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

6

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23 **Running title:** Intermediary metabolism in seabass during fasting and refeeding

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32 **Abstract**

33 We hypothesized that the analysis of mRNA level and activity of key enzymes in amino acid
34 and carbohydrate metabolism in a feeding/fasting/refeeding setting could improve our
35 understanding of how a carnivorous fish, like the European seabass (*Dicentrarchus labrax*),

36 responds to changes in dietary intake at the hepatic level. To this end cDNA fragments encoding
37 genes for cytosolic and mitochondrial alanine aminotransferase (cALT; mALT), pyruvate kinase
38 (PK), glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase
39 (6PGDH) were cloned and sequenced. Measurement of mRNA levels through quantitative real-
40 time PCR performed in livers of fasted seabass revealed a significant increase in cALT (8.5-fold
41 induction) while promoting a drastic 45-fold down-regulation of PK in relation to the levels
42 found in fed seabass. These observations were corroborated by enzyme activity meaning that
43 during food deprivation an increase in the capacity of pyruvate generation happened via alanine
44 to offset the reduction in pyruvate derived via glycolysis. After a 3-day refeeding period cALT
45 returned to control levels while PK was not able to rebound. No alterations were detected in the
46 expression levels of G6PDH while 6PGDH revealed to be more sensitive specially to fasting, as
47 confirmed by a significant 5.7-fold decrease in mRNA levels with no recovery after refeeding.
48 Our results indicate that in early stages of refeeding, the liver prioritized the restoration of
49 systemic normoglycemia and replenishment of hepatic glycogen. In a later stage, once regular
50 feeding is re-established, dietary fuel may then be channeled to glycolysis and *de novo*
51 lipogenesis.

52

53 **1. Introduction**

54

55 Aquaculture is highly dependent on capture fisheries to provide fishmeal required to produce
56 high-protein feeds (Tacon and Metian, 2008), especially for carnivorous fish (Kaushik and
57 Seiliez, 2010; Oliva-Teles, 2000). Thus, the development of well-suited and cost-effective feeds
58 has become a matter of high importance to the sustainability and profitability of the sector. Our
59 understanding of how carnivorous fish, such as the European seabass (*Dicentrarchus labrax* L.),
60 metabolize different dietary nutrients came to some extent from determining the activity of key
61 enzymes involved in carbohydrate and amino acid metabolism. This was done either by
62 subjecting fish to different dietary compositions (Dias et al., 2004; Enes et al., 2006; Moreira et
63 al., 2008) or to feeding/fasting/refeeding protocols (Pérez-Jiménez et al., 2007; Viegas et al.,
64 2013). Studies with fed/fasted/refed fish have often helped to clarify the underlying alterations
65 in hepatic intermediary metabolism in each setting and in its in-between transitions. Besides, in
66 aquaculture similar refeeding maneuvers are associated with a phase of accelerated growth,
67 known as compensatory growth (Ali et al., 2003). The physiological mechanisms behind this
68 process are still unclear in seabass (Dupont-Prinet et al., 2010; Türkmen et al., 2012).

69 Transamination reactions play an important role in amino acid metabolism and among the
70 transaminases, aspartate aminotransferase (AST; EC 2.6.1.1) and alanine aminotransferase

71 (ALT; EC 2.6.1.2) are the most representative (Cowey and Walton, 1989). The latter, by
72 catalyzing the exchange of alanine with pyruvate and ammonium ion, is considered to be one of
73 the most responsive to changes in dietary protein utilization (Gaye-Siessegger et al., 2006;
74 González et al., 2012; Metón et al., 1999; Pérez-Jiménez et al., 2007). In fish, alanine is an
75 important metabolite whose energy can be obtained either directly by oxidation of the carbon
76 skeleton (Pereira et al., 1995) or indirectly after conversion to glucose through gluconeogenesis
77 (French et al., 1981). Besides, it has been used as probe for muscle growth in tracer studies
78 (Gasier et al., 2009) and as indicator to assess the effects of food deprivation in muscle and liver
79 in metabolomic studies (Kullgren et al., 2010). Since the equilibrium constant of ALT is
80 approximately 1.0, the direction of alanine-pyruvate exchange is highly influenced by glycolytic
81 production of pyruvate via pyruvate kinase (PK; EC 2.7.1.40). PK is one of the key control
82 enzymes of glycolysis, and its expression and activity is highly sensitive to cellular energy
83 charge and glycolytic flux. The activity of PK along with 6-phosphofructo 1-kinase (PFK-1; EC
84 2.7.1.11) serve primarily to increase the equilibrium constant of the glycolytic pathway and to
85 commit the carbon skeletons to pyruvate production. However, in other carnivorous fish, PK
86 mRNA was irresponsive after refeeding in the peak of postprandial absorption (8 h) (Skiba-
87 Cassy et al., 2013) while activity levels only recover after 8 days of refeeding (Metón et al.
88 2003; Soengas et al. 2006; Furné et al. 2012; Pérez-Jiménez et al. 2012). This suggests that a
89 long-term stimulation by food intake is required in order to metabolize excess glucose towards
90 pyruvate production. By addressing how the interconversion of alanine and pyruvate is related
91 with the status of PK/PFK-1 gene expression and activity could provide insight into the hepatic
92 regulation during fasting and consequent refeeding.

93 Aside from glycolysis, the other principal fate of glucose carbons is metabolism via the pentose
94 phosphate pathway (PPP) (Dias et al., 1998). The PPP is a principal provider of NADPH for
95 biosynthetic pathways such as lipogenesis and for regeneration of reduced glutathione, as well
96 as 5-carbon precursors for nucleotide biosynthesis. The oxidative branch of the PPP, involving
97 glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and 6-phosphogluconate
98 dehydrogenase (6PGDH; EC 1.1.1.43), is highly regulated and reflects the cellular demand for
99 NADPH and/or nucleotide and lipid biosynthesis (Sandén et al. 2003). Thus, as for ALT and PK,
100 the mRNA levels and activity of these enzymes should be sensitive to nutritional and growth
101 states. This set of enzymes has often been used to assess the effects of food deprivation and
102 refeeding in seabass (Pérez-Jiménez et al., 2007; Viegas et al., 2013) and other species (Furné et
103 al., 2012; Metón et al., 1999; Pérez-Jiménez et al., 2012). Despite the valuable information
104 withdrawn from these enzymes' activity, the nutritional regulation of their gene expression at
105 transcriptional level still remains to be addressed in seabass. Given this, we hypothesized that an

106 integrated analysis of mRNA levels and activities for the ALT, PK, G6PDH and 6PGDH
107 enzymes would provide a sensitive biomarker of nutrient availability in seabass. To test this
108 hypothesis, we isolated cDNA fragments from *D. labrax* liver encoding ALT, PK and G6PDH
109 and 6PGDH in order to design specific molecular probes to measure expression at mRNA level,
110 while the corresponding enzymatic activities were also assayed. These measurements were
111 performed in cDNA from livers of seabass reared under three different conditions: regular
112 feeding, fasting (21 days) and fasting (21 days) followed by refeeding (3 days).

113

114 **2. Material & methods**

115

116 *2.1. Fish sampling and handling*

117 Farmed European seabass (*Dicentrarchus labrax* L.) were maintained as previously described
118 (Viegas et al., 2012). Briefly, a total of 18 fish provided by a local farm were transported to the
119 lab, and distributed in 200 L tanks supplied with aerated filtered seawater from a recirculation
120 system equipped with a central filtering unit and a UV unit (n = 6 per tank; initial mean length
121 of 28.0±1.7 cm and initial mean body weight of 218.0±43.0 g). The system was maintained at
122 18°C and 30‰ salinity throughout the experiment. After acclimation, fish from one of the tanks
123 were provided with a commercial diet (the same used in the farm: Dourasoja Ultra 5, SORGAL,
124 S.A; 44% crude protein, 18% crude fat, 2.2% starch, 9.2% ash, 5 mm standard pellet; 20 kJ g⁻¹
125 dry weight gross energy) once a day with ration of 2% mean body weight per day. Fish in the
126 remaining two tanks were fasted for 21 days. After this period fish from one of those tanks were
127 provided with food again, once a day with the same commercial diet for 3 days. Regularly fed
128 fish and refeed fish were provided with last meal 24 h before sacrifice. Fish were anaesthetized in
129 saltwater containing 0.1 g L⁻¹ of MS-222, and sampled for blood from the caudal vein with
130 heparinized syringes. After sacrifice by cervical section, the liver was excised, weighed, freeze-
131 clamped in liquid N₂, ground and stored at -80 °C until further analysis.

132

133 *2.2. Total RNA extraction and reverse transcription (RT)*

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

140

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 °C for 30 s, annealing for 30 s at 55 °C and DNA synthesis at 72 °C 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 37°C. 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

175 alongside GeneRuler™ 100 bp DNA ladder (Fermentas) for length confirmation and discard
176 presence of secondary bands.
177 Extraction of mRNA and subsequent synthesis of single strand cDNA templates was performed
178 as mentioned above from frozen liver samples. The reaction product was diluted 10x in milliQ
179 water and real-time quantitative PCR was performed in a StepOnePlus™ Real-Time PCR
180 System (Applied Biosystems®) using 0.4 µM of each oligonucleotide, 10 µL of Power SYBR®
181 Green (Applied Biosystems®) and 1.6 µL of diluted cDNA. The temperature cycle protocol for
182 amplification was: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles with 95 °C for 15 s
183 and 62 °C for 1 min. Ribosomal subunit 18 (18S) from *D. labrax* was chosen as the reference
184 gene to normalize expression levels of targets between different samples. Among the reference
185 (housekeeping) genes tested, 18s did not reveal changes in expression levels between
186 experimental conditions studied and presented the lowest standard deviation within conditions.
187 Variations in gene expression and n-fold induction were calculated relative to fed fish using the
188 standard $\Delta\Delta C_t$ method including the efficiencies for the experimental gene and 18S. Specific
189 details for each amplified transcript such as oligonucleotides used, length of bands generated
190 and GenBank accession numbers are listed in Table 2.

191

192 2.5. Enzyme activities

193 The assays to determine enzyme activities were carried out as previously described (Metón et al.,
194 1999), with minor modifications. Crude extracts were obtained by mixing the powdered frozen
195 liver in a buffer containing 50 mM Tris-HCl pH 7.5, 4 mM EDTA acid, 50 mM NaF, 0.5 mM
196 phenylmethylsulfonyl fluoride, 1 mM 1,4-dithiothreitol and 250 mM sucrose (1/5, w/v),
197 followed by homogenization using a PTA-7 Polytron mixer (Kinematica GmbH, Switzerland;
198 position 3, 30 s), and centrifugation at 15,800 g for 40 min at 4 °C. PK activity was assayed in a
199 final volume of 250 µL of final volume containing 70 mM glycylglycine pH 7.4, 10 mM MgCl₂,
200 100 mM KCl, 0.15 mM NADH, 2.8 mM phosphoenolpyruvate, 21 U/mL lactate dehydrogenase
201 and 4 µL of crude extract. The reaction was triggered after addition of 2.5 mM ADP. PFK-1
202 activity was assayed in a final volume of 200 µL containing 100 mM Tris HCl pH 8.25, 5 mM
203 MgCl₂, 50 mM KCl, 0.15 mM NADH, 4 mM ammonium sulfate, 12 mM 2-mercaptoethanol, 10
204 mM fructose 6-phosphate, 30 mM glucose 6-phosphate, 0.675 U/mL fructose biphosphate
205 aldolase, 5 U/mL triose-phosphate isomerase, 2 U/mL glycerol-3-phosphate dehydrogenase and
206 4 µL crude extract. The reaction was triggered after addition of 10 mM ATP. G6PDH activity
207 was assayed in a final volume of 200 µL of final volume containing 77.5 mM imidazole-HCl pH
208 7.7, 5 mM MgCl₂, 1 mM NADP and 1 mM glucose 6-phosphate and 4 µL of crude extract.
209 6PGDH activity was assayed in a final volume of 200 µL of final volume containing 82.7 mM

210 imidazole-HCl pH 7.7, 3 mM MgCl₂, 0.5 mM NADP, 2 mM 6-phosphogluconate and 4 μL of
211 crude extract. ALT and AST activity were assayed both in cytosolic (cALT/cAST) and
212 mitochondrial (mALT/mAST) fractions. Mitochondrial fraction was prepared as described by
213 Metón et al. (2004) at 4°C, from powdered frozen liver in buffer A (50 mM Tris-HCl, pH 7.5,
214 0.5 mM EDTA, 50 mM NaF, 0.5 mM PMSF, 1 mM DTT, 200 mM mannitol and 70 mM
215 sucrose) (1/5, w/v), followed by homogenization using a Dounce homogenizer, and
216 centrifugation at 500 g for 10 min to remove nuclear and cell debris. The mitochondrial fraction
217 was pelleted by centrifugation at 9000 g for 20 min. The resulting supernatant contained the
218 cytosolic fraction. The pellet was yet washed twice with buffer A followed by another
219 centrifugation at 9000 g for 20 min. Mitochondria were resuspended and disrupted in buffer A
220 prepared without mannitol and sucrose. Activity for ALT and AST were determined with a
221 commercial kit (Cromatest, Linear Chemicals, Spain). All enzymatic assays were carried out at
222 30°C and followed at 340 nm. The total protein content in liver crude extracts was determined
223 by the Bradford method (Bio-Rad, Spain) at 30 °C using bovine serum albumin as a standard
224 and followed at 600 nm. All assays were adapted for automated measurement using a Cobas
225 Mira S spectrophotometric analyzer (Hoffman-La Roche, Switzerland). Enzyme activities were
226 expressed per mg of soluble protein (specific activity). One unit of enzyme activity was defined
227 as the amount of enzyme necessary to transform 1 μmol of substrate per min.

228

229 2.6. Statistical analysis

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

235

236 3. Results

237

238 Blood glucose and hepatic glycogen levels (data not shown) plummeted after fasting and the
239 refeeding period allowed full recovery to levels found in fed fish (blood glucose in mM:
240 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
241 fed / fasted / refeed fish, respectively) as previously reported (Viegas et al., 2013).

242 A 599 bp cDNA fragment encoding cALT was isolated from the liver of *D. labrax* by RT-PCR.
243 The nucleotide sequence shared an 89% identity with *S. aurata* cALT messenger (GenBank
244 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**

280

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

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

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

350 supported mainly via the gluconeogenic or so-called "indirect" pathway (Viegas et al., 2012).
351 These observations are consistent with a reduced role of glycolysis and the weak correlation of
352 hepatic PK and PFK-1 expression and activities with the fasting to feeding transition.
353 The decreased hepatic activity level of *D. labrax* G6PDH and 6PGDH after fasting was
354 consistent with observations in other species (Metón et al., 2003; Morales et al., 2004; Pérez-
355 Jiménez et al., 2012) reflecting the downregulation of biosynthetic and growth activities and its
356 corresponding reduction in demand for NADPH and nucleotide precursors. However, as
357 previously reported for G6PDH activity in fasted fish (Furné et al., 2012; Ibarz et al., 2007;
358 Sangiao-Alvarellos et al., 2005), we observed that G6PDH mRNA levels remained unchanged
359 in every condition possibly indicating an active role in cellular equilibrium, for example, on
360 maintaining the redox state of the hepatocytes (Morales et al., 2004). The decrease in cALT
361 activity after refeeding could lead to increased alanine which has been proven to modulate the
362 oxidative part of the PPP, significantly increasing G6PDH activity in primary cultures of
363 Atlantic salmon *Salmo salar* hepatocytes (Sandén et al. 2003). However the lack of rebound of
364 G6PDH and 6PGDH after refeeding with a high-protein diet supports the role of the PPP as
365 NADPH-generating system rather than a component of nutrient conversion into lipids (Barroso
366 et al., 1998). A study with 2-day fasted *O. mykiss*, where refeeding was followed through 8
367 time-points within 24 h after meal seemed to support this assumption (Mennigen et al., 2012).
368 Despite the significant increase observed in the expression of various genes involved in hepatic
369 lipid metabolism (e.g. SREBP1c, FAS, and ACLY), G6PDH expression remained unaltered.
370 Interestingly, after refeeding seabass with a high-protein diet (49% crude protein), Pérez-
371 Jiménez et al. (2007) reported full recovery of G6PDH activity in after 1 d, something that was
372 not observed using a lower protein diet (41% crude protein; same study) or with the diet used in
373 the present work (44% crude protein) even after 3 days of refeeding. Although different
374 protein/lipid ratios may explain such discrepancy, the mRNA levels confirmed to some extent
375 the obtained activities suggesting that *de novo* lipogenesis occurs in a later phase of the recovery.
376 In this study we report for the first time partial sequences and nutritional variations on mRNA
377 levels for cALT, mALT, PK, G6PDH and 6PGDH in seabass. This enabled a more
378 comprehensive analysis of how this species copes with food deprivation and consequent
379 refeeding. In conclusion, the counterbalance between cALT and PK during fasting, both at
380 activity and mRNA level, ensure a steady supply of pyruvate to the TCA cycle. cAST also
381 participated by increasing its activity during fasting and supplying metabolic intermediates for
382 gluconeogenesis. After a 3-day refeeding period rather than rebooting lipogenesis and enhancing
383 downstream glycolysis, the liver prioritizes the production of glucose 6-phosphate (via GK) for
384 glycogen replenishment (Viegas et al., 2013). At this point, the fasting-refeeding transition in

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

388

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396

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524

525 **Figure captions**

526
527 **Fig. 1** Effects of feeding, fasting (21 days), and refeeding (21 days fasting, 3 days refeeding) on
528 mRNA levels (grey bars; in arbitrary units) and specific activity (black bars; in mU mg^{-1}
529 protein) of cALT (left) and mALT (right) in liver of *D. labrax*. Mean values \pm S.D. ($n = 6$) are
530 presented. Significant differences between conditions are indicated by different lower case
531 letters for mRNA levels and by different upper case letters for activity (one-way ANOVA
532 followed by Tukey's test).

533
534 **Fig. 2** Effects of feeding, fasting (21 days), and refeeding (21 days fasting, 3 days refeeding) on
535 specific activity (black bars; in mU mg^{-1} protein) of cAST (left) and mAST (right) in liver of *D.*
536 *labrax*. Mean values \pm S.D. ($n = 6$) are presented. Significant differences between conditions are
537 indicated by different letters (one-way ANOVA followed by Tukey's test).

538
539 **Fig. 3** Effects of feeding, fasting (21 days), and refeeding (21 days fasting, 3 days refeeding) on
540 mRNA levels (grey bars; in arbitrary units) and specific activity (black bars; in mU mg^{-1}
541 protein) of PK (left) and PFK-1 (right - specific activity only) in liver of *D. labrax*. Mean values
542 \pm S.D. ($n = 6$) are presented. Significant differences between conditions are indicated by
543 different lower case letters for mRNA levels and by different upper case letters for activity (one-
544 way ANOVA followed by Tukey's test).

545
546 **Fig. 4** Effects of feeding, food deprivation (21 days), and refeeding (21 days fasting, 3 days
547 refeeding) on mRNA levels (grey bars; in arbitrary units) and specific activity (black bars; in
548 mU mg^{-1} protein) of G6PDH (left) and 6PGDH (right) in liver of *D. labrax*. Mean values \pm S.D.
549 ($n = 6$) are presented. Significant differences between conditions are indicated by different
550 lower case letters for mRNA levels and by different upper case letters for activity (one-way
551 ANOVA followed by Tukey's test).

552

553 **Table 1**

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

555

Gene	RT-PCR		Expected band extension (bp)
		Primer sequence (5'-3')	
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	

556

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

558

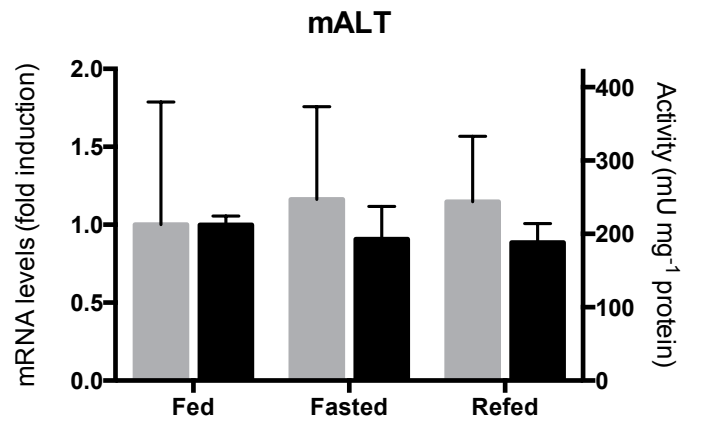
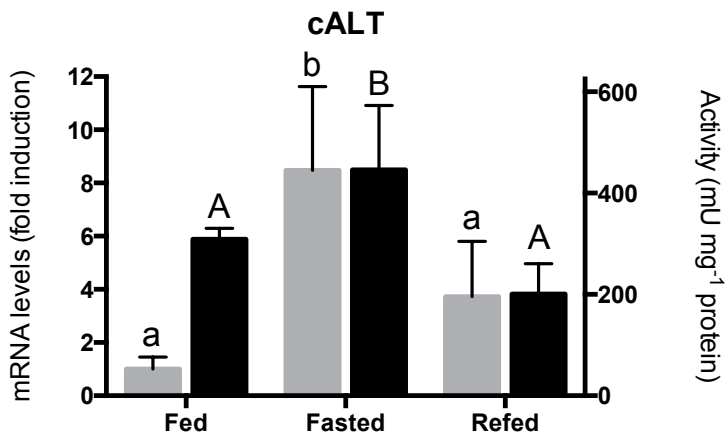
559 **Table 2**

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.

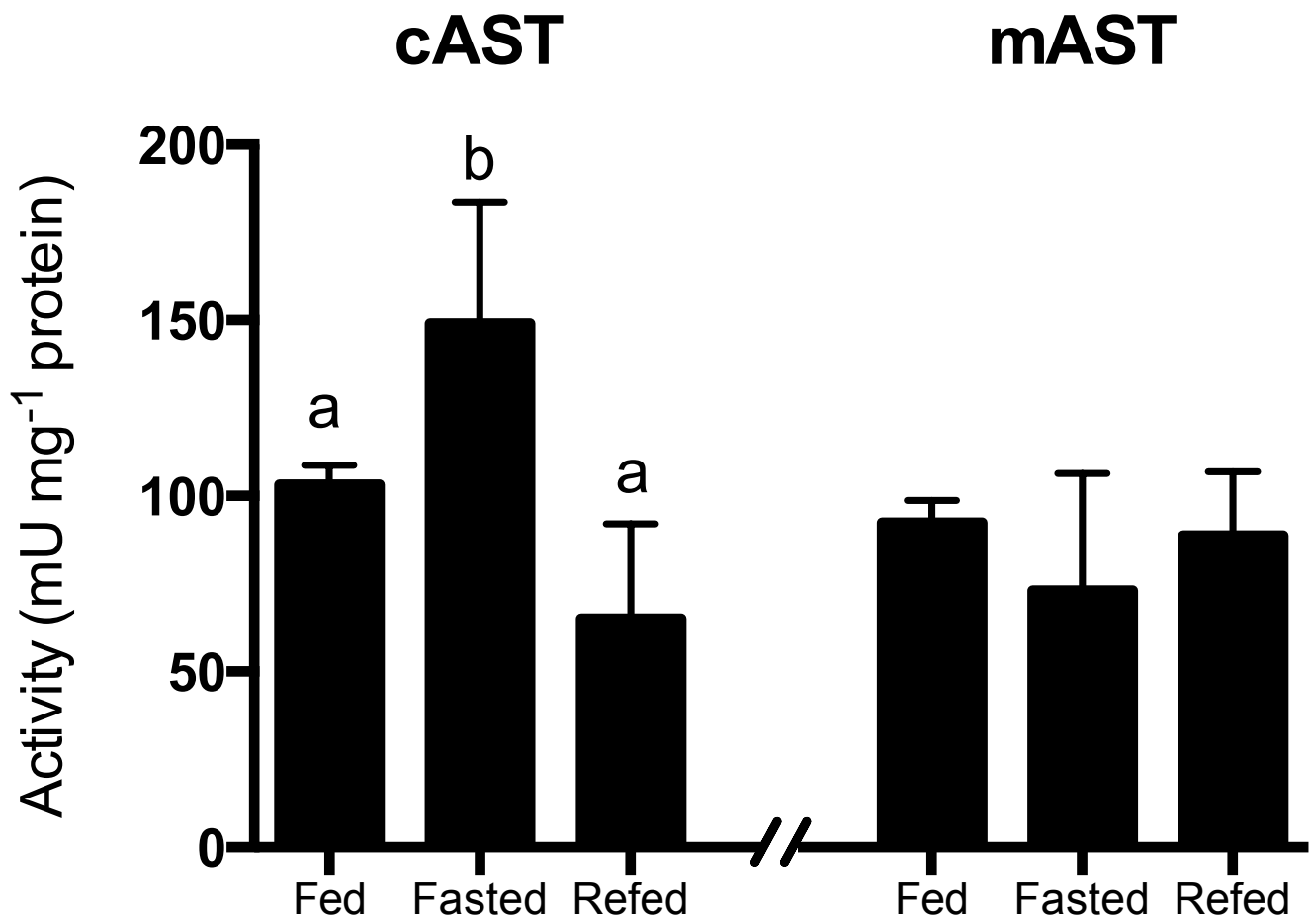
562

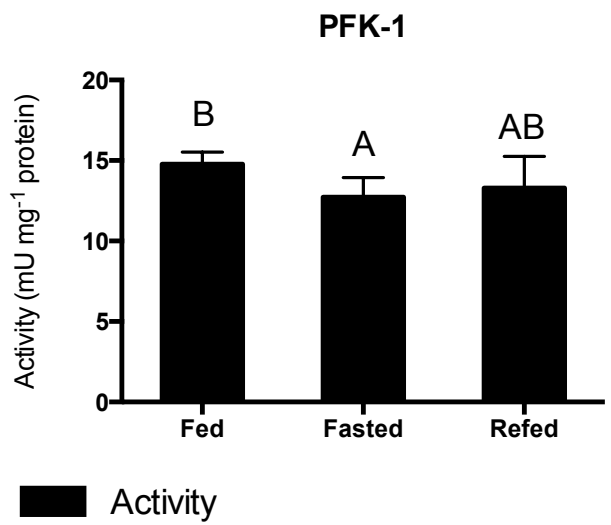
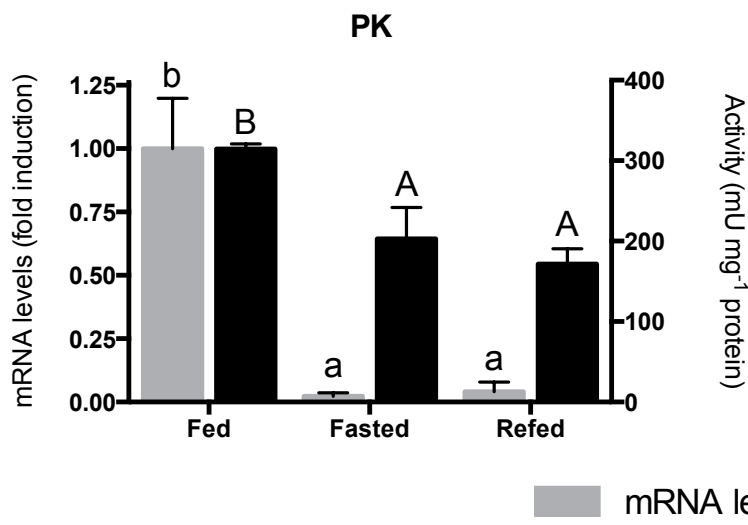
Gene		Quantitative real time RT-PCR			GenBank accession no.
		Primer sequence (5'–3')	Length of sequence (bp)	Length of amplified fragment (bp)	
Housekeeping gene					
18s	Forward	ACGGACGAAAGCGAAAGCA	406	91	<u>AM419038</u>
	Reverse	GGAACTACGACGGTATCTGATC			
Amino acid metabolism					
cALT	Forward	TGAAGGAGGGGGTCAAGAAA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

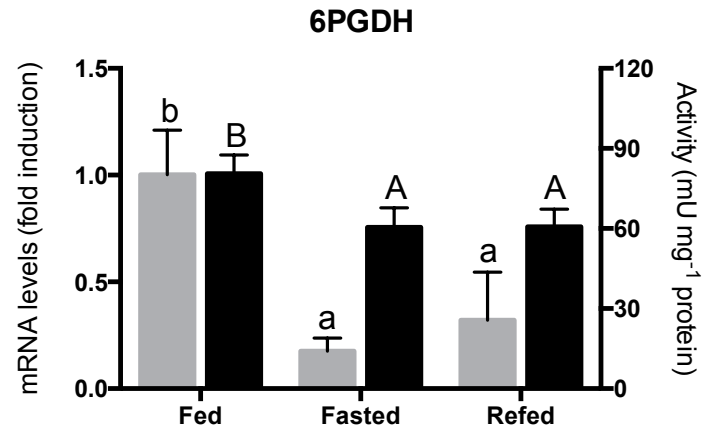
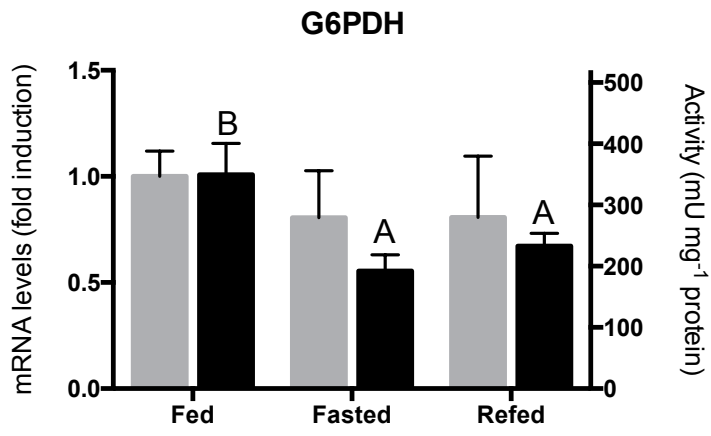
563



■ mRNA levels ■ Activity







■ mRNA levels ■ Activity