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# Dietary LC-PUFA and environmental salinity modulate the fatty acid biosynthesis capacity of the euryhaline teleost thicklip grey mullet (*Chelon labrosus*)

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**Abbreviations**

α-linolenic acid (ALA); arachidonic acid (ARA); average body weight (ABW); butylated hydroxytoluene (BHT); complementary DNA (cDNA); docosahexaenoic acid (DHA); eicosapentaenoic acid (EPA); Ethics and Animal Welfare Committee (CEIBA); fatty acid (FA); fatty acyl elongase (Elovl); fatty acid-free bovine serum albumin (FAF-BSA); fatty acid methyl ester (FAME); fatty acyl desaturases (Fads); fish oil (FO); Hanks Balanced Salt Solution (HBSS); linoleic acid (LA); long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA); monounsaturated fatty acid (MUFA); oleic acid (OA); parts per thousand (ppt); saturated fatty acids (SFA); stearidonic acid (SDA); tetracosahexaenoic acid (THA); thin layer chromatography (TLC); total lipid (TL); vegetable oil (VO).

**Abstract**

The capacity to biosynthesise long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA) depends upon the complement and function of key enzymes commonly known as fatty acyl desaturases and elongases. The presence of a Δ5/Δ6 desaturase enabling the biosynthesis of docosahexaenoic acid (22:6n-3, DHA) through the "Sprecher pathway" has been reported in *Chelon labrosus*. Research in other teleosts have demonstrated that LC-PUFA biosynthesis can be modulated by diet and ambient salinity. The present study aimed to assess the combined effects of partial dietary replacement of fish oil (FO) by vegetable oil (VO) and reduced ambient salinity (35 ppt vs 20 ppt) on the fatty acid composition of muscle, enterocytes and hepatocytes of *C. labrosus* juveniles. Moreover, the enzymatic activity over radiolabelled [1-$^{14}$C] 18:3n-3 (α-linolenic acid, ALA) and [1-$^{14}$C] 20:5n-3 (eicosapentaenoic acid, EPA) to biosynthesise n-3 LC-PUFA in hepatocytes and enterocytes, and the gene regulation of the *C. labrosus* fatty acid desaturase-2 (*fads2*) and elongation of very long chain fatty acids protein 5 (*elovl5*) in liver and intestine was also investigated. Recovery of radiolabelled products including stearidonic acid (18:4n-3, SDA), 20:5n-3, tetracosahexaenoic acid (24:6n-3, THA) and 22:6n-3 in all treatments except FO35-fish, provided compelling evidence that a complete pathway enabling the biosynthesis of EPA and DHA from ALA is present and active in *C. labrosus*. Low salinity conditions upregulated *fads2* in hepatocytes and *elovl5* in both cell types, regardless of dietary composition. Interestingly, FO20-fish showed the highest amount of n-3 LC-PUFA in muscle, while no differences in VO-fish reared at both salinities were found. These results demonstrate a compensatory capacity of *C. labrosus* to biosynthesise n-3 LC-PUFA under reduced dietary supply, and emphasise the potential of low salinity conditions to stimulate this pathway in euryhaline fish.

**Keywords:** LC-PUFA biosynthesis; low salinity; diet; *Chelon labrosus*

## Introduction

Marine fish are hypotonic to the medium, passively gaining salt and losing water. They compensate for such imbalance by drinking seawater and actively excreting monovalent and divalent ions through gills and kidney, respectively (Moyle and Cech 2000; Wen et al. 2020). Additionally, intestine plays a direct critical role in response to salinity changes avoiding the passive loss of water through regulation of absorption (Scott et al. 2006; Grosell et al. 2007; Ruiz-Jarabo et al. 2017). In this context, euryhaline teleosts possess mechanisms to mobilise large amounts of energy for maintaining osmoregulatory homeostasis. These include the exchange of electrolytes between the intracellular and extracellular spaces, as well as remodelling fatty acid (FA) composition in cell membranes (Soengas et al. 2007).

Several studies have reported that ambient salinity may also affect growth performance in teleosts. Thus, salinities close to the isosmotic point can improve the growth rate of some teleost species (Laiz-Carrión et al. 2005; Fonseca-Madrigal et al. 2012; Barany et al. 2021), while others exhibit a higher growth when salinity is slightly over that of the isosmotic point (Arjona et al. 2009); and growth appears not to be affected by salinity in other species (Li et al. 2008; Sarker et al. 2011). Salinity variations can also influence the FA composition of certain tissues. This is particularly true for the content of the physiologically essential long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ARA) (Khériji et al. 2003; Li et al. 2008, 2022; Sarker et al. 2011; Imen et al. 2013; Bao et al. 2022). LC-PUFA are abundant in fish oils (FO) and, to a lesser extent, fishmeal, which have been traditionally used as ingredients in aquafeeds (Turchini et al. 2010, 2022). However, the global supply of marine raw materials is finite and insufficient to support the increasing demand of aquaculture and

other industries (Tocher et al. 2019). Indeed, the rapid expansion of aquaculture has prompted interest in the search for alternatives to FO, with vegetable oils (VO) becoming nowadays widely used in aquafeed formulations (Aas et al. 2022; Turchini et al. 2022). VO are devoid of LC-PUFA but typically rich in their $C_{18}$ biosynthetic precursors linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA). Consequently, in species with limited capacity to bioconvert LA and ALA into LC-PUFA, inclusion of VO in the feed can lower their nutritional value associated with a reduction in contents of the health-promoting EPA and DHA in the fillet. Concomitantly, the overall fish health and performance might be also compromised by the deficient dietary supply of these essential nutrients (Monroig et al. 2018). Drawbacks linked to dietary VO can be counteracted in some teleosts with the ability to biosynthesise LC-PUFA from $C_{18}$ precursors contained in VO (Garrido et al. 2019, 2020; Galindo et al. 2021; Marrero et al. 2021, 2022; Xie et al. 2021; Monroig et al. 2022). Hence, the search for fish species with high LC-PUFA biosynthetic capacity that are able to tolerate high VO inclusion levels arises as a reasonable strategy for the sustainable expansion of the finfish farming industry. Such a strategy becomes especially pertinent for species that, along being able to endogenously produce LC-PUFA, are naturally tolerant to salinity changes. For such species, the regulatory effects of salinity alluded to above could be used to develop culture protocols that maximise the de novo production of LC-PUFA.

Grey mullets are members of the Mugilidae family that include a group of marine polyphagous fish highly appreciated for culture purposes (Cardona 1994; Strydom 2003; García-Márquez et al. 2021). In particular, the thick-lipped grey mullet (*Chelon labrosus*) has been suggested as a good candidate for the diversification of European aquaculture, being omnivorous during early life-cycle stages and switching to an

herbivorous feeding behaviour in later stages (Heras et al. 2012). Moreover, *C. labrosus* exhibits rapid growth and robustness (Abellan and Arnal 2013; Khemis et al. 2013; García-Márquez et al. 2021). Similarly to other mullets, *C. labrosus* can live in environments with changing salinity conditions such as estuaries and coastal water during its life cycle without compromising its growth performance (Cardona 2006; Pujante et al. 2018; García-Márquez et al. 2021). Importantly, *C. labrosus* has been shown to possess the enzymatic machinery to biosynthesise the health beneficial n-3 LC-PUFA including EPA and DHA (Garrido et al. 2019; Galindo et al. 2021). Collectively, the abovementioned biological features make *C. labrosus* a valid model to test the potential of combining dietary and salinity modulation on LC-PUFA biosynthesis. The present study aimed to investigate the combined effects of variable dietary LC-PUFA contents and environmental salinity, on the LC-PUFA biosynthetic capacity of *C. labrosus* juveniles. For this purpose, impacts on FA composition, the enzymatic activity of hepatocytes and enterocytes incubated with radiolabelled FA substrates, and the regulation of the LC-PUFA biosynthetic genes (*fads2* and *elovl5*) in liver and intestine were determined.

## 2. Materials and methods

This study follows the Guidelines of the European Union Council (2010/63/EU) and the Spanish Government (RD1201/2005; RD53/2013 and law 32/2007) for the use of experimental animals for scientific purposes. The experimental procedures were authorised by the Ethics and Animal Welfare Committee (CEIBA, 2015-0165) of University of La Laguna (Spain).

### *2.1 Experimental design*

*C. labrosus* juveniles with an average body weight (ABW) of 14.6 ± 1.6 g were randomly distributed into four quadrangular flat bottom 1000 L tanks (containing 15 fish each) and cultured in a recirculating aquaponic system at Fundación Neotrópico (Tenerife, Spain) for 10 weeks. Throughout the experiment, fish were maintained under natural photoperiod conditions at an average water temperature of 19.5 ± 0.4 °C (18.4-20.7 ºC), with a 2-3% water renewal per day. During the experiment, pH ranged between 7.9 and 8.2.

Fish from two tanks were fed with a "FO" supplemented commercial diet (TI-5 Tilapia; Skretting + 4% Croda Incromega™ high DHA content oil), while the other two groups of fish received a "VO" supplemented diet (TI-5 Tilapia; Skretting + 4% olive oil). For manufacturing the experimental diets, the commercial pellets were triturated, the corresponding oil added and mixed, and the resultant mixture finally repelletised. The lipid and FA composition of both diets is presented in Table 1. Juveniles were fed twice daily at a rate of 3-5% of their total biomass. Each diet was tested at two different salinities, 35 ppt and 20 ppt, resulting in four different experimental conditions (FO35, FO20, VO35 and VO20).

Table 1. Proximate composition (g $kg^{-1}$ dry matter), total fatty acid (FA) (mg $g^{-1}$ dry matter) and main FA composition (% of total FA) of experimental diets "FO" (supplemented with a marine oil) and "VO" (supplemented with a vegetable oil).

| | FO | VO |
|---|---|---|
| Dry matter (g $kg^{-1}$) | 937.6 ± 4.0 | 935.1 ± 5.0 |
| Crude protein | 404.0 ± 4.0 | 407.0 ± 3.5 |
| Crude lipid | 120.8 ± 2.5 | 123.4 ± 0.3 |
| Ash | 58.0 ± 2.3 | 57.0 ± 1.7 |
| Crude fibre | 45.2 ± 1.2 | 45.0 ± 0.9 |
| Nitrogen free extract | 309.6 ± 3.3 | 302.7 ± 2.9 |
| Total FA | 74.17 ± 1.78 | 82.31 ± 1.01 |
| 14:0 | 1.97 ± 0.03 | 1.73 ± 0.01 |
| 16:0 | 13.23 ± 0.15 | 15.01 ± 0.07 |
| 18:0 | 4.21 ± 0.05 | 3.92 ± 0.01 |
| Σ SFA | 20.85 ± 0.23 | 21.80 ± 0.12 |
| 16:1n-7 | 3.15 ± 0.02 | 3.00 ± 0.01 |
| 18:1n-9 | 23.58 ± 0.09 | 40.98 ± 0.13 |
| 18:1n-7 | 3.16 ± 0.16 | 3.49 ± 0.05 |
| 20:1n-9 | 1.29 ± 0.00 | 0.87 ± 0.00 |
| Σ MUFA | 33.90 ± 0.33 | 49.87 ± 0.27 |
| 18:2n-6 | 15.73 ± 0.05 | 16.27 ± 0.04 |
| 18:3n-6 | nd | nd |
| 20:3n-6 | nd | nd |
| 20:4n-6 | 1.18 ± 0.01 | 0.57 ± 0.01 |
| 22:4n-6 | nd | nd |
| 22:5n-6 | 1.00 ± 0.02 | nd |
| Σ n-6 PUFA | 18.24 ± 0.09 | 16.95 ± 0.10 |
| 18:3n-3 | 2.68 ± 0.04 | 2.67 ± 0.03 |
| 18:4n-3 | 0.58 ± 0.04 | 0.52 ± 0.03 |
| 20:4n-3 | 0.37 ± 0.01 | 0.16 ± 0.01 |
| 20:5n-3 | 4.92 ± 0.03 | 2.55 ± 0.06 |
| 22:5n-3 | 0.94 ± 0.01 | 0.34 ± 0.00 |
| 22:6n-3 | 14.81 ± 0.23 | 2.38 ± 0.03 |
| Σ n-3 PUFA | 24.57 ± 0.36 | 8.67 ± 0.10 |
| Σ n-3 LC-PUFA | 21.30 ± 0.28 | 5.49 ± 0.11 |
| n-3/n-6 | 1.35 ± 0.01 | 0.51 ± 0.00 |

Values are means ± SE (n = 3). Nitrogen free extract calculated as dry matter − (crude protein + crude lipid + ash + crude fibre). nd, not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain ($\geq C_{20}$) polyunsaturated fatty acids. Totals (Σ) include other minor components not shown.

146

147 *2.2 Tissue collection*

148 *C. labrosus* juveniles were fasted for 24 h prior being sacrificed by an anaesthetic
149 overdose (immersion in >1 mL $L^{-1}$ 2-phenoxyethanol). Then, due to the small size of
150 the specimens (for details, see Supplementary Table 1), fish from each experimental

151 treatment (i.e., tank) were pooled into five groups of three individuals each (n=5 per
152 tank). Pooled samples of muscle, and a fraction of pooled enterocyte and hepatocyte
153 suspensions (control groups in Section 2.4) were used for lipid determinations.
154 Additionally, two further fractions of both enterocyte and hepatocyte suspensions
155 (Section 2.4) were incubated with [1-$^{14}$C] 20:5n-3 and [1-$^{14}$C] 18:3n-3 for *in vitro*
156 metabolism studies. Finally, a pooled section of intestine and liver were collected (~100
157 mg wet weight), preserved in RNAlater®, and kept at 4 $^{o}$C for the first 24 h, and
158 subsequently frozen at -20 $^{o}$C until gene expression analysis.
159
160 *2.3. Lipid analysis*
161 Total lipids (TL) were determined from muscle, isolated cells (hepatocytes and
162 enterocytes) and diets following Folch et al. (1957) with some modifications (Christie
163 and Han 2010). Briefly, muscle samples were homogenised in 2.5 mL of 0.88% KCl
164 (w/v) and 10 mL of chloroform/methanol (2:1, v/v). The mixture was shaken and
165 centrifuged (716 × *g* for 5 min) to obtain two phases. The lower phase containing the
166 organic solvent and the lipids was collected and filtered. Finally, the organic solvent
167 was evaporated under a flow of nitrogen to determine the TL gravimetrically. To extract
168 TL from hepatocytes and enterocytes, cell samples were also diluted in 2.5 mL of
169 0.88% KCl and 10 mL of chloroform/methanol (2:1), containing 0.01% (w/v) butylated
170 hydroxytoluene (BHT) as antioxidant. Finally, ~100 mg of ground pellets from the
171 experimental diets (FO and VO) were used for TL extraction following the method
172 described by Marrero et al. (2021). Pellets were hydrated with 0.5 mL of dd$H_2O$ for 30
173 min at 4 $^{o}$C. Subsequently, 5 mL of chloroform/methanol (2:1) were used to
174 homogenise with a Virtis rotor homogeniser (Virtishear, Virtis, Gardiner, New York,
175 USA). To prevent oxidation, samples were stored overnight under a nitrogen

atmosphere. After this period, a further 5 mL of chloroform/methanol (2:1) were added to the solution and the mixture re-homogenised, prior to the addition of 2.5 mL of 0.88% KCl. To prevent sample degradation all processes were conducted under an ice-cold environment.

Fatty acid methyl esters (FAME) were prepared from 1 mg of TL extract and purified by thin-layer chromatography (TLC) according to Christie and Han (2010) (Macherey-Nagel, Düren, Germany). Then, the FAME were separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Scientific, Milan, Italy) equipped with a fused silica capillary column Supelcowax TM 10 (30 m × 0.32 mm ID, df 0.25 μm) (Supelco Inc., Bellefonte, PA, USA), following the procedures described by Marrero et al. (2021). A mixture of different standards (Mix $C_4$-$C_{24}$ and PUFA No. 3 from menhaden oil, Supelco Inc.) and a well-characterised cod roe oil were used to identify each specific FAME. Results for each FA are expressed as percentage of total FA. Furthermore, total FA were calculated as μg FA mg protein$^{-1}$ for enterocyte and hepatocyte samples, and mg FA 100 g wet weight$^{-1}$ for muscle samples (Section 2.4).

### *2.4. Isolation and incubation of cells with [1-$^{14}$C] 18:3n-3 and [1-$^{14}$C] 20:5n-3*

Enterocytes and hepatocytes from *C. labrosus* juveniles were isolated as described by Rodríguez et al. (2002) and Díaz-López et al. (2010). Briefly, the liver was perfused through the hepatic portal vein with a solution of marine Ringer (1 mM $CaCl_2$, 6 mM KCl, 116 mM NaCl, 10 mM $NaHCO_3$, 1 mM $MgSO_4$, 1 mM $NaH_2PO_4$, 10 mM $K_2SO_4$ and 10 mM HEPES, at pH 7.4), and the gut was cleaned from faeces and food. Tissues were minced with Hanks Balanced Salt Solution (HBSS) (NaCl 1.75%, 1.73 mM $NaHCO_3$, 9.69 mM HEPES), and incubated with agitation at 20 ºC for 40 min with collagenase (10 mg mL$^{-1}$). To obtain the isolated enterocytes and hepatocytes, the

solutions with the cell suspensions were filtered through a 100 µm nylon mesh with HBSS including 1% fatty acid-free bovine serum albumin (FAF-BSA). Then, cells were collected by centrifugation, washed with HBSS and re-centrifuged (716 × *g* for 10 min). The trypan blue exclusion test was used to assess the cell viability (>90% in all cases). Later, triplicates of each cell preparation were incubated, for 3 h with 0.20 µCi of [1-$^{14}$C] 18:3n-3 or 0.20 µCi of [1-$^{14}$C] 20:5n-3 with specific activities of 114.8 and 122.1 dpm pmol$^{-1}$, respectively. Each cell type was also incubated without radiolabelled FA as control groups. After incubation, the cell suspensions were centrifuged and washed twice to discharge non-incorporated radioactive substrate, and the resultant cell pellets stored at -80 $^{o}$C until further analysis.

TL were extracted as described in Section 2.3. The protein content of enterocytes and hepatocytes was established according to Lowry (1951) using FAF-BSA as standard. A liquid scintillation β-counter (TRI-CARB 4810TR, Perkin Elmer, Jurong, Singapore) was used to measure the radioactivity incorporated in 100 µg-aliquot of TL from cells incubated with radiolabelled FA. The measure of dpm were associated to protein contents and TL, and converted to pmol mg protein$^{-1}$ h$^{-1}$ taking into consideration the specific activities of each substrate.

Next, 1 mg-aliquot of the TL extracted from each incubated cell type was transmethylated by acid-catalysis and separated by argentation TLC using plates previously impregnated with silver nitrate as described by Rodríguez et al. (2002). To determine the elongation and desaturation products of [1-$^{14}$C] 18:3n-3 and [1-$^{14}$C] 20:5n-3, TLC plates were loaded with 50 µL of a standard mixture of commercial radiolabelled FA. Then, TLC were developed in a toluene/acetonitrile solution. Later, the plates were placed in closed cassettes (Exposure Cassette-K, BioRad, Madrid, Spain), and incubated in contact with a phosphorus screen sensitive to radioactivity

(Image Screen-K, BioRad). After two weeks, the screens were visualised by a scanner (Molecular Imager FX, BioRad). The radioactivity corresponding to the bands of the unmodified FA substrates and their transformation products was quantified by an image analysis software (Image Lab Software for PC Version 6.1, BioRad). Since not all the fish were metabolically active, the number of the fish pooled-cell samples displaying elongation/desaturation activities was also determined, and is given in the corresponding result tables.

*2.5. RNA extraction*

The RNA TRI Reagent extraction protocol (Sigma-Aldrich, Saint Louis, MO, USA) was used to extract total RNA. A Mini-Beadbeater (Bio Spec Products Inc., Bartlesville, OK, USA) was used to homogenise the pools of tissue samples (~100 mg) in 1 mL of TRI Reagent (Sigma-Aldrich, USA). RNA pellets were purified from the homogenised samples as described by Marrero et al. (2021). The NanoDrop® (ND-1000 spectrophotometer, LabTech International, Uckfield, UK) was used to assess the quality and concentration of total RNA extracts. An aliquot (~500 ng) of total RNA extracts was run on an agarose gel (1%, w/v) to assess the integrity of the RNA samples. Finally, total RNA solutions were kept at -70 $^{o}$C until further analysis.

*2.6. First strand cDNA synthesis and quantitative real-time PCR (qPCR)*

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, CA, USA) was used to synthesise the first strand complementary DNA (cDNA) as described by Marrero et al. (2021). PCR tubes (0.2 mL) containing 1 µg of total RNA dissolved in 10 µL of dd$H_2O$, were heated at 75 $^{o}$C (5 min) to denature the RNA. Later, the cDNA reverse transcriptase mix (Applied Biosystems$^{TM}$) composed of reverse

transcriptase (1 µL), Oligo dT (0.5 µL), reverse transcriptase random primers (1.5 µL), reverse transcriptase buffer (2 µL), dNTP mix (0.8 µL) and dd$H_2O$ (4.2 µL), was added to the denatured RNA. The cDNA synthesis was carried out in a Biometra TOptical thermocycler (Analytik Jena, Jena, Germany) set at 25 $^{o}$C for 10 min, 37 $^{o}$C for 2 h, 85 $^{o}$C for 5 min and 12 $^{o}$C for 4 min.

Quantitative real-time PCR (qPCR) were carried out to determine the expression of the *C. labrosus fads2* and *elovl5* in liver and intestine. The number of replicates per treatment was 5 for each gene and tissue, and elongation factor 1 α (*ef1α*) and *β-actin* were used as reference genes to normalise the expression of *elovl5* and *fads2*. Primer pairs are presented in Table 2. Serial dilutions of cDNA were carried out to determine their efficiency. The Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Carlsbad, CA, USA) were used to carry out the qPCR in a thermocycler using 96-well plates in duplicates. Each well was filled with 1 µL of each primer (10 µM), 5 µL or 2 µL of cDNA (1/20 dilution) for target genes and reference respectively, as well as 3 or 6 µL of molecular biology grade water and 10 µL of qPCR Master Mix. No template control (NTC) with 5 µL molecular biology grade water, instead of templates were also run. The qPCR conditions included a first step of activation at 50 $^{o}$C for 2 min, then 95 $^{o}$C for 10 min followed by 35 cycles of the denaturation step at 95 $^{o}$C for 15 s, the annealing temperature (Table 2) for 30 s, and a final step of extension at 72 $^{o}$C for 30 s. After amplification, a melting curve with 1 $^{o}$C increments during 6 s from 60 to 95 $^{o}$C was performed, to check the presence of a single product in each reaction. The results of the target gene expression by fold change were normalised and calculated following the method of Pfaffl (2001) using the reference housekeeping genes (*ef1α* and *β-actin*), which were considered stable according to geNorm (M-value < 0.5, https://genorm.cmgg.be/). Arbitrary units were calculated for each tissue and target gene

(*elovl5* and *fads2*) from the ratio among the expression level of each of them and the average of the control treatment (FO35).

Table 2. Primers used for real-time quantitative PCR (qPCR) analysis of gene expression (Garrido et al. 2019; Galindo et al. 2021). Details of primer sequence, amplicon size, annealing temperature (Ta), and reaction efficiency are included.

| Transcript | Primer sequence (5′–3′) | Amplicon size | Ta | Efficiency (%) | Reference |
|---|---|---|---|---|---|
| *fads2* | GTGTCAAGGCTTCGCTGATG<br>AACGTCACTCCTTTCGCATACA | 120 bp | 60°C | 96 | Garrido et al., 2019 |
| *elovl5* | AGAACGGCTCCTCCCTATCA<br>CAGCATTAGCTAACACGCTACA | 125 bp | 60°C | 95 | Galindo et al., 2020 |
| *β-actin* | CAGGGAGAAGATGACCCAGA<br>CCCTCGTAGATGGGCACTGT | 159 bp | 70°C | 97 | Garrido et al., 2019 |
| *ef1α* | GTCGAGATGCACCACGAGTC<br>GGGTGGTTCAGGATGATGAC | 176 bp | 70°C | 98 | Garrido et al., 2019 |

*2.7. Statistical analysis*

The Shapiro–Wilk and Levene tests were used to examine the normal distribution of data (relative expression and FA composition) and the homogeneity of the variances, respectively. The ln (x) or arcsine square root transformation was used when homoscedasticity and/or normality was not achieved. To evaluate the combined effects of the factors, namely salinity (35 or 20 ppt) and diet (FO or VO), and their interaction, a two-way ANOVA was applied with the significant differences established for $P < 0.05$. IBM SPSS statistics 25.0 for Windows (SPSS Inc., Armonk, NY, USA) was used to carry out all statistical analyses.

## 3. Results

*3.1. Fatty acid composition of muscle, hepatocytes and enterocytes*

The TL content of *C. labrosus* muscle did not vary among treatments (1.41-1.73% DW; Table 3). Total saturated fatty acids (SFA) showed the highest levels in FO35 fish, mainly associated to a significant increase in palmitic acid (16:0). Independently of salinity, fish fed the VO diet presented the highest monounsaturated fatty acid (MUFA) contents (VO35: 38.30%; VO20: 39.90%, *vs.* FO35: 33.02%; FO20: 30.64%). The lowest proportions of total n-6 PUFA and their most abundant FA 18:2n-6 (LA), were found in FO35-fish, while ARA remained unchanged among groups. On the other hand, n-3 PUFA were highest in FO20-fish based on the increased level of DHA in this experimental group (11.25%) compared to the others (3.84-5.56%). Meanwhile, EPA did not vary among treatments (Table 3).

Table 3. Total lipid (% wet weight), total fatty acids (FA) (mg FA 100 g wet weight$^{-1}$) and main FA composition (% of total FA) of muscle from *Chelon labrosus*.

| | FO 35 | FO 20 | VO 35 | VO 20 | Diet | Salinity | Interact. |
|---|---|---|---|---|---|---|---|
| Total lipid | 1.46 ± 0.17 | 1.53 ± 0.12 | 1.41 ± 0.30 | 1.73 ± 0.21 | | | |
| Total FA | 870.29 ± 228.23 | 1012.61 ± 70.43 | 1043.22 ± 270.85 | 1431.10 ± 117.66 | | | |
| 14:0 | 3.02 ± 0.22 | 2.49 ± 0.28 | 2.15 ± 0.15 | 2.31 ± 0.09 | * | | |
| 16:0 | 26.11 ± 1.94 | 20.49 ± 0.49 | 22.01 ± 0.35 | 21.84 ± 0.34 | | | * |
| 18:0 | 4.26 ± 0.48 | 4.06 ± 0.18 | 3.85 ± 0.24 | 3.75 ± 0.09 | | | |
| Σ SFA | 36.00 ± 2.54 | 28.45 ± 0.71 | 29.61 ± 0.15 | 29.36 ± 0.37 | | | * |
| 16:1n-7 | 6.80 ± 0.37 | 5.09 ± 0.08 | 5.11 ± 0.23 | 6.32 ± 0.24 | | | * |
| 18:1n-9 | 20.02 ± 0.42 | 21.21 ± 0.84 | 28.02 ± 1.77 | 28.49 ± 0.68 | * | | |
| 18:1n-7 | 4.00 ± 0.17 | 3.00 0.10 | 3.41 ± 0.10 | 3.35 ± 0.09 | | * | * |
| 20:1n-9 | 0.85 ± 0.04 | 0.65 ± 0.17 | 0.83 ± 0.05 | 0.81 ± 0.04 | | | |
| Σ MUFA | 33.02 ± 1.13 | 30.64 ± 0.98 | 38.30 ± 1.94 | 39.90 ± 0.79 | * | | |
| 18:2n-6 | 6.74 ± 0.88 | 10.50 ± 0.45 | 10.88 ± 0.17 | 11.05 ± 0.13 | * | * | * |
| 18:3n-6 | nd | 0.31 ± 0.03 | 0.33 ± 0.04 | 0.32 ± 0.01 | * | * | * |
| 20:3n-6 | nd | 0.23 ± 0.06 | 0.33 ± 0.05 | 0.30 ± 0.02 | * | * | * |
| 20:4n-6 | 2.74 ± 0.43 | 2.67 ± 0.26 | 2.24 ± 0.46 | 2.35 ± 0.29 | | | |
| 22:4n-6 | 0.25 ± 0.10 | 0.15 ± 0.09 | 0.36 ± 0.06 | 0.32 ± 0.02 | | | |
| 22:5n-6 | 0.66 ± 0.08 | 0.95 ± 0.05 | 0.45 ± 0.04 | 0.48 ± 0.04 | * | * | * |
| Σ n-6 PUFA | 10.39 ± 1.34 | 15.14 ± 0.42 | 14.94 ± 0.49 | 15.10 ± 0.46 | * | * | * |
| 18:3n-3 | 2.19 ± 0.26 | 3.28 ± 0.30 | 3.13 ± 0.47 | 2.29 ± 0.10 | | | * |
| 18:4n-3 | 1.50 ± 0.33 | 0.81 ± 0.10 | 0.92 ± 0.17 | 0.81 ± 0.02 | | | |
| 20:4n-3 | 0.25 ± 0.12 | 0.31 ± 0.01 | 0.31 ± 0.02 | 0.11 ± 0.05 | | | |
| 20:5n-3 | 3.81 ± 0.61 | 4.66 ± 0.26 | 3.44 ± 0.33 | 3.89 ± 0.20 | | | |
| 22:5n-3 | 0.78 ± 0.25 | 1.51 ± 0.08 | 1.28 ± 0.09 | 1.29 ± 0.03 | | * | * |
| 22:6n-3 | 5.56 ± 0.85 | 11.25 ± 0.70 | 4.31 ± 0.30 | 3.84 ± 0.20 | * | * | * |
| Σ n-3 PUFA | 14.10 ± 2.01 | 21.83 ± 0.80 | 13.39 ± 1.12 | 12.30 ± 0.48 | * | * | * |
| Σ n-3 LC-PUFA | 10.41 ± 1.57 | 17.74 ± 0.92 | 9.35 ± 0.71 | 9.20 ± 0.39 | * | * | * |
| n-3/n-6 | 1.34 ± 0.06 | 1.44 ± 0.06 | 0.89 ± 0.05 | 0.81 ± 0.02 | * | | |

Values are means ± SE (n = 5); nd, not detected. FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain ($\geq C_{20}$) polyunsaturated fatty acids. Totals include other minor components not shown. * Significant differences ($P < 0.05$).

Regardless of the dietary treatment or salinity, the TL content of hepatocytes was similar in all experimental groups (Table 4). With respect to the FA profiles, only total n-3 PUFA varied between groups, with the highest levels found in the hepatocytes of fish fed FO (FO35: 15.26%; FO20: 17.20% *vs.* VO35: 12.56%; VO20: 13.62%). Thus, liver cells from FO-fed fish contained higher amounts of 18:3n-3 and 18:4n-3, while

22:5n-3 was more abundant in VO-fed fish. By contrast, there were not significant differences in the abundance of EPA and DHA in hepatocytes.

Table 4. Total lipid (mg lipid mg protein$^{-1}$), total fatty acids (FA) (μg FA mg protein$^{-1}$) and main FA composition (% of total FA) of hepatocytes from *Chelon labrosus*.

| | FO 35 | FO 20 | VO 35 | VO 20 | Diet | Salinity | Interact. |
|---|---|---|---|---|---|---|---|
| Total lipid | 0.74 ± 0.01 | 0.87 ± 0.05 | 0.78 ± 0.10 | 0.74 ± 0.06 | | | |
| Total FA | 230.57 ± 9.49 | 296.43 ± 18.44 | 265.06 ± 34.60 | 230.42 ± 23.52 | | | |
| 14:0 | 1.45 ± 0.07 | 1.62 ± 0.16 | 1.06 ± 0.06 | 1.37 ± 0.09 | * | | |
| 16:0 | 22.09 ± 0.92 | 22.46 ± 2.23 | 24.63 ± 1.89 | 21.84 ± 0.71 | | | |
| 18:0 | 8.07 ± 0.59 | 7.04 ± 0.36 | 11.62 ± 1.06 | 9.07 ± 0.26 | * | * | |
| Σ SFA | 34.44 ± 1.69 | 33.37 ± 2.79 | 38.75 ± 2.87 | 34.38 ± 0.88 | | | |
| 16:1n-7 | 3.84 ± 0.13 | 4.99 ± 0.66 | 3.13 ± 0.09 | 4.27 ± 0.17 | | * | |
| 18:1n-9 | 22.46 ± 2.80 | 22.35 ± 1.32 | 23.41 ± 1.02 | 23.53 ± 1.05 | | | |
| 18:1n-7 | 2.52 ± 0.22 | 3.01 ± 0.25 | 2.46 ± 0.20 | 2.86 ± 0.09 | | * | |
| 20:1n-9 | 1.00 ± 0.04 | 1.16 ± 0.08 | 1.21 ± 0.07 | 1.16 ± 0.01 | | | |
| Σ MUFA | 30.51 ± 2.65 | 32.54 ± 1.83 | 30.80 ± 1.33 | 32.55 ± 0.88 | | | |
| 18:2n-6 | 10.33 ± 1.08 | 9.12 ± 1.21 | 8.88 ± 0.65 | 9.53 ± 0.43 | | | |
| 18:3n-6 | 0.96 ± 0.35 | 1.49 ± 0.99 | 0.32 ± 0.03 | 0.26 ± 0.01 | | | |
| 20:3n-6 | nd | nd | nd | nd | | | |
| 20:4n-6 | 2.07 ± 0.34 | 1.89 ± 0.34 | 3.02 ± 0.21 | 3.54 ± 0.21 | * | | |
| 22:4n-6 | nd | nd | nd | nd | | | |
| 22:5n-6 | 0.40 ± 0.05 | 0.44 ± 0.13 | 0.42 ± 0.02 | 0.42 ± 0.02 | | | |
| Σ n-6 PUFA | 13.77 ± 0.81 | 12.94 ± 1.71 | 12.62 ± 0.87 | 13.76 ± 0.44 | | | |
| 18:3n-3 | 4.66 ± 0.98 | 5.55 ± 2.71 | 2.23 ± 0.19 | 2.02 ± 0.24 | * | | |
| 18:4n-3 | 1.38 ± 0.52 | 2.02 ± 1.22 | nd | nd | | | |
| 20:4n-3 | nd | nd | nd | nd | | | |
| 20:5n-3 | 1.95 ± 0.28 | 2.07 ± 0.32 | 2.12 ± 0.22 | 2.89 ± 0.20 | | | |
| 22:5n-3 | 0.81 ± 0.06 | 0.65 ± 0.10 | 1.14 ± 0.10 | 1.21 ± 0.06 | * | | |
| 22:6n-3 | 6.47 ± 1.54 | 6.91 ± 1.05 | 7.07 ± 0.44 | 7.50 ± 0.34 | | | |
| Σ n-3 PUFA | 15.26 ± 1.51 | 17.20 ± 2.61 | 12.56 ± 0.89 | 13.62 ± 0.44 | * | | |
| Σ n-3 LC-PUFA | 9.22 ± 1.81 | 9.62 ± 1.40 | 10.33 ± 0.75 | 11.60 ± 0.56 | | | |
| n-3/n-6 | 1.15 ± 0.18 | 1.34 ± 0.09 | 1.00 ± 0.03 | 0.99 ± 0.03 | * | | |

Values are means ± SE (n = 5); nd, not detected. FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain ($\geq C_{20}$) polyunsaturated fatty acids. Totals include other minor components not shown. * Significant differences ($P < 0.05$).

The TL content of fish enterocytes increased under low salinity conditions regardless of the dietary treatment (Table 5). Fish fed the FO diet at 35 ppt had the highest proportion of total SFA in enterocytes mainly associated to high levels of 14:0, and the lowest content of total MUFA (23.87% *vs.* 31.48-35.99%), due to their reduced amount of

18:1n-9. Enterocytes from FO35 fish also presented the lowest 18:2n-6 and n-6 PUFA levels. Regardless of dietary composition, ARA was more abundant in enterocytes from fish reared at 35 ppt (FO35: 2.60%; VO35: 2.69% *vs.* FO20: 1.62%; VO20: 2.18%). On the other hand, DHA was higher in FO treatments compared to VO treatments (FO35: 7.32%; FO20: 9.45% *vs.* VO35: 4.63%; VO20: 5.16%), which also presented the highest levels of total n-3 PUFA. Similarly to muscle and hepatocytes, EPA from enterocytes did not vary among groups (Table 5).

Table 5. Total lipid (mg lipid mg protein$^{-1}$), total fatty acids (FA) (μg FA mg protein$^{-1}$) and main FA composition (% of total FA) of enterocytes from *Chelon labrosus*.

| | FO 35 | FO 20 | VO 35 | VO 20 | Diet | Salinity | Interact. |
|---|---|---|---|---|---|---|---|
| Total lipid | 1.48 ± 0.20 | 2.45 ± 0.18 | 1.64 ± 0.22 | 2.12 ± 0.39 | | * | |
| Total FA | 734.21 ± 52.97 | 1088.23 ± 136.75 | 773.65 ± 127.94 | 1117.64 ± 293.30 | | * | |
| 14:0 | 10.18 ± 0.97 | 2.88 ± 0.47 | 1.58 ± 0.11 | 4.51 ± 1.17 | * | | * |
| 16:0 | 16.43 ± 1.41 | 19.92 ± 0.28 | 19.53 ± 0.91 | 19.25 ± 0.50 | * | | * |
| 18:0 | 7.47 ± 1.08 | 5.56 ± 0.08 | 8.00 ± 0.94 | 6.30 ± 0.19 | | * | |
| Σ SFA | 42.70 ± 0.86 | 30.53 ± 0.78 | 31.63 ± 0.72 | 33.71 ± 1.91 | * | | * |
| 16:1n-7 | 5.53 ± 0.68 | 4.74 ± 0.22 | 4.33 ± 0.38 | 5.19 ± 0.34 | | | |
| 18:1n-9 | 13.32 ± 1.06 | 22.03 ± 0.24 | 27.85 ± 1.18 | 23.80 ± 1.95 | * | | * |
| 18:1n-7 | 3.53 ± 0.12 | 3.35 ± 0.05 | 3.38 ± 0.30 | 3.08 ± 0.23 | | | |
| 20:1n-9 | 1.49 ± 0.14 | 1.14 ± 0.03 | 0.43 ± 0.05 | 1.24 ± 0.19 | * | | * |
| Σ MUFA | 23.87 ± 1.22 | 31.48 ± 0.22 | 35.99 ± 1.79 | 34.12 ± 1.73 | * | | * |
| 18:2n-6 | 7.81 ± 0.74 | 12.15 ± 0.59 | 12.00 ± 0.50 | 10.59 ± 1.23 | | | * |
| 18:3n-6 | nd | 0.24 ± 0.06 | 0.55 ± 0.20 | 0.49 ± 0.18 | * | | |
| 20:3n-6 | nd | nd | nd | 0.11 ± 0.07 | | | |
| 20:4n-6 | 2.60 ± 0.18 | 1.62 ± 0.08 | 2.69 ± 0.50 | 2.18 ± 0.34 | | * | |
| 22:4n-6 | nd | nd | nd | nd | | | |
| 22:5n-6 | nd | nd | nd | 0.10 ± 0.06 | | | |
| Σ n-6 PUFA | 11.31 ± 0.36 | 14.01 ± 0.66 | 15.24 ± 0.78 | 13.65 ± 1.76 | | | * |
| 18:3n-3 | 2.80 ± 0.25 | 3.67 ± 0.23 | 3.45 ± 0.17 | 2.81 ± 0.25 | | | * |
| 18:4n-3 | 1.28 ± 0.07 | 0.85 ± 0.04 | 0.76 ± 0.06 | 0.78 ± 0.08 | * | | |
| 20:4n-3 | nd | nd | nd | nd | | | |
| 20:5n-3 | 2.76 ± 0.15 | 2.95 ± 0.12 | 2.47 ± 0.34 | 2.51 ± 0.05 | | | |
| 22:5n-3 | 0.76 ± 0.07 | 1.11 ± 0.05 | 1.07 ± 0.23 | 0.95 ± 0.10 | | | |
| 22:6n-3 | 7.32 ± 1.43 | 9.45 ± 0.39 | 4.63 ± 0.99 | 5.16 ± 0.86 | * | | |
| Σ n-3 PUFA | 14.91 ± 1.15 | 18.03 ± 0.65 | 12.39 ± 1.59 | 12.20 ± 0.61 | * | | |
| Σ n-3 LC-PUFA | 10.84 ± 1.29 | 13.51 ± 0.49 | 8.18 ± 1.55 | 8.61 ± 0.76 | * | | |
| n-3/n-6 | 1.32 ± 0.11 | 1.29 ± 0.03 | 0.80 ± 0.06 | 1.01 ± 0.22 | * | | |

Values are means ± SE (n = 5); nd, not detected. FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain ($\geq C_{20}$) polyunsaturated fatty acids. Totals include other minor components not shown. * Significant differences ($P < 0.05$).

### *3.2. Incorporation of radioactivity into cell total lipids and bioconversion of radiolabelled fatty acids*

The incorporation rate of both radiolabelled FA, namely [1-$^{14}$C] 18:3n-3 and [1-$^{14}$C] 20:5n-3, into cell TL was higher in enterocytes (143.67±2.49 to 184.74±7.72 pmol mg protein$^{-1}$ h$^{-1}$) than in hepatocytes (78.69±3.13 to 110.74±3.34 pmol mg protein$^{-1}$ h$^{-1}$) (Table 6). The radioactivity recovered as unmodified substrate varied from 79.17% to

100% and, as a consequence, the bioconversion activity over radiolabelled FA was not detectable in all fish. For both radiolabelled FA assayed, the number of fish with elongation+desaturation activities was higher in enterocytes (20) than hepatocytes (8). Moreover, FO35 fish were the sole group that did not show desaturase activity over the radiolabelled FA substrates in any cellular type assayed (Table 6).

Table 6. Incorporation of radioactivity into total lipids (pmol mg protein$^{-1}$ h$^{-1}$) and bioconversion rates (% of total radioactivity) registered in isolated hepatocytes and enterocytes from *C. labrosus* incubated with [1-$^{14}$C] 18:3n-3 and [1-$^{14}$C] 20:5n-3.

| | | | | |
|---|---|---|---|---|
| | | **Hepatocytes** | | |
| **[1-$^{14}$C] 18:3n-3** | FO35 | FO20 | VO35 | VO20 |
| Incorporation | 101.95 ± 7.63 (5) | 85.03 ± 9.30 (5) | 110.74 ± 3.34 (5) | 90.01 ± 4.98 (5) |
| 18:3n-3 recovery | 100 ± 0.00 (5) | 97.16 ± 1.22 (5) | 96.28 ± 0.74 (5) | 92.26 ± 2.33 (5) |
| Elongation | nd | 4.89 ± 0.15 (2) | 3.29 ± 0.63 (5) | 2.37 (1) |
| Desaturation | nd | 0.36 ± 0.01 (2) | 0.53 ± 0.15 (4) | nd |
| E+D | nd | 1.86 ± 1.51 (2) | nd | 7.15 ± 2.79 (5) |
| De novo | nd | nd | nd | nd |
| Unknown | nd | nd | nd | nd |
| **[1-$^{14}$C] 20:5n-3** | FO35 | FO20 | VO35 | VO20 |
| Incorporation | 86.19 ± 9.08 (5) | 78.69 ± 3.13 (5) | 101.31 ± 6.18 (5) | 88.61 ± 19.17 (5) |
| 20:5n-3 recovery | 100 ± 0.00 (5) | 99.14 ± 0.86 (5) | 96.18 ± 0.82 (5) | 98.84 ± 1.16 (5) |
| Elongation | nd | 3.57 (1) | 2.84 ± 0.56 (5) | 3.99 (1) |
| Desaturation | nd | nd | nd | nd |
| E+D | nd | nd | 0.47 (1) | nd |
| De novo | nd | nd | nd | nd |
| Unknown | nd | 0.72 (1) | 1.11 ± 0.32 (4) | 1.81 (1) |
| | | **Enterocytes** | | |
| **[1-$^{14}$C] 18:3n-3** | FO35 | FO20 | VO35 | VO20 |
| Incorporation | 181.71 ± 14.43 (5) | 184.74 ± 7.72 (5) | 173.01 ± 11.67 (5) | 179.44 ± 5.80 (5) |
| 18:3n-3 recovery | 98.36 ± 0.54 (5) | 92.34 ± 1.51 (5) | 94.58 ± 1.05 (5) | 93.42 ± 1.55 (5) |
| Elongation | 2.05 ± 0.44 (4) | 5.49 ± 0.78 (5) | 3.96 ± 0.56 (5) | 4.53 ± 0.69 (5) |
| Desaturation | nd | 0.44 ± 0.17 (2) | 0.23 ± 0.01 (3) | 0.50 ± 0.09 (3) |
| E+D | nd | 0.94 ± 0.21 (3) | 0.64 ± 0.11 (4) | 1.23 ± 0.26 (3) |
| De novo | nd | nd | nd | nd |
| Unknown | nd | 1.67 ± 0.05 (3) | 1.36 ± 0.44 (3) | 1.69 ± 0.28 (3) |
| **[1-$^{14}$C] 20:5n-3** | FO35 | FO20 | VO35 | VO20 |
| Incorporation | 147.79 ± 6.61 (5) | 143.67 ± 2.49 (5) | 154.71 ± 10.12 (5) | 155.28 ± 9.86 (5) |
| 20:5n-3 recovery | 94.77 ± 2.12 (5) | 79.17 ± 7.60 (5) | 83.05 ± 7.85 (5) | 85.36 ± 2.42 (5) |
| Elongation | 6.54 ± 2.16 (4) | 9.86 ± 4.08 (5) | 9.86 ± 2.53 (4) | 10.69 ± 0.95 (5) |
| Desaturation | nd | nd | nd | nd |
| E+D | nd | 2.92 ± 0.90 (4) | 2.90 ± 0.45 (3) | 1.85 ± 0.24 (3) |
| De novo | nd | nd | nd | nd |
| Unknown | nd | 8.05 ± 3.76 (4) | 12.20 ± 6.62 (3) | 3.42 ± 1.81 (3) |

Values are means ± SE. E+D, products which combine elongation and desaturation processes; nd, not detected. Values in brackets represent the number of pooled fish cell samples with bioconversion detected.

The metabolic products obtained from hepatocytes and enterocytes incubated with [1-$^{14}$C] 18:3n-3 and [1-$^{14}$C] 20:5n-3 are shown in Table 7. Overall, elongation of [1-$^{14}$C]

18:3n-3 up to 20:3n-3 was the most common enzymatic activity in both cell types, as well as elongation over [1-$^{14}$C] 20:5n-3 to 22:5n-3 (Table 7). Interestingly, all assayed fish from the VO20 treatment (5) produced EPA in their hepatocytes when incubated with [1-$^{14}$C] 18:3n-3, whereas 18:4n-3, the direct Δ6 desaturation product from 18:3n-3, was more frequently recovered in VO35 fish. Also for VO35 treatment, all fish (5) were able to transform [1-$^{14}$C] 20:5n-3 to 22:5n-3 in hepatocytes, with one individual even producing 24:6n-3 (Table 7). The biosynthesis of EPA and DHA was more commonly detected in enterocytes than in hepatocytes, especially in fish receiving the VO diet (Table 7). Incubation of enterocytes from FO-fed fish reared at 35 ppt with [1-$^{14}$C] 18:3n-3 and [1-$^{14}$C] 20:5n-3 resulted in the production of elongation products, but not desaturation products were detected. However, enterocytes from FO-fed fish reared at 20 ppt contained biosynthetic products resulting from the combined action of both elongases and desaturases (e.g., 20:5n-3, 22:6n-3 and 24:6n-3). Likewise, such biosynthetic products requiring the action of both elongases and desaturases were also detected in enterocytes from both VO treatments (VO35 and VO20).

Table 7. Radioactive products obtained (% of total radioactivity) from the incubation of isolated hepatocytes and enterocytes from *C. labrosus* with [1-$^{14}$C] 18:3n-3 and [1-$^{14}$C] 20:5n-3.

| | | | | |
|---|---|---|---|---|
| **Hepatocytes** | | | | |
| **[1-$^{14}$C] 18:3n-3** | FO35 | FO20 | VO35 | VO20 |
| 18:4n-3 | nd | 0.36 ± 0.01 (2) | 0.53 ± 0.15 (4) | nd |
| 20:3n-3 | nd | 2.04 ± 0.12 (2) | 1.56 ± 0.36 (5) | 0.86 (1) |
| 20:4n-3 | nd | nd | nd | nd |
| 20:5n-3 | nd | 1.86 ± 1.51 (2) | nd | 7.15 ± 2.79 (5) |
| 22:3n-3 | nd | 1.8 ± 0.05 (2) | 1.05 ± 0.14 (5) | 1.51 (1) |
| 22:6n-3 | nd | nd | nd | nd |
| 24:3n-3 | nd | 1.06 ± 0.01 (2) | 0.68 ± 0.2 (5) | nd |
| 24:6n-3 | nd | nd | nd | nd |
| **[1-$^{14}$C] 20:5n-3** | FO35 | FO20 | VO35 | VO20 |
| 22:5n-3 | nd | 2.49 (1) | 2.20 ± 0.38 (5) | 2.93 (1) |
| 22:6n-3 | nd | nd | nd | nd |
| 24:5n-3 | nd | 1.08 (1) | 0.79 ± 0.18 (4) | 1.07 (1) |
| 24:6n-3 | nd | nd | 0.47 (1) | nd |
| **Enterocytes** | | | | |
| **[1-$^{14}$C] 18:3n-3** | FO35 | FO20 | VO35 | VO20 |
| 18:4n-3 | nd | 0.44 ± 0.17 (2) | 0.23 ± 0.01 (3) | 0.50 ± 0.09 (3) |
| 20:3n-3 | 0.72 ± 0.08 (4) | 1.47 ± 0.16 (5) | 1.23 ± 0.17 (5) | 1.40 ± 0.15 (5) |
| 20:4n-3 | nd | nd | nd | nd |
| 20:5n-3 | nd | 0.40 ± 0.15 (2) | 0.44 ± 0.17 (4) | 0.75 ± 0.18 (3) |
| 22:3n-3 | 1.46 ± 0.29 (3) | 1.95 ± 0.33 (5) | 1.75 ± 0.10 (5) | 1.77 ± 0.11 (5) |
| 22:6n-3 | nd | 0.68 ± 0.21 (3) | 0.26 ± 0.01 (3) | 0.48 ± 0.08 (3) |
| 24:3n-3 | 0.31 ± 0.04 (3) | 0.89 ± 0.17 (5) | 0.82 ± 0.12 (3) | 0.80 ± 0.05 (4) |
| 24:6n-3 | nd | nd | nd | nd |
| **[1-$^{14}$C] 20:5n-3** | FO35 | FO20 | VO35 | VO20 |
| 22:5n-3 | 5.19 ± 1.72 (4) | 4.00 ± 1.25 (5) | 4.75 ± 0.96 (4) | 4.10 ± 0.31 (5) |
| 22:6n-3 | nd | nd | nd | nd |
| 24:5n-3 | 1.81 ± 0.08 (3) | 3.23 ± 1.31 (5) | 2.98 ± 0.59 (4) | 3.40 ± 0.42 (5) |
| 24:6n-3 | nd | 2.92 ± 0.90 (4) | 2.90 ± 0.45 (3) | 1.85 ± 0.24 (3) |

Values are means ± SE. nd, not detected. Values in brackets represent the number of pooled fish cell samples with bioconversion detected.

### *3.3. Regulation of LC-PUFA biosynthetic genes through diet and salinity*

Salinity affected the expression of *fads2* and *elovl5* in the liver of *C. labrosus* juveniles (Figure 1). More specifically, hepatic *fads2* (Figure 1a) and *elovl5* (Figure 1b) were upregulated in fish reared at 20 ppt, regardless of the dietary treatment. A similar response was shown by *elovl5* but not *fads2* in the intestine (Figures 1d and 1c). Moreover, no dietary regulation of *fads2* or *elovl5* was detected in either enterocytes or hepatocytes (Figure 1).

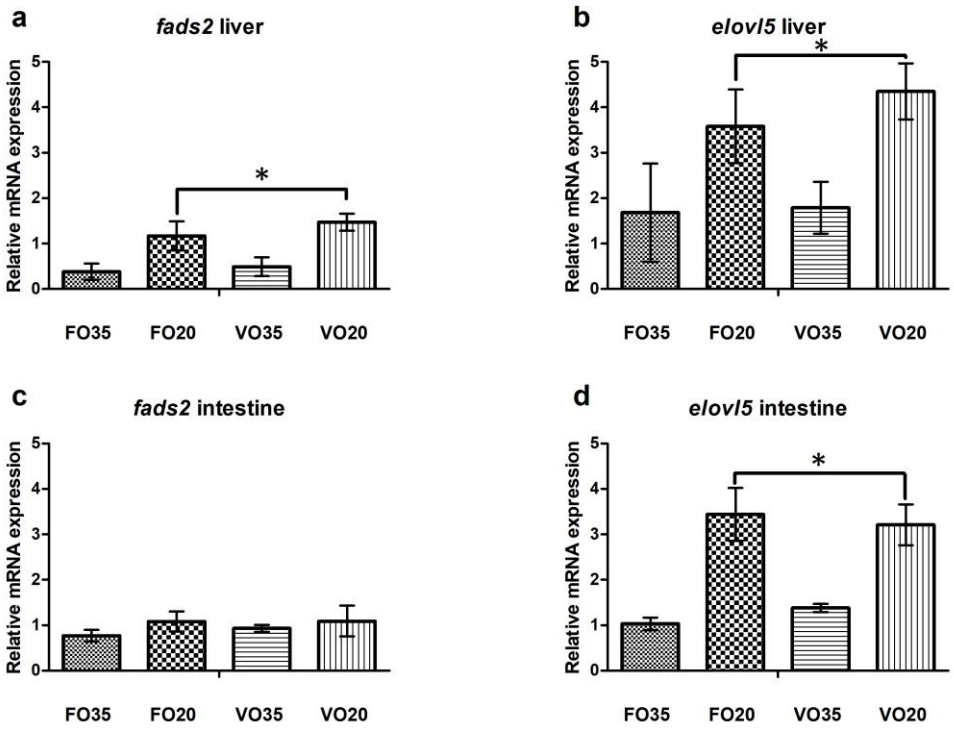


Figure 1. Distribution of *fads2* and *elovl5* mRNA levels in liver (a, b) and intestine (c, d) of *C. labrosus* juveniles. The relative expression is shown as geometric mean normalised expression ratios ± SE (n = 5). * Significant differences ($P < 0.05$).

## 4. Discussion

It has been previously reported that *C. labrosus* possess the genes encoding desaturase and elongase enzymes that enable all the reactions to produce the physiologically essential ARA, EPA and DHA from their $C_{18}$ precursors (Garrido et al. 2019; Galindo et al. 2021). However, the actual activity of the encoded enzymes, as well as their regulation through diet and environmental factors such as salinity, remains largely unknown. In the present work, fish hepatocytes presented similar levels of EPA and DHA despite the FO diet providing 1.9-fold more EPA and 6.2-fold more DHA than the VO diet. Besides, ALA, the $C_{18}$ PUFA precursor of the biosynthetic pathways leading to the production of EPA and DHA (Monroig et al., 2022), was more abundant in hepatocytes from FO-fed fish despite being equally supplied in both diets. These

371 compensatory mechanisms of hepatocytes to obtain the same levels of EPA and DHA
372 in all treatments, along with reduced ALA in VO-fed fish, suggest a strong dietary
373 modulation over the biosynthetic activity to produce n-3 LC-PUFA (Steinberg 2022;
374 Turchini et al. 2022). Consistently, hepatocytes from fish fed the VO diet had higher
375 elongation and desaturation activities than those of fish fed the FO diet, particularly
376 FO35-fish, where no activity was detected.
377 The enzymatic activity of elongases and desaturases involved in the LC-PUFA
378 biosynthetic pathways is often supported by increased expression of the corresponding
379 genes (Xie et al., 2021). In the present study, the relative expression of hepatic *fads2*
380 and *elovl5* was high in fish reared at 20 ppt, indicating that salinity is also a key
381 parameter in the modulation of these genes, independently of the diet. These results are
382 in agreement with those obtained in other marine teleost showing higher mRNA levels
383 in fish reared at lower salinity than that of sea water (Sarker et al. 2011; Xie et al. 2015;
384 Luo et al. 2021). Indeed, Khériji et al. (2003) reported a LC-PUFA increase in *Mugil*
385 *cephalus* reared at low salinity, suggesting that the herein observed response in *C.*
386 *labrosus* can extend to other members of the Mugilidae family composed of species
387 tolerant to a wide range of salinities (Thomson 1966; Cardona 2006; Khemis et al.
388 2013; Pujante et al. 2018; García-Márquez et al. 2021). Even though only low salinity
389 resulted in increased expression of *fads2* and *elovl5*, VO35-cells but not FO35-cells
390 showed certain rate of bioconversion of radiolabelled ALA to EPA and DHA. In this
391 regard, some discrepancies between transcriptome and proteome have been stated in
392 previous studies comparing enzymatic activity and mRNA levels in teleosts (Péres et al.
393 1998; Gawlicka and Horn 2006), including *C. labrosus* (Pujante et al. 2018). A low
394 expression does not necessarily imply a low enzymatic activity, since several factors

such as the efficiency of translation or post-translational processes that affect the protein efficiency could be taking place (Macdonald 2001; Glanemann et al. 2003).

Enterocytes from FO-fed fish contained about two-fold more DHA than enterocytes from fish fed the VO diet despite dietary DHA being 6.2-fold higher in the FO diet compared to the VO diet. Moreover, both elongation and desaturation activities of enterocytes were detected in all treatments except FO35, which presented elongation products but not desaturation products. Therefore, these results suggest that the low dietary supply of n-3 LC-PUFA and reduced salinity are promoting the compensatory biosynthesis of both EPA and DHA in the intestinal epithelial cells, and confirming that intestine is an active site in LC-PUFA biosynthesis (Díaz-López et al. 2009, 2010; Marrero et al. 2021). Moreover, the increased TL content in 20 ppt-enterocytes might indicate that decreasing salinity enhances the lipid deposition in intestine as previously reported in *C. labrosus* (Imen et al. 2013). Osmoregulation is a big challenge for marine fish and, as a consequence, a lower β-oxidation rate of FA may take place in gut epithelial cells when fish are reared under less stressing environmental conditions, i.e. 20 ppt rather than 35 ppt. The lower contents of MUFA (good β-oxidation substrates) and high values of 14:0 (β-oxidation product) found in the enterocytes of the FO35-fish suggest greater β-oxidation in this treatment (Sangiao-Alvarellos et al. 2003; Soengas et al. 2007). Consistently, and similarly to the liver, intestinal expression of LC-PUFA biosynthetic genes such as *fads2* and *elovl5* was higher at 20 ppt, although it was only significant for *elovl5*. However, diet prevailed over salinity in enterocytes from VO-fed fish incubated with ALA where the same number of fish pooled-cells (3) synthesised DHA, regardless of the salinity conditions. This is consistent with previous studies by Monroig et al. (2018) and Xie et al. (2021) where diet was regarded as the most

419 influential factor in the modulation of LC-PUFA biosynthesis in teleosts, over ambient
420 factors such as salinity and temperature.
421 Muscle FA composition is one of the most relevant indicators when evaluating the
422 nutritional value of fish as a product for human consumption. In our study, *C. labrosus*
423 juveniles were able to compensate for the varying composition of the experimental diets
424 to maintain similar levels of n-3 LC-PUFA in the muscle from fish reared at 35 ppt.
425 LC-PUFA biosynthesis was potentiated under low salinity conditions in FO20
426 treatment, and agrees well to what happened in hepatic and intestinal cells incubated
427 with radiolabelled FA. Consistently, Rabeh et al. (2015) reported that *C. labrosus*
428 juveniles reared at fresh water contained a higher level of n-3 LC-PUFA than those
429 reared at sea water.
430 Overall, results from both the FA analyses and *in vitro* assays evidenced that the effect
431 of salinity towards LC-PUFA biosynthesis depends upon the dietary supply of n-3 LC-
432 PUFA. For both salinity conditions tested, *C. labrosus* juveniles fed the VO diet were
433 able to produce n-3 LC-PUFA from ALA, resulting in no significant differences in the
434 final n-3 LC-PUFA composition of either muscle, hepatocytes or enterocytes. This is in
435 accordance with the number of fish that were able to transform radiolabelled ALA in the
436 VO treatments regardless of salinity. However, *C. labrosus* specimens fed with FO diet
437 presented higher amounts of n-3 LC-PUFA when reared at reduced salinity. The higher
438 capacity for n-3 LC-PUFA biosynthesis in enterocytes and hepatocytes from FO20 fish
439 in comparison to that of FO35 fish reflects the effect of salinity on LC-PUFA
440 biosynthesis in *C. labrosus*. Similar results were obtained in a dietary and salinity trial
441 conducted with the herbivore *Siganus canaliculatus*, although the effect of reduced
442 salinity was more evident in the specimens fed with the VO-based diet (Xie et al. 2015).
443 The relatively limited capacity of the thicklip grey mullet for the biosynthesis of n-3

LC-PUFA compared to *S. canaliculatus*, can be partly explained by the increased desaturation abilities of *S. canaliculatus*. Thus, while *S. canaliculatus* has Δ4, Δ5 and Δ6 desaturase capacities contained in two distinct *fads2*-like desaturases (Li et al. 2008, 2010), *C. labrosus* only possess one sole *fads2* with Δ5 and Δ6 activities (Garrido et al. 2019; Galindo et al. 2021).

## 5. Conclusions

In conclusion, our results demonstrate that the biosynthetic capacity of *C. labrosus* to produce n-3 LC-PUFA is partially dependent upon rearing conditions. Moreover, the present study shows that *C. labrosus* can operate compensatory mechanisms leading to increased LC-PUFA biosynthesis and thus counteract potential detrimental effects of limited supply of dietary n-3 LC-PUFA associated with VO-rich feed. The combination of a moderate dietary supply of n-3 LC-PUFA and a reduced salinity is an adequate strategy to maintain the nutritional value of *C. labrosus* juveniles. Both FA profiles and *in vitro* assays confirm that the enzymatic machinery involved in the biosynthesis of LC-PUFA from $C_{18}$ precursors is active in *C. labrosus* juveniles.

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## Data availability statement

Data are available on request to the corresponding author.

## Author contributions

Conceptualization, C.R. and Ó.M.; methodology, M.M., M.B., J.A.P., A.G., N.G.A. and C.R.; formal analysis, M.M.; investigation, M.M.; resources, C.R., A.B. and M.B.; writing—original draft preparation, M.M.; writing—review and editing, M.M., Ó.M., J.A.P. and C.R.; project administration, C.R.; funding acquisition, C.R. All authors have read and agreed to the published version of the manuscript.

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**Declaration of Competing Interest**

The authors have no conflicts of interest to declare regarding this work.

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**Supplementary Table 1.** Growth parameters of juveniles *Chelon labrosus* fed the fish oil (FO) and vegetable oil (VO) experimental diets and reared at 35 ppt and 20 ppt for 10 weeks.

| **Growth parameters** | ***FO 35ppt*** | ***FO 20ppt*** | ***VO 35ppt*** | ***VO 20ppt*** |
|---|---|---|---|---|
| FBW (g) | 25.0 ± 5.9 | 25.8 ± 4.6 | 26.0 ± 3.7 | 25.1 ± 4.3 |
| WG (g) | 9.2 ± 1.8 | 10.5 ± 1.7 | 11.8 ± 1.6 | 9.3 ± 1.9 |
| SGR (% day $^{-1}$) | 0.9 ± 0.2 | 1.1 ± 0.3 | 1.2 ± 0.4 | 0.9 ± 0.1 |
| FCR | 1.6 ± 0.3 | 1.5 ± 0.6 | 1.3 ± 0.4 | 1.6 ± 0.5 |

Data are expressed as mean ± SE (n = 15). FBW, Final Body Weight; WG, Weight Gain = final weight - initial weight; SGR, Specific Growth Rate [(ln final weight − ln initial weight)/time] × 100; FCR, Feed Conversion Ratio = feed intake / weight gain.

**Highlights of manuscript (3 or 4)**

Dietary regulation of the LC-PUFA biosynthesis is possible in *Chelon labrosus*.

Overexpression of *fads2* and *elovl5* shows the role of salinity in LC-PUFA production.

Low salinity is a convenient strategy to produce DHA enriched specimens.