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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 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 (Kaushik and Seiliez, 2010; Oliva-Teles, 2000). 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 (Gaye-Siessegger et al., 2006; González et al., 2012; Metón et al., 1999; Pérez-Jiménez et al., 2007). In fish, alanine is an important metabolite whose energy can be obtained either directly by 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) serve 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 irresponsive after refeeding in the peak of postprandial absorption (8 h) (Skiba-Cassy et al., 2013) while activity levels only recover 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 towards pyruvate production. By addressing how the interconversion of alanine and pyruvate is related with 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 (Furné et al., 2012; Metón et al., 1999; 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 (*Dicentrarchus 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 anaesthetized 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 ºC 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 ºC for 1 h, according to supplier's instructions.

141 *2.3. RT-PCR analysis*

142 The pairs of oligonucleotides and expected length of bands generated in the amplification by
143 RT-PCR for the different enzymes are presented in Table 1. The oligonucleotides were designed
144 from highly conserved regions in the nucleotide sequences published in GenBank for each of the
145 enzymes, aligning whenever possible sequences from fish species such as zebrafish *Danio rerio*,
146 rainbow trout *Oncorhynchus mykiss*, flounder *Platichthys flesus*, common carp *Cyprinus carpio*,
147 and gilthead seabream *Sparus aurata*. The sequences were aligned using EMBL-EBI ClustalW2
148 - Multiple Sequence Alignment Tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) for
149 comparing homologies and oligonucleotides were designed with Oligo Explorer 1.2 Software
150 (Gene Link). For mALT and cALT, oligonucleotides were designed according to previous
151 studies in gilthead seabream *Sparus aurata* (Metón et al., 2004). RT-PCR mixtures (30 µL total
152 volume) consisted of 3 µL of single-strand cDNA product, 3 µL of 10x PCR buffer, 200 µM
153 dNTPs, 0.5 µM of oligonucleotides (forward and reverse), and 1 U of Expand High Fidelity
154 PCR System (Roche). The amplification reaction was conducted through 39 cycles of
155 denaturation at 94 ºC for 30 s, annealing for 30 s at 55 ºC and DNA synthesis at 72 ºC for 2 min,
156 followed by a final extension step of 5 min. The PCR products were separated
157 electrophoretically on 1% agarose gel alongside a 1 kbp ladder (Biotools). Bands of appropriate
158 size were excised, purified using High Pure PCR Cleanup Micro Kit (Roche), ligated into
159 pGEM-T Easy plasmid (Promega), transformed by thermic shock in DH5α competent cells and
160 selected colonies were allowed to grow overnight in LB/ampicillin broth at 37ºC. Recombinant
161 plasmid DNA was isolated using GenElute™ Plasmid Miniprep Kit (Sigma) and sequenced with
162 ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems®).
163 Identity of insert sequences were verified using BLASTx against the National Center for
164 Biotechnology Information (NCBI) public databases and aligned amongst different cDNA
165 clones. The new *D. labrax* obtained sequences were introduced in the GenBank from the
166 National Center for Biotechnology Information (NCBI) (length of the fragment and accession
167 numbers for each enzyme are listed in Table 2).

168

169 *2.4. qRT-PCR analysis*

170 Specific oligonucleotides for quantitative real time RT-PCR (qRT-PCR) were designed with
171 Oligo Explorer 1.2 (Gene Link) (Table 2). Linearity and efficiency of amplification on qRT-
172 PCR for the chosen oligonucleotides were tested by generating standard curves with consecutive
173 dilutions of a cDNA test sample. PCR product from a non-diluted cDNA sample and a blank
174 (with milliQ water instead of cDNA) were separated electrophoretically on 2% agarose gel

alongside GeneRuler™ 100 bp DNA ladder (Fermentas) for length confirmation and discard presence of secondary bands.

Extraction of mRNA and subsequent synthesis of single strand cDNA templates was performed as mentioned above from frozen liver samples. The reaction product was diluted 10x in milliQ water and real-time quantitative PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems®) using 0.4 µM of each oligonucleotide, 10 µL of Power SYBR® Green (Applied Biosystems®) and 1.6 µL of diluted cDNA. The temperature cycle protocol for amplification was: 50 ºC for 2 min, 95 ºC for 10 min, followed by 40 cycles with 95 ºC for 15 s and 62 ºC for 1 min. Ribosomal subunit 18 (18S) from *D. labrax* was chosen as the reference gene to normalize expression levels of targets between different samples. Among the reference (housekeeping) genes tested, 18s did not reveal changes in expression levels between experimental conditions studied and presented the lowest standard deviation within conditions. Variations in gene expression and n-fold induction were calculated relative to fed fish using the standard ΔΔCt method including the efficiencies for the experimental gene and 18S. Specific details for each amplified transcript such as oligonucleotides used, length of bands generated and GenBank accession numbers are listed in Table 2.

2.5. Enzyme activities

The assays to determine enzyme activities were carried out as previously described (Metón et al., 1999), with minor modifications. Crude extracts were obtained by mixing the powdered frozen liver in a buffer containing 50 mM Tris-HCl pH 7.5, 4 mM EDTA acid, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM 1,4-dithiothreitol and 250 mM sucrose (1/5, w/v), followed by homogenization using a PTA-7 Polytron mixer (Kinematica GmbH, Switzerland; position 3, 30 s), and centrifugation at 15,800 *g* for 40 min at 4 ºC. PK activity was assayed in a final volume of 250 µL of final volume containing 70 mM glycylglycine pH 7.4, 10 mM MgCl2, 100 mM KCl, 0.15 mM NADH, 2.8 mM phosphoenolpyruvate, 21 U/mL lactate dehydrogenase and 4 µL of crude extract. The reaction was triggered after addition of 2.5 mM ADP. PFK-1 activity was assayed in a final volume of 200 µL containing 100 mM Tris HCl pH 8.25, 5 mM $MgCl_2$, 50 mM KCl, 0.15 mM NADH, 4 mM ammonium sulfate, 12 mM 2-mercaptoethanol, 10 mM fructose 6-phosphate, 30 mM glucose 6-phosphate, 0.675 U/mL fructose bisphosphate aldolase, 5 U/mL triose-phosphate isomerase, 2 U/mL glycerol-3-phosphate dehydrogenase and 4 µL crude extract. The reaction was triggered after addition of 10 mM ATP. G6PDH activity was assayed in a final volume of 200 µL of final volume containing 77.5 mM imidazole-HCl pH 7.7, 5 mM $MgCl_2$, 1 mM NADP and 1 mM glucose 6-phosphate and 4 µL of crude extract. 6PGDH activity was assayed in a final volume of 200 µL of final volume containing 82.7 mM

imidazole-HCl pH 7.7, 3 mM MgCl2, 0.5 mM NADP, 2 mM 6-phosphogluconate and 4 µL of crude extract. ALT and AST activity were assayed both in cytosolic (cALT/cAST) and mitochondrial (mALT/mAST) fractions. Mitochondrial fraction was prepared as described by Metón et al. (2004) at 4°C, from powdered frozen liver in buffer A (50 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 50 mM NaF, 0.5 mM PMSF, 1 mM DTT, 200 mM mannitol and 70 mM sucrose) (1/5, w/v), followed by homogenization using a Dounce homogenizer, and centrifugation at 500 *g* for 10 min to remove nuclear and cell debris. The mitochondrial fraction was pelleted by centrifugation at 9000 *g* for 20 min. The resulting supernatant contained the cytosolic fraction. The pellet was yet washed twice with buffer A followed by another centrifugation at 9000 *g* for 20 min. Mitochondria were resuspended and disrupted in buffer A prepared without mannitol and sucrose. Activity for ALT and AST were determined with a commercial kit (Cromatest, Linear Chemicals, Spain). All enzymatic assays were carried out at 30ºC and followed at 340 nm. The total protein content in liver crude extracts was determined by the Bradford method (Bio-Rad, Spain) at 30 ºC using bovine serum albumin as a standard and followed at 600 nm. All assays were adapted for automated measurement using a Cobas Mira S spectrophotometric analyzer (Hoffman-La Roche, Switzerland). Enzyme activities were expressed per mg of soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme necessary to transform 1 µmol of substrate per min.

2.6. Statistical analysis

Normality (Kolmogorov-Smirnov's test) and homogeneity of variance (Bartlett's test) were verified prior to the analysis. Log transformation was performed during the statistical analysis if Bartlett's test > 0.05. Analysis of variance (ANOVA) was used to test the statistical differences between nutritional conditions. *A posteriori* Tukey's test was performed when significant differences were found (differences were considered statistically significant at $P<0.05$).

3. Results

Blood glucose and hepatic glycogen levels (data not shown) plummeted after fasting and the refeeding period allowed full recovery to levels found in fed fish (blood glucose in mM: 10.7±6.3 / 4.8±1.2 / 9.3±1.4; hepatic glycogen in g 100 g^{-1} liver: 3.0±0.9 / 0.7±0.4 / 3.5±0.4, for fed / fasted / refed fish, respectively) as previously reported (Viegas et al., 2013).

A 599 bp cDNA fragment encoding cALT was isolated from the liver of *D. labrax* by RT-PCR. The nucleotide sequence shared an 89% identity with *S. aurata* cALT messenger (GenBank accession no. **AY206502**). To isolate a cDNA fragment of *D. labrax* mALT, a forward

245 oligonucleotide was designed from a portion exclusively found in the *S. aurata* mitochondrial
246 ALT gene (Metón et al. 2004). The obtained nucleotide sequence of 465 bp was 94% identical
247 to its homolog fragment in *S. aurata* mALT (GenBank accession no. **AY206503**). Protein
248 identity between *D. labrax* ALT isoforms was 70%. Availability of cALT and mALT *D. labrax*
249 cDNA sequences allowed us to address the effect of nutritional status on the hepatic expression
250 of both genes at transcriptional level. qRT-PCR showed that fasting promoted a significant
251 increase in the mRNA levels of cALT (a 8.5-fold induction) while return to control values was
252 established after a 3-day refeeding period. The nutritional status did not affect mALT mRNA
253 levels. ALT activity was assayed in both cytosolic and mitochondrial liver extracts (Fig. 1) and
254 corroborated the results obtained at mRNA level for both genes. Although mALT mRNA levels
255 and activity showed no differences among the different feeding conditions, the cALT:mALT
256 activity ratio decreased after refeeding (19:1 for fed fish; 16:1 for fasted fish; 9:1 for refed fish).
257 Activity for cAST and mAST was also assayed and a similar trend to that observed for cALT
258 and mALT activity, respectively, was obtained (Fig. 2).
259 A cDNA sequence of 347 bp encoding glycolytic enzyme PK was isolated and cloned from *D.*
260 *labrax* liver. This fragment was ~73% identical to *Takifugu rubripes* (GenBank accession no.
261 **AB074495**), *Salmo salar* (**NM_001141703**) and *Oreochromis niloticus* (**XM_003443869**).
262 qRT-PCR performed on cDNA from livers of fasted *D. labrax* showed a drastic 45-fold down-
263 regulation of PK mRNA levels in relation to control and this condition was maintained after a 3-
264 day refeeding period. These observations were consistent with enzyme activity which revealed
265 significantly higher values for PK in fed fish compared to fasted and refed fish (Fig. 3). Activity
266 values determined for the other glycolytic enzyme, PFK-1 (Fig. 3), revealed a similar decrease
267 to PK during fasting. The 3-day refeeding period however, was comparatively more effective in
268 triggering a response as confirmed by the partial recovery to levels found in fed fish.
269 To analyze gene expression at mRNA level, cDNA encoding *D. labrax* G6PDH and 6PGDH
270 were isolated (497 bp and 410 bp, respectively) and sequenced. The conditions studied in this
271 report did not allow to detect mRNA changes of G6PDH, while 6PGDH revealed to be more
272 sensitive specially to fasting, as confirmed by a significant 5.7-fold decrease in mRNA levels.
273 This down-regulation was slightly alleviated by refeeding but with no statistical significance
274 which was in agreement with the observed activity for this enzyme. Both G6PDH and 6PGDH
275 responded similarly in terms of enzyme activity with significantly higher values in the fed state
276 than in the fasted state. The 3-days refeeding period was not sufficient to recover control values
277 for both activities (Fig. 4).
278
279 **4. Discussion**

The liver is the main site for amino acid transamination where ALT and AST are quantitatively the most important aminotransferases in teleost fish. Their activity is correlated with scenarios of enhanced gluconeogenesis (Cowey and Walton, 1989), a major pathway for utilization of amino acids. The response of ALT to starvation does not follow a clear pattern between different fish species (Furné et al., 2012; Metón et al., 1999; Pérez-Jiménez et al., 2012). Moreover, its correlation with dietary protein content and its coordination with other transaminases such as AST for the supply of gluconeogenic precursors, may contribute to variable responses even within the same species as it seems to be the case of seabass (Pérez-Jiménez et al. 2007). Our findings differ from those of Pérez-Jiménez et al. (2007) that reported different responses for both ALT and AST in seabass fed a high- and a low- protein diet (49% and 41% crude protein, respectively) after a 9-day fasting period. These observations revealed that the behavior of ALT and AST to regular feeding was dependent on protein content. The influence the past nutritional history (different levels of dietary protein) on the observed metabolic responsiveness to refeeding after a fasting period was also evident. Our findings, using an intermediate level of crude protein (44%), revealed that cALT responded to a 21-day fasting period with a significant increase in both mRNA levels and activity. After refeeding, ALT returned to the levels found in fed fish. During fasting, net flux through cALT lies in the direction of alanine to pyruvate thereby providing carbon for both oxidative and anaplerotic tricarboxylic acid (TCA) cycle fluxes that sustain energy generation and gluconeogenesis, respectively (Mommsen 1986). Increased cALT activity under these conditions may represent an increase in the capacity of pyruvate generation from alanine in order to offset the reduction in pyruvate derived via glycolysis, as indicated by the observed decrease in expression and activity of PK during fasting. The opposite setting also seems to confirm such assessment. *S. aurata* subjected to a diet supplemented with amino-oxyacetate (an inhibitor of pyridoxal phosphate-dependent transaminases) balanced the decrease in ALT activity with a significant increase in PK activity (González et al., 2012). In contrast to cALT, none of the conditions induced significant changes in mALT expression or activity. Its contribution to total ALT activity was found to be residual as in *S. aurata* (Metón et al., 2004), hence its effect on the net flux between alanine and pyruvate under different nutritional states was considered to be insignificant, reinforcing the premise that mRNA and activity of mALT are not sensitive indicators of nutritional status for carnivorous saltwater fish.

It was previously demonstrated that plasma glucose was highly sensitive to fasting/refeeding conditions in seabass (Viegas et al., 2013) so that after a 21-day fasting period there was a significant decrease in glycemia to about half of that observed in fed fish. In the present study

glycolytic enzymes like PK and PFK-1 fell significantly along with glycemia, as expected. The slight decrease in glucokinase (GK; EC 2.7.1.2) coupled to the lack of up-regulation of gluconeogenic enzymes like glucose 6-phosphatase (G6Pase, EC 3.1.3.9) was also consistent with these observations (Viegas et al., 2013). This pattern has also been reported in some fish species such as common dentex *Dentex dentex* (Pérez-Jiménez et al., 2012), *S. aurata* (Bonamusa et al., 1992; Metón et al., 1999) and *O. mykiss* (Furné et al., 2012) despite some other cases where fasting caused no alterations in PK activity (Soengas et al., 2006) or mRNA levels (Kirchner et al., 2003; Panserat et al., 2001). Alanine is a strong allosteric inhibitor of hepatic PK (Fenton and Hutchinson, 2009; González et al., 2012), so increased levels of cALT could also have contributed to the decrease observed in PK activity. The restoration of PK activity to control levels after refeeding takes longer than the 3-day refeeding interval of our study as reported for other fish species (Furné et al., 2012; Metón et al., 2003; Pérez-Jiménez et al., 2012; Skiba-Cassy et al., 2013). Yet, surprisingly, dietary input after food deprivation affects differently glycolytic enzymes. GK is very responsive to refeeding as reported for this experiment (Viegas et al., 2013), other studies with *D. labrax* (Pérez-Jiménez et al., 2007) and other fish species (Skiba-Cassy et al., 2009; Soengas et al., 2006). This highlights the importance of rapidly phosphorylating exogenous (dietary) glucose to glucose 6-phosphate (G6P) since it is a key branchpoint metabolite for glycolysis, glycogen synthesis, and PPP. It has also been hypothesized that after refeeding, the overshoot in GK associated with the lack of inhibition of the gluconeogenic enzyme G6Pase may be the result of futile glucose-G6P cycling (Kamalam et al., 2012; Skiba-Cassy et al., 2013; Viegas et al., 2013), an argument previously raised while using stable isotopes (Viegas et al., 2011). Contrary to GK, PFK-1 and PK, located downstream of G6P in the glycolytic pathway, seem to be poorly regulated by short-term refeeding in seabass. This is consistent with the findings for *O. mykiss* (Dai et al., 2013; Skiba-Cassy et al., 2009) and *D. rerio* (Seiliez et al., 2013) further supporting the argument of upstream glycolysis futile cycling. Methodologies on how to resolve this have been recently developed using labeled glucose (Martins et al., 2013; Nunes et al., 2013). The low control coefficients of these enzymes in fish glycolytic metabolism is not well understood, but may also involve post-translational regulation, as suggested for *O. mykiss* (Panserat et al., 2001; Skiba-Cassy et al., 2009), *S. aurata* (Mediavilla et al., 2008) and *D. rerio* (Dai et al., 2013). Nutrient availability was expected to favor glycolysis over gluconeogenesis during the early stages of refeeding, nevertheless in this setting the liver seemed to prioritize the restoration of systemic normoglycemia and replenishment of the hepatic glycogen pool. It has been demonstrated through the use of stable isotopes that gluconeogenesis is the main contributor to blood glucose production in refed seabass (Viegas et al., 2013), while hepatic glycogen replenishment is

supported mainly via the gluconeogenic or so-called "indirect" pathway (Viegas et al., 2012). These observations are consistent with a reduced role of glycolysis and the weak correlation of hepatic PK and PFK-1 expression and activities with the fasting to feeding transition.

The decreased hepatic activity level of *D. labrax* G6PDH and 6PGDH after fasting was consistent with observations in other species (Metón et al., 2003; Morales et al., 2004; Pérez-Jiménez et al., 2012) reflecting the downregulation of biosynthetic and growth activities and its corresponding reduction in demand for NADPH and nucleotide precursors. However, as previously reported for G6PDH activity in fasted fish (Furné et al., 2012; Ibarz et al., 2007; Sangiao-Alvarellos et al., 2005), we observed that G6PDH mRNA levels remained unchanged in every condition possibly indicating an active role in cellular equilibrium, for example, on maintaining the redox state of the hepatocytes (Morales et al., 2004). The decrease in cALT activity after refeeding could lead to increased alanine which has been proven to modulate the oxidative part of the PPP, significantly increasing G6PDH activity in primary cultures of Atlantic salmon *Salmo salar* hepatocytes (Sanden et al. 2003). However the lack of rebound of G6PDH and 6PGDH after refeeding with a high-protein diet supports the role of the PPP as NADPH-generating system rather than a component of nutrient conversion into lipids (Barroso et al., 1998). A study with 2-day fasted *O. mykiss*, where refeeding was followed through 8 time-points within 24 h after meal seemed to support this assumption (Mennigen et al., 2012). Despite the significant increase observed in the expression of various genes involved in hepatic lipid metabolism (e.g. SREBP1c, FAS, and ACLY), G6PDH expression remained unaltered. Interestingly, after refeeding seabass with a high-protein diet (49% crude protein), Pérez-Jiménez et al. (2007) reported full recovery of G6PDH activity in after 1 d, something that was not observed using a lower protein diet (41% crude protein; same study) or with the diet used in the present work (44% crude protein) even after 3 days of refeeding. Although different protein/lipid ratios may explain such discrepancy, the mRNA levels confirmed to some extent the obtained activities suggesting that *de novo* lipogenesis occurs in a later phase of the recovery.

In this study we report for the first time partial sequences and nutritional variations on mRNA levels for cALT, mALT, PK, G6PDH and 6PGDH in seabass. This enabled a more comprehensive analysis of how this species copes with food deprivation and consequent refeeding. In conclusion, the counterbalance between cALT and PK during fasting, both at activity and mRNA level, ensure a steady supply of pyruvate to the TCA cycle. cAST also participated by increasing its activity during fasting and supplying metabolic intermediates for gluconeogenesis. After a 3-day refeeding period rather than rebooting lipogenesis and enhancing downstream glycolysis, the liver prioritizes the production of glucose 6-phosphate (via GK) for glycogen replenishment (Viegas et al., 2013). At this point, the fasting-refeeding transition in

seabass could be further clarified by assessing the expression of genes involved in TCA cycle to confirm the oxidation of endogenous vs. dietary substrates and assessing the expression of genes involved in lipogenesis to narrow the discrepancies described for the role of the PPP.

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References

Barroso, J.B., Peragón, J., Contreras-Jurado, C., García-Salguero, L., Corpas, F.J., Esteban, F.J., Peinado, M.A., De La Higuera, M., Lupiáñez, J.A., 1998. Impact of starvation-refeeding on kinetics and protein expression of trout liver NADPH-production systems. Am J Physiol Regul Integr Comp Physiol 274, R1578-R1587.

Bonamusa, I., García de Frutos, P., Fernández, F., Baanante, I.V., 1992. Nutritional effects on key glycolytic-gluconeogenic enzyme activities and metabolite levels in the liver of the teleost fish *Sparus aurata*. Mol Mar Biol Biotechnol 1, 113-125.

Cowey, C., Walton, M., 1989. Intermediary Metabolism, in: E. John (Ed.), Fish Nutrition (Second Edition). Academic Press, New York, 259-329.

Dai, W., Panserat, S., Mennigen, J., Terrier, F., Dias, K., Seiliez, I., Skiba-Cassy, S., 2013. Postprandial regulation of hepatic glucokinase and lipogenesis requires the activation of TORC1 signaling in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol.

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.

Dias, J., Rueda-Jasso, R., Panserat, S., Conceição, L.E.C., Gomes, E.F., Dinis, M.T., 2004. Effect of dietary carbohydrate-to-lipid ratios on growth, lipid deposition and metabolic hepatic enzymes in juvenile Senegalese sole (*Solea senegalensis*, Kaup). Aquaculture Res 35, 1122-1130.

Dupont-Prinet, A., Chatain, B., Grima, L., Vandeputte, M., Claireaux, G., McKenzie, D.J., 2010. Physiological mechanisms underlying a trade-off between growth rate and tolerance of feed deprivation in the European sea bass (*Dicentrarchus labrax*). J Exp Biol 213, 1143-1152.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2006. Rapid metabolic adaptation in European sea bass (*Dicentrarchus labrax*) juveniles fed different carbohydrate sources after heat shock stress. Comp Biochem Physiol A Mol Integr Physiol 145, 73-81.

Fenton, A.W., Hutchinson, M., 2009. The pH dependence of the allosteric response of human liver pyruvate kinase to fructose-1,6-bisphosphate, ATP, and alanine. Arch Biochem Biophys 484, 16-23.

French, C.J., Mommsen, T.P., Hochachka, P.W., 1981. Amino acid utilisation in isolated hepatocytes from rainbow trout. Eur J Biochem 113, 311-317.

Furné, M., Morales, A., Trenzado, C., García-Gallego, M., Carmen Hidalgo, M., Domezain, A., Sanz Rus, A., 2012. The metabolic effects of prolonged starvation and refeeding in sturgeon and rainbow trout. J Comp Physiol B 182, 63-76.

Gasier, H.G., Previs, S.F., Pohlenz, C., Fluckey, J.D., Gatlin, D.M., Buentello, J.A., 2009. A novel approach for assessing protein synthesis in channel catfish, *Ictalurus punctatus*. Comp Biochem Physiol B Biochem Mol Biol 154, 235-238.

Gaye-Siessegger, J., Focken, U., Becker, K., 2006. Effect of dietary protein/carbohydrate ratio on activities of hepatic enzymes involved in the amino acid metabolism of Nile tilapia, *Oreochromis niloticus* (L.). Fish Physiol Biochem 32, 275-282.

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

Ibarz, A., Beltrán, M., Fernández-Borràs, J., Gallardo, M.A., Sánchez, J., Blasco, J., 2007. Alterations in lipid metabolism and use of energy depots of gilthead sea bream (*Sparus aurata*) at low temperatures. Aquaculture 262, 470-480.

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.

Kaushik, S.J., Seiliez, I., 2010. Protein and amino acid nutrition and metabolism in fish: Current knowledge and future needs. Aquaculture Res 41, 322-332.

Kirchner, S., Kaushik, S., Panserat, S., 2003. Low protein intake is associated with reduced hepatic gluconeogenic enzyme expression in rainbow trout (*Oncorhynchus mykiss*). J Nutr 133, 2561-2564.

Kullgren, A., Samuelsson, L.M., Larsson, D.G.J., Björnsson, B.T., Bergman, E.J., 2010. A metabolomics approach to elucidate effects of food deprivation in juvenile rainbow trout (*Oncorhynchus mykiss*). Am J Physiol Regul Integr Comp Physiol 299, R1440-R1448.

Martins, F.O., Rito, J., Jarak, I., Viegas, I., Pardal, M.A., Macedo, M.P., Jones, J.G., 2013. Disposition of [U-2H7]glucose into hepatic glycogen in rat and in seabass. Comp Biochem Physiol A 166, 316-322.

Mediavilla, D., Metón, I., Baanante, I.V., 2008. Purification and Kinetic Characterization of 6-Phosphofructo-1-kinase from the Liver of Gilthead Sea Bream (*Sparus aurata*). J Biochem 144, 235-244.

Mennigen, J.A., Panserat, S.p., Larquier, M.l., Plagnes-Juan, E., Medale, F.o., Seiliez, I., Skiba-Cassy, S., 2012. Postprandial regulation of hepatic microRNAs predicted to target the insulin pathway in rainbow trout. PLoS ONE 7, e38604.

Metón, I., Egea, M., Fernández, F., Eraso, M.C., Baanante, I.V., 2004. The N-terminal sequence directs import of mitochondrial alanine aminotransferase into mitochondria. FEBS Letts 566, 251-254.

Metón, I., Fernández, F., Baanante, I.V., 2003. Short- and long-term effects of refeeding on key enzyme activities in glycolysis-gluconeogenesis in the liver of gilthead seabream (*Sparus aurata*). Aquaculture 225, 99-107.

Metón, I., Mediavilla, D., Caseras, A., Cantó, M., Fernández, F., Baanante, I.V., 1999. Effect of diet composition and ration size on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). Br J Nutr 82, 223-232.

Morales, A.E., Pérez-Jiménez, A., Carmen Hidalgo, M., Abellón, E., Cardenete, G., 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp Biochem Physiol C Toxicol Pharmacol 139, 153-161.

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.

Nunes, P.M., Jarak, I., Heerschap, A., Jones, J.G., 2013. Resolving futile glucose cycling and glycogenolytic contributions to plasma glucose levels following a glucose load. Magn Reson Med. doi: 10.1002/mrm.24789

Oliva-Teles, A., 2000. Recent advances in European sea bass and gilthead sea bream nutrition. Aquaculture Int 8, 477-492.

Panserat, S., Plagnes-Juan, E., Kaushik, S., 2001. 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.

Pereira, C., Vijayan, M.M., Moon, T.W., 1995. In vitro hepatocyte metabolism of alanine and glucose and the response to insulin in fed and fasted rainbow trout. J Exp Zool 271, 425-431.

Pérez-Jiménez, A., Cardenete, G., Hidalgo, M., García-Alcázar, A., Abellán, E., Morales, A., 2012. Metabolic adjustments of *Dentex dentex* to prolonged starvation and refeeding. Fish Physiol Biochem 38, 1145-1157.

Pérez-Jiménez, A., Guedes, M.J., Morales, A.E., Oliva-Teles, A., 2007. Metabolic responses to short starvation and refeeding in *Dicentrarchus labrax*. Effect of dietary composition. Aquaculture 265, 325-335.

Sangiao-Alvarellos, S., Guzmán, J.M., Láiz-Carrión, R., Míguez, J.M., Martín Del Río, M.P., Mancera, J.M., Soengas, J.L., 2005. Interactive effects of high stocking density and food deprivation on carbohydrate metabolism in several tissues of gilthead sea bream *Sparus auratus*. J Exp Zool A Comp Exp Biol 303, 761-775.

Seiliez, I., Médale, 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.

Skiba-Cassy, S., Lansard, M., Panserat, S., Médale, F., 2009. Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. Am J Physiol Regul Integr Comp Physiol 297, R1421-R1429.

Skiba-Cassy, S., Panserat, S., Larquier, M., Dias, K., Surget, A., Plagnes-Juan, E., Kaushik, S., Seiliez, I., 2013. Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (*Oncorhynchus mykiss*). Br J Nutr 109, 1359-1372.

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.

Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285, 146-158.

Türkmen, S., Eroldoğan, O.T., Yılmaz, H.A., Ölçülü, A., Inan, G.A.K., Erçen, Z., Tekelioğlu, N., 2012. Compensatory growth response of European sea bass (*Dicentrarchus labrax* L.) under cycled starvation and restricted feeding rate. Aquaculture Res 43, 1643-1650.

Viegas, I., Mendes, V.M., Leston, S., Jarak, I., Carvalho, R.A., Pardal, M.A., Manadas, B., Jones, J.G., 2011. Analysis of glucose metabolism in farmed European sea bass (*Dicentrarchus labrax* L.) using deuterated water. Comp Biochem Physiol A Mol Integr Physiol 160, 341-347.

Viegas, I., Rito, J., González, J.D., Jarak, I., Carvalho, R.A., Metón, I., Pardal, M.A., Baanante, I.V., Jones, J.G., 2013. Effects of food-deprivation and refeeding on the regulation and sources of blood glucose appearance in European seabass (*Dicentrarchus labrax* L.). Comp Biochem Physiol A Mol Integr Physiol 166, 399-405.

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

Figure captions

Fig. 1 Effects of feeding, fasting (21 days), and refeeding (21 days fasting, 3 days refeeding) on mRNA levels (grey bars; in arbitrary units) and specific activity (black bars; in mU mg^{-1} protein) of cALT (left) and mALT (right) in liver of *D. labrax*. Mean values ± S.D. (n = 6) are presented. Significant differences between conditions are indicated by different lower case letters for mRNA levels and by different upper case letters for activity (one-way ANOVA followed by Tukey's test).

Fig. 2 Effects of feeding, fasting (21 days), and refeeding (21 days fasting, 3 days refeeding) on specific activity (black bars; in mU mg^{-1} protein) of cAST (left) and mAST (right) in liver of *D. labrax*. Mean values ± S.D. (n = 6) are presented. Significant differences between conditions are indicated by different letters (one-way ANOVA followed by Tukey's test).

Fig. 3 Effects of feeding, fasting (21 days), and refeeding (21 days fasting, 3 days refeeding) on mRNA levels (grey bars; in arbitrary units) and specific activity (black bars; in mU mg^{-1} protein) of PK (left) and PFK-1 (right - specific activity only) in liver of *D. labrax*. Mean values ± S.D. (n = 6) are presented. Significant differences between conditions are indicated by different lower case letters for mRNA levels and by different upper case letters for activity (one-way ANOVA followed by Tukey's test).

Fig. 4 Effects of feeding, food deprivation (21 days), and refeeding (21 days fasting, 3 days refeeding) on mRNA levels (grey bars; in arbitrary units) and specific activity (black bars; in mU mg^{-1} protein) of G6PDH (left) and 6PGDH (right) in liver of *D. labrax*. Mean values ± S.D. (n = 6) are presented. Significant differences between conditions are indicated by different lower case letters for mRNA levels and by different upper case letters for activity (one-way ANOVA followed by Tukey's test).

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.

559 **Table 2**

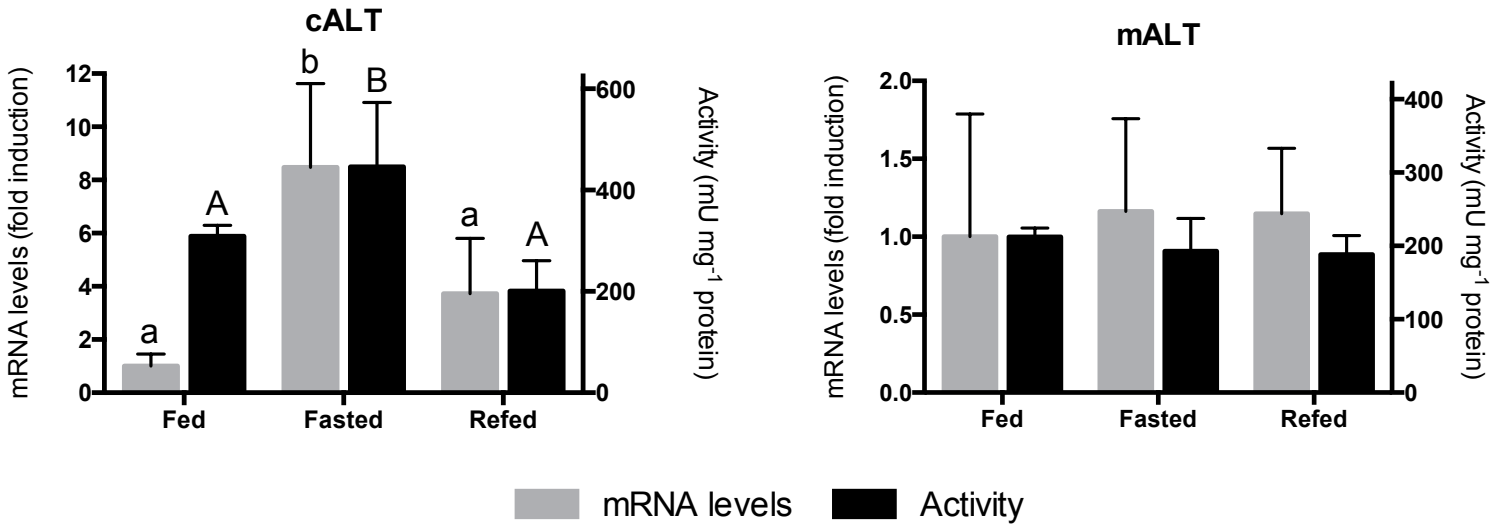
560 Primer pairs used to assess mRNA levels by quantitative real-time RT-PCR analysis, length of

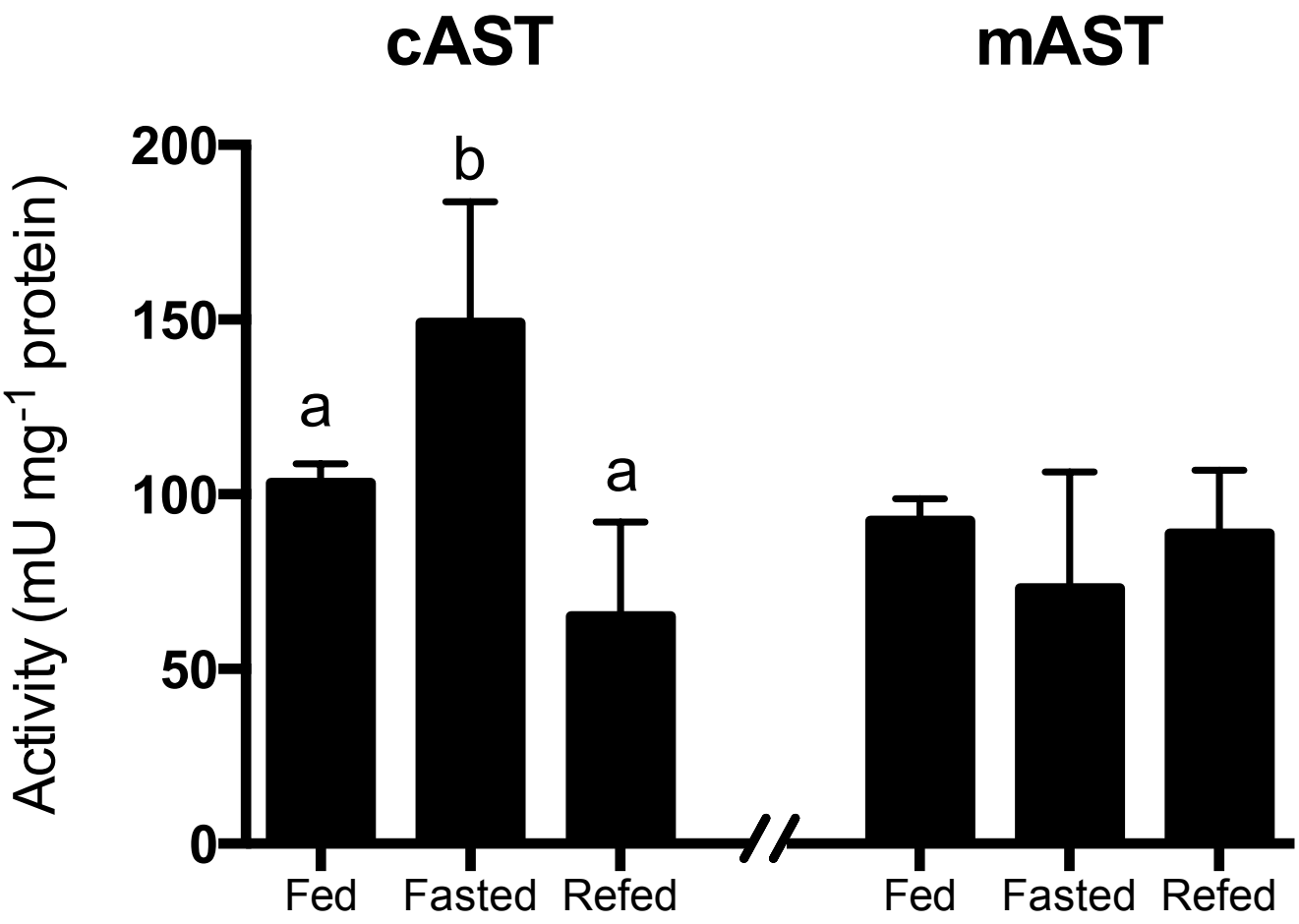
561 amplified fragments total length of the sequence and respective GenBank accession numbers.

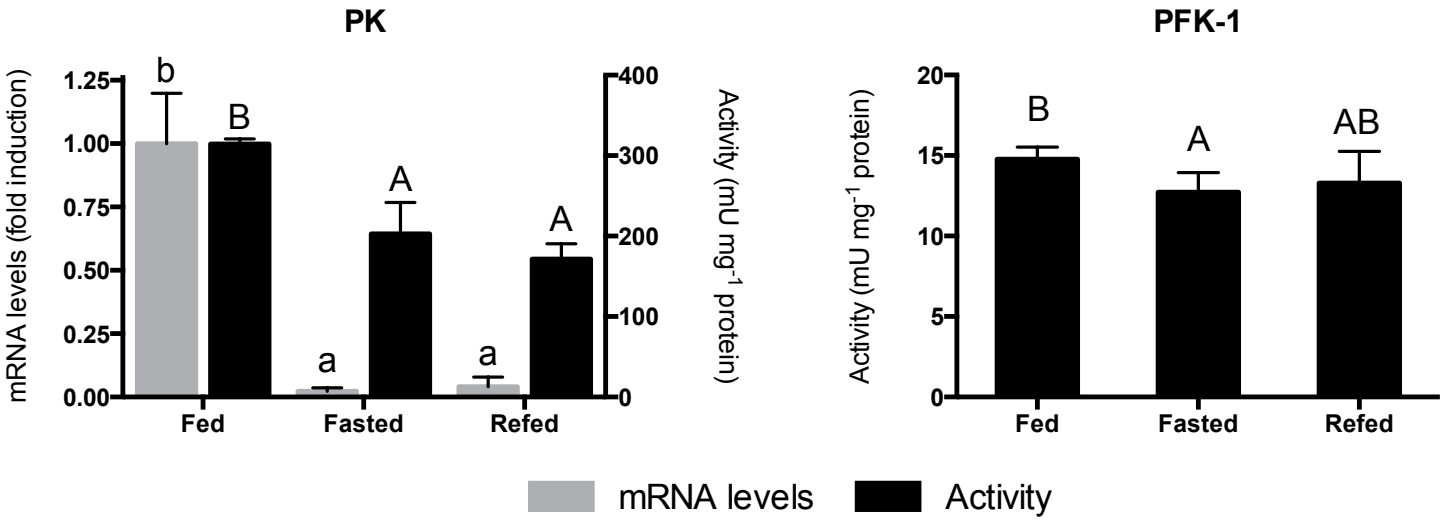
562

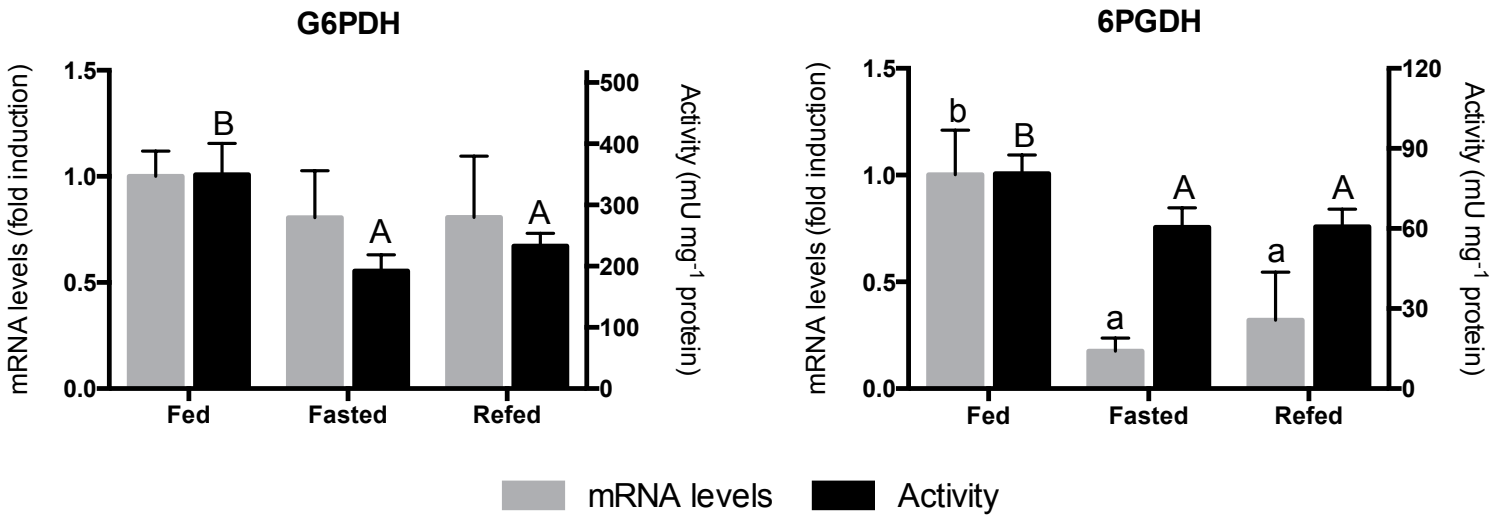
Gene		Quantitative real time RT-PCR			
		Primer sequence (5′–′3)	Length of sequence (bp)	Length of amplified fragment (bp)	GenBank accession no.
Housekeeping gene					
18s	Forward	ACGGACGAAAGCGAAAGCA	406	91	**AM419038**
	Reverse	GGAACTACGACGGTATCTGATC			
Amino acid metabolism					
cALT	Forward	TGAAGGAGGGGGTCAAGAAA	559	123	**JX073702**
	Reverse	AGGGTAAGAACACAGAGCCA			
mALT	Forward	GCAGCCAATCACTTTCTTCCG	465	106	**JX073703**
	Reverse	AATGCGGCGTGCTCTACTTTT			
Glycolysis					
PK	Forward	CAAGGTGGAAAGCCGGCAAGGC	347	83	**KF857578**
	Reverse	GGTCACCCCTGGCAACCATCA			
Pentose phosphate pathway					
G6PDH	Forward	TGGAACAGGGACAGCGTGG	497	132	**JX073705**
	Reverse	GAGCATCTGGAGCAAGTGGTT			
6PGDH	Forward	CGGTGACAAGGCTGCGTTC	410	110	**JX073706**
	Reverse	CGAACTCTTTGGCTGCCTGC			

563


cALT
mALT
mRNA levels (fold induction)
Activity (mU mg-1 protein)
a
A
b
B
a
A
Fed
Fasted
Refed
mRNA levels
Activity


cAST
mAST
Activity (mU mg-1 protein)
200
150
100
50
0
a
b
a
Fed
Fasted
Refed
Fed
Fasted
Refed


PK
mRNA levels (fold induction)
1.25
1.00
0.75
0.50
0.25
0.00
b
B
a
A
a
A
400
300
200
100
0
Activity (mU mg-1 protein)
Fed
Fasted
Refed
PFK-1
Activity (mU mg-1 protein)
20
15
10
5
0
B
A
AB
Fed
Fasted
Refed
mRNA levels
Activity


G6PDH
6PGDH
mRNA levels (fold induction)
Activity (mU mg-1 protein)
1.5
1.0
0.5
0.0
500
400
300
200
100
0
120
90
60
30
B
A
A
b
B
a
A
a
A
Fed
Fasted
Refed
mRNA levels
Activity