, pp. 125-132.

The importance of critical windows for nutritional programming

Chapter III

Glucose metabolism and gene expression in juvenile zebrafish (*Danio rerio*) challenged with a high carbohydrate diet: effects of an acute glucose stimulus during late embryonic life

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Glucose metabolism and gene expression in juvenile zebrafish (*Danio rerio*) challenged with a high carbohydrate diet: effects of an acute glucose stimulus during late embryonic life

Abstract

Knowledge on the role of early nutritional stimuli as triggers of metabolic pathways in fish is extremely scarce. A study was undertaken to assess long-term effects of glucose injection in yolk (early stimulus) on the carbohydrate metabolism and gene regulation of zebrafish juveniles challenged with high-carbohydrate low-protein (HC) diet. Eggs were microinjected at 1 dpf (day post-fertilization) with either glucose (2 M) or saline solutions. Up to 25 dpf, fish were fed a low- carbohydrate high-protein (LC) control diet, which was followed by a challenge with the HC-diet. Survival and growth of 35 dpf juveniles were not affected by injection or HC diet. Glucose stimulus induced some long-term metabolic changes in the juveniles, as shown by the altered expression of genes involved in glucose metabolism. On glycolysis, the expression levels of hexokinase 1 (*HK1*) and phosphofructokinase-6 (*6PFK*) were up-regulated in visceral and muscle tissues, respectively, of juveniles exposed to glucose, indicating a possible improvement of glucose oxidation. On gluconeogenesis, PEPCK inhibition in fish injected with glucose suggested a lower hepatic glucose production. Unexpectedly, FBP expression was induced and 6PFK reduced by glucose stimulus, leaving the possibility of a specific regulation of the FBP-6PFK metabolic cycle. Glucose metabolism in juveniles was estimated using a [^{14}C]glucose tracer; fish previously exposed to stimulus showed lower retention of [^{14}C]glucose in visceral tissue (but not in muscle) and accordingly a higher glucose catabolism, in comparison to the saline group. Globally, our data suggest that glucose stimulus at embryo stage has the potential to alter particular steps of the glucose metabolism in zebrafish juveniles.

Keywords: Carbohydrates, Nutritional programming, Nutrigenomics, Zebrafish

3.1. Introduction

The ability of fish to use dietary carbohydrates is widely variable among and within species and in close association to their feeding habits. Fish species showing high dietary protein requirements and therefore usually presented as “carnivorous” fish are generally considered to be poor utilizers of dietary carbohydrates (Enes et al., 2009; Hemre et al., 2002; Moon, 2001; Polakof et al., 2012). In spite of the significant research efforts devoted to this theme, the physiological basis for such apparent glucose intolerance in fish is not fully understood. Despite having the whole enzymatic machinery required for carbohydrate utilization (Enes et al., 2009) fish react with a prolonged postprandial hyperglycaemia after the ingestion of carbohydrate-rich diets (Moon, 2001). Various hypotheses have been proposed to explain this poor utilisation of carbohydrates by fish. Insulin deficiency, lack of insulin-dependent glucose transporters and lack of an inducible hepatic glucokinase were proven to be false (Caruso and Sheridan, 2011; Mommsen and Plisetskaya, 1991; Panserat et al., 2000a; Planas et al., 2000). On the other hand, the lower potency of glucose over amino acids as insulin secretagogues; a relatively low number of insulin receptors; the lack of an adequate balance between hepatic glucose uptake (glycolysis) and production (gluconeogenesis); and poor hepatic lipogenesis from glucose were proven to be valid hypotheses (Andoh, 2007; Ekmann et al., 2013; Mommsen and Plisetskaya, 1991; Navarro et al., 1999; Navarro et al., 2002; Panserat et al., 2000b; Polakof et al., 2012). Furthermore, the ubiquitous presence of most key enzymes involved in carbohydrate metabolic pathways among fish species indicate that the poor utilisation may be due to an anomalous hormonal and nutritional regulation caused by evolutionary adaptation (Polakof et al., 2011; Polakof et al., 2012).

The concept of early nutritional programming is being largely studied in mammals to understand how nutritional events during critical periods of development can result in persistent physiological changes in adulthood (Lucas, 1998). In fish nutrition, this raises the possibility of tailoring specific metabolic pathways or functions in juvenile fish, such as the improvement in the use of dietary carbohydrates as energy substrates (Fang et al., 2014 ; Rocha et al., 2014). Glucose, as a simple monosaccharide, is a primary source of energy for cells. Immediately after the egg fertilization, glucose derived from maternal glycogen reserves is the first nutrient to be catabolized for cellular division. However, glycogen stores in fertilized fish eggs are extremely low and rapidly depleted (Hoar and Randall, 1988; Kamler, 2008). Under these circumstances, glucose is not expected to play a pivotal role in nutrient

sensing pathways during embryogenesis. However, recent studies suggest that glucose levels, used as a cofactor, can induce modifications on certain epigenetic mechanisms like histone acetylation and contribute to genomic imprinting, which in some cases can be transgenerational (Badeaux and Shi, 2013; Patel et al., 2009). Combined, these factors support glucose as a suitable nutrient for studying the effectiveness of nutritional programming in fish.

Recently, early zebrafish embryos at 0·2 days post-fertilization (dpf) were successful enriched with glucose by means of direct microinjection into the yolk reserves (Rocha et al., 2014). In this previous study, we reported that glucose conditioning had no marked beneficial effects on the ability of juvenile fish to cope with high dietary glucose levels, at least at a molecular level; yet, a short-term effect (although transient) related to the early stimulus was observed with the down-regulation of several metabolic-related genes. Despite being rather weak effects and based only on molecular data, zebrafish juveniles conditioned by an early glucose stimulus showed some indications of enhanced capacity for glucose storage in muscle, lower glucose production in liver and lower glucose transport in intestinal lumen. However, doubts subsisted if such effects would be more pronounced if we had exerted the glucose stimulus at a later development stage with fully functional metabolic pathways and with a lower supplemental dose of glucose to avoid a potential overload status and consequently cellular damage. In the meanwhile, Fang et al. (2014) showed recently that a high dietary carbohydrate stimuli exerted at the first feeding stages (3-5 dpf) significantly altered the molecular regulation of carbohydrate utilization, production, digestion and transport of adult zebrafish.

In this context, the objective of the present study was to explore the effects of glucose injection (nutritional stimulus) in the egg at the late embryo stage of 1 dpf on gene expression of target metabolic pathways and ^{14}C-glucose metabolism of juvenile zebrafish challenged with a high carbohydrate diet. Additionally, the effect of early glucose stimulus on gene regulation of yolk-sac feeding larvae was also assessed in order to identify possible short-term effects.

3.2. Materials and methods

3.2.1. Microinjection procedure

Fertilized zebrafish eggs were obtained from natural spawning of wild-type breeding fish (Centre of Marine Sciences, Faro, Portugal). Embryos were injected into the yolk with 4·6 nl of either a saline solution (Danieau) or 2 M glucose solution, at late embryo stage of 1 dpf (during pharyngula period, according to Kimmel et al. (1995)). Solutions were prepared according to Rocha et al. (2014). Microinjection was performed using a 0.5 mm diameter glass capillary inserted on a nanoliter injector (World Precision Instruments, Sarasota, USA) following the same procedures as described by Rocha et al. (2014).

3.2.2. Fish rearing and experimental feeds

This experiment was carried out in compliance with the Guidelines of the European Union Council (2010/63/EU) legislation for the use of vertebrate animals (Commission, 2010). After glucose and saline injections (described above), embryos and larvae were raised in triplicate tanks (n=200) at an initial density of 100 larvae per litre, under standardized conditions (28ºC) as described in Westerfield (2000). An additional group of non-injected embryos was reared in simultaneous to monitorize egg quality and embryonic development. From 5 dpf, larvae were fed *Artemia* nauplii, which was gradually replaced by and inert diet from 10 to 15 dpf. After day 15, larvae were fed exclusively on a low-carbohydrate high-protein (LC) diet used as control (Figure 3.1). From 25 to 35 dpf, all fish from both injection treatments were submitted to a dietary challenge with a high-carbohydrate low-protein (HC) diet (Figure 3.1). Throughout the trial, larvae and juvenile zebrafish were fed by hand (4 meals per day) until visual satiation. Both diets were well accepted by fish. Fish mortality was daily monitored and survival rate was determined at the end of the trial.

Formulation of the experimental diets was based on the use of purified ingredients in order to guarantee a high control of nutritional changes among the diets (Table 3.1). The control diet (LC) had a high incorporation level of concentrated protein sources (casein, soy isolate, wheat gluten and fish gelatine), guaranteeing a high crude protein level (70%) and a low level of carbohydrates (6%). In the high carbohydrate diet (HC) or challenge diet, the crude protein level was drastically reduced (25%), whereas carbohydrates level (51%) was increased through the incorporation of corn dextrin, a highly digestible carbohydrate. Both diets were isolipidic (12%) and dully supplemented with selected crystalline indispensable amino acids

and monocalcium phosphate to avoid essential amino acid or phosphorus imbalance. Experimental diets were manufactured by SPAROS Lda. (Olhão, Portugal). Powder ingredients were grinded (below 100 µm) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Powder ingredients and oil sources were then mixed accordingly to the target formulation in a mixer (SAMMIC, BM5E, Spain) and the mixture was humidified with 25% water. Diets were manufactured by temperature controlled-extrusion (pellet size: 2.0 mm) by means of a low shear extruder (Italplast P55, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF, LTE Scientifics, UK) for 3 h at 40°C. Dry feed pellets were then grinded in a coffee mill and sieved manually to retrieve the desired particle size (100 – 200 µm and 200 – 400 µm). Diets were analysed for proximate composition according to the following procedures: dry matter after drying at 105 °C for 24 h; ash content by incineration in a muffle furnace at 500ºC for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428, St. Joseph, MI, USA); fat by dichloromethane extraction (Soxhlet); gross energy in an adiabatic bomb calorimeter (IKA C2000, Germany); total phosphorus according to the ISO/DIS 6491 method using the vanado-molybdate reagent.

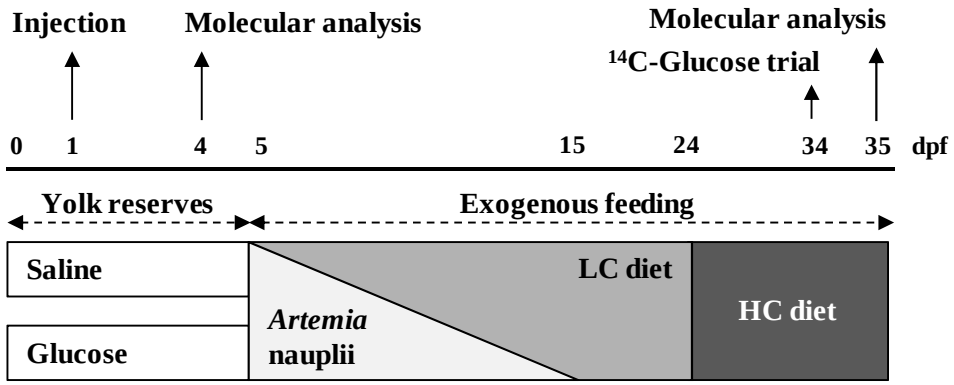

Figure 3.1 - Experimental setup for zebrafish rearing and feeding regime up to 35d post-fertilization (dpf); Embryos were injected into the yolk at 1 dpf either with a saline or 2 M-glucose solution. At the beginning of exogenous feeding, larvae were fed with *Artemia* nauplii which were gradual replaced by a low-carbohydrate high-protein (LC) control diet. Juveniles were subjecetd to a 10d dietary challenge, being fed exclusively on a high-carbohydrate low-protein (HC) diet. Sampling points for metabolic trial and gene expression were marked. Age is given as dpf at 28º C.

Table 3.1 - Formulation and composition of the low-carbohydrate high-protein diet (LC) control and and the high-carbohydrate low-protein (HC) challenge diets

Ingredients (%)	**LC control**	**HC challenge**
Fish gelatine[1]	17.00	5.00
Casein[2]	25.00	5.00
Soy protein isolate[3]	25.00	5.00
Wheat gluten[4]	12.80	5.00
Yellow corn dextrin[5]	–	50.10
Fish oil[6]	11.70	12.00
Vitamin and mineral premix[7]	2.00	2.00
Mono-calcium phosphate[8]	3.00	3.50
Guar gum[9]	2.00	2.00
L-Arginine[9]	–	1.50
L-Histidine[9]	–	0.60
L-Isoleucine[9]	–	0.50
L- Leucine[9]	–	2.30
L-Lysine[9]	1.00	2.80
L-Threonine[9]	–	0.80
L-Tryptophan[9]	–	0.20
DL-Methionine[9]	0.50	1.20
L-Valine[9]	–	0.50
Proximate composition (as fed basis)		
Moisture (%)	6.32 ± 0.12	6.57 ± 0.27
Crude protein (%)	70·12 ± 0.43	25.32 ± 0.31
Crude fat (%)	12.34 ± 0.82	12.23 ± 0.46
Carbohydrates (%)*	5.93 ± 0.12	51.56 ± 0.32
Ash (%)	5.29 ± 0.03	4.32 ± 0.16
Total phosphorus (%)	1.21 ± 0.11	0.98 ± 0.05
Gross energy (kJ/g)	22.11 ± 0.22	19.35 ± 0.17

[1] Pharma Grade bloom 240: 92% CP, LAPI Gelatine SPA, Italy; [2] Edible acid casein 90 mesh: 85% CP, EPI Ingredients, France; [3] SEAH Soy Instant: 87 CP%, Seah International, France; [4] VITEN: 86% CP, 1.3%, Roquette, France; [5] TACKIDEX C070: Roquette, France; [6] Marine oil omega 3: Henry Lamotte Oils GmbH, Germany; [7] PREMIX Lda, Portugal. Vitamins (kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 6.9mg; DL-cholecalciferol, 0.05mg; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings; [8]Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy; [9]Sigma Aldrich Quimica SA, Portugal; *Carbohydrate content calculated as: 100–(moisture+protein+fat+ash).

3.2.3. Biological and analytical sampling

Immediately after injection, samples (n=30) of glucose and saline injected eggs were collected for analysis of glucose levels by fluorescent spectroscopy using a commercial kit (Amplit Glucose Quantitation Kit, AAT Bioquest, USA). Fluorescence readings were performed in triplicate using a Synergy^{TM} 4 Multi-Mode Microplate Reader controlled by Gen5^{TM} software (BioTek Instruments, USA). At the end of the experiment (35 dpf), juveniles (n=20 per treatment) were individually sampled for growth determination based on dry weight and total length parameters. Total length was determined using the AxioVision 4.8.2 (Carl Zeiss Ltd., United Kingdom) program for image analysis and dry weight measurements were obtained from freeze-dried samples using a precision scale. For gene expression analysis, samples of whole larvae (n=20) from each replicate of glucose and saline injected treatments were collected during the endogenous feeding period (4 dpf). At the end of the trial, liver and muscle from individual fish (n=6 per treatment) were sampled 6 h after feeding for the same purpose. All samples were randomly collected, snap-frozen in liquid nitrogen and kept at –80ºC until analysis.

3.2.4. Metabolic trial: tube-feeding method

One day prior to the final sampling (34 dpf), juveniles from each treatment (n= 10) were randomly harvested from the tanks and transferred to the flux laboratory for overnight acclimatization at room temperature (28ºC). Fish were deprived from feed for 16 h prior to the metabolic trail. The *in vivo* method of controlled tube-feeding, as described by Rust et al. (1993) and later modified by Rønnestad et al. (2001), for marine fish was adapted to supply nutrients to freshwater species. This approach was used to assess the effects of both nutritional conditioning (early glucose injection) and increase of dietary carbohydrates on the metabolic handling of glucose by zebrafish juveniles. Following a 16 h fasting period, zebrafish juveniles were allowed to feed for a period of 40 min the HC diet. This feeding period was suitable for the uptake of a full meal, confirmed by observation of the gastrointestinal tract, and within the beginning of zebrafish gastrointestinal transit (Field et al., 2009). Following this single meal, fish were anaesthetised with 33 µM of tricaine methanesulfonate (Sigma-Aldrich, Germany). Subsequently, the radioactive label D-[^{14}C(U)] glucose (9·25 MBq, American Radiolabeled Chemicals Inc., The Netherlands) was added to Ringer salt solution and tube-fed to all fish using a 0·19 mm diameter plastic capillary (Sigma-Aldrich, Germany) inserted on a nanoliter injector (World Precision Instruments,

Sarasota, USA). Three consecutive injections of 4·6 nl were done into the fish gut. This injection volume (13·8 nl) was in the range of that previously used for marine species in late larval stages (> 25 days after hatching): Senegalese sole (Aragão et al., 2004; Morais et al., 2005), gilthead seabream (Pinto et al., 2010), white seabream (Saavedra et al., 2008). After capillary withdrawal, fish were gently rinsed for spillage through two successive wells filled with clean freshwater and transferred into sealed incubation chambers containing 6·5 ml of freshwater. The incubation water was considered to contain all labeled ^{14}C resultant from fish evacuation (evacuated fraction). In addition, an airflow connection was provided between each incubation chamber and a KOH trap (5 ml, 0.5 M), in order to collect all $^{14}CO_2$ released by fish through glucose metabolism (catabolized fraction). After an incubation period of 24 h, juveniles from each injection treatment were sampled individually for muscle and viscera, in order to determine the amount of ^{14}C retained in tissues (retained fraction). Tissue samples were immediately solubilised with Solvable (500 µl, Perkin Elmer, USA) and kept at 50ºC for 24 h. Following larval sampling, the incubation chambers were resealed and 1 ml of HCl (0.1 M) was added in a series of gradual steps, resulting in a progressive decrease of pH that causes the rapid diffusion of any remaining $^{14}CO_2$ from the water into the metabolic trap (catabolized fraction) (Rønnestad et al., 2001). For radioactive counting, disintegrations per minute (DPM) were determined in all samples by adding Ultima Gold XR scintillation cocktail (Perkin Elmer, USA) and counting in a TriCarb 2910TR Low activity liquid scintillation analyser (Perkin Elmer, USA). Metabolic budgets were calculated after subtracting blanks of each fraction (evacuated, catabolized, retained). Results for each fraction were expressed as a percentage of total label tube-fed, i.e. the sum of DPM in all compartments of metabolic chamber and fish.

3.2.5. Gene expression analysis by real time PCR

Analyses of mRNA levels were performed at two distinctive periods and sample types: 4 dpf in whole body larvae, for assessing the short-term effect of early glucose stimulus (injection) and at 35 dpf in visceral and muscle tissues of juveniles, for assessing both effects of early glucose stimulus and dietary challenge. Juvenile fish were sampled 6 h after the last meal, based on previous data identifying this period as relevant for examining the postprandial response of genes in zebrafish (Amaral and Johnston, 2011; Seiliez et al., 2013). Total RNA was extracted from all samples using 1 ml of TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). From the resulting total RNA, 1 µg was reverse transcribed into cDNA using the

SuperScript III RNase H Reverse Transcriptase kit (Invitrogen) using random primers (Promega, Charbonniéres, France). The molecular analysis was focused on the expression of target genes related to glycolysis (GK – glucokinase; HK1 – hexokinase 1; 6PFK – phosphofructokinase-6; PK-L, PK-M – pyruvate kinase, both liver and muscle isoforms), gluconeogenesis (PEPCK – phosphoenolpyruvate carboxykinase, both cytosolic and mitochondrial isoforms; FBP – fructose1,6bisphosphatase; G6Pase – glucose-6-phosphatase), lipogenesis (FAS – fatty acid synthase; G6PDH – glucose-6-phosphate deshydrogenase, MEc – cytosolic malic enzyme) and glycogen metabolism (GS – glycogen synthase; GP – glycogen phosphorylase). These primers were referred as good molecular markers for nutritional studies in zebrafish (Rocha et al., 2014; Seiliez et al., 2013). Gene expression levels were determined by quantitative real-time RT-PCR performed by means of the iCycler iQ (BIO-RAD, Hercules, CA, USA). Analyses were performed on 5µl of the diluted cDNA using the iQ SYBR® Green supermix in a total PCR reaction volume of 15 µl containing, 200 nM of each primer. Thermal cycling was initiated with the incubation at 95ºC for 90 s for Taq DNA polymerase activation, then 35 steps of PCR were performed, each one consisting of heating at 95ºC for 20 s for denaturing and at 55ºC or 62ºC for 30 s for annealing and extension, depending on the primers. After the final cycle of the PCR, melting curves were systematically monitored (55ºC temperature gradient at 0·5ºC/s from 55 to 94ºC). Each q-PCR run included duplicates of samples (reverse transcription) and negative controls (samples without reverse transcriptase or mRNA or cDNA). Target gene expression analysis of whole larval bodies and visceral tissue was performed using elongation factor-1 (EF1α) as reference gene while 18S rRNA gene was used as reference for muscle samples, once EF1α was not being stably expressed in this tissue. Both EF1α and 18S were employed as non-regulated reference genes and their gene expression values did not significantly changed over the respective time frame or tissue type (McCurley and Callard, 2008) (data not shown). Relative quantification of gene expression was performed using the mathematical model described by Pfaffl (2001).

3.2.6. Statistical analysis

Data are presented as mean with their standard errors of the mean. Criteria expressed as a percentage were arcsine transformed previously to the statistical analysis. The effects of glucose injection on the several analysed parameters in larvae and juveniles fish were tested using SPSS® statistics software 16·0 for Windows (SPSS Inc.) by means of an unpaired two-

tailed Student's t-test. Differences were considerate significant at $P<0{\cdot}05$. For relative quantification of gene expression in juvenile fish, the control group was set as the saline-injected HC diet-fed group.

3.3. Results

3.3.1. Glucose supplementation, growth performance and survival

In comparison to eggs of the saline treatment, those microinjected with a 2 M glucose solution (9·2 nmol/ egg) showed a 6-fold increase of glucose levels. No permanent damage on egg chorion or signs of leakage through the puncture hole was recorded after the injection. Zebrafish growth and survival was not affected by early glucose stimulus neither by the dietary challenge with high carbohydrates ($P>0{\cdot}05$). At the end of the experiment, juveniles showed similar mean values for dry weight (4·7 – 4·8 mg) and total length (10·0 - 11·1 mm) (Table 3.2). Survival varied between 61% and 68% and values were within the expected range for zebrafish fed with live feed and purified diets. Carvalho et al. (2006) showed that 27-day-old zebrafish fed from mouth opening with a semi-purified diet reached a good growth (7·0 (SD 0·2) mm) and survival rate (55%).

Table 3.2 – Survival (n=3) and growth (n=20) of zebrafish juveniles initially injected with a saline or glucose solution and challenged with a high-carbohydrate low-protein diet*

	Saline		**Glucose**	
	Mean	SEM	Mean	SEM
Survival (%)	68·0	6·1	61·1	5·8
Total length (mm/ fish)	11·1	0·5	10·0	0·5
TL variation coefficient (%)	21·7		21·4	
Dry weight (mg/ fish)	4.8	0·7	4·7	0·6

Values are means and their standard errors; TL, total length; * Student's *t*-test

3.3.2. Effect of early glucose stimulus on [^{14}C]glucose metabolism in fish fed with carbohydrates

Survival rate of zebrafish after 24 h of incubation in the metabolic chambers was 89% and 100% for the glucose and saline injected treatments (respectively). Results showed that in juvenile fish, glucose evacuation and absorption was not significantly affected by the early glucose conditioning (P>0·05) (Figure 3.2-A). Zebrafish presented high levels of glucose absorption (over 87%) under the same intake amount of carbohydrates. Juveniles subjected to the early glucose stimulus showed significantly lower (P<0·05) retention of glucose in visceral tissue (but not in muscle tissue) and consequently a higher glucose catabolism (P=0·072) in comparison to the saline injected group. The retention efficiency in visceral tissue was significantly reduced on juveniles injected with glucose compared to the saline injected group (6·0% and 11·5%, respectively) (Figure 3.2-B). Although no significant differences were found, glucose catabolism shows a trend to increase on the glucose injected fish rather than in the saline injected fish (75·8 % and 68%, respectively) (Figure 3.2-B).

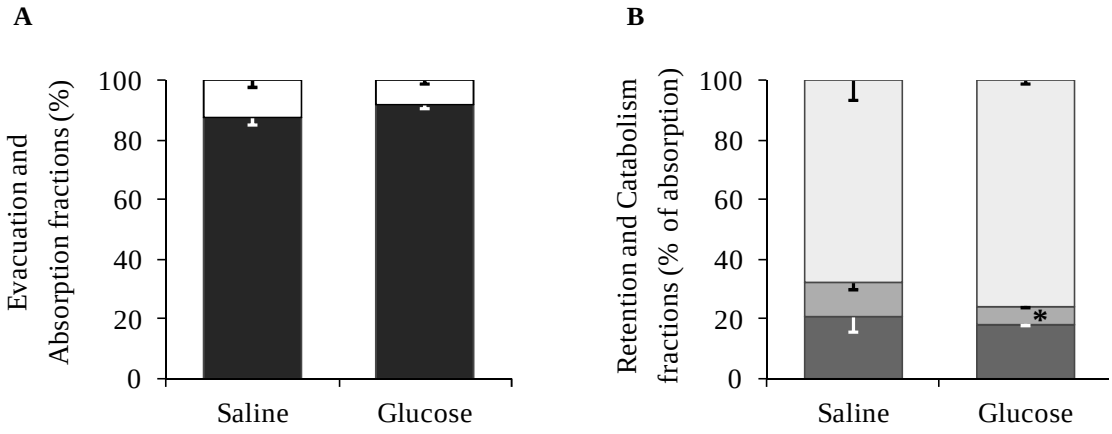

Figure 3.2 – Study of glucose metabolism in zebrafish juveniles. (A) Percentage of the absorbed (black bar) and evacuated (white bar) [^{14}C]glucose of tube-fed zebrafish juveniles initially subjected to either a saline or glucose injection (stimulus) and fed a high-carbohydrate low-protein (HC) diet. (B) Percentage of [^{14}C]glucose retained in muscle tissue (dark-grey bar) and visceral tissue (medium-grey bar), and percentage of [^{14}C]glucose catabolized (light-grey bar) in tube-fed zebrafish juveniles initially subjected to either a saline or glucose injection (stimulus) and fed a HC diet. Retention and catabolism fractions are associated with the absorbed label in the fish. Values (absorption, evacuation, retention and catabolism) are mean with their standard errors represented by vertical bars (n=10). * mean value was significantly different from that of saline-injected group (P < 0·05, Student's t-test).

3.3.3. Effect of early glucose stimulus on metabolic gene expression in yolk-sac larvae

The RNA levels of enzymes involved in intermediary metabolism of yolk-sac larvae (4 dpf) were compared between saline and glucose injected groups (Table 3.3). For the majority of analised genes, mRNA levels remained unaltered between the saline injection and the 2 M glucose injection treatments. Only PK gene (muscle isoform) was significantly up-regulated in glucose injected larvae ($P<0{\cdot}05$; Table 3.3) while MEc gene showed a slight but not significant decrease of its expression ($P=0{\cdot}056$). These results denote that glucose stimulus had no marked effects on gene regulation of 4 dpf larvae.

3.3.4. Effect of early glucose stimulus on metabolic gene expression in fish fed with carbohydrates

The same set of target-genes was analised in two tissues types of juvenile fish (35 dpf): viscera and muscle. The long-term effects on gene expression of fish injected with glucose at early embryo stage and later submitted to a feeding challenge with high carbohydrates are shown in Table 3.3.

In viscera, the expression of GK was unchanged regardless of the injection treatment (glucose stimulus). In contrast, HK1 gene was significantly up-regulated ($P=0{\cdot}04$) while 6PFK was down-regulated ($P=0{\cdot}009$) on the glucose injected fish, in comparison to the control group (saline injected). Also PK-L transcript levels were not affected by the early glucose stimulus at a long-term. On the gluconeogenic pathway, PEPCKc and PEPCKm expression levels were inhibited by glucose injection ($P=0{\cdot}000$; $P=0{\cdot}002$, respectively) while G6Pase gene was not affected at long-term. Surprisingly, the gene expression level of FBP was reverse-regulated as expected for this pathway: juveniles that were exposed to glucose stimulus showed higher levels of expression than the saline group ($P=0{\cdot}034$). For genes involved in lipogenesis and energy production FAS, G6PDH and MEc no long-term effects were found related to the injection of glucose (Table 3.3). Similar observations were found for GS and GP genes responsible for glycogen metabolism (Table 3.3).

In muscle tissue of juvenile fish, only 6PFK gene was found to be significantly up-regulated ($P=0{\cdot}038$) by early glucose injection (Table 3.3). Therefore, glucose injection increased the expression of 6PFK in juvenile fish whereas genes encoding for glycolysis (HK1 and PK-M) and glycogen metabolism (GS and GP) were not affected, at long-term, by early glucose conditioning (Table 3.3).

Table 3.3 – Relative expression of metabolic genes involved in glycolysis (A), gluconeogenesis (B), lipogenesis (C) and glycogen metabolism (D) in whole larval body (4 dpf) and visceral and muscle tissues from zebrafish juveniles (35 dpf), initially injected with a saline (control) or glucose (stimulus) solution and challenged with a high-carbohydrate low-protein diet*

		Saline injection		**Glucose injection**		
Pathways	Gene	Mean	SEM	Mean	SEM	*P*
Whole body larvae (4 dpf)						
A	GK	1·1	0·23	1·5	0·32	0·351
A	HK1	1·0	0·09	0·9	0·04	0·280
A	6PFK	1·0	0·16	0·7	0·04	0·094
A	PK-L	1·8	0·14	1·8	0·12	0·955
A	PK-M	1·0	0·05	1·2	0·06	0·038
B	PEPCKc	1·0	0·09	0·9	0·07	0·290
B	PEPCKm	1·0	0·10	0·8	0·02	0·159
B	FBP	1·0	0·03	1·0	0·08	0·979
B	G6Pase	1·0	0·13	0·8	0·07	0·138
C	FAS	1·1	0·19	0·9	0·07	0·333
C	G6PDH	1·0	0·14	0·9	0·04	0·250
C	MEc	1·0	0·03	0·8	0·07	0·056
D	GS	1·0	0·04	1·1	0·06	0·111
D	GP	1·0	0·03	1·0	0·07	0·936
Viscera tissue (35 dpf)						
A	GK	1·1	0·18	0·9	0·24	0·657
A	HK1	1·0	0·16	2·5	0·58	0·040
A	6PFK	1·0	0·08	0·7	0·06	0·009
A	PK-L	1·0	0·10	0·8	0·14	0·219
B	PEPCKc	1·0	0·05	0·5	0·06	0·000
B	PEPCKm	1·0	0·06	0·6	0·07	0·002
B	FBP	1·1	0·24	39·5	12·28	0·034
B	G6Pase	1·0	0·11	0·8	0·06	0·171
C	FAS	1·3	0·36	1·9	0·37	0·292
C	G6PDH	1·1	0·11	1·0	0·15	0·830
C	MEc	1·1	0·25	0·9	0·11	0·374
D	GS	1·1	0·24	1·6	0·38	0·291
D	GP	1·1	0·15	1·3	0·20	0·424
Muscle tissue (35 dpf)						
A	HK1	1·1	0·19	0·9	0·15	0·537
A	6PFK	1·0	0·15	1·6	0·18	0·048
A	PK-M	1·0	0·10	0·7	0·06	0·055
B	GS	1·0	0·08	0·9	0·08	0·315
B	GP	1·8	1·02	0·4	0·09	0·252

*Expression values of larvae and visceral tissue were normalized with α-elongation factor 1 transcripts (EF1α) while that of muscle tissue was normalized with 18S transcripts. Relative fold differences between the treatments were analised by Student's *t*-test ($P < 0·05$). Age is given as dpf at 28º C. Values are means with their standard errors (n=6). For the description of gene symbols, refer to the "Gene expression analysis" section.

3.4. Discussion

Fish nutritionists struggle to establish balanced formulations where large amounts of plant ingredients could be included without compromising the nutritional requirements and a good acceptance of the diet by the fish (Gatlin et al., 2007). The inclusion of plant ingredients, being it dietary protein or starch sources enhances the overall intake of carbohydrates. However, the ability of fish to use dietary carbohydrates as an energy yielding substrate is variable among species with different feeding habits. New trends in the field of fish nutrition begin to emerge, such as the concept of early nutritional programming, as a promising strategy to enhance the use of alternative feedstuffs (Geurden et al., 2007; Vagner et al., 2009). We believe that such approach has the potential to improve specific metabolic pathways, through actions during embryonic development that could permanently alter the capacity of adult fish to cope with dietary sources rich in carbohydrates. However, studies in mammals have shown that the period in which the programming stimulus occurs is usually confined to early stages (pre or post-natal) which influence the “imprinting” effect as well as the long lasting outcomes into adulthood (Hanley et al., 2010; Srinivasan and Patel, 2008). The goal of the present study was to gain further knowledge on the effects of a hyper-glucidic stimulus during the final stage of fish embryogenesis on growth, nutrients metabolism and gene expression regulation, of zebrafish juveniles submitted to a drastic increase in the ratio of dietary carbohydrates/ proteins.

3.4.1. Effects of early glucose stimulus on growth and survival of zebrafish

In our previous study, the glucose injection used as a trigger for nutritional programming in zebrafish eggs (0·2 dpf) was found to suppress in the embryos (4 dpf) the transcription level of several genes involved in glycolysis, glucose transport and lipogenesis (Rocha et al., 2014). The inhibition of these metabolic pathways was somehow unexpected since the reverse (stimulation) was observed in several fish species and at later life stages, after the intake of high dietary carbohydrates(Polakof et al., 2012). We therefore considered the possibility that glucose overload at this incipient developmental stage induced cellular damages or compromised key metabolic regulators. Here we explore a new “metabolic window” for glucose delivery (stimulus) at a later period of embryonic development (1 dpf). This period was selected based on two important features: the level of endogenous glucose in the yolk and the stage of embryo development. Zebrafish dynamically regulates glucose during

embryogenesis reaching the highest endogenous levels at 1 dpf, prior to hatching (Jurczyk et al., 2011; Rocha et al., 2014; Soanes et al., 2011). We hypothesized that supplementation of high doses of glucose (9·2 nmol/ egg) while endogenous glucose levels are elevated would contribute to a better adaptation of embryos for hyperglycaemia conditions. A lower dose may mask the stimulus effect by not altering enough the ratio of carbohydrate over the other macro-nutrients, taking in account the overall low glucose level in cyprinid eggs (Lahnsteiner et al., 2001). The glucose injections (9·2 nmol/ egg) increased by 6-fold the glucose levels relative to those in 1 dpf embryos of the saline injected group (control). In absolute levels, the amount of glucose injected into the egg was similar to basal glycaemia levels found in adult zebrafish fasted for 24 h (about 2·5 mM; 40-45 mg/L) (Eames et al., 2010). Thus, the achieved glucose supplementation can be considered within the physiological range for this species. At 1 dpf stage, major processes of organogenesis are ongoing in several systems, like the emergence of hepatic and pancreatic buds (future key metabolic organs) (Tao and Peng, 2009; Tehrani and Lin, 2011), the functioning of heart and circulatory system and the appearance of muscle spontaneous contractions (Kimmel et al., 1995; Saint-Amant and Drapeau, 1998). In addition, the transcriptional regulation of the insulin gene and the gluconeogenic genes fructose-bisphosphate aldolase and phosphoenolpyruvate carboxykinase is already occurring at this early stage (Papasani et al., 2006; Soanes et al., 2011). Therefore, the stage of 1 dpf can be considered of high genetic plasticity, once important metabolic pathways are newly-established or in process of becoming active, suggesting that possible epigenetic alterations can occur during this period due to early nutritional events (Lillycrop and Burdge, 2012).

To avoid the influence of sexual dimorphism on hepatic gene transcription already reported in zebrafish (Robison et al., 2008) our trial was conducted up to 35 dpf (5 weeks) so that fish could remain immature. Evidences on the proteomic field had demonstrated that zebrafish can mature before reaching 3 months (90 dpf), in few cases from 30 dpf onwards (Gomez-Requeni et al., 2010), suggesting that fish length (growth) can have a stronger effect on sexual maturation rather than age (Spence et al., 2008). We found that glucose supplementation by microinjection did not impair embryonic development and that the stimulus along with a drastic increase on carbohydrate intake had no detrimental effects on growth and survival of juvenile fish. The lack of negative effects on physiological parameters is in conformity with other nutritional programming studies performed in fish (Fang et al., 2014 ; Geurden et al., 2007; Rocha et al., 2014).

3.4.2. Immediate and persistent effects of early glucose stimulus on gene expression and metabolic utilization of glucose in juvenile fish

In mammals pre-natal exposure to high glucose can permanently alter adult metabolism and trigger diet-related diseases such as diabetes, metabolic syndrome and obesity (Burdge and Lillycrop, 2010). Despite the lack of full knowledge on the mechanisms involved in metabolic regulation by nutritional factors, there are strong evidences that the genome can be "imprinted" to store the memory of the early nutritional event (Lucas, 1998). But caution should be taken in extrapolate the programming concept from mammals to fish. Mammals and fish exhibit a very distinct embryonic development (in uterus vs. ex uterus) thus, stimuli delivery during a sensitive phase becomes more restricted to control and manipulate in fish, mostly because fish embryos operate as energetically closed systems during yolk reserves consumption (Kamler, 2008). Furthermore, fish have a poor control over glucose homeostasis and exhibit slow metabolic rates for glucose utilization compared to mammals (Hemre et al., 2002). Therefore, the results from the present study addressing metabolic programming by the early glucose stimulus will be cautiously discussed in comparison to mammals, whenever possible.

3.4.2.1. Short-term effect of early glucose stimulus on metabolic gene expression

The short-term effect linked to glucose injection (stimulus) was assessed on gene expression of free-swimming 4 dpf larvae that still rely exclusively on endogenous yolk reserves for nutrient supply. From the metabolic genes analised, only PK expression (muscle isoform) was up-regulated in the glucose-injected group. In mammals, the expression of PK is regulated by both dietary carbohydrates and hormones (insulin, glucagon) at a pre- and post-translational level (Yamada and Noguchi, 1999). Such dynamic regulation is thought to occur in adult zebrafish according to different feeding conditions: refeeding a commercial diet poorly regulates the postprandial expression of PK while a high carbohydrate meal induces its expression (Seiliez et al., 2013), being this last in line with the higher PK mRNA levels after the delivery of glucose stimulus at 4 dpf. Overall, the early glucose stimulus had a poor short-term effect on the transcripts levels of zebrafish larvea, which may be due to the 3d gap between glucose injection (at 1 dpf) and sampling (at 4 dpf). This however does not exclude the possibility of a long-term effect of the glucose injection at a later life stage, when the fish are confronted again to a challenging nutritional condition. Indeed, sea bass larvae fed different dietary HUFA levels showed no change in the expression levels of several lipogenic

enzymes during the first days of stimulus, whereas long-term molecular changes were found in juvenile fish fed a HUFA-deficient diet (Vagner et al., 2009). In terms of short-term effects, we obtained less responsiveness of metabolic genes towards glucose stimulus delivered at 1 dpf in comparison to our previous study, where glucose was injected at 0·2 dpf (Rocha et al., 2014). Although the glucose stimulus was the same in both studies (9·2nmol/ egg) the shift of stimulus delivery towards a "window" of high embryonic development (1 dpf) did not inhibit gene expression which allowed excluding the hypothesis of cell damage due to glucose overload.

3.4.2.2. Long-term effect of early glucose stimulus on metabolic gene expression

The long-term effect was evaluated in juvenile fish (35 dpf) after being challenged for ten days with the high carbohydrate/ low protein diet (HC), with the idea that the early glucose injection may generate a “metabolic memory” for improving the future use of carbohydrates. The present study confirms the potential of a single glucose injection (at 1 dpf) to induce persistent molecular changes, as shown by the enhanced expression of genes involved in the first step of glycolysis and gluconeogenesis pathways in the visceral tissue of juveniles conditioned by the glucose stimulus. The increase of HK1 and simultaneous decrease of PEPCK genes expression suggests a higher capacity for glucose phosphorylation as well as a lower glucose production, and thus, the possibility of glucose stimulus to “program” these two major pathways towards a more efficient control of glucose homeostasis when subjected again to hyperglycemic conditions. Although not fully understood, the persistent hyperglycaemia observed in several fish species after high carbohydrates intakes has been ascribed to an atypical regulation of hepatic gluconeogenesis (Panserat, 2009; Panserat and Kaushik, 2010). More specifically, in contrast to mammals, fish transcriptional regulation of gluconeogenic genes does not seem to be down-regulated by high dietary carbohydrates intakes (Panserat et al., 2000b; Panserat et al., 2001c; Panserat and Kaushik, 2010; Pilkis and Granner, 1992). As such, the possibility to programme and decrease this pathway in conditions of hyperglycaemia (through an early glucose stimulus) is encouraging. Here we found that both mitochondrial and cytosolic isoforms of PEPCK gene were affected by the stimulus at the late juvenile stage. A similar down-regulation of PEPCKc was also reported in zebrafish juveniles from our previous study following glucose injections at 0·2 dpf (Rocha et al., 2014), which reinforces our hypothesis of early nutritional programming. Also the expression of glycolytic HK1 gene, known to be poorly regulated by dietary carbohydrates in

fish (Gonzalez-Alvarez et al., 2009; Soengas et al., 2006) but found here to be enhanced by the early stimulus, appears as a relevant indication for the occurrence of genomic imprinting. Further, the beneficial effects of glucose injection at 1 dpf upon HK1 regulation contrast with our data obtained after glucose injection at 0·2 dpf, where HK1 was down-regulated (Rocha et al., 2014). The analysis of HK1 enzyme activity in viscera is however needed to confirm the physiological relevance. A recent study on metabolic programming of adult zebrafish, but exerting the nutritional stimulus at the larval first-feeding stage, showed the same molecular pattern of glycolysis stimulation and gluconeogenesis inhibition following early high carbohydrate intakes (Fang et al., 2014). In contrast, expression of the GK gene which is highly responsive in fish to rises in dietary carbohydrate intake (Gonzalez-Alvarez et al., 2009; Panserat et al., 2000a; Panserat et al., 2001a; Panserat et al., 2001b; Seiliez et al., 2013) was not persistently affected by the glucose stimulus, in line with our previous data (Rocha et al., 2014). The lack of programming effects for the GK gene could be related to the lower sensitivity of GK enzyme to punctual hyper-glucidic stimuli delivered during early stages of fish development, as observed in zebrafish and rainbow trout (Fang et al., 2014 ; Geurden et al., 2007)

On the downstream reactions of both pathways, the long-term effect of glucose stimulus on the expression of 6PFK (key glycolytic enzyme) and FBP (the opposing gluconeogenic enzyme) genes was unexpected. Results in viscera demonstrated that both genes were regulated in the reverse way of what was anticipated: the glycolytic flux was reduced (6PFK down-regulated) whereas gluconeogenesis was promoted (FBP up-regulated). Given the similarities between fish and mammals on this enzyme-substrate cycle, the programming effect of glucose stimulus upon these genes could have been masked by other factors such as hormonal or allosteric control (Enes et al., 2009; Pilkis and Granner, 1992). However, 6PFK gene was regulated differently according to tissue type: being down-regulated in viscera but up-regulated in the muscle by the glucose injection. In addition, it was the only gene to be positively altered in muscle tissue, as a long-term consequence of the glucose stimulus. So far, these results present the first indication that 6PFK – FBP loop can be target of nutritional programming by early glucose stimulus, but the reason why the "memory" stored at the genome after the stimulus resulted in such unexpected programming of 6PFK – FBP remains unclear. Finally, the last step of both metabolic pathways regulated by PK (glycolysis) and G6Pase (gluconeogenesis) enzymes was not affected at a molecular level by the early glucose injection, suggesting that the stimulus was not suitable for a permanent imprinting of these

genes. For PK gene, the short-term induction recorded at 4 dpf might indicate that some short-term effects related to the stimulus can be reversible at later stages of development. Likewise, previous studies showed no long-term effects of early glucidic stimuli on G6Pase and PK mRNA levels in juvenile rainbow trout and zebrafish (Fang et al., 2014 ; Geurden et al., 2007; Rocha et al., 2014). However, it has been shown that PK gene expression can be promoted by the increase of dietary carbohydrates, as in mammals (Kamalam et al., 2012; Seiliez et al., 2013; Yamada and Noguchi, 1999), contrary to G6Pase which, depending on the fish species, appears to be poorly or even not regulated (Caseras et al., 2002; Panserat et al., 2000b; Panserat et al., 2001a; Panserat et al., 2002). Therefore, the regulation of a certain metabolic gene in response to higher carbohydrate intake cannot be used as indicator of possible long-term effects related to early glucidic stimulus. The different susceptibility of each gene to epigenetic modifications as well as all the different epigenetic mechanisms that can be involved after an early nutritional event are important factors to consider for possible programming effects (Canani et al., 2011; Lillycrop and Burdge, 2012).

Concerning the genes related to lipogenesis and glycogen metabolism, the early glucose stimulus had no long-term effects as all the analised genes had the same level of expression between saline and glucose injected fish. This result with respect to lipogenesis is in line with our earlier data on zebrafish conditioned to glucose at 0·2 dpf (Rocha et al., 2014), but disagrees with the hypothesis of increased lipogenesis as a consequence of the early glucose stimulus as anticipated from feeding studies with fish using high dietary carbohydrates (Kamalam et al., 2012; Shimeno et al., 1995). Studies on the time course of adaptation of lipogenic enzymes on coho salmon revealed that at least 2 – 3 weeks are required to cause changes in the activities of these enzymes in response to dietary changes (Lin et al., 1977). For lipogenesis, our sampling after 10 days of HC dietary challenge may therefore not represent metabolically steady-state conditions fully under the influence of the nutritional history of the glucose stimulus. On the other hand, since the stimulus did not improve glycogenesis (glucose storage in muscle) as occurred previously in zebrafish (Rocha et al., 2014), one could consider that glycogen synthesis may have been affected by the reduced amino acids levels in the HC diet. Knowing that amino acids are potent insulin secretagogues in mammals (Armstrong et al., 2001) as in fish (Andoh, 2007; Navarro et al., 2002) glycogen metabolism could be regulated in a way similar to mammalian. This way, the drastic reduction of protein level towards carbohydrate incorporation in the HC challenge diet and

the subsequent lowering of amino acids may have limited the full potential of glucose stimulus to programming the glycogen metabolism of juvenile zebrafish.

3.4.2.3. Long-term effect of early glucose stimulus on glucose use

The metabolic fate of the forced-fed [^{14}C]glucose tracer revealed that juvenile zebrafish can achieve high absorption levels (over 87 %) of dietary glucose for subsequent retention in tissues or catabolism, regardless the early nutritional history. Juveniles that were exposed to glucose stimulus had significantly lower [^{14}C]glucose retention in visceral tissue and accordingly higher catabolism, in comparison to the saline injected group, suggesting an enhancement of glucose oxidation and even a possible decrease in glycogen storage. However, given that no further analysis on glycogen or lipids content in tissues was performed, our statements concerning ^{14}C-glucose retention in tissues are limited. Nevertheless, these results agree with those observed at a molecular level, as fish injected with glucose showed an up-regulation of HK1 (viscera) and 6PFK (muscle) genes involved in glucose oxidation. Clearly, glucose is an important substrate for oxidation in zebrafish, however its metabolic utilization can be enhanced through means of early programming, as demonstrated here. Such an approach raises the possibility of even greater results upon carnivorous species with less capacity to use dietary carbohydrates as an energy source.

3.5. Conclusion

We were able to demonstrate that late embryo stage is a period of high genetic plasticity and better suitable for nutritional stimulus delivery in zebrafish, when compared to incipient stages of 0·2 dpf. Our data suggest that, at least at a molecular level, the two major pathways for glucose metabolism were permanently modified by the early glucose stimulus at specific key metabolic steps. Although few genes were modified by the early stimulus, juvenile fish fed high carbohydrates showed an improved capacity for glucose phosphorylation and a lower glucose production in viscera. The metabolic fate of dietary carbohydrates showed that the early glucose injection lowered the retention of [^{14}C]glucose in visceral tissue, thereby promoting a higher catabolism by oxidative processes. Our study contributes to the generation of new knowledge on nutritional programming on fish following glucose injection during embryogenesis.

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3.7. References

Amaral, I.P., Johnston, I.A., 2011. Insulin-like growth factor (IGF) signalling and genome-wide transcriptional regulation in fast muscle of zebrafish following a single-satiating meal. J Exp Biol. 214, 2125-2139.

Andoh, T., 2007. Amino acids are more important insulinotropins than glucose in a teleost fish, barfin flounder (Verasper moseri). Gen Comp Endocrinol. 151, 308-317.

Aragão, C., Conceição, L.E.C., Martins, D., Rønnestad, I., Gomes, E., amp, x, dio, Dinis, M.T., 2004. A balanced dietary amino acid profile improves amino acid retention in post-larval Senegalese sole (Solea senegalensis). Aquaculture. 233, 293-304.

Armstrong, J.L., Bonavaud, S.M., Toole, B.J., Yeaman, S.J., 2001. Regulation of glycogen synthesis by amino acids in cultured human muscle cells. J Biol Chem. 276, 952-956.

Badeaux, A.I., Shi, Y., 2013. Emerging roles for chromatin as a signal integration and storage platform. Nat Rev Mol Cell Biol. 14, 211-224.

Burdge, G.C., Lillycrop, K.A., 2010. Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr. 30, 315-339.

Canani, R.B., Di Costanzo, M., Leone, L., Bedogni, G., Brambilla, P., Cianfarani, S., Nobili, V., Pietrobelli, A., Agostoni, C., 2011. Epigenetic mechanisms elicited by nutrition in early life. Nutr Res Rev. 24, 198-205.

Caruso, M.A., Sheridan, M.A., 2011. New insights into the signaling system and function of insulin in fish. Gen Comp Endocrinol. 173, 227-247.

Carvalho, A.P., Araujo, L., Santos, M.M., 2006. Rearing zebrafish (Danio rerio) larvae without live food: evaluation of a commercial, a practical, and a purified starter diet on larval performance. Aquac Res. 37, 107 -111.

Caseras, A., Meton, I., Vives, C., Egea, M., Fernandez, F., Baanante, I.V., 2002. Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (Sparus aurata). Br J Nutr. 88, 607-614.

Commission, E., 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purpose. Official Journal of the European Commission.

Eames, S.C., Philipson, L.H., Prince, V.E., Kinkel, M.D., 2010. Blood sugar measurement in zebrafish reveals dynamics of glucose homeostasis. Zebrafish. 7, 205-213.

Ekmann, K.S., Dalsgaard, J., Holm, J., Campbell, P.J., Skov, P.V., 2013. Glycogenesis and de novo lipid synthesis from dietary starch in juvenile gilthead sea bream (Sparus aurata) quantified with stable isotopes. Br J Nutr. 109, 2135-2146.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2009. Nutritional regulation of hepatic glucose metabolism in fish. Fish Physiol Biochem. 35, 519-539.

Fang, L., Liang, X.F., Zhou, Y., Guo, X.Z., He, Y., Yi, T.L., Liu, L.W., Yuan, X.C., Tao, Y.X., 2014 Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, Danio rerio. Br J Nutr. 111, 808-818.

Field, H.A., Kelley, K.A., Martell, L., Goldstein, A.M., Serluca, F.C., 2009. Analysis of gastrointestinal physiology using a novel intestinal transit assay in zebrafish. Neurogastroenterol Motil. 21, 304-312.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., J Souza, E., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac Res. 38, 551-579.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (Oncorhynchus mykiss) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am J Physiol Regul Integr Comp Physiol. 292, 2275-2283.

Gomez-Requeni, P., Conceicao, L.E., Olderbakk Jordal, A.E., Ronnestad, I., 2010. A reference growth curve for nutritional experiments in zebrafish (Danio rerio) and changes in whole body proteome during development. Fish Physiol Biochem. 36, 1199-1215.

Gonzalez-Alvarez, R., Ortega-Cuellar, D., Hernandez-Mendoza, A., Moreno-Arriola, E., Villasenor-Mendoza, K., Galvez-Mariscal, A., Perez-Cruz, M.E., Morales-Salas, I., Velazquez-Arellano, A., 2009. The hexokinase gene family in the zebrafish: structure, expression, functional and phylogenetic analysis. Comp Biochem Physiol B Biochem Mol Biol. 152, 189-195.

Hanley, B., Dijane, J., Fewtrell, M., Grynberg, A., Hummel, S., Junien, C., Koletzko, B., Lewis, S., Renz, H., Symonds, M., Gros, M., Harthoorn, L., Mace, K., Samuels, F., van Der Beek, E.M., 2010. Metabolic imprinting, programming and epigenetics - a review of present priorities and future opportunities. Br J Nutr. 104 Suppl 1, S1-25.

Hemre, G.I., Mommsen, T.P., Krogdahl, A., 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquacult Nutr. 8, 175-194.

Hoar, W.S., Randall, D.J., 1988. Fish Physiology, The Physiology of Developing Fish - Eggs and Larvae. Academic Press, San Diego, California.

Jurczyk, A., Roy, N., Bajwa, R., Gut, P., Lipson, K., Yang, C., Covassin, L., Racki, W.J., Rossini, A.A., Phillips, N., Stainier, D.Y., Greiner, D.L., Brehm, M.A., Bortell, R., diIorio, P., 2011. Dynamic glucoregulation and mammalian-like responses to metabolic and developmental disruption in zebrafish. Gen Comp Endocrinol. 170, 334-345.

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S., 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J Exp Biol. 215, 2567-2578.

Kamler, E., 2008. Resource allocation in yolk-feeding fish. Rev Fish Biol Fisheries. 18, 143-200.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev Dyn. 203, 253-310.

Lahnsteiner, F., Urbanyi, B., Horvath, A., Weismann, T., 2001. Bio-markers for egg quality determination in cyprinid fish. Aquaculture. 195, 331-352.

Lillycrop, K.A., Burdge, G.C., 2012. Epigenetic mechanisms linking early nutrition to long term health. Best Pract Res Clin Endocrinol Metab 26, 667-676.

Lin, H., Romsos, D.R., Tack, P.I., Leveille, G.A., 1977. Effects of fasting and feeding various diets on hepatic lipogenic enzyme activities in coho salmon (Oncorhynchus kisutch (Walbaum)). J Nutr. 107, 1477-1483.

Lucas, A., 1998. Programming by early nutrition: an experimental approach. J Nutr 128, 401-406.

McCurley, A.T., Callard, G.V., 2008. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. BMC Mol Biol. 9, 102.

Mommsen, T.P., Plisetskaya, E.M., 1991. Insulin in fishes and agnathans: history structure and metabolic regulation. Rev Aquat Sci 4, 225-260.

Moon, T.W., 2001. Glucose intolerance in teleost fish: fact or fiction? Comp Biochem Physiol B Biochem Mol Biol. 129, 243-249.

Morais, S., Koven, W., Rønnestad, I., Dinis, M.T., Conceição, L.E.C., 2005. Dietary protein/lipid ratio affects growth and amino acid and fatty acid absorption and metabolism in Senegalese sole (Solea senegalensis Kaup 1858) larvae. Aquaculture. 246, 347-357.

Navarro, I., Leibush, B., Moon, T.W., Plisetskaya, E.M., Baños, N., Méndez, E., Planas, J.V., Gutiérrez, J., 1999. Insulin, insulin-like growth factor-I (IGF-I) and glucagon: the evolution of their receptors. Comp Biochem Physiol B Biochem Mol Biol 122, 137-153.

Navarro, I., Rojas, P., Capilla, E., Albalat, A., Castillo, J., Montserrat, N., Codina, M., Gutiérrez, J., 2002. Insights into Insulin and Glucagon Responses in Fish. Fish Physiol Biochem. 27, 205-216.

Panserat, S., Medale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R., Kaushik, S., 2000a. Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. Am J Physiol Regul Integr Comp Physiol. 278, 1164-1170.

Panserat, S., Medale, F., Breque, J., Plagnes-Juan, E., Kaushik, S., 2000b. Lack of significant long-term effect of dietary carbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout (Oncorhynchus mykiss). J Nutr Biochem. 11, 22-29.

Panserat, S., Capilla, E., Gutierrez, J., Frappart, P.O., Vachot, C., Plagnes-Juan, E., Aguirre, P., Breque, J., Kaushik, S., 2001a. Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (Oncorhynchus mykiss) by a single meal with glucose. Comp Biochem Physiol B Biochem Mol Biol. 128, 275-283.

Panserat, S., Fontagne, S., Bergot, P., Kaushik, S., 2001b. Ontogenesis of hexokinase I and hexokinase IV (glucokinase) gene expressions in common carp (Cyprinus carpio) related to diet. Br J Nutr. 85, 649-651.

Panserat, S., Plagnes-Juan, E., Breque, J., Kaushik, S., 2001c. Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol. 204, 359-365.

Panserat, S., Plagnes-Juan, E., Kaushik, S., 2002. Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). Biochim Biophys Acta. 1579, 35-42.

Panserat, S., 2009. Molecular regulation of intermediary metabolism focusing on utilization of dietary carbohydrates. in: Overturf, K. (Ed.), Molecular research in aquaculture. Wiley-Blackwell, John Wiley & Sons, Ames, Iowa, USA, pp. 408.

Panserat, S., Kaushik, S., 2010. Regulation of gene expression by nutritional factors in fish. Aquac Res. 41, 751-762.

Papasani, M.R., Robison, B.D., Hardy, R.W., Hill, R.A., 2006. Early developmental expression of two insulins in zebrafish (Danio rerio). Physiol Genomics. 27, 79-85.

Patel, M.S., Srinivasan, M., Laychock, S.G., 2009. Metabolic programming: Role of nutrition in the immediate postnatal life. J Inherit Metab Dis. 32, 218-228.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Pilkis, S.J., Granner, D.K., 1992. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. Annu Rev Physiol. 54, 885-909.

Pinto, W., Rodrigues, V., Dinis, M., Aragão, C., 2010. Can dietary aromatic amino acid supplementation be beneficial during fish metamorphosis? Aquaculture. 310, 200-205.

Planas, J.V., Capilla, E., Gutiérrez, J., 2000. Molecular identification of a glucose transporter from fish muscle. FEBS Letters. 481, 266-270.

Polakof, S., Mommsen, T.P., Soengas, J.L., 2011. Glucosensing and glucose homeostasis: From fish to mammals. Comp Biochem Physiol B Biochem Mol Biol. 160, 123-149.

Polakof, S., Panserat, S., Soengas, J.L., Moon, T.W., 2012. Glucose metabolism in fish: a review. J Comp Physiol B.

Robison, B.D., Drew, R.E., Murdoch, G.K., Powell, M., Rodnick, K.J., Settles, M., Stone, D., Churchill, E., Hill, R.A., Papasani, M.R., Lewis, S.S., Hardy, R.W., 2008. Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (Danio rerio). Comp Biochem Physiol Part D Genomics Proteomics. 3, 141-154.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M.T., Panserat, S., 2014. Glucose overload in yolk has little effect on the long-term modulation of carbohydrate metabolic genes in zebrafish (Danio rerio). J Exp Biol. 217, 1139-1149.

Rønnestad, I., Rojas-García, C.R., Tonheim, S.K., Conceição, L.E.C., 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture. 201, 161-175.

Rust, M.B., Hardy, R.W., Stickney, R.R., 1993. A new method for force-feeding larval fish. Aquaculture. 116, 341-352.

Saavedra, M., Conceição, L.E.C., Helland, S., Pousão-Ferreira, P., Dinis, M.T., 2008. Effect of lysine and tyrosine supplementation in the amino acid metabolism of Diplodus sargus larvae fed rotifers. Aquaculture. 284, 180-184.

Saint-Amant, L., Drapeau, P., 1998. Time course of the development of motor behaviors in the zebrafish embryo. J Neurobiol. 37, 622-632.

Seiliez, I., Medale, F., Aguirre, P., Larquier, M., Lanneretonne, L., Alami-Durante, H., Panserat, S., Skiba-Cassy, S., 2013. Postprandial regulation of growth- and metabolism-related factors in zebrafish. Zebrafish. 10, 237-248.

Shimeno, S., Kheyyali, D., Shikata, T., 1995. Metabolic Response to Dietary Carbohydrate to Protein Rations in Carp. Fish Sci. 61, 277-281.

Soanes, K.H., Achenbach, J.C., Burton, I.W., Hui, J.P., Penny, S.L., Karakach, T.K., 2011. Molecular characterization of zebrafish embryogenesis via DNA microarrays and multiplatform time course metabolomics studies. J Proteome Res. 10, 5102-5117.

Soengas, J.L., Polakof, S., Chen, X., Sangiao-Alvarellos, S., Moon, T.W., 2006. Glucokinase and hexokinase expression and activities in rainbow trout tissues: changes with food deprivation and refeeding. Am J Physiol Regul Integr Comp Physiol. 291, R810-821.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biol Rev Camb Philos Soc. 83, 13-34.

Srinivasan, M., Patel, M.S., 2008. Metabolic programming in the immediate postnatal period. Trends Endocrinol Metab. 19, 146-152.

Tao, T., Peng, J., 2009. Liver development in zebrafish (Danio rerio). J Genet Genomics. 36, 325-334.

Tehrani, Z., Lin, S., 2011. Endocrine pancreas development in zebrafish. Cell Cycle. 10, 3466-3472.

Vagner, M., Robin, J.H., Zambonino-Infante, J.L., Tocher, D.R., Person-Le Ruyet, J., 2009. Ontogenic effects of early feeding of sea bass (Dicentrarchus labrax) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. Br J Nutr. 101, 1452-1462.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 4th ed. University of Oregon Press, Eugene.

Yamada, K., Noguchi, T., 1999. Nutrient and hormonal regulation of pyruvate kinase gene expression. Biochem J. 337 (Pt 1), 1-11.

Early nutritional programming in marine fish species

Chapter IV

Hyperglucidic feeding of gilthead seabream (*Sparus aurata*) larvae: effects on molecular and metabolic pathways

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Hyperglucidic feeding of gilthead seabream (*Sparus aurata*) larvae: effects on molecular and metabolic pathways.

Abstract

Nutritional programming begins to arouse interest as a novel tool to alter specific metabolic pathways or functions in farmed animals. The aim of the present study was to explore the potential of early hyperglucidic stimuli to induce changes in nutrient metabolism of gilthead seabream. Nutritional conditioning was performed by delivering glucose-rich feed at three distinct recurrent periods of larval feeding regime: during first-feeding with rotifers (3 days after hatching, DAH) and mid-feeding with *Artemia metanauplii* (20DAH) and the beginning of inert diet feeding (30DAH), called the Recurrent treatment (REC). As opposed, the control treatment (CTRL) did not experience any glucidic stimuli. At post-larval stage (from 50 to 60DAH), both treatments were challenged with a high-carbohydrate diet (50%). The immediate response to the early stimuli was assessed through gene expression of metabolic markers and by nutrient metabolism using [^{14}C] tracers. Each dietary stimulus induced metabolic changes on REC larvae, shown by altered expression of some genes, including those involved in glycolysis, and by a different pattern of glucose utilization. However, none of the molecular adaptations (except G6PDH gene) were persistent in the viscera and muscle of challenged post-larvae from REC group. In contrast, the glucose metabolism of challenged REC post-larvae revealed a shift towards a higher catabolism and lower glucose retention in tissues, compared to the CTRL group, suggesting an improvement of glucose oxidation pathways. Also, the REC group showed a higher bio-conversion of glucose into lipids, indicating enhanced hepatic lipogenesis. The early stimuli did not affect the relative retention or use of amino acids or the growth and survival of challenged fish, up to 60DAH. In summary, although not substantiated at a molecular level, our data reveal that a recurrent dietary glucidic stimulus during larval stages affects the short-term modulation of pathways for glucose utilization in gilthead seabream.

Keywords: Carbohydrates, Glucose metabolism, Nutrigenomics, Nutrient flux, Nutritional programming

4.1. Introduction

Gilthead seabream (*Sparus aurata*) is the main produced marine fish species in the Mediterranean region and over 583 million gilthead sea bream juveniles were produced in 2013, mainly in Greece, Turkey, Italy, Spain and France (FEAP, 2014). Nutrition of marine fish larvae still relies on the use of live preys (rotifers and *Artemia*) and a gradual transition into inert diets, taking into account the significant ontogenetically determined morphological and physiological changes, during these first-feeding stages (Hamre et al., 2013; Rønnestad et al., 2013). To support the high rates of energy needs essential for fast growth, fish larvae require a protein- and lipid-rich feeding regime (Hamre et al., 2013; Rønnestad et al., 2013). Information about the digestion and metabolic use of carbohydrates by marine fish larvae is extremely scarce and has received much less attention than protein and lipid. Although with variable patterns among species, the activity of α-amylase, a key enzyme for the digestion of complex carbohydrates, has been detected in the larvae of several fish species, including seabream (Moyano et al., 1996; Naz, 2009; Zambonino-Infante et al., 2008). In general, species with more carnivorous habits, like seabream, tend to reduce amylase activity when the stomach is becoming functional (Cara et al., 2003; Zambonino-Infante et al., 2008), while herbivorous and omnivorous species seem to exhibit an increase in activity as they approach the juvenile stage (Zouiten et al., 2008). Despite the detrimental effects on growth and survival, Peres et al. (1996) showed in European seabass larvae that the normal decline in amylase activity was reduced when larvae were fed increasing levels of dietary carbohydrates, supplied as maltose and pre-cooked starch. Seabream larvae reared in glucose-enriched sea water immediately after mouth opening showed an enhanced accumulation of glycogen in the hepatocytes (Diaz et al., 1994). The addition of glycerol, a known gluconeogenic precursor, to the rearing water and rotifer culture medium, also resulted in a significant increase of hepatic glycogen content in seabream larvae (Maurizi et al., 2000). Although limited and fragmented, these studies demonstrate that carbohydrates serve as metabolic substrates in marine fish larvae.

It is now clearly accepted that performance in grow-out fish is directly linked to the quality of the larvae (Valente et al., 2013). In the grow-out stage, feeds tend to have a lower reliance on marine-derived protein sources and progressively incorporate higher levels of plant-based ingredients (Gatlin et al., 2007). The inclusion of these plant ingredients, being it dietary protein or starch sources, increases the overall intake of carbohydrates. However, the ability

of fish to use dietary carbohydrates as an energy yielding substrate is variable among and within species and closely associated with their natural feeding habits. New trends in the field of fish nutrition begin to emerge, such as the concept of early nutritional programming, as a promising strategy to enhance the use of alternative feedstuffs (Geurden et al., 2007; Geurden et al., 2014; Rocha et al., 2015; Vagner et al., 2009). Nutritional programming can be defined as an early nutritional event (or stimulus) exerted at a critical period of development that may have long-term consequences on later physiological functions (Burdge and Lillycrop, 2010; Harder et al., 1998; Lucas, 1998; Metges et al., 2014; Patel and Srinivasan, 2002). The perspective of applying this nutritional programming concept to fish larvae in order to tailor specific metabolic pathways or functions in juvenile fish, such as improving the use of dietary carbohydrates, is highly attractive yet extremely challenging. Understanding how the "memory" of an early nutritional event can be "stored" throughout life involves further analysis on the following mechanisms: adaptive changes on gene expression pattern or cellular phenotype (epigenetic mechanisms), nutrient-sensitive signaling pathways and adaptive clonal selection (Jaenisch and Bird, 2003; Lucas, 1998; Symonds et al., 2009; Waterland and Jirtle, 2004).

Knowledge on the role of early nutritional stimuli as modulators of metabolic pathways in fish is still scarce. However, in the past few years the number of studies performed on this topic has been growing, as the concept gains more notability for fish nutrition research. Recent studies exploring the short- and long-term effects of carbohydrate stimulus on the modulation of metabolic pathways were performed: at different stages of zebrafish embryogenesis through direct supplementation of the embryo yolk reserve (Rocha et al., 2014; 2015) and at the onset of exogenous feeding in rainbow trout (Geurden et al., 2007; Geurden et al., 2014), zebrafish (Fang et al., 2014) and Siberian sturgeon (Gong et al., 2015). With variable extent, all these studies showed that some effects at molecular and/or metabolic level were related to the early nutritional conditioning. To the best of our knowledge, the concept of using early dietary carbohydrate stimulus as modulator of metabolic pathways has never been tested in a marine fish species. Moreover, the use of tracer methodologies in fish larvae is a powerful tool to measure the metabolic plasticity and adaptation capacity to new nutrients and/or feeding regimes (Conceição et al., 2007; Conceição et al., 2010; Engrola et al., 2010; Morais et al., 2006) and abiotic factors (Campos et al., 2013).

In this context, the objective of the present study is to assess the effect of a recurrent early-feeding glucidic stimulus, exerted at several periods of gilthead seabream larval development, on the modulation of growth, nutrient metabolism and gene expression of post-larvae challenged with a high carbohydrate diet. Also, we investigated, at a metabolic and molecular level, the immediate responses of the larvae to each hyperglucidic stimulus.

4.2. Material and Methods

4.2.1. Larval rearing

The experiment was carried out in compliance with the Guidelines of the European Union Council (2010/63/EU) legislation for the use of vertebrate animals. Gilthead seabream eggs were obtained from MARESA - Mariscos de Estero S.A. (Huelva, Spain) and the experiment was conducted at CCMAR facilities (Faro, Portugal). Newly hatched larvae were reared in 100 L cylindro-conical tanks in a closed recirculation system with an initial density of 173 larvae L^{-1}. The experimental system was equipped with a mechanical filter, a submerged biological filter, a protein skimmer and a UV sterilizer. Photoperiod was set at 12:12h (L:D) cycle, temperature averaged 18 ± 1 ºC, salinity 33 ± 2 ppt and dissolved oxygen in water was maintained above 95% of saturation. Larvae were maintained in green-water conditions, provided by daily addition of frozen microalgae *Nannochloropsis oculata* to the rearing tanks (150 mg L^{-1}) (Nannochloropsis 18% FP 472/180909, Acuicultura Y Nutrición de Galicia SL, Spain). A daily monitoring of environmental parameters and larval mortality was performed; also the rearing tanks were cleaned regularly to preserve water quality.

4.2.2. Experimental design and feeding plan

Two treatments were randomly assigned to 8 tanks: Control – standard live feed feeding regime (CTRL treatment) and Recurrent – standard live feeding regime except for three 5-day periods (stimuli) during which glucose was offered to the larvae (REC treatment) (Fig. 4.1). Each treatment was done in quadruplicate tanks. The feeding plan in both groups was initiated with small-sized prey, rotifers (*Brachionus rotundiformis*), followed by *Artemia* AF *nauplii* (Inve, Belgium) then *Artemia* EG *metanauplii* (Inve, Belgium) and, at later developmental stage (30 days after hatching, DAH), the inert diet was gradually introduced by co-feeding regime up to total replacement of live prey, as shown in Figure 4.1. Briefly, larvae from

Control treatment (CTRL), started to feed at 3DAH on rotifers previously enriched with the STD emulsion (Table 4.1), which was rich in PUFAs and protein. From day 15, A*rtemia nauplii* were supplied in a co-feeding regime and at 20DAH, A*rtemia metanauplii* previously enriched with the STD emulsion were fed to larvae up to 35DAH. Live prey started to be gradually replaced at 30DAH by the control diet (HPD, Table 4.2), which presented high levels of protein. From 36DAH onwards larvae were fed exclusively on HPD diet (Figure 4.1). Rotifers were enriched with 0.1 g L^{-1} of STD emulsion which was delivered in two doses, at 3 h and 6 h before the harvesting of the rotifers; *Artemia metanauplii* were enriched with 0.6 g L^{-1} of STD emulsion, also delivered in two doses at 4 h and 8 h before harvest. Once enriched, the preys were fed directly to the larvae, three times a day and always in excess. Inert diet was distributed by automatic feeders (Fish mate F14, PET MATE Lda, England) eight times a day in a semi-continuous way (in cycles of 2 h of feeding followed by 1 h of pause) and pellets were hydrated before entering the tank.

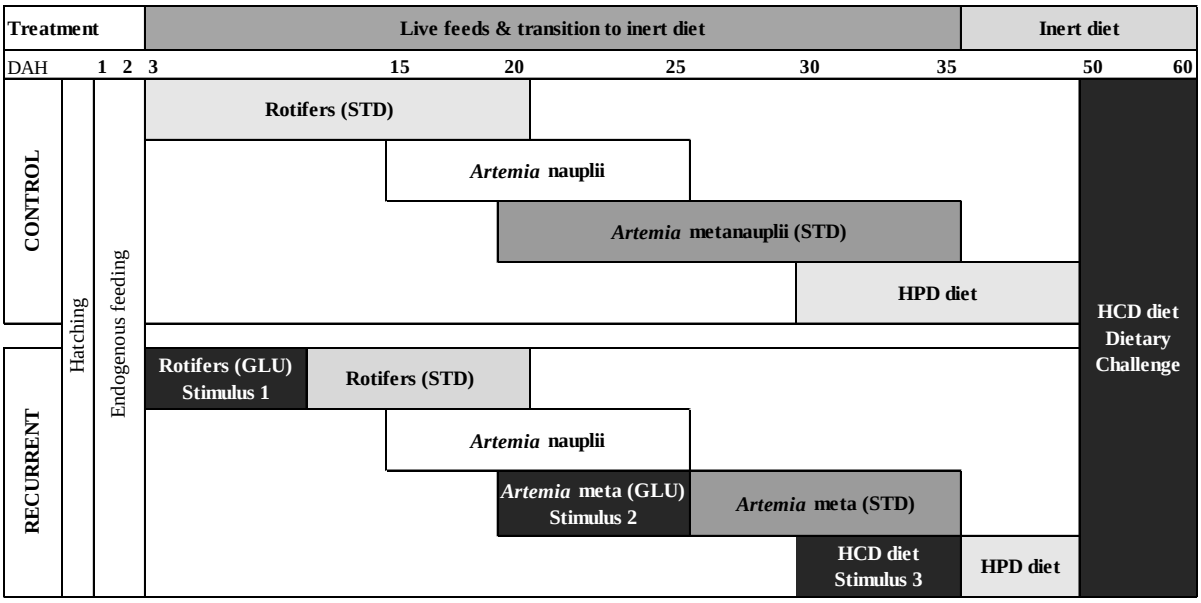

Figure. 4.1 - Experimental design, feeding plan and stimuli delivery periods for gilthead seabream larvae up to 60 DAH.

In the Recurrent treatment (REC), seabream larvae were exposed to repeated hyperglucidic stimuli at different stages of early development (Figure 4.1), using i) glucose-enriched rotifers at the onset of exogenous feeding (3DAH; stimulus 1), ii) glucose-enriched *Artemia metanauplii* (20DAH; stimulus 2) and iii) a high-carbohydrate diet HCD (30DAH; stimulus

3). The three stimuli were delivered to the REC larvae in all of the meals delivered, during a period of 5 days each. Both rotifers and *Artemia* used for stimuli delivery were enriched with glucose using the GLU emulsion (Table 4.1), following the same procedure described for live prey STD enrichment. To avoid the metabolic consumption of glucose by the prey, a short-term enrichment of 1 h was performed three times a day, to assure that glucose remained bioencapsulated in the live prey. Outside the glucose stimuli periods, larvae from the REC treatment followed the same feeding regime as the CTRL treatment, characterized by a high protein intake. From 50 to 60DAH, post-larvae of both CTRL and REC treatments were nutritionally challenged with a high carbohydrate-low protein diet (HCD diet - Table 4.2).

4.2.3. Experimental feeds

Live prey enrichments and inert diets were manufactured exclusively for this trial by SPAROS Lda (Olhão, Portugal). The standard emulsion (STD) was formulated according to existing commercial products to be highly rich in lipids, especially in essential fatty acids (EFA), thus containing 70.5% crude lipids, 16.0% crude protein and no carbohydrates (on a dry matter basis). The glucose-rich emulsion (GLU) was formulated to contain a high carbohydrate level (57.3%), which was achieved by the incorporation of D-glucose at the expenses of all protein sources and a concomitant reduction of crude lipids. In both emulsions, the essential fatty acid contents were maintained at recommended levels for seabream larvae (Rodríguez et al., 1998). Experimental emulsions were manufactured by progressively mixing the powder ingredients (<20 micron) with the oils and water (3:7 w/w ratio) with the aid of a high-shear mixing (Silverson L5T, United Kingdom). Emulsions were kept refrigerated throughout the experimental period. Formulation and proximate composition of both emulsions is presented in Table 4.1.

For the feeding phase with inert diets (after 30DAH), two experimental diets were formulated with distinctive protein/carbohydrate ratios: a high-protein diet (HPD) with 71% crude protein (derived from fish meal, fish soluble concentrate, squid meal, wheat gluten and fish gelatin) and trace levels of carbohydrates; and a high-carbohydrate diet (HCD) with high levels of carbohydrates (50%, supplied as D-glucose) and a drastic reduction of crude protein level (34%). Powder ingredients and oil sources were mixed and extruded at 1.0 mm in a low shear extruder (Italplast P55, Italy). Upon extrusion, feeds were dried in a convection oven (LTE Scientifics OP 750-UF, United Kingdom) for 3 h at 40 °C. Dry feed pellets were then crumbled and sieved manually to retrieve the desired particle size (200–400 and 400–600

micron, used according to larvae size). Formulation and proximate composition of diets is presented in Table 4.2.

Table 4.1 - Formulation and proximate composition of the control (STD) and glucose (GLU) emulsions used to enrich live preys (rotifers and *Artemia* metanauplii) before larval feeding and stimuli delivery, respectively.

	Emulsions	
Ingredients (%)	**STD**	**GLU**
Micronized fishmeal[1]	5.0	-
Fish solubles concentrate[2]	5.0	-
Krill hydrolisate[3]	10.0	-
D-Glucose[4]	-	55.0
Tuna oil[5]	40.0	12.0
Concentrated DHA oil (70%)[6]	12.0	7.5
Marine phospholipids[7]	5.0	5.0
Vitamin and mineral premix[8]	1.0	1.0
Vitamin C	2.0	2.0
Vitamin E	1.0	1.0
Choline chloride	1.0	1.0
Soy lecithin	5.0	5.0
Tween 80[4]	3.0	3.0
Sodium alginate	2.5	2.5
Antioxidant[9]	2.0	2.0
NaH_2PO_4	3.0	3.0
L-Tryptophan	0.5	-
DL-Methionine	0.5	-
L-Taurine	1.5	-
Proximate composition		
Dry matter (DM, %)*	29.1 ± 0.6	27.7 ± 0.3
Crude protein (% DM)	16.0 ± 0.0	1.5 ± 0.1
Crude fat (% DM)	70.5 ± 0.1	33.3 ± 0.2
Total carbohydrates** (% DM)	3.1 ± 0.5	57.2 ± 0.2
Ash (% DM)	10.4 ± 0.4	8.0 ± 0.0
Gross energy (MJ/kg DM)	32.1 ± 0.1	23.3 ± 0.1

* Emulsions were prepared at a proportion of 3/7 (w/w) ingredient mix/water ratio. **Total carbohydrates calculated as: 100 – (protein + fat + ash). [1] MicroNorse: 70.6% crude protein (CP), 9.9% crude fat (CF), K/S Tromsø Fiskeindustri A/S & Co, Norway; [2] CPSP 90: 84% CP, 12% CF, Sopropêche, France; [3] KPH: 73% CP, 2.2% CF, Sopropêche, France; [4] Sigma-Aldrich, Portugal; [5] Omegavie tuna oil 25 DHA TG, Polaris, France; [6] Algatrium 70 DHA, Brudy Technologies, Spain; [7] PhosphoNorse, K/S Tromsø Fiskeindustri A/S & Co, Norway; [8] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings; [9] Paramega PX, Kemin Europe NV, Belgium.

Table 4.2: Formulation and proximate composition of the High protein diet (HPD), delivered during larval growth and the High carbohydrate diet (HCD), delivered during the periods of stimulus 3 and dietary challenge.

	Diets	
Ingredients (%)	**HPD**	**HCD**
Micronized fishmeal[1]	56.4	9.0
Fish solubles concentrate[2]	10.0	10.0
Squid meal[3]	7.5	10.0
Fish gelatin[4]	2.0	2.0
Wheat gluten[5]	10.0	10.7
D-Glucose[6]	0.0	45.0
Tuna oil[7]	5.3	5.5
Vitamin and mineral premix[8]	2.0	2.0
Vitamin C	0.1	0.1
Vitamin E	0.05	0.05
Betaine	0.5	0.5
Soy lecithin	2.5	0.0
Antioxidant[9]	0.2	0.2
NaH_2PO_4	2.0	5.0
L-Threonine	0.7	0.0
DL-Methionine	0.3	0.0
L-Taurine	0.5	0.0
Proximate composition		
Dry matter (DM, %)	97.4 ± 0.1	95.5 ± 0.0
Crude protein (% DM)	71.0 ± 0.2	33.7 ± 0.4
Crude fat (% DM)	15.8 ± 0.3	9.1 ± 0.1
Total carbohydrates* (% DM)	0.8 ± 0.7	49.8 ± 0.6
Ash (% DM)	12.4 ± 0.2	7.5 ± 0.1
Total phosphorus (% DM)	1.9 ± 0.0	1.4 ± 0.1
Gross energy (MJ/kg DM)	23.1 ± 0.0	20.0 ± 0.1

* Total carbohydrates calculated as: 100 – (protein + fat + ash). [1] MicroNorse: 70.6% crude protein (CP), 9.9% crude fat (CF), K/S Tromsø Fiskeindustri A/S & Co, Norway; [2] CPSP 90: 84% CP, 12% CF, Sopropêche, France; [3] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France; [4] Pharma grade bloom 240: 92% CP, LAPI Gelatine SPA, Italy; [5] VITEN: 84.3% CP, 1.3% CF, ROQUETTE, France; [6] Sigma-Aldrich, Portugal; [7] Omegavie tuna oil 25 DHA TG, Polaris, France; [8] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings; [9] Paramega PX, Kemin Europe NV, Belgium.

4.2.4. Biological sampling

At the end of the experimental trial (60DAH) survival rate from each treatment was determined by direct counting of individuals, relative to the initially stocked number of larvae and excluding the individuals sampled over the trial. Dry weight and total length were determined at the end of each stimulus period, stimulus 1 (n=60), stimulus 2 (n=60), stimulus 3 (n=60), sampled at 8, 25 and 35DAH, respectively and at the end of dietary challenge (n=60). Growth was determined based on individual dry weight and total length measurements (except for larvae at 8DAH that were pooled). Total length was determined using the AxioVision 4.8.2 software (Carl Zeiss Ltd, Cambridge, UK) for digital image analysis, and dry weight measurements were obtained from freeze-dried samples using a precision scale (±0.001 mg). For assessing the composition of enriched-live prey, samples of rotifers (n=2, each with 1,500,000 rotifers) and *Artemia* (n=2, each with 150,000 metanauplii) were collected and immediately frozen at –80ºC. Samples for gene expression analysis were randomly collected from each treatment 2h after the last meal, at the larval stages of 8, 25, 35DAH and 4h after the last meal at the post-larval stage of 60DAH. These times were considered suitable to evaluate the peak of nutrient absorption based on previous tracer studies in marine fish species (Morais et al., 2006). Thus, whole larval bodies were collected after the stimulus 1 (n=6 in pools of 40 larvae), stimulus 2 and 3 (n=6 in pools of 20 larvae) and at the end of the dietary challenge, where each post-larvae (n=6 per treatment) was dissected for separate collection of viscera (all abdominal content) and muscle. All samples were then snap-frozen in liquid nitrogen and kept at −80 ºC until molecular analysis. For the metabolic trials, samples were collected at the end of hyperglucidic stimulus 3 (n=20) and at the end of the trial (n=20 per treatment of which n=10 per radiolabeled tracer).

4.2.5. Metabolic Trials

A method combining the use of live food and radiolabeled tracers was used to determine the metabolic fate of glucose and amino acids at two stages of seabream development, corresponding to the major periods of exposure to high levels of dietary glucose (HCD diet): the end of stimuli 3 (35 DAH) and the end of the dietary challenge (60 DAH).

4.2.5.1. *Artemia* [^{14}C] labeling

Artemia metanauplii were enriched with glucose using the GLU emulsion and radiolabeled with [U–^{14}C] D-glucose (9.25 MBq; American Radiolabeled Chemicals Inc., The

Netherlands) according to a modified version of the method developed by Morais et al. (2004a). *Artemia nauplii* were enriched at 28 ºC, with a dose of 0.5 µL of the [U–^{14}C] D-glucose per mL of seawater. The radiolabeling process lasted for 2 h and was performed right before the beginning of the metabolic trial. After incubation, *Artemia metanauplii* were washed in seawater several times and sampled (n = 4, 3 mL each sample) in order to determine the amount of radiolabel per prey. For the second metabolic trial performed with post-larvae, glucose enriched *Artemia* were also labeled with [U–^{14}C] L-Amino acid mixture (37 MBq; American Radiolabeled Chemicals Inc., The Netherlands), following the protocol developed by Morais et al. (2004a) . The dose of [U–^{14}C] L-Amino acid mixture was 1.6 µL per mL of seawater and *Artemia* was incubated for 9 h.

4.2.5.2. Metabolic trial in seabream larvae at stimulus 3

This trial was conducted in seabream larvae (35DAH) after having received the third glucose stimulus through the HCD diet, to evaluate the immediate metabolic response of larvae. On the evening prior to the radiolabeling trial, larvae from the CTRL and REC treatments (n=20) were randomly harvested and transferred to the flux laboratory for overnight acclimatization (18±1 °C). Larvae were stocked in 1L tanks and deprived from feed for 16 h. After fasting, larvae were allowed to eat *ad libitum* the ^{14}C-D-glucose *Artemia* during 45 to 60 min; this period was considered suitable for the uptake of a full meal based on previous ingestion rate trials with seabream larvae of similar age (Kolkovski et al., 1993; Morais et al., 2006). Following, larvae were carefully transferred, one by one, through two tanks with clean seawater (to eliminate any ^{14}C-tracer that could be present on the surface of the larvae), and subsequently transferred to an incubation vial. The incubation setup was described previously by Rønnestad et al. (2001). In brief, the incubation setup consists of sealed vials containing 6 mL of seawater with gentle air flow where the larva was placed. The air was forced through a capillary from the incubation vial to a CO_2 trap (5 mL of 0.5 M KOH). After a 24 h incubation period, each seabream larva was rinsed with clean seawater and individually sampled (the whole body) to determine the amount of ^{14}C retained in tissues (retained fraction). The bodies were immediately solubilised with Solvable (Perkin Elmer, USA) and kept at 50ºC for 24 h. Samples collected from the labeled *Artemia* were also solubilised together with the bodies. Following larval sampling, the incubation chambers were resealed and 1 mL of HCl (0.1 M) was added in a series of gradual steps, resulting in a progressive decrease of pH that causes the rapid diffusion of any remaining $^{14}CO_2$ from the incubation

water into the KOH trap (catabolized fraction) (Rønnestad et al., 2001). The incubation water was considered to contain all labeled ^{14}C resultant from fish excretion (evacuated fraction). No larval mortality was registered during the incubation period.

4.2.5.3. Metabolic trial in seabream post-larvae after dietary challenge

The second trial was conducted in seabream post-larvae (60DAH) after being submitted to the 10-day dietary challenge with HCD diet. The scope was to assess possible effects of early glucose-stimuli on nutrient metabolism of post-larvae seabream fed exclusively on a high carbohydrate diet. Post-larvae from both CTRL and REC treatments were randomly collected and transferred to the flux laboratory for overnight acclimatization (18±1 °C) and fasting period (16 h). For this experiment, *Artemia metanauplii* were labeled, in separate, with two radioactive tracers: ^{14}C-D-glucose or ^{14}C-L-Amino acid mixture (^{14}C-AA). After the fasting period, 10 post-larvae from the CTRL treatment were fed with the labeled ^{14}C-D-glucose *Artemia*, while another group (n=10) of the same treatment was fed with the ^{14}C-Amino acid *Artemia*, for a period of 45 min to 60 min. An identical feeding scheme was applied to two groups of 10 post-larvae each from REC treatment. Then, all individuals were transferred to the incubator set-up and the experiment was carried out as previously described. After the incubation period of 24 h, samples from the incubation water and CO_2 trap were collected as formerly indicated. For the post-larvae, a series of extraction procedures of organic compounds, such as free amino acids (FAA), protein and fat were performed in order to get a more detailed analysis of the glucose and amino acid retention. The larval bodies were individually placed in 1 mL of 6 % trichloroacetic acid (TCA, Sigma Chemical Co., St. Louis, MO) for 24 h at 4 ºC, with periodical stirrings, for FAA extraction (Campos et al., 2013; Morais et al., 2004b). After each body was removed from the TCA solution and placed in a clean vial for tissue homogenization in distilled water (0.8 mL), using an Ultra-turrax homogenizer (IKA, Germany). Total body lipid was extracted from a known aliquot volume using a method modified from Bligh and Dyer (1959), suitable for small volume samples (Conceição et al., 2002; Morais et al., 2004b). Total protein was extracted from the upper phase of methanol/water recovered from lipid extraction, based on a TCA precipitation method, adapted and modified from Panchout et al. (2013). Briefly, 24% TCA was added to each sample to a final concentration of 6%, after samples were vortex and incubated at 4 ºC for 1 h. Precipitated proteins were pelleted by centrifugation at 6000 G for 10 min at 4 ºC. The supernatant was collected to a clean vial for DPM (disintegrations per minute) counting

and the precipitated protein was washed with ice-cold acetone and centrifuged at 6000 G for 10 min at 4 ºC. The acetone was removed to a clean vial for DPM counting and the protein pellet was re-suspended in Solvable (Perkin Elmer, USA) and kept at 50 º C for 24 h for complete solubilization. The two supernatants obtained from this protocol were not discarded since they represent the fraction of non-extracted metabolites from the larval body, where glycogen was considered to be present. No mortality was registered during the incubation period.

4.2.5.4. Radiolabel measurements

Larvae that did not ingest any labeled *Artemia* during the feeding period (at the beginning of the metabolic trial) were excluded from the analysis, after visual confirmation of empty stomachs. Samples from incubation seawater, KOH-CO_2 traps and *Artemia metanauplii*, collected during the two metabolic trials, were counted for radioactivity (DPM) by adding Ultima Gold XR scintillation cocktail (Perkin Elmer, USA). For the first trial (35DAH), whole larval bodies were counted, while for the second trial with post-larvae (60DAH), the bodies were fractionated in lipids, protein, FAA and other metabolites and each fraction was separately counted for DPM. All samples were counted on a Tri-Carb 2910TR Low activity liquid scintillation analyzer (Perkin Elmer, USA). The metabolic budgets were calculated after subtraction of blanks for quench and lumex correction. Results for each component (evacuation, catabolism, retention in whole larvae and retention in several fractions of organic compounds) were expressed as a percentage of total ^{14}C-label, i.e. the sum of DPM in all compartments of metabolic chambers and fish. Glucose utilization in seabream larvae (35DAH) was determined based on Absorption (*A*, %), Evacuation (*E*, %), Retention efficiency (*R*, %) and Catabolism fraction (*C*, %). These estimates were determined as:

$A = [(R_{body} + R_{CO_2\,trap}) / (R_{\text{body}} + R_{CO_2\,trap} + R_{water})] \times 100;$

$E = [R_{water} / (R_{body} + R_{CO_2\,trap} + R_{water})] \times 100;$

$R = [R_{body} / (R_{body} + R_{CO_2\,trap})] \times 100;$

$C = [R_{CO_2\,trap} / (R_{body} + R_{CO_2\,trap})] \times 100;$

where R_{body} is the total radioactivity in larval body (DPM), $R_{CO_2\,trap}$ is the total radioactivity per CO_2 trap (DPM) and R_{water} is the total radioactivity in the incubation seawater (DPM).

The metabolic fate of glucose and amino acids in seabream post-larvae (60 DAH) was determined based on nutrient Absorption (*A*, %), Evacuation (*E*, %), Retention efficiency (*R*,

%) and Catabolism fraction (*C*, %), as previously described. Additionally, it was determined the relative retention in lipid fraction (rR_{Lip}, %), protein fraction (rR_{Prot}, %), FAA fraction (rR_{FAA}, %) and other metabolites fraction where glycogen was included (rR_{Other}, %). The several relative retentions were determined as:

$rR_{Lip} = (R_{Lipids} / R_{body}) \times 100$

$rR_{prot} = (R_{Protein} / R_{body}) \times 100$

$rR_{FAA} = (R_{FAA} / R_{body}) \times 100$

$rR_{other} = (R_{Other} / R_{body}) \times 100$

where R_{body} is the sum of all fractions of compounds extracted from body (DPM), R_{Lipids} is the total radioactivity in chloroform fraction (DPM), $R_{Protein}$ is the total radioactivity in protein precipitate (DPM), R_{FAA} is the total radioactivity in TCA soluble fraction (DPM) and R_{Other} is the total radioactivity in the washes performed during protein extraction (DPM).

4.2.6. Gene expression analysis by qRT-PCR

Relative gene expression of mRNA was determined by quantitative real-time RT-PCR on RNAs extracted from whole body larvae (pooled samples) or visceral and muscle tissues of seabream post-larvae. Total RNA was extracted from all samples (larvae and tissues) using 1 mL of Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. An amount of 2 µg of total RNA was reverse transcribed into complementary DNA (cDNA) using the SuperScript III RNaseH Reverse Transcriptase Kit (Invitrogen, Carlsbad, USA) with random primers (Promega, France). Molecular analysis was focused on genes involved in intermediate metabolism and energy production. The transcripts analized were glucokinase (GK), hexokinase 1 (HK1), 6-phosphofructo-1-kinase liver isoform (6PFK-L) and muscle isoform (6PFK-M) and pyruvate kinase muscle isoform (PK-M) for glycolysis; glucose-6-phosphatase (G6Pase) and phosphoenopyruvate carboxykinase (PEPCK) for gluconeogenesis; fatty acid synthase (FAS), acetyl CoA carboxylase (ACCα) for lipid metabolism; glucose-6-phosphate dehydrogenase (G6PDH) for the pentose phosphate pathway, cytochrome oxidase subunit IV (COX4) and citrate synthase (CS) for energy metabolism; facilitative glucose transporter (GLUT9) for glucose transporter and amylase (AMY) for carbohydrate digestion. The amino acid catabolism was also studied based on the mitochondrial alanine aminotransferase (ALTm) and cytosolic aspartate aminotransferase (ASTc) transcripts. The genes COX4, CS, ALTs and ASTs were only analised at the time points related to the metabolic studies (35 and 60 DAH). For the remaining sampled points,

the analised gene panel was identical. The primer sequences of GK, G6Pase, COX4, CS and elongation factor 1 (EF1) genes used for real-time qPCR have previously been published for this species (Bermejo-Nogales et al., 2014; Enes et al., 2008a; Perez-Sanchez et al., 2013).
For the other analized genes, specific primers were designed for gilthead seabream using the Primer3 software (http://primer3.ut.ee/) and the available seabream sequences from the nucleotide GenBank (http://www.ncbi.nlm.nih.gov/genbank) or the EST SIGENAE (http://www.sigenae.org) databases (Table 4.3). PCR product resulting from the newly designed primer pairs was controlled by sequencing to confirm the nature of the amplification. For quantification of target gene expression levels the Roche Lightcycler 480 system was used (Roche Diagnostics, France). Analyses were performed on 2 µL of the diluted cDNA using 3 µL of Light Cycler 480 SYBR® Green I Master mix (Roche), in a total PCR reaction volume of 6µL, containing 100 nM of each primer. The PCR protocol was initiated at 95 °C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a two-step amplification programme (15 s at 95 °C; 40 s at 60 °C). Melting curves were systematically monitored (temperature gradient at 1.1 °C/10 s from 65-94 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (samples without reverse transcriptase and samples without RNA). Relative quantification of gene expression was performed using the ΔC_T method described by Pfaffl (2001). EF1 gene was elected as non-regulated reference gene, being used for the normalization of measured mRNAs since its expression levels were stable over the several developmental stages of seabream larvae and post-larvae (data not shown). In all cases, PCR efficiency was measured by the slope of a standard curve using serial dilutions of cDNA, and ranged between 1.8 and 2.1.

4.2.7. Analytical methods

Experimental diets, freeze-dried emulsions and freeze-dried samples of rotifers and *Artemia metanauplii* enriched with the experimental emulsions were analised for proximate composition according to the following procedures: dry matter after drying at 105 °C for 24 h; ash content by incineration in a muffle furnace at 500ºC for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428, St. Joseph, MI, USA); crude fat in diets and emulsions was quantified by dichloromethane extraction (Soxhlet method), while in rotifers and *Artemia*

it was performed according to Segura and Lopez-Bote (2014); gross energy in an adiabatic bomb calorimeter (IKA C2000, Germany); total phosphorus according to the ISO/DIS 6491 method using the vanado-molybdate reagent. Total carbohydrates were estimated by difference of other constituents: total carbohydrates (%) = 100 – (% protein + % lipid + % ash).

Table 4.3 – Real time PCR primer sequences

Gene	Forward primer (5'-3')	Revese primer (5'-3')	Database (accession no.)
EF1[a]	CATGCTGGAGACCAGTGAAA	CGGGTACAGTTCCAATACCG	GeneBank (AF184170)
GK[a]	CAAGAGACGAGGGGACTCG	TCCTCGCCTTCTACCAGCTC	GeneBank (AF053330)
HK1	AGATCCATCCTGCAGCACTT	CCAGTCCTCGGTTCTCTCTG	Sigenae (AM955654.p.sb.5)
6PFK-L	CATGTGTGATTGGCCTCAAC	AGGGAGCCTAAAACCCAGAG	Sigenae (AM968607.p.sb)
6PFK-M	ACGGTCGTATCTTTGCCAAC	GTGTGTTCGGATGTGTCCAG	Sigenae (FM147562.p.sb.5)
PK-M	GATCCTGGCACAAAGCTCTC	TGTCTGCTGGACATCGACTC	Sigenae (FG590446.p.sb.5)
G6Pase[a]	CGCTGGAGTCATTACAGGCGT	CAGGTCCACGCCCAGAACTC	GeneBank (AF151718)
PEPCK	GCAACACAGAGAGGGAGGAG	TATCCTCCAGTGCCTTCAGC	Sigenae (CV133734.p.sb.5)
GLUT9	GAGGACTACCCAGGTGACCA	GGTGACTGTCTCTGCTGCAA	Sigenae (FM145240.p.sb.5)
AMY	CAGATGGCGTCAGATCAAGA	GTCCAGGTTCCAGTCGTCAT	Sigenae (FG268830.p.sb.5)
FAS	TGAAACTGAAGCCCTGTGTG	TCTCGGCTGATGTTCTTGTG	Sigenae (AM952430.p.sb.5)
ACC	ATCAGAGGTGGCGATGGTAG	TCGTCATGCAGTTAGCCAAG	Sigenae (FP332814.p.sb.5)
G6PDH	GCAGCCAGATGCACTTTGTA	GCGAAATCCAACTCTCTTCG	Sigenae (AM951965.p.sb.5)
COX4[b]	ACCCTGAGTCCAGAGCAGAAGTCC	AGCCAGTGAAGCCGATGAGAAAGAAC	GeneBank (JQ308835.1)
CS[c]	TCCAGGAGGTGACGAGCC	GTGACCAGCAGCCAGAAGAG	GeneBank (JX975229)
ALTm	CGTGGAGGCTACATGGAGAT	AGCTTGGCCTTCTCTGCTAA	GeneBank (AY206503.1)
ASTc	AGTGTCTTGGAGGTACAGGC	CCAAGGAAACCAGCCAAGTC	Sigenae (CB176687.p.sb.5)

[a] From Enes et al. (2008a)
[b] From Perez-Sanchez et al. (2013)
[c] From Bermejo-Nogales et al. (2014)

4.2.8. Statistical analysis

Data are presented as means with their standard deviation. Criteria expressed as a percentage were arcsine-square-root transformed prior to statistical analysis (Ennos, 2007). The effect of the early feeding glucose stimuli on growth, survival, nutrient metabolism and gene expression of seabream larvae was tested using SPSS® statistics software 16.0 for Windows

(SPSS Inc.) by means of an unpaired two-tailed Student's *t*-test. For relative quantification of gene expression, the control group was established by the group that did not receive any glucose stimuli. Differences were considerate significant at $P<0.05$.

4.3. Results

4.3.1. Efficacy of glucose supplementation in live prey

The composition of live preys, rotifers and *Artemia*, after the enrichment with both STD and GLU emulsions are presented in Table 4.4. On a dry matter basis, rotifers enriched with the STD emulsion had high protein content (53.7%), lipids (10.1%) and total carbohydrates (27.3%). In comparison, rotifers enriched with the GLU emulsion showed a relative reduction of -20% on their protein content (43.1%) and a relative increase of +38.6% on the total carbohydrates content (37.8%). Crude lipid content was similar among both treatments (10%). Similarly, *Artemia* enriched with the STD emulsion were protein-rich (57.4%) and contained a low level of total carbohydrates (9.2%), while those enriched with the GLU emulsion had lower protein content (36.8%) and higher total carbohydrates (36.4%). On a relative basis to the STD treatment, these changes represented a 294% increase of total carbohydrates levels in *Artemia* enriched with the GLU emulsion.

Table 4.4 – Proximate composition (% of dry weight) of the live prey Rotifers and *Artemia metanauplii* after enrichment with a lipid-rich (STD) or a glucose-rich (GLU) emulsions.

	Rotifers STD	Rotifers GLU	% Relative of STD	*Artemia* STD	*Artemia* GLU	% Relative of STD
Crude protein (% dm)	53.7 ± 0.7	43.1 ± 0.8	- 19.8	57.4 ± 0.0	36.8 ± 0.1	- 35.8
Crude lipids (% dm)	10.1 ± 0.5	9.7 ± 0.2	- 4.1	23.9 ± 1.1	17.3 ± 0.1	- 27.8
Ash (% dm)	8.9 ± 0.3	9.4 ± 0.1	+ 6.0	9.5 ± 0.1	9.5 ± 0.2	- 0.2
Total carbohydrates*	27.3 ± 0.1	37.8 ± 1.1	+ 38.6	9.2 ± 1.0	36.4 ± 0.2	+ 294.4
Gross energy (MJ/kg dm)	21.3 ± 0.0	20.5 ± 0.1	- 4.0	24.5 ± 0.3	21.7 ± 0.1	- 11.3

Values are means ± SD for rotifers and *Artemia* enriched with the STD and GLU emulsions (n=2, for both live prey). STD: control emulsion containing a high lipid level; GLU: glucose emulsion containing a high carbohydrate level; *Total carbohydrates calculated as: 100 – (protein + fat + ash).

4.3.2. Immediate response of larval metabolic gene expression to early glucidic stimuli

The analysis of gene expression showed that early glucidic stimuli applied during critical periods of seabream larval development induced some changes on the regulation of genes related to intermediary metabolism. The intake of high glucose levels at the onset of exogenous feeding through enriched rotifers (stimulus 1) induced the expression of GK and FAS genes, but at the same time inhibited the expression of 6PFK-L and G6PDH on the REC group, in comparison to the control group (Figure 4.2). Two enzymes involved in glycolysis (GK and 6PFK-L) were oppositely regulated by the first stimulus in the REC larvae. Also, the same stimulus caused no effects in the expression of genes related to gluconeogenesis, lipogenesis, carbohydrate digestion (amylase) and glucose transporter (GLUT9) (Figure 4.2). Moreover, larvae at 25 DAH showed few changes on gene expression levels related to the stimulus of glucose enriched *Artemia* (stimulus 2). Only two genes were modified, being G6PDH up-regulated while AMY was repressed in REC larvae, compared to CTRL group. Somehow the second stimulus of *Artemia* may have led to an improvement of NADPH production as well as to a decrease of the digestive capacity of carbohydrates (Figure 4.3). At the last stimulus with the HCD diet (stimulus 3), larvae from REC group exhibited the induction of some genes involved in glycolysis (6PFK-L and PK-M), citric-acid cycle (CS) and amino acid catabolism (ASTc), in relation to the CTRL group. While the glycolytic pathway was being promoted in REC larvae by the up-regulation of 6PFK-L and PK-M genes, the gluconeogenic pathway was not differently regulated at this stage. Also, the expression of genes involved in lipogenesis (FAS, ACC), energy production (G6PDH, COX4), glucose transporter (GLUT9) and carbohydrate digestion (AMY) was not affected by stimulus 3 (Figure 4.4).

4.3.3. Immediate response of larval [^{14}C] glucose metabolism to early glucidic stimuli

The metabolic fate of glucose was assessed in the CTRL and REC larvae at 35DAH, after the feeding of stimulus 3 (HCD diet), using ^{14}C-glucose as a tracer. Results showed that 35DAH larvae had a nearly equal capacity for glucose absorption and evacuation (about 50%), and none was significantly affected by the early glucose conditioning (P>0·05) (Figure 4.5-A). Larvae submitted to the recurrent hyperglucidic stimuli (REC group) showed significantly lower glucose catabolism (47%) and higher retention of glucose in body tissues (53%), in comparison to the CTRL group (P<0·05). In the CTRL group, the glucose catabolism showed

to be 10% higher while body retention was 10% lower than in REC larvae. (Figure 4.5 -B). Survival rate was 100% for both treatments, after 24 h of incubation.

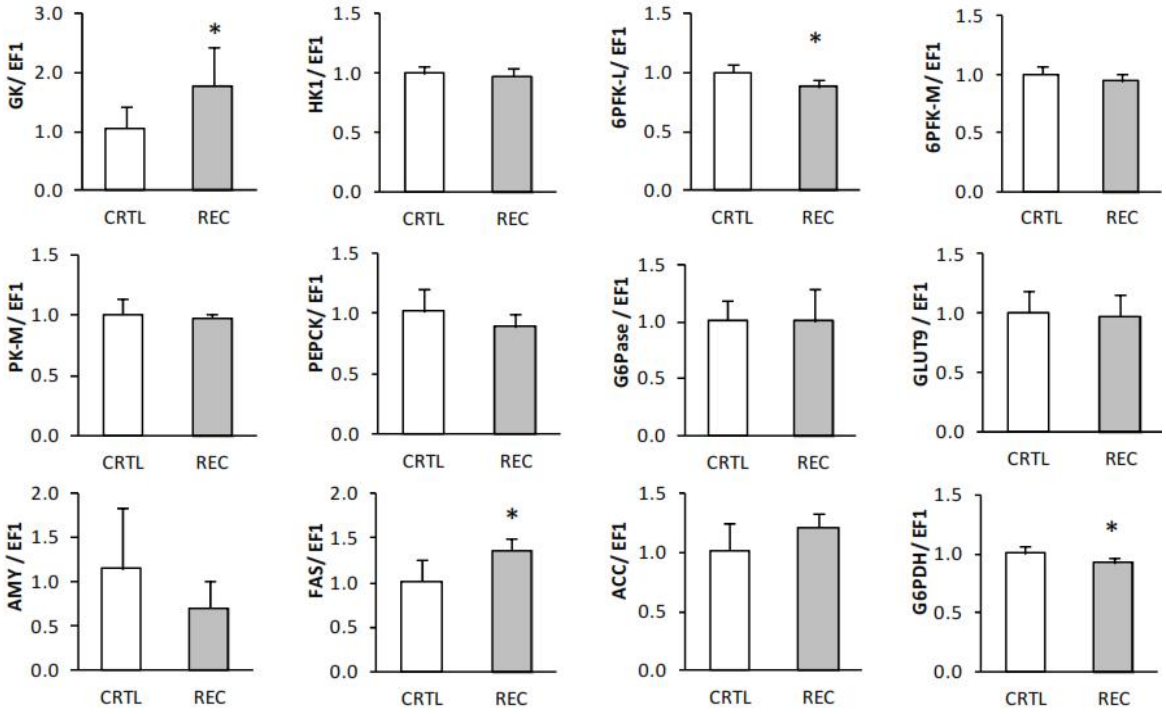

Figure 4.2 –- Immediate effect of stimulus 1 – glucose enriched rotifers – on gene expression of selected metabolic enzymes in whole-body seabream larvae at 8 DAH from the REC treatment, compared to the Ctrl. Expression values were measured using real-time RT-PCR and normalized with α-elongation factor 1 transcript (EF1α). Relative fold differences between treatments are presented as means + SD (n = 6 in pools of 40 larvae). Significant statistical differences are denoted by * ($P < 0{\cdot}05$, Student's *t*-test).

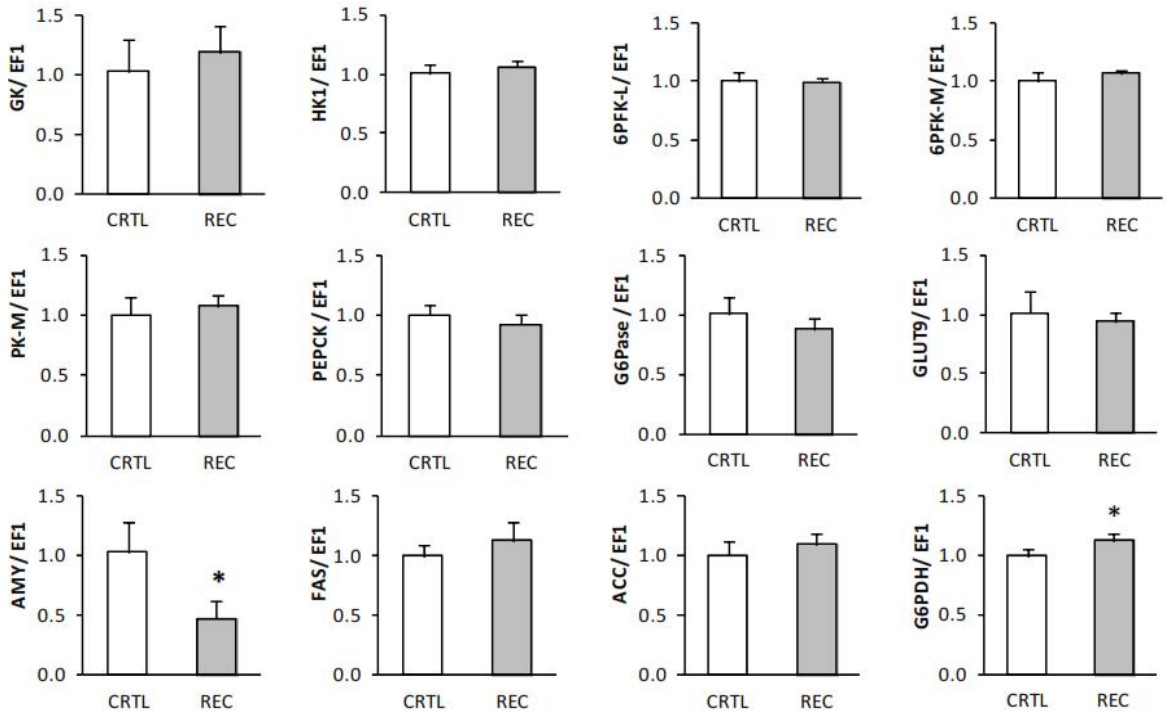

Figure 4.3 – Immediate effect of stimulus 2 – glucose enriched *Artemia* metanauplii – on gene expression of selected metabolic enzymes in whole-body seabream larvae at 25 DAH from the REC treatment, compared to the Ctrl. Expression values were measured using real-time RT-PCR and normalized with α-elongation factor 1 transcript (EF1α). Relative fold differences between treatments are presented as means + SD (n = 6 in pools of 20 larvae). Significant statistical differences are denoted by * ($P < 0{\cdot}05$, Student's *t*-test).

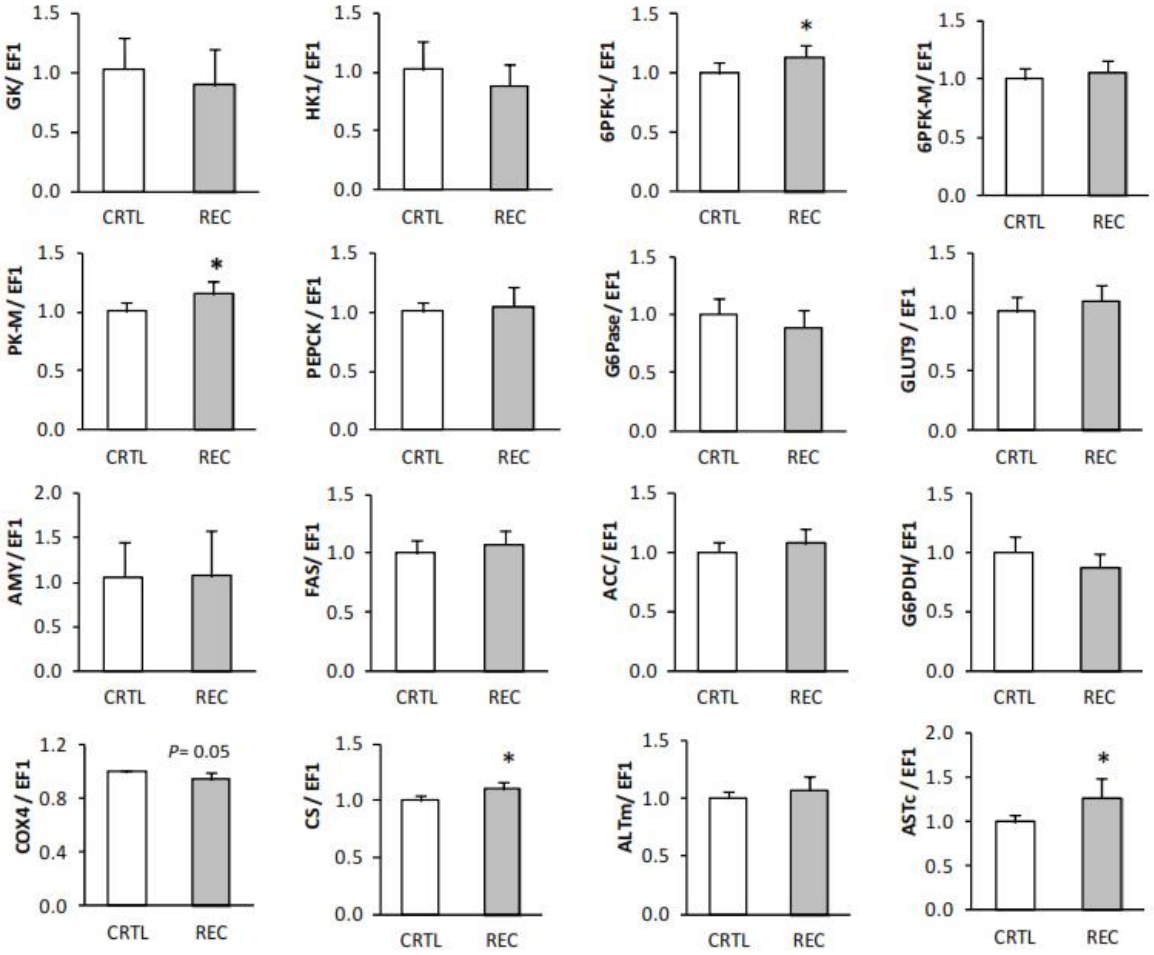

Figure 4.4 – Immediate effect of stimulus 3 – a high carbohydrate diet (HCD) – on gene expression of selected metabolic enzymes in whole-body seabream larvae at 35 DAH from the REC treatment, compared to the Ctrl. Expression values were measured using real-time RT-PCR and normalized with α-elongation factor 1 transcript (EF1α). Relative fold differences between treatments are presented as means + SD (n = 6 in pools of 20 larvae). Significant statistical differences are denoted by * ($P < 0{\cdot}05$, Student's *t*-test).

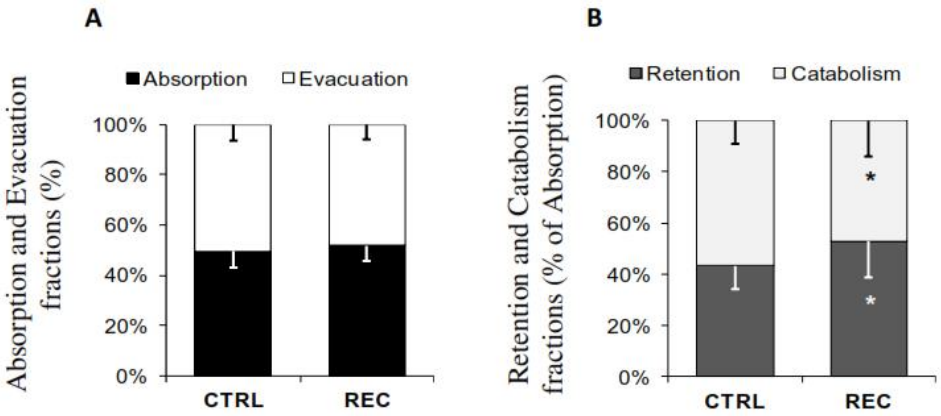

Figure 4.5 – Metabolic flux of ^{14}C-glucose in seabream larvae (35 DAH) after the delivery of stimulus 3 with a high carbohydrate diet **(A)** Proportion (%) of radiolabel absorbed (larvae and CO_2 trap) and evacuated (water) in relation to the total quantity of radiolabeled feed, after 24 h of incubation, in seabream larvae **(B)** Proportion (%) of radiolabel retained in tissues (larval body) and catabolized (CO_2 trap) in relation to the total absorbed label after 24 h of incubation, in seabream larvae. Values are means – SD of seabream absorption, evacuation, retention and catabolism fractions (n= 20 individuals). CTRL, control group (larvae never fed with glucose); REC, recurrent group (larvae fed the three glucose stimuli at early stages) *Denotes significant differences between treatments for the corresponding fraction ($P < 0{\cdot}05$, Student's *t*-test).

4.3.4. Effects of early glucidic stimuli in seabream post-larvae challenged with a high carbohydrate diet

The dietary challenge with the HCD diet (50% of carbohydrate content) was performed at a later stage of seabream development (60DAH) to analyse possible effects of the early-feeding stimuli on:

4.3.4.1 Growth performance and survival

At the end of a 60 day growth trial, no differences were found in growth and survival of seabream larvae, showing that early glucose stimuli were not detrimental for a correct larval development ($P> 0.05$) (Table 4.5). Larvae presented an exponential growth rate in both treatments during the experimental period and at the end of the trial, post-larvae showed similar dry weight (2.57 – 2.60 mg) and total length (13.11 – 13.13 mm). REC larvae had higher dry weight (0.90 mg) at the end of stimulus 3 when compared to the CTRL group (0.85 mg) ($P<0.05$), but this effect was not persistent until the end of the trial. At the end of the trial, survival ranged between 8.6% for the CTRL group and 8.1% for the REC group (Table 4.5).

Table 4.5 – Growth performance of the experimental groups of seabream larvae following the three periods of stimuli delivery and dietary challenge and survival rate at the end of the trial

		Stimulus 1	Stimulus 2	Stimulus 3	Dietary challenge
Dry weight (mg larvae^{-1})	*CTRL*	0.06 ± 0.01	0.30 ± 0.09	0.85 ± 0.22	2.57 ± 1.04
	REC	0.07 ± 0.01	0.31 ± 0.08	0.99 ± 0.28 *	2.60 ± 1.11
Total length (mm larvae^{-1})	*CTRL*	4.44 ± 0.30	7.78 ± 1.10	9.97 ± 0.23	13.13 ± 1.21
	REC	4.42 ± 0.37	8.20 ± 1.09	10.34 ± 0.28	13.11 ± 1.26
Survival (%)	*CTRL*				8.6 ± 3.60
	REC				8.1 ± 1.87

Values are means ± s.d. (n=60, per each sampled point). Significant differences between treatments are denoted by * ($P < 0.05$, Student's *t*-test). CTRL: control group (larvae never fed with glucose); REC: recurrent group (larvae fed the three glucose stimuli at early stages); Stimulus 1: glucose enriched rotifers (from 3 to 8DAH); Stimulus 2: glucose enriched *Artemia metanauplii* (from 20 to 25DAH); Stimulus 3: high carbohydrate diet, HCD (from 30 to 35DAH); Dietary challenge: 10-day period of feeding the HCD diet (from 50 to 60DAH).

4.3.4.2 Regulation of metabolic gene expression

The same panel of genes tested after the delivery of stimulus 3 (35DAH) was analysed in visceral and muscle tissues of challenged seabream post-larvae (60DAH). For the majority of analised genes, mRNA levels remained unaltered between the CTRL and REC treatments. No differences were detected, in both viscera and muscle, for the expression of glycolytic enzymes (GK, 6PFK-L, PK-M), lipogenic and energy-related enzymes (FAS, CS) and amino acid catabolising enzymes (ASTc) that were previously shown to be regulated by stimuli 3 (Figure 4.6-A, B). Only G6PDH gene was significantly down-regulated in viscera of REC larvae ($P<0.05$) while CS gene (also involved in energy metabolism) showed a slight but not significant increase of its expression ($P=0.06$) (Figure 4.6-A). Amylase gene was considered not expressed in visceral tissue of post-larvae, since CT values of real time PCR were higher than 30. All other metabolic genes that did not respond differently to the early stimulus were still unchanged after the dietary challenge (data not shown).

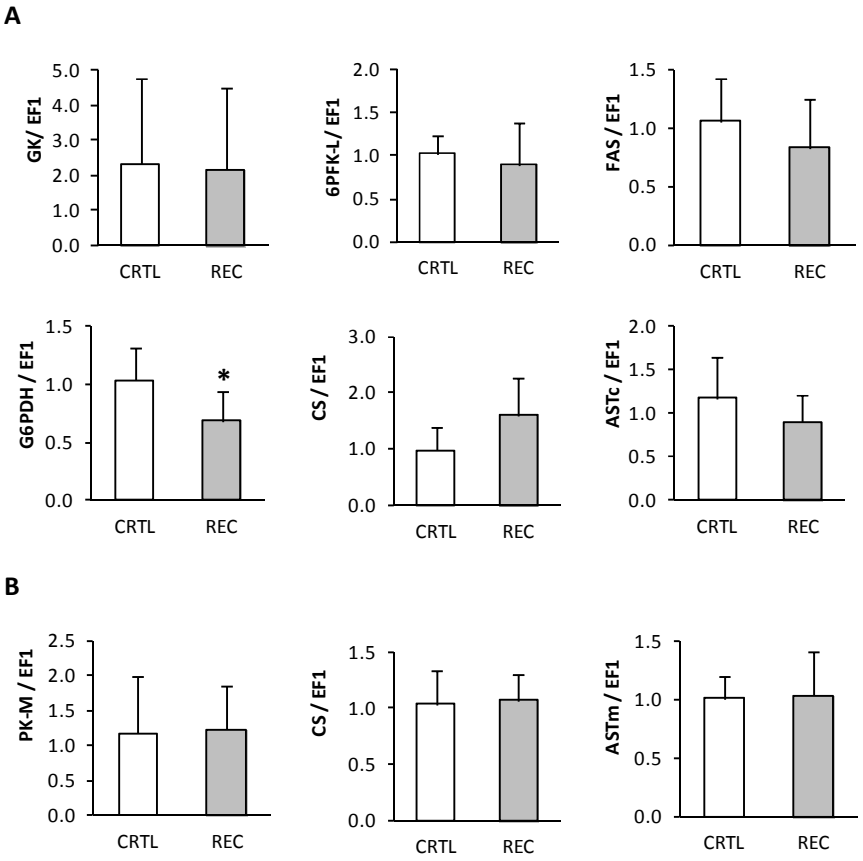

Figure 4.6 – Effects induced by the early-feeding stimuli on gene expression of selected metabolic enzymes in **(A)** visceral and **(B)** muscle tissues from seabream post-larvae (60 DAH) challenged with a high carbohydrate diet. The presented genes were selected based on the short-term effects of stimuli delivery previously observed (see Figures 2, 3 and 4). The expression of the other genes tested in larvae was not significantly affected by the early stimuli at this stage (data not shown). Expression values were measured using real-time RT-PCR and normalized with α-elongation factor 1 transcript (EF1α). Relative fold differences between treatments are presented as means + SD (n=6 individuals). Significant statistical differences are denoted by * ($P < 0{\cdot}05$, Student's *t*-test).

4.3.4.3. Regulation of carbohydrate and amino acid metabolism

Effects related to the early nutritional stimuli were found in both glucose and amino acid metabolic studies performed with ^{14}C-glucose and ^{14}C-AA mixture tracers. In the glucose flux study, results showed that larvae from REC treatment had a lower capacity for glucose absorption in the gut (50%) which was reflected in a higher evacuation of the nutrient (50%) when compared to the CTRL larvae (55% and 45%, respectively, $P<0.05$) (Figure 4.7-A). Larvae from REC treatment showed an improved utilization of the absorbed glucose by promoting a higher catabolism (59%) and lower body glucose retention (41%) when compared to the CTRL group ($P<0.05$). These results were the opposite of what was observed after the delivery of stimulus 3, meaning the glucose utilization pattern drastically changed towards higher glucose breakdown (Figure 4.7-B). Glucose was preferentially converted into protein and FAA in both treatments and no differences were found for these fractions between CTRL and REC treatments ($P>0.05$). Together these fractions held almost 77% of all retained glucose. Significant differences were found for the bio-conversion of glucose into lipids, larvae from REC group had a higher lipid fraction than the CTRL group ($P<0.05$). Of the retained ^{14}C-label in REC larvae, 38% was found in the protein fraction, 39% in the FAA, 16% in lipids and only 7% in the other metabolites fraction, which included glycogen. For CTRL larvae the retention of glucose was fractionated into 47% protein, 31 % FAA, 12 % lipids and 10% of other metabolites (Figure 4.7-C). From the amino acid flux study, results showed that neither absorption, evacuation, catabolism nor retention of amino acids were affected by the early glucidic stimuli in larvae of CTRL and REC treatments (Figure 4.8). Seabream post-larvae presented a moderate amino acid absorption in the gut of around 65%, which reflects the capacity at this stage to digest *Artemia* protein (Figure 4.8-A). From the absorbed amino acids, nearly 60% was retained in body tissues and 40% was catabolized (Figure 4.8-B). As expected, the amino acids were mostly retained as protein and FAA (near 93% of the retained label), in both treatments (Figure 4.8-C). The fraction retained as FAA was significantly lower in the REC group (20%), indicating a reduction of the FAA pool in post-larvae from this treatment when compared to the CTRL larvae (27%). No differences were found for the other retained fractions.

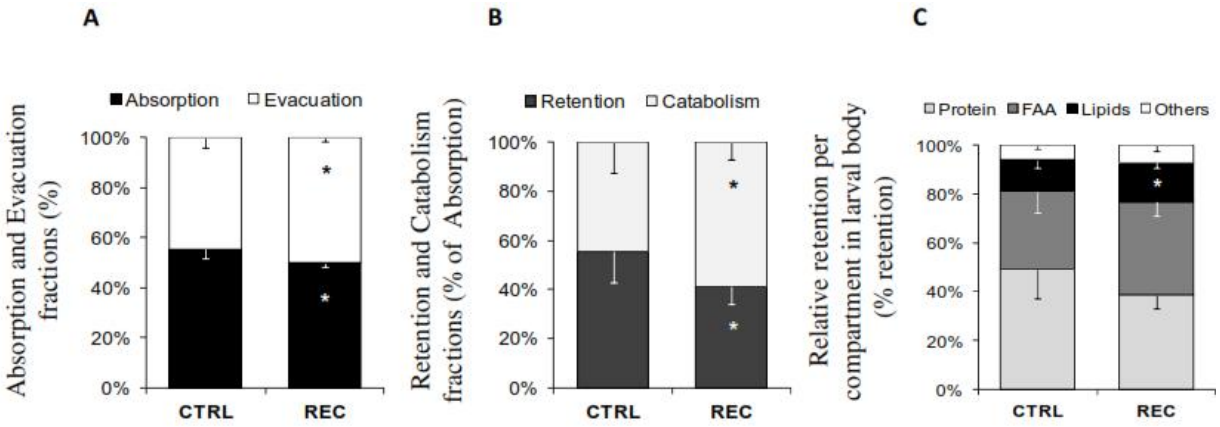

Figure 4.7 – Metabolic flux of ^{14}C-glucose in seabream post-larvae (60DAH) submitted to the dietary challenge with a high carbohydrate diet for 10 days **(A)** Proportion (%) of radiolabel absorbed (larvae and CO_2 trap) and evacuated (water) in relation to the total quantity of radiolabeled feed; **(B)** Proportion (%) of radiolabel retained in tissues (larval body) and catabolized (CO_2 trap) in relation to the total absorbed label; **(C)** Proportion (%) of relative retention of radiolabel in each fraction of larval body (protein, FAA, lipids and other metabolites) in relation to total retained label, in seabream post-larvae after 24 h of incubation. Values are means – s.d. of seabream absorption, evacuation, retention, catabolism and relative retention fractions (n= 10 individuals). *Denotes significant differences between treatments for the corresponding fraction ($P < 0{\cdot}05$, Student's *t*-test).

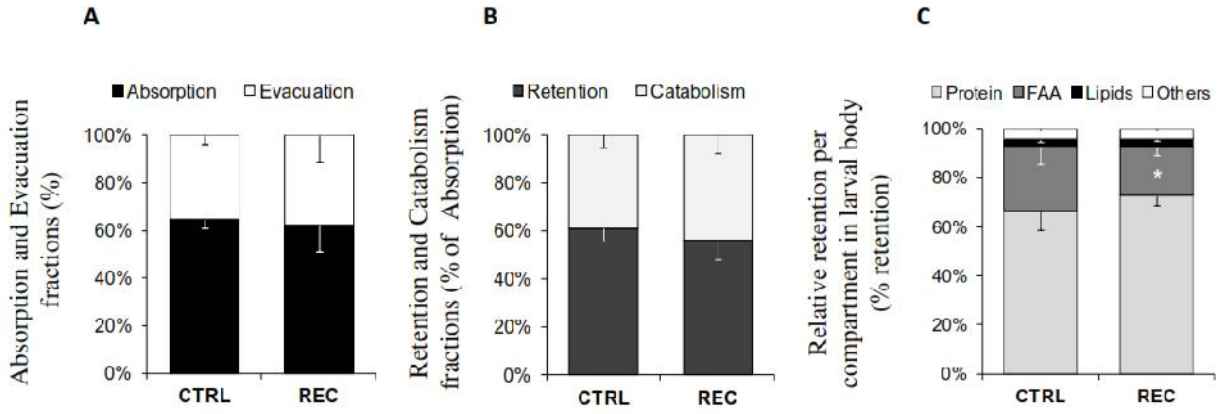

Figure 4.8 – Metabolic flux of ^{14}C-amino acid mixture in seabream post-larvae (60DAH) submitted to the dietary challenge with a high carbohydrate diet for 10 days **(A)** Proportion (%) of radiolabel absorbed (larvae and CO_2 trap) and evacuated (water) in relation to the total quantity of radiolabeled feed; **(B)** Proportion (%) of radiolabel retained in tissues (larval body) and catabolized (CO_2 trap) in relation to the total absorbed label, **(C)** Proportion (%) of relative retention of radiolabel in each fraction of larval body (protein, FAA, lipids and other metabolites) in relation to total retained label, in seabream post-larvae after 24 h of incubation. Values are means – SD of seabream absorption, evacuation, retention, catabolism and relative retention fractions (n= 10 individuals). *Denotes significant differences between CTRL and REC groups for the corresponding fraction ($P < 0{\cdot}05$, Student's *t*-test).

4.4. Discussion

A major endeavour in fish nutrition is to understand and improve digestibility and metabolic utilization of dietary nutrients by fish juveniles. Fish diets are generally protein-rich and approaches used to reduce the dietary protein cost involved in fish production rely on: a) the optimization of dietary protein utilization (sparing it for muscle growth) through an enhanced catabolic use of the non-protein energy supply (fats or digestible carbohydrates) (NCR, 2011); and b) a reduction of dietary inclusion levels of expensive protein sources (fishmeal) through the use of alternative protein sources (e.g. plant proteins) (Gatlin et al., 2007; Naylor et al., 2009). However, both scenarios imply an increased intake of carbohydrates, which is not well tolerated by most finfish with carnivorous feeding habits. In our study, we have explored the concept of nutritional programming as a tool to enhance the metabolic use of dietary carbohydrates by a marine fish species, gilthead seabream. Since mammals and fish are physiologically different in terms of embryonic development (*in uterus* vs *ex uterus*), the embryos are exposed to external stimuli in distinct ways, meaning that for fish the "windows" of high metabolic plasticity suitable for stimuli action are probably at embryogenesis and early larval development (e.g. during first-feeding stage). Promising studies upon applying the concept of nutritional programming in fish to improve their capacity to cope with high dietary carbohydrates have been conducted, mostly in fresh water species (Fang et al., 2014 ; Geurden et al., 2007; Geurden et al., 2014; Gong et al., 2015; Rocha et al., 2014; 2015). In contrast, only few were targeted to marine species (Vagner et al., 2007; Vagner et al., 2009) of which none was aimed to program the carbohydrate utilization. Fish larvae, the vertebrate organism with the highest growth potential (up to 100%/day) (Conceição et al., 1998) may be an optimal model organism to study the effect of a stimulus or event at a critical window of development and to assess rapidly a shift of the metabolic pathways.

Marine fish larvae´s high vulnerability in early stages of development must be considered when nutritional stimuli are exerted. So by offering enriched live preys as "vehicles" for glucose supplementation during early larval stages, it was ensured a high intake of glucose without introducing new stress factors to those already existing during the larval rearing. The bioencapsulation of glucose with the GLU enrichment was well succeeded in rotifers and *Artemia*, showing an increase of 38.6% and 294%, respectively, of their level of total carbohydrates in comparison to those fed a standard enrichment. These results validate the use of live prey as effective delivery vectors of glucidic stimuli to fish larvae. Previous studies

had shown that changes on the emulsions or microalgae used to enrich live preys allows us to tailor the nutritional content of both rotifers and *Artemia* in terms of overall protein and lipid levels, specific amino acids and fatty acids, vitamins and trace elements (Hamre et al., 2013; Hawkyard et al., 2014; Matsumoto et al., 2009).

Our results also show that each five-day hyperglucidic stimulus (stimulus 1, 2 and 3) modified metabolic gene expressions in the seabream larvae. Although few, some genes involved in glycolysis, lipogenesis and energy production pathways were enhanced: GK and FAS (by stimulus 1), G6PDH (by stimulus 2) and 6PFK-L, PK and CS genes (by stimulus 3). These data are similar to the postprandial induction of gene expression observed in rainbow trout, gilthead sea bream and zebrafish, after a high carbohydrate intake (Enes et al., 2008b; Kamalam et al., 2012; Meton et al., 2004; Panserat et al., 2000; Polakof et al., 2012b; Seiliez et al., 2013), comparable to what occurs in mammals (Kersten, 2001; Pilkis and Granner, 1992; Yamada and Noguchi, 1999). Moreover, a similar short-term effect in the up-regulation of the expression of glycolytic enzymes (GK and HK1) was observed in rainbow trout and zebrafish fed at first-feeding with a high level (50 to 65%) of dietary carbohydrates (Fang et al., 2014 ; Geurden et al., 2007; Geurden et al., 2014). In several fish species, glucose in excess stimulates the action of hepatic glucokinase enzyme (GK) for the conversion and storage of glucose into lipids (also glycogen) and eventually, also enhances the pathways involved in lipogenesis and NADPH/energy production (Panserat et al., 2014; Polakof et al., 2012a), suggesting that the glucidic stimuli may had induced a similar modulation of the lipogenesis pathway in REC larvae. The action of stimulus 1 revealed to be of great interest since it allowed the nutritional manipulation of the first exogenous meal, at the end of the lecithotrophic larval phase, and resulted in the only stimulus capable of inducing GK expression in the seabream larvae. Nevertheless, the glucidic stimuli delivered by live feed (stimulus 1 and 2) also decreased the expression of genes involved in the second step of glycolysis (6PFK-L), in NADPH production (G6PDH) and in carbohydrate digestion (AMY). The latter enzyme was found up-regulated after early-feeding a complex carbohydrate stimulus during 3-day, showing that different sources of carbohydrates in the diet could enhance the transcript levels of digestive enzymes (Fang et al., 2014 ; Geurden et al., 2007). In the present study glucose was used as source for the glucidic stimuli, and we observed a negative acute effect on amylase expression, which may be related to an excess (overload) of the final product of this enzyme, as it was seen previously in zebrafish larvae (Rocha et al., 2014). It has been shown that amylase expression may be repressed in lower organism when

high levels of glucose are present: the bacterial amylase transcription is regulated by a catabolite repression mechanism (Nicholson et al., 1987) and in *Drosophila* the amylase expression is repressed after high glucose intakes (Benkel and Hickey, 1986). Seabream larvae were fed for 5-days with high-glucose *Artemia* in the present study, so a sudden increase of circulating glucose levels may have occurred leading to a down-regulation of AMY expression, analogous to the regulation observed in lower organisms. Finally, the absence of regulation of genes involved in gluconeogenesis (G6Pase and PEPCK) observed after the delivery of the three stimuli, is in agreement with the lack of transcriptional regulation of this pathway by dietary carbohydrates, observed in several fish species, including juvenile seabream (Enes et al., 2008a; Panserat et al., 2001; Polakof et al., 2012a; Seiliez et al., 2013). Recent findings in rainbow trout, Siberian sturgeon and zebrafish larvae fed from first feeding with a high carbohydrate diet (50% to 65%) showed that the immediate action of stimulus could affect (inhibit) or not the expression of gluconeogenic genes (G6Pase, PEPCK and FBPase) (Fang et al., 2014 ; Geurden et al., 2014; Gong et al., 2015). As far as we know, these are the first data for gilthead seabream confirming a molecular regulation of some steps of glucose metabolism, lipogenesis and energy production by the early glucidic stimuli.

We also analised whether the early nutritional event could change the metabolic phenotype of glucose utilization, by means of a tracer study in larvae after stimulus 3, using labeled ^{14}C-glucose. Seabream larvae showed a similar capacity to absorb glucose (50%) at this stage of development (35DAH), regardless of the early nutritional experience. Indeed this ability for glucose absorption in the gut may be an indicator of full maturation of the intestine at this age (Zambonino-Infante et al., 2008). Of interest, larvae from the recurrent stimuli (REC) had a significantly lower catabolism, and a concomitantly higher body retention of the absorbed ^{14}C- glucose, in comparison with the control group (CTRL), suggesting the preferential steering of glucose towards storage pathways instead of towards energy production. Given that no further analysis on protein, lipid or glycogen retention was performed at this stage of development, our hypothesis on how the glucose-derived ^{14}C was retained in the larval tissues is limited. However, our data collected at the later post-larvae stage revealed that glucose was preferentially retained as protein and free AA. It is known that fish larvae are able to discriminate the use of amino acids for energy purposes (dispensable AA) or for protein deposition and growth (indispensable AA) (Aragão et al., 2004; Conceição et al., 2002) during periods of fast growth as found before metamorphosis (Russo et al., 2007). Thus, we

believe that REC larvae were able to discriminate glucose use similarly to AA utilization. The higher glucose-derived ^{14}C-retention in the REC larvae (35DAH) may reflect an improved storage and later use of this substrate for protein accretion and growth, rather than use as energy source. The observed up-regulation of 6PFK-L, PK and CS genes in the REC larvae does not necessarily mean a direct glucose catabolism, but an adaptation to generate anaplerotic intermediates to replenish the citric acid cycle. Even so, seabream larvae seemed to tolerate hyperglucidic stimuli without jeopardizing survival and our findings suggest some molecular and metabolic adaptations to the early glucose stimuli that might be related to a possible nutritional programming.

Besides characterizing changes on molecular and metabolic pathways immediately after the hyperglucidic stimuli, our study further assessed possible persistency of effects, by challenging both the REC vs CTRL groups at the later post-larval stage with a high carbohydrate diet (HCD). By the end of the dietary challenge, we found that both the early stimuli and the high carbohydrate intake associated to the challenge caused no detrimental effects on final growth and survival of seabream post-larvae, up to 60DAH. The lack of the negative effects on physiological parameters is in conformity with other nutritional programming studies performed in fish (Fang et al., 2014 ; Geurden et al., 2007; Geurden et al., 2014; Rocha et al., 2014; 2015). Also, both growth (around 2.6 mg and 13.13 mm) and survival rate (around 8.6%) were within the expected range for seabream larvae reared under experimental conditions up to 60DAH, with a similar feeding regime. Costa (2012) achieved a survival rate of 12.5% and a total length of larvae of 15.9 mm for 60-day seabream, fed from mouth opening with life prey; likewise, Dimitrios et al. (2010) showed a good growth and survival rate (12 mm and 11%, respectively) for seabream fed exclusively with *Artemia* up to 40DAH. For seabream, as for other marine species, one of the most limiting factors for a successful larval rearing is related to larvae nutrition and their feeding physiology. Whereas in the present study, larvae were repeatedly exposed to high levels of dietary glucose the survival rate, even though reduced (8.6%), was still comparable to other experimental studies free of any early nutritional manipulation.

Concerning the molecular effects, we found that most of the metabolic genes that were differently expressed immediately after the exposure to glucidic stimuli (GK, FAS, PK, CS and ASTc up-regulation, AMY down-regulation and 6PFK-L and G6PDH double regulation) were no longer regulated in the tissues of seabream post-larvae, except for the G6PDH gene.

Indeed, the regulation of gene expression was less pronounced at later stages of seabream development, showing that short-term effects of the early stimuli might have faded with time. These observations denote that the early nutritional history had no marked effects on modulating, at a molecular level, the three major pathways involved in glucose metabolism: glycolysis, gluconeogenesis and lipogenesis. Opposite to the present findings, zebrafish embryos submitted to a glucose stimulus (using microinjection) showed a poor molecular response to glucose at the early larval stages, whereas at the juvenile stage, there were several long-term modifications on the expression of genes involved in glycolysis (HK1, 6PFK) and gluconeogenesis (PEPCK, FBP) (Rocha et al., 2015). Also, in rainbow trout an early carbohydrate feeding induced some persistent changes in the gene expression of HK1, PK (glycolysis) and GLUT4 (transporter) of juvenile fish, which surprisingly were not the same genes modified immediately after stimulus delivery (Geurden et al., 2014). It seems thus that glucidic stimuli can affect differently the molecular regulation at short- or long-term, indicating that adaptations might be linked to the stimulus nature, duration, period of interventions and/or to fish species. The analysis of gene expression pattern is considered as a useful tool to understand possible "imprinting" effects in adulthood in response to early nutritional events, since one of the proposed mechanisms for nutritional programming in mammals is the epigenetic regulation of gene expression (Lucas, 1998; Symonds et al., 2009). But other mechanisms may also be involved such as differential cellular proliferation, that occurs when the quantity or proportion of cells in a tissue is permanently affected by a nutritional event, as illustrated by the decrease of islet size and cell proliferation in the offspring of rats fed a low protein diet during pregnancy (Snoeck et al., 1990). Therefore, the lack of a strong molecular modulation in the post-larvae by the early stimuli does not exclude the possibility of nutritional programming effects in this study. Indeed, it was shown in mammals that the particular type of mechanism involved in nutritional programming seems to vary between tissues, according to the duration and timing of the nutritional intervention through pregnancy and/or lactation (Symonds et al., 2009)

Using a labeled glucose tracer, we assessed the effects of the early glucose stimuli on the use of ^{14}C-glucose by the seabream post-larvae at the end of the high carbohydrate diet challenge. Post-larvae from the REC group showed a reduced capacity for glucose absorption in the gut. From the absorbed fraction, REC compared to Ctrl group post-larvae showed a higher catabolism of ^{14}C-glucose and a concomitantly lower retention, suggestive of enhanced glucose oxidation or allocation to other metabolic pathways. Similarly, in zebrafish, the

supplementation of yolk-sac embryos with glucose was associated with reduced glucose retention in visceral tissue of juvenile fish, after a high dietary carbohydrate intake (Rocha et al., 2015). As mentioned before, glucose may be an important anabolic substrate during periods of high larval growth (35DAH), being used for protein synthesis. Moreover, the analysis of the retention of glucose-derived ^{14}C in the post-larval body fractions (during a period of incubation of 24 h) showed the ^{14}C carbon was mainly recovered in the FAA and protein fraction, for both REC and CRTL treatments. This clearly demonstrates the capacity of seabream post-larvae to use glucose as a precursor of amino acid synthesis and protein accretion. These two fractions (protein and FAA) represented almost 77% of all retained ^{14}C-glucose while the conversion into lipids and glycogen was much lower than was expected. Earlier studies on defining the essentiality of amino acids in fish, also relying in isotopic-labeling approaches, showed the incorporation of glucose-derived ^{14}C skeletons in dispensable amino acid pool by *de novo* synthesis, which might occur from the citric-acid cycle intermediates, as observed in mammals; for instance aspartate may be synthesized from oxaloacetate, whereas glutamate and glutamine may be synthesized from α-ketoglutarate (Bequette et al., 2006; Cowey et al., 1970; Hellman and Larsson, 1961). Although relatively well studied in juvenile and adult fish, the balance between anaplerosis (replenishment of the pools of metabolic intermediates in the citric-acid cycle) and cataplerosis (removal of citric-acid cycle intermediates) pathways is little known in fish larval stages, which typically present extremely high rates of growth and protein synthesis (Hamre et al., 2013). The REC post-larvae presented also a higher relative retention of glucose carbon in the whole body lipid fraction in comparison to the CTRL group. Such modification may indicate that larval conditioning to hyperglucidic stimuli directed the ^{14}C-glucose proportionally more towards lipogenesis in the REC relative to the CTRL post-larvae challenged with excess dietary glucose. The stimulation of hepatic lipogenesis by a high intake of dietary carbohydrates was described in rainbow trout (Polakof et al., 2011), in a similar way to what occurs in mammals (Towle et al., 1997). Also, seabream fed with ^{13}C-starch labeled diets showed that increased proportions of dietary starch stimulated *de novo* lipogenesis in both whole-fish and liver tissues (Ekmann et al., 2013). Taken together, the finding that the early stimuli acted as short-term modulators of several metabolic pathways, reinforce the initial hypothesis of nutritional programming at later stages of seabream development.

The effects of early hyperglucidic stimuli were also analised in terms of dietary amino acid use, in order to see possible effects on the AA retention efficiency in the post-larvae, at the

end of the challenge period. Post-larvae from both the CTRL and REC treatments displayed a 65% absorption of the ^{14}C-amino acid mixture, which reflects a moderate *Artemia* protein digestibility. Also, only 56% to 61% of the absorbed label was retained in the tissues of the 60 DAH seabream. No previous tracer studies using the *Artemia* labeling method in seabream larvae focussed before on AA metabolism. However, similar studies in 35DAH Senegalese sole larvae using ^{14}C-AA revealed a high *Artemia* protein digestibility (80%) as well as an efficient protein retention (77%) (Engrola et al., 2009). Similar values for ^{14}C-AA gut absorption and tissue retention were reported by Morais et al. (2004b), Engrola et al. (2010) and Campos et al. (2013) at 35DAH, 21DAH and 49DAH sole post-larvae, respectively. The lack of differences in amino acid absorption, retention and catabolism between the treatments indicates that early glucose conditioning did not affect the gluconeogenesis from amino acids (in conformity with the molecular data) and also did not cause a protein sparing effect in the seabream post-larvae. Further, a major amount of the retained label was first recovered into the FAA pool and later into the protein fraction, corresponding to nearly 93% of the absorbed amount. The REC post-larvae showed a significantly lower pool of ^{14}C-FAA compared to the CTRL group. This may be related to a faster synthesis of protein by the REC post-larvae, which also had a slightly though not significantly higher ^{14}C incorporation in the protein fraction. Possibly the incubation period (24 h) should be extended in order to better determine all *de novo* synthesis of protein from dietary AA.

4.5. Conclusions

The seabream larvae tolerated the recurrent hyperglucidic stimuli, during critical stages of development, without compromising growth or survival. The glucidic stimuli caused some immediate responses at a molecular level, but these tended to fade over larval ontogeny. However, the early glucidic stimuli induced some short-term changes in the post-larval glucose metabolic phenotype, characterized by an increase in glucose oxidation, and also a proportionally higher use of glucose in lipogenesis. Our data suggest that repeated exposures to high-glucose stimuli can promote the modulation of specific pathways involved in glucose utilization in seabream larvae, unlocking the possibility for nutritional programming of adult fish. Still, further investigation is needed to fully understand the interaction between the

genomic and metabolomic regulation pathways, which are extremely relevant to the concept of nutritional programming in fish.

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4.7. References

Aragão, C., Conceição, L.E.C., Martins, D., Rønnestad, I., Gomes, E., amp, x, dio, Dinis, M.T., 2004. A balanced dietary amino acid profile improves amino acid retention in post-larval Senegalese sole (Solea senegalensis). Aquaculture. 233, 293-304.

Benkel, B.F., Hickey, D.A., 1986. Glucose Repression of Amylase Gene Expression in *Drosophila melanogaster*. Genetics. 114, 137-144.

Bequette, B.J., Sunny, N.E., El-Kadi, S.W., Owens, S.L., 2006. Application of stable isotopes and mass isotopomer distribution analysis to the study of intermediary metabolism of nutrients. J Anim Sci. 84 Suppl, E50-59.

Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G.F., Folkedal, O., Olsen, R.E., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014. Metabolic and transcriptional responses of gilthead sea bream (Sparus aurata L.) to environmental stress: New insights in fish mitochondrial phenotyping. Gen Comp Endocrinol. 205, 305-315.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purificaton. Can J Biochem Phys. 37, 911-917.

Burdge, G.C., Lillycrop, K.A., 2010. Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr. 30, 315-339.

Campos, C., Castanheira, M.F., Engrola, S., Valente, L.M., Fernandes, J.M., Conceicao, L.E., 2013. Rearing temperature affects Senegalese sole (Solea senegalensis) larvae protein metabolic capacity. Fish Physiol Biochem. 39, 1485-1496.

Cara, J.B., Moyano, F.J., Cárdenas, S., Fernández-Díaz, C., Yúfera, M., 2003. Assessment of digestive enzyme activities during larval development of white bream. J Fish Biol 63, 48-58.

Conceição, L.E.C., Dersjant-Li, Y., Verreth, J.A.J., 1998. Cost of growth in larval and juvenile African catfish (Clarias gariepinus) in relation to growth rate, food intake and oxygen consumption. Aquaculture. 161, 95-106.

Conceição, L.E.C., Rønnestad, I., Tonheim, S.K., 2002. Metabolic budgets for lysine and glutamate in unfed herring (Clupea harengus) larvae. Aquaculture. 206, 305-312.

Conceição, L.E.C., Morais, S., Rønnestad, I., 2007. Tracers in fish larvae nutrition: A review of methods and applications. Aquaculture. 267, 62-75.

Conceição, L.E.C., Aragão, C., Richard, N., Engrola, S., Gavaia, P., Mira, S., Dias, J., 2010. Novel methodologies in marine fish larval nutrition. Fish Physiol Biochem. 36, 1-16.

Costa, J.R., 2012. Sea bream (Sparus aurata) intensive larval rearing: effects of microalgae and phages addition on the performance of the larvae. Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, pp. 90.

Cowey, C.B., Adron, J., Blair, A., 1970. Studies on the nutrition of marine flatfish the essential amino acid requirements of plaice and sole. J. Mar. Biol. Assoc. U.K. 50, 87-95.

Diaz, J.P., mani-Ponset, L., Guyot, E., Connes, R., 1994. Assimilation of dissolved glucose from sea water by the sea bass Dicentrarchus labrax and the sea bream Sparus aurata. Mar Biol 120, 181-186.

Dimitrios, D., Panagiota, P., Eleni, G., Emmanuil, M., Olga, D., Petros, M., Athanassios, E., Spyridon, K., Christos, N., 2010. Partial replacement of fresh algae with dry, in intensive seabream larval production. International Aquatic Research. 2, 163-166.

Ekmann, K.S., Dalsgaard, J., Holm, J., Campbell, P.J., Skov, P.V., 2013. Glycogenesis and de novo lipid synthesis from dietary starch in juvenile gilthead sea bream (Sparus aurata) quantified with stable isotopes. Br J Nutr. 109, 2135-2146.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008a. Hepatic glucokinase and glucose-6-phosphatase responses to dietary glucose and starch in gilthead sea bream (Sparus aurata) juveniles reared at two temperatures. Comp Biochem Physiol A Mol Integr Physiol. 149, 80-86.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008b. Rearing temperature enhances hepatic glucokinase but not glucose-6-phosphatase activities in European sea bass (Dicentrarchus labrax) and gilthead sea bream (Sparus aurata) juveniles fed with the same level of glucose. Comp Biochem Physiol A Mol Integr Physiol. 150, 355-358.

Engrola, S., Mai, M., Dinis, M.T., Conceição, L.E.C., 2009. Co-feeding of inert diet from mouth opening does not impair protein utilization by Senegalese sole (Solea senegalensis) larvae. Aquaculture. 287, 185-190.

Engrola, S., Dinis, M.T., Conceição, L.E.C., 2010. Senegalese sole larvae growth and protein utilization is depressed when co-fed high levels of inert diet and Artemia since first feeding. Aquacult Nutr. 16, 457-465.

Ennos, A.R., 2007. Statistical and data handling skills in biology, 2nd ed. Pearson Prentice Hall, Harlow, England.

Fang, L., Liang, X.F., Zhou, Y., Guo, X.Z., He, Y., Yi, T.L., Liu, L.W., Yuan, X.C., Tao, Y.X., 2014 Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, Danio rerio. Br J Nutr. 111, 808-818.

FEAP, 2014. Annual Report 2014. Federation of European Aquaculture Producers, Belgium.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., J Souza, E., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac Res. 38, 551-579.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (Oncorhynchus mykiss) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am J Physiol Regul Integr Comp Physiol. 292, 2275-2283.

Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D., Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S., Panserat, S., 2014. High or low dietary carbohydrate:protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout. J Exp Biol. 217, 3396-3406.

Gong, G., Xue, M., Wang, J., Wu, X.-f., Zheng, Y.-h., Han, F., Liang, X.-f., Su, X.-o., 2015. The regulation of gluconeogenesis in the Siberian sturgeon (Acipenser baerii) affected later in life by a short-term high-glucose programming during early life. Aquaculture. 436, 127-136.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E.C., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Reviews in Aquaculture. 5, S26-S58.

Harder, T., Plagemann, A., Rohde, W., Dorner, G., 1998. Syndrome X-like alterations in adult female rats due to neonatal insulin treatment. Metabolism. 47, 855-862.

Hawkyard, M., Laurel, B., Langdon, C., 2014. Rotifers enriched with taurine by microparticulate and dissolved enrichment methods influence the growth and metamorphic development of northern rock sole (Lepidopsetta polyxystra) larvae. Aquaculture. 424–425, 151-157.

Hellman, B., Larsson, S., 1961. The glucose metabolism in the islets of Langerhans. I. In vitro studies of the fate of uniformly 14C-labelled glucose and fructose in Cottus quadricornis L. Acta Endocrinol (Copenh). 38, 303-314.

Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 33 Suppl, 245-254.

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S., 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J Exp Biol. 215, 2567-2578.

Kersten, S., 2001. Mechanisms of nutritional and hormonal regulation of lipogenesis. EMBO Rep. 2, 282-286.

Kolkovski, S., Tandler, A., Wm. Kissil, G., A., G., 1993. The effect of dietary exogenous digestive enzymes on ingestion, assimilation, growth and survival of gilthead seabream (Sparus aurata, Sparidae, Linnaeus) larvae. Fish Physiol Biochem 12, 203-209.

Lucas, A., 1998. Programming by early nutrition: an experimental approach. J Nutr 128, 401-406.

Matsumoto, S., Satoh, S., Kotani, T., Fushimi, H., 2009. Examination of a practical method for zinc enrichment of euryhaline rotifers (Brachionus plicatilis). Aquaculture. 286, 113-120.

Maurizi, A., Diaz, J.P., Divanach, P., Papandroulakis, N., Connes, R., 2000. The effect of glycerol dissolved in the rearing water on the transition to exotrophy in gilthead sea bream Sparus aurata larvae. Aquaculture. 189, 119-131.

Metges, C.C., Gors, S., Lang, I.S., Hammon, H.M., Brussow, K.P., Weitzel, J.M., Nurnberg, G., Rehfeldt, C., Otten, W., 2014. Low and high dietary protein:carbohydrate ratios during pregnancy affect materno-fetal glucose metabolism in pigs. J Nutr. 144, 155-163.

Meton, I., Caseras, A., Fernandez, F., Baanante, I.V., 2004. Molecular cloning of hepatic glucose-6-phosphatase catalytic subunit from gilthead sea bream (Sparus aurata): response of its mRNA levels and glucokinase expression to refeeding and diet composition. Comp Biochem Physiol B Biochem Mol Biol. 138, 145-153.

Morais, S., Conceição, L., amp, x, s, E.C., Dinis, M.T., Rønnestad, I., 2004a. A method for radiolabeling Artemia with applications in studies of food intake, digestibility, protein and amino acid metabolism in larval fish. Aquaculture. 231, 469-487.

Morais, S., Lacuisse, M., Conceição, L.E.C., Dinis, M.T., Rønnestad, I., 2004b. Ontogeny of the digestive capacity of Senegalese sole (Solea senegalensis), with respect to digestion, absorption and metabolism of amino acids from Artemia. Mar Biol 145, 243-250.

Morais, S., Torten, M., Nixon, O., Lutzky, S., Conceição, L.E.C., Dinis, M.T., Tandler, A., Koven, W., 2006. Food intake and absorption are affected by dietary lipid level and lipid source in seabream (Sparus aurata L.) larvae. J Exp Mar Biol Ecol 331, 51-63.

Moyano, F.J., Díaz, M., Alarcón, F.J., Sarasquete, M.C., 1996. Characterization of digestive enzyme activity during larval development of gilthead seabream (Sparus aurata). Fish Physiol Biochem. 15, 121-130.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D., 2009. Feeding aquaculture in an

era of finite resources. Proceedings of the National Academy of Sciences USA 106, 15103-15110.

Naz, M., 2009. Ontogeny of Biochemical Phases of Fertilized Eggs and Yolk Sac Larvae of Gilthead Seabream (Sparus aurata L.). Turk J Fish Aquat Sci. 9, 77-83.

NCR, 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washington, DC.

Nicholson, W.L., Park, Y.-K., Henkin, T.M., Won, M., Weickert, M.J., Gaskell, J.A., Chambliss, G.H., 1987. Catabolite repression-resistant mutations of the Bacillus subtilis alpha-amylase promoter affect transcription levels and are in an operator-like sequence. J. Mol. Biol. 198, 609-618.

Panchout, F., Letendre, J., Bultelle, F., Denier, X., Rocher, B., Chan, P., Vaudry, D., Durand, F., 2013. Comparison of protein-extraction methods for gills of the shore crab, Carcinus maenas (L.), and application to 2DE. J Biomol Tech. 24, 218-223.

Panserat, S., Medale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R., Kaushik, S., 2000. Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. Am J Physiol Regul Integr Comp Physiol. 278, 1164-1170.

Panserat, S., Plagnes-Juan, E., Breque, J., Kaushik, S., 2001. Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol. 204, 359-365.

Panserat, S., Rideau, N., Polakof, S., 2014. Nutritional regulation of glucokinase: a cross-species story. Nutr Res Rev. 27, 21-47.

Patel, M.S., Srinivasan, M., 2002. Metabolic programming: causes and consequences. J Biol Chem. 277, 1629-1632.

Peres, A., Cahu, C.L., Zambonino Infante, J.L., Le Gall, M.M., Quazuguel, P., 1996. Amylase and trypsin responses to intake of dietary carbohydrate and protein depend on the developmental stage in sea bass (Dicentrarchus labrax) larvae. Fish Physiol Biochem. 15, 237-242.

Perez-Sanchez, J., Borrel, M., Bermejo-Nogales, A., Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J.A., Kaushik, S., 2013. Dietary oils mediate cortisol kinetics and the hepatic mRNA expression profile of stress-responsive genes in gilthead sea bream (Sparus aurata) exposed to crowding stress. Implications on energy homeostasis and stress susceptibility. Comp Biochem Physiol Part D Genomics Proteomics. 8, 123-130.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Pilkis, S.J., Granner, D.K., 1992. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. Annu Rev Physiol. 54, 885-909.

Polakof, S., Medale, F., Larroquet, L., Vachot, C., Corraze, G., Panserat, S., 2011. Regulation of de novo hepatic lipogenesis by insulin infusion in rainbow trout fed a high-carbohydrate diet. J Anim Sci. 89, 3079-3088.

Polakof, S., Panserat, S., Soengas, J.L., Moon, T.W., 2012a. Glucose metabolism in fish: a review. J Comp Physiol B.

Polakof, S., Skiba-Cassy, S., Kaushik, S., Seiliez, I., Soengas, J.L., Panserat, S., 2012b. Glucose and lipid metabolism in the pancreas of rainbow trout is regulated at the molecular level by nutritional status and carbohydrate intake. J Comp Physiol B. 182, 507-516.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M.T., Panserat, S., 2014. Glucose overload in yolk has little effect on the long-term modulation of carbohydrate metabolic genes in zebrafish (Danio rerio). J Exp Biol. 217, 1139-1149.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M.T., Panserat, S., 2015. Glucose metabolism and gene expression in juvenile zebrafish (Danio rerio) challenged with a high carbohydrate diet: effects of an acute glucose stimulus during late embryonic life. Br J Nutr. 113, 403-413.

Rodríguez, C., Pérez, J.A., Badía, P., Izquierdo, M.S., Fernández-Palacios, H., Hernández, A.L., 1998. The n−3 highly unsaturated fatty acids requirements of gilthead seabream (Sparus aurata L.) larvae when using an appropriate DHA/EPA ratio in the diet. Aquaculture. 169, 9-23.

Rønnestad, I., Rojas-García, C.R., Tonheim, S.K., Conceição, L.E.C., 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture. 201, 161-175.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev Aquaculture. 5, S59-S98.

Russo, T., Costa, C., Cataudella, S., 2007. Correspondence between shape and feeding habit changes throughout ontogeny of gilthead sea bream Sparus aurata L., 1758. J Fish Biol 71, 629-656.

Segura, J., Lopez-Bote, C.J., 2014. A laboratory efficient method for intramuscular fat analysis. Food Chem. 145, 821-825.

Seiliez, I., Medale, F., Aguirre, P., Larquier, M., Lanneretonne, L., Alami-Durante, H., Panserat, S., Skiba-Cassy, S., 2013. Postprandial regulation of growth- and metabolism-related factors in zebrafish. Zebrafish. 10, 237-248.

Snoeck, A., Remacle, C., Reusens, B., Hoet, J.J., 1990. Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate. 57, 107-118.

Symonds, M.E., Sebert, S.P., Hyatt, M.A., Budge, H., 2009. Nutritional programming of the metabolic syndrome. Nat Rev Endocrinol. 5, 604-610.

Towle, H.C., Kaytor, E.N., Shih, H.M., 1997. Regulation of the expression of lipogenic enzyme genes by carbohydrate. Annu Rev Nutr. 17, 405-433.

Vagner, M., Zambonino-Infante, J.L., Robin, J.H., Person, J., 2007. Is it possible to influence European sea bass (Dicentrarchus labrax) juvenile metabolism by a nutritional conditioning during larval stage? Aquaculture. 267, 165-174.

Vagner, M., Robin, J.H., Zambonino-Infante, J.L., Tocher, D.R., Person-Le Ruyet, J., 2009. Ontogenic effects of early feeding of sea bass (Dicentrarchus labrax) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. Br J Nutr. 101, 1452-1462.

Valente, L.M.P., Moutou, K.A., Conceição, L.E.C., Engrola, S., Fernandes, J.M.O., Johnston, I.A., 2013. What determines growth potential and juvenile quality of farmed fish species? Reviews in Aquaculture. 5, S168-S193.

Waterland, R.A., Jirtle, R.L., 2004. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 20, 63-68.

Yamada, K., Noguchi, T., 1999. Nutrient and hormonal regulation of pyruvate kinase gene expression. Biochem J. 337 (Pt 1), 1-11.

Zambonino-Infante, J.L., Gisbert, E., Sarasquete, C., Navarro, I., Gutiérrez, J., Cahu, C.L., 2008. Ontogeny and Physiology of the Digestive System of Marine Fish Larvae. in: Cyrino, J.E.P., Bureau, D., Kapoor, B.G. (Eds.), Feeding and Digestive Functions in Fishes. CRC Press, pp. 281-348.

Zouiten, D., Khemis, I.B., Besbes, R., Cahu, C., 2008. Ontogeny of the digestive tract of thick lipped grey mullet (Chelon labrosus) larvae reared in “mesocosms”. Aquaculture. 279, 166-172.

Long-lasting programming effects in marine fish species

Chapter V

Dietary glucose stimulus at larval stage modifies the carbohydrate metabolic pathway in gilthead seabream (*Sparus aurata*) juveniles: an *in vivo* approach using ^{14}C-starch

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Dietary glucose stimulus at larval stage modifies the carbohydrate metabolic pathway in gilthead seabream (*Sparus aurata*) juveniles: an *in vivo* approach using ^{14}C-starch

Abstract

The present study aims at the tailoring of specific metabolic pathways or physiological functions in fish for a better use of carbohydrates as energy substrate in juvenile seabream, following the concept of early nutritional programming. We assessed the long-term effects of hyperglucidic stimuli, exerted at the larval stage, on the growth performance, nutrient digestibility and metabolic utilization and gene expression of gilthead seabream juveniles, challenged with a high carbohydrate intake. During early development, larvae were fed either a standard feeding regime characterized by high protein and lipid intake (control group, CTRL) or were subjected to a series of recurrent hyperglucidic stimuli delivered at various periods over the standard feeding regime (GLU). Apart from these short periods of high-carbohydrate exposure, larvae from both CTRL and GLU treatments were exclusively fed with a protein-rich diet. At the later juvenile stage, triplicate groups of 35 fish (IBW: 2.5 g) from each of the fish stocks were fed a high-protein (59.4%) / low-carbohydrate (2.0%) diet for 33 days before being subjected to a dietary challenge with a low-protein (43.0%)/ high-carbohydrate (33.0%) diet during 36 days. At the end of the trial, fish had a 8-fold increase of their initial body weight, but neither growth rate, feed intake, feed and protein efficiency, nutrient retention (except lipids) nor whole-body composition were affected ($P > 0.05$) by the early nutritional history of the fish. Nutrient digestibility was also similar among both groups. The metabolic fate of dietary carbohydrates and proteins was estimated using ^{14}C-starch and ^{14}C-amino acids as tracers; GLU juveniles showed higher absorption of starch-derived glucose in the gut, suggesting an enhanced digestion of carbohydrates. Moreover, glucose-derived ^{14}C skeletons were less used for *de novo* synthesis of proteins (in the liver) and glycogen (in muscle) in GLU fish than in CTRL fish ($P<0.05$). No persistent modifications related to the early glucose conditioning were found in ^{14}C-amino acid use or in metabolic gene regulation ($P>0.05$). Based on metabolic flux results, our data suggests that early hyperglucidic interventions at larval stages had the potential to permanently modify some pathways of carbohydrate utilization in seabream juveniles.

Keywords: Seabream, Carbohydrates, Nutritional programming, Tracer studies, Metabolism

5.1. Introduction

Aquaculture is currently the fastest growing animal food production sector, but its future expansion requires the reinforcement of sustainable practices. Marine fish diets are generally protein-rich. For more than thirty years now, two approaches have been used to reduce the protein cost involved in fish production: a) optimization of dietary protein allocation for growth; and b) reduction of inclusion levels of finite marine protein sources through the use of adequate substitutes of plant origin. The inclusion of plant ingredients, being it as dietary protein or starch sources results in an increased overall intake of carbohydrates (Naylor et al., 2009). However, the ability of fish to use dietary carbohydrates as an energy yielding substrate is variable among species and closely associated with their feeding habits. Gilthead seabream (*Sparus aurata*) is the major farmed marine fish species in the Mediterranean region (FEAP, 2014); during the juvenile and grow-out stage this species presents a high dietary protein requirement (46-50%), close to the range reported for other carnivorous species (Basurco et al., 2011; Enes et al., 2011). Research has devoted great efforts to assess the feasibility of reducing and replacing dietary protein by other less expensive energy sources, such as lipids and carbohydrates (Enes et al., 2011; Santinha et al., 1999; Velázquez et al., 2006; Vergara et al., 1996). In seabream, starch digestibility ranges from 70 to 99% (Couto et al., 2012; Enes et al., 2008b; Venou et al., 2003) and maximum recommended dietary levels of digestible carbohydrates is 20% (Enes et al., 2011). Additionally, an enhancement in the activity of key-glycolytic enzymes together with an increase of liver glycogen content was observed in seabream fed with carbohydrates (Couto et al., 2008; Enes et al., 2008b; Panserat et al., 2000), suggesting that dietary carbohydrates were partially used as an energy yielding substrate. Research promoting an enhanced utilization of dietary carbohydrate sources in fish is needed.

Studies in mammals and humans show that dietary stimulus exerted at critical developmental stages early in life (neonatal or post-natal nutrition) may have long-term consequences on physiological functions in later life (Burdge and Lillycrop, 2010; Harder et al., 1998; Lucas, 1998; Metges et al., 2014; Patel and Srinivasan, 2002). This phenomenon, known as nutritional programming, is largely studied in mammalian models for the understanding of diseases such as the metabolic syndrome or diabetes (Burdge and Lillycrop, 2010; Gluckman et al., 2007). Possible biological mechanisms for “imprinting” the nutritional programming event until adulthood include epigenetic regulation of gene expression (e.g. DNA methylation

and histone modifications), nutrient-sensitive signaling pathways, altered cell number, cell type or structural changes in organs, impaired mitochondrial function and adaptive clonal selection (Jaenisch and Bird, 2003; Lucas, 1998; Symonds et al., 2009; Tarry-Adkins and Ozanne, 2011; Waterland and Jirtle, 2004). The perspective of applying this novel concept to fish nutrition provides numerous possibilities mainly focused on tailoring specific metabolic pathways or functions in juvenile fish, such as the improvement of the inefficient use of dietary carbohydrates in some omnivore and most carnivore fish.

Knowledge on the long-term effects of a nutritional event exerted at early stages of fish development is still limited. Some studies aimed to induce persistent metabolic adaptations to better cope with high levels of dietary carbohydrates (Fang et al., 2014 ; Geurden et al., 2007; Geurden et al., 2014; Gong et al., 2015; Rocha et al., 2015) or low levels of long-chain polyunsaturated fatty acids (Vagner et al., 2007; Vagner et al., 2009). In these various studies, the concept of early nutritional conditioning was demonstrated, with variable extent, through some long-term modifications at a molecular and/or metabolic level. Even so, the long-term modulation of carbohydrate metabolic pathways by means of nutritional programming was never been demonstrated in a marine species, such as gilthead seabream.

The objective of the present study was to assess the long-term effects of early hyperglucidic stimuli, exerted at the larvae stage, on the growth performance, nutrient digestibility and metabolic utilization and gene expression of gilthead seabream juveniles, challenged with a high carbohydrate diet.

5.2. Material and Methods

5.2.1. Experimental diets

The trial comprised two experimental diets, which were fed to seabream juveniles at distinct feeding periods. Diet HP (high-protein) was formulated with both marine and plant protein concentrates, guaranteeing a high crude protein level (59.4% DM) and a very low level of starch (2.1% DM). Additionally, diet HS (high-starch) was formulated with lower crude protein level (43.2% DM) and higher starch content (33.3% DM). Both diets were kept isolipidic (crude fat: 20.0% DM) and were supplemented with selected crystalline indispensable amino acids and mono-calcium phosphate to avoid any essential amino acids or

phosphorus imbalance. Formulation and proximate composition of both diets are presented in Table 5.1. Diets were manufactured by SPAROS Lda (Olhão, Portugal). Powder ingredients were grinded (<200 micron) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Ingredients and oil sources were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain) and the mixture was humidified with approximately 25% water. Diets were manufactured by temperature controlled-extrusion (pellet sizes: 1.2 and 2.0 mm) by means of a low-shear extruder (Italplast, P55, Italy). Upon extrusion, all feed batches were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). Throughout the duration of the trial, experimental feeds were stored at a cool and aerated environment. To measure the apparent digestibility of protein and energy by the indirect method, a portion of the HS diet was supplemented with 1% chromium oxide

5.2.2. Zootechnical trials

The growth trial was conducted at the Ramalhete Experimental Research Station of the University of Algarve - CCMAR (Faro, Portugal). Experiment was conducted according to the European guidelines on protection of vertebrate animals used for scientific purposes (Directive 2010/63/UE of European Parliament and of the European Union Council).

5.2.2.1. Nutritional background of fish

Gilthead seabream juveniles used in the trial originated from two distinct groups (CTRL and GLU), were established based on their early nutritional history at the larval stage. Fish from the CTRL group were reared throughout larval development with a standard feeding protocol for seabream, which comprised live preys (rotifers and *Artemia*) enriched in protein and marine lipids until 35 days after hatching (DAH). Live preys were gradually replaced at 30DAH by a high-protein diet suitable for marine fish larvae (crude protein: 71.0% DM; crude fat: 16.0% DM; total carbohydrates: 0.8% DM). From 36DAH onwards, larvae from the CTRL group were fed exclusively on this high-protein diet. Fish from the GLU group were reared with a feeding protocol similar to the one described for CTRL fish, but comprising four recurrent periods of exposure (5-days) to high dietary carbohydrates. These feeding periods were: at the onset of exogenous feeding (3-8DAH) using rotifers enriched with glucose; from 20-25DAH using glucose-enriched *Artemia* metanauplii; at 30-35DAH and 50-60DAH using an inert diet with high carbohydrates (crude protein: 34.0% DM; crude fat: 9.0% DM; total carbohydrates: 50.0% DM). In between the periods of dietary exposure to

carbohydrates, GLU fish were fed exactly the same regime as CTRL fish. From 60DAH onwards, post-larvae from both CTRL and GLU treatments were exclusively fed with the experimental diet HP. A schematic view of the experimental design for glucose stimuli delivery at the larval stages and dietary challenge at the juvenile stage with a high-starch diet is presented in Figure 5.1.

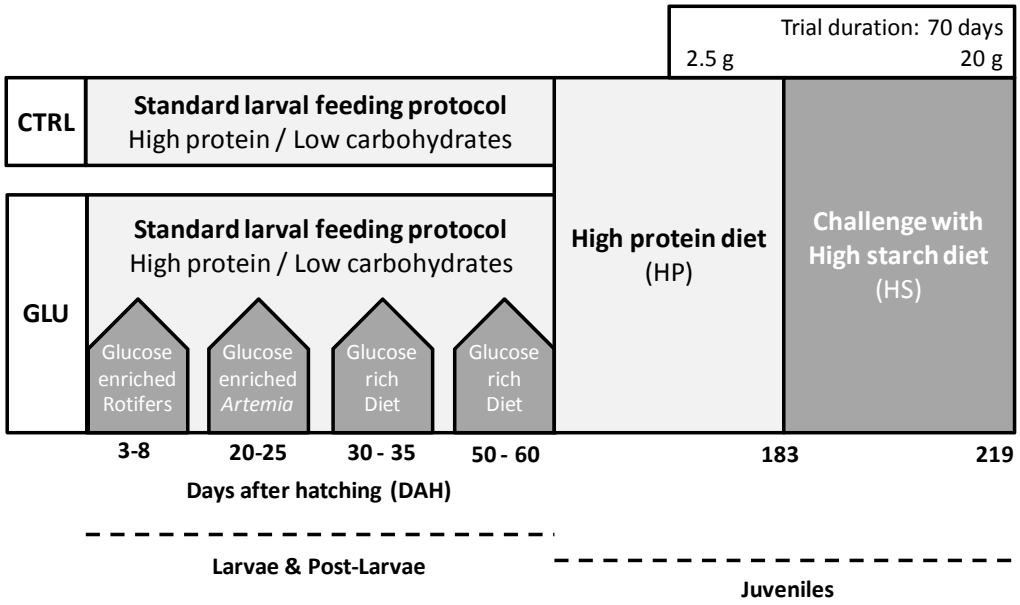

Figure 5.1 – Experimental design for glucose stimuli intervention in seabream larvae and later dietary challenge with high-starch diet.

5.2.2.2. Rearing conditions

Six homogenous groups of 35 gilthead seabream juveniles, with a mean initial body weight of 2.46 ± 0.43 g were stocked in 100 L cylinder-conical fiberglass tanks. The CTRL and GLU treatments were reared in triplicate groups. Rearing tanks were supplied with flow-through gravel-filtered, aerated seawater (salinity: 35 part per thousand; temperature: 22.5 ± 2.3°C; dissolved oxygen levels were kept above 6.9 ± 1.1 $mg \cdot L^{-1}$). All experimental groups were fed with diet HP (high-protein) over 33 days and afterwards, from day 34 to 70, all fish were solely fed the HS diet (high-starch) (Figure 5.1). Throughout the entire trial, fish were fed to apparent satiety, by hand once a day (09:00 to 10:00 am) and by means of automated feeders (8 meals per day) during the remaining day. Rearing tanks were equipped with a solids decantation trap that allowed a visual assessment and minimization of any feed losses. At the

start (10 fish from each initial stock, CTRL and GLU) and at the end of the trial, five fish from each tank were sampled for analysis of whole-body composition. At the end of the trial, three fish from each replicate tank were anaesthetized and sampled 6 h after the last meal. Blood was quickly removed from the caudal vein using heparinized syringes, centrifuged (3000 *g*, 5 min) and the recovered plasma was frozen in liquid nitrogen and stored at -80ºC until glycaemia analysis. The liver and muscle of the same individuals were dissected, snap-frozen and kept at –80ºC for subsequent gene expression analysis. Blood of unfed fish was also collected, on the next day.

5.2.3. Apparent digestibility measurements

Following the growth trial, duplicate groups of fish from the CTRL and GLU treatments were maintained in their rearing tanks and used to measure the apparent digestibility coefficients (ADC) of protein, lipid, starch and energy of diet HS, by indirect method. After 5 days of adaptation to the chromium oxide marked HS diet, feces were collected during 6 consecutive days in a solids decantation trap. After daily collection, feces were frozen at -20ºC. Pooled feces from each group of fish were freeze-dried prior to analysis. ADC of the dietary nutrients and energy were calculated as follows:

$$\text{ADC } (\%) = 100 \times \left[1 - \frac{\text{dietary } Cr_2O_3 \text{ level}}{\text{faecal } Cr_2O_3 \text{ level}} \times \frac{\text{faecal nutrient or energy level}}{\text{dietary nutrient or energy level}} \right]$$

5.2.4. Glucose tolerance test

At the end of the growth trial, fish from both CTRL and GLU treatments were fasted for 24 h and subjected to a glucose tolerance test (GTT). For this, 3 fish per replicate tank (n=9) were lightly anaesthetized with 2-phenoxyethanol (Sigma-Aldrich, Spain), weighed and injected intraperitoneally (10 ml·kg^{-1} body weight) with either a glucose solution (125 µM) or a saline solution (NaCl 0.9%). The glucose dose was set at 250 mg·kg^{-1} body weight. Three hours after injection, blood was collected from the caudal vein with a heparinized syringe, centrifuged (3000 *g*, 5 min) and the recovered plasma was frozen at –80ºC until glycaemia analysis. The time interval for blood collection was determined based on the peak of plasma glucose observed in seabream after a glucose load (Peres et al., 1999).

Table 5.1 – Ingredients and composition of experimental diets.

Ingredients, g·Kg^{-1}	**HP diet**	**HS diet**
Fishmeal LT70[1]	50.00	
Fish protein concentrate[2]	50.00	
Squid meal[3]	100.00	100.00
Fish gelatin[4]	25.00	25.00
Soy protein concentrate[5]	129.40	
Pea protein concentrate[6]	50.00	50.00
Potato protein concentrate[7]	100.00	120.00
Wheat gluten[8]	105.00	125.00
Corn gluten meal[9]	75.00	
Rice protein concentrate[10]	60.00	
Pea starch[11]		321.40
Glycerol[12]	30.00	30.00
Fish oil[13]	40.00	43.00
Soybean oil[13]	40.00	50.00
Rapeseed oil[13]	20.00	20.00
Linseed oil[13]	40.00	43.00
Vitamin and Mineral Premix[14]	10.00	10.00
Soy lecithin	20.00	20.00
Binder[15]	15.00	15.00
Antioxidant[16]	3.600	3.60
Mono calcium phosphate[17]	30.00	40.00
L-Lysine	2.00	5.00
L-Threonine		4.00
L-Taurine	5.00	5.00
Chromium oxide[18]		10.00
Proximate composition		
Dry matter (DM), %	95.9 ± 0.10	93.5 ± 0.00
Crude protein, % DM	59.4 ± 0.10	43.2 ± 0.30
Crude fat, % DM	20.2 ± 0.40	20.4 ± 0.00
Starch, % DM	2.1 ± 0.20	33.3 ± 0.40
Ash, % DM	7.2 ± 0.10	5.3 ± 0.10
Total phosphorus, % DM	1.4 ± 0.020	1.4 ± 0.010
Gross energy, MJ·kg^{-1} DM	23.8 ± 0.30	23.3 ± 0.00
Chromium oxide, % DM		1.02 ± 0.01

[1] Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), Exalmar, Peru; [2] CPSP 90: 84% CP, 12% CF, Sopropêche, France; [3] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France; [4] Pharma grade bloom 240: 92% CP, LAPI Gelatine SPA, Italy; [5] Soycomil P: 62% CP, 0.8% CF, ADM, The Netherlands; [6] Lysamine GP: 78% CP, 8% CF, Roquette, France; [7] Protastar: 81% CP, 3.1% CF, Avebe, The Netherlands; [8] Viten: 84.3% CP, 1.3% CF, Roquette, France ; [9] Corn gluten meal: 61% CP, 6% CF, Copam, Portugal. [10] Rico 50: 50.4% CP, 7.2% CF, Sopropêche, France; [11] 86% starch, Roquette, France; [12] Rapeseed-derived crude glycerol: 82% glycerol, Iberol, Portugal; [13] Henry Lamotte Oils GmbH, Germany; [14] Premix for marine fish, Premix Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings; [15] Kielseguhr: Ligrana GmbH, Germany; [16] Paramega PX, Kemin Europe NV, Belgium; [17] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy; [18] Chromium oxide was only incorporated in a fraction of the HS diet used for digestibility measurements.

5.2.5. Metabolic study with radiolabeled tracers

The method of tube-feeding specific radiolabeled nutrients was performed in seabream juveniles of both CTRL and GLU treatments, at the end of the trial. Two ^{14}C-tracers, starch-[^{14}C(U)] (1.85 MBq; Perkin- Elmer, USA) and L-Amino acid mixture-[^{14}C(U)] (37.0 MBq; American Radiolabeled Chemicals Inc., The Netherlands) were used, separately, to assess the metabolic fate of dietary glucose and amino acids, respectively.

5.2.5.1. Diet labeling with ^{14}C-nutrients

The ^{14}C-starch or the ^{14}C-AA mixture tracers were diluted in an alcohol solution (70%) and a known volume deposed with a micropipette on individual pellets from the HS diet. These "hot" pellets were dried at 50ºC for 30 min and reserved for subsequent tube-feeding procedure. Pellets presented a mean value of disintegrations per minute (DPM) of 391705 DPM, for ^{14}C-starch and 168623 DPM for ^{14}C-AA mixture solution. Additionally, another set of HS pellets were also labeled with a non-radioactive solution of blue food colorant ("cold" pellets), used to monitor possible feed regurgitation.

5.2.5.2 Tube-feeding trial

Random fish from the CTRL and GLU treatments (n=6, form each treatment) were transferred to the flux laboratory after being fasted for 18 h. The *in* vivo method of tube-feeding was adapted to seabream juveniles based on the modified method described by Costas et al. (2011), which in turn was an up scaling of the method firstly described by Rust et al. (1993) and modified for marine fish larvae by Rønnestad et al. (2001). In brief, anesthetized fish were tube-fed 0.5% body weight, which corresponded to a total of 16 pellets of the HS diet (3 "hot" from ^{14}C-starch or ^{14}C-AA mixture, 2 "cold" and 11 not manipulated pellets). For tube-feeding, a hollow plastic tube of 1.5 mm inner diameter and a solid piece with minor diameter placed inside as a plunger was used. The diameter and length of the plastic tube were previously tested to avoid injuries in the esophagus and stomach of seabream juveniles, with approximately 20 g of body weight. Tube-fed fish were allowed to recover in clean seawater to eliminate residual anesthetic in skin and gills and monitored for eventual pellet regurgitation. After this period, fish were transferred to the incubation chambers. The total handling time per fish between tube-feeding and the sealing of the incubation chamber was within 3 min. The incubation setup consists in sealed cylindrical incubation chambers, filled with 2 L of seawater, where the juveniles were placed individually. A gentle oxygen flow was

forced through a capillary from the incubation chamber to a battery of three CO_2 traps build in series (each 10 mL of 0.5 M KOH). After the incubation period of 18 h, oxygen flow was stopped and fish were sacrificed inside the chambers by a lethal dose of MS-222 (Sigma-Aldrich, Spain). Then, fish were taken for sampling and the incubation chambers were resealed for the addition of 130 mL of HCl (0.1 M) in a series of gradual steps as suggested by Rønnestad et al. (2001). The lowered pH led to rapid diffusion of any remaining CO_2 from the water and into the KOH traps (catabolized fraction). From each CO_2 trap, 5 mL aliquots were collected (n=3) for radioactive counting. The incubation water was considered to contain all labeled ^{14}C resultant from fish excretion (evacuated fraction). Due to the high volume of incubation water (2 L), aliquots of 5 mL were collected from each incubation chamber (n=5) for radioactive counting. Concerning fish sampling, each individual was weighted and dissected for liver and muscle (two fillets) collection. A series of extraction procedures were performed to extract free amino acids (FAA), protein and lipids from liver and muscle, in an attempt to determine how the ^{14}C-starch and ^{14}C-AA were retained in the various tissues. Therefore, the liver and one muscle fillet were placed individually in 4 mL of 6.0 % trichloroacetic acid (TCA, Sigma- Aldrich, Spain) for 24 h at 4ºC, with periodical stirrings, for FAA extraction (Campos et al., 2013; Morais et al., 2004). Then, each tissue was individually transferred to a clean vial for homogenization in distilled water, using an Ultra-turrax homogenizer (IKA, Germany). The TCA solutions were stored for radioactive counting (DPM). Total lipids from liver and muscle were extracted from a known aliquot volume using a method modified from Bligh and Dyer (1959) (Conceição et al., 2002; Morais et al., 2004). Liver and muscle total protein was extracted from the upper phase of methanol/water recovered from lipid extraction, based on a TCA precipitation method, adapted and modified from Panchout et al. (2013). Briefly, 24.0% TCA was added to each sample to a final concentration of 6%, after samples were vortex and incubated at 4 ºC for 1 h. Precipitated proteins were pelleted by centrifugation at 6000 *g* for 10 min at 4ºC. The supernatant was collected to a clean vial for DPM counting and the precipitated protein was washed with ice-cold acetone and centrifuged at 6000 *g* for 10 min at 4ºC. The acetone was removed to a clean vial for DPM counting and the protein pellet (either the liver or muscle) was re-suspended in Solvable (Perkin Elmer, USA) and kept at 50º C for 24 h for complete solubilization. The two supernatants obtained from the protein protocol were not discarded since they represent, for each tissue, the fraction of non-extracted metabolites, where glycogen was considered to be present.

5.2.5.3. Metabolic budget determination

For each ^{14}C-tracer, samples from incubation seawater, KOH-CO_2 traps, liver and muscle fractions were counted for radioactivity (DPM) by adding Ultima Gold XR scintillation cocktail (Perkin Elmer, USA). All samples were counted on a Tri-Carb 2910TR Low activity liquid scintillation analyzer (Perkin Elmer, USA). The metabolic budgets were calculated after subtraction of blanks for quench and lumex correction. Results for each component (evacuation, catabolism, retention in tissues and relative retention in several fractions of organic compounds) were expressed as a percentage of total ^{14}C-label, i.e. the sum of DPM in all compartments of metabolic chambers and fish. Glucose and amino acids utilization in seabream juveniles was determined based on nutrient Absorption (*A*, %), Evacuation (*E*, %), Retention fraction (*R*, %) and Catabolism fraction (*C*, %). The Retention fraction was determined for liver and muscle tissues based on the retention in lipid fraction (R_{Lip}, DMP/100 mg tissue), protein fraction (R_{Prot}, DMP/100 mg tissue), FAA fraction (R_{FAA}, DMP/100 mg tissue) and other metabolites fraction, where glycogen was considered included (R_{Other}, DMP/100 mg tissue). Nutrient retention was normalized for 100 mg of each tissue, liver and muscle, to allow comparisons between them. These estimates were determined as:

$$A = [(R_{tissues} + R_{CO_2trap})/(R_{tissues} + R_{CO_2trap} + R_{water})] \times 100$$

$$E = [R_{water} / (R_{tissues} + R_{CO_2\,trap} + R_{water})] \times 100$$

$$C = [R_{CO_2trap} / (R_{tissues} + R_{CO_2\,trap})] \times 100$$

Where $R_{tissues}= (R_{muscle} + R_{liver})$; R_{CO_2trap} is the total radioactivity in the battery of CO_2 trap (DPM) and R_{water} is the total radioactivity in the incubation seawater (DPM). For nutrient retention in liver or muscle tissues (R_{muscle} or R_{liver}):

$$R_{Lip} = [R_{lip\text{-}Tissue} \times (R_{Tissue} / P_{Tissue} \times 100)] / R_{Tissue}$$

$$R_{Prot} = [R_{prot\text{-}Tissue} \times (R_{Tissue} / P_{Tissue} \times 100)] / R_{Tissue}$$

$$R_{FAA} = [R_{FAA\text{-}Tissue} \times (R_{Tissue} / P_{Tissue} \times 100)] / R_{Tissue}$$

$$R_{Other} = [R_{other\text{-}Tissue} \times (R_{Tissue} / P_{Tissue} \times 100)] / R_{Tissue}$$

Where $R_{Tissue}= R_{muscle}$ or R_{liver}, is the sum of radioactivity (DPM) counted in all nutrient fractions of each tissue i.e., $R_{muscle}= R_{lip} + R_{prot} + R_{FAA} + R_{other}$ and $R_{liver}= R_{lip} + R_{prot} + R_{FAA} + R_{other}$. Also, $R_{lip\text{-}Tissue}$ is the total radioactivity in chloroform fraction of liver or muscle tissues (DPM), $R_{prot\text{-}Tissue}$ is the total radioactivity in protein precipitate from liver or muscle tissues

(DPM), $R_{FAA\text{-}Tissue}$ is the total radioactivity in TCA soluble fraction of liver or muscle tissues (DPM), $R_{other\text{-}Tissue}$ is the total radioactivity in the washes performed during protein extraction in liver and muscle tissues (DPM) and P_{Tissue} is either the weight of liver or the two muscle fillets (mg).

5.2.6. Gene expression analysis by real-time PCR

Analyses of mRNA levels were performed on samples from the liver and muscle tissue of fish sampled 6 h after the last meal, the time interval found suitable for examining the postprandial response of genes in juvenile seabream (Caseras et al., 2000; Enes et al., 2008c; Panserat et al., 2000; Panserat et al., 2002). Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. From the extracted RNA, 2 µg were reverse transcribed into complementary DNA (cDNA) using the SuperScript III RNaseH Reverse Transcriptase Kit (Invitrogen, Carlsbad, USA) and random primers (Promega, France). mRNA levels were determined by real-time qPCR using the Roche Lightcycler 480 system (Roche Diagnostics, France). Analyses were performed on 2 µL of the diluted cDNA using 3 µL of Light Cycler 480 SYBR® Green I Master mix (Roche), in a total PCR reaction volume of 6µL, containing 100 nM of each primer. Specific primers were designed for gilthead seabream using the Primer3 software (http://primer3.ut.ee/) and the available seabream sequences from the nucleotide GenBank or the EST SIGENAE databases (http://www.ncbi.nlm.nih.gov/genbank/; http://www.sigenae. org/, respectively) except for GK, G6Pase, COX4, CS and elongation factor 1 (EF1) genes whose sequences were already published (Bermejo-Nogales et al., 2014; Enes et al., 2008c; Perez-Sanchez et al., 2013) (Table 5.2). The transcripts analised were: glucokinase (GK), hexokinase 1 (HK1), 6-phosphofructo-1-kinase liver isoform (6PFK-L) and muscle isoform (6PFK-M) and pyruvate kinase muscle isoform (PK-M) for glycolysis; glucose-6-phosphatase (G6Pase) and phosphoenopyruvate carboxykinase (PEPCK) for gluconeogenesis; fatty acid synthase (FAS), acetyl CoA carboxylase (ACCα) and glucose-6-phosphate dehydrogenase (G6PDH) for lipid metabolism; cytochrome oxidase subunit IV (COX4) and citrate synthase (CS) for energy metabolism; facilitative glucose transporter (GLUT9) for glucose transporter; mitochondrial alanine aminotransferase (ALTm) and cytosolic aspartate aminotransferase (ASTc) for amino acid catabolism transcripts.

Table 5.2 – Real time PCR primer sequences

Gene	Forward primer (5'-3')	Revese primer (5'-3')	Database (accession no.)
EF1[a]	CATGCTGGAGACCAGTGAAA	CGGGTACAGTTCCAATACCG	GeneBank (AF184170)
GK[a]	CAAGAGACGAGGGGACTCG	TCCTCGCCTTCTACCAGCTC	GeneBank (AF053330)
HK1	AGATCCATCCTGCAGCACTT	CCAGTCCTCGGTTCTCTCTG	Sigenae (AM955654.p.sb.5)
6PFK-L	CATGTGTGATTGGCCTCAAC	AGGGAGCCTAAAACCCAGAG	Sigenae (AM968607.p.sb)
6PFK-M	ACGGTCGTATCTTTGCCAAC	GTGTGTTCGGATGTGTCCAG	Sigenae (FM147562.p.sb.5)
PK-M	GATCCTGGCACAAAGCTCTC	TGTCTGCTGGACATCGACTC	Sigenae (FG590446.p.sb.5)
G6Pase[a]	CGCTGGAGTCATTACAGGCGT	CAGGTCCACGCCCAGAACTC	GeneBank (AF151718)
PEPCK	GCAACACAGAGAGGGAGGAG	TATCCTCCAGTGCCTTCAGC	Sigenae (CV133734.p.sb.5)
GLUT9	GAGGACTACCCAGGTGACCA	GGTGACTGTCTCTGCTGCAA	Sigenae (FM145240.p.sb.5)
FAS	TGAAACTGAAGCCCTGTGTG	TCTCGGCTGATGTTCTTGTG	Sigenae (AM952430.p.sb.5)
ACC	ATCAGAGGTGGCGATGGTAG	TCGTCATGCAGTTAGCCAAG	Sigenae (FP332814.p.sb.5)
G6PDH	GCAGCCAGATGCACTTTGTA	GCGAAATCCAACTCTCTTCG	Sigenae AM951965.p.sb.5)
Cox4[b]	ACCCTGAGTCCAGAGCAGAAGTCC	AGCCAGTGAAGCCGATGAGAAAGAAC	GeneBank (JQ308835.1)
CS[c]	TCCAGGAGGTGACGAGCC	GTGACCAGCAGCCAGAAGAG	GeneBank (JX975229)
ALTm	CGTGGAGGCTACATGGAGAT	AGCTTGGCCTTCTCTGCTAA	GeneBank (AY206503.1)
ASTc	AGTGTCTTGGAGGTACAGGC	CCAAGGAAACCAGCCAAGTC	Sigenae (CB176687.p.sb.5)

[a] From (Enes et al., 2008c)
[b] From (Perez-Sanchez et al., 2013)
[c] From (Bermejo-Nogales et al., 2014)

PCR product resulting from the newly designed primer pairs was controlled by sequencing to confirm the nature of the amplification. Relative quantification of the target gene transcript was done using EF1 RNA level, which was stably expressed in both tissues (data not shown). The PCR protocol was initiated at 95 °C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a two-step amplification programme (15 s at 95 °C; 40 s at 60 °C). Melting curves were systematically monitored (temperature gradient at 1.1 °C/10 s from 65-94 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (samples without reverse transcriptase and samples without RNA). Relative quantification of gene expression was performed using the ΔC_T method described by Pfaffl (2001). In all cases, PCR efficiency was measured by the slope of a standard curve using serial dilutions of cDNA, and ranged between 1.8 and 2.1.

5.2.7. Analytical methods

Experimental diets, freeze-dried whole-fish and freeze-dried feces were analised for biochemical composition according to the following procedures: dry matter after drying at 105ºC for 24 h; ash content by incineration in a muffle furnace at 500ºC for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428, USA); fat by dichloromethane extraction (Soxhlet); starch by the glucoamylase-glucose oxidase method (Thivend et al., 1972); gross energy in an adiabatic bomb calorimeter (IKA C2000, Germany); chromium oxide according to Bolin et al. (1952), after perchloric acid digestion and total phosphorus according to the ISO/DIS 6491 method using the vanado-molybdate reagent. Glycaemia analysis was performed using an enzymatic-colorimetric method (glucose kit, reference 1001190; SPINREACT, Spain).

5.2.8. Statistical analysis

Data are presented as means and standard deviation. Data expressed as percentage were arcsine-square-root transformed prior to statistical analysis (Ennos, 2007). The effect of the early nutritional history (recurrent glucose stimuli) on the several analised parameters in seabream juveniles was tested using SPSS® statistics software 16.0 for Windows (SPSS Inc.) by means of an unpaired two-tailed Student's *t*-test. To determine the relative quantification of gene expression using the ΔC_T method, the control group was established by the CTRL fish. Differences were considered significant at $P<0.05$

5.3. Results

5.3.1. Growth, feed efficiency and nutrient utilization

At the end of the trial, fish from CTRL and GLU treatments showed nearly an 8-fold increase of their initial weight, reaching a final body weight of between 19.2 - 20.3 g, respectively (Table 5.3). Daily growth index (DGI) varied between 1.90 and 1.97, while feed efficiency (FE) was around 0.90 for both treatments. Overall growth performance criteria (FBW, DGI, FE, feed intake and protein efficiency ratio) were not significantly affected ($P>0.05$) by the nutritional background of fish, namely the recurrent exposure to glucose intake in the larval stages of the GLU treatment.

Table 5.3 – Growth performance, apparent digestibility coefficients (ADC), whole-body composition and nutrient retention of fish from the CTRL and GLU groups, at the end of the experimental trial (IBW[1]: 2.46 ± 0.43 g).

	CTRL group	**GLU group**	***P* value**
FBW[2]	19.20 ± 0.08	20.30 ± 1.29	0.214
VFI[3]	2.83 ± 0.08	2.96 ± 0.43	0.725
DGI[4]	1.90 ± 0.01	1.97 ± 0.07	0.203
FE[5]	0.90 ± 0.01	0.91 ± 0.08	0.894
PER[6]	2.23 ± 0.03	2.25 ± 0.19	0.846
ADC Protein, %	93.6 ± 0.60	93.5 ± 0.1	1.000
ADC Fat, %	93.0 ± 0.80	93.5 ± 0.4	0.592
ADC Starch, %	83.1 ± 0.10	84.3 ± 0.4	0.095
ADC Energy, %	77.2 ± 1.60	78.7 ± 0.7	0.493
Body composition[7] (%)			
Moisture	68.7 ± 0.40	68.5 ± 0.40	0.565
Protein	16.4 ± 0.50	16.5 ± 0.80	0.813
Lipid	11.0 ± 0.60	11.3 ± 0.90	0.626
Ash	4.0 ± 0.01	4.0 ± 0.20	0.775
Energy ($kJ \cdot g^{-1}$)	8.0 ± 0.10	8.0 ± 0.20	0.866
Retention[8] (% of intake)			
Protein	25.9 ± 1.50	27.6 ± 2.50	0.440
Lipid	47.9 ± 1.30	54.8 ± 2.00	0.015
Energy	28.1 ± 1.40	30.5 ± 0.60	0.099

Values are means ± standard deviation (n=3). [1] Initial mean body weight (g); [2] Final mean body weight (g);[3] Voluntary feed intake: crude feed intake/(IBW+FBW)/2/days; [4] Daily growth index: ($FBW^{1/3}$ –$IBW^{1/3}$)/days)x100; [5] Feed efficiency: wet weight gain/dry feed intake; [6] Protein efficiency ratio: wet weight gain/crude protein intake; [7] Initial fish, CTRL group: moisture 78.5%, protein 15.5%, lipid 2.1%, ash 3.9%, energy 4.3 $kJ \cdot g^{-1}$; [7] Initial fish, GLU group: moisture 79.2%, protein 14.8%, lipid 2.0%, ash 4.1%, energy 4.1 $kJ \cdot g^{-1}$; [8] Retention: 100 x (FBWxfinal carcass nutrient content-IBWxinitial carcass nutrient content)/nutrient intake.

Similarly, no differences were found (P>0.05) on the whole-body moisture, protein, lipid, ash and energy contents of fish (Table 5.3). Data on weight gain, feed intake and whole-body composition of fish allowed the estimation of nutrient retention (% of intake). GLU fish showed a significantly higher fat retention (54.8%) compared to the CTRL fish (47.9%) (P=0.01). Protein retention was 25.9% and 27.6% while energy retention was 28.1% and 30.5% in the CTRL and GLU fish, respectively (Table 5.3). Protein and energy retention were not affected by the nutritional background of fish (P>0.05). Both treatments showed a high (>90.0%) apparent digestibility of protein and fat, while starch digestibility ranged 83-84%.

The nutritional background of fish had no effect (P>0.05) on the ADC of protein, fat, starch and energy.

5.3.2. Plasma glucose levels

Plasma glucose levels measured at 0 h (unfed) and 6 h after the HS meal are presented in Table 5.4. The CTRL fish (9.5 mM) had a postprandial plasma glucose level at 6 h after the meal similar to that of GLU fish (10.2 mM), therefore postprandial glycaemia was not influenced by the nutritional history of the fish (P>0.05). Similar observations were found for the basal levels of plasma glucose, measured in unfed fish of both treatments (ranging from 5.6 to 6.7 mM). Similarly, no significant effect (P>0.05) of CTRL and GLU nutritional history was found for the plasma glucose levels after the intraperitoneal injection of glucose or saline solutions. In both nutritional groups, glucose administration resulted in a slightly higher glycaemia (6.1 mM, mean value of both treatments) in comparison to the saline injected fish (4.4 mM, mean value).

Table 5.4 – Plasma glucose (mM) of juvenile seabream coming from CTRL and GLU fish groups measured before and after a meal (postprandial) and after a glucose tolerance test, all determined at the end of the experimental trial

	CTRL group	**GLU group**	***P* value**
Postprandial			
0 h (unfed)	5.6 ± 1.06	6.3 ± 1.99	0.424
6 h	9.5 ± 3.68	10.2 ± 4.46	0.509
Glucose tolerance test			
Saline	4.5 ± 0.86	4.3 ± 0.55	0.499
Glucose	5.6 ± 2.27	6.5 ± 1.72	0.338

Data are presented as means and standard deviation (SD) (n=9 individuals). A Student's *t*-test (P < 0·05) was performed to analysed data from the CTRL and GLU group.

5.3.3. Carbohydrate and protein metabolic utilization

The metabolic fate of dietary carbohydrates and protein in the CTRL and GLU treatments was assessed by a tube-feeding method, using ^{14}C-starch and ^{14}C-AA mixture tracers. Long-term effects related to the early nutritional history of glucose stimuli were found in starch metabolism. Results showed that juveniles from the GLU treatment had a higher capacity for

starch absorption in the gut (74%) and concomitant lower evacuation of the nutrient (26%) when compared to the CTRL fish (62% and 38%, respectively, $P<0.05$) (Figure 5.2-A). After starch absorption, the metabolic use of glucose was assessed by the flux of ^{14}C-skeletons. In both treatments, glucose resulting from dietary starch was preferentially catabolized (over 80%) rather than retained in body tissue (Figure 5.2-B). At this stage, the metabolic use of glucose was not affected by the nutritional history ($P>0.05$). Glucose was mostly retained in the form of protein and FAA, in the liver and muscle tissue ($P<0.05$). Together these two fractions represent more than 70% of the glucose retained in both tissues (Figure 5.2-C, D). The GLU juveniles showed a lower retention of ^{14}C-skeletons from glucose in the protein fraction of liver (19%) when compared to the CTRL fish (27%) (Figure 5.2-C). No significant differences ($P>0.05$) were found, between treatments, for the hepatic bio-conversion of glucose into FAA, lipids and glycogen, although the FAA fraction was slightly, but not significant ($P=0.06$), higher in the GLU fish (Figure 5.2-C). In muscle, the fraction of ^{14}C-skeletons from starch tracer retained as other metabolites (mostly glycogen) was significantly lower ($P<0.05$) in GLU fish (19%) compared to the CTRL (25%) (Figure 5.2-D). The muscle bio-conversion of glucose into protein, FAA and lipids was not significantly different between the treatments ($P>0.05$), however the FAA fraction was again slightly higher in the GLU fish ($P=0.06$) (Figure 5.2-D). Between the two tissues (in a basis of 100 mg), glycogen retention was higher in the muscle (19-25%) than in the liver (7%). Although not different among treatments ($P>0.05$), *de novo* synthesized fatty acids from ^{14}C-starch were found in the liver (19-24%) and muscle (5-7%). From the ^{14}C-amino acid flux study, we found that none of the assessed parameters for protein utilization - absorption, evacuation, catabolism or tissue retention of AA - were affected, at long-term, by the early nutritional history of the fish (Figure 5.3). Results showed that seabream juveniles from CTRL and GLU groups had nearly the same capacity for amino acid absorption (71 % and 67%) and evacuation (29% and 33%), not being significantly different between the two treatments ($P>0{\cdot}05$) (Figure 5.3-A). The AA retention in body tissues was 47.1% and 41.3%, while catabolism was of 52.0% and 58.9% in the CTRL and GLU fish, respectively (Figure 5.3-B). Most of amino acids were retained as protein and/ or FAA in the liver and muscle tissues of both fish groups, being at least 80% the total retained label (Figure 5.3-C, D). The bio-conversion of amino acids into lipids was low, still higher in the liver (14% CTRL and 8% GLU) than in muscle (4.0%), and inversely the bio-conversion of amino acids into glycogen was higher in the muscle (9.0%) than in liver (4%) (Figure 5.3-C, D). Nevertheless, none of the retained fractions of organic compounds were significantly affected by the nutritional history of the fish ($P>0{\cdot}05$).

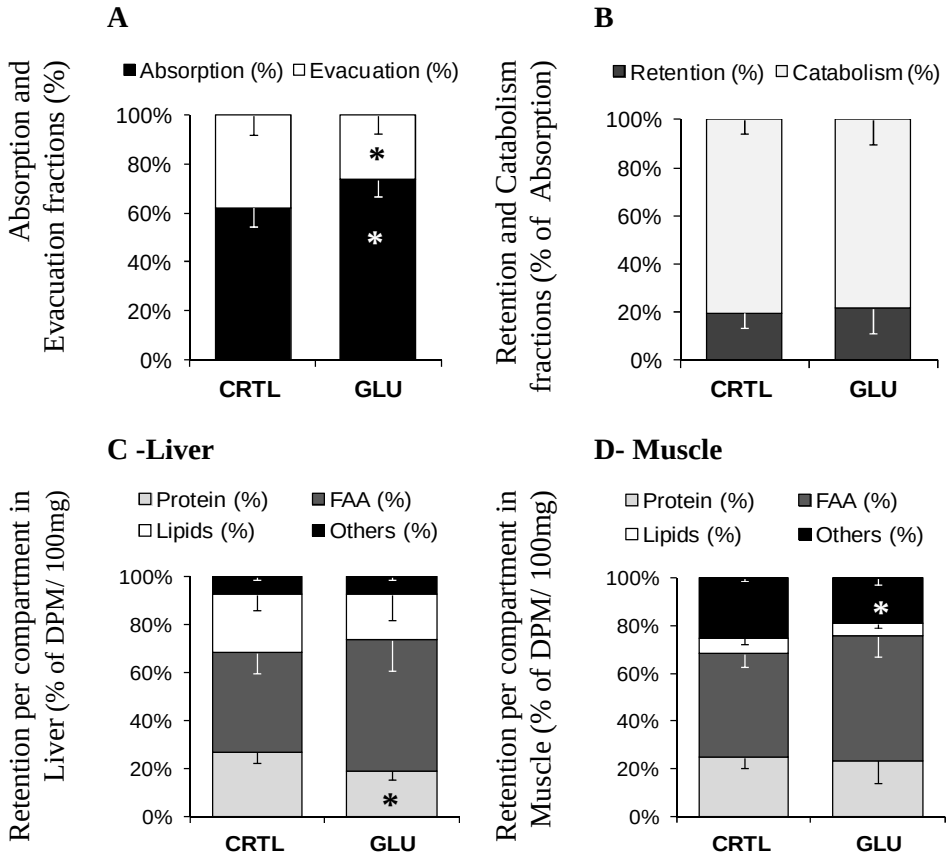

Figure 5.2 – Metabolic flux of ^{14}C-Starch in seabream juveniles submitted to a dietary challenge with a high-starch (HS)diet **(A)** Proportion (%) of radiolabel absorbed (fish and CO_2 trap) and evacuated (water) in relation to the total quantity of radiolabeled feed to the juvenile; **(B)** Proportion (%) of radiolabel retained in tissues and catabolized (CO_2 trap) in relation to the total absorbed label; **(C)** Proportion (%) of relative retention of radiolabel in each fractions of liver tissue (protein, FAA, lipids and other metabolites) in relation to total retained label; **(D)** Proportion (%) of relative retention of radiolabel in each fractions of muscle tissue (protein, FAA, lipids and other metabolites) in relation to total retained label. Data was determined after an incubation period of 18 h. Values are means – SD of seabream absorption, evacuation, retention, catabolism and relative retention fractions (n= 9 individuals). *Denotes significant differences between CTRL and GLU treatments for the corresponding fraction ($P < 0{\cdot}05$, Student's *t*-test).

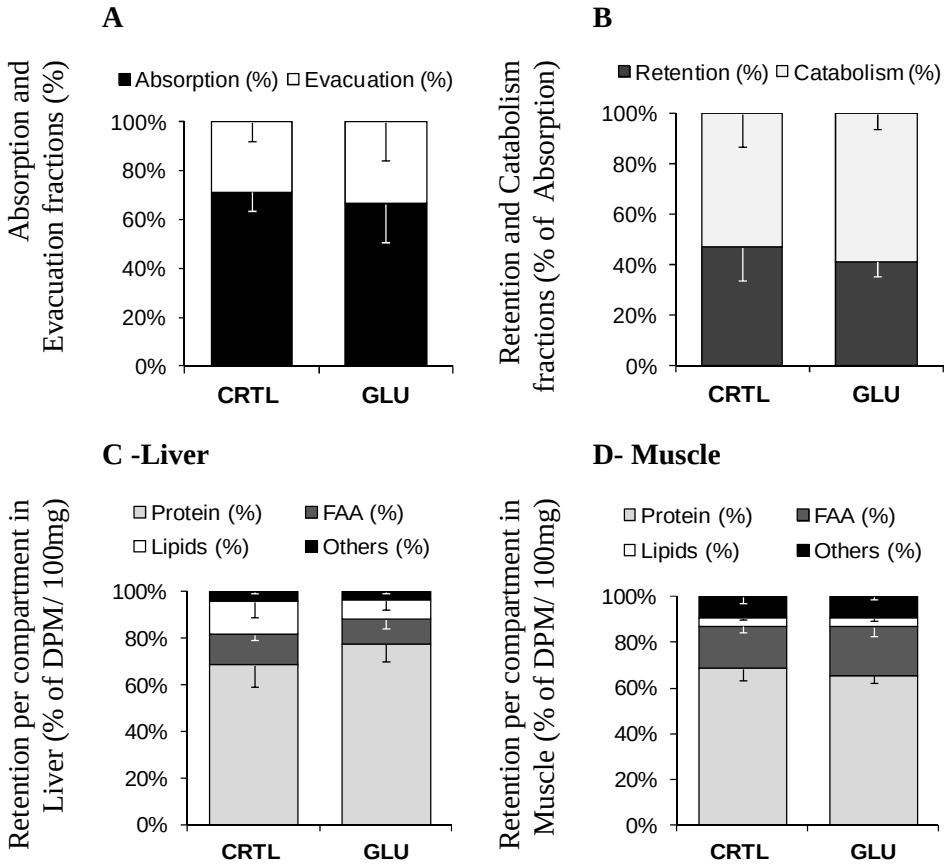

Figure 5.3 – Metabolic flux of ^{14}C-Amino acid mixture in seabream juveniles submitted to a dietary challenge with a high-starch (HS)diet **(A)** Proportion (%) of radiolabel absorbed (fish and CO_2 trap) and evacuated (water) in relation to the total quantity of radiolabeled feed to the juvenile; **(B)** Proportion (%) of radiolabel retained in tissues and catabolized (CO_2 trap) in relation to the total absorbed label; **(C)** Proportion (%) of relative retention of radiolabel in each fractions of liver tissue (protein, FAA, lipids and other metabolites) in relation to total retained label; **(D)** Proportion (%) of relative retention of radiolabel in each fractions of muscle tissue (protein, FAA, lipids and other metabolites) in relation to total retained label. Data was determined after an incubation period of 18 h. Values are means – SD of seabream absorption, evacuation, retention, catabolism and relative retention fractions (n= 9 individuals). *Denotes significant differences between CTRL and GLU treatments for the corresponding fraction ($P < 0{\cdot}05$, Student's *t*-test).

5.3.4. Expression of hepatic and muscle metabolism-related genes

The relative expression of genes involved in several metabolic pathways of the challenged juvenile fish from the CTRL and GLU groups, is presented in Table 5.5. mRNA levels of hepatic genes related to glycolysis, gluconeogenesis, glucose transporter, lipogenesis, energy metabolism and amino acids catabolism remained unaltered on the CTRL and GLU treatments (P>0.05). Further, the expression level of muscle genes involved in glycolysis, energy metabolism and amino acid catabolism was also not affected (P>0.05) by the early nutritional history of fish (Table 5.5).

Tabel 5.5 – Relative expression of genes involved in several metabolic pathways in the liver and muscle tissues of seabream juveniles from the CTRL and GLU groups, after being submitted to a dietary challenge with a high-starch (HS) diet.

		CTRL group		**GLU group**		
Pathways	Gene	Mean	SD	Mean	SD	*P* value
Liver tissue						
Glycolysis	GK	1.1	0.43	0.9	0.33	0.389
Glycolysis	HK1	n.d.		n.d.		
Glycolysis	6PFK-L	1.0	0.23	0.8	0.20	0.096
Gluconeogenesis	PEPCK	1.0	0.20	0.8	0.19	0.130
Gluconeogenesis	G6Pase	1.1	0.41	1.0	0.27	0.853
Lipogenesis	FAS	1.2	0.70	0.8	0.34	0.273
Lipogenesis	ACC	1.1	0.42	0.8	0.31	0.179
Energy	G6PDH	1.0	0.56	0.8	0.50	0.287
Energy	COX4	1.0	0.12	0.9	0.10	0.063
Energy	CS	1.1	0.55	1.0	0.59	0.710
Transporter	GLUT9	1.0	0.23	1.0	0.30	0.710
Amino acid catabolism	ALTm	1.0	0.19	0.9	0.15	0.354
Amino acid catabolism	ASTc	1.0	0.18	0.9	0.14	0.056
Muscle tissue						
Glycolysis	HK1	1.0	0.24	0.9	0.40	0.413
Glycolysis	6PFK-M	1.2	0.77	1.2	0.74	0.946
Glycolysis	PK-M	1.0	0.27	1.0	0.25	0.658
Energy	COX4	1.0	0.23	0.8	0.33	0.298
Energy	CS	1.1	0.39	1.0	0.37	0.719
Amino acid catabolism	ALTm	1.0	0.18	0.9	0.31	0.343
Amino acid catabolism	ASTc	n.d.		n.d.		

Expression values of liver and muscle tissues were normalized with α-elongation factor 1 (EF1α) transcripts. Relative fold differences between CTRL and GLU group are presented as means and standard deviation (SD) (n=6 individuals) and were analysed using Student's *t*-test ($P < 0{\cdot}05$). n.d., expression was not detected

5. 4. Discussion

The optimization of dietary protein allocation for growth and the increase of plant ingredients utilization, either as a source of dietary protein or starch, is a priority for the sustainable development of fish feeds. Under this scenario, the overall intake of carbohydrates will substantially increase and for carnivorous fish species exhibiting a poor utilization of carbohydrates, this trend can become a serious disadvantage (Polakof et al., 2012). The concept of altering the animal's ability to use nutrients by means of nutritional programming is under increasing attention. In order to evaluate this hypothesis in gilthead seabream, we conducted early nutritional interventions by recurrently feeding seabream larvae with high glucose levels (8, 25, 35 and 60 DAH) and then assessed its long-term effects upon changing some metabolic pathways of juvenile fish (219 DAH). Since our main goal was to program the metabolic pathways related to carbohydrates, the juvenile fish were submitted to an intense 5-week regime of high-carbohydrate intake (challenge) to trigger any "metabolic memory" seeded by the early glucidic stimuli. Raw pea starch was selected as the main source of carbohydrate in the challenge diet (HS) because of its high amylose content (35%) (FAO, 1998), and its fairly low digestibility in mammals and fish (Butterworth et al., 2011; Dias et al., 1998; Venou et al., 2003). This choice was made in an attempt not to dismiss a possible positive effect of the early nutritional interventions in improving carbohydrate digestion, in addition to those related to glucose utilization, at a molecular or metabolic level. It was also considered the risk that a highly digestive starch may mask the possible positive effects of the early stimuli. To our knowledge, so far no other study in a marine fish reported the long-term effects of hyperglucidic stimuli during early life on carbohydrate metabolic use.

After the dietary challenge with the HS diet, fish from both nutritional groups showed similar growth, feed intake and feed efficiency. This indicates that the nutritional history (the early glucidic stimuli) did not cause long-term effects on the overall growth-related criteria at the late juvenile stage. The lack of persistent effects of the early nutritional interventions on growth and feed efficiency was also reported in other studies performed in the field of metabolic programming in fish, as seen in rainbow trout, challenged with a high intake of carbohydrates (Geurden et al., 2007; Geurden et al., 2014) and in European seabass, challenged with a low n-3 polyunsaturated fatty acids diet (Vagner et al., 2007; Vagner et al., 2009). In the present study, fish from CTRL and GLU groups were able to digest the raw pea starch fairly efficiently, showing an ADC of 84.0% at a dietary inclusion level of 30.0%.

These starch digestibility values are close to those reported in other works for gilthead seabream using similar raw starch inclusion levels. For example, seabream fed diets containing 30-31% of raw wheat starch, also displayed starch ADC values between 80% to 91%, whereas for raw maize starch the ADC varied from 88% to 95% (Enes et al., 2011). Another study conducted with seabream fed on diet similar to our HS formulation (45% protein, 21% fat, 20% starch from dextrin source) showed a starch ADC of 81.2%; but it was also shown that changes in the lipid level affected starch digestibility as well the enzymatic activity of amylase (Fountoulaki et al., 2005). Therefore, to compare starch digestibilities within a particular species one must be consider not only the botanical origin, processing treatment, inclusion levels of the starch but also how nutrients are available in the diet, their balance and contribution for energy ratios (Enes et al., 2011; Krogdahl et al., 2005). Despite the similarity in the apparent starch digestibility, independent of the nutritional background, the metabolic tracer data using a ^{14}C-starch showed a higher capacity for starch-derived glucose absorption in the gut of GLU vs CTRL juveniles. This discrepancy between the starch digestion and absorption capacity data can probably be explained by the sensitivity of each methodological approach. Given the inherent difficulties of assessing true digestibility coefficients in fish, starch digestibility data was measured as apparent digestibility, and did not account for potential endogenous losses. On the other hand, tube feeding of radiolabeled starch, while allowing a higher sensitivity on detection of effects, may not necessarily represent the normal digestive process of an undisturbed fish feeding *ad libitum* in a culture system (Conceição et al., 2007). Although speculative, an improvement of glucose uptake in GLU fish could potentially be related to an increment of intestinal brush border tissue mass, as previously observed in carp and tilapia in response to high intake of carbohydrates (Krogdahl et al., 2005).

By tube-feeding the juvenile fish with the ^{14}C-starch labeled diet, we examined the utilization of the absorbed ^{14}C-glucose, the end product of starch digestion, over a time frame of 18 h. From the absorbed fraction of ^{14}C-starch, fish from the two nutritional groups presented no effect of the early nutritional experience in the metabolic utilization of the ^{14}C-skeletons, showing that dietary starch was mainly catabolized (up to 80%). Compared to the carnivorous rainbow trout, known for their poor utilization of dietary carbohydrates and to the omnivorous common carp, which easily deals with high carbohydrate intake, seabream (marine carnivorous) revealed to have an intermediate phenotype for the use of carbohydrates (Panserat et al., 2000; Peres et al., 1999). This pattern might be reflected here by the rather

elevated capacity for glucose oxidation (80%) compared with the low rate of glucose oxidation found in the highly carnivorous plaice (12.3%) (Cowey et al., 1975). Glucose tolerance tests (GTT) can also be used as an indicator of any alteration on glucose metabolism. Again, no effects of the early nutritional history were observed after the GTT, as both fish groups were able to metabolize the injected glucose in similar way. The plasma glucose peaked at 5.6 and 6.5 mM in juveniles (20 g body weight) of the CTRL and GLU treatments, respectively, being this response considerably lower than the observed in seabream (194 g body weight) of 20 mM, after receiving a dose of 1 g of glucose kg^{-1} (Peres et al., 1999). Perhaps the dose used in our study for glucose injection (250 mg glucose·kg^{-1}) was not enough to induce a more pronounced increase of glycaemia level; however, this dose was corrected for the stage of development, size and weight of the fish. Concerning the postprandial glycaemia levels, fish from both CTRL and GLU treatments had a higher level of plasma glucose after the intake of dietary carbohydrates, regardless of the nutritional history. The observed postprandial glycaemia levels are in agreement with other studies performed in gilthead seabream, being closer to levels achieved after the intake of dietary glucose (Enes et al., 2008a; c) than to those obtained with diets containing maize starch (Enes et al., 2008b; c).

Regarding the use and bioconversion of the retained glucose originating from the dietary starch, we observed that the ^{14}C-carbon was mainly recovered into the FAA fraction in both liver and muscle. Previous studies performed in flatfish, using a radiolabeled tracer, also observed the incorporation of ^{14}C-skeletons derived from glucose in nonessential amino acids, which confirms that glucose may serve as precursor for amino acid synthesis in fish (Cowey et al., 1970). The *de novo* synthesis of protein (in liver) and glycogen (in muscle) was lower in the GLU than in the CTRL juvenile. This suggests that the early glucidic events permanently affected some pathways involved in glucose bioconversion, yet possible underlying mechanisms susceptible to be altered by the early glucose stimuli remain uncertain. One possible way to elucidate which pathways were modified is through the addition of more incubation periods in future tracer studies, in order to get a dynamic overview during nutrient absorption and retention. Extra sampling points may also help to unravel the pathways involved, since the glucose sensing system engages a complex network of enzymes and transcription factors, acting in several nutrient-sensitive tissues, such the liver, intestine and adipose tissue (Efeyan et al., 2015; Polakof et al., 2011b).

At the end of the experimental feeding period, fish displayed a relativly good growth compared to that in other studies in seabream juveniles (Mihelakakis et al., 2002), despite being challenged with a diet having a dietary protein level (43%) below that recommended for juvenile seabream (50-55%) (Vergara et al., 1996). The finding that the drastic reduction of crude protein did not jeopardize growth suggests that the requirements of amino acids were all covered. Hence, the protein efficiency ratio (PER, 2.23 - 2.25) and protein retention (25.9 - 27.6% of intake) were high, in relation to other studies with seabream fed diets up to 30% inclusion of gelatinized, extruded or raw starches (PER values from 0.85 to 1.6) (Couto et al., 2008; Enes et al., 2008b; Venou et al., 2003). The similarity in PER and protein retention in both CTRL and GLU fish indicates that the early glucose stimuli did not promote extra protein-sparing by high dietary carbohydrates in the juveniles. The metabolic flux of amino acids, assessed by using a radiolabeled mixture of amino acids, confirmed that the early glucidic stimuli at larval stages failed to cause long-lasting modifications on amino acid metabolism. These results are further supported by the molecular data of genes involved in gluconeogenesis (G6Pase, PEPCK) and amino acid catabolism (ALTm, ASTc) that remained unchanged among the two fish groups.

Seabream from the GLU treatment had higher lipid retention (58.4%) in comparison to the CTRL fish (47.9%), which indicates a long-term effect of the early glucidic stimuli on lipid metabolic pathways. In fish, as in mammals (Kersten, 2001), there exist a strong interrelationship between carbohydrate and lipid metabolism. This is often reflected through the stimulation of lipogenesis by high carbohydrate intake, as illustrated by enhanced lipogenic enzyme activities, enhanced lipogenic genes expressions, and high plasma metabolites or lipid retention ratios in rainbow trout (Alvarez et al., 2000; Figueiredo-Silva et al., 2012; Kamalam et al., 2012), European seabass (Dias et al., 1998) and even in seabream (Bou et al., 2014). However, in our study the higher lipid retention or enhanced lipogenesis in the GLU juveniles was not accompanied by a higher expression of genes coding for hepatic lipogenic enzymes (FAS, ACCα, G6PDH) nor by an enhanced allocation of starch-derived ^{14}C-skeletons towards *de novo* synthesis of fatty acid in the liver or muscle. In fish, the liver plays a central role in controlling glucose homeostasis while the white muscle is an important peripheral tissue for glucose utilization (Moon, 2001). A recent study in rainbow trout demonstrated that the adipose tissue may act not only as lipid depot but also as a lipogenic organ, being involved in glucose homeostasis through insulin regulation (Polakof et al., 2011a). Therefore, the increased lipid retention in the GLU fish could possibly be better

explained if a detailed molecular analysis of the white adipose tissue had been performed in the juvenile fish. Moreover, our tracer studies followed the metabolic fate of starch for a period of only 18 h, which may not be long enough to allow a clear portrait of hepatic fatty acid biosynthesis and its subsequent transport and deposition into lipid storage.

Our molecular analyses which focused on the expression of genes involved in glycolysis, gluconeogenesis, energy metabolism and amino acid metabolism did not reveal persistent molecular changes related with the higher glucose absorption and lower bioconversion of glucose ^{14}C into protein and glycogen between the GLU and CTRL juveniles. In contrast, the few studies that explored the concept of early nutritional programming in fish were able to demonstrate some permanent molecular modifications that supported the possibility of programming specific metabolic pathways (Fang et al., 2014 ; Geurden et al., 2007; Geurden et al., 2014; Rocha et al., 2014; 2015; Vagner et al., 2009). A long-term response to early nutritional stimuli would have been of interest as a possible mechanism for storing "metabolic memory" of the stimulus by epigenetic regulation of the genome (Lillycrop and Burdge, 2012; Lucas, 1998; Vickers, 2014). Nevertheless, other mechanisms have also been proposed to operate during the nutritional programming of an organism, such as permanent changes in a tissue or organ structure (Tarry-Adkins and Ozanne, 2011). It has been demonstrated in rat models that the endocrine pancreas is particularly susceptible to changes in nutrition during fetal life that permanently alter the structure of this tissue by impairing vascularization and reducing β-cell regeneration (Garofano et al., 2000; Snoeck et al., 1990). Thus, further studies are warranted to better understand the mechanisms involved in the long-term phenotypic changes induced by the early glucose stimuli in seabream and to validate the concept of nutritional programming.

5.5. Conclusions

The present study showed that early glucidic stimuli exerted at the larval stage positively affected lipid retention and the absorptive capacity of dietary starch-derived glucose of the seabream juveniles fed a starch-rich diet. The early stimuli also modified the recovery of the absorbed starch-derived carbon skeletons among different nutrient fractions (protein, lipids, FAA, glycogen) suggesting long-term changes in the respective bioconversion pathways. Yet, several other parameters like growth, apparent digestibility, feed efficiency, amino acid

metabolism as well as the transcript levels of metabolic genes were not permanently affected at the juvenile stage. Further work is needed to determine which mechanisms or which pathways of glucose metabolism were changed by the early nutritional events at the long-term. Our study is the first to present permanent modifications, although few, on carbohydrate utilization in a marine species, through the action of early nutritional events.

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5.7. References

Alvarez, M.J., Diez, A., Lopez-Bote, C., Gallego, M., Bautista, J.M., 2000. Short-term modulation of lipogenesis by macronutrients in rainbow trout (Oncorhynchus mykiss) hepatocytes. Br J Nutr. 84, 619-628.

Basurco, B., Lovatelli, A., García, B., 2011. Current status of Sparidae aquaculture. in: Pavlidis, M., Mylonas, C. (Eds.), Sparidae: Biology and aquaculture of gilthead sea bream and other species. Wiley-Blackwell, pp. 1-50.

Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G.F., Folkedal, O., Olsen, R.E., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014. Metabolic and transcriptional responses of gilthead sea bream (Sparus aurata L.) to environmental stress: New insights in fish mitochondrial phenotyping. Gen Comp Endocrinol. 205, 305-315.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purificaton. Can J Biochem Phys. 37, 911-917.

Bolin, D.W., King, R.P., Klosterman, E.W., 1952. A simplified method for the determination of chromic oxide (Cr2O3) when used as an index substance. Science. 116, 634-635.

Bou, M., Todorcevic, M., Fontanillas, R., Capilla, E., Gutierrez, J., Navarro, I., 2014. Adipose tissue and liver metabolic responses to different levels of dietary carbohydrates in gilthead sea bream (Sparus aurata). Comp Biochem Physiol A Mol Integr Physiol. 175, 72-81.

Burdge, G.C., Lillycrop, K.A., 2010. Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr. 30, 315-339.

Butterworth, P.J., Warren, F.J., Ellis, P.R., 2011. Human α-amylase and starch digestion: An interesting marriage. Starch - Stärke. 63, 395-405.

Campos, C., Castanheira, M.F., Engrola, S., Valente, L.M., Fernandes, J.M., Conceicao, L.E., 2013. Rearing temperature affects Senegalese sole (Solea senegalensis) larvae protein metabolic capacity. Fish Physiol Biochem. 39, 1485-1496.

Caseras, A., Meton, I., Fernandez, F., Baanante, I.V., 2000. Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (Sparus aurata). Biochim Biophys Acta. 1493, 135-141.

Conceição, L.E.C., Rønnestad, I., Tonheim, S.K., 2002. Metabolic budgets for lysine and glutamate in unfed herring (Clupea harengus) larvae. Aquaculture. 206, 305-312.

Conceição, L.E.C., Morais, S., Rønnestad, I., 2007. Tracers in fish larvae nutrition: A review of methods and applications. Aquaculture. 267, 62-75.

Costas, B., Aragão, C., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arjona, F., Dinis, M., Mancera, J., Conceição, L.C., 2011. Feed deprivation in Senegalese sole (Solea senegalensis Kaup, 1858) juveniles: effects on blood plasma metabolites and free amino acid levels. Fish Physiol Biochem. 37, 495-504.

Couto, A., Enes, P., Peres, H., Oliva-Teles, A., 2008. Effect of water temperature and dietary starch on growth and metabolic utilization of diets in gilthead sea bream (Sparus aurata) juveniles. Comp Biochem Physiol A Mol Integr Physiol. 151, 45-50.

Couto, A., Enes, P., Peres, H., Oliva-Teles, A., 2012. Temperature and dietary starch level affected protein but not starch digestibility in gilthead sea bream juveniles. Fish Physiol Biochem. 38, 595-601.

Cowey, C.B., Adron, J., Blair, A., 1970. Studies on the nutrition of marine flatfish the essential amino acid requirements of plaice and sole. J. Mar. Biol. Assoc. U.K. 50, 87-95.

Cowey, C.B., Adron, J.W., Brown, D.A., 1975. Studies on the nutrition of marine flatfish. The metabolism of glucose by plaice (Pleuronectes platessa) and the effect of dietary energy source on protein utilization in plaice. Br J Nutr. 33, 219-231.

Dias, J., Alvarez, M.J., Diez, A., Arzel, J., Corraze, G., Bautista, J.M., Kaushik, S.J., 1998. Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (Dicentrarchus labrax). Aquaculture. 161, 169-186.

Efeyan, A., Comb, W.C., Sabatini, D.M., 2015. Nutrient-sensing mechanisms and pathways. Nature. 517, 302-310.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008a. Rearing temperature enhances hepatic glucokinase but not glucose-6-phosphatase activities in European sea bass (Dicentrarchus labrax) and gilthead sea bream (Sparus aurata) juveniles fed with the same level of glucose. Comp Biochem Physiol A Mol Integr Physiol. 150, 355-358.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008b. Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (Sparus aurata) juveniles. Aquaculture. 274, 101-108.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008c. Hepatic glucokinase and glucose-6-phosphatase responses to dietary glucose and starch in gilthead sea bream (Sparus aurata) juveniles reared at two temperatures. Comp Biochem Physiol A Mol Integr Physiol. 149, 80-86.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2011. Dietary carbohydrate utilization by European sea bass (Dicentrarchus labrax L.) and gilthead sea bream (Sparus aurata L.) juveniles. Reviews in Fisheries Science. 19, 201-215.

Ennos, A.R., 2007. Statistical and data handling skills in biology, 2nd ed. Pearson Prentice Hall, Harlow, England.

Fang, L., Liang, X.F., Zhou, Y., Guo, X.Z., He, Y., Yi, T.L., Liu, L.W., Yuan, X.C., Tao, Y.X., 2014 Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, Danio rerio. Br J Nutr. 111, 808-818.

FAO, 1998. Carbohydrates in human nutrition. Report of a Joint FAO/ WHO Expert Consultation. FAO- Food Nutr Paper 66, 1-140.

FEAP, 2014. European aquaculture production report 2004-2013. Federation of European Aquaculture Producers, Belgium, pp. 53.

Figueiredo-Silva, A.C., Panserat, S., Kaushik, S., Geurden, I., Polakof, S., 2012. High levels of dietary fat impair glucose homeostasis in rainbow trout. J Exp Biol. 215, 169-178.

Fountoulaki, E., Alexis, M.N., Nengas, I., Venou, B., 2005. Effect of diet composition on nutrient digestibility and digestive enzyme levels of gilthead sea bream (*Sparus aurata* L.). Aquaculture Research. 36, 1243-1251.

Garofano, A., Czernichow, P., BrÉAnt, B., 2000. Impaired β-cell regeneration in perinatally malnourished rats: a study with STZ. The FASEB Journal. 14, 2611-2617.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (Oncorhynchus mykiss) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am J Physiol Regul Integr Comp Physiol. 292, 2275-2283.

Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D., Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S., Panserat, S., 2014. High or low dietary carbohydrate:protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout. J Exp Biol. 217, 3396-3406.

Gluckman, P.D., Hanson, M.A., Beedle, A.S., 2007. Early life events and their consequences for later disease: a life history and evolutionary perspective. Am J Hum Biol. 19, 1-19.

Gong, G., Xue, M., Wang, J., Wu, X.-f., Zheng, Y.-h., Han, F., Liang, X.-f., Su, X.-o., 2015. The regulation of gluconeogenesis in the Siberian sturgeon (Acipenser baerii) affected

later in life by a short-term high-glucose programming during early life. Aquaculture. 436, 127-136.

Harder, T., Plagemann, A., Rohde, W., Dorner, G., 1998. Syndrome X-like alterations in adult female rats due to neonatal insulin treatment. Metabolism. 47, 855-862.

Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 33 Suppl, 245-254.

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S., 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J Exp Biol. 215, 2567-2578.

Kersten, S., 2001. Mechanisms of nutritional and hormonal regulation of lipogenesis. EMBO Rep. 2, 282-286.

Krogdahl, A., Hemre, G.I., Mommsen, T.P., 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquacult Nutr. 11, 103-122.

Lillycrop, K.A., Burdge, G.C., 2012. Epigenetic mechanisms linking early nutrition to long term health. Best Pract Res Clin Endocrinol Metab 26, 667-676.

Lucas, A., 1998. Programming by early nutrition: an experimental approach. J Nutr 128, 401-406.

Metges, C.C., Gors, S., Lang, I.S., Hammon, H.M., Brussow, K.P., Weitzel, J.M., Nurnberg, G., Rehfeldt, C., Otten, W., 2014. Low and high dietary protein:carbohydrate ratios during pregnancy affect materno-fetal glucose metabolism in pigs. J Nutr. 144, 155-163.

Mihelakakis, A., Tsolkas, C., Yoshimatsu, T., 2002. Optimization of Feeding Rate for Hatchery-Produced Juvenile Gilthead Sea Bream Sparus aurata. Journal of the World Aquaculture Society. 33, 169-175.

Moon, T.W., 2001. Glucose intolerance in teleost fish: fact or fiction? Comp Biochem Physiol B Biochem Mol Biol. 129, 243-249.

Morais, S., Lacuisse, M., Conceição, L.E.C., Dinis, M.T., Rønnestad, I., 2004. Ontogeny of the digestive capacity of Senegalese sole (Solea senegalensis), with respect to digestion, absorption and metabolism of amino acids from Artemia. Mar Biol 145, 243-250.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D., 2009. Feeding aquaculture in an era of finite resources. Proceedings of the National Academy of Sciences USA 106, 15103-15110.

Panchout, F., Letendre, J., Bultelle, F., Denier, X., Rocher, B., Chan, P., Vaudry, D., Durand, F., 2013. Comparison of protein-extraction methods for gills of the shore crab, Carcinus maenas (L.), and application to 2DE. J Biomol Tech. 24, 218-223.

Panserat, S., Medale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R., Kaushik, S., 2000. Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. Am J Physiol Regul Integr Comp Physiol. 278, 1164-1170.

Panserat, S., Plagnes-Juan, E., Kaushik, S., 2002. Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). Biochim Biophys Acta. 1579, 35-42.

Patel, M.S., Srinivasan, M., 2002. Metabolic programming: causes and consequences. J Biol Chem. 277, 1629-1632.

Peres, H., Gonçalves, P., Oliva-Teles, A., 1999. Glucose tolerance in gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax). Aquaculture. 179, 415-423.

Perez-Sanchez, J., Borrel, M., Bermejo-Nogales, A., Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J.A., Kaushik, S., 2013. Dietary oils mediate cortisol kinetics and the hepatic mRNA expression profile of stress-responsive genes in gilthead sea bream (Sparus aurata) exposed to crowding stress. Implications on energy homeostasis and stress susceptibility. Comp Biochem Physiol Part D Genomics Proteomics. 8, 123-130.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Polakof, S., Medale, F., Larroquet, L., Vachot, C., Corraze, G., Panserat, S., 2011a. Insulin stimulates lipogenesis and attenuates Beta-oxidation in white adipose tissue of fed rainbow trout. Lipids. 46, 189-199.

Polakof, S., Mommsen, T.P., Soengas, J.L., 2011b. Glucosensing and glucose homeostasis: From fish to mammals. Comp Biochem Physiol B Biochem Mol Biol. 160, 123-149.

Polakof, S., Panserat, S., Soengas, J.L., Moon, T.W., 2012. Glucose metabolism in fish: a review. J Comp Physiol B.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M.T., Panserat, S., 2014. Glucose overload in yolk has little effect on the long-term modulation of carbohydrate metabolic genes in zebrafish (Danio rerio). J Exp Biol. 217, 1139-1149.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M.T., Panserat, S., 2015. Glucose metabolism and gene expression in juvenile zebrafish (Danio rerio) challenged with a high carbohydrate diet: effects of an acute glucose stimulus during late embryonic life. Br J Nutr. 113, 403-413.

Rønnestad, I., Rojas-Garcı́a, C.R., Tonheim, S.K., Conceição, L.E.C., 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture. 201, 161-175.

Rust, M.B., Hardy, R.W., Stickney, R.R., 1993. A new method for force-feeding larval fish. Aquaculture. 116, 341-352.

Santinha, P.J.M., Medale, F., Corraze, G., Gomes, E.F.S., 1999. Effects of the dietary protein : lipid ratio on growth and nutrient utilization in gilthead seabream (Sparus aurata L.). Aquaculture Nutrition. 5, 147-156.

Snoeck, A., Remacle, C., Reusens, B., Hoet, J.J., 1990. Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol Neonate. 57, 107-118.

Symonds, M.E., Sebert, S.P., Hyatt, M.A., Budge, H., 2009. Nutritional programming of the metabolic syndrome. Nat Rev Endocrinol. 5, 604-610.

Tarry-Adkins, J.L., Ozanne, S.E., 2011. Mechanisms of early life programming: current knowledge and future directions. Am J Clin Nutr. 94, 1765S-1771S.

Thivend, P., Mercier, C., Guilbot, A., 1972. Determination of starch with glucoamylase. in: Whistler, R.L., BeMiller, J.N. (Eds.), Methods in Carbohydrate Chemistry. Academic Press, New York, pp. 100-105.

Vagner, M., Zambonino-Infante, J.L., Robin, J.H., Person, J., 2007. Is it possible to influence European sea bass (Dicentrarchus labrax) juvenile metabolism by a nutritional conditioning during larval stage? Aquaculture. 267, 165-174.

Vagner, M., Robin, J.H., Zambonino-Infante, J.L., Tocher, D.R., Person-Le Ruyet, J., 2009. Ontogenic effects of early feeding of sea bass (Dicentrarchus labrax) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. Br J Nutr. 101, 1452-1462.

Velázquez, M., Zamora, S., Martínez, F.J., 2006. Effect of dietary energy content on gilthead sea bream (*Sparus aurata*) feeding behaviour and nutritional use of the diet. Aquacult Nutr 12, 127-133.

Venou, B., Alexis, M.N., Fountoulaki, E., Nengas, I., Apostolopoulou, M., Castritsi-Cathariou, I., 2003. Effect of extrusion of wheat and corn on gilthead sea bream (Sparus aurata) growth, nutrient utilization efficiency, rates of gastric evacuation and digestive enzyme activities. Aquaculture. 225, 207-223.

Vergara, J.M., Robain, L., Izquierdo, M., Higuera, M.d.l., 1996. Protein Sparing Effect of Lipids in Diets for Fingerlings of Gilthead Sea Bream. Fisheries Science. 62, 624-628.

Vickers, M.H., 2014. Early life nutrition, epigenetics and programming of later life disease. Nutrients. 6, 2165-2178.

Waterland, R.A., Jirtle, R.L., 2004. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 20, 63-68.

General Discussion

Chapter VI

6.1 Which scientific challenges did we tackled?

Currently, little is known about the triggering effects of early nutrition on fish in particular during embryonic stages, where the only nutritional source for development is confined to the egg yolk. We proposed to investigate, for the first time in fish, the potential role of glucidic stimuli as modulators of metabolic pathways, following the concept of early nutritional programming. In terms of novelty, this thesis contributed towards a better understanding of fish nutritional programming, by exploring new approaches and tools that can change both the yolk reserves of embryos and the first feeding experience of early larvae. Former studies focused on nutritional manipulation during early stages of fish development were aimed at either the period previous to conception, through maternal nutrition or the post-hatching period, through the onset of exogenous feeding. The period between conception, spawning and mouth opening, corresponding to fish embryogenesis, was never investigated before as a potential period to perform nutritional interventions, perhaps due to the high fragility of embryos to external factors. In this thesis, we took advantage of this knowledge gap to explore the embryonic period as a new "developmental window" for programming in fish.

In this context, the **first part** of this thesis included several experiments aimed to the direct supplementation of the embryo's yolk with glucose, by means of microinjection, to verify the initial hypothesis of permanent modifications in growth potential, survival and regulation of the intermediary metabolism of juvenile fish. For these purposes, we considered the zebrafish a suitable species to fulfil all the requirements necessary to assess the technical and biological feasibility of manipulating the nutritional content of embryos, and later to validate the nutritional programming at embryonic stages. The selection of this species was based on its relevant features as a research model on studies of developmental biology and molecular genetics, in particular to its high resilience to manipulations during embryonic development and a elevated number of eggs in each spawning (Goldsmith and Jobin, 2012), which facilitated the performance of this studies. Nevertheless, the fact that zebrafish is an omnivorous cyprinid and exhibits a relative higher tolerance to dietary carbohydrates compared to other species with more carnivorous feeding habits was not neglected, but was also not considered relevant for the action of glucose stimulus. In our studies, the nutritional event with a glucidic stimulus was performed during embryogenesis and for the majority of fish species, carbohydrates are not a relevant energetic substrate, except for the first cellular divisions (Heming and Buddington, 1988).

Given the variability among fish species on the depletion patterns of nutritional substrates during the yolk-embryo stage and their ability to use dietary carbohydrates is mainly linked to their natural feeding behaviour, this doctoral study also targeted a farmed species, with preferred carnivorous feeding habits and of relevance in the context of marine aquaculture, specially of the Mediterranean region - the gilthead seabream. The **second part** of this research study comprised two experiments focused on assessing the immediate and persistent effects of first-feeding gilthead seabream larvae with high levels of glucose on growth, survival, gene expression, nutrient metabolism and several zootechnical parameters related to feed utilization and digestibility, all under the scope of the nutritional programming concept. For the first time during a larval rearing, live preys (rotifers and *Artemia*) were used as vehicles for delivering a glucose stimulus, in order to ensure a good acceptance of the stimulus by the larvae from the moment of mouth opening. These nutritional studies relying on marine fish species are of great interest for the expansion of sustainable feeding practices in fish farming, since they contribute to clarify some metabolic bottlenecks on the use of dietary carbohydrates as energy sources. The results presented in the previous chapters are going to be discussed in a more consolidated and integrative way for a better comprehension of the possible achievement of fish metabolic programming through early nutritional stimuli.

6.2. Is embryogenesis a suitable period to program fish metabolism?

In mammals, the prenatal period is a time of major changes in the fetus characterized by rapid growth, development and maturation of organs and systems. It is now well established that changes in the macro- and/or micronutrients of the maternal diet, at critical stages of fetus development, can exert permanent and powerful effects upon developing tissues. This phenomenon is known as nutritional programming and it has been extensively studied in mammalian models for the understanding of non-communicable diseases, such as the metabolic syndrome, diabetes and obesity (Armitage et al., 2004; Burdge and Lillycrop, 2010; Langley-Evans, 2015; Vieau, 2011). Programming effects are likely to be more efficient in adulthood when the stimulus is exerted in cells and tissues poorly differentiated, that still retain high levels of plasticity and pluripotency, thereby enabling the physiological adaptation of the embryo or fetus to their new uterine environment (Gluckman et al., 2011). Analogous to the prenatal period of mammals, the embryonic stage in fish development also comprises high levels of developmental plasticity, being thus considered in this thesis as an important

window for testing the nutritional programming concept in fish. Since most fish are oviparous and with external fertilization, the embryo development is not dependent on the maternal nutrition but solely on their endogenous yolk reserves, (Heming and Buddington, 1988). In contrast, mammals have evolved with a reproductive pattern totally opposite of fish, the gestation is performed inside the uterus and the fetus is fed by the mother through the placenta (Blackburn, 1999). Therefore, given these marked differences in the embryonic development, one should be caution in extrapolating the programming concept from mammals to fish, since the embryos are exposed to the nutritional stimulus in distinct ways, which may influence the mechanisms and processes underlying the imprinting of the early event.

A fertilized fish egg operates like a contained physiological system, once exchange of nutrients and other solutes with its surrounding are limited or inexistent. As a consequence, the nutritional composition of the yolk reserve formed after fertilization, is only changed by the natural depletion pattern of the embryos, throughout their development. The attempt of altering the nutritional composition of the yolk by direct action during embryogenesis is highly difficult, mainly due to the fragility of embryos to excessive handling and invasive procedures. Microinjection is one of the most effective and precise methods for *in ovo* interventions. This technique is particularly well known and applied in zebrafish, within the piscine models (Xu, 1999). Its applications extend to the field of genomics (DNA, RNA and protein fragments introduction), cryopreservation, toxicology, infectious diseases and *in vitro* fertilization (Amacher, 2008; Hornung et al., 1996; Janik et al., 2000; Kopeika et al., 2006; Poleo et al., 2001; Xu, 1999), however it has never been tested as a tool for nutrient supplementation at early developmental stages. In addition, zebrafish was recently proposed as a model organism for nutritional research, being emphasized its use in nutrigenomic studies (Ulloa et al., 2011). Given the great potential of zebrafish, we have tested and validated the efficiency of microinjection, as an accurate technique to increase glucose levels in the yolk of zebrafish embryos, which had never been demonstrated before. **It was found that immediately after the injections with a 2M glucose solution, embryos at the stage of 0.2 dpf (blastula period) or 1 dpf (pharyngula period) had an increase up to 28- or 6-fold of their basal levels of glucose, respectively (Chapter 2 and 3).** These results indicate the feasibility of microinjecting at different stages of embryo development as well as the dynamic regulation of glucose utilization throughout embryogenesis, demonstrated by the variation in the supplementation levels reached with the same injected dose. Alter the composition of embryo's yolk and assess embryo capacity to cope with excess glucose were important

achievements on this thesis, to propose an additional developmental window for testing the nutritional programming concept in fish. In **Chapter 2**, we performed a tolerance test using a range of glucose concentrations, from 0.2 to 2.2M, to establish the highest dose to be injected in early embryos (0.2 dpf). **Zebrafish embryos were able to tolerate the gradual increase of glucose in the yolk up to hyperglucidic levels (1.5mM) without impairing embryonic development, growth and survival until the stage of total consumption of the yolk (10 dpf).** This knowledge on the manipulation of egg-yolk composition may contribute to develop new nutritional studies on the role of specific nutrients supplementation.

Although it was shown that zebrafish embryos possess a high degree of tolerance to high manipulation, it does not mean that the same features are found in other fish species, especially in marine fish that present an altricial development. After a total of five experiments performed with seabream embryos at different stages of development (gastrula, segmentation and pharyngula) it became clear that embryos were extremely sensitive not only to handling, sorting and puncturing but also to the glucose loading in the yolk, which resulted in a high mortality rate shortly after the microinjection procedure (data not presented). Based on these results, an additional approach to deliver the early glucidic stimulus in seabream was explored, apart from the embryonic stages. **Chapter 4** was dedicated to investigate a new strategy for stimulus delivery in seabream, and these findings are discussed later on this thesis.

Teleost fish have a much faster embryonic development than mammals, in terms of duration, probably due to their ex-uterus development that increases their vulnerability to predation, random dissemination and environmental changes. The period between the fertilization of the egg and its hatching can last for 3 to 4 days in zebrafish or 2 days in seabream, taking in account the rearing temperatures of 28º C and 18º C, respectively. (Kimmel et al., 1995; Lawrence, 2007; Polo et al., 1991). During embryogenesis, several important ontogenic events begin to occur focusing on the development of organs buds and the organization of key regulatory pathways, as demonstrated in zebrafish through several genetic screening studies (Amsterdam et al., 2004; Kudoh et al., 2001; Mathavan et al., 2005; Soanes et al., 2011). Our results concerning the ontogenetic expression of key metabolic genes in zebrafish are in line with these findings. **The majority of the pathways involved in glucose utilization remain poorly activated or inactivated at embryonic stages (0.2 and 1 dpf) becoming progressively functional before the onset of exogenous feeding (4 - 6 dpf). This is**

coincident with completion of several physiological steps, including the formation of functional organs (Chapter 2). Based on these results, we have considered that during incipient stages of development (0.2 dpf), where most of the glucose-related genes were not being expressed, is likely to occur higher levels of plasticity for "memorizing" the nutritional stimulus and provoke permanent changes in adulthood. Contrary to what was expected, we saw in **Chapter 2** that **the enrichment of the yolk with high levels of glucose at early stages led to an acute inhibitory effect on the expression of several metabolic genes.** This short-term effect of glucose injection on gene expression observed at 4 dpf was not permanent, since it faded away along larval development, until it was no longer observed at 10 dpf. Therefore, it seems that an overload of glucose in the yolk at such early embryonic stages may have compromised some physiological or molecular mechanisms involved in nutritional regulation; however, this did not impair the ability of future juvenile fish to cope with dietary carbohydrates. From this point, we begin to question if a) the selected developmental window was not too premature to exert a glucidic stimulus, since important regulatory pathways were still switched-off, or / and b) the glucose dose was not too excessive to be used as energy source to the embryo.

In order to investigate these hypotheses and gain further knowledge on the concept of nutritional programming in fish, we performed another experiment with zebrafish (**Chapter 3**). We explored a new developmental window for the delivery of the glucose stimulus, at a late embryo stage (1 dpf), based on previous studies in mammalian nutritional programming. Studies revealed that the timeframe of exposure to potential programming stimuli during pregnancy may be critical in determining the outcome of the event (Symonds et al., 2007). This can be expected, considering that the different fetal organs have critical and precise developmental stages, where they seem to be more sensitive to nutritional cues and thus, to permanent changes (Langley-Evans, 2015). Evidences of critical developmental windows more susceptible to the programming of the fetus have been documented over decades. McCance (1962) shown that a decrease on the volume of milk during rat's nursing led to significant differences on growth rate, only if the restriction was applied during the early suckling period and not after. In a similar way, **we have observed different short- and long-term effects of the same glucidic stimulus (2M solution) depending on whether it was delivered at an early (0.2 dpf) or late (1 dpf) stage of embryo development**. The shift of stimulus delivery towards a window in late embryogenesis did not inhibit the expression of genes in the yolk-sac larvae (4 dpf) instead it caused no significant changes in the

transcription of the metabolic genes (**Chapter 3**). These results clearly demonstrate how is decisive the relationship between nutrient supply and the developmental stage in the long-term metabolic programming. Also, they revealed the ability of zebrafish embryos to dynamically regulate glucose levels after the supplementation, as it was suggested by Jurczyk et al. (2011), which can possibly occur in other species, if we extrapolate this situation.

Based on the zebrafish research model, embryogenesis represents an important developmental period, where embryos undergo dramatic morphological and physiological changes. The high sensitiveness found during this developmental period is particularly interesting to study changes in the nutritional status. Indeed, it was shown that nutrient restriction by partial yolk depletion affected the expression of metabolic genes and hormonal signaling of zebrafish embryos (Huang and Linsen, 2015). Moreover, at early larval stages (post-hatching) evidences of nutritional conditioning were also found by the increase of β-cells number after an over-nutrition (glucose excess) of larvae (Maddison and Chen, 2012). These findings together with our results obtained in **Chapter 2 and 3** are strong indicators that the nutritional composition of the egg yolk can be manipulated to induce immediate and long-lasting effects in fish metabolism. However, attention should be paid to the fact that features like embryonic robustness of the species and timing for stimulus delivery were crucial in determining the programming effects. Even so, the concept of programming was not totally proved based on our findings, but some molecular evidences support its potential occurrence

In this thesis we have presented microinjection as a novel technique to deliver specific nutrients directly into the egg yolk of fish embryos and its efficiency was confirmed by the increase of glucose level after injecting the embryos. Although we saw that gilthead seabream, like most marine fish species, are extremely sensitive to manipulations during embryogenesis, this does not compromises the attempt to change their yolk reserves during embryogenesis. In these cases, other strategies for *in ovo* supplementation of nutrients may be explored aiming a less stressful and harmful methodology to the embryo, such as sonophoresis (low-frequency ultrasounds). The efficacy of such technique in enhancing the transport of certain compounds across skin epithelium, gills and embryo or yolk-feeding larvae membranes have been reported in fish (Bart et al., 2001; Silakes and Bart, 2010; Wang et al., 2008; Zhou et al., 2002), but still remain poorly investigated. Recent studies within the nutritional programming concept were conducted using sonophoresis as a membrane permeability enhancer to certain nutrients, in fish eggs and larvae. Results confirmed that this

technique had the potential to incorporate some amino acids levels in seabream embryos, but revealed to be inefficient in changing the glucose levels (Engrola et al., 2014a; Engrola et al., 2014b). Novel methods and technologies capable of manipulating the nutritional reserves of fish embryos would be useful to gain a better insight on the nutritional regulation of key biological and metabolic processes occurring in early stages of fish development.

6.3. Programming the metabolism of carbohydrates in marine fish species: how challenging can be?

Glucose plays a pivotal role during mammalian gestation in ensuring fetal growth and development and determining metabolic requirements of other micro- and macro-nutrients. The extent to which an increase or decrease of glucose supply from the mother to the fetus may modulate the late metabolic response of the offspring still remains to be established, yet strong evidences suggest that epigenetic mechanism can be involved (Symonds et al., 2007). Indeed, it has been shown that glucose can operate as a cofactor in some epigenetic mechanism, like histone acetylation, and participate during the genome imprinting (Badeaux and Shi, 2013; Friis et al., 2009). A different scenario is found in fish, glucose does not seem to play a pivotal energetic role during embryogenesis, since the few amounts found in the yolk of some species are rapidly consumed during the first cellular divisions, after fertilization, becoming afterwards unavailable for the rest of embryo development (Heming and Buddington, 1988; Kamler, 2008). Therefore, under these circumstances we have assumed that glucose could act as a potential programming nutrient during fish early development, and tailor specific metabolic functions or pathways in juvenile fish towards a better use of dietary carbohydrates.

Such goal becomes even more challenging when aimed to a marine fish species, with carnivorous feeding habits and economically interesting. On the run towards sustainability, the demands for a higher use of plant-based ingredients and non-protein energy substrates (lipids and carbohydrates) led to the overall increase of carbohydrates in fish diets, which are not well tolerated by most carnivorous fish (Klinger and Naylor, 2012). One of the major constrains on performing a glucidic supplementation during the embryogenesis of a marine fish is linked to the fact that most species exhibit an altricial development strategy, characterized by the spawning of small eggs, short embryonic period and hatching of larvae

still underdeveloped and in lecithotrophic phase (Balon, 1981). In fact, seabream embryogenesis revealed to be a highly sensitive to microinjection procedure. Based on these facts, our opportunities to exert a glucidic stimulus during a critical window of seabream development were narrowed to the early larval period, more precisely the onset of exogenous feeding (**Chapter 4**). The vast literature on nutritional programming of mammals have demonstrated that the period of susceptibility/ plasticity to environmental cues can be extended up to the immediate postnatal life (Patel et al., 2009; Srinivasan and Patel, 2008). For instance, changes in neonatal nutrition were found to cause permanent alterations on carbohydrate uptake and metabolism, as it was observed in suckling rats fed on a high-carbohydrate milk formula, which induced a hyperinsulinemic condition and predisposing to obesity into adulthood (Srinivasan et al., 2008)

In the fish nutrition field, the few studies that have approached the concept of nutritional programming also selected the period of mouth opening to deliver the nutritional stimulus to the larvae during their first exogenous feeding experience. These studies investigated the long-term effects on metabolism of early feeding on: a) high levels of carbohydrates, focusing exclusively in freshwater species (Fang et al., 2014 ; Geurden et al., 2007; Geurden et al., 2014; Gong et al., 2015), or b) low levels of highly unsaturated fatty acids, focusing in a marine fish species (Vagner et al., 2007; Vagner et al., 2009). In all these cases, the nutritional stimulus was provided to the first-feeding larvae in the form of an inert diet. However, at the exception of salmonid species like rainbow trout, most fish larvae hardly accept the replacement of live preys by inert diets at mouth opening, due in part to their lack of attractiveness (e.g. palatability, mobility) that reduces the rate of feed intake and compromises the growth and survival (Hamre et al., 2013). Despite the many efforts done in formulating well balanced diets for larvae, there are still important gaps on the knowledge of larval nutritional requirements, especially on marine fish species such as gilthead seabream (Yufera et al., 2000). Thus, in order to overcome these difficulties related to early larval nutrition and insure a good acceptance of the glucidic stimulus, we kept the standard feeding plan of seabream larvae and used the live prey (rotifers and *Artemia*) as vehicles for the glucose supplementation. The study described in **Chapter 4** was pioneer not only in investigating the programming effects of glucose stimuli in a marine fish species, but also by doing so through the enrichment of live prey. It was demonstrated that a nutritional stimulus may be delivered using the feed resource that best suits the larvae, at a specific stage. This knowledge may help to improve the formulation of new diets for larvae toward a better acceptance at early stages.

The efficiency of each glucidic stimuli, namely the enriched rotifers, enriched *Artemia* and inert diet, was assessed immediately after their delivery to the larvae, by means of gene expression analyses and metabolic studies with radiolabeled glucose (**Chapter 4**). **Each hyperglucidic stimulus induced immediate changes on the expression of some genes involved in the glycolytic, lipogenic, energy and carbohydrate digestion pathways. It was also verified a different pattern of glucose utilization in the larvae after receiving the last stimulus with a glucose-rich diet. Moreover, the induction of GK gene in larvae was only achieved after the first stimulus with enriched rotifers,** yet it was expected a similar response to all stimuli, since the ingestion of high levels of glucose stimulated the action of this enzyme in most vertebrates, including fish (Panserat et al., 2014). In recent studies over nutritional programming in fish, the up-regulation of GK enzyme was also observed as an immediate response to hyperglucidic stimuli (Fang et al., 2014 ; Geurden et al., 2007; Geurden et al., 2014). It is worthy to highlight the fact that seabream larvae at 30 DAH still showed to be sensitive to the last stimulus with a glucose-rich diet, demonstrated by the changes in gene expression and glucose flux, indicating that this species is still sensitive to nutritional events at a period distant from the first- feeding window. However, Geurden et al. (2007) clearly showed that the short-term effects of a hyperglucidic stimulus on gene expression are diminished when the stimulus is delivered 3-weeks after the opening of rainbow trout mouth, suggesting a lower plasticity of the alevins at that later stage of development. Therefore, it is likely that the extension and duration of critical developmental windows may be specific for each fish species. Although few, the changes obtained on gene expression and ^{14}C-glucose flux after each individual stimulus are valid indicators of their efficacy in seabream larvae, showing also a great potential of recurrent strategies for the performance of the early feeding events.

The next step was to evaluate the short-term effects of the recurrent stimuli on the regulation of gene expression and nutrient metabolism of seabream post-larvae (60DAH). In order to trigger the pathways for carbohydrate metabolism and those involved in the intermediary metabolism, the post-larvae were submitted to a 10-day challenge and fed with a high-carbohydrate low-protein diet. Short-term effects can be good indicators of a possible "metabolic memory" of the early stimuli that may be imprinted in the genome as a result of an epigenetic mechanism**. In Chapter 4 we have seen that, at a late stage of post-larvae, the glucose metabolism of programmed fish revealed a shift towards a higher catabolism and lower glucose retention in tissues. These fish also showed a higher bio-conversion of**

glucose into lipids, indicating enhanced hepatic lipogenesis. Nevertheless, the regulation of amino acid metabolism as well as the expression of several metabolic-related genes showed not to be affected by the early conditioning to high glucose levels. Even with no substantial modifications in gene regulation, these short-term outcomes may anticipate a positive metabolic adaptation of seabream towards a better use of dietary carbohydrates. Further investigation is needed to determine which mechanisms were involved in the short-term effects, set by the early exposure to glucidic stimuli.

6.4. Can an early glucidic stimulus induce permanent changes in the metabolism of juvenile fish?

One major goal of this thesis was to pave the way towards a better use of dietary carbohydrates by fish and open a whole set of opportunities to be explored in the field of nutritional programming. However, we are dealing with a multifactorial concept on which a combination of developmental plasticity, genetics and abiotic events work together to shape the phenotype in adult life.

It has been demonstrated that several critical elements can influence the long-term effects of an early nutrient exposure in fish: the time-frame for stimulus delivery – certain developmental windows can be more sensitive for a certain trait; b) the duration of the stimulus – the exposure to the stimulus can be either punctual or recurrent, brief or prolonged, and all these possibilities can influence their action (Hanley et al., 2010; Wells, 2014); c) the method for stimulus delivery – maternal nutritional, direct manipulation of the embryo yolk content (microinjection) or first exogenous feeding at the larvae stage; d) the supplemental dose of the nutrient – should avoid an excessive dose that cause overloads or a highly reduce dose that can mask the effects. In a certain way, this unpredictability of the long-term results was observed in the two experiments with zebrafish and gilthead seabream. Changing the time-frame for stimulus delivery in zebrafish embryos (**Chapters 2 and 3**) resulted in distinct short- and long-term effects on gene expression, yet the expected beneficial effects related to a later nutritional event were only partially achieved. **In both zebrafish (Chapter 3) and seabream (Chapter 5) we saw that the promising molecular and metabolic adaptations obtained immediately after each glucidic stimuli at the larval stage, failed to persist at the same magnitude in the juvenile fish.** We should not forget that the concept of nutritional

programming in fish is still blooming and available knowledge is highly fragmented and often species-specific. Although with very encouraging results, up to now few studies have shown clear evidences of a long-term persistence of “programmed” effects or its intergenerational transmission in fish. Further studies are needed to fully validate the concept of nutritional programming in fish.

Evidence from both human and animal studies suggests that programmed effects might not be limited to the first (directly exposed) generation but could also be transmitted to the subsequent generation (Drake and Liu, 2010). This suggests that mechanisms by which environmentally or nutritionally induced changes in parental phenotype are transmissible to offspring. Programmed responses to environmental cues appear to occur rapidly (within a generation or two), and in many studies the magnitude of programmed effects diminish with successive generations; however, the mechanisms which underpin the imprinting and transmission of programmed phenotype across generations remain unclear (Benyshek et al., 2008; Harrison and Langley-Evans, 2009). A possible mechanism underlying the changes of an organism's phenotype in response to early life events may be related to the epigenetic regulation of genes. Epigenetic studies the changes in gene function that are not explained by changes in DNA sequence and which might be mitotically and/or meiotically heritable (Dupont et al., 2009). The major epigenetic mechanism known for gene expression regulation are DNA methylation, which is the most widely investigated epigenetic marker, histone modifications and non-coding RNAs (Dupont et al., 2009). Currently there is a large and varied volume of work within the mammalian programming field that is focused on these epigenetic mechanisms and its potential consequences in adulthood and subsequent generations (Canani et al., 2011; Desai et al., 2015; Lillycrop and Burdge, 2015). Comparatively to terrestrial vertebrates, the epigenetic research performed in fish is less extensive, even so it has already been applied to several topics of fish biology (Anderson et al., 2012; Li and Leatherland, 2013; Moghadam et al., 2015). Moreover, there are now strong evidences indicating that environmental factors, like temperature, can induce genomic imprinting through epigenetic mechanisms in fish (Campos et al., 2013; Navarro-Martín et al., 2011; Piferrer et al., 2012). If environmental cues can be link to epigenetic programming of fish the same could be expected from nutritional cues, since early nutrition can influence DNA methylation and the one-carbon metabolism pathway, which is dependent on dietary methyl donors and cofactors, including methionine, folate and choline (Anderson et al., 2012).

The effects of the initial programming event may be, in some cases, transient and reversible after a lifetime exposure to a range of other external factors. Little is known about the mechanisms involved in the reversibility of the programmed traits, but theoretically the phenotype acquired from a non-genomic event (epigenome) can be "erased", as an adaptive response of the organism to new environmental conditions (Li and Leatherland, 2013). **None of the molecular changes found in the seabream larvae after the delivery of the three stimuli, i.e. the suppression or enhancement of key-genes involved in the glucose-related metabolic pathway, were detected in the juvenile fish (Chapter 5).** The opposite pattern of gene regulation was found in zebrafish injected with high-glucose levels at 1 dpf. **During the yolk-sac stage, the glucose-injected larvae showed no significant changes in gene expression (except for PK gene) whereas at the juvenile stage, several genes from the glycolytic and gluconeogenic pathways were differently regulated (Chapter 3).** Different scenarios were observed in response to early glucidic stimulus. In zebrafish, the efficiency of the stimulus was not assessed immediately after the injection being performed, but rather during the period of exclusive consumption of the supplemented yolk. This time gap may have masked the potential molecular changes in gene expression of the late embryo, leading to think that no change occurred in the epigenome. Yet, further work needs to be done to unravel which mechanisms caused the changes in gene expression of the zebrafish juveniles. Most DNA methylation and chromatin remodeling occurs during ontogenic periods, thus one could question whether or not the glucidic stimuli fed to the seabream larvae acted as modulators of the epigenome, even if transiently. However, the lability of imprinted genes is not limited to the early embryonic period. It was recently demonstrated that a methyl-donor-deficient diet in postnatal life permanently affects the expression of IGF2, resulting in growth retardation (Waterland et al., 2006). This suggests that nutrition during postnatal development can also alter the epigenetic regulation of imprinted genes. Nevertheless, **the early glucidic stimuli caused some long-lasting alteration in the metabolic flux of starch-derived glucose, by improving the absorption of glucose in the gut of juvenile seabream (Chapter 5).** Other mechanisms, besides epigenetic, can be involved in storing the "memory" of the programmed trait until adulthood; tissue remodelling and differential cellular proliferation are two possibilities (Hershkovitz et al., 2007; Tarry-Adkins and Ozanne, 2011) already discussed in more detail in the previous chapters.

To date, the few studies performed in nutritional programming of fish have not investigated the possible mechanisms underlying the long-term effects. Future works on this topic should

attempt to identify possible epigenetic markers involved in the long-lasting outcomes and also analise in more detail the molecular and metabolic machinery behind the programmed phenotype.

6.5. References

Amacher, S.L., 2008. Emerging gene knockout technology in zebrafish: zinc-finger nucleases. Brief Funct Genomic Proteomic. 7, 460-464.

Amsterdam, A., Nissen, R.M., Sun, Z., Swindell, E.C., Farrington, S., Hopkins, N., 2004. Identification of 315 genes essential for early zebrafish development. Proc Natl Acad Sci U S A. 101, 12792-12797.

Anderson, O.S., Sant, K.E., Dolinoy, D.C., 2012. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J Nutr Biochem. 23, 853-859.

Armitage, J.A., Khan, I.Y., Taylor, P.D., Nathanielsz, P.W., Poston, L., 2004. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol. 561, 355-377.

Badeaux, A.I., Shi, Y., 2013. Emerging roles for chromatin as a signal integration and storage platform. Nat Rev Mol Cell Biol. 14, 211-224.

Balon, E.K., 1981. Saltatory Processes and Altricial to Precocial Forms in the Ontogeny of Fishes. American Zoologist. 21, 573-596.

Bart, A.N., Kindschi, G.A., Ahmed, H., Clark, J., Young, J., Zohar, Y., 2001. Enhanced transport of calcein into rainbow trout, *Oncorhynchus mykiss*, larvae using cavitation level ultrasound. Aquaculture. 196, 189-197.

Benyshek, D.C., Johnston, C.S., Martin, J.F., Ross, W.D., 2008. Insulin sensitivity is normalized in the third generation (F3) offspring of developmentally programmed insulin resistant (F2) rats fed an energy-restricted diet. Nutrition & Metabolism. 5, 26-26.

Blackburn, D., 1999. Viviparity and oviparity: evolution and reproductive strategies. in: Knobil, T.E., Neill, D.J. (Eds.), Encyclopedia of Reproduction. Academic Press, London, pp. 994-1003.

Burdge, G.C., Lillycrop, K.A., 2010. Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr. 30, 315-339.

Campos, C., Valente, L., Conceicao, L., Engrola, S., Fernandes, J., 2013. Temperature affects methylation of the myogenin putative promoter, its expression and muscle cellularity in Senegalese sole larvae. Epigenetics. 8.

Canani, R.B., Di Costanzo, M., Leone, L., Bedogni, G., Brambilla, P., Cianfarani, S., Nobili, V., Pietrobelli, A., Agostoni, C., 2011. Epigenetic mechanisms elicited by nutrition in early life. Nutr Res Rev. 24, 198-205.

Desai, M., Jellyman, J.K., Ross, M.G., 2015. Epigenomics, gestational programming and risk of metabolic syndrome. Int J Obes (Lond). 39, 633-641.

Drake, A.J., Liu, L., 2010. Intergenerational transmission of programmed effects: public health consequences. Trends in Endocrinology & Metabolism. 21, 206-213.

Dupont, C., Armant, D.R., Brenner, C.A., 2009. Epigenetics: definition, mechanisms and clinical perspective. Semin Reprod Med. 27, 351-357.

Engrola, S., Colen, R., Rocha, F., Barrios, C., Conceição, L.E.C., Aragão, C., Gavaia, P.J., Laizé, V., Valente, L., Dias, J., 2014a. Emerging tools for *in ovo* delivery of nutrients and regulatory peptides in fish: effects on larval growth, feed intake and protein metabolism. Oral presentation. 16th International Symposium on Fish Nutrition and Feeding, Queensland, Australia.

Engrola, S., Colen, R., Rocha, F., Barrios, C., Rema, P., Conceição, L.E.C., Aragão, C., Dias, J., 2014b. *In ovo* delivery of nutrients using a sonophoresis protocol. Oral Presentation. Aquacultue Europe 14 (EAS 2014), San Sebastian, Spain.

Fang, L., Liang, X.F., Zhou, Y., Guo, X.Z., He, Y., Yi, T.L., Liu, L.W., Yuan, X.C., Tao, Y.X., 2014 Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, Danio rerio. Br J Nutr. 111, 808-818.

Friis, R.M.N., Wu, B.P., Reinke, S.N., Hockman, D.J., Sykes, B.D., Schultz, M.C., 2009. A glycolytic burst drives glucose induction of global histone acetylation by picNuA4 and SAGA. Nucleic Acids Res. 37, 3969-3980.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (Oncorhynchus mykiss) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am J Physiol Regul Integr Comp Physiol. 292, 2275-2283.

Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D., Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S., Panserat, S., 2014. High or low dietary carbohydrate:protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout. J Exp Biol. 217, 3396-3406.

Gluckman, P.D., Hanson, M.A., Low, F.M., 2011. The role of developmental plasticity and epigenetics in human health. Birth Defects Res C Embryo Today. 93, 12-18.

Goldsmith, J.R., Jobin, C., 2012. Think Small: Zebrafish as a Model System of Human Pathology. J Biomed Biotechnol. 2012, 12.

Gong, G., Xue, M., Wang, J., Wu, X.-f., Zheng, Y.-h., Han, F., Liang, X.-f., Su, X.-o., 2015. The regulation of gluconeogenesis in the Siberian sturgeon (Acipenser baerii) affected later in life by a short-term high-glucose programming during early life. Aquaculture. 436, 127-136.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E.C., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Reviews in Aquaculture. 5, S26-S58.

Hanley, B., Dijane, J., Fewtrell, M., Grynberg, A., Hummel, S., Junien, C., Koletzko, B., Lewis, S., Renz, H., Symonds, M., Gros, M., Harthoorn, L., Mace, K., Samuels, F., van Der Beek, E.M., 2010. Metabolic imprinting, programming and epigenetics - a review of present priorities and future opportunities. Br J Nutr. 104 Suppl 1, S1-25.

Harrison, M., Langley-Evans, S.C., 2009. Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy. Br J Nutr. 101, 1020-1030.

Heming, T.A., Buddington, R.K., 1988. Yolk Absorption in Embryonic and Larval Fishes. in: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, The Physiology of Developing Fish - Eggs and Larvae. Academic Press, San Diego, California, pp. 407-446.

Hershkovitz, D., Burbea, Z., Skorecki, K., Brenner, B.M., 2007. Fetal Programming of Adult Kidney Disease: Cellular and Molecular Mechanisms. Clinical Journal of the American Society of Nephrology. 2, 334-342.

Hornung, M.W., Zabel, E.W., Peterson, R.E., 1996. Toxic equivalency factors of polybrominated dibenzo-p-dioxin, dibenzofuran, biphenyl, and polyhalogenated diphenyl ether congeners based on rainbow trout early life stage mortality. Toxicol Appl Pharmacol. 140, 227-234.

Huang, Y., Linsen, S.E., 2015. Partial depletion of yolk during zebrafish embryogenesis changes the dynamics of methionine cycle and metabolic genes. BMC Genomics. 16, 427.

Janik, M., Kleinhans, F.W., Hagedorn, M., 2000. Overcoming a permeability barrier by microinjecting cryoprotectants into zebrafish embryos (Brachydanio rerio). Cryobiology. 41, 25-34.

Jurczyk, A., Roy, N., Bajwa, R., Gut, P., Lipson, K., Yang, C., Covassin, L., Racki, W.J., Rossini, A.A., Phillips, N., Stainier, D.Y., Greiner, D.L., Brehm, M.A., Bortell, R., diIorio, P., 2011. Dynamic glucoregulation and mammalian-like responses to metabolic and developmental disruption in zebrafish. Gen Comp Endocrinol. 170, 334-345.

Kamler, E., 2008. Resource allocation in yolk-feeding fish. Rev Fish Biol Fisheries. 18, 143-200.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev Dyn. 203, 253-310.

Klinger, D., Naylor, R., 2012. Searching for Solutions in Aquaculture: Charting a Sustainable Course. Annual Review of Environment and Resources. 37, 247-276.

Kopeika, J., Zhang, T., Rawson, D., 2006. Zebrafish embryos (Danio rerio) using microinjection. Cryo Letters. 27, 319-328.

Kudoh, T., Tsang, M., Hukriede, N.A., Chen, X., Dedekian, M., Clarke, C.J., Kiang, A., Schultz, S., Epstein, J.A., Toyama, R., Dawid, I.B., 2001. A gene expression screen in zebrafish embryogenesis. Genome Res. 11, 1979-1987.

Langley-Evans, S.C., 2015. Nutrition in early life and the programming of adult disease: a review. J Hum Nutr Diet. 28 Suppl 1, 1-14.

Lawrence, C., 2007. The husbandry of zebrafish (Danio rerio): A review. Aquaculture 269, 1-20.

Li, M., Leatherland, J.F., 2013. The implications for aquaculture practice of epigenomic programming of components of the endocrine system of teleostean embryos: lessons learned from mammalian studies. Fish and Fisheries. 14, 528-553.

Lillycrop, K.A., Burdge, G.C., 2015. Maternal diet as a modifier of offspring epigenetics. J Dev Orig Health Dis. 6, 88-95.

Maddison, L.A., Chen, W., 2012. Nutrient Excess Stimulates β-Cell Neogenesis in Zebrafish. Diabetes.

Mathavan, S., Lee, S.G.P., Mak, A., Miller, L.D., Murthy, K.R.K., Govindarajan, K.R., Tong, Y., Wu, Y.L., Lam, S.H., Yang, H., Ruan, Y., Korzh, V., Gong, Z., Liu, E.T., Lufkin, T., 2005. Transcriptome Analysis of Zebrafish Embryogenesis Using Microarrays. PLoS Genet. 1, e29.

McCance, R.A., 1962. Food, growth, and time. Lancet. 2, 671-676.

Moghadam, H., Mørkøre, T., Robinson, N., 2015. Epigenetics—Potential for Programming Fish for Aquaculture? Journal of Marine Science and Engineering. 3, 175-192.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., Piferrer, F., 2011. DNA Methylation of the Gonadal Aromatase (cyp19a) Promoter Is Involved in Temperature-Dependent Sex Ratio Shifts in the European Sea Bass. PLoS Genet. 7, e1002447.

Panserat, S., Rideau, N., Polakof, S., 2014. Nutritional regulation of glucokinase: a cross-species story. Nutr Res Rev. 27, 21-47.

Patel, M.S., Srinivasan, M., Laychock, S.G., 2009. Metabolic programming: Role of nutrition in the immediate postnatal life. J Inherit Metab Dis. 32, 218-228.

Piferrer, F., Ribas, L., Diaz, N., 2012. Genomic approaches to study genetic and environmental influences on fish sex determination and differentiation. Mar Biotechnol (NY). 14, 591-604.

Poleo, G.A., Denniston, R.S., Reggio, B.C., Godke, R.A., Tiersch, T.R., 2001. Fertilization of eggs of zebrafish, Danio rerio, by intracytoplasmic sperm injection. Biol Reprod. 65, 961-966.

Polo, A., Yúfera, M., Pascual, E., 1991. Effects of temperature on egg and larval development of Sparus aurata L. Aquaculture. 92, 367-375.

Silakes, S., Bart, A.N., 2010. Ultrasound enhanced permeation of methanol into zebrafish, *Danio rerio*, embryos. Aquaculture. 303, 71-76.

Soanes, K.H., Achenbach, J.C., Burton, I.W., Hui, J.P., Penny, S.L., Karakach, T.K., 2011. Molecular characterization of zebrafish embryogenesis via DNA microarrays and multiplatform time course metabolomics studies. J Proteome Res. 10, 5102-5117.

Srinivasan, M., Mitrani, P., Sadhanandan, G., Dodds, C., Shbeir-ElDika, S., Thamotharan, S., Ghanim, H., Dandona, P., Devaskar, S.U., Patel, M.S., 2008. A high-carbohydrate diet in the immediate postnatal life of rats induces adaptations predisposing to adult-onset obesity. Journal of Endocrinology. 197, 565–574.

Srinivasan, M., Patel, M.S., 2008. Metabolic programming in the immediate postnatal period. Trends Endocrinol Metab. 19, 146-152.

Symonds, M.E., Stephenson, T., Gardner, D.S., Budge, H., 2007. Long-term effects of nutritional programming of the embryo and fetus: mechanisms and critical windows. Reprod Fertil Dev. 19, 53-63.

Tarry-Adkins, J.L., Ozanne, S.E., 2011. Mechanisms of early life programming: current knowledge and future directions. Am J Clin Nutr. 94, 1765S-1771S.

Ulloa, P.E., Iturra, P., Neira, R., Araneda, C., 2011. Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes. Rev Fish Biol Fisheries 21, 646-666.

Vagner, M., Zambonino-Infante, J.L., Robin, J.H., Person, J., 2007. Is it possible to influence European sea bass (Dicentrarchus labrax) juvenile metabolism by a nutritional conditioning during larval stage? Aquaculture. 267, 165-174.

Vagner, M., Robin, J.H., Zambonino-Infante, J.L., Tocher, D.R., Person-Le Ruyet, J., 2009. Ontogenic effects of early feeding of sea bass (Dicentrarchus labrax) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. Br J Nutr. 101, 1452-1462.

Vieau, D., 2011. Perinatal nutritional programming of health and metabolic adult disease. World J Diabetes. 2, 133-136.

Wang, R.Y., Guan, M., Rawson, D.M., Zhang, T., 2008. Ultrasound enhanced methanol penetration of zebrafish (*Danio rerio*) embryos measured by permittivity changes using impedance spectroscopy. European Biophysics Journal. 37, 1039-1044.

Waterland, R.A., Lin, J.R., Smith, C.A., Jirtle, R.L., 2006. Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus. Hum Mol Genet. 15, 705-716.

Wells, J.C.K., 2014. Adaptive variability in the duration of critical windows of plasticity: Implications for the programming of obesity. Evolution, Medicine, and Public Health. 2014, 109-121.

Xu, Q., 1999. Microinjection into zebrafish embryos. in: Guille, M. (Ed.), Molecular Methods in Developmental Biology. Humana Press, pp. 125-132.

Yufera, M., Fernández-Díaz, C., Pascual, E., Sarasquete, M.C., Moyano, F.J., Díaz, M., Alarcón, F.J., García-Gallego, M., Parra, G., 2000. Towards an inert diet for first-feeding gilthead seabream *Sparus aurata* L. larvae. Aquaculture Nutrition. 6, 143-152.

Zhou, Y.-C., Huang, H., Wang, J., Zhang, B., Su, Y.-Q., 2002. Vaccination of the grouper, Epinephalus awoara, against vibriosis using the ultrasonic technique. Aquaculture. 203, 229-238.

Conclusions and Future Perspectives

Chapter VII

7.1. Conclusions

In terrestrial vertebrates, a relevant number of research studies has established the concept that perinatal events (e.g. maternal nutrition, environmental conditions, neonatal feeding) can have significant contributions to postnatal growth potential, health resistance and welfare status in farmed animals. Over recent years, this concept, known as nutritional (or in some cases metabolic) programming, has also been assessed in fish.

The work undertaken in this thesis highlights the following conclusions:

- Nutritional interventions *in ovo* can be performed at different stages of the embryonic development of fish by means of microinjection technique. The feasibility of this methodology seems to be linked to the degree of resilience of the target fish species, toward stressful situations and intensive handling.

- By microinjection, we successfully enriched zebrafish egg yolk with glucose (a 43-fold increase of basal levels) without detrimental effects on larval weight, embryonic development (length and yolk volume) and survival.

- Embryogenesis is a period of high developmental plasticity. From the zebrafish model it was observed that the selection of the embryonic window to exert the glucose stimulus was determinant for the short- and long-term outcomes. To avoid the risk of repressing the expression of key-metabolic genes and later damages in the functionality of metabolic pathways, the glucose load should be exerted at a late embryo stage, where the primary organs and metabolic pathways begin to be activated.

- Nutritional interventions during the onset of exogenous feeding are likely more advantageous to marine fish species that exhibit a sensitive embryonic and larval development. It is possible to provide glucidic stimuli right at mouth opening, without altering the standard feeding plan of larvae, through the incorporation of glucose in the live preys.

- Nutritional programming is a multifactorial phenomenon on which a wide range of concepts converge. Several factors such as the timing and duration of stimulus

exposure, the molecular complexity and dose of the nutrient, the method for delivery, all combined determine the possible programming effects at later life.

- In juvenile zebrafish, the glucidic stimulus exerted in late embryos (1 dpf) led to some positive molecular and metabolic adaptations, contrary to the observed after the incipient stimulus delivery (0.2 dpf). The long-term effects suggested an improved capacity for glucose phosphorylation and lower glucose production as well an altered phenotype toward a reduced retention of ^{14}C-glucose in the visceral tissue.

- The juvenile gilthead seabream exposed to the early-feeding of repeated glucidic stimuli showed few, but positive, metabolic adaptations in the absorptive capacity of dietary starch. Also demonstrated a distinct use of carbon-skeletons from starch for the biosynthesis of proteins and possibly glycogen, compared to the unconditioned fish. However the lack of persistent changes in gene expression, amino acid metabolism and several zootechnical parameters suggest that the concept of nutritional programming was not fully established for seabream.

- Our data on zebrafish and seabream suggests that early glucidic stimuli (us) have the potential to alter, at a molecular level, some pathways involved in the intermediary metabolism, as well as to regulate differently the phenotype for carbohydrate utilization.

Although not fully validated in our work, this thesis generated relevant knowledge and evidences supporting the applicability of the nutritional programming concept in fish.

7.2. Future Perspectives

The concept of nutritional programming in fish is still prematurely investigated and available knowledge is highly fragmented and often species-specific. Nevertheless, this thesis together with some other recent works has demonstrated the possibility to successfully alter fish phenotypes in response to certain nutritional environments, by early life exposure to dietary stimulus. However, we feel that the concept of nutritional programming is not yet fully validated in fish. Moreover, little is known on the mechanisms underlying such changes.

Future studies are now aware that embryo developmental stage, stimulus dosage, duration and frequency of application (e.g. acute vs recurrent) are all aspects that can condition the triggering effect of the stimulus, and most probably the long-term persistency of the phenotypical changes. The disadvantages inherent to a still ill-defined topic in fish nutrition may be view as beneficial, in the sense that provide almost an unlimited set of new opportunities to be explored.

Although not exclusive, a key element often associated to nutritional programming effects are epigenetic changes. However, few studies in fish have assessed if the observed changes could be associated to heritable changes in gene function that occur without a modification in the DNA sequence. In order to validate an “imprinting” effect, future works should try to integrate also an assessment of the various epigenetic mechanisms such as DNA methylation patterns and histone modifications leading to chromatin remodeling. Undoubtedly that the field of nutritional epigenetic will stand out and make the difference, not only to understand the adaptive response at the molecular level but also to discover possible genetic predispositions to specific nutritional conditions that may conduct to new selections of fish genotypes.

Currently, the opportunities to exert a nutritional stimulus during fish embryogenesis are restricted to maternal transfer and the onset of exogenous feeding. Further studies are necessary to explore the positive effects of supplementing the embryos with important nutrients for growth and energy supply (amino acids), to protect endogenous lipids from oxidation (vitamins), or even to understand the role of minerals, hormones and other regulatory peptides (e.g. ghrelin, follistatin) during embryogenesis. Extending this knowledge to important farmed fish species, either from marine or freshwater production, could help to

minimize some difficulties encountered during larval nutrition on finding the right nutritional requirements that promote a correct growth and development for the production of high quality juveniles.

From a practical perspective of a fish maternity facility, the microinjection technique is far from being suitable to large batches of eggs. Alternative methodologies are needed to deliver nutrients or other compounds simultaneously to a great amount of fish eggs and/or embryos, and therefore maximize the supplementation. Innovative technologies associating the chemical structure, electrical properties and acoustic cavitation properties of molecules (e.g. iontophoresis, electroporation, sonophoresis) have progressively being introduced as tools to enhance and control the delivery of substances through cell membranes in various fish tissues. Recently, some promising results showed the potential of sonophoresis on the incorporation of specific nutrients in fish eggs, which encourages even more the alliance of such peculiar methodologies with the research on fish nutritional programming.

Stimulus delivered through the live preys at the onset of exogenous feeding opened a whole set of opportunities to investigate the concept of programming in fish species highly vulnerable during their early larval stages, that usually show a reduce acceptance of inert diets. However, attention should be paid to guarantee an effective and controlled delivery of the target nutritional stimuli, which in some cases would imply the use of innovative delivery vectors, such as microencapsulated forms to avoid uncontrollable leaching losses. In a broader sense, nutritional approaches within the programming concept may be tested at a major scale of production, by adjusting the feeding plan of the reared larvae to include specific periods of stimulus delivery. This allows its application to several fish species, regardless the rearing systems, tank capacity, larval density, among other zootechnical parameters.

This thesis aimed the nutritional programming of fish through the action of a nutritional stimulus. It would be also interesting to study the potential of non-nutritional stimuli, like abiotic factors (temperature, hypoxia, salinity) in the modulation of fish nutritional metabolism.

Aquaculture is currently the fastest growing animal food production sector. By 2030, nearly two-thirds of the seafood we eat will be farm-raised. However, the future growth of the aquaculture sector faces several sustainability challenges In nutritional terms, the main

challenges are associated to the capacity of marine fish species to cope efficiently with alternative feeds with higher levels of plant proteins and vegetable oil sources, and all the inherent aspects associated to it (Figure 7.1.).

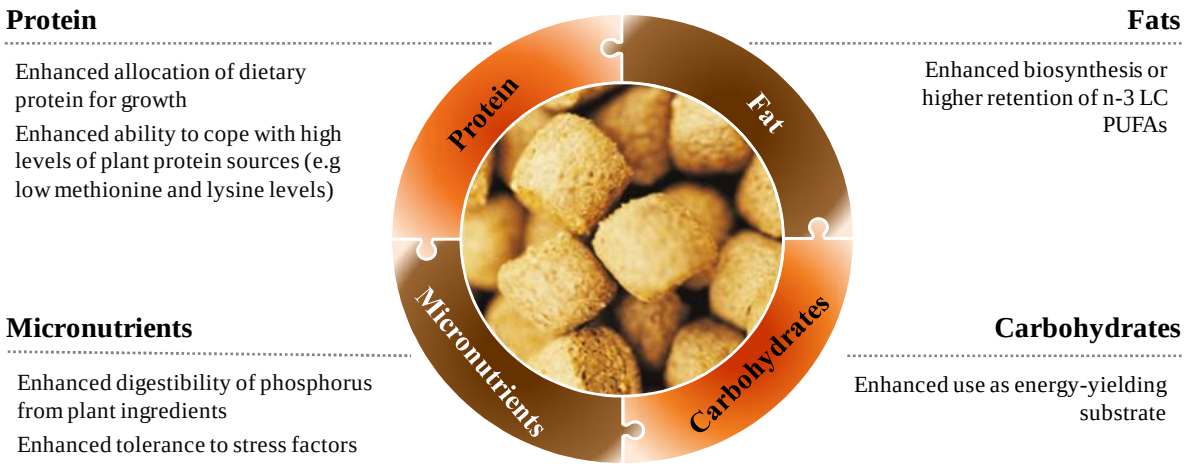

Figure 7.1 – Major challenges faced by the aquaculture sector toward a sustainable nutrition and feeding practices

In this context, nutritional programming could play a pivotal role in identifying nutritional triggers that could alter the metabolic utilization of several nutrients and possibly open new avenues for a more efficient utilization of plant ingredients in fish feeds.

Given the expected intensification of aquaculture farming systems, one aspect that should not be neglected is the potential programming of important traits in fish. The improvement of quality parameters such as fillet texture and colour, the enhancement of resistance to diseases, and a higher copying ability to stress factors are some potentialities that might be achieved through a nutrigenomic programming approaches. The wide range of possible physiological features or functions that can be hypothetically improved in fish augments the need of building solid knowledge upon this concept of programming. High expectations rely now upon how far this concept can evolve in the field of fish nutrition to revolutionize the fish farming sector.