Capítulo 5

Starving/re-feeding processes induces metabolic modifications in thick-lipped grey mullet (*Chelon labrosus*, Risso 1827)

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ABSTRACT

The effects of starvation and re-feeding on metabolites and tissue composition, GH/IGF-I axis, and digestive enzyme activities in juvenile thick-lipped grey mullet (*Chelon labrosus*) were evaluated. Fish were divided into three feeding groups (n= 72, 82.00 ± 4.09 g initial body mass). The control group was fed 1 % of their body mass once a day throughout the experiment with commercial pellets. The other two groups were deprived of feed for 21 days (starved), or re-fed during 7 days after 14 days of food deprivation (re-fed). Full-length cDNAs from pituitary GH and hepatic IGF-I were cloned by screening a cDNA library or by PCR techniques. Furthermore, changes in their mRNA expressions were assessed by real time PCR in specimens maintained under the different feeding patterns. Results showed a negative growth in starved and re-feeding groups. Starvation induced a significant increase in plasma triglycerides as well as a decrease in liver glucose and glycogen. Re-feeding increased plasma glucose, lactate and protein, as well as liver glucose and glycogen. In addition, starvation significantly increased pituitary GH expression, while re-feeding down-regulated it. No significant changes were observed in hepatic IGF-I expression in any dietary treatment. Digestive enzyme activities were not significantly affected either by starvation or by re-feeding. The results of the present work suggest that juveniles of the thick-lipped grey mullet may easily adjust their metabolism under situations characterized by a short-term starvation and re-feeding.

Key words: *Chelon labrosus*, food deprivation, metabolism, GH/IGF-I system

1. INTRODUCTION

Many fish species are routinely affected in the wild by periods of starvation of a variable length (Pérez-Jimenez et al., 2007; Furné et al., 2009; Bayir et al., 2011). This situation has clear effects on their metabolism, being reflected in different responses mainly related to the type of substrates used to provide energy, the growth rates observed, and in the functionality of their digestive system (Jobling, 2001). Re-feeding after starvation may also induce different responses compared to fasting or continual feeding situations, since recovery from food deprivation depends on several factors such as the species, environmental conditions, length of the period of food deprivation, and previous feeding history (Navarro & Gutiérrez, 1995; McCue, 2010). In general, during re-feeding fish show a fast weight recovery, known as compensatory growth, and a rapid restoration of the initial metabolic profile (Metón et al., 2003; Morales et al., 2004). Different metabolic strategies to provide energy during fasting periods have been described in fish. In some species, glycogen is the first energy store mobilized in liver to face food deprivation (Figueiredo-Garutti et al., 2002; Metón et al., 2003). Some others maintain the hepatic glycogen reserve by means of gluconeogenesis using amino acids mobilized from skeletal muscle (Navarro & Gutiérrez, 1995; Echevarría et al., 1997; Metón et al., 2003).

Growth Hormone (GH) and Insulin-like Growth Factor-I (IGF-I) appear to mediate many of the key processes required for normal tissue growth and repair. In addition, GH and IGF-I promote tissue amino acid uptake, enhance protein synthesis and/or decrease protein degradation, and thus facilitate tissue anabolic processes (Estivariz & Ziegler, 1997). It seems that fish are able to cope with long periods of nutrient restriction that may occur in the wild partly by modulating the expression of a variety of genes associated with the GH/IGF-I system (Wood et al., 2005; Gabillard et al., 2006). When food supplies are restricted, energy is diverted away from growth and storage to support other essential physiological processes related to maintenance and conservation of the basal metabolism. Interactions between these hormones during different nutritional states have been reported in several teleosts (Small & Peterson, 2005; Gabillard et al., 2006). In several fish, starvation decreases hepatic IGF-I expression while re-feeding increases it, suggesting that nutritional status regulates IGF-I production (Moriyama, et al, 2000).

Cortisol is an important glucocorticoid hormone that affects several metabolic processes. Starvation is considered a chronic stress condition that elevates plasma cortisol levels (Wendelaar Bonga, 1997, Mommsen et al., 1999). However, its role during starving/re-feeding is not clear, since the responses seem to be species-dependent (Wunderink et al., 2012).

In addition, when facing starvation or poor food quality, fish may reduce the function of their gastrointestinal tract to conserve energy. The activity of digestive enzymes is closely connected to the availability of essential nutrients providing energy (Johnston et al., 2004). Some fish species submitted to starvation showed a decrease in the activity of trypsin and lipase, which were recuperated after re-feeding (Krogdahl & Bakke-McKellep, 2005). However, other species showed a different effect after a brief starvation, with an increase in enzymatic activities in the digestive tract (Gildberg, 2004). It follows that the analysis of these activities may be an easy and reliable method that can be used as indicator of the nutritional condition in a given species of fish (Hidalgo et al., 1999; Sunde et al., 2004; Cara et al., 2007).

Herbivorous species have been described as continuous feeders, while piscivorous fish may suffer periods of starvation, thus they need a rapid metabolic and digestive adaptation to support this situation (Pérez-Jiménez et al., 2007; Kelli et al., 2011; Wunderink et al., 2012). Thick-lipped grey mullet (*Chelon labrosus*, Risso, 1827) is a marine teleost of the family Mugilidae, very common in the estuaries and natural earthen ponds of the Bay of Cadiz (Spain) (Drake & Arias, 1984). Thick-lipped grey mullet culture is often associated to the culture of other fish species, like sea bream and sea bass, in the salt marshes and earthen ponds from Southern Spain. Consequently, the intensive production of *C. labrosus* could be a good option for diversification in aquaculture in this area and in others of the Mediterranean Sea (Calderer-Reig, 1993). This euryhaline species is characterized by amphidromic life cycles, where fry enter estuarine areas for growth and feeding, and adults return to the sea for reproduction (Crosetti & Cataudella, 1995; Cardona, 2006). During these migrations, *C. labrosus* can suffer some periods of starving and recovering after feeding. In addition, mullets have been described as omnivorous species in the early stages and they tend to become herbivorous over time (Wassef et al., 2001). *C. labrosus* also changes its feeding habits when increases the size, being zooplanktophagus in early stages, and phytobentonic in adult stages (Albertini-Berhaut, 1973; Castel, 1985; Tosi & Torricelli, 1988). It is

known that herbivorous species are continuous feeders, while carnivorous are less restricted to food availability, with a rapid metabolic adaptation to long periods of starvation (Uchida et al., 2003; Pérez-Jiménez et al., 2007).

The aim of this study was to assess the physiological changes in *C. labrosus* when facing a short period of starvation and subsequent re-feeding using a multi-factorial approach comprising the evaluation of changes in different metabolites and enzymes involved in energy supply pathways, expression of GH and IGF-I and activity of some digestive enzymes. From an applied point of view, a better knowledge on those aspects may result in the formulation of better diets and feeding patterns for this species.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

Juvenile thick-lipped grey mullet *C. labrosus*, Risso 1827 ($n = 72$, 82.00 ± 4.09 g initial body mass) were kindly provided by IFAPA "El Toruño" (El Puerto de Santa Maria, Cádiz, Spain) and transferred to the wet laboratories of *Servicios Centrales de Investigación de Cultivos Marinos* (SC-ICM) (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Operational Code REGA ES11028000312), where they were acclimated for 15 days to 38 ppt salinity ($1,049 \text{ mOsm} \cdot \text{kg}^{-1} \text{ H}_2\text{O}$ osmolality) in 5 m^3 -tank in a flow-through system. Three experimental groups (control, starved and re-fed) were randomly distributed in three 1 m^3 -tanks and maintained in a flow-through system circuit under natural conditions of water temperature ($18 \pm 1 \text{ }^\circ\text{C}$), salinity (40 ppt) and photoperiod (June 2011) for the latitude ($36^\circ 31' 44'' \text{ N}$) for 21 days. Control group was fed once a day with a ration of 1 % of their body weight with commercial pellets (Élite-Skretting). Starved fish were not fed during the trial. At day 14, the re-feeding group was re-fed and maintained as described for the control group. No mortality was observed in any of the groups during experimentation. The duration of the experiment was similar to that used in analogous studies dealing with food deprivation in other teleost fish as in *Dicentrarchus labrax* (Pérez-Jiménez et al., 2007), *Oreochromis mossambicus* (Fox et al., 2010), or *Solea senegalensis* (Wunderink et al., 2012), In addition, previous studies performed by our research group with this species showed that a two weeks period of starvation is enough to produce stress, confirmed by changes in several endocrine and metabolic indicators, whereas shorter periods did not produce this effect (unpublished data).

All experimental procedures complied with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD53/2013 and law 32/2007) for the use of laboratory animals.

2.2. Sampling

On day 0 (control time 0), as well as at days 14 and 21, twelve fish from each experimental group were anaesthetized with 2-phenoxyethanol ($0.3 \text{ mL} \cdot \text{L}^{-1}$), weighed, measured and sampled. All the groups were fasted for 24 hours before each sampling point. Blood was obtained from the caudal vein using ammonium-heparinized syringes (25,000 units of ammonium heparin Sigma H6279; 3 mL saline solution 0.6 % NaCl). Plasma was obtained after blood centrifugation (3 min using ALC micro centrifuge 4204 at $\approx 13,000 \times g$) and stored at -80°C until subsequent analysis. For mRNA expression (qPCR) analysis, the pituitary gland and several biopsies from the liver of each animal were preserved in 500 % (w/v) RNAlater® (Ambion, LifeTechnologies), kept overnight at 4°C and then stored at -20°C until being processed for RNA extraction. Moreover, the complete liver from each specimen was extracted and weighed to calculate the hepatosomatic index (HSI), immediately frozen in liquid nitrogen and stored at -80°C . Frozen liver samples were divided into two parts to assess both enzymatic activities and levels of different metabolites. The tissues used for the assessment of metabolites were finely minced on an ice-cold Petri dish and subsequently homogenized by mechanical disruption (Ultra-Turrax, T 25 basic, IKA®-WERKE) with 7.5 vol. (w/v) of ice-cooled 0.6 N perchloric acid and neutralized after the addition of the same volume of 1 M KHCO_3 . The tissues used for the assessment of enzyme activities were homogenized by mechanical disruption (Ultra-Turrax, T 25 basic, IKA®-Werke) in 10 vol. of ice-cold stopping-buffer containing 50 mM imidazole-HCl (pH 8.5), 1 mM 2-mercaptoethanol, 50 mM NaF, 4 mM EDTA, 250 mM sucrose, and 0.5 mM p-methyl-sulphonylfluoride (Sigma Chemical Co., St. Louis, MO, USA), the latter added as dry crystals immediately before homogenization. The homogenates were centrifuged (30 min, $13,000 \text{ g}$, 4°C , Eppendorf 5415R) and the supernatants were stored in different aliquots at -80°C until use in metabolite or enzymatic assays.

For digestive enzymatic activities, the complete digestive systems of fish were obtained by dissection, immediately frozen in liquid nitrogen and stored at -80°C until processing. Before analysis, the digestive system from each fish was thawed and the

stomach and intestine were individually separated. Samples were homogenized in distilled water (1:5) and centrifuged (12,000 g, Eppendorf 5415 R) at 4 °C for 15 min. Supernatants were used to measure pepsin, α -amylase and trypsin activities. Concentration of soluble protein in samples was determined by Bradford method using bovine serum albumin ($1 \text{ mg} \cdot \text{mL}^{-1}$) as a standard.

2.3. Assay of metabolites in plasma and liver

Plasma glucose, lactate and triglycerides were measured using commercial kits (Spinreact SA, Sant Esteve d'en Bas, Girona, Spain: Glucose HK Ref 1001200; Lactate Ref 1001330; Triglycerides Ref 1001311). Plasma protein was determined using the bicinchoninic acid method with BCA protein kit (Pierce, Rockford, USA) for microplates, with bovine serum albumin as standard. Those assays were adapted to 96-well microplates and run on a PowerWaveTM 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft[®] Windows XP. In all protocols involving commercial kits cited here and elsewhere in this study, the manufacturer's instructions were followed, except where noted.

Plasma cortisol levels were measured by Enzyme Immune-Assay (EIA) using microtiter plates (MaxiSorpTM, Nunc, Roskilde, Denmark) as previously described for testosterone (Rodriguez et al., 2000; Martos-Sitcha et al., 2014). Steroids were extracted from 5 μL plasma in 100 μL RB (10 % v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01 % w/v NaN_3 , 2.34 % w/v NaCl, 0.037 % w/v EDTA, 0.1 % w/v BSA (Bovine Serum Albumin)) and 1.2 mL methanol (Panreac) and evaporated during 48-72 hours at 37 °C. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #4000002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate. Standard curve was run from $2.5 \text{ ng} \cdot \text{mL}^{-1}$ to $9.77 \text{ pg} \cdot \text{mL}^{-1}$ ($R^2 = 0.996$). The lower limit of detection (98.29 % of binding, ED_{92.87}) was 2 pg/mL. The percentage of recovery was 95 %. The inter- and intra-assay coefficients of variation (calculated from the sample duplicates) were $1.02 \pm 0.35 \%$ and $4.12 \pm 0.27 \%$, respectively. Cross-reactivity for specific antibody with intermediate products involved in steroids synthesis was given by the supplier (cortexolone (1.6 %),

11-deoxycorticosterone (0.23 %), 17-hydroxyprogesterone (0.23 %), cortisol glucurinoide (0.15 %), corticosterone (0.14 %), cortisone (0.13 %), androstenedione (<0.01 %), 17-hydroxypregnenolone (<0.01 %), testosterone (<0.01 %)).

Tissue lactate and triglyceride levels were determined spectrophotometrically in liver samples using the same commercial kits as described for plasma. The amount of tissue glycogen was measured using the method described by Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with the commercial kit described above (Spinreact).

Enzymatic activities (HK: hexokinase; G6PDH: glucose-6-phosphate dehydrogenase; FBPase: fructose-2, 6-biphosphatase; GDH: glutamate dehydrogenase; G3PDH: glycerol-3-phosphate dehydrogenase; LDH: lactate dehydrogenase; PK: pyruvate kinase; GPase: glycogen phosphorylase; GPT: glutamic pyruvate transaminase; and GOT: glutamic oxaloacetic transaminase) were assessed. Specific conditions for the enzymes were previously described by Laiz-Carrión et al. (2002) and Sangiao-Alvarellos et al. (2003). Spectrophotometric determinations were performed with a PowerWave™ 340 microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft® Windows XP.

2.4. Expression of GH and IGF-I

Cloning of partial GH and IGF-I cDNAs from C. labrosus

Firstly, a set of degenerate primers (Invitrogen™, Life Technologies; Table 12) was designed according to the most highly conserved sequences of cDNAs between different species of teleosts for GH, *Mugil platanus* (GenBank accession. no. [AY775148](#)), *Acanthopagrus schlegelii* (GenBank accession. no. [AY714371](#)); *Cyprinus carpio* (GenBank accession no. [M27000](#)) and *Sparus aurata* (GenBank accession no. [S54890](#)); *A. schlegelii* (GenBank accession no. [AF030573](#)), and for IGF-I *Ictalurus punctatus* (GenBank accession no. [AY353852](#)), *Oreochromis mossambicus* (GenBank accession no. [AF033797](#)) and *Mugil cephalus* (GenBank accession. no. [AY427954](#)). Total RNA was prepared from complete digestive system (≈ 1 g) or brain of *C. labrosus* in each case using the NuceloSpin® RNA II kit (Macherey-Nagel). Samples were homogenized in a volume of RA1 buffer with β -mercaptoethanol 1 % v/v (Sigma)

proportional to the tissue mass, using an Ultra-Turrax® T25 (IKA®-Werke), and on-column RNase-free DNase digestion was performed according to the manufacturer's protocol. At this point and further, RNA quality was checked in a Bioanalyzer 2100 with the RNA 6000 Nano kit, whereas RNA quantity was measured spectrophotometrically at 260 nm in a BioPhotometer Plus (Eppendorf). Total RNA (2 µg) were reverse-transcribed in a 20 µL reaction using 250 ng random primers (Invitrogen LifeTechnologies) and 200 U Superscript III reverse transcriptase (Invitrogen LifeTechnologies), with the manufacturer's first strand buffer (1x final concentration), DTT (5 mM final concentration) and dNTPs (0.5 mM final concentration) at 25 °C for 5 min, 50 °C for 60 min, and 70 °C for 15 min. PCR reaction was performed with 1 U Platinum Taq DNA polymerase (Invitrogen LifeTechnologies) with the first strand cDNA (corresponding to 100 ng of input total RNA), manufacturer's PCR buffer (1x final concentration), 200 nM each sense and antisense primers, 200 µM dNTPs mixture, and 1.5 mM MgCl₂ in a total volume of 20 µL. Samples were cycled at 94 °C for 1 min, followed by 35 cycles at [94 °C for 30 s, 50-60 °C gradient for 30 s, and 72 °C for 1 min], and a final step at 72 °C for 10 min, in a Mastercycler® proS vapoprotect (Eppendorf). PCR products were identified in a 1.5 % agarose gel electrophoresis and ligated with the TOPO TA Cloning® Kit for Sequencing (Invitrogen™, Life Technologies) into the pCR®4 TOPO® vector and were sequenced by the dideoxy method in a Biotechnology company (Newbiotechnique S.A., Seville, Spain). PCR products had a nucleotide identity of 100 % with IGF-I from *M. cephalus* (GenBank accession no. [AY427954](#)), *Epinephelus lanceolatus* (GenBank accession no. [EU280323](#)), *Solea senegalensis* (GenBank accession no. [AB248825](#)) and 99 % with GH from *M. platanus* (GenBank accession no. [AY775148](#)) and *Oreochromis niloticus* (GenBank accession no. [M26916](#)).

For the preparation of the IGF-I probe, around 2 µg of the plasmid DNA containing the partial IGF-I sequence was digested with 10 U of *EcoRI* (Takara) in a volume of 40 µL, the digestion product was separated on an 1 % agarose gel and the band of about 400 pb was excised and purified with the QIAquick kit (Qiagen). The cDNA fragment was diluted till a final concentration of 25 ng µL⁻¹ in TE buffer (10 mM Tris.HCl, 1 mM EDTA Na₂, pH 8.0) and stored at -20 °C afterwards.

Construction and screening of a gastro-intestinal tract (GIT) cDNA library and cloning of IGF-I full length cDNA

Total RNA was prepared from 1 g of *C. labrosus* fresh gastrointestinal tract containing liver, spleen and pancreas using an Ultra-Turrax® T25 (IKA®-Werke) and the RNeasy maxi kit (Qiagen), while mRNA was isolated from total RNA using the Oligotex Midi Kit (Qiagen). The digestive cDNA library was constructed using the lambda ZAP-cDNA/Gigapack III Kit (Stratagene, Agilent Technologies; product already discontinued), with few modifications to the manufacturer's protocol, consisting in the use of the Agilent 2100 Bioanalyzer (Agilent Technologies) instead of labelling cDNAs with ^{32}P , avoiding the agarose and polyacrilamide gel electrophoresis, or the use of the ethidium bromide plate assay for the final quantification of the cDNA fractions.

Approximately 250,000 pfu from *C. labrosus* digestive library were plated in a 240 x 240 mm NZY agar plates (Nunc), and transferred onto a Hybond-N Nylon membrane (GE LifeSciences). Blots were prehybridized for 1 h at 42 °C in prehybridization buffer (50 % formamide, 6x SSPE, 5 % SDS 10x, 5x Denhart's solution, 0.1 mg/mL yeast RNA type III). For hybridization, 25 ng of partial IGF-I was radiolabeled using the RadPrime DNA Labeling System (Invitrogen, LifeTechnologies) and [$\alpha^{32}\text{P}$] dCTP (PerkinElmer), and allowed to hybridize with the Nylon membranes overnight at 42 °C. Blots were washed twice in 2X SSC-0.1 % SDS for 30 min each at room temperature, twice in 1X SSC-0.1 % SDS for 30 min at 42 °C, and twice in 0.5X SSC-0.1 % SDS for 30 min at 60 °C, till background was very low. Membranes were exposed to autoradiography film (Amershan, GE LifeSciences) for two days with intensifying screens at -80 °C. Positive plaques were isolated and subjected to further two round of hybridization/isolation. After the third round of the screening, several putative clones from the digestive library were isolated and excised *in vivo* excision using *E. coli* XL1-Blue MRF' and SOLR strains (Stratagene, Agilent Technologies LifeSciences). Then, positive colonies from each clone were picked up and plasmid DNA prepared in a mini-prep column (GenElute™ Five-Minute Miniprep Kit; SIGMA®). Plasmids were double digested by *Eco*RI and *Xho*I (Takara), and the products were revealed in a 1 % agarose gel stained GelRed™ (Biotium). Positive clones were sequenced as above. Final sequences were compiled and analysed with eBiox software (v 1.5.1).

Obtaining GH 5' and 3' ends by Rapid Amplification of cDNA ends (RACE)

Using total RNA as template, the 5' and 3' ends of GH mRNA was amplified using 5' and 3' Rapid Amplification of cDNA Ends (FirstChoice® RLM-RACE kit, Life Technologies™). Specific forward primers were designed in the fragment previously cloned (see above) at two different positions (Table 12) and used in combination with the 3'RACE Outer or Inner primers supplied in the kit to amplify the 3' ends. For 5' RACE amplifications, specific reverse primers for each fragment were also designed (Table 12) and used in combination with the 5'RACE Outer or Inner primers supplied in the kit. Primers were designed to achieve an overlap of at least 150 bp between RACE clones and previously obtained partial cDNA. Cloning and sequencing of PCR products were performed as described above, and eBiox (v1.5.1) software was used for fragment assemblage and analysis. Homology analysis of putative protein sequence was run and compared with other teleost sequences using BLAST tools at the NCBI website (<http://www.ncbi.nlm.nih.gov>).

Table 12. Degenerate and specific primers designed for molecular identification of partial cDNA GH and IGF-I sequences, and for semi-quantitative expression by QPCR. The size of each pair of amplified primers is presented.

Primer	Direction	Sequence (5'-3')	Amplicon length
IGF-I	Forward 1	GCTCTCTCCTTTCAGTGGCA	490 bp
	Reverse 1	TGAACGATGAATGACTATGT	
	Forward Nested	ATGTGCTGTATCTCCTCTAG	399 bp
	Reverse Nested	TGTCTTGTCCGGCTGCTG	
GH-3' RACE	Forward 1	CCAGGATTTCTGCAACTCTGA	538 bp
	Forward 2	GAAGCTGCTGCGTATCTCCTA	466 bp
GH-5' RACE	Reverse 5	TAGGAGATACGCAGCAGCTTC	362 bp
	Reverse 6	TCAGAGTTGCAGAAATCCTGG	290 bp
qPCR-IGF-I	Forward	CTAAATCCGTCTCCTGTTTCGC	128 bp
	Reverse	GAAGTCATTAAAAACGGGGAGA	
qPCR-GH	Forward	CGTTATCTGTCCGGAGGTTCT	178 bp
	Reverse	AGGTTTCGCCTCAGTGACTCTT	
qPCR-β-ACT	Forward	CAGGGAGAAGATGACCCAGA	163 bp
	Reverse	GAGCGTAGCCCTCGTAGATG	

Analysis of GH and IGF-I mRNA levels by qPCR

In liver, total RNA was isolated using an Ultra-Turrax ® T8 (IKA®-Werke) from 20 mg of tissue using the NucleoSpin®RNA II kit (Macherey-Nagel), while in

pituitary total RNA was isolated from the complete gland using the NucleoSpin RNA XS kit (Macherey-Nagel). Only RNA samples with a RNA integrity number (RIN) higher than 8.0 were used for expression quantification.

Total RNA (500 ng for liver and 100 ng for pituitary) was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta Bioscience). In short, each reaction was performed using qScript Reaction Mix (1x final concentration) and qScript Reverse Transcriptase (2.5x final concentration). The reverse transcription program consisted in 5 min at 22 °C, 30 min at 42 °C and 5 min at 85 °C. Optimization of qPCR conditions was made on primers annealing temperature (50 to 60 °C), primers concentration (100 nM, 200 nM and 400 nM) and template concentration (five 1:10 step dilutions from 10 ng to 1 pg of input ARN). The resulting curves had amplification efficiencies and r^2 of 1.02 and 0.996 for GH, and of 0.99 and 0.999 for IGF-I, respectively. The primer sequences used for qPCR were designed with Primer3 software v. 0.4.0 (available in <http://frodo.wi.mit.edu/>) and were synthesized and HPLC purified by Invitrogen™ Life Technologies (Table 12). To confirm the correct amplification of GH and IGF-I, the obtained PCR amplicons were cloned and sequenced. qPCR was carried out with Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep *realplex* 2 S). Each reaction mixture (10 µL) contained 0.5 µL at 200 nM of each specific forward and reverse primers, and 5 µL of PerfeCTa SYBR® Green FastMix™ (Quanta BioSciences). Reactions were conducted in semi-skirted twin.tec real-time PCR plates 96 (Eppendorf) covered with adhesive Masterclear real-time PCR Film (Eppendorf). The PCR profile was as follows: 95 °C, 10 min; [95 °C, 30 s; 60 °C, 45 s] x 40 cycles; *melting curve* [60 °C to 95 °C, 20 min], 95 °C, 15s. The melting curve was used to ensure that a single product was amplified and to check for the absence of primer-dimer artefacts. Results were normalized to β -actin (acc. no. AY836368), owing its low variability (less than 0.3 C_T) under experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001).

2.5. Evaluation of the activity of digestive enzymes

Variations in the activity of pepsin, trypsin and amylase were used as indicators of possible changes in the nutritional status of the fish. Pepsin activity was determined by the method of Anson (1938): 20 µL of diluted enzyme sample into 1 mL of 0.5 %

acid-denatured bovine haemoglobin that had been extensively dialyzed against 0.1 M HCl-glycine buffer (pH 2.0). After incubation at 37 °C for 30 min, the reaction was terminated by adding 0.5 mL of 20 % trichloroacetic acid (TCA) and cooled at 4 °C during 15 min. Then, centrifuged at 8,000 g for 10 min. The absorbance of the resulting supernatant containing proteolytic digested small peptides was measured spectrophotometrically at 280 nm).

Trypsin activity was measured using 1.25 mM N-benzoyl-DL-arginine p-nitroanilide (BAPNA) in 200 mM Tris-HCl pH 8.5. Substrate stock solution (125 mM) was prepared in DMSO and brought to working concentration by diluting with buffer prior the assay. Ten microliters of enzyme extract were mixed with 200 µL of substrate and the liberation of p-nitroaniline was kinetically followed at 405 nm in a microplate reader ELx808 IU (BioTek). Assays were run in triplicate. The protein content of enzyme extracts was measured using BSA as standard. Trypsin activity was expressed as arbitrary units (Abs/min) per mL or per mg protein as needed.

Amylase activity was determined according to the Somogy-Nelson method using soluble starch (2 % w:v) as substrate, as described in Robyt & Whelan (1968). Briefly, 50 µL of enzyme extract and 125 µL of 100 mM phosphate-citrate pH 7.5 were incubate with 125 µL of starch for 30 min. Activity was measured by calculating the reducing sugars released at 600 nm.

2.6. Statistical analysis

Statistical differences related to feeding status and time were analysed by two-way ANOVA between control and starved groups, and one-way ANOVA at day 21 between all the groups. ANOVA analyses were followed by post-hoc comparison made with the Tukey's test. Significance was taken at $P < 0.05$. The information of all the metabolic variables was integrated using a cluster multivariate analysis, performed using group averages and standardized data. A hierarchical and agglomerative method was selected, and the number of clusters was not pre-established but randomly generated by the software. Values of all the metabolic parameters were considered as a single variable among data previously standardized in order to give the same relevance to all them in the analysis performed, independently of how different (in magnitude) were their values. Software packages GraphPad Prism[®] (v.6.0b), Statistica 10.0 Soft, Inc., and Statgraphics software v 4.0 were used in the analysis. Statistical parameters

(*P*-value and *F*) obtained from one-way and two-way ANOVA analyses in the experiments are provided in Table 13.

Table 13. Statistical parameters (*P*-value and *F*) obtained from two-way ANOVA analysis in fish maintained under different feeding situations (control and starving), and from one-way ANOVA analysis at the day 21 (control, starving and re-feeding). (A) Growth index, (B) plasma metabolites, (C) liver metabolites, and (D) digestive enzyme activities. In bold and italics, *P*-value showing significant differences in each case.

A	Time		Fed condition		Interaction	
Parameter	P-value	F	P-value	F	P-value	F
Growth	0.925	0.155	0.010	5.232	0.420	0.888
HSI	0.518	0.768	<0.001	21.391	0.634	0.459
B	Time		Fed condition		Interaction	
Parameter	P-value	F	P-value	F	P-value	F
Glucose	0.713	0.457	0.047	3.156	0.008	5.060
Lactate	0.115	2.030	0.052	3.056	0.865	0.144
Triglycerides	0.339	1.137	<0.001	8.669	0.037	3.411
Proteins	0.349	1.111	0.001	7.001	0.922	0.081
C	Time		Fed condition		Interaction	
Parameter	P-value	F	P-value	F	P-value	F
Glucose	<0.001	7.958	0.146	2.013	<0.001	10.356
Glycogen	0.073	2.506	<0.001	30.599	0.743	0.298
Triglycerides	<0.001	7.523	0.033	3.714	<0.001	10.158
D	Time		Fed condition		Interaction	
Parameter	P-value	F	P-value	F	P-value	F
Pepsin	0.279	1.361	0.449	0.833	0.756	0.098
Trypsin	0.840	0.175	0.067	3.058	0.663	0.194
Amylase	0.048	3.369	0.003	6.789	0.068	3.596

3. RESULTS

No mortality was observed during the experimental period. Zoometric indicators (weight and hepatosomatic index; HSI) were assessed (Table 14). Fish submitted to starvation showed a significantly ($p < 0.05$) lower weight (63.78 ± 4.39) and HSI (0.55 ± 0.02) than the other groups while the re-fed group showed no significant differences when compared to the control.

Table 14. Influence of starving/re-feeding processes on growth and hepatosomatic index (HSI) of juvenile *C. labrosus* specimens along the experimental period. HIS: hepatosomatic index. Values are represented as mean $\pm$ S.E.M. ($n = 12$ fish per group). Values not sharing a common superscript are significantly different with $p < 0.05$. Lowercase letters refer to comparison within a group, capital letters between groups.

Parameters	Day	Control	Starving	Re-feeding
Weight (g)	0	81.8 ± 4.4^a		
	14	75.1 ± 2.0^a	72.3 ± 3.5^a	
	21	83.1 ± 6.1^{aA}	63.8 ± 4.4^{aB}	73.4 ± 3.6^{AB}
HSI (%)	0	0.81 ± 0.06^a		
	14	0.91 ± 0.04^{aA}	0.58 ± 0.03^{aB}	
	21	0.79 ± 0.05^{aA}	0.55 ± 0.02^{aB}	0.83 ± 0.10^A

3.1. Metabolites and tissue composition

Results showed a significant reduction of hepatic glycogen and triglycerides in the starved group, together with an increase of these latter in plasma (Table 15). Values of plasma glucose and proteins measured in the re-feeding group were significantly higher than those in the starved group, and similar to the values in the control group (Table 15). However, lactate values were significantly higher in re-feeding group but not similar to controls. Same results were obtained in hepatic glucose, glycogen and triglycerides in re-feeding group (Table 15).

Table 15. Effect of starving/re-feeding processes on plasma and liver metabolites of juvenile *C. labrosus* specimens along experimental period. Values are represented as mean $\pm$ S.E.M. ($n = 6$ fish per group). Values not sharing a common superscript are significantly different with $p < 0.05$. Lowercase letters refer to comparison within a group, capital letters between groups.

Parameters	Day	Control	Starving	Re-feeding
PLASMA				
Glucose (mM)	0	3.3 ± 0.1^a		
	14	3.0 ± 0.1^a	3.4 ± 0.1^a	
	21	3.7 ± 0.1^{aAB}	3.1 ± 0.1^{aB}	3.8 ± 0.3^A
Lactate (mM)	0	2.7 ± 0.2^a		
	14	3.5 ± 0.2^a	3.1 ± 0.3^a	
	21	3.4 ± 0.3^{aB}	3.6 ± 0.3^{aB}	5.6 ± 0.7^A
Triglycerides (mM)	0	1.2 ± 0.1^a		
	14	1.3 ± 0.0^a	1.4 ± 0.1^{ab}	
	21	1.1 ± 0.0^{aB}	2.0 ± 0.3^{bA}	1.1 ± 0.1^B
Proteins (mg/mL)	0	34.9 ± 1.0^a		
	14	35.0 ± 1.6^a	33.0 ± 1.0^a	
	21	35.2 ± 1.4^{aAB}	33.8 ± 1.0^{aB}	41.1 ± 2.9^A
LIVER				
Glucose (mg/U of tissue)	0	2.3 ± 0.5^a		
	14	1.5 ± 0.3^a	2.4 ± 0.4^a	
	21	2.4 ± 0.2^{aA}	1.7 ± 0.3^{aB}	2.6 ± 0.4^{AB}
Glycogen (mg/U of tissue)	0	51.1 ± 11.2^a		
	14	46.2 ± 9.9^{aA}	26.4 ± 7.8^{aB}	
	21	60.4 ± 4.1^{aA}	20.8 ± 5.3^{aB}	64.7 ± 11.9^{AB}
Triglycerides (mg/U of tissue)	0	2.3 ± 0.4^a		
	14	2.0 ± 0.1^a	2.5 ± 0.3^a	
	21	1.6 ± 0.2^a	1.3 ± 0.2^b	2.1 ± 0.4

The effect of starving in plasma cortisol levels was significantly evidenced only after 21 days (Figure 27).

Figure 27. Effect of starving/re-feeding processes on plasma cortisol of juvenile *C. labrosus* specimens along experimental period. Values are represented as mean $\pm$ S.E.M. ($n = 6$ fish per group). Values not sharing a common superscript are significantly different with $p < 0.05$. Lowercase letters refer to comparison within a group, capital letter between groups.

The effect of starving/re-feeding on the activities of hepatic enzymes period is detailed in Figures 28 and 29. No significant differences between experimental groups were observed for values of HK, PK, G6PDH, G3PDH, GDH, and GPT. On the other hand, activity of GPase was significantly lower and GOT significantly higher in starved fish when compared to the control group, while FBPase and LDH were significantly higher in control fish.

Figure 28. Box-and-Whisker plot analysis of enzymes involved in the (A) glycolysis (HK and PK), (B) glycogenolysis (Total GPase), and (C) glyconeogenesis (FBPase and LDH) after experimental period (U/mg protein). HK: hexokinase; PK: pyruvate kinase; T GPase: total glycogen phosphorylase; FBPase: fructose-2,6-biphosphatase; LDH: lactate dehydrogenase. Values are represented as mean $\pm$ S.E.M. ($n = 6$ fish per group). Values not sharing a common superscript are significantly different with $p < 0.05$.

Figure 29. Box-and-Whisker plot analysis of enzymes involved in the (A) glycerol route (G3PDH), (B) phosphate pentose pathway (G6PDH), and (C) amino acids metabolism (GDH, GPT and GOT) after experimental period (U/mg protein). G3PDH: glycerol-3-phosphate dehydrogenase; G6PDH: glucose-6-phosphate dehydrogenase; GDH: glutamate dehydrogenase; GPT: glutamic pyruvate transaminase; GOT: glutamic oxaloacetic transaminase. Values are represented as mean $\pm$ S.E.M. ($n = 6$ fish per group). Values not sharing a common superscript are significantly different with $p < 0.05$.

The multivariate cluster's analysis, performed considering all the enzymes as variables, showed the clear influence of feeding on hepatic parameters after 21 days of experimental treatment (Figure 30).

Figure 30. Cluster analysis considering all previous variables. Values are represented as mean $\pm$ S.E.M. ($n = 6$ fish per group). C0 and C21; control; RF: re-feeding after 21 days; and ST: starving after 21 days.

3.2. Description of GH and IGF-I sequences and mRNA expression in *C. labrosus*

Thick-lipped grey mullet full-length GH and IGF-I cDNA sequences consisted of 819 bp and 2,858 bp respectively (GenBank accession numbers **KC195966**, Figure 31; and **KC195967**, Figure 32, respectively).

5' ctatcagaacattagaactcctgctctcaggtcggactgagctcctccagaacc	54
M D K V V L L L S V V F	12
tgaatgaatcccacaccagcc catg gacaaagttgtttctcctgctgtcagtcgtgttt	111
L G V S P Q P I T D S Q R L F S I A V	31
ctcggcgtctccccctcagccaatcacagacagccagcgtctgtttctccatcgcagtc	168
S R V Q H L H L L A Q R L F T D F E S	50
agcaggggtccagcacctccacctgctcgcccagagactcttcaactgactttgagagc	225
S L Q T E E Q R Q L N K I F L Q D F C	69
tctctgcagacggaggagcagcgtcaactcaacaagatctttctccaggattttctgc	282
N S D Y I I S P I D K H E T Q R S S V	88
aactctgactacatcatcagccccatcgacaagcatgagacgcaacgcagttccgctc	339
L K L L R I S Y Q L V E S W E Y P S R	107
ctgaagctgctgctgtatctcctaccaactggctcgagtcctgggagtatcccagccgt	396
Y L S G G S A P R N Q I S M K L S E L	126
tatctgtccggagggttctgctccgaggaaccagattttcaatgaaactgtctgaaactg	453
K T G I L L L F R A N Q D A A E I F P	145
aagacgggaatcctgctgcttttcagggccaatcaggatgcagccgaaatcttccct	510
D D S A I Q L G T Y G N Y E E S L R R	164
gacgactctgccatccagctgggtacttacggaaactacgaagagtcactgaggcgga	567
T Y E L L A C F K K D M H K V E T Y L	183
acctacgaactgctggcatgcttcaagaaggacatgcacaaggtggagacatacctg	624
T V A K C R L S P E A N C T L	198
acggtggctaaatgtcggctctctccggaagcaaaactgcactctg tag ccccggacc	681
ctgatgatacgcgatcatgtgtgtgtgtcattcaagtattagcattagcatagccctgct	738
cgggtggtttgttggtggtttttctgatgatgtcatactgtcttaaagtgtgaaataaag	795
tgtgttgcatagaaaaaaaaaaaaa 3'	819

Figure 31. Nucleotide and deduced amino acid (in italic) sequences from *Chelon labrosus* GH cDNA. The signal peptide (61 aa) is underlined. The start and stop codons are represented in italics and bold. The four cysteine residues are boxed. GenBank accession number KC195966.

Nucleotide and deduced amino acid (in italic) sequences from *Chelon labrosus* IGF-I cDNA. The signal peptide (37 aa) is underlined, the mature region (68 aa) consisting of B, C, A, and D domains is boxed, and the six cysteine residues of mature peptide are boxed. The E domain is composed of 74 aa. Start and stop codons are represented in italics and bold. GenBank accession number KC195967.

GH nucleotide sequence comprises an open reading frame (ORF) of 597 bp encoding a 198 amino acid (aa) protein, with a signal peptide of 61 aa and mature protein of 137 aa residues. IGF-I presented an ORF of 558 bp encoding a 186 aa. This is composed of 37 aa for the signal peptide, 68 aa for mature peptide comprising B, C, A, and D domains, and 74 aa for E domain. Both sequences were preceded and followed by 5' and 3' untranslated regions of 75 bp and 135 bp, respectively, for the GH, and 134 bp and 689 bp, respectively, for IGF-I.

mRNA expression of GH in control group were kept constant along the trial, and lower than the rest of treatments. Expression measured in starved group were significantly higher at 14 days, but it equalized to the control at 21 days (Figure 32). No significant effects of dietary treatment on IGF-I were found (Figure 32).

3.3. Digestive enzymatic activities

Starving and re-feeding did not affect activity of digestive enzymes except for trypsin (Table 16). A significant reduction of the values with time was observed in the starved group.

Table 16. Effect of starving/re-feeding processes on digestive enzymatic activities of juvenile *C. labrosus* specimens along experimental period. Values are represented as mean $\pm$ S.E.M. ($n = 6$ fish per group). Values not sharing a common superscript are significantly different with $p < 0.05$. Lowercase letters refer to comparison within a group, capital letters between groups.

Parameters	Day	Control	Starving	Re-feeding
Pepsin (U/g tissue)	0	65.4 $\pm$ 8.1 ^a		
	14	74.3 $\pm$ 8.1 ^a	48.21 $\pm$ 11.52 ^a	
	21	60.3 $\pm$ 5.7 ^a	47.90 $\pm$ 8.99 ^a	39.99 $\pm$ 5.26
Trypsin (U/g tissue)	0	1.4 $\pm$ 0.2 ^a		
	14	1.2 $\pm$ 0.1 ^a	0.8 $\pm$ 0.2 ^a	
	21	1.4 $\pm$ 0.3 ^a	0.8 $\pm$ 0.1 ^a	1.2 $\pm$ 0.1
Amylase (U/g tissue)	0	0.23 $\pm$ 0.02 ^a		
	14	0.32 $\pm$ 0.03 ^a	0.30 $\pm$ 0.03 ^a	
	21	0.32 $\pm$ 0.08 ^a	0.15 $\pm$ 0.03 ^a	0.27 $\pm$ 0.03

4. DISCUSSION

4.1. Growth

Results presented in Table 14 show a clear effect of a short-term starvation in the growth of *C. labrosus*, as well as the positive effect of re-feeding on this parameter, this latter reflecting a sort of compensatory growth response. Compensatory growth is the phase of rapid growth, greater than normal or control growth, which occurs upon adequate re-feeding following a period of malnutrition. In contrast with other marine teleost such as *Salmo salar* or *O. mossambicus* (Fox et al., 2010; Imsland et al., 2011) in the present study full compensation was not observed in fish undergoing starvation and re-feeding (Table 14). These results are in agreement with those obtained in *Gadus morhua* by Jobling et al. (1994), on which the weight was not compensated after three weeks of re-feeding. This may indicate that the end-point of the present experiment occurred during a phase when the fish were at the beginning of a compensatory growth state. In fact, it has been suggested that the total recovery of growth rates should require a much longer period (Hayward & Wang, 2001). The liver plays a key role in this compensatory response, as reported in other species like *Pagrus pagrus* and *Sparus aurata*, where the rapid recovery of hepatic reserves due to re-feeding process indicated the importance of the liver during periods of food deprivation (Tufan et al., 2008; Laiz-Carrión et al. 2012). Results obtained with the values of HSI agreed with the described general trend.

4.2. Energy metabolism

In fish, as in other organisms, food restriction reduces energy storage, redirecting it towards the maintenance of the metabolic homeostasis. These problems are reflected in a wide variety of physiological responses like a reduction in growth rate, changes in the weight of different organs and adipose tissue, as well as in increased mobilization of metabolites/nutrients (Navarro & Gutiérrez, 1995).

Whereas during starvation, plasma glucose levels are maintained from the beginning in few species, like in *Sparus aurata* or *Solea senegalensis* (Polakof et al., 2006; Costas et al., 2011), in some other teleost species, like *Dicentrarchus labrax* or *Oncorhynchus mykiss*, plasma glucose decreased in food-deprived fish (Kelli et al., 2011). In this study, plasma glucose decreased in starved fish, recovering its values in

the re-feeding group afterwards, which suggest a liver glycogen mobilization. Thus, changes of glucose values in re-feeding fish may be due to: i) a reduction in the rate of glucose utilization; ii) an increase of glyconeogenic and glycogenolytic potential; and iii) the enhancement in liver glucose exporting (Costas et al., 2011). During starving phase, liver glycogen levels were lower than those in fed fish, suggesting that *C. labrosus* specimens mobilized liver glycogen depots. De Pedro et al. (2003) reported that the concomitant decrease in both plasma glucose and liver glycogen observed after starving indicated that the active glycogenolysis produced to counteract fasting was not enough to maintain the glycaemia. This strategy for supplying energy during short starvation periods is in line with other species that also use liver glycogen for the keeping of metabolic functions (Figueroa et al., 2000). Pérez-Jiménez et al. (2007) suggested that the large reduction in liver glycogen induced by starvation was a readily available energy reserve for *D. labrax*. Results obtained in the present work, with a significantly reduced amount of glycogen in the liver of starved fish, also support this idea.

In homoeothermic species, plasma cortisol levels are consistently reported to be elevated in response to starving or malnutrition (Chang et al., 2002). In the present work, the increase in cortisol were observed at day 21 of starving, suggesting that fish were responding to a stressful situation by the general adaptation syndrome, which has different stages according to the length of the stressor (Mommsen et al. 1999; Barton 2002; Marrero-Hernández, 2008). It seems that during the last week, fish reached the resistance phase, where the stress situation is continuous, then being adapted to the new condition increasing the release of cortisol. This high plasma cortisol levels is related to the redistribution of energy during a stress response. Similar results were found in *S. senegalensis* suggesting that cortisol may play a functional role in the energetic turnover (Polakof et al., 2006; Costas et al., 2011; Wunderink et al., 2012; Martos-Sitcha et al., 2014).

HK and PK activities were maintained during 21 days of food deprivation. This fact suggests that liver would be a simple producer of glucose but not a consumer of it. However, in other species *S. aurata*, *Acipenser naccarii* or *O. mykiss* PK and HK showed lower activities values after 10 days without food supply (Polakof et al., 2006; Furné et al., 2012).

Starved fish showed a significant decrease in the activity of total GPase. However, and according to hepatic glycogen reduction observed after 21 days of food restriction that indicated an enhancement of glycogen utilization, a stimulation of this activity could be expected. In this way, it will be necessary to know the glycogen synthase activity in order to get a complex picture on dynamic of glycogen synthesis and degradation (as well as hepatic storage). Nevertheless, re-fed fish showed a significant increase in the activity of total GPase, it might due to the use of other energy sources than glycogen. Collins and Anderson (1995) postulated that hepatic GPase activation in fed fish is indicative of not only regeneration of glycogen reserves within this tissue, but also of the regeneration of the animal's carbohydrate metabolism in general.

On the other hand, the high levels of hepatic FBPase measured in the control group should reflect a normal pattern of synthesis of hepatic glucose, while in contrast the significantly low levels measured in the starved fish should be linked to a consumption of the hepatic reserves. It appears that the reduced rate of gluconeogenesis in the liver of starved *C. labrosus* is sufficient to meet the liver depressed metabolic demands (Collins & Anderson, 1997). LDH activity showed significantly lower values in starved fish when compared to those in the control group, this possibly reflecting a decrease in the anaerobic pathways linked to the general decrease of metabolism with the absence of food.

In the present work, hepatic G6PDH, G3PDH, GDH, GPT activities revealed that protein and lipid metabolism in juvenile *C. labrosus* is not altered by starving. However, on day 21 there was an increase in GOT levels in unfed fish, suggesting that proteins were degraded to amino acids and were used for energy production. This is further supported by the GPT and GDH activities, which remained almost unchanged during food restriction.

4.3. GH/IGF-I system

GH full-length cDNA from *C. labrosus* was obtained by PCR combining 5'- and 3'-Rapid Amplification cDNA Ends (RACE) due to the low representativeness of this hypophyseal hormone in the whole brain. This sequence consisted in 807 bp excluding the poly-A tail. The open reading frame (ORF) codes for a prehormone of 198 aa. Conserved cysteine residues that are present in all GH peptides are also conserved in the

thick-lipped grey mullet GH protein (amino acid residues 69, 171, 188, 196). The identity of *C. labrosus* GH is much closer to GHs of other perciform fishes (91-100 %), like *Epinephelus coiodes*.

181 amino acids spanning IGF-I translation product was identified from liver of *C. labrosus*. As is typical for the IGFs, it contains a signal peptide and B, C, A, D and E domains, and shows 6 cysteine residues (amino acids 49, 62, 89, 90, 94, 103) responsible for the maintenance of tertiary structure. At protein level, thick-lipped grey mullet IGF-I showed 81-100 % identity to other teleosts, like *E. coiodes* and *O. mossambicus*.

Fish are frequently exposed to extended periods of starvation. Thus, it can be assumed that food intake and endocrine function are strongly related. The effects of starving on the GH/IGF-I axis in fish have been recently reviewed (Reindl & Sheridan, 2012). These studies suggest that nutritional status plays an important role in the regulation of physiological function of GH/IGF system. Results obtained in this study showed that nutritional regulation of pituitary GH mRNA and hepatic IGF-I mRNA levels could be similar to other fish species. As shown previously in several fish, the typical response to starving is an increase in pituitary GH mRNA and a decrease in hepatic IGF-I mRNA. These data suggest that nutritional status can regulate circulating IGF-I levels, in part by modulating transcriptional activity of IGF-I gene, although other regulatory mechanism have not been ruled out at this time (Wood et al., 2005). In *O. masou*, the response of hepatic IGF-I mRNA was consistent to changes in nutritional status. Several previous reports have indicated that hepatic IGF-I mRNA expression were decreased by feed restriction and recovered by re-feeding, like in *O. mossambicus* (Fox et al., 2010) and in the *I. punctatus* (Peterson & Waldbieser, 2009). However, in this study no significant differences in hepatic IGF-I mRNA expression were observed before and after re-feeding processes suggesting that more time is necessary to see any influence of both processes on hepatic IGF-I expression in this specie. On the other hand, Duan and Hirano (1992) evidenced that hepatic IGF-I mRNA expression became lower relative to fed controls after 2 weeks in *Anguilla japonica*. According to Pierce et al. (2005), the increase of GH that accompanies starving usually occurs after 2 weeks, indicating that it takes a long time for the effect to be manifested. Similarly, in *C. labrosus* after 2 weeks of starving, GH mRNA enhanced significantly its expression.

Starving resulted in no significant decreased hepatic IGF-I mRNA level whereas a significant increase in pituitary GH mRNA levels can be observed. Furthermore, this study demonstrated that nutritional regulation of pituitary GH mRNA and hepatic IGF-I mRNA levels were altered by starving but not by re-feeding processes, or at least more re-feeding time is required. This fact is similar to several other fish species.

4.4. Digestive enzyme activities

Starving and re-feeding are processes that affect enzyme activities, mass and protein content of the intestine, with a pattern of a rapid decrease the first two days of starvation and a rapid increase when feed was made available in carnivorous species like the *S. salar* (Krogdahl & Bakke-McKellep, 2005). In contrast, results obtained in the present work showed no clear effect of starvation or re-feeding on the activity of the selected enzymes (Table 16), this suggesting that the feeding habits (carnivorous vs herbivorous) may have an influence on this response. *C. labrosus* is considered an omnivorous species, and in other omnivorous, like *Rutilus rutilus caspicus*, trypsin activity did not change significantly during starvation and re-feeding periods (Abolfathi et al., 2012). These authors propose that since none of the protease measurements gave a significant reduction in activity by increasing the starving period, assuming that maintenance of digestive protein capacity is still present in an advanced state of starvation; this would enable these fish to digest the main nutrients. Furthermore, amylase activity did not change in any experimental group. As in other omnivorous species, like *R. rutilus caspicus* and *A. naccarii*, the performance of amylase consists in a decrease of the activity during the starvation period and maintaining it during re-feeding (Abolfathi et al., 2012). This confirms the greater digestive capacity for carbohydrates cataloguing it closer to the habits of an omnivorous fish (Hidalgo et al., 1999).

5. CONCLUSIONS

1. Juvenile *C. labrosus* adapts to changes in feeding conditions without altering its physiology overly.
2. Results demonstrate that liver metabolism is significantly influenced by these changes in food availability and that energy metabolism is cortisol-dependent in starved fish.
3. mRNA expression of GH and IGF-I and activity of digestive enzyme did not evidence clear modifications this suggesting thus they may be not sensitive indicators of feeding status in this species.
4. Results of the present study could be of great interest for fish farming when under certain circumstances fish could undergo starvation periods.

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