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Expressional regulation of key hepatic enzymes of intermediary metabolism in European seabass (*Dicentrarchus labrax*) during food deprivation and refeeding

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ABSTRACT

We hypothesized that the analysis of mRNA level and activity of key enzymes in amino acid and carbohydrate metabolism in a feeding/fasting/refeeding setting could improve our understanding of how a carnivorous fish, like the European seabass (*Dicentrarchus labrax*), responds to changes in dietary intake at the hepatic level. To this end cDNA fragments encoding genes for cytosolic and mitochondrial alanine aminotransferase (cALT; mALT), pyruvate kinase (PK), glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) were cloned and sequenced. Measurement of mRNA levels through quantitative real-time PCR performed in livers of fasted seabass revealed a significant increase in cALT (8.5-fold induction) while promoting a drastic 45-fold down-regulation of PK in relation to the levels found in fed seabass. These observations were corroborated by enzyme activity meaning that during food deprivation an increase in the capacity of pyruvate generation happened via alanine to offset the reduction in pyruvate derived via glycolysis. After a 3-day refeeding period cALT returned to control levels while PK was not able to rebound. No alterations were detected in the expression levels of G6PDH while 6PGDH was revealed to be more sensitive specially to fasting, as confirmed by a significant 5.7-fold decrease in mRNA levels with no recovery after refeeding. Our results indicate that in early stages of refeeding, the liver prioritized the restoration of systemic normoglycemia and replenishment of hepatic glycogen. In a later stage, once regular feeding is re-established, dietary fuel may then be channeled to glycolysis and de novo lipogenesis.

1. Introduction

Aquaculture is highly dependent on capture fisheries to provide fishmeal required to produce high-protein feeds (Tacon and Metian, 2008), especially for carnivorous fish (Oliva-Teles, 2000; Kaushik and Seiliez, 2010). Thus, the development of well-suited and cost-effective feeds has become a matter of high importance to the sustainability and profitability of the sector. Our understanding of how carnivorous fish, such as the European seabass (*Dicentrarchus labrax* L.), metabolize different dietary nutrients came to some extent from determining the activity of key enzymes involved in carbohydrate and amino acid metabolism. This was done either by subjecting fish to different dietary compositions (Dias et al., 2004; Enes et al., 2006; Moreira et al., 2008) or to feeding/fasting/refeeding protocols (Pérez-Jiménez et al., 2007; Viegas et al., 2013). Studies with fed/fasted/refed fish have often helped to clarify the underlying alterations in hepatic intermediary metabolism in each setting and in its in-between transitions. Besides, in aquaculture similar refeeding maneuvers are associated with a phase of accelerated growth, known as compensatory growth (Ali et al., 2003). The physiological mechanisms behind this process are still unclear in seabass (Dupont-Prinet et al., 2010; Türkmen et al., 2012).

Transamination reactions play an important role in amino acid metabolism and among the transaminases, aspartate aminotransferase (AST; EC 2.6.1.1) and alanine aminotransferase (ALT; EC 2.6.1.2) are the most representative (Cowey and Walton, 1989). The latter, by catalyzing the exchange of alanine with pyruvate and ammonium ion, is considered to be one of the most responsive to changes in dietary protein utilization (Metón et al., 1999; Gaye-Siessegger et al., 2006; Pérez-Jiménez et al., 2007; González et al., 2012). In fish, alanine is an important metabolite whose energy can be obtained either directly by

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oxidation of the carbon skeleton (Pereira et al., 1995) or indirectly after conversion to glucose through gluconeogenesis (French et al., 1981). Besides, it has been used as probe for muscle growth in tracer studies (Gasier et al., 2009) and as indicator to assess the effects of food deprivation in muscle and liver in metabolomic studies (Kullgren et al., 2010). Since the equilibrium constant of ALT is approximately 1.0, the direction of alanine-pyruvate exchange is highly influenced by glycolytic production of pyruvate via pyruvate kinase (PK; EC 2.7.1.40). PK is one of the key control enzymes of glycolysis, and its expression and activity is highly sensitive to cellular energy charge and glycolytic flux. The activity of PK along with 6-phosphofructo 1-kinase (PFK-1; EC 2.7.1.11) serves primarily to increase the equilibrium constant of the glycolytic pathway and to commit the carbon skeletons to pyruvate production. However, in other carnivorous fish, PK mRNA was unresponsive after refeeding at the peak of postprandial absorption (8 h) (Skiba-Cassy et al., 2013) while activity levels recover only after 8 days of refeeding (Metón et al., 2003; Soengas et al., 2006; Furné et al., 2012; Pérez-Jiménez et al., 2012). This suggests that a long-term stimulation by food intake is required in order to metabolize excess glucose toward pyruvate production. By addressing how the interconversion of alanine and pyruvate is related to the status of PK/PFK-1 gene expression and activity could provide insight into the hepatic regulation during fasting and consequent refeeding.

Aside from glycolysis, the other principal fate of glucose carbons is metabolism via the pentose phosphate pathway (PPP) (Dias et al., 1998). The PPP is a principal provider of NADPH for biosynthetic pathways such as lipogenesis and for regeneration of reduced glutathione, as well as 5-carbon precursors for nucleotide biosynthesis. The oxidative branch of the PPP, involving glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.43), is highly regulated and reflects the cellular demand for NADPH and/or nucleotide and lipid biosynthesis (Sanden et al. 2003). Thus, as for ALT and PK, the mRNA levels and activity of these enzymes should be sensitive to nutritional and growth states. This set of enzymes has often been used to assess the effects of food deprivation and refeeding in seabass (Pérez-Jiménez et al., 2007; Viegas et al., 2013) and other species (Metón et al., 1999; Furné et al., 2012; Pérez-Jiménez et al., 2012). Despite the valuable information withdrawn from these enzymes' activity, the nutritional regulation of their gene expression at transcriptional level still remains to be addressed in seabass. Given this, we hypothesized that an integrated analysis of mRNA levels and activities for the ALT, PK, G6PDH and 6PGDH enzymes would provide a sensitive biomarker of nutrient availability in seabass. To test this hypothesis, we isolated cDNA fragments from *D. labrax* liver encoding ALT, PK and G6PDH and 6PGDH in order to design specific molecular probes to measure expression at mRNA level, while the corresponding enzymatic activities were also assayed. These measurements were performed in cDNA from livers of seabass reared under three different conditions: regular feeding, fasting (21 days) and fasting (21 days) followed by refeeding (3 days).

2. Material & methods

2.1. Fish sampling and handling

Farmed European seabass (*D. labrax* L.) were maintained as previously described (Viegas et al., 2012). Briefly, a total of 18 fish provided by a local farm were transported to the lab, and distributed in 200 L tanks supplied with aerated filtered seawater from a recirculation system equipped with a central filtering unit and a UV unit ($n = 6$ per tank; initial mean length of 28.0 ± 1.7 cm and initial mean body weight of 218.0 ± 43.0 g). The system was maintained at 18 °C and 30‰ salinity throughout the experiment. After acclimation, fish from one of the tanks were provided with a commercial diet (the same used in the farm: Dourasoja Ultra 5, SORGAL, S.A.; 44% crude protein, 18% crude fat, 2.2% starch, 9.2% ash, 5 mm standard pellet; 20 kJ g^{-1} dry weight gross energy) once a day with ration of 2% mean body weight per day. Fish in the remaining two tanks were fasted for 21 days. After this period fish from one of those tanks were provided with food again, once a day with the same commercial diet for 3 days. Regularly fed fish and refed fish were provided with last meal 24 h before sacrifice. Fish were anesthetized in saltwater containing 0.1 g L^{-1} of MS-222, and sampled for blood from the caudal vein with heparinized syringes. After sacrifice by cervical section, the liver was excised, weighed, freeze-clamped in liquid N_2, ground and stored at −80 °C until further analysis.

2.2. Total RNA extraction and reverse transcription (RT)

Total mRNA was isolated from frozen liver samples using the Speedtools Total RNA Extraction Kit (Biotools, Spain). The RNA obtained served as template for RT-PCR. RNA was spectrophotometrically quantified using a NanoDrop ND-1000 (Thermo Scientific) and quality was determined using the ratio of absorbance at 260 and 280 nm. Single strand cDNA templates for PCR amplification were synthesized from 1 μg of total RNA by incubation with M-MLV RT (Promega, Spain) at 37 °C for 1 h, according to the supplier's instructions.

2.3. RT-PCR analysis

The pairs of oligonucleotides and expected length of bands generated in the amplification by RT-PCR for the different enzymes are presented in Table 1. The oligonucleotides were designed from highly conserved regions in the nucleotide sequences published in GenBank for each of

Table 1
Primer pairs used for the partial cDNA cloning by RT-PCR and expected band extension.

Gene		RT-PCR	
		Primer sequence (5′–3′)	Expected band extension (bp)
Amino acid metabolism			
cALT	Forward	GCTGACTGTTGACACCATGAAC[a]	792
	Reverse	CAGCCCTCTGCGTACACATTATCCTG[b]	
mALT	Forward	AACATGTCGGCTACAAGGATG[c]	1028
	Reverse	CAGCCCTCTGCGTACACATTATCCTG[b]	
Glycolysis			
PK	Forward	GATGCTGGAGAGTATGGTGCACCACG	347
	Reverse	GCCTCTCTGCAGATCGAGTGCAT	
Pentose phosphate pathway			
G6PDH	Forward	GAGATGGTGCAGAACCTCATGG	725
	Reverse	CCACAGAAGACATCCAGGATGAG	
6PGDH	Forward	GGGGACATGCAGCTGATCTGTGAGGC	727
	Reverse	GTCTGTACCCGTCATAGAAGGA	

[a]IMAL10, [b]IMAL07 and [c]shorter version of IMAL23 from Metón et al. (2004).