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Effects of Short-Term Intermittent Fasting on Growth Performance, Fatty Acids Profile, Glycolysis and Cholesterol Synthesis Gene Expression in European Seabass *Dicentrarchus labrax*

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Abstract: The present study was applied to evaluate the effects of alternate feeding and feed restriction on gene expression, growth, proximate composition and biochemical indices in European seabass, *Dicentrarchus labrax*. Fish were randomly divided into six indoor tanks with 90 fish per tank in a recirculating aquaculture system. Two feeding strategies were applied, in which the first group was fed daily to satiation and the second was intermittently fed (8 days feeding to satiation–2 days starvation) for 40 days. At the end of the experiment, outlier fish were sorted as fast growers (FG) and slow growers (SG) according to their final body weight. The differential gene expression tested was related to glycolysis (*pk*, *ldha*, *hk*, *g3pdh*, *eno1* and *alda*), fatty acid metabolism (*lpl* and *acc*) and cholesterol synthesis (*7dhcr* and *sqle*). In addition, muscle *ldha* and *gpi* expressions were positively correlated with fish weight. The concentrations of glucose, triglycerides, cholesterol and non-esterified fatty acids (NEFA) were not affected by the dietary treatments. Glucose and NEFA differed significantly between SG and FG fed groups. Overall, the physiological responses of glucose and fatty acid metabolism in fish, as recorded by gene expression assays, were triggered by minor interventions in feeding rather than the different growth rates. Expression of specific genes and biochemical parameters could be used as potential biomarkers to improve aquaculture practices and benefit fish husbandry through selective breeding, feeding strategies and farm management. The study provides new insights on the impact of intermittent feeding of European seabass, with gene markers and their potential effects, for European seabass aquaculture.

Keywords: *Dicentrarchus labrax*; intermittent feeding; gene expression; growth; body composition; biochemical indices

Key Contribution: Our study complements the expanding research regarding the potential effects of feed restriction on gene expression related to the nutritional status of European seabass. Quantification of specific genes and certain biochemical parameters may identify potential biomarkers to assess dietary status and/or fish growth.

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1. Introduction

In the wild, fish regularly must deal with a lack of food and, consequently, have evolved mechanisms of coping with limited food availability. Most fish species can survive periods of fasting by altering their resting metabolic rate [1]. When food is limited, fish

tend to reduce their physiological activity, which is upregulated again when there is an abundance of nourishment [2]. Feed restriction is tested in aquaculture settings to reduce feed utilization without compromising production by leveraging physiological adaptations of fish [3–7].

Fish nutrition plays a key role in the production cycle as it constitutes the most important growth factor and is the major operating cost of the aquaculture production. Enhanced growth has been targeted by aquaculture for many years; for example, fasting and refeeding regimes, which have been used to accelerate growth [8], have been extensively reviewed [9]. Compensatory growth, during a specific period of time, is considered significantly faster than the growth rate of fish that have not experienced feed deprivation [10]. Intermittent fasting has been proposed for achieving compensatory growth in various economically important fish species such as the Atlantic salmon (*Salmo salar*) [11,12], rainbow trout (*Oncorhynchus mykiss*) [10], Nile tilapia (*Oreochromis niloticus*) [13], European seabass (*Dicentrarchus labrax*) [3,4,6], gilthead seabream (*Sparus aurata*) [14,15], gibel carp (*Carassius auratus gibelio*) and Chinese longsnout catfish (*Leiocassis longirostris*) [16].

The nutritional status of fish influences the muscle growth pathways. Low food intake impairs growth proportionally to deprivation intensity and duration of fasting. Growth is regulated by hormones such as growth hormone (GH) in the central nervous system, which, in turn, controls feeding behavior [17]. In addition, many other physiological and environmental factors, such as fish density, food intake, temperature, diet formulation, size or sex, moderate the growth rate. During fasting, the catabolism of lipids and the release of fatty acids are of paramount importance for fish to cope with restriction in food intake. These fatty acids are transported across the plasma membrane into different tissues for oxidation or storage. *Ad libitum* feeding of fish tends to increase lipid accumulation in the viscera, liver and muscle, which are mobilized during fasting [18].

The role of many interacting environmental and genetic factors can explain the physiological routes of fish growth. An analytical approach to mRNA levels provides important and useful insights into growth performance in fish given that expression of specific genes could affect fish muscle growth [19]. Expression levels of specific mRNAs can explain variation in growth rates and therefore can be used as valid biomarkers for fish selection. Although fish husbandry could have an effect on gene expression, the basal regulation of various genes is strongly correlated with productive traits [20]. For example, in rainbow trout selected for fat muscle content, lipogenic genes were both affected by refeeding and selection [21]. Furthermore, muscle recovery was correlated with upregulation of genes related to RNA processing, translation and maturation of proteins, ribosome biogenesis, cell proliferation and mitochondrial bioenergetics at an early stage, while, in the later phase, several genes regulating Golgi and reticulum dynamics and genes involved in muscle remodeling were induced [22]. In European seabass, fasting activates lipolytic genes in the adipose tissue, liver and muscle; to the contrary, lipogenesis is downregulated in the liver and adipose tissue. Phospholipid- and oxidative-metabolism-related genes are differentially expressed in the liver and the skeletal muscle of fasted European sea bass, and their regulation returns back to normal 12 days after refeeding [23]. Fasting affects growth through direct inhibition of the GH/IGF axis, by decreasing IGF-I and IGF-II mRNA levels, which are restored during refeeding [24].

European seabass, *Dicentrarchus labrax*, is an important Mediterranean aquaculture species and is considered of great commercial importance [25]. European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), with estimated productions of, respectively, 208,000 and 200,000 tons per year, are the most important aquaculture finfish species of the Mediterranean Sea [26]. European seabass accounts for 30% of the value of all the finfish species produced in the Mediterranean [27]. The present study aims to identify biochemical markers and target genes regulated by growth and nutritional status in short-term intermittent feeding of European seabass that can be used as potential biomarkers for breeding programs.

2. Materials and Methods

European seabass (*Dicentrarchus labrax*) juveniles were transferred from the fish farm Ecloserie Marine de Gravelines (Gravelines, France) to the CARUS research facility of Wageningen University and Research in the Netherlands. Fish were acclimatized to laboratory conditions before the start of the trial and fed on formulated diets (Altech Coppens, Helmond, The Netherlands) to fully satisfy the known nutritional requirements of the species. After acclimatization, 540 graded fish weighing 87.5 ± 16.2 g were uniformly dispersed to six cylindrical tanks of 1000 L in a recirculating aquaculture system (RAS). More specifically, fish were randomly distributed into two groups with three replicate tanks per group. Fish were hand fed with commercial 3 mm pellets twice daily (09:00 and 16:00). Fish of the first group (fed) were fed continuously to apparent satiation for 40 days, whereas the second group was fed in a feeding–fasting scheme (fasted) of 80–20% (2 days of fasting followed by 8 days of refeeding to apparent satiation) for 40 days. Water quality parameters were monitored and controlled, including NH_4^+, NO_2^-, NO_3^-, dissolved oxygen, temperature, salinity and pH. Fish were kept under a controlled light regime (12:12 h light/dark) at 21 °C. During sampling, fish were graded in two groups according to their final weight (FG—fast growers and SG—slow growers from both fed and fasted groups) and sacrificed by phenoxyethanol overdose (1 ppt). Blood from the caudal vein was drawn with heparinized syringes and centrifuged (4000 rpm for 10 min at 4 °C), and the plasma was separated and stored at −80 °C for biochemical analysis. Tissues from the liver and the white muscle were dissected and stored in RNAlater (Thermofisher Scientific, MA, USA) at −20 °C for downstream analysis. Whole carcasses were stored at −20 °C for proximate composition analysis and fatty acid analysis.

Growth parameters were calculated according to the following formulas:

Weight gain rate (WGR, %) = 100 × (final body weight-initial body weight)/initial body weight;

Specific growth rate (SGR, % day−1) = 100 × (Ln final body weight − Ln initial body weight)/number of days;

Condition factor (CF, g/cm^3) = 100 × (body weight, g)/(body length, cm^3);

Hepatosomatic index (HSI, %) = 100 × (liver weight, g)/(whole body weight, g);

Viscerosomatic index (VSI, %) = 100 × (viscera weight, g)/(whole body weight, g);

Intestine somatic index (ISI, %) = 100 × (intestine weight, g)/(body weight, g);

Spleen somatic index (SI, %) = 100 × (spleen weight, g)/(whole body weight, g);

Intraperitoneal fat rate (IFR, %) = 100 × (intraperitoneal fat weight, g)/(whole body weight, g).

Whole carcasses of nine fish per group were ground prior to proximate analysis, and moisture content was analyzed by drying the samples to a constant weight at 105 °C for 24 h. Crude fat was determined by the Soxhlet method, based on the extraction of the lipids, using a Soxtherm Multistat/SX PC (Sox-416 Macro, Gerhard, Germany) (AOAC, 1996). Crude protein content was determined using Kjeldahl method by measuring total nitrogen (N × 6.25; Behr Labor-Technik, Germany). To determine ash quantity, samples were placed in a muffle furnace (Nabertherm L9/12/C6, Lilienthal, Germany) at 600 °C for 5 h. Gross energy was assessed adiabatically utilizing an IKA oxygen bomb calorimeter (C5000, IKA Werke, Staufen, Germany).

Serum glucose, cholesterol, triglycerides, non-esterified fatty acids (NEFA) and lactate dehydrogenase (LDH) activities were measured colorimetrically according to standard commercial protocols. Glucose (Cayman Chemical Company, Ann Arbor, MI, USA) values were extracted from absorbance at 505 nm using a standard curve in the range of 0–25 mg/dL. Triglycerides (Cayman Chemical Company) were quantified at 540 nm. LDH activity (Abcam, Cambridge, UK) was measured at 450 nm in a kinetic mode at 37 °C for 30 min while protected from light. Serum cholesterol was quantified with a commercial fluorometric assay kit (BIOSIS Ltd., Athens, Greece) with absorbance wavelength at 510 nm according to the manufacturer's instructions. NEFA were measured enzymatically (Instruchemie B.V., Delfzijl, the Netherlands) at 540 nm at 37 °C for 4.5 min. All biochemical

analyses were carried out with a BMG Fluostar Omega microplate reader with different set-ups. To determine fatty acids profiles, the Folch extraction method was used, followed by esterification and separation using a capillary column in an Agilent GC 6890.

Levene and Shapiro–Wilk tests were used to assess homogeneity of variance and normality, respectively. Data were tested with nested ANOVA where growth (random factor) was nested within treatment (fixed factor).

Total RNA was extracted with Nucleospin®RNA (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Isolated RNA quantity and quality were determined via spectrophotometry, using a Quawell Q5000 UV–Vis spectrophotometer and on a 1% denaturing agarose gel, respectively. For cDNA synthesis, 500 ng of RNA was reverse transcribed with PrimeScript RT Reagent Kit according to the manufacturer's instructions, diluted 10-fold and stored at −20 °C. The qPCR analysis was carried out with RT^2 SYBR Green qPCR Mastermix (Qiagen) in a Rotor-Gene Q 5plex using 1ml of cDNA per reaction and 300 nM of each primer in a final volume of 10 mL. The qPCR assay was designed for the profiling of six glycolytic genes (*hk*, *gpi*, *eno1*, *g3pdh*, *alda*, *pk),* two genes related to fatty acid metabolism (*lpl* and *acc*) and two genes involved in cholesterol biosynthesis (*sqle*, *7dhcr*). The primers designed for the qPCR experiment are presented in Table 1. β-actin (*actb*) and elongation factor 1 (*ef1*) were used as reference genes (geometric mean). Each sample was analyzed for primer dimer, contamination or mispriming with melting curve analysis and 2.5% agarose gel electrophoresis. Relative gene expression was determined with the Pfaffl method (REST) [28], and fold change (FC) values were transformed to log2FC.

Table 1. Primers specifications for qPCR analysis.

Pathway	Gene Name	NCBI Acc. No.	Primer Name	Primer Sequence (5′–3′)	Tm (°C)	Product Length (bp)
Glucolysis	L-lactate dehydrogenase A chain	CBXY010014016	LDHAF	TTGGCCTTAACTCAGCCTGT	60	86
			LDHAR	ATACAGTACACAGAGTATAT	58.5	
Glucolysis	Hexokinase I	CBXY010013088	HKF	GATGAGTGCTGCTCCTTTCC	60	96
			HKR	GTCTCTGTCTAGTTTCTCTG	61	
Glucolysis	Glucose-6-phosphate isomerase	CBXY010012918	GPIF	CTCACACAGGACCCCAACTT	60	87
			GPIR	TTGTTGAATCTCTCTTTGTCA	59	
Glucolysis	Enolase 1	CBXY010009925	ENO1F	AGATCGTCATTGGCATGGAT	60	85
			ENO1R	AGGGGAGATGTAGCGGCTGG	60	
Glucolysis	Glyceraldehyde-3-phosphate dehydrogenase	CBXY010005389	G3PDHF	TGTAACCCAGCACTCCCTTC	60	84
			G3PDHR	GTGGACCTGACATGCCGTCT	61	
Glucolysis	Aldolase A	CBXY010008418	ALDAF	CTGTCCGACCACCATGTCTA	60	80
			ALDAR	GATCTCCTGGTTGCTGTACT	60	
Glucolysis	Pyruvate kinase	KF857578	PKF	GGCGTTCAGAATTTTGAGGA	60	109
			PKR	TTGCAGCGTCCAATCATCAT	60	
Fatty acid metabolism	Lipoprotein lipase	AM411614	LPLF	GTAACGGGGATGTTCGAGAG	59	89
			LPLR	CTGGTTGGCGCGGGTCAGCC	59	
Fatty acid metabolism	Acetyl-CoA carboxylase	CBXY010003615	ACCF	AGTACCTGCACAGCCAGGAT	60	83
			ACCR	GCAAGTTGACATCAGCCACC	60	
Cholesterol biosynthesis	Squalene epoxidase	CABK01002385	SQLEF	GAATCGACCGTGATGGAAAG	60	112
			SQLER	AGGGTCTGGATGCCCATCTG	60	
Cholesterol biosynthesis	7-dehydrocholesterol reductase	CBXY010012845	7DHCRF	TCGGCCACATACTCCCATAC	60	116
			7DHCRR	GGTAAGGCACAGTGTCTGTG	60	
Reference gene	Beta-actin	AY148350	ACTBF	ATCAAGATCATTGCCCCACCT	63	92
			ACTBR	TCATACTCCTGCTTGCTGA	59	
Reference gene	Elongation factor 1	AJ866727	EF1F	CGCTCTGTGGAAGTTTGAGA	59	102
			EF1R	GATCAGCACAGCGCAGTCAG	61	

3. Results

3.1. Growth Performance and Proximate Composition

Fasted and fed fish exhibited a feeding rate of 2.5 ± 0.1% and 2.9 ± 0.1%, and the FCR was calculated at 2.98 ± 0.2 and 3.23 ± 0.3, respectively. Growth performance body indices and proximate composition of the European seabass fed with two feeding regimes and divided into weight groups (FG and SG) are presented in Table 2. The experimental feeding regime did not significantly affect final body weight of either FG or SG groups. In general, all the somatometric indices were not significantly affected by short-term fasting, except

for the ISI. In addition, CF and IFR were substantially higher in the FG group than the SG group ($p < 0.001$), in both fasted and fed fish. Fasting did not affect proximate composition.

Table 2. Growth parameters and proximate composition of the experimental groups. Values represent mean ± standard error ($n = 9$). Nested ANOVA. T: treatment, G: growth. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

	SG		FG		T	G
	Fed	Fasted	Fed	Fasted		
Final Weight (g)	73.08 ± 4.12	84.43 ± 5.00	163.88 ± 1.80	164.57 ± 2.30	ns	***
Final Length (cm)	20.26 ± 0.09	19.77 ± 0.09	23.18 ± 0.07	22.68 ± 0.08	ns	***
WGR (%)	42.1 ± 2.06	39.70 ± 1.79	47.53 ± 0.60	47.20 ± 0.30	ns	*
SGR (% day^{-1})	0.86 ± 0.04	0.83 ± 0.04	0.97 ± 0.01	0.97 ± 0.005	ns	**
CF	1.04 ± 0.06	1.18 ± 0.07	1.35 ± 0.03	1.41 ± 0.03	ns	***
HSI (%)	0.48 ± 0.14	0.51 ± 0.09	0.76 ± 0.09	0.58 ± 0.08	ns	ns
VSI (%)	2.56 ± 0.15	2.72 ± 0.22	2.50 ± 0.10	2.64 ± 0.18	ns	ns
ISI (%)	1.56 ± 0.16	0.9 ± 0.11	1.41 ± 0.06	1.05 ± 0.06	*	ns
SSI (%)	0.12 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	ns	ns
IFR (%)	1.65 ± 0.67	2.91 ± 0.67	5.5 ± 0.45	5.37 ± 0.63	ns	***
Moisture (%)	68.15 ± 1.71	60.73 ± 2.68	50.53 ± 1.13	60.60 ± 1.35	ns	***
Crude protein (%)	54.77 ± 2.54	49.33 ± 2.86	41.37 ± 0.98	41.06 ± 3.92	ns	***
Crude lipid (%)	25.72 ± 2.61	30.86 ± 2.60	32.36 ± 0.84	33.03 ± 1.28	ns	*
Ash (%)	14.51 ± 1.04	11.39 ± 1.41	7.51 ± 0.30	11.21 ± 0.96	ns	***
Gross energy (KJ/g)	22.40 ± 0.57	23.38 ± 0.72	21.94 ± 0.28	24.14 ± 0.53	ns	ns

All fasted fish, both SG and FG, had similar ($p > 0.05$) proximate composition, but moisture, crude protein, crude lipid and ash contents were significantly increased in SG compared to FG fish.

3.2. Biochemical Parameters

The values of plasma glucose, triglycerides, cholesterol, non-esterified fatty acids (NEFA) and LDH activity are presented in Table 3. Significantly lower NEFA levels were found in FG compared to SG fish, and the levels of triglycerides and cholesterol were higher in fed fish ($p < 0.05$). On the other hand, growth did not affect their blood concentration levels.

Table 3. Biochemical indicators of the experimental groups. Values represent means ± standard error ($n = 9$). Nested ANOVA. T: treatment, G: growth. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$.

	SG		FG		T	G
	Fed	Fasted	Fed	Fasted		
Glucose (mg/dL)	16.9 ± 1.70	21.1 ± 3.20	27.6 ± 1.10	26.4 ± 3.40	ns	ns
Triglycerides (mg/dL)	238.0 ± 50.70	110.0 ± 47.5	117.3 ± 21.01	78.1 ± 14	*	ns
Cholesterol (mg/dL)	216.6 ± 20.20	161.3 ± 12	189.7 ± 23.20	157.8 ± 9.10	*	ns
NEFA (mmol/L)	8.5 ± 1.90	7.9 ± 1.80	5.9 ± 0.40	3.7 ± 0.40	ns	**
LDH (U/L)	1348.9 ± 64.60	554.6 ± 134.10	415.7 ± 127.30	541.3 ± 165	ns	ns

3.3. Fatty Acids (FA) Profiles

Nutritional status did not significantly affect FA profiles. On the other hand, growth significantly affected the FA profile since most of the FAs (10 out 16) showed different carcass concentrations between growth groups. The lipids of SG contained significantly lower proportions of 18:2n-6, 18:3n-3 and Σn-6 and higher proportions of 14:0, 18:4n-3, 20:1n-9, 22:1n-11, EPA and DHA compared to the FG (Table 4).

Table 4. Fatty acid profiles of experimental groups. Nested ANOVA. T: treatment, G: growth. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

	SG		FG		T	G
	Fed	**Fasted**	**Fed**	**Fasted**		
14:0	3.5 ± 0.18	3.07 ± 0.15	2.76 ± 0.11	2.59 ± 0.08	ns	***
16:0	17.05 ± 0.36	16.83 ± 0.21	17.64 ± 0.69	16.34 ± 0.51	ns	ns
16:1n-7	5.21 ± 0.20	4.98 ± 0.13	5.02 ± 0.20	4.55 ± 0.17	ns	ns
18:0	4.04 ± 0.11	3.87 ± 0.07	3.73 ± 0.33	3.7 ± 0.12	ns	ns
18:1n-9	25.71 ± 1.03	28.57 ± 1.31	29.69 ± 2.57	31.24 ± 1.05	ns	ns
18:1n-7	3.16 ± 0.08	3 ± 0.06	2.94 ± 0.11	2.82 ± 0.08	ns	ns
18:2n-6	9.55 ± 0.45	10.53 ± 0.52	11.8 ± 0.50	11.52 ± 0.41	ns	**
18:3n-3	2.08 ± 0.17	2.46 ± 0.19	2.93 ± 0.13	2.87 ± 0.10	ns	***
18:4n-3	0.86 ± 0.03	0.76 ± 0.02	0.73 ± 0.03	0.66 ± 0.02	ns	***
20:1n-9	3.03 ± 0.10	2.72 ± 0.12	2.64 ± 0.12	2.65 ± 0.08	ns	*
20:2n-6	0.73 ± 0.02	0.66 ± 0.02	0.69 ± 0.02	0.76 ± 0.03	ns	*
20:4n-6	0.13 ± 0.01	0.1 ± 0.02	0.11 ± 0.02	0.12 ± 0.02	ns	ns
20:5n-3	5.31 ± 0.33	4.28 ± 0.20	3.77 ± 0.16	3.44 ± 0.12	ns	***
22:1n-11	1.68 ± 0.09	1.45 ± 0.10	1.09 ± 0.11	1.19 ± 0.04	ns	***
22:5n-3	1.35 ± 0.08	1.06 ± 0.05	0.98 ± 0.08	0.89 ± 0.03	ns	***
22:6n-3	7.36 ± 0.46	5.46 ± 0.41	4.56 ± 0.17	4.17 ± 0.13	ns	***
SFA	33.45 ± 0.96	31.39 ± 0.49	31.4 ± 1.37	30.89 ± 0.77	ns	ns
MUFA	41.36 ± 0.6	43.1 ± 0.81	43.37 ± 2.07	44.67 ± 0.93	ns	ns
Σn-3	13.42 ± 0.90	12.89 ± 0.99	11.07 ± 0.63	10.3 ± 0.56	ns	*
Σn-3 HUFA	10.12 ± 0.93	9.37 ± 1.15	7.15 ± 0.61	6.54 ± 0.62	ns	**
Σn-6	11.08 ± 0.45	11.89 ± 0.48	13.27 ± 0.49	12.99 ± 0.37	ns	**
EPA + DHA	8.14 ± 0.95	7.68 ± 1.09	5.61 ± 0.61	5.12 ± 0.59	ns	*
SFA/MUFA	0.81 ± 0.03	0.73 ± 0.02	0.76 ± 0.10	0.7 ± 0.03	ns	ns

3.4. Gene Expression

Differential gene expression (log2FC) among the different treatments and fish groups is presented in Figure 1 for muscle tissue and Figure 2 for liver. In muscle, all genes demonstrated significant differential expression among fish group treatments, while, in the liver, 5 genes (*g3pdh*, *alda*, *gpi*, *hk*, *7dhcr*) out of 11 exhibited no significant difference ($p > 0.05$). Transcription levels of *g3pdh*, *alda*, *gpi* and *ldha* showed an increased trend in fed FG compared to fed SG, although such a trend was not observed for fasted fish groups. Furthermore, mRNA levels of *g3pdh*, *hk*, *pk*, *lpl*, *acc*, *sqle* and *7dhcr* were significantly different ($p < 0.05$) between fish groups with different dietary statuses (fed versus fasted), regardless of the growth group (SG or FG). In the liver, only pk was significantly upregulated ($p < 0.05$) in fed fish compared with the fasted ones, for both growth groups. *ldha*, *lpl* and *acc* mRNA levels were differentially expressed between fed and fasted fish, only for the SG group.

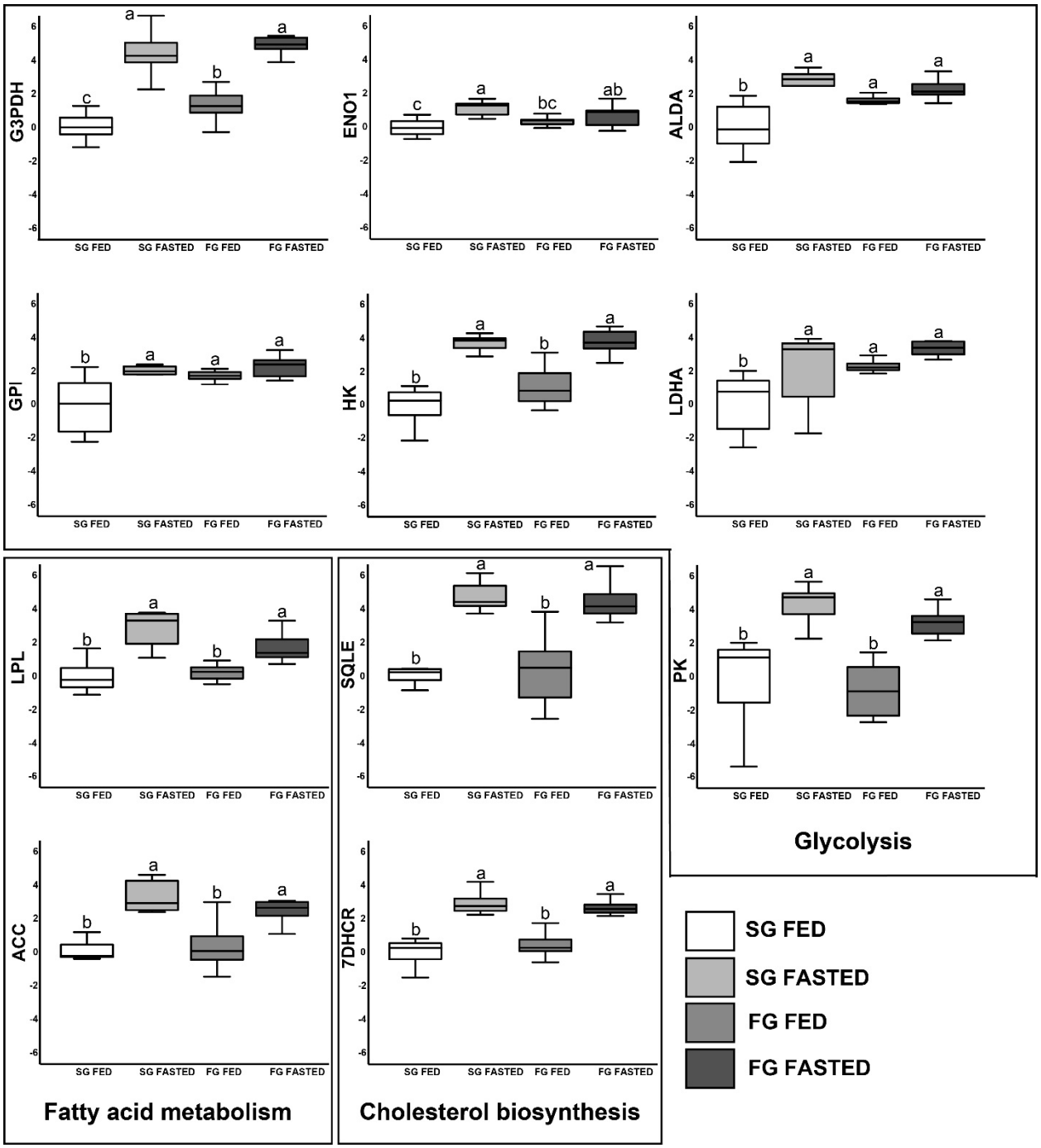

Figure 1. Differential gene expression in muscle. Different letters denote significant difference ($p < 0.05$).

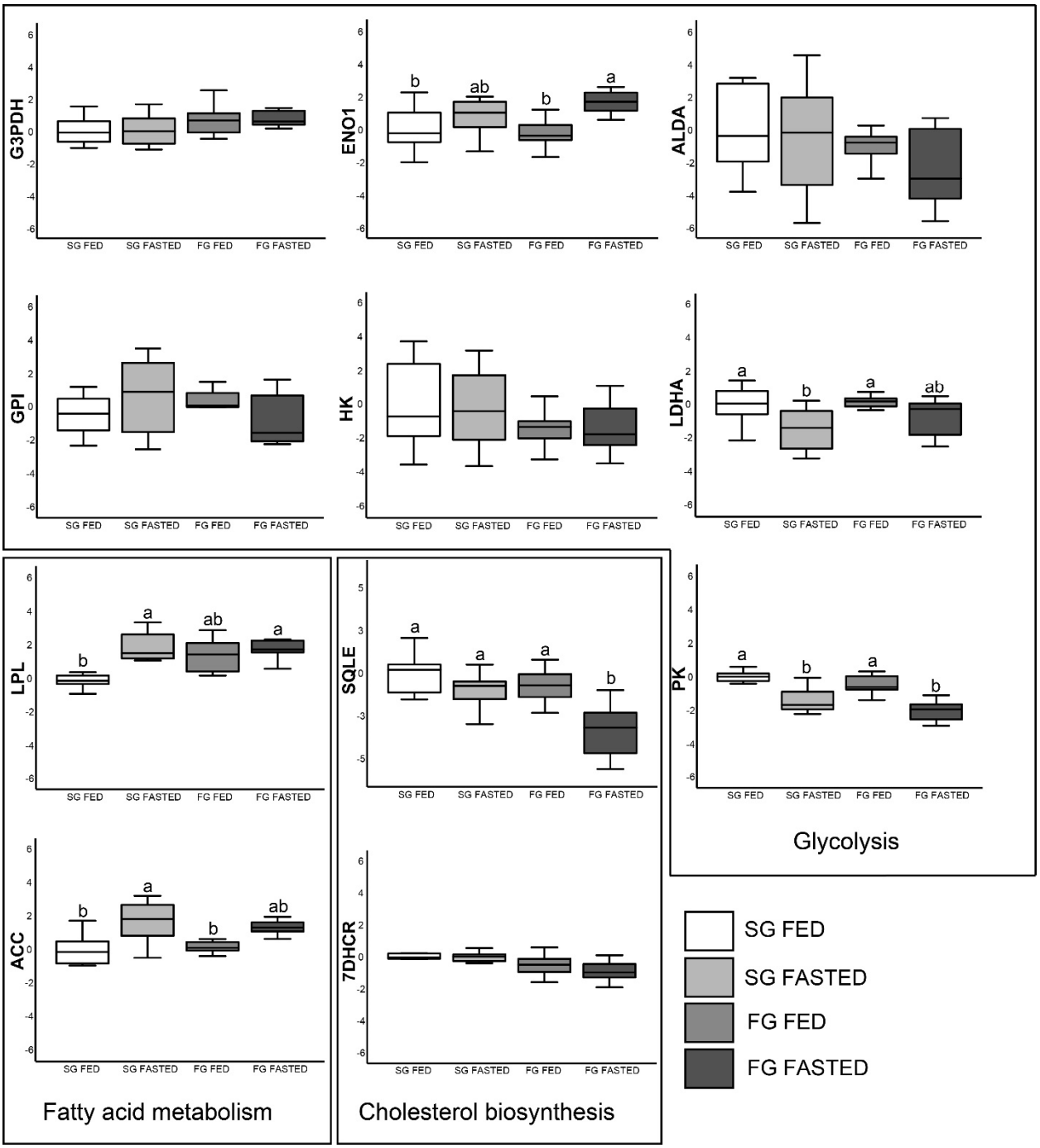

Figure 2. Differential gene expression in liver. Different letters denote significant difference ($p < 0.05$).

4. Discussion

In aquaculture, feeding strategies are of critical importance as feeds and feeding represent 57–59% of the production cost [29], and both underfeeding and overfeeding can have negative consequences for the production [30–32]. Thus, successful farming strongly relies on feed management [3]. Many feeding methods are being practiced, such as *ad libitum* feeding, restricted feeding and intermittent feeding, to achieve the best feeding scheme for each species and developmental stage [33]. A key factor in view of obtaining the desired growth compensation after feed deprivation for a specific species is to manage the appropriate duration and intensity of feed deprivation.

In the present study, the growth performance (final weight and length, WGR and SGR) within the fast growers (FG) and slow growers (SG) groups was not significantly affected by the short-term food deprivation periods. Any potential loss of weight during the 2-day (out of 10 days) fasting period was compensated during the refeeding-to-satiation periods. Under the current experimental conditions, feed restriction for 2 days followed by an 8-day refeeding period seemed to be an adequate management strategy for European seabass. Both duration and intensity of feed restriction in compensatory growth responses need to be further evaluated to be incorporated in commercial production of the species.

This short-term intermittent fasting did not have a substantial effect either on condition factors or other morphometric indices. It is noteworthy that intraperitoneal fat (IFR) was significantly higher in FG than SG, independently of the dietary regime followed. This was due to intraperitoneal fat accumulation in the FG fish, which is not a desirable trait, supported by the fact that these fish (either fed or fasted) had also an increased body lipid content, although this was not significant, compared to the SG fish. Also, no significant difference was observed in any parameter tested between fasted SG and fasted FG groups concerning the whole-body proximate composition. Similar results were reported in other studies, where there was no significant difference in the proximate composition of the fish under different feeding regimes [34] or feeding ratios/feeding rates [35–37]. In addition, in the present study, whole-body protein, lipid, moisture, dry matter and ash were greatly reduced in fed FG groups compared to fed SG. The values of protein, moisture and ash were not found to be statistically different in terms of nutritional composition, but only lipid values were affected by the feeding strategies in barramundi (*Lates calcarifer*) experiencing starvation and refeeding cycles [38]. Also, other studies reported that the body lipid content of fish decreased proportionally to feed consumption [39,40]. The proximate composition of fish generally fluctuates with food quantity, with lipid deposition likely to be increased with elevated food supply [41]. It is a fact that utilization of lipid stores is the most usual response of fish subjected to feed deprivation followed by refeeding in order to satisfy energy requirements [15,42–45]. In the present study, the deprivation period was short and did not substantially affect fatty acids (FAs) contents of the fish. FAs such as 20:1n-9 and 22:1n-11 are heavily catabolized for energy in the growth of farmed fish; therefore, FG tend to have lower amounts of these FAs. The same fact stands for EPA, which can be readily β-oxidized, and DHA, which requires peroxisomal and mitochondrial β-oxidation. Through this process, these FAs serve as energy deposits in fish if necessary for homeostasis [46].

Blood biochemical indices of reared fish are determined to evaluate the physiological status of fish [47] and to investigate conditions that might lead to adaptations due to the their nutritional status [48]. The biochemical features of fish blood could be adequate to monitor the metabolic balance and health status in intensive aquaculture conditions [49]. In this study, we used the biochemical profile of *D. labrax* to assess the physiological status during short-term restricted feeding. Differences between fed and fasted groups were not observed, a fact that supports the hypothesis that short-term intermittent fasting (2 days out of 10) does not affect fish biochemical indices in the long term. Some biochemical parameters were significantly different between SG and FG fish, and thus were related to growth, within the fasted or fed group. During short-term starvation of seabass, the prediction and prognosis of the nutritional or physiological status, measured by biochemical blood parameters, seem to have potential as useful tools which could manage and monitor feeding practices during production of the species [48,50–52].

Plasma cholesterol concentration was not affected, possibly due to the short-term nature of the fasting period, in contrast to more prolonged periods of fasting where differences in plasma cholesterol do occur [3]. LDH activity in plasma was not affected either by fasting or different growth, although it has been established that LDH activity in muscle increases in larger fish [53–58]. Although plasma LDH activity was considered as equal among groups, significant differences in plasma glucose and NEFA content between FG and SG imply growth-related divergence in glucose and lipid metabolism. LDH is a key

enzyme of anaerobic glycolysis that catalyzes pyruvate reduction to lactate and has been used as a dietary status biomarker even for wild fish populations [59].

A higher conversion rate of glucose into pyruvate in muscle to increase growth rate is depicted by increased expression of *gpi*, *alda* and *g3pdh*. In turbot, Scophthalmus maximus, a comparison between fast- and slow-growing fish revealed a set of 16 upregulated genes in muscle coding for enzymes of the glycolytic pathway [60]. Larger rainbow trout individuals possessed many transcripts with functions in glycolysis and gluconeogenesis, as well as lipid metabolism, compared to smaller individuals [61]; this upregulation has been associated with an increased muscle energy demand in fast-growing fish. Glucose homeostasis in general is critical for the fish in order to meet these demands [62]. When rainbow trout juveniles were subjected to high dietary carbohydrate first feeding, glucose metabolism glucose transport and glycolysis-related genes were upregulated, and their expression was permanently modified, demonstrating efficient programming of the glucose metabolism [63]. Therefore, *gpi*, *alda* and *g3pdh* muscle expression can be used as a reliable biomarker for growth estimation of fish individuals.

Pk and *ldha* gene expression in liver was suppressed after 40 days of intermediate fasting. Both enzymes play an essential role in glucose homeostasis, with PK being the final enzyme for ATP production and pyruvate production, while LDHA regulates interconversion of pyruvate and L-lactate. Therefore, these enzymes are strongly inter-related, and their gene expression is positively correlated. Glycolysis and gluconeogenesis are metabolic processes that degrade and synthesize glucose and are essential for fish survival. The gluconeogenic pathway, which results in the generation of glucose, uses ATP that is supplied from NEFA catabolism [64] and may be associated with increased feed intake [65]. G3PDH and LDH are significantly downregulated under prolonged hypoxia stress [66]. Significant feed deprivation has been shown to have a profound downregulating effect in genes involved in glycolysis [67]. Among others, glycolysis genes are differentially regulated between growth hormone transgenic and wild-type coho salmon during periods of long-term food shortage [68].

Cholesterol synthesis genes (*7dhcr* and *sqle*) were differentiated by dietary status. In Atlantic salmon, dietary substitution of fish oil with vegetable oils regulated cholesterol biosynthesis genes such as *7dhcr* and *sqle* [69]. Furthermore, liver transcriptome and tissue lipid composition are driven by cholesterol synthesis upregulation in fish fed with animal by-products and vegetable-based diets [70]. In tilapia, cholesterol biosynthesis and fatty acid metabolism genes have been associated with growth [71]. Cholesterol biosynthetic gene expression was not associated with plasma cholesterol, which did not exhibit differences among groups.

Positive correlation of growth and *ldha* gene expression implies the promotion of accelerated cell growth and replication. A positive correlation of LDH activity and growth has been demonstrated in the past for salmon [72] and rainbow trout [73]. Furthermore, transgenic fish that perform better in terms of growth exhibit increased LDH activity [74]. Although, correlations between gene expression and weight could be more complex than those for *ldh* and *gpi*, these correlations depict glycolytic rates.

Research on mRNA quantification may identify potential biomarkers and use gene expression profiles either to assess dietary status or predict fish growth. Results of the present study showed that body weight growth of *Dicentrarchus labrax* was not affected by repeated intermitted fasting for 40 days. The study provides new insights on the impact of intermittent feeding of European seabass, with gene markers and their potential effects, for European seabass aquaculture. Expression of specific genes and blood biochemical parameters could be used as potential biomarkers to improve aquaculture practices and benefit fish husbandry through selective breeding, feeding strategies and farm management.

5. Conclusions

Research on mRNA quantification may identify potential biomarkers and use gene expression profiles either to assess dietary status or predict fish growth. Results of the

present study showed that body weight growth of *Dicentrarchus labrax* was not affected by repeated intermitted fasting. The study provides new insights on the impact of intermittent feeding of European seabass, with gene markers and their potential effects, for European seabass aquaculture. Expression of specific genes and blood biochemical parameters could be used as potential biomarkers to improve aquaculture practices and benefit fish husbandry through selective breeding, feeding strategies and farm management.

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