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High-glucose feeding of gilthead seabream (*Sparus aurata*) larvae: Effects on molecular and metabolic pathways

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ABSTRACT

Nutritional programming has begun 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 glucose 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 glucose 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. In addition, 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 high-glucose stimulus during larval stages affects the short-term modulation of pathways for glucose utilization in gilthead seabream.

Statement of relevance

Carnivorous fish, such as rainbow trout and gilthead seabream exhibit a poor utilization of dietary carbohydrates as energy substrates. The concept of nutritional programming raises the possibility of tailoring specific metabolic pathways or functions in fish, for example to improve the use of dietary carbohydrates and promote the development of cereal based-diets for a more sustainable aquaculture.

1. Introduction

Gilthead seabream (*Sparus aurata*) is the main produced marine fish species in the Mediterranean region where over 583 million gilthead sea bream juveniles were produced in 2013, mainly in Greece, Turkey, Italy, Spain and France (FEAP, 2014). Fish larvae require a protein- and lipid-rich feeding regime in order to support the high energy needs essential for fast growth (Hamre et al., 2013; Rønnestad et al., 2013). Knowledge regarding 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 at early stages of marine fish larvae, 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

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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). In European seabass (*Dicentrarchus labrax*) larvae, the usual decline in amylase activity was reduced when larvae were fed increasing levels of dietary carbohydrates, supplied as maltose and pre-cooked starch (Peres et al., 1996). Also, 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 may serve as metabolic substrate in marine fish larvae, suggesting a possible modulation of the carbohydrate metabolic pathways.

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, 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.

Knowledge on the role of early nutritional stimuli as modulators of metabolic pathways in fish is scarce. However, in the past years the number of studies performed in 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 (*Danio rerio*) embryogenesis through direct supplementation of the embryo yolk reserve (Rocha et al., 2014, 2015) and at the onset of exogenous feeding in rainbow trout (*Oncorhynchus mykiss*) (Geurden et al., 2007, 2014), zebrafish (Fang et al., 2014) and Siberian sturgeon (*Acipenser baerii*) (Gong et al., 2015). With variable extent, all these studies showed some effects at molecular and/or metabolic level related to early nutritional conditioning. To the best of our knowledge, the concept of an early dietary carbohydrate stimulus used 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., 2010, 2007; Engrola et al., 2010; Morais et al., 2006) and abiotic factors (Campos et al., 2013). The possibility to assess the metabolic flux of several nutrients, namely amino acids, may allow a better understanding of the protein sparing potential of non-protein energy sources, such as dietary carbohydrates (Hemre et al., 2002).

In this context, the objective of the present study is to assess the effect of a recurrent early-feeding glucose 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 high-glucose stimulus.

2. Materials and methods

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 12:12 h (L:D) cycle, temperature averaged 18 ± 1 °C, salinity 33 ± 2 ppt and dissolved oxygen in water was maintained above 95% of saturation and larvae were maintained in green-water conditions. A daily monitoring of environmental parameters and larval mortality was performed; also the rearing tanks were cleaned regularly to preserve water quality.

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. 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 Fig. 1. Briefly, larvae from Control treatment (CTRL), started to feed at 3DAH on rotifers previously enriched with the STD emulsion (Table 1), which was rich in PUFAs and protein. From day 15, A. *nauplii* were supplied in a co-feeding regime and at 20DAH, A. *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 2), which presented high levels of protein. From 36DAH onwards larvae were fed exclusively on HPD diet (Fig. 1). Rotifers and Artemia were enriched with the STD emulsion, following the procedures described for the commercial enrichment. Live preys were fed directly to the larvae, three times a day while the inert diet was automatically distributed in the tanks eight times a day.

In the Recurrent treatment (REC), seabream larvae were exposed to repeated high-glucose stimuli at different stages of early development (Fig. 1), using i) glucose-enriched rotifers at the onset of exogenous feeding (3DAH; stimulus 1), ii) glucose-enriched *A. 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 1). To assure high live prey glucose bioencapsulation, a short-term enrichment protocol of 1 h (for rotifers) and 2 h (for *Artemia*) was performed with the GLU emulsion before each meal. This period is a trade-off between the time necessary for a high bioencapsulation and to avoid significant losses by prey absorption. The enrichment period was determined after a preliminary test that assessed the live prey glucose uptake (data not shown). Hence, samples were collected from the glucose enrichment media, at time 0 and every 30 min afterwards, up to 120 min and at 180 min based on the previous data from Li et al. (1993). Samples were washed and prey were filtered in order to determine glucose bioencapsulation. 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