



Gene markers of dietary macronutrient composition and growth in the skeletal muscle of gilthead sea bream (*Sparus aurata*)

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ABSTRACT

To increase our current knowledge on the nutritional regulation of growth and gene expression pattern in fish skeletal muscle, the effect of dietary macronutrient composition was assessed on digestibility, nutrient retention, growth performance, and the mRNA levels of key genes involved in functionality, growth and development of the skeletal muscle in gilthead sea bream (*Sparus aurata*). Long-term starvation decreased the expression of myogenic regulatory factors such as Myod2, Myf5, myogenin (Myog) and Myf6 in the skeletal muscle of *S. aurata*. The supply of high or medium protein, low carbohydrate diets enhanced growth parameters, feed efficiency ratio, feed conversion ratio and significantly upregulated *myod2*. However, the supply of low protein, high carbohydrate diets restricted growth and stimulated the mRNA levels of *myostatin*, while downregulated *follistatin (fst)*, *igf1*, *mtor* and *rps6*. Microarray analysis revealed *igfals*, *tnni2*, and *gadd45a* as gene markers upregulated by diets enriched with protein, lipids and carbohydrates, respectively. The results of the present study show that in addition to *myod2*, *fst*, *igf1*, *mtor* and *rps6*, the expression levels of *igfals*, *tnni2* and remarkably *gadd45a* in the skeletal muscle can be used as markers to evaluate the effect of dietary macronutrient changes on fish growth and muscle development in *S. aurata*.

1. Introduction

The development of the skeletal muscle follows a well-ordered structure that is highly adaptable to changing conditions. The process of myogenesis has a marked plasticity, which constitutes a fundamental event for proliferation, differentiation, migration and fusion of new myoblasts (Johnston, 2006). This complex and dynamic process leads to fusion of myocytes with existing myofibers (hypertrophy) and increasing proliferation of myocytes (hyperplasia) (Zhu et al., 2014). Myogenesis is primarily controlled by myogenic regulatory factors (MRFs) which include Myod, Myf5, myogenin (Myog) and Myf6, among others (Braun and Gautel, 2011; Rescan, 2001; Alami-Durante et al., 2019). MRFs are highly conserved proteins from teleosts to mammals that belong to a larger group of transcription factors containing a basic DNA-binding motif and a helix-loop-helix dimerisation domain (Olson

and Klein, 1994; Rossi and Messina, 2014). Myod and Myf5 are essential for initiation of the myogenic program, while Myog and Myf6 are expressed later, during myofibre differentiation (Holterman and Rudnicki, 2005; Rescan, 2001; Tan and Du, 2002). Additional factors, such as myostatin (Mstn) and follistatin (Fst), which belong to the transforming growth factor beta (Tgfb) superfamily, were also shown to have a major role in skeletal muscle growth and development. Mstn is a potent negative regulator of skeletal muscle growth (McPherron et al., 1997). Knockout mice for Mstn present 2 to 3-fold greater skeletal muscle mass than wild-type animals. Fst is an activin-binding protein essential for muscle fibre formation (Medeiros et al., 2009), and a potent antagonist of several members of the Tgfb superfamily, including Mstn (Amthor et al., 2004). Overexpression of Fst in mice and rainbow trout (*Oncorhynchus mykiss*) evidences the capacity of Fst to increase muscle mass (Lee and McPherron, 2001; Medeiros et al., 2009).

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Somatotropic compounds such as insulin-like growth factor 1 (Igf1) exert an important role in nutritional regulation of metabolism. Igf1 expression increases muscle mass and decreases muscle atrophy (Christoffolete et al., 2015; Glass, 2003a, 2003b; Vélez et al., 2014). Protein synthesis and glucose and amino acid uptake is stimulated by Igf1 in rainbow trout (*O. mykiss*) and gilthead sea bream (*S. aurata*) myocytes (Castillo et al., 2004; Montserrat et al., 2012). Igf1 and amino acids activate the phosphatidylinositol 3-kinase (Pi3k)-Akt pathway, which leads to phosphorylation of the serine/threonine protein kinase mechanistic target of rapamycin (Mtor) and activation of a critical pathway involved in cellular processes such as apoptosis, protein synthesis, gene transcription and cell proliferation (Vélez et al., 2016). Indeed, Mtor is an essential sensor of nutrient and amino acid availability through phosphorylation of 40S ribosomal protein S6 (Rps6) (Glass, 2005; Schiaffino and Mammucari, 2011).

Despite the fact that carnivorous fish are considered glucose intolerant (Panserat et al., 2019; Rashidpour et al., 2019), we previously showed that partial replacement of dietary protein by carbohydrates stimulates glucose oxidation via glycolysis and pentose phosphate pathway in the liver of *S. aurata* (Fernández et al., 2007; Metón et al., 1999). Indeed, the administration of chitosan-tripolyphosphate-DNA to overexpress *srebp1a* stimulates conversion of dietary carbohydrates into lipids in *S. aurata* through enhanced hepatic glycolysis, pentose phosphate pathway and lipogenesis (Silva-Marrero et al., 2019; Wu et al., 2020). Dietary carbohydrates upregulate the expression of glycolytic genes in the muscle of rainbow trout (Song et al., 2018), while it was claimed that glucose regulates protein synthesis and growth-related mechanisms in myogenic precursor cells from this species (Latimer et al., 2019). Although the fundamental events in muscle growth and development were well conserved during vertebrate evolution, fishes have unique features such as continuous growth and different proportion of white and red muscle fibres than mammals. Indeed, the molecular events that mediate the effect of dietary nutrients on signaling pathways that control growth of fish skeletal muscle remain unclear. With the aim to increase our current knowledge about the nutritional regulation of skeletal muscle growth and development in fish, we addressed the effect of dietary macronutrient composition on nutrient retention, growth performance and the gene expression pattern in the skeletal muscle of *S. aurata*, and identified novel gene markers for nutritional studies in cultured fish.

2. Materials and methods

2.1. Animals, feeding trial and sampling

S. aurata juveniles were obtained from Piscicultura Marina Mediterranea (Burriana, Castellón, Spain). A total of 330 fish (8.22 g ± 0.26 body weight) were transported to the laboratory, and distributed in 12 aquaria of 260 l supplied with running seawater at 21 °C in a closed system with active pump filter and UV lamps. The photoperiod was adjusted to a 12 h: 12 h dark-light cycle. Fish maintenance conditions were as previously described (Fernández et al., 2007). Three diets were formulated with gross energy at 20–22 kJ/g and macronutrient composition at levels above and below those in commercially available aquafeeds: HLL (high protein, low lipid, low carbohydrate), MHL (medium protein, high lipid, low carbohydrate) and LLH (low protein, low lipid, high carbohydrate) (Table 1). Following acclimation to our facilities, three groups of fish were fed twice a day (9:30 a.m. and 15:30 p.m.) with 25 g/kg body weight of the corresponding experimental diet for 23 days for microarray analysis and 37 days for evaluating growth, digestibility and gene expression (Fig. 1). Sampling points were selected according to previous studies showing that a period of 18–20 days is long enough for producing significant changes due to diet composition in the intermediary metabolism and gene expression profile of *S. aurata* juveniles (Metón et al., 1999; Silva-Marrero et al., 2017), while longer periods are necessary to obtain significant changes in growth and

Table 1
Composition of the diets supplied in this study to *S. aurata*.

	HLL	MHL	LLH
Formulation (%)			
Fish meal ^a	81.6	67.5	54.3
Fish oil ^b	0.8	13.1	6.0
Starch ^c	15.0	16.7	37.1
Carrageenan ^d	1.5	1.5	1.5
Mineral mixture ^e	0.9	0.9	0.9
Vitamin mixture ^f	0.2	0.2	0.2
Proximate Composition (%)			
Crude protein	59.5	50.1	40.6
Crude lipid	7.2	17.5	8.2
Carbohydrates ^g	18.4	20.9	40.9
Ash	14.9	11.5	10.3
Gross energy (kJ/g) ^h	20.2	22.4	19.9

^a Corpesca S.A. Super-Prime fish meal (Santiago de Chile, Chile).

^b Fish oil from A.F.A.M.S.A. (Vigo, Spain).

^c Pregelatinised corn starch from Brenntag Química S.A. (St. Andreu de la Barca, Barcelona, Spain).

^d Iota carrageenan (Sigma-Aldrich).

^e Mineral mixture provided (mg/kg): CaHPO₄·2 H₂O, 7340; MgO, 800; KCl, 750; FeSO₄·7 H₂O, 60; ZnO, 30; MnO₂, 15; CuSO₄·5 H₂O, 1.7; CoCl₂·6 H₂O, 1.5; KI, 1.5; Na₂SeO₃, 0.3.

^f Vitamin mixture provided (mg/kg): choline chloride, 1200; myo-inositol, 400; ascorbic acid, 200; nicotinic acid, 70; all-rac-tocopherol acetate, 60; calcium pantothenate, 30; riboflavin, 15; pyridoxin, 10; folic acid, 10; menadione, 10; thiamin-HCl, 8; all-trans retinol, 2; biotin, 0.7; cholecalciferol, 0.05; cyanocobalamin, 0.05.

^g Carbohydrates were calculated by difference.

^h Calculated from gross composition (protein 24 kJ/g, lipids 39 kJ/g, carbohydrates 17 kJ/g).

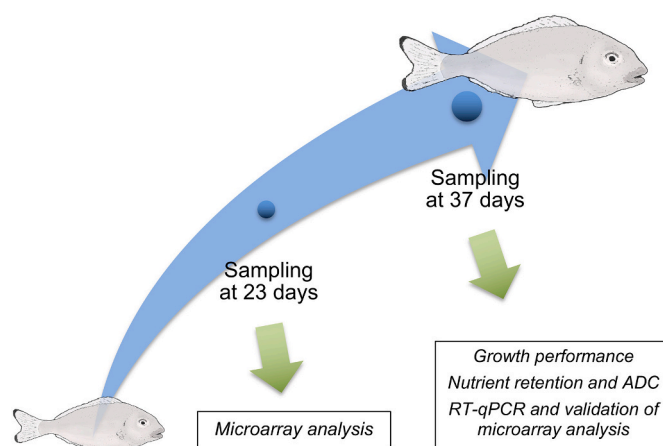


Fig. 1. Schematic workflow of sampling points and analysis carried out in the present study. ADC: apparent digestibility coefficient. RT-qPCR: reverse transcription coupled to quantitative real-time PCR.

nutrient retention parameters (Fernández et al., 2007). A fourth group of fish was submitted to starvation during the same period of time. Each dietary condition was assayed in 3 aquaria. All fish were weighed at the beginning of the experiment and every 12 days during the experiment. To calculate apparent digestibility coefficient (ADC), 0.1% yttrium oxide (Y₂O₃) was included in each experimental diet and used as inert marker the last week of the experiment. To determine body composition, 30 fish from the initial stock and 5 fish per aquarium at the end of the experiment were sampled and stored at −20 °C for subsequent analysis. Prior to sampling, fish were starved for 24 h and sacrificed by anaesthesia overdose with 1:12,500 tricaine methanesulfonate (MS-222) diluted in seawater followed by cervical section. Samples of skeletal

muscle extracted from the middle/dorsal region were dissected out, immediately frozen in liquid nitrogen and kept at -80 °C until analysis. For fecal collection, the posterior intestine was dissected out and the intestinal content was obtained by stripping. Posterior intestine samples were pooled (pools of 5 fish per aquarium), dried at 70 °C and kept at -20 °C. Experimental procedures involving fish complied with the guidelines of the University of Barcelona's Animal Welfare Committee and EU Directive 2010/63/EU for animal experiments.

2.2. Diet and body composition

To determine moisture, samples were dried at 70 °C until constant weight was obtained (Busacker et al., 1990; Lucas, 1996). Samples were analysed for carbon (C) and nitrogen (N) with a Carlo Erba NA 2100 elemental analyser (CE Instruments, Wigan, UK). Lipid content was extracted with petroleum ether using a Foss Tecator Soxtec HT 1043 extraction system (Hillerød, Denmark). Ash content was determined after incineration of samples in muffle furnace at 450 °C for 12 h (Busacker et al., 1990; Lucas, 1996). Protein was calculated from N content, using a factor of 6.25. Gross energy was calculated on the basis of dietary protein (24 kJ/g), lipid (39 kJ/g) and carbohydrate (17 kJ/g) (Bradfield and Llewellyn, 1982). For assaying Ca, P and Y, samples were digested and analysed with an inductively coupled plasma spectrometer (Polyscan 61E, Thermo Jarrell Ash Corporation, Waltham, MA, USA).

2.3. Apparent digestibility and growth parameters

Specific growth rate (SGR) was calculated as $(\ln W_f - \ln W_i) \cdot 100 / T$, where $\ln W_f$ and $\ln W_i$ are the natural logarithms of the final and initial mean fish weight in grams per aquarium, respectively, and T is time in days. Feed conversion ratio (FCR) was calculated as dry feed intake/wet weight gain. ADC of a given nutrient was calculated from the following equation (De Silva and Anderson, 1995):

$$ADC = 100 - [100 \cdot (\text{Nutrient}_{\text{Feces}} / \text{Nutrient}_{\text{Diet}}) \cdot (\text{Y}_{\text{Diet}} / \text{Y}_{\text{Feces}})]$$

For dry matter, the equation became:

$$ADC = 100 - [100 \cdot (\text{Y}_{\text{Diet}} / \text{Y}_{\text{Feces}})]$$

Other parameters calculated for each aquarium included: protein efficiency ratio (PER = g weight gain/g feed protein); protein retention (PR = g protein gain*100/g feed protein); lipid retention (LR = g lipid gain*100/g feed lipid) and hepatosomatic index (HSI = liver fresh weight*100/ fish body weight).

2.4. RNA isolation

Total RNA was extracted from the skeletal muscle using the RNeasy fibrous tissue Mini Kit (Qiagen, Sussex, UK) according to the manufacturer's recommendations. Concentration and purity were determined

spectrophotometrically at 260/280 nm using Nanodrop ND-1000 (Thermo Fischer Scientific, Waltham, MA, USA). RNA integrity was determined with an Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA Integrity Number (RIN) > 9.2 were used for subsequent studies.

2.5. Quantitative real-time PCR

The mRNA levels of key genes involved in skeletal muscle growth and development, and differentially expressed genes selected from the microarray analysis were assayed by reverse transcription coupled to quantitative real-time PCR (RT-qPCR). One microgram of total RNA isolated from white skeletal muscle of *S. aurata* was reverse-transcribed to cDNA with Moloney murine leukemia virus RT (Life Technologies, Carlsbad, CA, USA) for 1 h at 37 °C in the presence of random hexamer primers. The cDNA product was used for subsequent qPCR. The mRNA levels of *S. aurata* genes listed in Table 2 were determined in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 0.4 μM of each specific primer (Table 2), 10 μl of SYBR Green (Applied Biosystems Foster City, CA, USA), and 1.6 μl of the diluted cDNA product in a final volume of 18 μl. The temperature cycle protocol for amplification was: 1 cycle of initial activation at 95 °C for 10 min and 40 cycles of 15 s at 95 °C, 1 min at 62 °C. To validate the amplification efficiency of primers, standard curves with serial dilutions of a control cDNA were generated, and PCR amplicons were separated electrophoretically on 2% agarose gel for band size confirmation. 18S ribosomal RNA (18 s) and elongation factor 1 alpha (ef1α) were selected to normalise the amount of mRNA for the genes of interest in each sample. Variations in gene expression were calculated by the standard ΔΔCt method (Pfaffl, 2001). Results are presented as mean ± SD (n = 4–6 fish).

2.6. Microarray hybridisation and data analysis

A microarray analysis was performed to detect differentially expressed genes with potential interest for nutritional and diet formulation studies in *S. aurata*. To this end, labelling, hybridisation and scanning of an Agilent custom high-density oligonucleotide microarray (8 × 60 k; ID 079501; Agilent Technologies, Santa Clara, CA, USA), previously described to contain 2 60-mer probes for each of 25,392 assembled unique sequences of *S. aurata* transcriptome (Silva-Marrero et al., 2017), were performed with the Two-Color Microarray-Based Gene Expression Analysis v. 6.5 kit (Agilent Technologies, Santa Clara, CA, USA). Total RNA isolated from the skeletal muscle of four fish was analysed for each condition (starved fish and fish fed diets HLL, MHL and LLH for 23 days). For each sample, 200 ng of total RNA was labelled with Cy3 or Cy5 using Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA), and microarray workflow for linearity, sensitivity and accuracy was monitored using The Two-Color and RNA

Table 2 Primers used to analyse gene expression by qPCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')	GenBank accession nos.
18 s	TTACGCCCATGTTGCTCGAG	AGGATTCTGCATGATGGTCACC	AM490061
ef1a	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTCCGTTAGC	AF184170
fst	GGAACGACGGGATCATCTATGC	CGACTTGGCCCTTGATGATTTTCC	AY544167
gadd45a	AGCGGGTCTGTTTTTATTTCTTC	AGGAAGTGTGGTGTGTACCC	XM_030401611
igf1	ACTGCTGTGCGTCTCACCGTGA	GTGCATTGGGGCCGTAGCCA	AY996779
igfals	TGTGGTGAACGCCAGAGCTTTG	GAGGCCAGAGAATGATGGGTTGTGAG	XM_030407223
mstn	GGATGCAGGAACACACACAC	AGACGACGAAGGACGAGAAA	AF258448.1
mtor	GGAGACTGTTTGGAGTCCGCC	ACCTCATCACCGTGTGGCA	MH594580
myf5	CGACGGCATGGTTGACAGCA	TCCGGCTGTCTTATCGCCA	JN034420
myf6	TCATCCCACAGCTTTAAAGGCA	AGTGAATCTTCGGCGTCTCTC	JN034421
myod2	CACTACAGCGGGATTCAGAC	CCGTTTGTCTTCTCTGGACT	AF478569
myog	TTCCCTGACACGCGCTCCTA	TCTGTTCTGTCAACCCCAAC	EF462191.1
rps6	CAGCAAGATCCGCAAGTCTT	CTTCTGGGTGCGCTGTCTCT	MN172174
tni2	GCCCTGAAGAAAGTACGTATGTCTGC	CTCCCTCTCTTGTACCTCTCTC	XM_030426857

Spike-In Kit, Two-Color. The *RNeasy Mini Kit* (Qiagen, Hilden, Germany) was used to purify labelled cRNA. Microarray hybridisation was performed using 2.5 µg of each labelled sample at 65 °C for 17 h following the *Gene Expression Hybridization Kit* instructions. A double loop hybridisation with dye swap experimental design was followed (Kerr and Churchill, 2001), and included eight hybridisations to maximise discovery of significant changes among assayed conditions (starvation and feeding with diets HLL, MHL and LLH; $n = 4$ per condition). Scanning was performed using an Agilent Microarray Scanner G2565BA, and outlier spots and spot intensity for Cy3 and Cy5 channels were extracted with Agilent Feature Extraction software v. 10.7. Loess and Aquantile normalisation for within-and inter-array normalisation, respectively, was performed with R-Bioconductor package (Gentleman et al., 2004). Data analysis was only considered for unique sequences involved in growth and development with E -value $< 1e-10$ and HSP/hit > 30 .

2.7. Statistics

Data concerning growth performance, nutrient retention, apparent digestibility and qPCR were analysed using SPSS Version 25 (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by the Duncan post-hoc test was performed to identify significant differences between treatments. Statistical significance was considered when $P < 0.05$. For microarray data, a linear model analysis using Limma (Smyth, 2004) was conducted to determine differentially expressed genes with adjusted $P < 0.05$. Correlation coefficient values were calculated to assess relationships between growth, nutrient retention, ADC and gene expression variables, considering statistical significance when $P < 0.05$.

3. Results

3.1. Growth performance, nutrient retention and digestibility

The effect of dietary macronutrient composition on growth parameters and nutrient retention was studied in *S. aurata* juveniles fed 37 days with diets HLL (macronutrient composition similar to the diet of wild *S. aurata*), MHL (composition similar to commercial diets) and LLH (composition similar to HLL but with partial substitution of protein by carbohydrates). Fish fed the high protein, low lipid, low carbohydrate diet (HLL) and the medium protein, high lipid, low carbohydrate diet (MHL) presented significantly higher values of final body weight and SGR than fish fed the low protein, low lipid, high carbohydrate diet (LLH; Table 3). The highest FCR value was found in fish fed the LLH diet, while no significant difference was found between diets HLL and MHL. Fish fed the LLH diet also presented the highest LR value. The lowest PER levels were found for fish fed diet HLL, which were significantly different than in fish fed the MHL diet (Table 3). Diet composition did not significantly affect HSI or PR.

No significant differences were found in ADC values for calcium, carbon and dry matter in the posterior intestine of fish fed 37 days with

Table 3
Growth performance and nutrient retention of *S. aurata* fed diets HLL, MHL and LLH for 37 days.

	HLL	MHL	LLH
Initial BW (g)	8.20 ± 0.04	8.52 ± 0.06	8.11 ± 0.21
Final BW (g)	17.69 ± 0.22 ^b	18.36 ± 0.18 ^b	14.76 ± 0.71 ^a
SGR	2.08 ± 0.04 ^b	2.08 ± 0.02 ^b	1.61 ± 0.10 ^a
FCR	1.58 ± 0.01 ^a	1.44 ± 0.04 ^a	1.96 ± 0.16 ^b
HSI	1.22 ± 0.08	1.11 ± 0.05	1.33 ± 0.11
PR (% intake)	17.92 ± 0.72	20.52 ± 1.05	20.34 ± 1.79
LR (% intake)	24.90 ± 4.14 ^a	33.21 ± 2.15 ^{ab}	41.95 ± 4.30 ^b
PER	1.06 ± 0.01 ^a	1.39 ± 0.04 ^b	1.27 ± 0.10 ^{ab}

Data are means ± SEM ($n = 3$ tanks). Different superscript letters indicate significant differences among dietary conditions ($P < 0.05$). BW: body weight.

different diets. However, fish fed LLH revealed a trend to present lower values than fish fed diets HLL and MHL (Table 4). ADC levels for protein were not affected by diet composition, while the highest values for phosphorus, calcium, carbon and dry matter were found in fish fed MHL. No significant correlation was found between ADC values and growth and nutrient retention parameters (Table 5).

3.2. Effect of diet composition and starvation on the expression of MRFs, *mstn* and *fst* in the skeletal muscle

Dietary macronutrient composition and food deprivation significantly affected the expression of MRFs in the white skeletal muscle of *S. aurata*. Starvation for 37 days significantly downregulated the mRNA levels of all MRFs assayed. Concerning fed animals, the supply of HLL and MHL significantly enhanced 1.7–1.9 fold the expression levels of *myod2* compared to fish fed with diet LLH (Fig. 2a). In a significant manner but to a lesser extent than *myod2*, feeding fish with the diet containing the lowest protein/carbohydrate ratio (LLH) decreased *myf5* and *myog* mRNA levels when compared to fish fed with medium protein, high lipid, low carbohydrate (MHL) and high protein, low lipid, low carbohydrate (HLL) diets, respectively (Fig. 2b-c). The only MRF assayed whose mRNA levels did not significantly differ as a result of diet composition was *Myf6* (Fig. 2d). The mRNA levels of *myod2* and *myog* correlated positively with final body weight and SGR, and negatively with FCR (Table 5).

In regard of genes with a major role in regulation of skeletal muscle growth, we also analysed the expression of *mstn* and *fst*. Feeding with LLH significantly upregulated *mstn* 2.1–2.9 fold over the values observed in starved fish and *S. aurata* fed with HLL and MHL (Fig. 3a). A different expressional pattern was observed for *fst*. The expression levels of *fst* were 1.8–2.0-fold significantly higher in fish fed HLL and MHL than in fish fed LLH and submitted to starvation (Fig. 3b). The expression levels of *mstn* and *fst* exhibited significant but opposite correlation with final body weight, SGR, FCR, HSI and LR (Table 5).

3.3. Effect of diet composition and starvation on the expression of *igf1*, *mtor* and *rps6* in the skeletal muscle

The effect of diet composition and starvation on *igf1* mRNA levels in the skeletal muscle of *S. aurata* was also addressed. Starvation for 37 days significantly downregulated 2.9–6.0 fold *igf1* expression depending on the diet supplied. Among fed fish, significant upregulation of *igf1* (1.8–2.0 fold) was found in fish fed diets with improved growth parameters (MHL and HLL) (Fig. 4a).

Previous studies indicated that in addition to posttranslational regulation, the nutritional status regulates Mtor and downstream proteins at the mRNA level in fish (Lavajoo et al., 2020; Qin et al., 2019). We therefore investigated the effects of starvation and feeding diets differing in macronutrient composition on *mtor* and *rps6* expression in the skeletal muscle of *S. aurata*. Among fed fish, the mRNA levels of *mtor* and *rps6* showed a significant dependence on the protein/carbohydrate ratio in the diet. For *mtor*, diet HLL promoted the highest expression values, which were 1.7-fold and 2.4-fold greater than in fish fed diets MHL and LLH, respectively. Likewise, fish fed HLL presented 1.3-fold

Table 4
Apparent digestibility coefficient (ADC) values obtained for *S. aurata* fed HLL, MHL and LLH diets.

	HLL	MHL	LLH
Phosphorus	39.96 ± 1.11	50.97 ± 3.48	44.74 ± 6.27
Calcium	54.71 ± 1.98	59.25 ± 5.34	45.07 ± 6.72
Carbon	70.53 ± 6.78	79.94 ± 3.44	65.08 ± 8.17
Protein	79.24 ± 2.38	78.08 ± 3.45	77.46 ± 0.73
Dry matter	64.76 ± 1.54	67.21 ± 4.30	57.18 ± 5.24

Data are means ± SEM ($n = 3$ tanks).

Table 5
Correlation coefficient values for growth and nutrient retention parameters versus ADC and gene expression values.

	Final BW	SGR	FCR	HSI	PR	LR	PER
Phosphorus	0.151	0.091	-0.202	-0.342	0.520	0.254	0.590
Calcium	0.628	0.639	-0.634	-0.621	-0.091	-0.430	0.103
Carbon	0.490	0.639	-0.512	-0.552	0.119	-0.210	0.278
Protein	0.134	0.000	-0.120	-0.072	-0.190	-0.204	-0.156
Dry matter	0.600	0.548	-0.601	-0.577	-0.134	-0.442	0.048
<i>myod2</i>	0.744**	0.822**	-0.746**	-0.715**	-0.167	-0.548	0.059
<i>myf5</i>	0.494	0.513	-0.508	-0.524	0.027	-0.273	0.183
<i>myog</i>	0.613*	0.616*	-0.578*	-0.442	-0.569	-0.703*	-0.428
<i>myf6</i>	-0.308	-0.140	0.318	0.336	-0.034	0.162	-0.137
<i>mstn</i>	-0.858**	-0.768**	0.857**	0.815**	0.220	0.650*	-0.040
<i>fst</i>	0.785**	0.804**	-0.766**	-0.688**	-0.385	-0.756**	-0.108
<i>igf1</i>	0.767**	0.837**	-0.768**	-0.735**	-0.244	-0.591	-0.017
<i>mtor</i>	0.662**	0.841**	-0.615*	-0.449	-0.725**	-0.857**	-0.544*
<i>rps6</i>	0.612*	0.737**	-0.565*	-0.400	-0.667**	-0.800**	-0.503
<i>tnni2</i>	0.519	0.462	-0.565	-0.676*	0.384	-0.052	0.562
<i>igfals</i>	0.249	0.444	-0.249	-0.249	-0.440	-0.552	-0.440
<i>gadd45a</i>	-0.645*	-0.539*	0.623*	0.528	0.435	0.649*	0.259

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

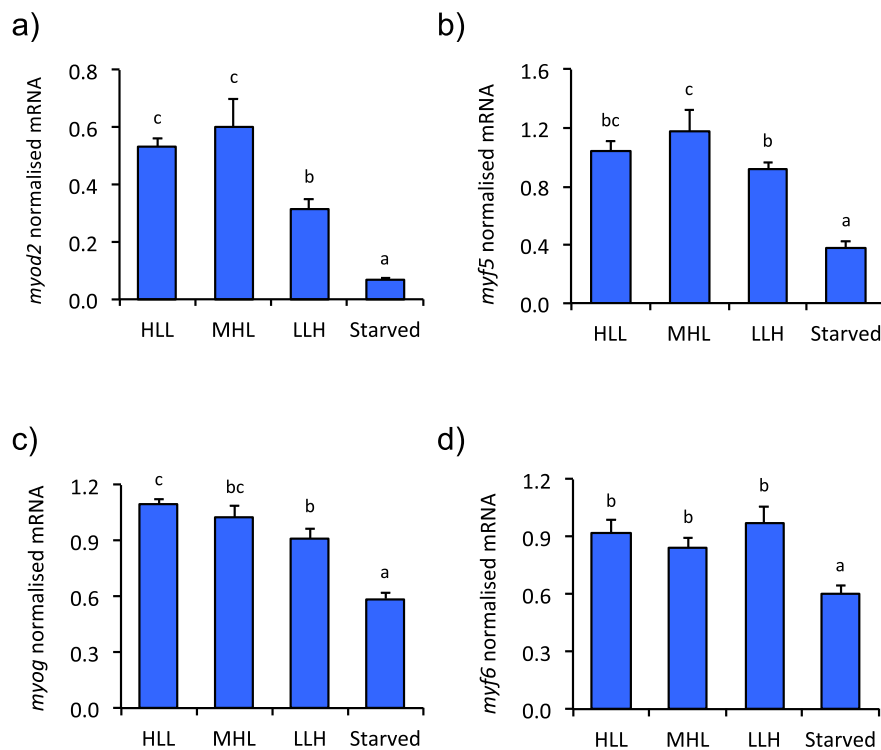


Fig. 2. Effect of diet composition and starvation on the expression of MRFs in the skeletal muscle of *S. aurata*. During 37 days, *S. aurata* juveniles were starved or fed with HLL, MHL and LLH diets. RT-qPCR analysis of *myod2* (a), *myf5* (b), *myog* (c) and *myf6* (d) expression in the skeletal muscle is shown. The mRNA levels for each gene were normalised with the geometrical mean of *S. aurata* 18 s and *ef1a*, which were used as housekeeping genes. Results are presented as mean \pm SEM ($n = 4-6$ fish). Different letters above deviation bars indicate significant differences ($P < 0.05$) among conditions.

and 1.6-fold higher *rps6* mRNA levels than fish supplied with MHL and LLH, respectively. The expression levels of *mtor* and *rps6* in starved fish were similar to those observed in the skeletal muscle of fish fed diet LLH (Fig. 4b-c). The mRNA levels of *igf1*, *mtor* and *rps6* positively correlated with final body weight and SGR, while negatively correlated with FCR (Table 5).

3.4. Microarray analysis

From a total of 247 genes involved in growth and development, the expression of 21 genes (included in the heat map hierarchical cluster shown in Fig. 5a) exhibited differential expression with an adjusted P value < 0.05 and at least 2-fold difference in the normalised intensity ratio (Cy5/Cy3 or Cy3/Cy5) between 2 or more dietary conditions (starvation and feeding for 23 days with diets HLL, MHL and LLH) in the

skeletal muscle of *S. aurata*. Starvation deeply affected the expression of most filtered genes. From the total of 21 genes selected, 3-6 genes (depending on the diet supplied) were significantly upregulated more than 2-fold in the skeletal muscle of starved fish, while food restriction downregulated 6-11 genes. Feeding upregulated genes associated with muscle contraction (*tnni2*, *tnnc2*, *stac3*, *ttn*, *actc1*, *calm*, *mpsf* and *cav3*), muscle development (*frg1*, *fgfr1* and *tagln*), as well as growth regulation and the growth hormone-Igf axis (*mstn*, *ghr1* and *igfals*), while downregulated genes were mostly involved in growth arrest and regulation (*gas6*, *ing3*, *gadd45a* and *naca*). Among upregulated genes by feeding with fold change > 2 , the greater values were found for *tnni2* (16.1, 39.1 and 29.0 fold change for fish fed HLL, MHL and LLH, respectively), followed by *stac3* (3.2, 3.5 and 2.8 fold change for fish supplied with HLL, MHL and LLH, respectively). The expression of *igfals* also increased in the skeletal muscle of fish fed diets HLL and MHL, but showed more

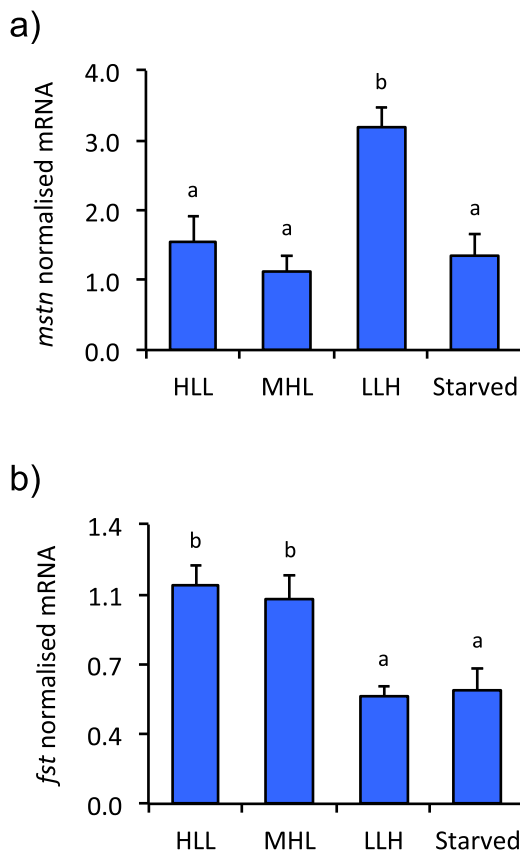


Fig. 3. Effect of diet composition and starvation on the expression of members of the Tgfb superfamily in the skeletal muscle of *S. aurata*. During 37 days, *S. aurata* juveniles were starved or fed with HLL, MHL and LLH diets. RT-qPCR analysis of *mstn* (a) and *fst* (b) expression in the skeletal muscle is shown. The mRNA levels for each gene were normalised with the geometrical mean of *S. aurata* *18 s* and *ef1a*, which were used as housekeeping genes. Results are presented as mean \pm SEM ($n = 4-6$ fish). Different letters above deviation bars indicate significant differences ($P < 0.05$) among conditions.

dependence on diet composition (5.3, 3.1 and 1.0 fold change for fish supplied with diet HLL, MHL and LLH, respectively). Similar expression patterns were found for *fgfr1* and *igfals*. In regard of downregulated genes by feeding, the greater fold changes were observed for *ing3* (4.6–6.7 fold change, depending on the diet supplied) and *gas6* (4.0–4.2 fold change, depending on the diet supplied), while *gadd45a* exhibited a higher dependence on diet composition (2.6, 2.3 and 1.6 fold change for fish supplied with HLL, MHL and LLH, respectively).

With the aim to identify gene markers regulated by dietary macronutrient composition, the mRNA levels of *gadd45a*, *igfals* and *tnni2* were assayed by RT-qPCR in the skeletal muscle of fish starved or fed diets HLL, MHL and LLH for 37 days (Fig. 5b). Starvation significantly upregulated *gadd45a* over the expression values observed in fish fed diets HLL, MHL and LLH (2.4, 1.7 and 1.4 fold, respectively). The mRNA levels of *gadd45a* negatively correlated with final body weight and SGR, while positively correlated with FCR and LR (Table 5). Similarly as *igf1*, the lowest *igfals* mRNA levels were promoted by starvation, while feeding with HLL significantly upregulated *igfals* 1.9 fold. In regard of *tnni2*, the lowest mRNA abundance was found in food-deprived animals. Among fed fish the highest *tnni2* expression was observed in the skeletal muscle of *S. aurata* fed diet MHL (2.0-, 4.2- and 45.3-fold increased levels than in fish fed diets HLL, LLH and under starvation, respectively). Significant negative correlation was found between *tnni2* expression and HSI (Table 5).

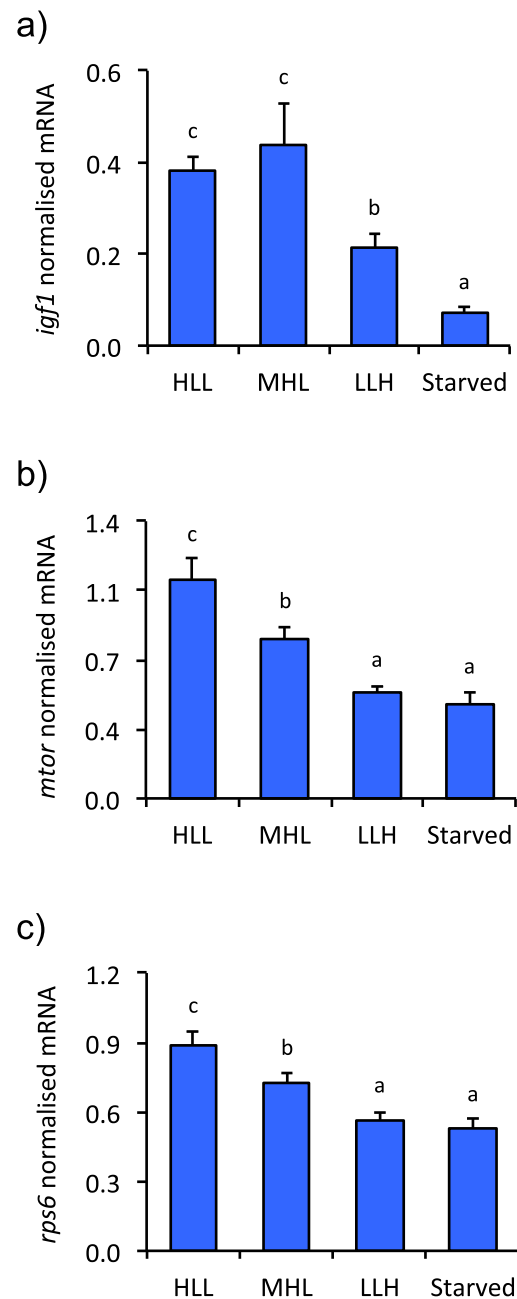


Fig. 4. Effect of diet composition and starvation on the expression of *igf1*, *mtor* and *rps6* in the skeletal muscle of *S. aurata*. During 37 days, *S. aurata* juveniles were starved or fed with HLL, MHL and LLH diets. RT-qPCR analysis of *igf1* (a), *mtor* (b) and *rps6* (c) expression in the skeletal muscle is shown. The mRNA levels for each gene were normalised with the geometrical mean of *S. aurata* *18 s* and *ef1a*, which were used as housekeeping genes. Results are presented as mean \pm SEM ($n = 4-6$ fish). Different letters above deviation bars indicate significant differences ($P < 0.05$) among conditions.

4. Discussion

Substitution of dietary protein by cheaper and sustainable nutrients is a challenging question in fish nutrition (Panserat et al., 2019). The results of this study showed that partial substitution of fish meal by carbohydrates had a strong negative impact on growth performance of *S. aurata* and nutrient retention parameters such as body weight, SGR and FCR. On the contrary, the supply of medium protein, high lipid, low carbohydrate diets (MHL) improved growth performance, although at

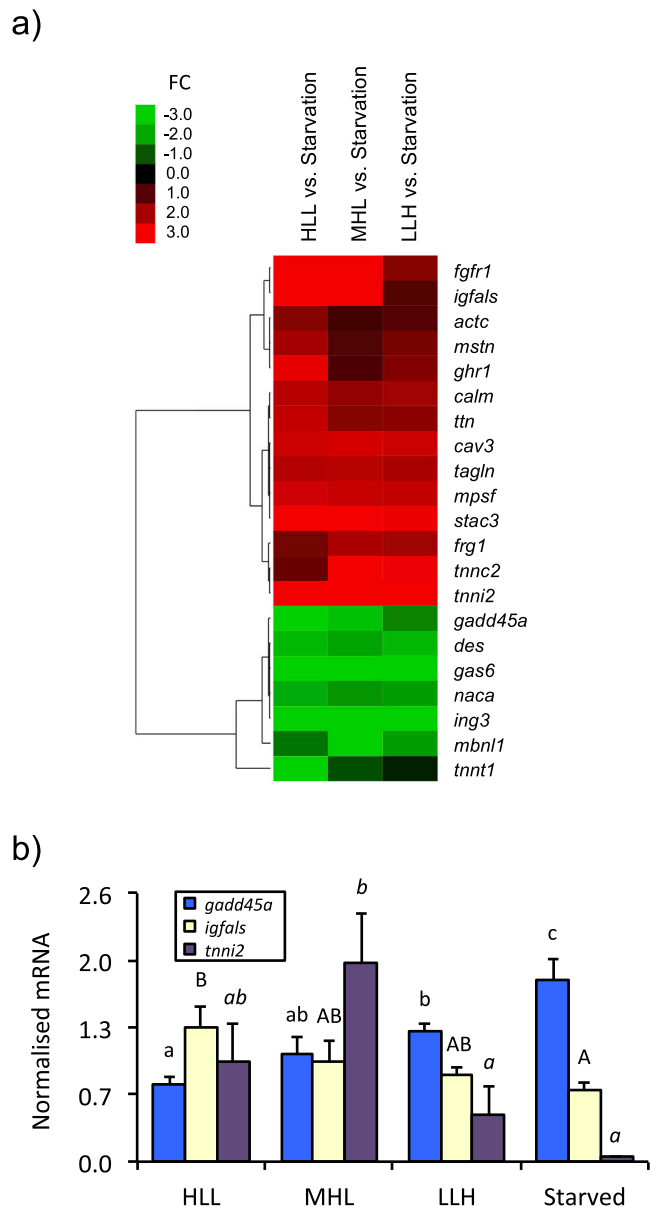


Fig. 5. Effect of diet composition and starvation on the expressional pattern of genes involved in skeletal muscle function, differentiation and growth, and validation of the expression of *gadd45a*, *igfals* and *tnni2*. (a) Heat map image of differentially transcribed genes involved in skeletal muscle function, differentiation and growth regulation. Three groups of fish were fed 23 days at a daily ration of 25 g/kg body weight with diets HLL, MHL and LLH, respectively. A fourth group of animals was submitted to starvation for the same period. Hierarchical clustering of differentially expressed genes in the skeletal muscle is represented from microarray data obtained from *S. aurata* fed with diets HLL, MHL and LLH versus starved fish, with an adjusted P value <0.05 and a difference of at least 2-fold in the normalised intensity ratio (Cy5/Cy3 or Cy3/Cy5) between two or more conditions. Results are presented as fold change (FC) mean value ($n = 4$ fish). Green color denotes downregulated genes and red color upregulated genes in fed animals. (b) RT-qPCR analysis of *gadd45a*, *igfals* and *tnni2* mRNA levels in the skeletal muscle of *S. aurata* following 37 days of starvation or feeding with diets HLL, MHL and LLH at a daily ration of 25 g/kg body weight. The mRNA levels for each gene were normalised with the geometrical mean of *S. aurata* 18 s and *ef1 α* , which were used as housekeeping genes. Results are presented as mean \pm SEM ($n = 4$ –5 fish). Different letters above deviation bars indicate significant differences ($P < 0.05$) among conditions for a given gene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

levels not far from those observed in fish fed high protein, low lipid, low carbohydrate diets (HLL). Increased PER values in fish fed MHL are in agreement with the increased weight gain exhibited by this group of fish. Consistent with our findings, diets with 54% of protein and 18% of carbohydrate, and therefore with macronutrient composition similar to MHL, are considered optimal to improve growth and nutritional parameters such as body weight and SGR in *S. aurata* (Fernández et al., 2007). A similar behaviour was observed in other fish species such as *Pagrus pagrus* (Schuchardt et al., 2008) and *O. mykiss* (Kamalam et al., 2012; Alami-Durante et al., 2019). A trend to present lower values for Ca, C and dry matter was observed in the group of fish fed LLH. Indeed, ADC levels for Ca, C and dry matter in turn showed a trend to correlate positively with final weight and negatively with FCR. Low tolerance of carnivorous fish to glucose (Polakof et al., 2012; Rashidpour et al., 2019), may determine the poor growth performance observed in fish fed the LLH diet.

Our observations also agree with the fact that long-term starvation and the supply of low protein, high carbohydrate diets in *S. aurata* affect similarly the expression of appetite-regulating peptides, leading to opposite effects than high protein, low carbohydrate diets on the expression of orexigenic and anorexigenic peptides (Babaei et al., 2017). Indeed, transcriptomic analysis revealed that both food deprivation and the supply of low protein, high carbohydrate diets also promoted similar effects on the expression levels of genes involved in mitochondrial oxidative phosphorylation in the skeletal muscle of *S. aurata* (Silva-Marrero et al., 2017).

In teleost fish, myoblast proliferation and hyperplasia occur mainly during the swim-up fry stage and through juvenile growth with a large increase in the number of white muscle fibres (Rowlerson and Veggetti, 2001). Herein, MRFs were measured to analyse myoblast proliferation and differentiation in *S. aurata* juveniles. Starvation downregulated *myod2*, *myf5*, *myog* and *myf6* in the skeletal muscle of *S. aurata*, which confirms previous observations in this species (García De La Serrana et al., 2014; Lavajoo et al., 2020). The strong reduction observed in the expression of *myod2* and *myf5* suggests that long-term starvation may promote muscular atrophy. Among fish fed different diets, the mRNA levels of *myod2* and to a lesser extent *myog* were more sensitive to the dietary protein to carbohydrate ratio than *myf5* and *myf6*. Therefore, *myod2* and *myog* mRNA levels significantly increased in fish that exhibited better growth performance (fish fed high or medium protein, low carbohydrate diets, MHL and HLL). However, low *myod2* mRNA levels in the skeletal muscle of fish fed LLH indicate that *myod2* can be used as sensitive marker of growth performance in *S. aurata* juveniles. In this regard, Igf-dependent expression of MRFs seems to play an important role in muscle differentiation and proliferation (Vélez et al., 2016). Indeed, in the present study, the expression of *igf1* positively correlated with growth rate and was highly dependent on nutritional status and diet composition. Consistent with our findings and the key role exerted in muscle growth, *igf1* was shown to stimulate growth and proliferation of *S. aurata* cultured myocytes (Vélez et al., 2014), while starvation markedly decreases *igf1* expression and Igf1 circulating levels in this species (Lavajoo et al., 2020; Metón et al., 2000; Pérez-Sánchez et al., 1995).

Upregulation of *mstn* expression inhibits cell growth and differentiation in the skeletal muscle of rainbow trout (Seilliez et al., 2012). Furthermore, high glucose supplementation inhibits protein synthesis in primary cultured muscle cells of olive flounder (*Paralichthys olivaceus*) by a mechanism involving downregulation of MRFs such as *myod* and *myog*, inhibition of Mtor signaling pathway and upregulation of *mstn1* (Liu et al., 2021). Similarly, *mstn* mRNA levels among fed fish were herein markedly higher in the skeletal muscle of *S. aurata* fed LLH, which was the diet that promoted the lowest growth performance. On the contrary, the lowest *mstn* expression was found in fish fed diet MHL, which in turn promoted better growth parameters. Therefore, downregulation of *mstn* in fish with enhanced growth performance may prevent *mstn*-dependent negative regulation of growth and induction of

muscle tissue hypertrophy. Consistent with this hypothesis, downregulation of *mstn* strongly enhances hyperplasia, hypertrophy and body size in zebrafish (*Danio rerio*) (Fuentes et al., 2013; Rossi and Messina, 2014). The *mstn* mRNA levels in *S. aurata* submitted to long-term starvation were even significantly lower than those presented by fish fed LLH. In this regard, *mstn* expression in food-deprived fish seems to depend on the species and the starvation period. For instance, 30 days of starvation upregulates *mstn* in the muscle of *S. aurata* (García De La Serrana et al., 2014), while 28 days and 10 weeks of starvation did not significantly affect *mstn* expression in the muscle of adult tilapia and rainbow trout, respectively (Chauvigné et al., 2003; Rodgers et al., 2003). Specific adaptations and dietary conditions previous to starvation may explain variations in *mstn* expression. Fst is a potent antagonist of Mstn (Amthor et al., 2004). Overexpression of *fst* increases muscle mass in rainbow trout through enhanced muscle hypertrophy and hyperplasia (Medeiros et al., 2009). Our findings support that *fst* may have a major role in regulating muscle growth in *S. aurata*. Accordingly, *fst* expression showed strong dependence on dietary macronutrient composition and was significantly downregulated by supplying low protein, high carbohydrate diets (LLH).

In agreement with previous results in *S. aurata* (Lavajoo et al., 2020), muscle mRNA levels of *mtor* and *rps6* decreased upon starvation. The results of the present study indicate that the Mtor signaling pathway may be activated in fish fed high or medium protein, low carbohydrate diets. Our findings are supported by the fact that Mtor activation triggers biological responses such as cell proliferation and contribution to muscle hypertrophy (Glass, 2003a, 2003b). In agreement with this hypothesis, fish fed MHL and HLL exhibited better growth performance. Given that Akt phosphorylation is induced by growth factors, such as Igf1 (Glass, 2005; Vélez et al., 2016), upregulation of *igf1* in fish fed MHL and HLL would facilitate Pi3k phosphorylation and induction of the Igf1/Pi3k/Akt signaling cascade, whose components are considered key mediators in muscle mass increase (Stitt et al., 2004). Consistently, the combined action of Igf1 and amino acids stimulated Mtor and Akt activity in *S. aurata* myocytes (Vélez et al., 2014), and low protein diets diminished growth performance and Mtor signaling pathway in yellow catfish (*Pelteobagrus fulvidraco*) by downregulating the mRNA levels of key genes such as *igf1*, *mtor* and *akt* (Qin et al., 2019).

A microarray analysis performed on RNA isolated from skeletal muscle of *S. aurata* allowed us to address the effect of nutritional status and diet composition on muscle expression pattern and identify novel gene markers of interest for nutritional studies and diet formulation. In general, feeding stimulated the expression of genes involved in growth, development and muscle function, while starvation upregulated genes implicated in growth regulation and arrest. Some of the differentially expressed genes were also dependent on dietary macronutrient composition. Remarkably, dietary carbohydrates, protein and lipids specifically upregulated the mRNA levels of *gadd45a*, *igfals* and *tnni2*, respectively, in the skeletal muscle of *S. aurata*, suggesting that their expression level can be used as marker for monitoring the effect of changes in dietary macronutrient composition and feeding regime in cultured fish. Indeed, the expression of *gadd45a* negatively correlated with final body weight and SGR, which suggests that *gadd45a* can be also used as sensitive marker of growth performance.

Gadd45a is a ubiquitously expressed protein belonging to the growth arrest and DNA damage 45 stress sensor gene family and is involved in cell response to DNA-damaging agents and growth cessation signals, such as starvation, by mediating cell cycle arrest and inhibition of cell entry into S phase (Salvador et al., 2013). Downregulation of *gadd45a* in the skeletal muscle of fed fish, particularly those supplied with high or medium protein diets (HLL and MHL) agrees with reports indicating increased *gadd45a* mRNA levels in human and rat muscle under starvation (Bongers et al., 2013; Ibrahim et al., 2020; Wijngaarden et al., 2014). Regulation of *gadd45a* expression by dietary macronutrients in fish remains largely unknown. However, a number of evidences point to *gadd45a* as sensitive stress sensor gene also in fish. Exposition to

cyanobacterial extracts, cadmium and DNA damage by acute exposure to hydrogen peroxide upregulate *gadd45* in zebrafish (Chen et al., 2014; Falfushynska et al., 2021; Reinardy et al., 2013). Moreover, the expression of *gadd45* increases in response to low temperature in rainbow trout, kelp grouper (*Epinephelus moara*) and large yellow croaker (*Larimichthys crocea*) (Borchel et al., 2017; Chen et al., 2020; Qian et al., 2020). Furthermore, the pattern of expression of *gadd45a* in tilapia (*Oreochromis* spp.) after infection with *Streptococcus agalactiae* and in grass carp (*Ctenopharyngodon idella*) following challenge with *Aeromonas hydrophila* suggests involvement of *gadd45a* in fish immunity against bacterial infection (Fang et al., 2018; Shen et al., 2016). In the present study, restricted-protein feeding (LLH diet) upregulated *gadd45a* to values not far from those observed in starved fish. Since *gadd45a* is critical mediator of muscle atrophy (Bongers et al., 2013; Ebert et al., 2012), the results of the present study suggest that protein restriction may shrink muscle growth in *S. aurata*.

Igfals encodes the insulin-like growth factor-binding protein complex acid labile subunit, which stabilises blood levels of Igf1, Igf2 and Igfbp proteins by binding binary complexes formed by Igf1 or Igf2 with Igfbp-3 or Igfbp-5 (Domené and Domené, 2020; Hwa et al., 2021). In the present study, changes in nutritional status and diet composition caused similar effects on *igfals* and *igf1* mRNA levels. Starvation downregulated *igfals*, while among fed fish the highest *igfals* expression was observed in *S. aurata* fed HLL. Our findings are consistent with reports in mammals showing that food deprivation reduces *igfals* expression and serum levels (Frystyk et al., 1999; Kong et al., 2002), while the supply of high protein diets and overfeeding increases Igfals circulating level (Khan et al., 2014; Rubio-Aliaga et al., 2011). Given that Igfals is well-established for its role in binding to Igf-Igfbp complexes and prolonge their half-life in serum (Boisclair et al., 2001), downregulation of *igfals* in fish fed low protein, high carbohydrate diets (LLH) would promote insulin insensitivity and growth impairment. Hence, Igfals deficiency decreases circulating levels of Igf and Igfbp proteins, leading to insulin insensitivity, growth impairment and puberty delay in humans (Domené and Domené, 2020; Hwa et al., 2021).

Tnni2 encodes the fast skeletal isoform of troponin I, which acts as the inhibitory subunit of the troponin complex during muscle contraction (Fu et al., 2009; Sheng and Jin, 2016). Knowledge of the effect of nutritional status and diet composition on *tnni2* expression in fish is scarce. However, our findings are consistent with previous observations that indicate downregulation of troponin in skeletal muscle of mandarin fish (*Siniperca chuatsi*) submitted to starvation (Liu et al., 2020). Accordingly, Lu et al. reported downregulation of troponin I in muscle of slow-growing grass carp (*C. idella*) (Lu et al., 2020). Conceivably, given that *S. aurata* is a carnivorous fish, starvation would promote the use of muscle protein as the major source of energy, while impairing muscle synthesis of proteins and contraction. However, the supply of dietary protein would avoid muscle breakdown, cessation of muscle contraction and facilitate muscle growth. Moreover, the fact that the highest *tnni2* expression was found in fish fed MHL suggests that dietary lipids could contribute to *tnni2* upregulation. In this regard, maternal dietary linoleic acid supplementation promotes muscle fibre transformation to type I fibre in a process that involves troponin I upregulation in the muscle of suckling piglets (Lu et al., 2017). Further studies are required to explore the hypothesis that the fatty acid profile of the diet can affect *tnni2* expression in fish.

5. Conclusions

The results of the present study show that the expression of MRFs and key genes in muscle growth and differentiation was markedly affected by nutritional status and dietary macronutrient composition in the skeletal muscle of *S. aurata*. In addition to the skeletal muscle mRNA levels of *myod2*, *fst*, *igf1*, *mtor* and *rps6*, our findings let us to report for the first time *gadd45a* and *igfals* as useful markers to study the effect of changes in feeding regime and diet composition on growth performance in fish.

Author contributions

Conception and design: Isidoro Metón, Isabel V. Baanante and Felipe Fernández. Material preparation, data collection and analysis were performed by Alberto Sáez-Arteaga, Yuanbing Wu, Jonás I. Silva-Marrero, Ania Rashidpour and María Pilar Almajano. Writing and original draft preparation: Isidoro Metón and Alberto Sáez-Arteaga. All authors read and approved the final manuscript.

CRedit authorship contribution statement

Alberto Sáez-Arteaga: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Yuanbing Wu:** Investigation, Formal analysis, Writing – review & editing. **Jonás I. Silva-Marrero:** Investigation, Formal analysis, Writing – review & editing. **Ania Rashidpour:** Investigation, Formal analysis, Writing – review & editing. **María Pilar Almajano:** Investigation, Formal analysis, Writing – review & editing. **Felipe Fernández:** Conceptualization, Methodology, Writing – review & editing. **Isabel V. Baanante:** Conceptualization, Methodology, Writing – review & editing. **Isidoro Metón:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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