Aquaculture 540 (2021) 736733

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

The lipid metabolism of Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae determined by ^{14}C *in vivo* incubations

Diana B. Reis [a,*], José A. Pérez [a], Kristin Hamre [b], Nieves G. Acosta [a], Birgitta Norberg [c], Torstein Harboe [c], Covadonga Rodríguez [a]

[a] Departamento de Biología Animal, Edafología y Geología, Universidad de La Laguna, 38206 San Cristóbal de La Laguna, Spain
[b] Institute of Marine Research, Nordnes, 5817, Bergen, Norway
[c] Institute of Marine Research, Austevoll Research Station, 5392 Storebø, Norway

ARTICLE INFO

Keywords:
Elongation/desaturation
Esterification
Fatty acids
Hippoglossus hippoglossus larvae
Lipid metabolism

ABSTRACT

The *in vivo* ability of Atlantic halibut (*Hippoglossus hippoglossus*) larvae to incorporate, de-acylate and re-acylate [1-^{14}C]fatty acids (FA) into lipid classes and to elongate and desaturate those substrates was elucidated. To this purpose, 30 days post-first-feeding (30 dpff) larvae were incubated in the presence of either free [1-^{14}C]-FAs (18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3), [1-^{14}C]18:1n-9 bound to mono- (MAG) and triacylglycerols (TAG), or [1-^{14}C]20:4n-6 bound to phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Halibut larvae can efficiently incorporate dietary FAs through MAG, while FA incorporation when provided as free FAs and especially bound to TAG or phospholipids seems to be very low. These results suggest that lipid digestion might be a limiting factor in the FA absorption and incorporation by 30 dpff Atlantic halibut. The present study also shows that esterification into phospholipids is a strong metabolic fate for dietary long-chain polyunsaturated fatty acids (LC-PUFAs), and the capacity of 30 dpff Atlantic halibut larvae to metabolize dietary FAs through elongation/desaturation processes. Nonetheless, neither EPA nor DHA were detected from [1-^{14}C]ALA, indicating a reduced capacity to biosynthesize these LC-PUFAs and the necessity for them to be adequately supplied through the diet for proper larval development.

1. Introduction

The Atlantic halibut (*Hippoglossus hippoglossus* Linnaeus, 1758) is a marine cold-water flatfish species inhabiting the boreal and subarctic waters in the North Atlantic, that can reach a weight over 300 kg in the wild (Roberts, 2018). Atlantic halibut populations have been defined as vulnerable, as they are sensitive to overfishing due to relatively slow growth and high age at maturation. Its high-quality meat and high market value make the halibut a good candidate for the aquaculture industry in the Northern hemisphere.

Despite the great interest on Atlantic halibut research and cultivation during the 1980s, its commercial production has been progressing slowly. One of the main obstacles for its production in captivity has been the unreliable supply of juveniles with an acceptable quality (Kongsvik, 2007). The survival rate of this species in captivity used to be less than 10% (Bjornsdottir et al., 2009) and there was a high incidence of abnormalities including malpigmentation, impaired eye migration, and skeletal deformities (Hamre et al., 2002; McEvoy et al., 1998; Næss and Lie, 1998). Nowadays, advances in live feed enrichment and larval rearing procedures have improved rearing success in Atlantic halibut larval culture (Hamre et al., 2020), it now being possible to achieve at least 40% survival through the larval stages and malformations in some cases reduced to less than 10%. Nonetheless, malformations remain as a major global problem in flatfish culture. Shao et al. (2017) showed that pigmentation and eye migration in Japanese flounder (*Paralichthys olivaceus*) was driven by light, setting up regulatory retinoic acid gradients. However, feeding strategy and nutrition may also be regarded as one of the major casual factors of malformations (Hamre et al., 2013), with deficiencies in n-3 long-chain polyunsaturated fatty acids (LC-PUFAs) being linked to the incidence of a plethora of abnormalities in these species (Estevez and Kanazawa, 1995; Hamre et al., 2005, 2007, 2013; McEvoy et al., 1998; Næss and Lie, 1998; Sargent et al., 1999).

Atlantic halibut is known to possess a long-lasting yolk sac stage of nearly 45 days at 6 °C, with first-feeding larvae being larger than most

* Corresponding author at: Departamento de Biología Animal, Edafología y Geología, Universidad de La Laguna, Avda, Astrofísico Francisco Sánchez s/n, 38206, La Laguna, Santa Cruz de Tenerife, Canary Islands, Spain.
E-mail address: dbotelho@ull.edu.es (D.B. Reis).

https://doi.org/10.1016/j.aquaculture.2021.736733
Received 27 November 2020; Received in revised form 22 March 2021; Accepted 2 April 2021
Available online 4 April 2021

other marine fish, at approximately 12 mm length (Harboe and Mangor-Jensen, 1998). Moreover, metamorphosis normally extends over a period of 45 days and larvae attain post-metamorphosis stage within 57 days after hatching (Sæle et al., 2004). Due to its large size, first-feeding larvae are normally fed with short-time enriched *Artemia* sp. nauplii up to 50–60 days before weaned onto a dry diet (Harboe and Mangor-Jensen, 1998). *Artemia* sp. is known to have a suboptimal nutritional profile for normal development of marine fish larvae, naturally presenting low contents of phospholipids and LC-PUFA such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3), essential during fish larval stages (Dhont et al., 2013; Monroig et al., 2003; Navarro et al., 1999; Sargent et al., 1999). Nonetheless, Hamre et al. (2020) showed the possibility to rear Atlantic halibut larvae with *Artemia* sp. nauplii. *Artemia* sp. is normally enriched with lipids in order to tailor its composition towards marine larvae requirements (Sorgeloos et al., 2001; van Stappen, 1996). However, the inherent metabolic activity of *Artemia* sp. towards the enrichment products and the variability associated with its enrichment protocols (Navarro et al., 1999; Reis et al., 2017) makes it difficult to targeting lipidic recommended levels. The complications on *Artemia* sp. enrichment with DHA is even more evident due to the preferential oxidation of this fatty acid (FA) and its retroconversion into EPA (Navarro et al., 1999; Reis et al., 2017), as this FA is directly related with the metamorphosis success of Atlantic halibut (Hamre et al., 2002). Moreover, Atlantic halibut larvae seem to have a low capacity to use DHA when it is bound to triacylglycerols (TAG; Evjemo et al., 2003; Mollan et al., 2008) which suggests that larvae may not efficiently metabolize TAG for *de novo* synthesis of phospholipids (Olsen et al., 2014). This limited ability of larvae to metabolize TAG may have adverse implications in halibut larviculture due to the preferential esterification of DHA into *Artemia* TAG even when it is provided in the form of phospholipids (Guinot et al., 2013).

A recent study showed that 28 days post-first-feeding (dpff) larvae seem to be sufficiently developed to feed on formulated diets (Hamre et al., 2019), which may allow for a reduction in the period during which halibut larvae are fed with *Artemia* sp. and the introduction of a dry-pellet diet at an earlier stage. The FA composition of halibut larvae seems to be highly influenced by dietary FA profile (Hamre et al., 2002). Therefore, the successful rearing of this species depends on optimized first-feeding regimes and the nutritional quality of starter diets. In this sense, it is essential to have a good knowledge of larval lipid requirements through development in order to improve larval and juvenile quality. *In vivo* incubation of larvae with radiolabeled FA markers has proven a reliable methodology for obtaining useful knowledge on lipid metabolism during early life stages of pikeperch (*Sander lucioperca;* Reis et al., 2020), European eel (*Anguilla anguilla*; Lund et al., 2021) and cephalopods species (*Octopus vulgaris* and *Sepia officinalis*; Reis et al., 2014, 2016a, 2016b), being observed a diversified LC-PUFA biosynthetic capability between species and specific incorporation or esterification patterns of FA substrates into lipid classes.

In order to gain information for the establishment of the FA requirements of Atlantic halibut larvae, to optimize first-feeding regimes and the nutritional quality of starter diets, the present study on lipid metabolism determined the ability of 30 dpff larvae to incorporate, esterify into lipid classes, and to de-acylate and re-acylate [1-^{14}C]free fatty acids (FFA), [1-^{14}C]18:1n-9 bound to monoacylglycerol (MAG) or TAG, and [1-^{14}C]20:4n-6 bound to phosphatidylcholine (PC) or phosphatidylethanolamine (PE).

2. Materials & methods

2.1. Larval rearing

Atlantic halibut larvae, initial wet weight 0.015 ± 0.001 g, were fed *Artemia* sp. nauplii enriched with LARVIVA Multigain (Biomar, Aarhus, Denmark) from day 1 post-first-feeding. Larvae were reared in triplicate tanks ($n = 3$) and reached 0.068 ± 0.013 g on 32 dpff. The first-feeding tanks were flat-bottomed, with a volume of 1100 L and a water flow of 5 L min^{-1}. Water temperature was 12 ± 0.3 °C during the whole experimental period. The tanks had shadow frames to avoid illumination of the walls and fluorescent (daylight) light sources were placed 70 cm above the water surface, giving a light intensity of approximately 400 lx at the surface. The tanks had central aeration near the bottom. The water outlet sieves were also in the centre of the tanks, reaching from the bottom to the surface. Water inlets were placed near the tank wall approximately 10 cm below the surface. Automatic cleaning devices (silicone wiper, Robert Bosch GmbH, Gerlingen-Schillerhöhe, Germany) were mounted in each tank and were run once a day. After one rotation, dead material was removed by a siphon. Water turbidity was created by the use of dissolved clay (Sibelco, Vingerling K148, white) to an initial turbidity of 2 NTU (Harboe and Reitan, 2005). Approximately 10 g of clay was dissolved in 1 L of freshwater and added to each tank twice a day. The survival on 40 dpff was 43 ± 4% ($n = 3$).

2.2. In vivo incubations with labeled [1-^{14}C] substrates

A total of 60 *H. hippoglossus* 30 dpff larvae were sampled and incubated in flat-bottom 6-wells tissue culture plates (SARSTEDT AG & CO., Nümbrecht, Germany) in triplicate (n = 3) at a density of 2 larvae per well in 10 mL of seawater (34‰). Incubations were performed with gentle stirring at 12 °C for 4 h, with 0.2 μCi (0.3 μM) of [1-^{14}C]labeled fatty acids (free FA molecule, labeled with ^{14}C in its first carbon from the carboxyl head), including 18:2n-6 (LA, linoleic acid), 18:3n-3 (ALA, alfa-linolenic acid) (PerkinElmer, Inc., Waltham, Massachusetts, USA), 20:4n-6 (ARA), 20:5n-3 (EPA), and 22:6n-3 (DHA) (American Radiolabeled Chemicals, Inc., St. Louis, Missouri, USA). Additionally, [1-^{14}C] 18:1n-9 (OA, oleic acid) esterified in the sn-2 position of MAG (2-Monooleyl-[1-^{14}C]glycerol) or at sn-1, sn-2 and sn-3 position of TAG (triolein-[carboxil-^{14}C]) (PerkinElmer, Inc.), as well as [1-^{14}C]ARA esterified in the sn-2 position of PC (L-α-palmitoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine) or PE (L-α-palmitoyl-2-[1-^{14}C]arachidonyl-phosphatidylethanolamine) (American Radiolabeled Chemicals) were also used as experimental substrates. In these last cases, both [1-^{14}C]OA and [1-^{14}C] ARA were used as a commercially-available FA model. Labeled substrates, dissolved in ethanol (absolute alcohol), were individually added to separate wells. Fish incubated without [1-^{14}C]substrates were used to assess lipid classes (LC) and FA profiles of 30 dpff *H. hippoglossus* larvae ($n = 3$). A total of 30 incubations were performed. A survival rate of 100% was obtained in all incubations. After incubation, larvae were sacrificed with an overdose MS222 (500 mg L^{-1}), thoroughly washed with seawater to remove excess of radiolabeled substrates, and immediately frozen at −80 °C.

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (EU Dir 2010/63) and the Norwegian Food Safety Authority, (FOTS application id: 15518).

2.3. Total lipid, lipid classes and fatty acid composition

Atlantic halibut total lipid (TL) content was obtained by homogenization of larvae with chloroform/methanol (2:1, *v*/v) according to the Folch method as described by Christie (2003). The organic solvent was evaporated under a stream of nitrogen and the lipid content determined gravimetrically. The TL extracts were stored at −20 °C in chloroform/methanol (2:1) with 0.01% butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10 mg mL^{-1} under an inert atmosphere of nitrogen until further analysis.

TL extracts of both larvae incubated without radiolabeled substrates and of *Artemia* sp. used as prey were used to determine LC and FA composition (n = 3). LC were separated by high-performance thin-layer chromatography (HPTLC) in a single-dimensional double-development using propanol/chloroform/methyl acetate/methanol/0.25%

potassium chloride (5:5:5:2:1.8, *v*/v) as developing solvent for polar lipid classes, and hexane/diethyl ether/acetic acid (22.5:2.5:0.25, v/v) for the neutral fractions on 10 cm × 10 cm HPTLC plates (Merck KGaA, Darmstadt, Germany). The different LC were visualized by charring at 160 °C after spraying with 3% (*w*/*v*) aqueous cupric acetate containing 8% (*v*/v) phosphoric acid and quantified by means of a CAMAG TLC Visualizer (Camag, Muttenz, Switzerland).

Fatty acid methyl esters (FAME) were obtained by acid-catalyzed transmethylation of 1 mg of TL extract during 16 h at 50 °C. FAME were purified by thin-layer chromatography (TLC; Christie, 2003) with hexane/diethyl ether/acetic acid (90:10:1, v/v) and then separated and quantified by means of a TRACE-GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The GC was equipped with an on-column injector, a flame ionization detector, and a fused silica capillary column, Supelcowax TM 10 (30 m × 0.32 mm I.D. x 0.25 μm; Sigma-Aldrich Co., St. Louis, Missouri, USA). Helium was used as carrier gas and temperature programming was 50–150 °C at 40 °C min^{-1} slope, then from 150 to 200 °C at 2 °C min^{-1}, to 214 °C at 1 °C min^{-1} and, finally, to 230 °C at 40 °C min^{-1}. When necessary, identification of individual FAME was confirmed by GC–MS chromatography (DSQ II, Thermo Fisher Scientific Inc.).

2.4. Incorporation and esterification of radiolabeled substrates into lipid classes

The incorporation of radiolabeled FAs into TL of Atlantic halibut larvae was determined as described by Reis et al. (2019). Briefly, an aliquot of 0.1 mg of radiolabeled lipid extracts were transferred to scintillation vials and radioactivity determined on a LKB Wallac 1214 Rackbeta liquid scintillation ß-counter (PerkinElmer, Inc.). Results in dpm were transformed into pmol mg $protein^{-1}$ h^{-1} taking into account the specific activity of each substrate, and sample TL and protein contents (Lowry et al., 1951).

The esterification pattern of incorporated [1-^{14}C]FAs (either supplied as FFA or bound to MAG, TAG, PC or PE) into the different LC of halibut larvae was determined as described in Section 2.3., for the separation of the LC. Then, developed HPTLC plates were placed for 2 weeks in closed exposure cassettes (Exposure Cassette-K, BioRad, Madrid, Spain) in contact with a radiation-sensitive phosphor screen (Image Screen-K, BioRad). The screens were then scanned with an image acquisition system (Molecular Imager FX, BioRad), and the bands were quantified in percentage by an image analysis software (Quantity One, BioRad).

2.5. Transformation of radiolabeled substrates by elongation/desaturation

Transformation of incubated [1-^{14}C]FAs by elongation/desaturation processes was determined using pre-coated TLC plates SIL G-25 (20 cm × 20 cm; Macherey-Nagel GmbH & Co. KG, Düren, Germany), pre-impregnated with a solution of 2 g of silver nitrate in 20 mL of acetonitrile as described by Reis et al. (2019). Plates were fully developed in toluene/acetonitrile (95:5, *v*/v) as solvents, which resolve the FAME into discrete bands based on both degree of unsaturation and chain length. Bands were then revealed and quantified as mentioned in Section 2.4. Identification of labeled bands was confirmed by radiolabeled standards formulated with a blend of commercially available ^{14}C-FA substrates (including 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, 20:4n-6, 20:5n-3 and 22:6n-3) at a concentration of 1 μCi mL^{-1} hexane each, simultaneously run on the same plate (Reis et al., 2019). Radioactivity of the standard mixture was previously validated by means of a scintillation β-counter. The capacity of Atlantic halibut larvae to elongate and desaturate polyunsaturated fatty acids was verified over 18:2n-6 as ARA precursor, 18:3n-3 as EPA precursor, and 20:5n-3 as DHA precursor.

2.6. Statistical analysis

Differences between the incorporation rates of radiolabeled FFAs into larvae TL, between their esterification into LC and between substrates after elongation/desaturation processes, were assessed by a one-way ANOVA followed by Tukey HSD *post hoc* test. Normal distribution of the data and homogeneity of the variances were verified with the one-sample Shapiro-Wilk test and the Levene test, respectively and, when necessary, appropriate variance-stabilizing transformations were performed. If transformations did not succeed, Welch test was performed, followed by T3 Dunnet. Kruskall-Wallis non-parametric test was applied in the case of non-normal distribution followed by pair-wise comparisons Mann-Whitney test with Bonferroni correction. Differences between the incorporation into TL and re-esterification rates of OA bound to MAG or TAG or between ARA bound to PC or PE, as well as *de novo* synthesis of <18C FAs from LA and ALA metabolism were analyzed by a Student's *t*-test. Results are presented as mean ± standard deviation (SD) and the statistical significance was established at $p < 0.05$. All statistical analyses were performed using IBM® SPSS Statistics 23.0 software package (IBM Corp., New York, USA) for Windows.

3. Results

3.1. Fatty acid composition of Artemia sp.

The FA profile of the enriched *Artemia* sp. nauplii used to feed Atlantic halibut larvae was PUFA (50.1%) > monounsaturated fatty acids (MUFA; 26.1%) > saturated fatty acids (SFA; 22.8%), where ALA was the most abundant individual FA, followed by OA and palmitic acid (16:0) (23.8, 16.4 and 13.6% of total FA, respectively; Table 1).

Table 1
Main fatty acid composition (% of total fatty acids) of *Artemia* sp. used as prey for Atlantic halibut (*Hippoglossus hippoglossus*) larvae.

	Artemia
16:0	13.6 ± 2.8
18:0	5.9 ± 1.2
Σ SFA	22.8 ± 2.4
16:1n-9	1.0 ± 0.2
16:1n-7	1.6 ± 0.2
18:1n-9 OA	16.4 ± 2.6
18:1n-7	6.0 ± 1.4
Σ MUFA	26.1 ± 4.6
18:2n-6 LA	5.3 ± 0.4
20:2n-6	0.2 ± 0.0
20:4n-6 ARA	2.2 ± 0.3
22:5n-6	2.7 ± 0.3
Σ n-6 PUFA	10.6 ± 1.4
Σ n-6 LC-PUFA	5.3 ± 0.1
18:3n-3 ALA	23.8 ± 3.3
20:3n-3	nd
20:5n-3 EPA	3.2 ± 1.3
22:5n-3	0.1 ± 0.0
22:6n-3 DHA	7.7 ± 1.1
Σ n-3 PUFA	38.5 ± 1.7
Σ n-3 LC-PUFA	11.0 ± 1.3
Σ PUFA	50.1 ± 2.9
n-3/n-6	3.6 ± 0.4
ARA/EPA	0.7 ± 0.3
DHA/EPA	2.4 ± 0.1

Results represent means ± SD; $n = 3$. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; Σ include some minor components not shown. nd, not detected.

3.2. Lipid and fatty acid composition of H. hippoglossus larvae

The TL content of 30 dpff Atlantic halibut larvae was 1.1 ± 0.4 mg larvae^{-1} (Table 2). Larvae presented higher proportions of polar lipids (PL) than neutral lipids (NL) (~60.0 *vs.* ~40%, respectively), with PC being the most abundant lipid fraction (22.8%) followed by PE (18.0%). Within NL, cholesterol was the major LC representing 16.4% of the TL (Table 2).

The larval FA distribution pattern was PUFA (46.7%) > MUFA (27.7%) > SFA (21.2%) (Table 3), mainly represented by ALA, OA and 16:0, respectively. Noteworthy, the n-3 LC-PUFA proportion represented around 20% of total FA, with DHA being the most abundant component (Table 3).

3.3. Incorporation of [1-^{14}C] fatty acids into H. hippoglossus larval tissues

[1-^{14}C]DHA showed the lowest incorporation rate (~2 pmol mg protein^{-1} h^{-1}) into larval lipids of all incubated [1-^{14}C]free FAs whereas [1-^{14}C]EPA was the most-incorporated FA (7.2 ± 3.1 pmol mg protein^{-1} h^{-1}) (Table 4). Larvae esterified a much larger proportion of [1-^{14}C]OA when incubated bound to MAG (16.9 pmol mg protein^{-1} h^{-1}) compared to TAG (0.8 ± 0.1 pmol mg protein^{-1} h^{-1}), while [1-^{14}C]ARA was incorporated to a higher extent when provided as FFA than when bound to PC or to PE (Table 4).

3.4. Esterification of [1-^{14}C] fatty acids into H. hippoglossus larval lipid classes

The [1-^{14}C]LC-PUFAs (ARA, EPA and DHA) were largely esterified into the different LC with a maximum of 16.1% of the radioactivity recovered as FFA while a larger proportion of [1-^{14}C]PUFA substrates (LA and ALA) remained unesterified (32%; $p < 0.05$; Table 5). The three LC-PUFAs were incorporated to a higher extent into phospholipids than the two C18 PUFAs (65–73% *vs.* 52–55%, respectively). In addition, [1-^{14}C]LA and [1-^{14}C]ALA were preferentially esterified into PC, PE, and PAG (partial-acylglycerols), [1-^{14}C]ARA into PI and PC, [1-^{14}C]EPA into PC and PI/PAG, and [1-^{14}C]DHA into PC and PE. Interestingly, neither [1-^{14}C]ARA nor [1-^{14}C]DHA were esterified into TAG. Overall, [1-^{14}C] C18 FAs, [1-^{14}C]EPA and [1-^{14}C]DHA were preferentially acylated into larval PC (32–43%), while [1-^{14}C]ARA presented the highest esterification rate into phosphatidylinositol (PI; 32.4% *vs.* 1.9–15.5% of the other radiolabeled FAs).

The esterification pattern of [1-^{14}C]OA differed notably depending upon the form of MAG or TAG that was provided (Table 6). When acylated to MAG, most [1-^{14}C]OA was recovered as FFA (37%) or re-esterified into PC (23%) whereas when bound to TAG, 50% was recovered as FFA and the remaining substrate re-esterified into PAG (21%) and TAG (29%). Interestingly, OA was not incorporated into phospholipids when presented as TAG (Table 6). The esterification of [1-^{14}C] ARA also varied depending on to which phospholipid it was originally acylated. Thus, when acylated into PC, [1-^{14}C]ARA was re-esterified into PC > PI > PAG > PE. By contrast, a preferential re-esterification into PE = PC > PI = PAG occurred when bound to PE.

Table 2
Total lipid content (mg larvae^{-1}) and main lipid class composition (% of total lipid) of 30 days post-first-feeding Atlantic halibut (*Hippoglossus hippoglossus*) larvae.

Total lipid content	1.1 ± 0.4
Lipid class	
Lysophosphatidylcholine	0.5 ± 0.1
Sphingomyelin	1.3 ± 0.1
Phosphatidylcholine	22.8 ± 1.0
Phosphatidylserine	4.9 ± 0.2
Phosphatidylinositol	4.9 ± 0.1
Phosphatidylglycerol	4.0 ± 0.3
Phosphatidylethanolamine	18.0 ± 0.6
Unknown	2.3 ± 0.6
Total polar lipids	58.6 ± 1.9
Monoacylglycerols	2.1 ± 0.2
Diacylglycerols	0.3 ± 0.0
Cholesterol	16.4 ± 0.5
Free Fatty Acids	6.5 ± 0.6
Triacylglycerols	13.6 ± 1.0
Sterol Esters	2.5 ± 0.5
Total neutral lipids	41.4 ± 1.9

Results represent means ± SD; n = 3.

Table 3
Main fatty acid composition (% of total fatty acids) of 30 days post-first-feeding (dpff) Atlantic halibut (*Hippoglossus hippoglossus*) larvae.

	30 dpff larvae
16:0	13.0 ± 0.2
18:0	7.2 ± 0.2
Σ SFA	21.2 ± 0.4
16:1n-9	1.1 ± 0.0
16:1n-7	1.4 ± 0.1
18:1n-9 OA	16.4 ± 0.5
18:1n-7	7.2 ± 0.2
Σ MUFA	27.7 ± 0.5
18:2n-6 LA	4.9 ± 0.0
20:2n-6	0.4 ± 0.0
20:4n-6 ARA	3.6 ± 0.2
22:5n-6	2.1 ± 0.1
Σ n-6 PUFA	11.2 ± 0.3
Σ n-6 LC-PUFA	5.6 ± 0.3
18:3n-3 ALA	14.1 ± 0.8
20:3n-3	2.9 ± 0.1
20:5n-3 EPA	6.9 ± 0.1
22:5n-3	0.5 ± 0.0
22:6n-3 DHA	8.2 ± 0.5
Σ n-3 PUFA	35.5 ± 0.6
Σ n-3 LC-PUFA	19.4 ± 0.5
Σ PUFA	46.7 ± 0.5
Σ DMA	1.0 ± 0.2
n-3/n-6	3.3 ± 0.1
ARA/EPA	0.5 ± 0.0
DHA/EPA	1.2 ± 0.1

Results represent means ± SD; n = 3. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; DMA, dimethylacetals. Σ include some minor components not shown.

Table 4
Incorporation of radiolabeled substrates into total lipid (pmol mg protein^{-1} h^{-1}) in 30 days post-first-feeding Atlantic halibut (*Hippoglossus hippoglossus*) larvae.

[1-^{14}C]Substrates			
18:2n-6 LA	3.3 ± 1.0	ab	
18:3n-3 ALA	5.0 ± 0.8	ab	
20:4n-6 ARA	4.1 ± 1.1	ab	y
20:5n-3 EPA	7.2 ± 3.1	b	
22:6n-3 DHA	2.2 ± 0.5	a	
18:1n-9-MAG	16.9 ± 5.0		*
18:1n-9-TAG	0.8 ± 0.1		
20:4n-6-PC	2.0 ± 0.8		x
20:4n-6-PE	1.2 ± 0.3		x

Results represent means ± SD; n = 3. MAG, monoacylglycerols; TAG, triacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Different letters within columns 3 and 4 indicate significant differences between incubated free fatty acids (a,b) or between ARA incubated as free fatty acid or bound to PC and PE (x,y) ($p < 0.05$). Asterisk (*) indicates significant difference between OA bound to MAG or TAG ($p < 0.05$).

Table 5
Esterification (% total incorporated substrate) of radiolabeled free fatty acids into lipid classes in 30 days post-first-feeding Atlantic halibut (*Hippoglossus hippoglossus*) larvae.

	^{14}C 18:2n-6		^{14}C 18:3n-3		^{14}C 20:4n-6		^{14}C 20:5n-3		^{14}C 22:6n-3	
LPC	2.6 ± 1.3		nd		nd		nd		nd	
PC	31.5 ± 2.1	b	43.3 ± 1.3	c	22.7 ± 2.2	a	32.0 ± 1.6	b	32.6 ± 2.0	b
PS	3.9 ± 0.5	ab	2.0 ± 0.3	a	5.2 ± 0.6	b	5.1 ± 0.2	b	4.4 ± 1.5	b
PI	4.5 ± 0.8	b	1.9 ± 0.8	a	32.4 ± 4.4	d	15.5 ± 1.6	c	11.6 ± 0.5	c
PE	9.7 ± 1.0	ab	7.4 ± 1.4	a	12.5 ± 1.4	b	12.8 ± 1.2	b	20.4 ± 0.9	c
TPL	52.2 ± 5.2	ab	54.6 ± 0.6	a	72.8 ± 2.2	c	65.4 ± 2.3	bc	69.0 ± 2.9	bc
PAG	9.8 ± 1.2	ab	6.7 ± 0.2	a	11.1 ± 0.4	b	16.1 ± 2.0	c	15.1 ± 2.0	c
FFA	32.6 ± 6.1	b	32.1 ± 1.6	b	16.1 ± 1.8	a	13.2 ± 1.4	a	15.9 ± 1.2	a
TAG	5.5 ± 0.6	b	6.6 ± 0.9	b	nd	a	5.3 ± 1.1	b	nd	a
TNL	47.8 ± 5.2	bc	45.4 ± 0.6	c	27.2 ± 2.2	a	34.6 ± 2.3	ab	31.0 ± 2.9	ab

Results represent means ± SD; n = 3. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TPL, total polar lipids; PAG, partial-acylglycerols; FFA, free fatty acids; TAG, triacylglycerols; TNL, total neutral lipids. nd, not detected. Different letters placed after the esterification values within the same row indicate significant differences between substrates ($p < 0.05$).

Table 6
Re-esterification (% total incorporated substrate) of radiolabeled fatty acids into lipid classes in 30 days post-first-feeding Atlantic halibut (*Hippoglossus hippoglossus*) larvae when provided bounded to MAG, TAG, PC, or PE.

	[1-^{14}C]18:1n-9			[1-^{14}C]20:4n-6		
	MAG	TAG		PC	PE	
LPC	2.1 ± 0.4	nd		nd	nd	
PC	22.8 ± 1.5	nd		30.2 ± 0.4	27.0 ± 2.1	
PS	2.1 ± 0.1	nd		7.1 ± 0.9	10.9 ± 0.6	#
PI	5.3 ± 0.4	nd		20.7 ± 1.1	13.4 ± 0.8	#
PE	8.3 ± 0.9	nd		10.6 ± 0.2	28.9 ± 2.0	#
TPL	40.6 ± 2.7	nd		68.7 ± 0.5	80.2 ± 0.3	#
PAG	9.1 ± 0.8	21.1 ± 8.1	*	15.4 ± 1.3	12.1 ± 0.2	#
FFA	37.4 ± 3.0	50.2 ±10.1		15.9 ± 0.9	7.8 ± 0.1	#
TAG	10.6 ± 1.1	28.7 ± 4.7	*	nd	nd	
SE	2.3 ± 0.6	nd		nd	nd	
TNL	59.4 ± 2.7	100.0 ± 0.0	*	31.3 ± 0.5	19.8 ± 0.3	#

Results represent means ± SD; n = 3. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TPL, total polar lipids; PAG, partial-acylglycerols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; TNL, total neutral lipids; MAG, monoacylglycerols; Asterisk (*) indicates significant difference between OA bound to MAG or TAG ($p < 0.05$). Hashtag (#) indicates significant difference between ARA bound to PC or PE ($p < 0.05$).

3.5. Transformation of [1-^{14}C] fatty acids by elongation/desaturation processes

Most radioactivity incorporated into larval lipids was recovered as unmodified substrate (56–93%) (Table 7). However, at least one elongation product was obtained from every [1-^{14}C]FA assayed, this being the only product detected from [1-^{14}C]EPA metabolism. Elongation/desaturation products from both [1-^{14}C]LA and [1-^{14}C]ALA were present in larval lipids where [1-^{14}C]LA was partially converted to 20:2n-6, 20:3n-6, 20:4n-6 and 22:5n-6, and [1-^{14}C]ALA was transformed into 20:3n-3 and 20:4n-3 (Table 7 and Fig. 1). Moreover, *de novo* synthesis of shorter chain-length FAs (<18 carbons) was also registered for the two [1-^{14}C] C18 FAs.

Table 7
Recovery (% total incorporated substrate) of [1-^{14}C]FA substrates and their elongation/desaturation metabolites in 30 days post-first-feeding Atlantic halibut (*Hippoglossus hippoglossus*) larvae.

Substrate	Product	Recovery	
[1-^{14}C]18:2n-6 (LA)			
	18:2n-6	56.4 ± 7.0	a
	20:2n-6	7.5 ± 1.8	
	20:3n-6	14.8 ± 1.8	
	20:4n-6	9.0 ± 0.5	
	22:5n-6	3.4 ± 2.3	
	de novo	8.8 ± 1.8	*
[1-^{14}C]18:3n-3 (ALA)			
	18:3n-3	74.5 ± 1.8	b
	20:3n-3	17.4 ± 1.9	
	20:4n-3	3.6 ± 1.4	
	de novo	4.6 ± 1.0	
[1-^{14}C]20:5n-3 (EPA)			
	20:5n-3	93.5 ± 2.0	c
	22:5n-3	6.5 ± 2.0	

Results represent means ± SD; n = 3. *de novo,* synthesis of fatty acids with shorter chain-length (less than 18 carbons). Different letters indicate significant differences between unmodified substrates ($p < 0.05$). Asterisk (*) indicates significant difference between *de novo* synthesis of <18C FA.

4. Discussion

4.1. Lipid and fatty acid composition of H. hippoglossus larvae

The successful rearing of *H. hippoglossus* larvae depends on the optimization of first-feeding regimes and the nutritional quality of starter diets, which requires a wide knowledge of larval lipid metabolism and requirements through development. The FA profile of wild Atlantic halibut eggs (Flak-Petersen et al., 1989), hatchlings (Rainuzzo et al., 1992), and larvae prior to first-feeding (Næss et al., 1995) is characterized by a high content of DHA (over 25% of total FA), 16:0 (~17% of total FA), EPA (over 11%), and OA (~10%). Interestingly, 30 dpff halibut larvae reflected *Artemia* FA profile, presenting high levels of OA (16.4% of total FA), ALA (14.1%) and 16:0 (13.0%), but reduced proportions of DHA (8.2%) and EPA (6.9%). A low content of ALA has been reported in wild Atlantic halibut eggs (0.3–0.4% of total FA; Flak-Petersen et al., 1989), hatchlings (~0.8%), and larvae prior to first-feeding (0.2–0.6%; Rainuzzo et al., 1992). Moreover, during first-feeding, Næss et al. (1995) reported a 0.4–0.7% of ALA in larvae fed wild zooplankton and values from 9.0 to 11.7% when fed with *Artemia* sp. In this sense, the high amount of ALA recorded in 30 dpff larvae seems to be a direct consequence of the dietary input through *Artemia* sp., which contained a 24% of C18 omega-3 FA (Table 1), making it more difficult to resemble the natural larvae fatty acid profiles.

4.2. Digestion, incorporation and esterification of [1-^{14}C] fatty acids into H. hippoglossus larval lipid classes

The incorporation and esterification of FA into animals tissues depend on their digestive and absorptive capacity, this being influenced by the enzymatic endowment of each species to digest phospholipids and TAGs as the main dietary components (Olsen et al., 2014). Prior to their absorption into enterocytes, phospholipids and TAG must be hydrolyzed by a different set of enzymes to produce lyso-phospholipids (from phospholipids digestion) and MAG (from TAG digestion) in

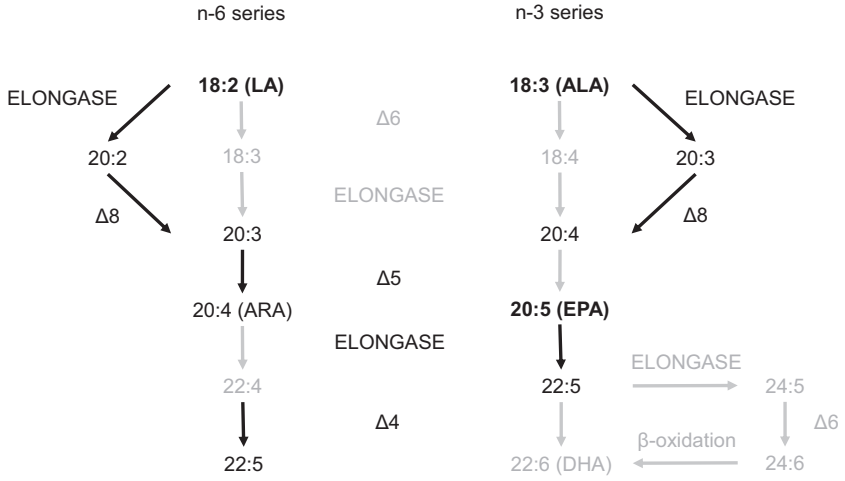

Fig. 1. The suggested biosynthetic pathway of long-chain polyunsaturated fatty acids found in 30 days post-first-feeding Atlantic halibut (*Hippoglossus hippoglossus*) larvae, considering previous metabolic routes reported in teleost species. Fatty acids in bold represent incubated ^{14}C-substrates. Fatty acids and line arrows in black represent detected routes. Fatty acids and line arrows in grey represent known compounds and routes that were not detected.

addition to the FFA released. In this sense, FA composition and its configuration into dietary phospholipids and TAG may affect lipid absorption and, consequently, lipid classes synthesis and fish larvae performance (Olsen et al., 2014).

Once absorbed, the esterification pattern of individual FAs into phospholipids is related to their specific role in membrane integrity and function (Sargent et al., 2002; Tocher et al., 2008). On the other hand, neutral lipids, and more precisely TAG, are presumably used to satisfy energy demands (Rainuzzo et al., 1997; Sargent et al., 2002), with SFA and MUFA being the main substrates. [1-^{14}C]OA bound to MAG, and [1-^{14}C]ALA and [1-^{14}C]LA provided as FFA, were mainly recovered as FFA or esterified into larval PC. PC is known as the most important reservoir of FAs among the polar lipids, FAs which are needed for phospholipid anabolism and turnover (Sargent et al., 2002; Tocher et al., 2008).

The metabolic fate of [1-^{14}C]FFAs demonstrate a consistent specificity in the esterification pattern of each LC-PUFA by halibut larvae. As previously reported, ARA presents a selective location/retention into PI in fish tissues (Bell and Dick, 1990; Bell and Tocher, 1989; Bell et al., 1997; Sargent et al., 2002; Tocher, 1995) whereas both [1-^{14}C]EPA and [1-^{14}C]DHA were highly esterified into PC, the major constituent of polar lipids in membranes and lipoproteins, with DHA being the major FA esterified into PE.

The preservation of the characteristic FA esterification pattern among lipid classes is not only controlled by the endogenous capability of organisms to complete the de-acylation/re-acylation turnover processes, but it is also influenced by the FA profile of the diet (Olsen et al., 2014; Tocher, 2003). Evjemo et al. (2003) reported a low capacity of Atlantic halibut larvae to incorporate DHA when fed short-term enriched *Artemia*. The high content of ALA in the *Artemia* and its esterification affinity for the larval PC (see Lund et al., 2021; Reis et al., 2020) could hamper the incorporation of DHA which is provided at a much lower rate than ALA by the enriched *Artemia* (Reis et al., 2017). This poorer incorporation of DHA might also be associated with the intrinsic *Artemia* metabolism, which shows a preferential oxidation of DHA and its esterification into TAG (Navarro et al., 1999; Reis et al., 2017), suggesting that halibut larvae cannot efficiently utilize DHA from dietary TAG (Evjemo et al., 2003; Mollan et al., 2008). In accordance to Mollan et al. (2008), [1-^{14}C]OA bound to TAG presented a much lower incorporation rate into larval tissues than when bound to MAG (0.8 *vs.* 16 pmol mg protein^{-1} h^{-1}, respectively). This difference might be explained by the direct absorption of MAG without previous digestion while two FAs must be hydrolyzed from the TAG molecule prior to being absorbed (Olsen et al., 2014). Similarly, labeled ARA bound to PC and PE presented lower incorporation rates than [1-^{14}C]OA bound to MAG (Table 4). Although a different set of digestive enzymes are responsible for the hydrolysis of phospholipids (see Olsen et al., 2014), associated FAs must also be hydrolyzed prior to their absorption by enterocytes. Altogether, these results indicate that, in line with Mollan et al. (2008), digestion might be the most limiting factor for lipid absorption in Atlantic halibut larvae and that halibut rearing performance may be improved by providing dietary pre-digested lipids. In this sense, Sæle et al. (2013) indicated that hydrolyzed phospholipids were toxic to cod larvae because FFAs and lysophospholipids seemed to act as detergents, solubilizing enterocyte membranes. Nonetheless, the use of marine TAG (with a high content of DHA and EPA) previous hydrolyzation to provide dietary MAG and FFAs for Atlantic halibut larvae would be an interesting line of research for the future.

The re-esterification pattern of incorporated [1-^{14}C]OA highly differed depending on whether it was originally bound to MAG or TAG. When bound to TAG, over 50% of [1-^{14}C]OA was recovered as FFA, which indicates some capacity of this species to digest TAG during the larval stage. Interestingly, no [1-^{14}C]OA was re-esterified into PL, although the reduced amount (<1 pmol mg protein^{-1} h^{-1}) of radiolabeled substrate incorporated could have hampered its detection. Even so, these results are in agreement with the findings of an inefficient *de novo* synthesis of PL from dietary TAG in fish larvae (Cahu et al., 2009; Daprà et al., 2011; Gisbert et al., 2005; Tocher et al., 2008). By contrast, [1-^{14}C]OA bound to MAG was esterified into both NL and PL. It is generally assumed that the FA esterified at sn-2 position in the MAG molecule is retained during absorption (Olsen et al., 2014). However, despite being bound into the sn-2 position, the majority of [1-^{14}C]OA was recovered as FFA (~37% of incorporated substrate), indicating its hydrolysis from the MAG molecule. Moreover, a high proportion of [1-^{14}C]OA was re-acylated into PC, similar to every assayed radiolabeled FFA with the exception of [1-^{14}C]ARA, which was mainly detected into PI.

The principal precursors of phospholipid synthesis in larvae are dietary phospholipids, producing lyso-phospholipids and FFAs after digestion that are later re-esterified in the enterocyte to form phospholipids (Olsen et al., 2014). In the present study, [1-^{14}C]ARA bound to PC or PE was re-acylated into other phospholipids, indicating that 30 dpff Atlantic halibut larvae may re-modulate dietary phospholipids through de-acylation/re-acylation processes. This phospholipid remodeling has an important role in maintaining the characteristic FA esterification pattern within LC (Tocher, 2003), being related to the specific

role of phospholipids in membrane structure and function (Tocher, 1995; Tocher et al., 2008), and therefore in larval development and performance. The esterification pattern of [1-^{14}C]ARA after PC and PE digestion and re-acylation into larval tissues was different from that observed when provided as [1-^{14}C]FFA (Tables 5 and 6). Thus, ARA was mainly recovered in the same phospholipid class in which it was provided. However, the capacity of 30 dpff halibut larvae to re-modulate dietary phospholipids seems to be hampered by the limited capacity of this species to digest phospholipids during the larval stage, which could lead to an insufficient delivery of FA for normal larval development. During the larval stage, Atlantic halibut lipid profile seems to be highly influenced by prey composition (Næss et al., 1995; Hamre et al., 2002) and consequently, dietary lipid composition supplied during this stage might be crucial to its development, defining the future performance of the culture of this species.

4.3. Transformation of [1-^{14}C] fatty acids by elongation/desaturation processes

The incubation of larvae with C18 FAs gave rise to *de novo* synthesis of FAs with less than 18 carbons of chain-length by the recycling of the labeled carbon (acetyl-CoA) obtained from their β-oxidation. FAs are the major energetic compounds in fish (Sargent et al., 1995), with both LA and ALA being considered as good substrates for β-oxidation (Brown, 2016; Chen et al., 2018; Freemantle et al., 2006). The data reported herein indicate that 30 dpff halibut larvae may effectively use dietary LA and ALA as energetic substrates. In addition to their role as energy sources, C18 FAs are also precursors of the physiologically-important LC-PUFA including ARA, EPA and DHA (Tocher, 2015). Higher dietary contents of DHA and EPA have been described to improve pigmentation and eye-migration success in halibut (Hamre and Harboe, 2008a; Hamre et al., 2002, 2005, 2007; McEvoy et al., 1998; Næss and Lie, 1998), and larval and juvenile rearing performance (Hamre and Harboe, 2008b; Martins et al., 2007; Mazorra et al., 2003; Olsen et al., 1999). The capacity of each species to biosynthesize LC-PUFA depends on their capacity to elongate and desaturate C18 PUFA precursors (Garrido et al., 2019; Monroig et al., 2018). The present *in vivo* metabolic study reveals that Atlantic halibut larvae have the ability to elongate and desaturate C18 and C20 FAs (Fig. 1). Although similar capacity has also been reported in other Pleuronectiform species, each species shows different LC-PUFA biosynthetic abilities. Thus, turbot (*Scophthalmus maximus*) has some capacity to biosynthesize DHA and EPA from ALA, and ARA from LA (Ghioni et al., 1999; Rodriguez et al., 2002). In Senegalese sole (*Solea senegalensis*), Morais et al. (2012, 2015) showed the activity of a Δ4 desaturase and of a elongation of very long-chain fatty acids proteins (Elov5) in hepatocytes and enterocytes, that allowed the conversion of EPA into DHA. Similar results were obtained by Galindo et al. (2021) and Garrido et al. (2019) for sand sole (*Pegusa lascaris*) where only Δ4 desaturase activity was recorded whereas Kabeya et al. (2017) reported the activity of Δ6 and Δ8 desaturases, and Elovl5 elongase in the Japanese flounder (*Paralichthys olivaceus*). Atlantic halibut larvae incubated with [1-^{14}C]LA and [1-^{14}C]ALA elongated these FAs into ^{14}C-20:2n-6 and ^{14}C-20:3n-3, respectively, which could have been subsequently converted into ^{14}C-20:3n-6 and ^{14}C-20:4n-3 through the addition of a double bond to the carboxylic acid chain by a Δ8 desaturase. Moreover, a Δ5 and a possible Δ4 desaturase activity was also recorded over ^{14}C-20:3n-6 to produce ^{14}C-20:4n-6 (ARA) and ^{14}C-22:5n-6, respectively (Fig. 1); although the production of this last FA through the action of an Elovl2 to elongate 22:4n-6 to 24:4n-6 followed by a Δ6 desaturase and a final ß-oxidation over 24:5n-6 by the Sprecher pathway (Sprecher, 2000) cannot be completely ruled out. As Elovl2 has only been described in a few teleost species (Machado et al., 2018; Monroig et al., 2016), its absence has been hypothesized to account for the limited DHA biosynthetic capacity in marine fish (Castro et al., 2016). By contrast, similar bioconversions were not registered when larvae were incubated with [1-^{14}C]ALA, where neither ^{14}C-EPA nor ^{14}C-DHA were obtained, indicating the reduced ability of this species to biosynthesize these FAs. As a consequence, EPA and DHA must be considered as essential FAs for Atlantic halibut larvae, and must be adequately supplied through the diet for proper larval development. The reported differences in the production of longer and more unsaturated FAs may be attributed to the possible n-6 preference/specificity of the enzymes involved. In accordance, Morais et al. (2015) did not find evidence for a Δ6 activity in their study on *S. senegalensis* where only [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 were used as substrates.

5. Conclusion

The present study shows that Atlantic halibut larvae can efficiently incorporate dietary FAs through MAG, while FA absorption when bound to TAG or even to phospholipids seems to be very low. In line with Mollan et al. (2008), the present results indicate that digestion might be the limiting factor on FAs absorption in Atlantic halibut and that halibut rearing performance may be improved by providing dietary pre-digested lipids to larvae. Moreover, the capacity of 30 dpff halibut larvae to metabolize dietary fatty acids through elongation/desaturation processes was demonstrated. Nonetheless, neither EPA nor DHA were obtained from [1-^{14}C]ALA, suggesting the absence or limited activity of the enzymatic machinery involved in the biosynthesis of these FAs and the necessity to be adequately supplied through the diet for proper larval development.

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.

Acknowledgements

This work was supported by the project DIVERSIFY (KBBE-2013-07single stage, GA 603121) founded by the European Union Seventh Framework Programme. Dr. C. Rodriguez belongs to the Institute of Biomedical Technologies (ITB) and Centro de Investigaciones Biomédicas de Canarias (CIBICAN), Canary Islands, Spain.

References

Bell, M.V., Dick, J.R., 1990. Molecular species composition of phosphatidylinositol from the brain, retina, liver and muscle of cod (*Gadus morhua*). Lipids 25, 691–694.
Bell, M.V., Tocher, D.R., 1989. Molecular species composition of the major phospholipids in brain and retina from rainbow trout (*Salmo gairdneri*). Occurrence of high levels of di-(n-3) polyunsaturated fatty acid species. Biochem. J. 264, 909–915.
Bell, J.G., Farndale, B.M., Bruce, M.P., Navas, J.M., Carillo, M., 1997. Effects of broodstock dietary lipid on fatty acid compositions of eggs from sea bass (*Dicentrarchus labrax*). Aquaculture 149, 107–119.
Bjornsdottir, R., Johannsdottir, J., Coe, J., Smaradottir, H., Agustsson, T., Sigurgisladottir, S., Gudmundsdottir, B.K., 2009. Survival and quality of halibut larvae (*Hippoglossus hippoglossus* L.) in intensive farming: possible impact of the intestinal bacterial community. Aquaculture 286, 53–63.
Brown, R.A., 2016. The crucial relevance of ALA and LA as primary peroxisomal betaoxidation substrates, of oxidised LA as the primary endogenous activator of PPAR gamma, and energy deficit as the primary activator of PPAR alpha. In: Hegde, M., Zanwar, A., Adekar, S. (Eds.), Omega-3 Fatty Acids. Springer, Cham, pp. 451–463.
Cahu, C.L., Gisbert, E., Villeneuve, L.A., Morais, S., Hamza, N., Wold, P.A., Zambonino-Infante, J.L., 2009. Influence of dietary phospholipids on early ontogenesis of fish. Aquac. Res. 40, 989–999.
Castro, L.F.C., Tocher, D.R., Monroig, Ó., 2016. Long-chain polyunsaturated fatty acid biosynthesis in chordates: insights into the evolution of fads and Elovl gene repertoire. Prog. Lipid Res. 62, 25–40.
Chen, C., Guan, W., Xie, Q., Chen, G., He, X., Zhang, H., Guo, W., Chen, F., Tan, Y., Pan, Q., 2018. N-3 essential fatty acids in Nile tilapia, *Oreochromis niloticus*: bioconverting LNA to DHA is relatively efficient and the LC-PUFA biosynthetic pathway is substrate limited in juvenile fish. Aquaculture 195, 513–522.
Christie, W.W., 2003. Lipid Analysis, 3rd ed. The Oily Press, Bridgewater, UK, pp. 205–224.
Daprà, F., Geurden, I., Corraze, G., Bazin, D., Zambonino-Infante, J.L., Fontagné-Dicharry, S., 2011. Physiological and molecular responses to dietary phospholipids

vary between fry and early juvenile stages of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 319, 377–384.
Dhont, J., Dierckens, K., Støttrup, J., Van Stappen, G., Wille, M., Sorgeloos, P., 2013. Rotifers, Artemia and copepods as live feeds for fish larvae in aquaculture. In: Advances in aquXaculture Hatchery Technology. Woodhead Publishing, pp. 157–202.
Estevez, A., Kanazawa, A., 1995. Effect of n-3 PUFA and vitamin A *Artemia* enrichment on pigmentation success of turbot, *Scophthalmus maximus* (L). Aquac. Nutr. 1, 159–168.
Evjemo, J.O., Reitan, K.I., Olsen, Y., 2003. Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value. Aquaculture 227, 191–210.
Flak-Petersen, S., Sargent, J.R., Fox, C., Flak-Petersen, I.-B., Haug, T., Kjøsvik, E., 1989. Lipids in Atlantic halibut (*Hippoglossus hippoglossus*) eggs from planktonic samples in northern Norway. Mar. Biol. 101, 553–556.
Freemantle, E., Vandal, M., Tremblay-Mercier, J., Tremblay, S., Blachère, J.C., Bégin, M. E., Brenna, J.T., Windust, A., Cunnane, S.C., 2006. Omega-3 fatty acids, energy substrates, and brain function during aging. Prostaglandins Leukot. Essent. Fat. Acids 75, 213–220.
Galindo, A., Garrido, D., Monroig, Ó., Perez, J.A., Betancor, M.B., Acosta, N.G., Kabeya, N., Marrero, M.A., Bolaños, A., Rodriguez, C., 2021. Polyunsaturated fatty acid metabolism in three fish species with different trophic level. Aquaculture 530, 735761.
Garrido, D., Kabeya, N., Betancor, M.B., Pérez, J.A., Acosta, N.G., Tocher, D.R., Rodríguez, C., Monroig, O., 2019. Functional diversification of teleost Fads2 fatty acyl desaturases occurs independently of the trophic level. Sci. Rep. 9, 11199.
Ghioni, C., Tocher, D.R., Bell, M.V., Dick, J.R., Sargent, J.R., 1999. Low C18 to C20 fatty acid elongase activity and limited conversion of stearidonic acid, 18: 4 (n–3), to eicosapentaenoic acid, 20: 5 (n–3), in a cell line from the turbot, *Scophthalmus maximus*. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1437, 170–181.
Gisbert, E., Villeneuve, L., Zambonino-Infante, J.L., Quazuguel, P., Cahu, C.L., 2005. Dietary phospholipids are more efficient than neutral lipids for long-chain polyunsaturated fatty acid supply in European sea bass *Dicentrarchus labrax* larval development. Lipids 40, 609.
Guinot, D., Monroig, O., Hontoria, F., Amat, F., Varó, I., Navarro, J.C., 2013. Enriched on-grown *Artemia* metanauplii actively metabolise highly unsaturated fatty acid-rich phospholipids. Aquaculture 412-413, 173–178.
Hamre, K., Harboe, T., 2008a. Critical levels of essential fatty acids for normal pigmentation in Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae. Aquaculture 277, 101–108.
Hamre, K., Harboe, T., 2008b. Artemia enriched with high n-3 HUFA may give large improvement in performance of Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae. Aquaculture 277, 239–243.
Hamre, K., Opstad, I., Espe, M., Solbakken, J., Hemre, G.I., Pittman, K., 2002. Nutrient composition and metamorphosis success of Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae fed natural zooplankton or *Artemia*. Aquac. Nutr. 8 (2), 139–148.
Hamre, K., Baeverfjord, G., Harboe, T., 2005. Macronutrient composition of formulated diets for Atlantic halibut (*Hippoglossus hippoglossus* L.) juveniles, II: protein / lipid levels at low carbohydrate. Aquaculture 244, 283–291.
Hamre, K., Holen, E., Moren, M., 2007. Pigmentation and eye migration in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae: new findings and hypotheses. Aquac. Nutr. 13, 65–80.
Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Rev. Aquac. 5, S26–S58.
Hamre, K., Erstad, B., Harboe, T., 2019. Early weaning of Atlantic halibut (*Hippoglossus hippoglossus*) larvae. Aquaculture 502, 268–271.
Hamre, K., Erstad, B., de Kok, J., Norberg, B., Harboe, T., 2020. Change in nutrient composition of *Artemia* grown for 3–4 days and effects of feeding on-grown *Artemia* on performance of Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae. Aquac. Nutr. 26 (5), 1542–1554.
Harboe, T., Mangor-Jensen, A., 1998. Time of first feeding of Atlantic halibut, *Hippoglossus hippoglossus* L., larvae. Aquac. Res. 29 (12), 913–918.
Harboe, T., Reitan, K.I., 2005. Halibut fry production. Larvi '05 – Fish and shellfish larviculture symposium. In: Hendry, C.I., Van Stappen, G., Wille, M., Sorgeloos, P. (Eds.), European Aquaculture Society, Special publication No. 36, Oostende, Belgium.
Kabeya, N., Chiba, M., Haga, Y., Satoh, S., Yoshizaki, G., 2017. Cloning and functional characterization of fads2 desaturase and elovl5 elongase from Japanese flounder *Paralichthys olivaceus*. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 214, 36–46.
Kongsvik, R., 2007. Produksjon av torsk og kveite i 2006. In: Fisken og havet, særnummer 2007 (Summary in English).
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biochem. Chem. 193, 265–275.
Lund, I., Reis, D.B., Tomkiewicz, J., Benini, E., Pérez, J.A., Kottmann, J.S., Politis, S.N., Rodríguez, C., 2021. Assessment of lipid uptake and fatty acid metabolism of European eel larvae (*Anguilla anguilla*) determined by ^{14}C in vivo incubation. Aquaculture 531, 735858.
Machado, A.M., Tørresen, O.K., Kabeya, N., Couto, A., Petersen, B., Felício, M., Campos, P.F., Fonseca, E., Bandarra, N., Lopes-Marques, M., Ferraz, R., Ruivo, R., Fonseca, M.M., Jentoft, S., Monroig, Ó., da Fonseca, R., Castro, L.F.C., 2018. "Out of the can": a draft genome assembly, liver transcriptome and nutrigenomics of the European sardine, *Sardina pilchardus*. Genes (Basel) 9 (10), 485.
Martins, D.A., Valente, L.M.P., Santosh, P.L., 2007. Effects of dietary lipid level on growth and lipid utilization by juvenile Atlantic halibut (*Hippoglossus hippoglossus*, L.). Aquaculture 263, 150–158.
Mazorra, C., Bruce, M., Bell, J.G., Davie, A., Alorend, E., Jordan, N., Rees, J., Papanikos, N., Porter, M., Bromage, N., 2003. Dietary lipid enhancement of broodstock reproductive performance and egg and larval quality in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 227, 21–33.
McEvoy, L.A., Næss, T., Bell, J.G., Lie, Ø., 1998. Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (*Hippoglossus hippoglossus*) fed enriched *Artemia*: a comparison with fry fed wild copepods. Aquaculture 163, 237–250.
Mollan, T.A., Tonheim, S.K., Hamre, K., 2008. Pre-hydrolysis improves absortion of neutral lipids in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. Aquaculture 275, 217–224.
Monroig, Ó., Navarro, J.C., Amat, I., González, P., Amat, F., Hontoria, F., 2003. Enrichment of *Artemia* nauplii in PUFA, phospholipids, and water-soluble nutrients using liposomes. Aquac. Int. 11, 151–161.
Monroig, Ó., Lopes-Marques, M., Navarro, J.C., Hontoria, F., Ruivo, R., Santos, M.M., Venkatesh, B., Tocher, D.R., Castro, L.F.C., 2016. Evolutionary functional elaboration of the Elovl2/5 gene family in chordates. Sci. Rep. 6, 20510.
Monroig, Ó., Tocher, D.R., Castro, L.F.C., 2018. Polyunsaturated fatty acid biosynthesis and metabolism in fish. In: Burdge, G.C. (Ed.), Polyunsaturated Fatty Acid Metabolism. Elsevier, Amsterdam, pp. 31–60.
Morais, S., Castanheira, F., Martinez-Rubio, L., Conceição, L.E., Tocher, D.R., 2012. Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity. Biochimica et Biophysica Acta (BBA) Mol. Cell Biol. Lipid 1821, 660–671.
Morais, S., Mourente, G., Martinez, A., Gras, N., Tocher, D.R., 2015. Docosahexaenoic acid biosynthesis via fatty acyl elongase and Δ4-desaturase and its modulation by dietary lipid level and fatty acid composition in a marine vertebrate. Biochimica et Biophysica Acta (BBA) Mol. Cell Biol. Lipid 1851, 588–597.
Næss, T., Lie, Ø., 1998. A sensitive period during first feeding for the determination of pigmentation pattern in Atlantic halibut, *Hippoglossus hippoglossus* L., juveniles: the role of diet. Aquac. Res. 29, 925–934.
Næss, T., Germain-Henry, M., Naas, K.E., 1995. First feeding of Atlantic halibut (*Hippoglossus hippoglossus*) using different combinations of *Artemia* and wild zooplankton. Aquaculture 130, 235–250.
Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid conversions during enrichment of *Artemia*. Aquaculture 174, 155–166.
Olsen, A.I., Attramadal, Y., Jensen, A., Olsen, Y., 1999. Influence of size and nutritional value of *Artemia franciscana* on growth and quality of halibut larvae (*Hippoglossus hippoglossus*) during the live feed period. Aquaculture 179, 475–487.
Olsen, Y., Evjemo, J.O., Kjørsvik, E., Larssen, H., Li, K., Overrein, I., Rainuzzo, J., 2014. DHA content in dietary phospholipids affects DHA content in phospholipids of cod larvae and larval performance. Aquaculture 428–429, 203–214.
Rainuzzo, J.R., Reitan, K.I., Jørgensen, L., 1992. Comparative study on the fatty acid and lipid composition of four marine fish larvae. Comp. Biochem. Physiol. B 103, 21–26.
Rainuzzo, J.R., Reitan, K.I., Olsen, Y., 1997. The significance of lipids at early stages of marine fish: a review. Aquaculture 155, 103–115.
Reis, D.B., Acosta, N.G., Almansa, E., Navarro, J.C., Tocher, D.R., Monroig, O., Andrade, J.P., Sykes, A.V., Rodriguez, C., 2014. *In vivo* metabolism of unsaturated fatty acids in *Octopus vulgaris* hatchlings determined by incubation with ^{14}C-labelled fatty acids added directly to seawater as protein complexes. Aquaculture 431, 28–33.
Reis, D.B., Acosta, N.G., Almansa, E., Tocher, D.R., Andrade, J.P., Sykes, A.V., Rodriguez, C., 2016a. Composition and metabolism of phospholipids in *Octopus vulgaris* and *Sepia officinalis* hatchlings. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 200, 62–68.
Reis, D.B., Rodriguez, C., Acosta, N.G., Almansa, E., Tocher, D.R., Andrade, J.P., Sykes, A.V., 2016b. *In vivo* metabolism of unsaturated fatty acids in *Sepia officinalis* hatchlings. Aquaculture 450, 67–73.
Reis, D.B., Acosta, N.G., Almansa, E., Navarro, J.C., Tocher, D.R., Andrade, J.P., Sykes, A. V., Rodríguez, C., 2017. Comparative study on fatty acid metabolism of early stages of two crustacean species: *Artemia* sp. metanauplii and *Grapsus adscensionis* zoeae, as live prey for marine animals. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 204, 53–60.
Reis, D.B., Acosta, N.G., Almansa, E., Garrido, D., Andrade, J.P., Sykes, A.V., Rodríguez, C., 2019. Effect of Artemia inherent fatty acid metabolism on the bioavailability of essential fatty acids for *Octopus vulgaris* paralarvae development. Aquaculture 500, 264–271.
Reis, D.B., Peréz, J.A., Lund, I., Acosta, N.G., Abdul-Jalbar, B., Bolaños, A., Rodríguez, C., 2020. Esterification and modification of [1-^{14}C] n-3 and n-6 polyunsaturated fatty acids in pikeperch (*Sander lucioperca*) larvae reared under linoleic or α-linolenic acid-based diets and variable environmental salinities. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 110449.
Roberts, J., 2018. Northeast Multispecies Fishery Management Plan Resource Guide: Atlantic Halibut (*Hippoglossus hippoglossus*). NOAA Central Library, Maryland.
Rodriguez, C., Pérez, J.A., Henderson, R.J., 2002. The esterification and modification of n-3 and n-6 polyunsaturated fatty acids by hepatocytes and liver microsomes of turbot (*Scophthalmus maximus*). Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 132 (3), 559–570.
Sæle, Ø., Solbakken, J.S., Watanabe, K., Hamre, K., Power, D., Pittman, K., 2004. Staging of Atlantic halibut (*Hippoglossus hippoglossus* L.) from first feeding through metamorphosis, including cranial ossification independent of eye migration. Aquaculture 239, 445–465.
Sæle, Ø., Nordgreen, A., Olsvik, P.A., Hjelle, J.I., Harboe, T., Hamre, K., 2013. Toxic effects of dietary hydrolysed lipids: an *in vivo* study on fish larvae. Br. J. Nutr. 109, 1071–1081.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J., Tocher, D.R., 1995. Requirement criteria for essential fatty acids. J. Appl. Ichthyol. 11, 183–198.
Sargent, J.R., McEvoy, L.A., Estevez, A., Bell, J.G., Bell, M.V., Henderson, R.J., Tocher, D. R., 1999. Lipid nutrition of marine fish during early development: current status and future directions. Aquaculture 179, 217–229.
Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition, 3rd ed.. Academic Press, San Diego, pp. 181–257.
Shao, C., Bao, B., Xie, Z., Chen, X., Li, B., Jia, X., Yao, Q., Ortí, G., Li, W., Li, X., Hamre, K., Xu, J., Wang, L., Chen, F., Tian, Y., Schreiber, A.M., Wang, N., Wei, F., Zhang, J., Dong, Z., Gao, L., Gai, J., Sakamoto, T., Mo, S., Chen, W., Shi, Q., Li, H., Xiu, Y., Li, Y., Xu, W., Shi, Z., Zhang, G., Power, D.M., Wang, Q., Schartl, M., Chan, S., 2017. The genome and transcriptome of Japanese flounder provide insights into flatfish asymmetry. Nat. Genet. 49 (1), 119–124.
Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture 200, 147–159.
Sprecher, H., 2000. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochimica et Biophysica Acta (BBA) Mol. Cell Biol. Lipid 1486, 219–231.
Tocher, D.R., 1995. Glycerophospholipid metabolism. In: Biochemistry and Molecular Biology of Fishes, Vol. 4. Elsevier, pp. 119–157.
Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. 11, 107–184.
Tocher, D.R., 2015. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 449, 94–107.
Tocher, D.R., Bendiksen, E.A., Campbell, P.J., Bell, G., 2008. The role of phospholipids in nutrition and metabolism of teleost fish. Aquaculture 280, 21–34.
van Stappen, G., 1996. Introduction, biology and ecology of *Artemia*. In: Lavens, P., Sorgeloos, P. (Eds.), Manual on the Production and Use of Live Food for Aquaculture, vol. 361. FAO, Rome, Italy, pp. 79–106.