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Assessment of lipid uptake and fatty acid metabolism of European eel larvae (Anguilla anguilla) determined by 14C in vivo incubation

Ivar Lund [a], [*], [1], Diana B. Reis [b], [1], Jonna Tomkiewicz [c] , Elisa Benini [c] , José A. Pérez [b] , Johanna S. Kottmann [c] , Sebastian N. Politis [c] , Covadonga Rodríguez [b]

[a] Section for Aquaculture, Institute of Aquatic Resources, Technical University, Hirtshals, Denmark

[b] Departamento de Biología Animal, Edafología y Geología, Universidad de La Laguna, Avenida Astrofísico Francisco Sánchez s/n, La Laguna, Spain

[c] Section for Marine Living Resources, Institute of Aquatic Resources, Technical University, Kgs. Lyngby, Denmark

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Abstract

Knowledge on dietary nutrient requirements of first-feeding European eel larvae (*Anguilla anguilla*) is very limited. This study provides first ever information on in vivo lipid uptake and fatty acid (FA) metabolism of European pre-leptocephalus eel larvae and advances directions for dietary lipid and FA inclusions. The in vivo capability of eel larvae to incorporate and metabolize unsaturated fatty acids was tested on larvae at different ontogenetic stages (4, 8 and 12 days post hatch, DPH). Larvae were incubated in 10 mL flat-bottom tissue culture plates, with [1-14C]-labelled FA (18:2n-6, ALA; 18:3n-3, LA; 20:4n-6, ARA and 20:5n-3, EPA) directly added to seawater. The capability of the larvae for de-acylation and re-acylation of [1-14C]arachidonic acid (ARA), initially bound to phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was also investigated. In all cases, control incubations without any radiolabelled substrate were performed for further lipid analysis. The results revealed that direct incubation with 14C-labelled FA is a feasible method to investigate in vivo FA and phospholipids metabolism of pre-leptocephalus stages of the European eel. No enzymatic elongation/desaturation activity towards [1-14C]C18 or [1-14C]C20 FA was detected. Consequently, ARA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) must be considered essential FA and thus provided firstly through female broodstock and later through diet at least during the first-feeding stage. Pre-leptocephalus larvae display a high capacity to remodel dietary phospholipids with a preferential esterification of all FA substrates into PC. The unexpectedly high esterification rate of [1-14C] ARA into PC and PE is supported by the individual FA profiles of the larval phospholipids. The high levels of ARA present in the European eel larvae denotes its physiological relevance for this species. It is therefore essential to consider this FA as particularly important when designing suitable broodstock – or first-feeding diets for this species.

1. Introduction

Nutritional requirements of first-feeding European eel larvae (Anguilla anguilla) are largely unknown and research within this area is highly necessary for obtaining viable leptocephalus larvae and a future sustainable production of glass eels in aquaculture, which until now relies entirely on wild caught specimens. Research in hatchery technology has raised European eel breeding from a state of reproductive failure to a stable production of viable eggs and yolk-sac larvae from hormonally induced wild caught and farmed eel broodstock (Tomkiewicz, 2012; Tomkiewicz et al., 2019; Butts et al., 2014). Reproductive method improvements imply progress in hormonal induction of eel maturation (Tomkiewicz et al., 2011; Mordenti et al., 2013; Müller et al., 2016; da Silva et al., 2018a, 2018b; Kottmann et al., 2020a); optimization and standardization of spawning, and fertilization and incubation protocols (Butts et al., 2014), including reduction of egg microbial activity (Sørensen et al., 2014) especially during the early life history stages that seem to be immunocompromised (Miest et al., 2019). Further achievements include improved larval culture technology in terms of light (Politis et al., 2014), temperature (Politis et al., 2017) and salinity (Sørensen et al., 2016b; Politis et al., 2018a), enhancing larval survival until the first feeding stage, i.e. the end of yolk-sac and lipid droplet depletion (Sørensen et al., 2016a). T To promote development of pre-leptocephalus larvae into the leptocephalus larval stage and ultimately transformation into glass eels, studies on DNA gut content of wild caught European eel leptocephalus larvae from the Sargasso Sea (Riemann et al., 2010; Ayala et al., 2018) have suggested potential preys but remain inconclusive. Inert diets based on shark egg powder, krill hydrolysate and soybean peptide as main components have been successful in generating glass eels of the Japanese eel (Anguilla japonica) (Tanaka, 2015; Tanaka et al., 2006). Feeding trials have been conducted on European eel larvae defining the first-feeding "window" (Butts et al., 2016; Politis et al., 2018b). However, the development into leptocephalus larvae and ultimately glass eels has still not been achieved, which complicates research on dietary nutrient requirements. Most strict marine carnivorous fish larvae have high and specific dietary requirements for lipids and long chain polyunsaturated fatty acids (LC–PUFA) during endogenous development and first-feeding phases, while euryhaline and freshwater species may display lower dietary FA requirements and higher metabolic capacities. European eel is catadromous with a complex life cycle and a unique migratory ability between sea- and freshwater and although spending most of its juvenility and adult life in freshwater, it suggests a high plasticity for physiological and metabolic adaptation. During its lifespan in freshwater, eels build up high stores of fat and lipids in tissues. Upon returning to the Sargasso Sea spawning area, silver eels likely cease feeding and undergo maturation while using freshwater obtained fat resources, as was reported for migrating Japanese silver eels (Saito et al., 2015). Not only tissue levels of fat seems of importance, but muscle, visceral fat and ovary of wild female silver eels have revealed significantly higher ARA content than farmed eels fed standard commercial fish meal based diet (Støttrup et al., 2013) and evidence of the significance of supplemented ARA in the reproductive success of farmed European and Japanese eels has been documented (Furuita et al., 2006; Støttrup et al., 2016; Kottmann et al., 2020b). These findings moreover suggest a significant importance of ARA for first-feeding of European eel larvae, although this has been questioned in Japanese pre-leptocephalus larvae for which a low lipid diet (i.e. defatted shark eggs) low in n-6:n-3 (0.22); low in ARA:EPA (0.32) and high in DHA:EPA (1.61) promoted better survival and growth compared with a similar not defatted diet (Furuita et al., 2014). Freshwater food webs are generally characterized by a higher n-6:n3 ratio, including higher abundance of ARA than marine food webs (Jobling, 2001; Olsen, 2009), and 18:2n-6 and 20:4n-6 are characteristically contained in freshwater fishes. This may likely explain the high ARA tissue content in wild silver eels (Saito et al., 2015), but may also reflect a certain ability to modify dietary FA precursors as elongation/ desaturation of dietary [1-14C] linoleic acid to ARA has been confirmed in forced-fed European glass eels (Kissil et al., 1987), and enzymatic expression of all key desaturase and elongase enzymes revealed the capacity to elongate C18 PUFA substrates in adult Japanese eels (Xu et al., 2020). Although a similar capacity for European eel adults cannot be precluded, it is also known that prior to migration to the Sargasso Sea area for spawning, silver eel females seem to carry out a particularly selective feeding on LC-PUFA in coastal lagoons habitats (Capoccioni et al., 2018), with preys such as the blue crab (Callinectes sapidus), which provides high levels of n-3 and n-6 LC-PUFA including EPA, DHA and ARA (Çelik et al., 2004). The extent to which European eel larvae can convert C18 PUFA to C20-C22 LC-PUFA, mainly EPA, DHA and ARA is at present unknown and complicated by the ability to manipulate and test this in experimental diets. In vivo incubation of larvae with radiolabelled nutrient markers has proven a reliable methodology for obtaining useful knowledge on nutrient metabolism during early ontogeny of both marine, -freshwater fishes as well as invertebrate species for which dietary nutritional requirements are limited (Reis et al., 2014; Reis et al., 2016a, 2016b, 2020; Lund et al., 2019). Thus, the present experiment studied the in vivo larval capability of European eel larvae to incorporate and metabolize a range of [1-14C] n-3 and n-6 PUFAs to provide first insights on eel larval FA requirements. Larvae at several ontogenetic stages without (4 and 8 days post hatch (DPH)) or with a functional feeding apparatus (12 DPH) were sampled from different parental combinations. Larval capacity for de-acylation and re-acylation of [1-14C] ARA, initially bound to phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was also investigated. Results are discussed related to the control larvae lipid class composition and the fatty acid profiles of total lipids, and individual PC, PE, phosphatidylinositol (PI) and phosphatidylserine (PS).

2. Materials and methods

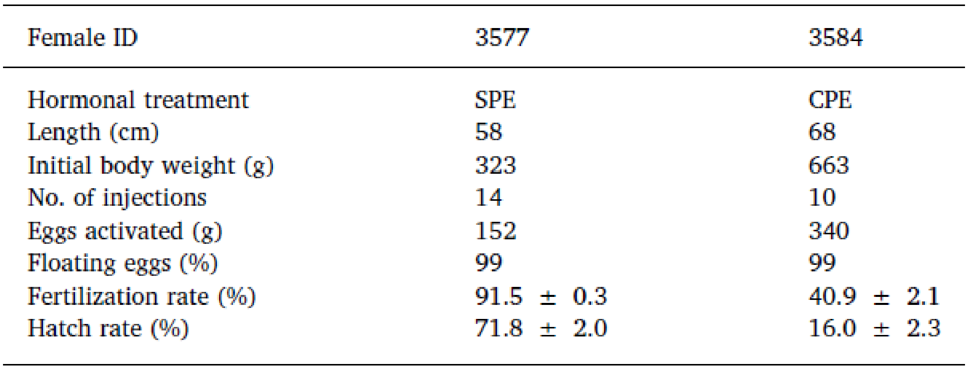
2.1. Broodstock maturation and husbandry

Female silver eels (length = 64.42 ± 1.21 cm; weight = 535.33 ± 39.93 g, n = 26) were obtained from Klitmøller Å, Lake Vandet (Denmark), and transported to the EEL-HATCH facility in Hirtshals (Denmark). Farmed male eels (length = 38.5 ± 2.1 cm, weight = 114.7 ± 15.8 g, n = 88) were raised from glass eels at a commercial eel farm (Stensgård Eel Farm A/S. Jutland. Denmark). The female broodstock were equally distributed among three 1150 L tanks and males among four 485 L tanks equipped with separate closed recirculation systems under a continuous flow rate of ~15 L min−1. A 12 h day/12 h night photoperiod was applied with a light intensity at ~20 lx. Acclimatization took place over 2 weeks in order to reach a salinity of 36 PSU and temperature of 20 °C. No feed was provided during the period of induced gametogenesis, as eels naturally undergo a fasting period from the onset of the silvering stage (Tesch, 2003). At the onset of reproduction, broodstock fishes were anesthetized (ethyl paminobenzoate, 20 mg L−1; Sigma-Aldrich Chemie, Steinheim, Germany) and tagged with a passive integrated transponder, while initial length and weight were recorded. Vitellogenesis was induced in the female broodstock via weekly injections of pituitary extract from carp (CPE) or salmon (SPE) based on whole freeze-dried glands (CPE: Ducamar Spain S.L.U., Cantabria, Spain; SPE: Argent Aquaculture L.L.C., Washington, USA) at a dose of 18.75 mg kg−1 initial body weight (Kottmann et al., 2020a). Follicular maturation was induced, using the maturation inducing steroid, i.e. 17α.20β-dihydroxy-4-pregnen-3-one (DHP crystalline, Sigma Aldrich Chemie), when female weight increased, which varied between 10 and 14 weeks. Length, initial body weight and number of injections for two females producing the offspring used in the present study are given in Table 1. Spermatogenesis in broodstock males was induced through weekly injection of human chorionic gonadotropin (hCG, Sigma Aldrich Chemie; 150 IU per male) for a period up to 17 weeks.

2.2. Gamete production and embryonic rearing

Prior to spawning, milt was collected from 4 to 5 randomly selected males per female. The sperm concentration was standardized instantly using an immobilizing medium (Peñaranda et al., 2010; Sørensen et al., 2014) with the diluents maintained at 4 °C until use. Females were strip-spawned and the eggs fertilized using a standardized sperm to egg ratio at a temperature of 20 °C (Butts et al., 2014). Upon mixing of gametes, artificial seawater (ASW), i.e. reverse osmosis water salted to ~36 PSU with Blue Treasure (Qingdao Sea-Salt Aquarium Technology Co., Ltd., Qingdao, China), was added for zygote activation, ensuring a salinity of 36 PSU (Sørensen et al., 2016a, 2016b). Eggs were incubated in 15 L of ASW for 1 h, from where the buoyant egg layer was gently moved into new 15 L of ASW. At 2 h post fertilization (HPF), buoyant eggs were transferred to 60 L conical egg incubators at a flow through rate of ~350 mL min−1. Gentle aeration was added after ~5 HPF, while temperature was lowered to 18 °C for improved development (Politis et al., 2017). Light was kept at a low intensity of ~10 lx (Politis et al., 2014) and twice a day sinking dead eggs were purged from the bottom valve of each incubator. At ~50 HPF, aeration was stopped and embryos hatched at ~56 HPF. Fertilization success was determined from representative egg aliquots obtained at 3 to 5 HPF. Three replicate samples were analyzed using presence of blastomeric cleavages (4–64 stages) as criterion for fertilization (Sørensen et al., 2016a, 2016b). For estimation of hatch success, triplicate flasks with ~600 eggs/embryos from the floating layer were incubated in darkness at 18 °C and 36 PSU in UV-treated filtered seawater with rifampicin and ampicillin (Sørensen et al., 2014; Politis et al., 2017). The proportion of hatched larvae was estimated at 69 HPF. Data on female egg production, fertilization and hatch success of each batch used in the present experiments are given in Table 1.

Table 1 Reproductive outcome of Anguilla anguilla broodstock according to female ID, hormonal treatment and body size.



Female ID	3577	3584
Hormonal treatment	SPE	CPE
Length (cm)	58	68
Initial body weight (g)	323	663
No. of injections	14	10
Eggs activated (g)	152	340
Floating eggs (%)	99	99
Fertilization rate (%)	91.5 ± 0.3	40.9 ± 2.1
Hatch rate (%)	71.8 ± 2.0	16.0 ± 2.3

Results represent means ± SD; n = 3.

2.3. Larval rearing and ontogenic development

Directly after hatch, larvae of each batch were stocked in separate 250 L rearing units connected to a recirculation system containing filtered seawater (FSW) at a salinity of 36 PSU. The system comprised a sump reservoir of ~1 m3 from where water passed to a 320 L combined bio-trickling filter (RK bioelements) and thereafter re-entered the sump. A protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic Gmbh., Bissendorf, Germany) was included for removal of waste protein. In each rearing unit, flow rate was kept at ~10 L min−1 and temperature at 18 °C (Politis et al., 2017), while light regime was set to a low intensity 12 light/12 dark photoperiod (Politis et al., 2014). Non-fed larvae at 4, 8 and 12 DPH were used for the subsequent incubations. Fig. 1 illustrates larval ontogenetic development at 18 °C. At this temperature the yolk is usually depleted at around 10 DPH, while the oil droplet may remain visible until 15–17 DPH (Politis et al., 2017).

2.4. Ethics statement

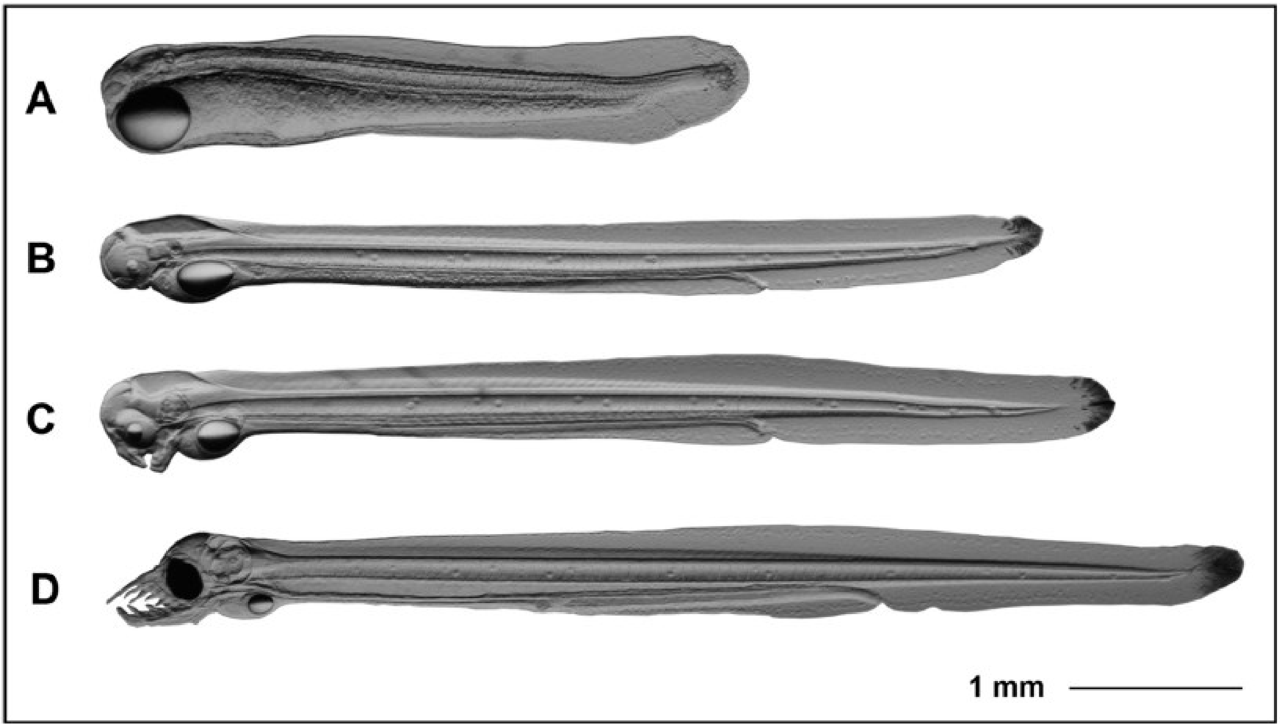
All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (EU Dir 2010/63) and the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15- 0201-00696). Briefly, broodstock eels were anesthetized using ethyl paminobenzoate (benzocaine, 20 mg L−1, Sigma Aldrich, Germany) before tagging and handling. Larvae were anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222, Sigma Aldrich, Germany), at a concentration of 7.5 and 15 mg L−1, respectively.

All efforts were made to minimize animal handling and stress. Permission to use 14C labelled FA was obtained by The Danish Health Board, The State Institute of Radiation Protection.

2.5. In vivo incubation with labelled [1-14C] fatty acids

Larval capability to incorporate and metabolize unsaturated FAs was studied by in vivo radio tracing of [1-^{14}C] FAs (Reis et al., 2014, 2016a, 2020). Larvae at different stages of ontogeny (4, 8, 12 DPH) were incubated in 10 mL flat-bottom tissue culture plates (SARSTEDT AG & Co., Nümbrecht, Germany) at a density of 150 larvae per incubation well (n = 3) for 4 h, with gentle stirring and 0.2 µCi (0.3 µM) of [1-^{14}C] labelled FAs (free fatty acid molecules labelled with ^{14}C in its first carbon counting from the carboxyl head) including either 18:3n-3 (LA) and 18:2n-6 (ALA; PerkinElmer Inc., Waltham, Massachusetts, USA), as well as [1-^{14}C] ARA molecule esterified into the sn-2 position of phosphatidylcholine (^{14}C-PC; L-∝-1-palmitoyl-2-arachidonyl-[arachidonyl-1-^{14}C]) and of phosphatidylethanolamine (^{14}C-PE; L-∝-1-palmitoyl-2-arachidonyl-arachidonyl-1-^{14}C]; American Radiolabelled Chemicals, Inc.). Additionally, 12 DPH larvae were also incubated under the same conditions with [1-^{14}C] 20:5n-3 (EPA), and 20:4n-6 (ARA; American Radiolabelled Chemicals Inc., St. Louis, Missouri, USA). The selection of 12 DPH to perform those incubations, was mainly based on mouth opening and functionality, likely ensuring the incorporation of the substrates by ingestion. All larvae were incubated in triplicates under similar water conditions as mentioned above and the radiolabelled substrates were directly added to the medium. Each age group of larvae was also incubated (n = 3) without radiolabelled substrate as a control group for larval lipid composition determination. After incubation, larvae were thoroughly washed twice with water to remove radiolabelled substrate excess. Subsequently, euthanized larvae were transferred to Eppendorf vials and stored at −80 °C until analysis.

Fig. 1. European eel larvae 0, 4, 8 and 12 days post hatch (DPH) illustrating developmental characteristics from hatch to first-feeding stage (reared at 18 °C and 36 PSU).



Larval characteristics, A: 0 DPH – head with initial optic vesicles, vertebrae, yolk-sac with oil globule, B: 4 DPH - larval head anterior to yolk-sac, and brain visible, heart, extended primordial fin, C: 8 DPH - upper and lower jaw distinguishable, D: 12 DPH – head with forward pointing jaws, prominent teeth and pigmented eyes. Scale bar: 1 mm.

The extraction of the total lipid (TL) was performed with chloroform/methanol (2:1, v/v) as an organic solvent according to the Folch method as described by Christie (2003). The lipid content was gravimetrically determined after evaporation of the organic solvent under a stream of nitrogen. TL extracts were stored at a concentration of 10 mg mL−1 in chloroform/methanol (2:1, v/v) with 0.01% of butylated hydroxytoluene (BHT; Sigma-Aldrich Co., St. Louis, Missouri, USA) as antioxidant, at −20 °C under an inert atmosphere of nitrogen. Lipid extracts of control group incubations (4, 8 and 12 DPH eel larvae; n = 3) were analyzed for lipid class (LC) and FA composition. Lipid classes were separated by high-performance thin-layer chromatography (HPTLC, Merck, Darmstadt, Germany) in a one-dimensional double-development, and quantified by calibrated densitometry, using a dual-wavelength flying spot scanner CAMAG TLC Visualizer (Camag, Muttenz, Switzerland), as described by Reis et al. (2019). Isolation of individual phospholipids (PC, PE, PS and PI) from a pool of 12 DPH larvae (n = 1) TL was performed by a one-dimension single-development TLC silica plates as described by Reis et al. (2016a). Briefly, polar lipid classes were separated with 1-propanol/chloroform/methyl acetate/methanol/0.25% KCL (25:25:25:10:9 by volume). The phospholipid classes were then visualized under UV light after brief exposure to dichlorofluorescein and separately scraped from the TLC plates. Fatty acid methyl esters (FAME) of TL extracts and isolated PC, PE, PS and PI fractions, were obtained by acid-catalysed transmethylation. FAME were purified by thin-layer chromatography (TLC) (Christie, 2003), separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) 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. Identity of individual FAME was confirmed by GC–MS chromatography (DSQ II, Thermo Fisher Scientific Inc.).

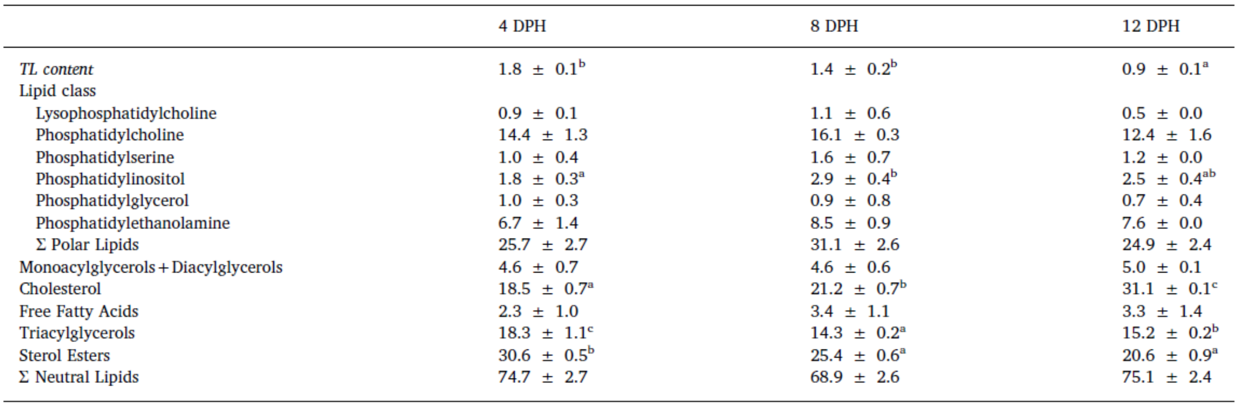
2.7. Metabolism of radiolabelled substrates

An aliquot of 0.1 mg of TL extracts from each [1-14C] incubated sample was transferred into scintillation vials, to determine radioactivity incorporated into eel larvae. Radioactivity was quantified on an LKB Wallac 1214 RackBeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, USA) following Reis et al. (2019). To determine the esterification pattern of each [1-14C] FA into the different LC and the capacity of larvae to remodel the metabolic fate of [1-14C] ARA when bound to PC or PE, another aliquot of 0.1 mg of eel TL extract was applied to HPTLC plates and separated in discrete lipid bands as mentioned in Reis et al. (2016a, 2019). The developed HPTLC plates were placed in closed exposure cassettes (Exposure Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Image Screen-K, BioRad) for 1 week. The screens were then scanned with an image acquisition system (Molecular Imager FX, BioRad) and the bands quantified in percentage by an image analysis software (Quantity One, BioRad). Finally, transformation of incubated [1-14C] FA substrates by desaturation/elongation processes were determined using pre-coated TLC plates G-25 (20 cm × 20 cm; MachereyNagel GmbH & Co. KG) pre-impregnated with a solution of 2 g silver nitrate in 20 mL of acetonitrile (Reis et al., 2019). Developed TLC plates were then placed in BioRad closed exposure cassettes in contact with Image Screen-K for 2 weeks. The screens were then scanned with a Molecular Imager FX and bands quantified by Quantity One software. The identification of labelled bands was confirmed by radiolabelled standards run on the same plate (Rodríguez et al., 2002).

2.8. Statistical analysis

Differences between larvae TL content, LC, FA composition and between the incorporation of radiolabelled substrates into eel larvae TL at 4, 8 and 12 DPH were assessed by a one-way ANOVA followed by the Tukey HSD post hoc test. This was also the statistical treatment performed to determine differences in the esterification rates of [1-^{14}C] LA, ALA, ARA and EPA into lipid classes of 12 DPH larvae, and of [1-^{14}C] LA, ALA, PC and PE esterification between different stages (4, 8 and 12 DPH). Differences between the esterification of radiolabelled substrates into LC within the same age, were assessed by t-student analysis. Normality and homogeneity of data were confirmed within groups and, where necessary, appropriate variance stabilizing transformations were performed. Results are presented as mean ± standard deviation (SD) and the statistical significance was established at $p < 0.05$. All statistical analyses were conducted using IBM® SPSS Statistics 25.0 software package (IBM Corp., New York, USA) for Windows.

Table 2 Total lipid content (mg 100 larvae−1) and main lipid class composition (% of total lipid) of Anguilla anguilla larvae at 4, 8 and 12 DPH.



	4 DPH	8 DPH	12 DPH
TL content	1.8 ± 0.1^{b}	1.4 ± 0.2^{b}	0.9 ± 0.1^{a}
Lipid class			
Lysophosphatidylcholine	0.9 ± 0.1	1.1 ± 0.6	0.5 ± 0.0
Phosphatidylcholine	14.4 ± 1.3	16.1 ± 0.3	12.4 ± 1.6
Phosphatidylserine	1.0 ± 0.4	1.6 ± 0.7	1.2 ± 0.0
Phosphatidylinositol	1.8 ± 0.3^{a}	2.9 ± 0.4^{b}	2.5 ± 0.4^{ab}
Phosphatidylglycerol	1.0 ± 0.3	0.9 ± 0.8	0.7 ± 0.4
Phosphatidylethanolamine	6.7 ± 1.4	8.5 ± 0.9	7.6 ± 0.0
Σ Polar Lipids	25.7 ± 2.7	31.1 ± 2.6	24.9 ± 2.4
Monoacylglycerols + Diacylglycerols	4.6 ± 0.7	4.6 ± 0.6	5.0 ± 0.1
Cholesterol	18.5 ± 0.7^{a}	21.2 ± 0.7^{b}	31.1 ± 0.1^{c}
Free Fatty Acids	2.3 ± 1.0	3.4 ± 1.1	3.3 ± 1.4
Triacylglycerols	18.3 ± 1.1^{c}	14.3 ± 0.2^{a}	15.2 ± 0.2^{b}
Sterol Esters	30.6 ± 0.5^{b}	25.4 ± 0.6^{a}	20.6 ± 0.9^{a}
Σ Neutral Lipids	74.7 ± 2.7	68.9 ± 2.6	75.1 ± 2.4

Results represent means ± SD;
n = 3.
Mean values with unlike superscript letters are significantly different (p < 0.05).

3. Results

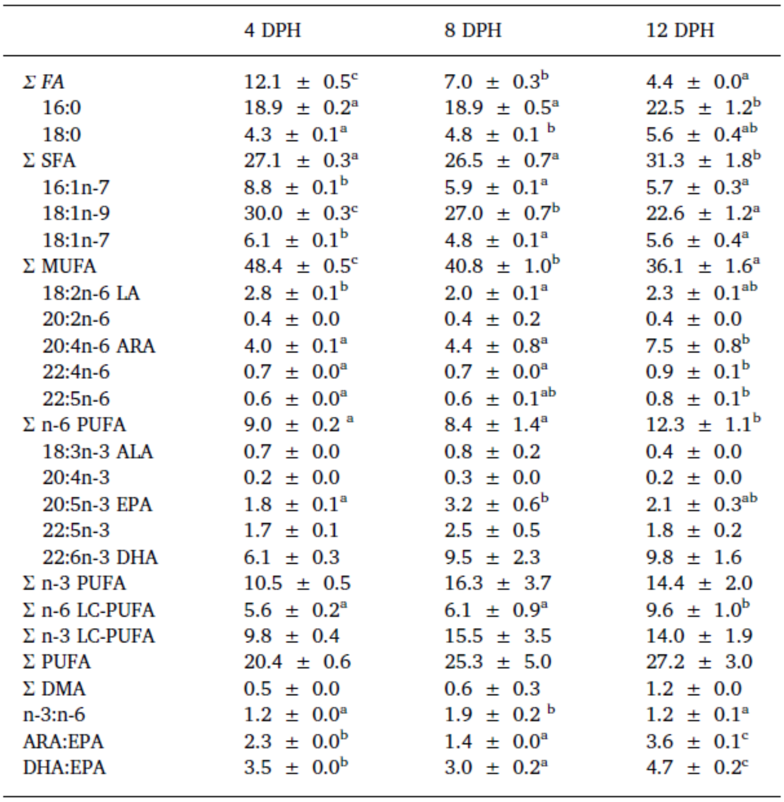
3.1. Larvae lipid and FA composition

Average wet weight (w.w.) of eel larvae at 4–12 DPH ranged between 0.32 and 0.46 mg, with TL contents gradually decreasing with development from 1.8 mg 100 larvae−1 (i.e. 5.5% w.w.) at 4 DPH larvae, to 1.4 mg 100 larvae−1 (i.e. 3.5% w.w.) at 8 DPH, and to 0.9 mg 100 larvae−1 (i.e. 1.9% w.w.) at 12 DPH (Table 2). Regardless of stage, eel larvae contained higher levels of neutral lipids (NL, 68.9–75.1%) than polar lipids (PL), with sterol esters (SE) being the most abundant LC (20.6–30.6%; Table 2). Assessment of the PL fraction profiles showed that PC and PE were the major lipid classes representing around 14 and 8%, respectively. A decrease in SE and triacylglycerol (TAG) contents was observed during larval development, while relative levels of cholesterol (CHO) increased significantly ($p < 0.05$; Table 2). At 8 DPH, there also seemed to be a relative prominence of other structural lipids including a significant increment of PI ($p < 0.05$; Table 2). Similarly, to the lipid content, total FA content of eel larvae decreased with age from 12.1 ± 0.5 μg larvae−1 at 4 DPH to 4.4 ± 0.0 μg larvae−1 at 12 DPH (Table 3). Larvae TL FA profile was mainly composed by monounsaturated fatty acids (MUFA, 36.1–48.4%) primarily 18:1n-9, followed by saturated fatty acids (SFA, 27.1–31.3%) especially 16:0 (Table 3). A higher content of n-3 LC-PUFA (9.8–15.5%) than n-6 LC-PUFA (5.6–9.6%) was also observed with DHA and EPA being the main n-3 PUFA, and ARA representing between 44 and 61% of total n-6 PUFA. A relative decrease of MUFA (from 48.4 ± 0.5% at 4 DPH to 36.1 ± 1.6% at 12 DPH), together with a relative increase of SFA and n-6 LC-PUFA proportions were evident with age ($p < 0.05$; Table 3). Both ARA/EPA and DHA/EPA ratios reached the highest values at 12 DPH. Regarding the FA profile of individual glycerophospholipids (Table 4), 18:1n-9 was also a relevant fatty acid together with 16:0, particularly in PC, whereas 18:0 became the most abundant saturate in PS and PI. DHA was by far the most prominent fatty acid by all lipid classes, with particular emphasis in PE. Interestingly, ARA was also a relevant fatty acid not only in PI, but also in PC and PE (Table 4).

3.2. Incorporation of radiolabelled substrates into larvae total lipids and its distribution among lipid classes

[1-^{14}C] ALA was incorporated to a higher extent into eel larval lipids at 12 DPH (31.0 ± 10.5 pmol mg prot−1 h−1) than at 4 and 8 DPH (8.8 ± 2.5 and 8.9 ± 3.5 pmol mg prot−1 h−1, respectively) whereas incorporation of [1-^{14}C]LA remained unchanged with respect to the initial value (15.0 ± 5.0 and 18.9 ± 0.9 pmol mg prot−1 h−1, for 4 and 12 DPH, respectively) (Table 5). At 12 DPH, not only ALA but also [1-^{14}C] EPA presented higher incorporation rates into larval tissues than both n-6 [1-^{14}C] PUFAs (LA and ARA). The incorporation of [1-^{14}C] ARA, when bounded to both PC and PE (^{14}C-PC and ^{14}C-PE, respectively), was the lowest at 12 DPH (1.5 ± 0.2 and 2.5 ± 0.7 pmol mg prot−1 h−1, respectively; $p < 0.05$) and much lower at this stage than that displayed, when the incubation was performed as free fatty acid (Table 5). Independently of larval stage, all [1-^{14}C] FAs were highly esterified into TL (only 5.8–17.0% of radioactivity present as FFA) with PL (62.0–83.1%), and more precisely PC (46.1–63.2%) and PE (6.6–10.7%) being the main metabolic target for esterification (Table 6). [1-^{14}C] ARA was the most incorporated substrate into phosphatidylinositol (PI) of 12 DPH larvae (9.2% vs 2.7–4.1% for the other [1-^{14}C] FAs; $p < 0.05$). Regardless of stage, the esterification pattern of all [1-^{14}C] FAs into NL was: Partial acylglycerols (PAG; monoacylglycerols + diacylglycerols) > TAG ≥ SE. Minor differences existed in the esterification of LA between developmental stages, whereas that of ALA remained unchanged (Table 6). Important differences were observed in the esterification pattern of [1-^{14}C] ARA, when this fatty acid was not provided to the larvae in its free form but bound to PC or PE. This was especially evident at 4 DPH, while these differences were less prominent at 12 DPH (Table 7). For instance, and regardless of stage, over 40% of [1-^{14}C] ARA was recovered into larval PC, when it was added to the culture media bound to PC, whereas when incubated as PE, [1-^{14}C] ARA was more equitably distributed into both PC and PE. Interestingly, PI was again a secondary target for the esterification of ARA (Table 7). In spite of the high incorporation and esterification rates, no elongation/desaturation products were obtained from incubated [1-^{14}C]FA. Only the incubation with [1-^{14}C]EPA displayed a reduced proportion of total radioactivity (4.5 ± 0.6%) as an unknown but shorter product detected further above the [1-^{14}C]EPA band (Table 8). The position of this product on the plate did not correspond to any known product, and its accurate identification was not possible through the available standards.

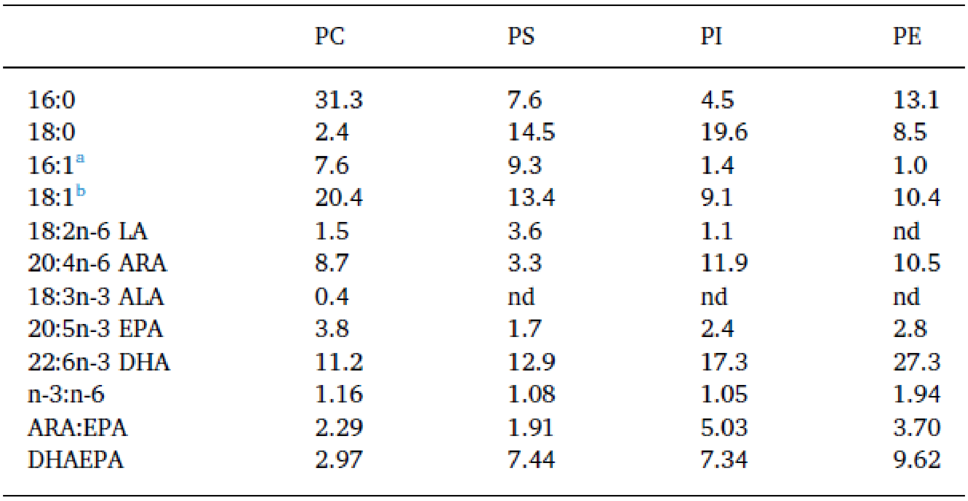
Table 3 *Total fatty acid content (µg larvae−1) and main fatty acid composition (% of total FA) of Anguilla anguilla larvae at 4, 8 and 12 DPH.*



	4 DPH	8 DPH	12 DPH
Σ *FA*	12.1 ± 0.5^{c}	7.0 ± 0.3^{b}	4.4 ± 0.0^{a}
16:0	18.9 ± 0.2^{a}	18.9 ± 0.5^{a}	22.5 ± 1.2^{b}
18:0	4.3 ± 0.1^{a}	4.8 ± 0.1 ^{b}	5.6 ± 0.4^{ab}
Σ SFA	27.1 ± 0.3^{a}	26.5 ± 0.7^{a}	31.3 ± 1.8^{b}
16:1n-7	8.8 ± 0.1^{b}	5.9 ± 0.1^{a}	5.7 ± 0.3^{a}
18:1n-9	30.0 ± 0.3^{c}	27.0 ± 0.7^{b}	22.6 ± 1.2^{a}
18:1n-7	6.1 ± 0.1^{b}	4.8 ± 0.1^{a}	5.6 ± 0.4^{a}
Σ MUFA	48.4 ± 0.5^{c}	40.8 ± 1.0^{b}	36.1 ± 1.6^{a}
18:2n-6 LA	2.8 ± 0.1^{b}	2.0 ± 0.1^{a}	2.3 ± 0.1^{ab}
20:2n-6	0.4 ± 0.0	0.4 ± 0.2	0.4 ± 0.0
20:4n-6 ARA	4.0 ± 0.1^{a}	4.4 ± 0.8^{a}	7.5 ± 0.8^{b}
22:4n-6	0.7 ± 0.0^{a}	0.7 ± 0.0^{a}	0.9 ± 0.1^{b}
22:5n-6	0.6 ± 0.0^{a}	0.6 ± 0.1^{ab}	0.8 ± 0.1^{b}
Σ n-6 PUFA	9.0 ± 0.2 ^{a}	8.4 ± 1.4^{a}	12.3 ± 1.1^{b}
18:3n-3 ALA	0.7 ± 0.0	0.8 ± 0.2	0.4 ± 0.0
20:4n-3	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
20:5n-3 EPA	1.8 ± 0.1^{a}	3.2 ± 0.6^{b}	2.1 ± 0.3^{ab}
22:5n-3	1.7 ± 0.1	2.5 ± 0.5	1.8 ± 0.2
22:6n-3 DHA	6.1 ± 0.3	9.5 ± 2.3	9.8 ± 1.6
Σ n-3 PUFA	10.5 ± 0.5	16.3 ± 3.7	14.4 ± 2.0
Σ n-6 LC-PUFA	5.6 ± 0.2^{a}	6.1 ± 0.9^{a}	9.6 ± 1.0^{b}
Σ n-3 LC-PUFA	9.8 ± 0.4	15.5 ± 3.5	14.0 ± 1.9
Σ PUFA	20.4 ± 0.6	25.3 ± 5.0	27.2 ± 3.0
Σ DMA	0.5 ± 0.0	0.6 ± 0.3	1.2 ± 0.0
n-3:n-6	1.2 ± 0.0^{a}	1.9 ± 0.2 ^{b}	1.2 ± 0.1^{a}
ARA:EPA	2.3 ± 0.0^{b}	1.4 ± 0.0^{a}	3.6 ± 0.1^{c}
DHA:EPA	3.5 ± 0.0^{b}	3.0 ± 0.2^{a}	4.7 ± 0.2^{c}

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. LA, linoleic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Σ include some minor components not shown. Mean values with unlike superscript letters are significantly different (p < 0.05).

Table 4 *Main fatty acid composition (% of total FA) of individual phospholipids from Anguilla anguilla larvae at 12 DPH.*



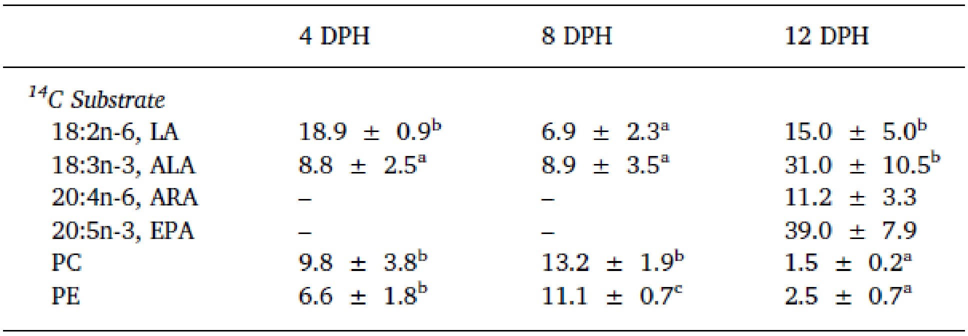
	PC	PS	PI	PE
16:0	31.3	7.6	4.5	13.1
18:0	2.4	14.5	19.6	8.5
16:1[a]	7.6	9.3	1.4	1.0
18:1[b]	20.4	13.4	9.1	10.4
18:2n-6 LA	1.5	3.6	1.1	nd
20:4n-6 ARA	8.7	3.3	11.9	10.5
18:3n-3 ALA	0.4	nd	nd	nd
20:5n-3 EPA	3.8	1.7	2.4	2.8
22:6n-3 DHA	11.2	12.9	17.3	27.3
n-3:n-6	1.16	1.08	1.05	1.94
ARA:EPA	2.29	1.91	5.03	3.70
DHAEPA	2.97	7.44	7.34	9.62

n = 1; PC, Phosphatidylcholine; PS, Phosphatidylserine; PI, Phosphatidylinositol; PE, Phosphatidylethanolamine.nd, not detected.

[a] *Mainly n-7 isomer.*

[b] *Mainly n-9 isomer.*

Table 5 *Incorporation of radiolabelled substrates into total lipid (pmol mg prot−1 h−1) of Anguilla anguilla larvae at 4, 8 and 12 DPH.*

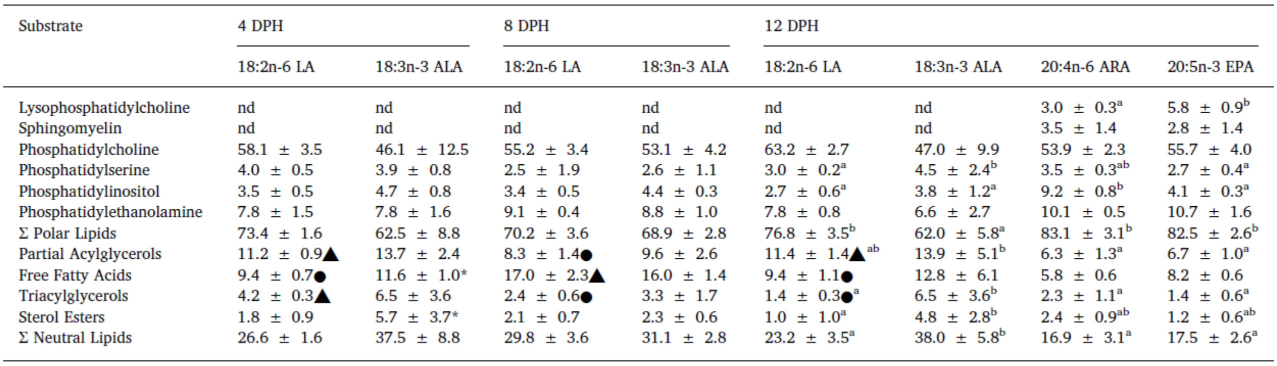


	4 DPH	8 DPH	12 DPH
^{14}C Substrate			
18:2n-6, LA	18.9 ± 0.9^{b}	6.9 ± 2.3^{a}	15.0 ± 5.0^{b}
18:3n-3, ALA	8.8 ± 2.5^{a}	8.9 ± 3.5^{a}	31.0 ± 10.5^{b}
20:4n-6, ARA	–	–	11.2 ± 3.3
20:5n-3, EPA	–	–	39.0 ± 7.9
PC	9.8 ± 3.8^{b}	13.2 ± 1.9^{b}	1.5 ± 0.2^{a}
PE	6.6 ± 1.8^{b}	11.1 ± 0.7^{c}	2.5 ± 0.7^{a}

Results represent means ± SD; n = 3. LA, linoleic acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Mean values with unlike superscript letters are significantly different ($p < 0.05$).

3.3. Transformation of radiolabelled fatty acids by elongation/desaturation processes

Table 6 *Esterification pattern (%) of [1-14C]FA substrates into the different lipid classes of Anguilla anguilla larvae at 4, 8 and 12 DPH.*



Substrate	4 DPH		8 DPH		12 DPH			
	18:2n-6 LA	18:3n-3 ALA	18:2n-6 LA	18:3n-3 ALA	18:2n-6 LA	18:3n-3 ALA	20:4n-6 ARA	20:5n-3 EPA
Lysophosphatidylcholine	nd	nd	nd	nd	nd	nd	3.0 ± 0.3^{a}	5.8 ± 0.9^{b}
Sphingomyelin	nd	nd	nd	nd	nd	nd	3.5 ± 1.4	2.8 ± 1.4
Phosphatidylcholine	58.1 ± 3.5	46.1 ± 12.5	55.2 ± 3.4	53.1 ± 4.2	63.2 ± 2.7	47.0 ± 9.9	53.9 ± 2.3	55.7 ± 4.0
Phosphatidylserine	4.0 ± 0.5	3.9 ± 0.8	2.5 ± 1.9	2.6 ± 1.1	3.0 ± 0.2^{a}	4.5 ± 2.4^{b}	3.5 ± 0.3^{ab}	2.7 ± 0.4^{a}
Phosphatidylinositol	3.5 ± 0.5	4.7 ± 0.8	3.4 ± 0.5	4.4 ± 0.3	2.7 ± 0.6^{a}	3.8 ± 1.2^{a}	9.2 ± 0.8^{b}	4.1 ± 0.3^{a}
Phosphatidylethanolamine	7.8 ± 1.5	7.8 ± 1.6	9.1 ± 0.4	8.8 ± 1.0	7.8 ± 0.8	6.6 ± 2.7	10.1 ± 0.5	10.7 ± 1.6
Σ Polar Lipids	73.4 ± 1.6	62.5 ± 8.8	70.2 ± 3.6	68.9 ± 2.8	76.8 ± 3.5^{b}	62.0 ± 5.8^{a}	83.1 ± 3.1^{b}	82.5 ± 2.6^{b}
Partial Acylglycerols	11.2 ± 0.9▲	13.7 ± 2.4	8.3 ± 1.4●	9.6 ± 2.6	11.4 ± 1.4▲^{ab}	13.9 ± 5.1^{b}	6.3 ± 1.3^{a}	6.7 ± 1.0^{a}
Free Fatty Acids	9.4 ± 0.7●	11.6 ± 1.0*	17.0 ± 2.3▲	16.0 ± 1.4	9.4 ± 1.1●	12.8 ± 6.1	5.8 ± 0.6	8.2 ± 0.6
Triacylglycerols	4.2 ± 0.3▲	6.5 ± 3.6	2.4 ± 0.6●	3.3 ± 1.7	1.4 ± 0.3●^{a}	6.5 ± 3.6^{b}	2.3 ± 1.1^{a}	1.4 ± 0.6^{a}
Sterol Esters	1.8 ± 0.9	5.7 ± 3.7*	2.1 ± 0.7	2.3 ± 0.6	1.0 ± 1.0^{a}	4.8 ± 2.8^{b}	2.4 ± 0.9^{ab}	1.2 ± 0.6^{ab}
Σ Neutral Lipids	26.6 ± 1.6	37.5 ± 8.8	29.8 ± 3.6	31.1 ± 2.8	23.2 ± 3.5^{a}	38.0 ± 5.8^{b}	16.9 ± 3.1^{a}	17.5 ± 2.6^{a}

Results represent means ± SD;

n = 3. Nd, not detected.

Different full symbols (●▲) within the same row represent significant differences within 18:2n-6 and 18:3n3 at different ages ($p < 0.05$).

** Represent differences between C18 substrates within 4 DPH and 8 DPH ($p < 0.05$).*

Different letters in superscript within the same row represent significant differences within 12 DPH incubated substrates ($p < 0.05$).

Table 7 Re-esterification pattern (%) of [1-14C]ARA into different lipid classes of Anguilla anguilla larvae

at 4, 8 and 12 DPH, when provided bounded to PC or PE.

Substrate	4 DPH		8 DPH		12 DPH	
	^{14}C PC	^{14}C PE	^{14}C PC	^{14}C PE	^{14}C PC	^{14}C PE
Phosphatidylcholine	65.5 ± 6.5	19.7 ± 1.3$^{*\circ}$	57.4 ± 2.7	38.5 ± 6.1$^{*\Delta}$	43.1 ± 17.8	25.1 ± 1.8○
Phosphatidylserine	3.5 ± 1.5	10.6 ± 4.1*	3.3 ± 0.5	4.0 ± 1.0	5.3 ± 0.6	8.4 ± 4.3
Phosphatidylinositol	7.4 ± 0.5▲	10.9 ± 1.8$^{*\circ\Delta}$	11.9 ± 0.1	15.7 ± 1.8$^{*\Delta}$	5.0 ± 0.3●	8.0 ± 2.0$^{*\circ}$
Phosphatidylethanolamine	8.7 ± 1.3	25.4 ± 5.3*	13.4 ± 0.2	16.8 ± 1.1*	8.7 ± 2.7	16.0 ± 9.0
Σ Polar Lipids	85.1 ± 5.4	66.5 ± 9.1$^{*\circ\Delta}$	86.0 ± 2.4	74.9 ± 5.5$^{*\Delta}$	62.1 ± 15.9	55.7 ± 7.1○
Partial Acylglycerlos	4.9 ± 1.8●	22.7 ± 5.7*	5.4 ± 1.0●▲	16.7 ± 0.8*	12.6 ± 8.6▲	23.0 ± 8.4
Free Fatty Acids	4.2 ± 0.7	3.5 ± 2.6	5.3 ± 0.5	5.9 ± 0.3	7.6 ± 4.7	3.7 ± 0.5
Triacylglycerols	2.4 ± 1.6●	4.1 ± 1.7	1.6 ± 0.7●	1.9 ± 1.5	8.0 ± 2.6▲	7.5 ± 3.8
Sterol Esters	3.4 ± 1.9●	3.2 ± 1.9	1.7 ± 0.9●	0.7 ± 0.2	9.8 ± 3.2▲	8.3 ± 4.7
Σ Neutral Lipids	14.9 ± 5.4	33.5 ± 9.1$^{*\circ\Delta}$	14.0 ± 2.4	25.1 ± 5.5$^{*\circ}$	37.9 ± 15.9	44.3 ± 7.1Δ

Results represent means ± SD; n = 3.

Different full symbols (●▲) within the same row represent significant differences within 14C PC ($p < 0.05$); Different clear symbols in superscript (○Δ) within the same row represent significant differences within 14C PE ($p < 0.05$);

** Represent significant differences between substrates within the same age ($p < 0.05$).*

Table 8 Distribution of recovered radioactivity (%) from [1-^{14}C]FA substrates as FA metabolites in 12 DPH

Anguilla anguilla larvae.

Substrate	Product	Recovery (12 DPH)
[1-^{14}C]18:2n-6	18:2n-6	100 ± 0.0
[1-^{14}C]18:3n-3	18:3n-3	100 ± 0.0
[1-^{14}C]20:4n-6	20:4n-6	100 ± 0.0
[1-^{14}C]20:5n-3	20:5n-3	95.5 ± 0.6
	UK	4.5 ± 0.6

Results represent means ± SD. UK, unknown product.

4. Discussion

Understanding the dietary requirements of lipids and FA in early eel larval stages is of fundamental importance and a first step in the development of artificial diets. So far, no studies on the European or the Japanese eel pre-leptocephalus larvae have determined requirements of macronutrients including lipids and FA. While pre-leptocephalus larvae of the European eel have never been found in the wild, the natural diet of wild caught leptocephalus larvae seems to be mainly composed of a variety of planktonic organisms with gelatinous zooplankton being highly important (Riemann et al., 2010; Ayala et al., 2018). To date, the success in the transformation of Japanese first-feeding eel larvae to glass eels has been obtained by use of shark-egg based diets (Tanaka et al., 2003; Furuita et al., 2014). Eel eggs are characterized by large oil droplets and an unusually high amount of lipids (Heinsbroek et al., 2013). At hatch, the yolk sac extends along the entire abdomen of the larva and generally lasts up to 2 weeks, coinciding with the full development of a functional feeding apparatus (Sørensen et al., 2016a). During the eel pre-leptocephalus stages examined here (until 12 DPH) an accentuated progressive decrease in the TL content occurred, indicating the exhaustion of the yolk sac. The high amount of NL (up to 75%) during this period, and the reduction of SE and TAG contents indicates the importance of these lipid classes as energy reserves during early growth as well as their likely role as FA reservoir for the processes of lipid synthesis in neural tissues, and digestive and osmoregulatory systems, which are under development (Sargent et al., 1999). Cholesterol also represented a high percentage of eel larval TL, increasing its preservation proportion up to 31% in 12 DPH larvae. Cholesterol is known to be fundamental during both temperature and salinity changes, due to its decisive role in the lipid-bilayer fluidity fluctuations (Kamat and Roy, 2016; Arashiki and Takakuwa, 2019; Biederman et al., 2019; Farhat et al., 2019). While migrating from the continental shelves of Europe to oceanic spawning areas (Tesch and Rohlf, 2003), the European eel undergoes drastic changes in salinity, temperature and feeding habitats with a surprisingly high adaptability (Righton et al., 2016) suggesting lipid restructuring to preserve membrane structure and function, where cholesterol levels play a pivotal role. An increase in cholesterol levels hardens the membrane by decreasing fluidity (Simopoulos and Cleland, 2003). Among PL, preservation of phosphoglycerides such as PC, PE and specifically PI seem to be also relevant during early development. Indeed, young fish contain abundant phospholipids received during embryonic and larval development either from endogenous yolk sac or exogenous lipids and are particularly important in fish larvae as necessary components for cellular bio-membranes and organelles formation among other functions. Additionally PI is involved in a complex signaling system controlling a wide range of biological processes such as, cytoskeleton regulation and motility or regulation of intracellular membrane traffic in early development in vertebrates (Tocher et al., 2008). The present study showed an accentuated decrease of all MUFA proportions with age, mainly 18:1n-9, indicating a preferred oxidation of this FA as source for metabolic energy during the pre-leptocephalus stage. Interestingly, eel larvae presented low n-3:n-6 ratios ranging between 1.2 (at 4 and 12 DPH) and 1.9 (at 8 DPH), similarly to values reported for freshwater fish species by Tocher (1995). The high n-6 content was mainly due to a high amount of ARA (up to 7.5 ± 0.8% at 12 DPH), which is not usually found in such ranges of abundance in strict stenohaline, marine species denoting the importance of this FA for eel larval development. Interestingly, this fatty acid was highly esterified not only in PI as widely described for fish and other vertebrates, but also in PC and PE (PC > PE > PI, Table 6). During the early nonfeeding pre-leptocephalus stage, larval development is fully supported by the yolk (Sørensen et al., 2016a), denoting that the high ARA content must have been exclusively provided by the parent broodstock. An adequate amount of dietary ARA is known to improve marine fish larval survival and growth rates (Atalah et al., 2011), fish adaptive physiological response to hypersalinity stress and hypo-osmoregulatory ability (Carrier III et al., 2011), recovery from infections (Khozin-Goldberg et al., 2006) and also to improve fish reproductive performance (Furuita et al., 2003; Norambuena et al., 2013). In this regard, ARA content and eicosanoids derivatives seem also important in the reproductive success of the European eel. A. anguilla females seem to have the ability to accumulate ARA into tissues, that can be later transferred to the ovaries during induced vitellogenesis (Støttrup et al., 2013, 2016). Moreover, an increase in the ARA content of broodstock, induces a higher production of fertilized eggs and a successful embryonic development and production of eel larvae (Støttrup et al., 2016; Kottmann et al., 2020b). During A. anguilla catadromous migration to the Sargasso Sea area of the North Atlantic Ocean, mature silver eels do not feed, thus energy and nutrients required for reproduction need to be built in before this period. In captivity, as performed in the present study, wild caught female silver eels are relocated and accustomed as broodstock for subsequent hormonal induction of ovarian development, and then transferred to seawater, where the fish are no longer fed. Thus, modifications into eel broodstock lipid composition should take place during the period prior to seawater and induction of vitellogenesis, being critical to supply an adequate ARA dietary content during this stage. A strategy not used here, however, may involve continuous feeding during seawater transfer until observation of a cease in voluntary feed intake to ensure adequate FA tissue levels during vitellogenesis. The high DHA:EPA ratios (3.0–4.7) observed here are common in marine fish eggs and larvae that have not yet started exogenous feeding and also denote the relevance of DHA during early life stages (Sargent et al., 1999; Olsen et al., 2014). Interestingly, DHA is provided by eel broodstock females which live for a long period (10–18 years) in a freshwater environment (van Ginneken and Maes, 2005; Bruijs and Durif, 2009). In this sense, the high DHA content of European eel broodstock tissues (Støttrup et al., 2013), eggs (Støttrup et al., 2016, Kottmann et al., 2020b) and larvae (Kottmann et al., 2020b) suggest that this species may have the enzymatic battery to endogenously biosynthesize EPA and DHA from ALA and in the same way of ARA from LA. A. anguilla glass eels have shown the capacity to biosynthesize ARA after being force-fed with [1-14C]18:2n-6, nonetheless, seven days after administration only 4% of radioactivity was recovered as ARA, showing a relatively slow conversion rate (Kissil et al., 1987). Recent studies on the functional characterization of fatty acyl desaturases and elongases in adults of Japanese eel (Anguilla japonica) revealed that this species has the complete enzymatic repertoire required for the biosynthesis of LC-PUFA from C18 PUFA (Wang et al., 2014; Xu et al., 2020). Interestingly, the LC-PUFA metabolism of this species presented neither a strict marine nor a freshwater pattern; while its FA requirements can be satisfied by C18 PUFA (like freshwater species), a higher enzymatic expression was detected in brain and eyes (similar to marine species), indicating the importance of LC-PUFA in eel neural tissues (Wang et al., 2014; Xu et al., 2020). The incorporation of the radiolabelled FA substrates by European eel larvae could be the result of the continuous intake of the surrounding incubation water within an osmoregulatory process typical of marine fish. Even though images at 0 and 4 DPH (Fig. 1) indicate an apparent lack of mouth formation and passage to the esophagus, studies on the Japanese eel larvae by use of scanning electron microscopy and flourescent dextran have revealed that the mouth appears as a slit with larvae being capable of drinking already at the day of hatching (Ahn et al., 2015*). This suggests a similar capacity for the European eel larvae examined here at 4 DPH. Although drinking or uptake through premature gills cannot totally be ruled out, images at DPH 4 indicate lack of mouth formation and no passage to esophagus. However, it cannot be completely ruled out, thus, that part of the lipid uptake observed is most likely explained by a high permeability of the naked integument of the A. anguilla, similar to cephalopod species (Boucaud-Camou and Roper, 1995; de Eguileor et al., 2000). Therefore, the experimental design developed in our study helps to elucidate some aspects of lipid metabolism during the first stages of development. Thus, radiolabelled substrates either as free FA, or bound to PC and PE, may also have entered the larvae via skin at 4 and at 8 DPH, encountering presumably a completely different set of enzymes for lipid metabolism to that of gut, while at 12 DPH a major component of ingestion by intake of the surrounding water is the most likely. Authors, however, consider it important to highlight the consistent results obtained here in comparison to previous studies applying the present methodology (e.g. FA composition of the different phospholipids and in vivo free FA esterification patterns; Reis et al., 2014, 2016a, 2016b, 2020). At 12 DPH, larvae did not show any elongase or desaturase activity towards incubated substrates (ALA, LA, ARA and EPA). These results indicate the absence or inactivity of elongase/desaturase enzymes during eel preleptocephalus stage, which might be related to the high endowment of newly hatched eel larvae with ARA, EPA and DHA necessary for their proper development. Moreover, it has been suggested that the natural diet of leptocephalus eel is mainly based on gelatinous zooplankton (Hydrozoa, Thaliacea and Ctenophora) fed on phytoplankton, especially photosynthetic, capable of synthesizing EPA, DHA and ARA very efficiently (Valenzuela et al., 1999). Eel larvae, besides likely being carnivorous, are born in marine environment with high salinity, which has been linked to the inactivation of desaturases enzymes especially in migratory species such as salmonids just after smoltification and adaptation to the marine environment has been performed (Tocher, 2015). In any case, the present data undoubtedly suggest that LC-PUFA (EPA, DHA and ARA) are essential FA not just during the A. anguilla preleptocephalus stage, but also at the time prior to and during mouth opening, indicating these FAs must be provided firstly through female broodstock and subsequently through diet at least during first-feeding stage. The notably higher incorporation of n-3 FA (LA and EPA) over n6 FA (ALA and ARA) in 12 DPH larvae evidenced here, agrees well with what has been described for marine species (Sargent et al., 1999) and must be also taken into account in dietary formulations for European eel larvae. Despite the absence of elongase/desaturase enzymatic activity during the pre-leptocephalus stage, eel larvae do present the capacity to re-modulate dietary phospholipids through de-acylation/re-acylation processes. Phospholipids catalytic hydrolysis is under the control of phospholipase A1 and A2 enzymes (Tocher, 1995). The [1-14C]ARA molecule esterified to phospholipids used for our experimental design was bound to the sn-2 position of PC and PE molecules. Therefore, the radioactivity detected into new lipid classes such as PS, PI, PAG or TAG evidences the presence of phospholipase A2 (PLA2) in larval tissues, which is generally believed to be the active digestive enzyme that hydrolyses phospholipids at the sn-2 position (for details see Olsen et al., 2014).

This phospholipid-remodeling process has important consequences in maintaining the proper FA distribution among phospholipids, and thus in its adaptation to environmental changes (Tocher, 1995; Tocher et al., 2008). In this sense, several authors have reported selective location/retention of ARA in PI in fish tissues (Bell and Tocher, 1989; Bell and Dick, 1990; Tocher, 1995; Tocher et al., 2008; Sargent et al., 2002). In our study, [1-14C]ARA presented the highest esterification rate into PI of all [1-14C]FFA assayed, although it was mainly incorporated into PC followed by PE. Moreover, when [1-14C]ARA was bound to PC, up to 65.5% of incorporated ARA was recovered as PC and when provided bound to PE a maximum of 25.4% was recovered as PE, with the majority of [1-14C]ARA being re-esterified into PC and PAG (mono and diacylglycerols), that are involved in de novo synthesis of phospholipids and TAG (for details see Tocher et al., 2008 and Olsen et al., 2014). This unusual esterification pattern of ARA found in eel is supported by the analysis of FA of individual phospholipids, where it is also evident the high content of ARA not only in PI but also in PC and PE. These results are also consistent with those found in common octopus and cuttlefish (Reis et al., 2016a). Both PC and PE are the dominant polar lipids in biological membranes (van Meer et al., 2008) and their influences on the membrane are likely to be particularly impactful. PC tends to provide a fluidizing effect within membranes, while PE can provide a rigidifying effect (Silvius et al., 1986; Fajardo et al., 2011). Thus, the preferential esterification of all [1-14C] substrates into PC registered in our radio-tracing design may be related to the maintenance of a high membrane fluidity associated to environmental changes. The high content of PC and PE is characteristic of larvae from numerous marine species of both fish (Cahu et al., 2009) and cephalopods such as O. vulgaris and S. officinalis (Reis et al., 2014, 2016b). These data, in addition to the high DHA and ARA content in eel larvae tissues and individual phospholipids, confirm the importance of these molecules, particularly essential in the formation of neural tissues and in the processes of metamorphosis that this species undergoes in its osmotic adaptation to progressively less saline environments (Sargent et al., 1999; de Silva et al., 2005; Olsen et al., 2014; Capoccioni et al., 2018).

5. Conclusion

The results of the present study showed that similarly to other marine organisms (Reis et al., 2014, 2016a, 2016b, 2017, 2020; Lund et al., 2019), direct incubation with 14C-labelled FA is a feasible method to investigate in vivo FA and phospholipids metabolism during the preleptocephalus stage of the European eel. As European pre-leptocephalus eel larvae have no desaturation/elongation capacity over PUFA, it is mandatory to provide diets with LC-PUFA such as ARA, DHA and EPA, as these are essential FA for this species at first-feeding stage. Although the capacity of eel larvae to metabolize TAG was not determined in the present study, larvae possess the ability to metabolize phospholipids such as PC and PE. In this sense, and considering that LC-PUFA esterified into phospholipids seems to improve its availability to larvae (Cahu et al., 2009), these FA, and particularly DHA and ARA, should be provided through phospholipids rather than TAG for optimal eel larval nutrition.

Declaration of competing interest

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

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Author's contribution

Ivar Lund and Cova Rodrigues conceived and designed the assimilation study. Covadonga Rodriguez; Diana B. Reis; Ivar Lund conceived and designed the lipid and fatty acid analyses of the study. Jonna Tomkiewicz conceived and designed the broodstock experiment providing larvae for the study. Johanna S. Kottman performed the broodstock experiment. Elisa Benini; Sebastian N. Politis; Johanna S. Kottman performed the assimilation experiment. Jonna Tomkiewicz; Covadonga Rodriguez; primary funding acquisition. Jonna Tomkiewicz; resources for the experimental work. Covadonga Rodriguez; contributed reagents/materials/analysis tools. Diana B. Reis and Jose A. Perèz performed lipid and FA analysis. Diana B. Reis; Ivar Lund; Jose A. Perèz; Covadonga Rodriguez analyzed the data. Ivar Lund; Diana B. Reis, Sebastian N. Politis writing/original draft. All authors contributed to review and editing. All authors gave final approval for publication.

References

Ahn, H., Lee, K.M., Inokuchi, M., Watanabe, S., Okamura, A., Tsukamoto, K., Kaneko, T., 2015. Observation of initial water ingestion and ion absorption in the digestive tract of the Japanese eel larvae. Fish Sci 81, 283–290.

Arashiki, N., Takakuwa, Y., 2019. Role of cholesterol in maintaining asymmetric distribution of phosphatidylserine in plasma membranes. In: Patel, V.B. (Ed.), The Molecular Nutrition of Fats. Academic Press, Cambridge, UK, pp. 77–86 Chapter 6.

Atalah, E., Hernández-Cruz, C.M., Benítez-Santana, T., Ganga, R., Roo, J., Izquierdo, M., 2011. Importance of the relative levels of dietary arachidonic acid and eicosapentaenoic acid for culture performance of gilthead seabream (Sparus aurata) larvae. Aquac. Res. 42, 1279–1288.

Ayala, D.J., Munk, P., Lundgreen, R.B.C., Traving, S.J., Jaspers, C., Jørgensen, T.S., Hansen, L.H., Riemann, L., 2018. Gelatinous plankton is important in the diet of European eel (Anguilla anguilla) larvae in the Sargasso Sea. Sci. Rep. 8, 6156. https:// doi.org/10.1038/s41598-018-24388-x.

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 (11), 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 (3), 909–915.

Biederman, A.M., Kuhn, D.E., O'Brien, K.M., Crockett, E.L., 2019. Physical, chemical, and functional properties of neuronal membranes vary between species of Antartic notothenioids differing in thermal tolerance. J. Comp. Physiol. B. 189, 213–222.

Boucaud-Camou, E., Roper, C.F.E., 1995. Digestive enzymes in paralarval cephalopods. Bull. Mar. Sci. 57 (2), 313–327.

Bruijs, M.C.M., Durif, C.M.F., 2009. Silver eel migration and behaviour. In: van den Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning Migration of the European Eel. Fish & Fisheries Series Vol. 30 Springer, Dordrecht. https://doi.org/10.1007/978-1- 4020-9095-0_4.

Butts, I.A.E., Politis, S.N., Sørensen, S.R., Tomkiewicz, J., 2014. Standardization of fertilization protocols for the European eel. Aquaculture 426, 9–13.

Butts, I.A.E., Sørensen, S.R., Politis, S.N., Tomkiewicz, J., 2016. First-feeding by European eel larvae: a step towards closing the life cycle in captivity. Aquaculture 464, 451–458.

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 (9), 989–999.

Capoccioni, F., Contò, M., Failla, S., Cataudella, S., Ciccotti, E., 2018. Fatty acid profiles of migrating female silver eel from Mediterranean coastal lagoons as integrative descriptors of spawners biological quality. Estuar. Coast. Shelf Sci. 210, 87–97.

Carrier III, J.K., Watanabe, W.O., Harel, M., Rezek, T., Seaton, P.J., Shafer, T.H., 2011. Effects of dietary arachidonic acid on larval performance, fatty acid profiles, stress resistance, and expression of Na+/K+ ATPase mRNA in black sea bass Centropristis striata. Aquaculture 319, 111–121.

Çelik, M., Türeli, C., Çelik, M., Yanara, Y., Erdem, Ü., 2004. Fatty acid composition of the blue crab (Callinectes sapidus Rathbun, 1896) in the north eastern Mediterranean. Food Chem. 88 (2), 271–273. Christie, W.W., 2003. Lipid Analysis, 3rd ed. The Oily Press, Bridgewater, UK, pp. 205–224.

da Silva, F.F.G., Jacobsen, C., Kjorsvik, E., Støttrup, J.G., Tomkiewicz, J., 2018a. Oocyte and egg quality indicators in European eel: lipid droplet coalescence and fatty acid composition. Aquaculture 496, 30–38.

da Silva, F.F.G., Tveiten, H., Maugars, G., Lafont, A.-G., Dufour, S., Støttrup, J.G., Kjørsvik, E., Tomkiewicz, J., 2018b. Differential expression of gonadotropin and estrogen receptors and oocyte cytology during follicular maturation associated with egg viability in European eel (Anguilla anguilla). Comp. Biochem. Physiol. A 221, 44–54.

de Eguileor, M., Leonardi, M.G., Grimaldi, A., Tettamanti, G., Fiandra, L., Giordana, B., Valvassori, R., Lanzavecchia, G., 2000. Integumental amino acid uptake in carnivorous predator mollusc (Sepia officinalis, Cephalopoda). Tissue Cell 32, 389–398.

de Silva, S.S., Gunasekera, R.M., Collins, R.O., 2005. Some morphometric and biochemical features of ready-to-migrate silver and pre-migratory yellow stages of the shortfin eel of south-eastern Australian waters. J. Fish Biol. 61 (4), 915–928.

Fajardo, V.A., McMeekin, L., Leblanc, P.J., 2011. Influence of phospholipid species on membrane fluidity: a meta-analysis for a novel phospholipidfluidity index. J. Membr. Biol. 244 (2), 97–103.

Farhat, E., Turenne, E.D., Choi, K., Weber, J.-M., 2019. Hypoxia-induced remodelling of goldfish membranes. Comp. Biochem. Physiol. B 237, 110326.

Furuita, H., Yamamoto, T., Shima, T., Suzuki, N., Takeuchi, T., 2003. Effect of arachidonic acid levels in broodstock diet on larval egg quality of Japanese flounder Paralichthys olivaceus. Aquaculture 220, 725–735.

Furuita, H., Unuma, T., Nomura, K., Tanaka, H., Okuzawa, K., Sugita, T., Yamamoto, T., 2006. Lipid and fatty acid composition of eggs producing larvae with high survival rate in the Japanese eel. J. Fish Biol. 69 (4), 1178–1189. https://doi.org/10.1111/j. 1095-8649.2006.01196.x.

Furuita, H., Murashita, K., Matsunari, H., Yamamoto, T., Nagao, J., Nomura, K., Tanaka, H., 2014. Decreasing dietary lipids improves larval survival and growth of Japanese eel Anguilla japonica. Fish. Sci. 80, 581–587. https://doi.org/10.1007/s12562-014- 0713-2.

Heinsbroek, L.T.N., Støttrup, J.G., Jacobsen, C., Corraze, G., Kraiem, M.M., Holst, L.K., TomKiewicz, J., Kaushik, S.J., 2013. A review on broodstock nutrition of marine pelagic spawners: the curious case of the freshwater eels (Anguilla spp.). Aquac. Nutr. 19, 1–24. Jobling, M., 2001. In:

Houlihan, D., Boujard, T. (Eds.), Food Intake in Fish. M. WileyBlackwell, Jobling. https://doi.org/10.1002/9780470999516. Kamat, S.G., Roy, R., 2016. Evaluation of the effect of n-3 PUFA-rich dietary fish oils on lipid profile and membrane fluidity in alloxan-induced diabetic mice (Mus musculus). Mol. Cell. Biochem. 416, 117–129.

Khozin-Goldberg, I., Cohen, Z., Pimenta-Leibowitz, M., Nechev, J., Zilberg, D., 2006. Feeding with arachidonic acid-rich triacylglycerols from the microalga Parietochloris incisa improved recovery of guppies from infection with Tetrahymena sp. Aquaculture 255 (1–4), 142–150.

Kissil, G.W., Youngson, A., Cowey, C.B., 1987. Capacity of the European eel (Anguilla anguilla) to elongate and desaturate dietary linoleic-acid. J. Nutr. 117, 1379–1384.

Kottmann, J.S., Jørgensen, M.G.P., Bertolini, F., Loh, A., Tomkiewicz, J., 2020a. Differential impacts of carp and salmon pituitary extracts on induced oogenesis, egg quality, molecular ontogeny and embryonic developmental competence in European eel. PLoS One 15 (7), 1–24. https://doi.org/10.1371/journal.pone.0235617. e0235617.

Kottmann, J.S., Tomkiewicz, J., Butts, I.A.E., Lund, I., Jacobsen, C., Støttrup, J.G., Holst, L., 2020b. Effects of essential fatty acids and feeding regimes on egg and offspring quality of European eel: comparing reproductive success of farm-raised and wild caught broodstock. Aquaculture 529, 1–16. https://doi.org/10.1016/j.aquaculture. 2020.735581. AQUA_2019_2873_R2Accepted.

Lund, I., Rodríguez, C., Izquierdo, M.S., El Kertaoui, N., Kestemont, P., Reis, D.B., Dominguez, D., Pérez, J.A., 2019. Influence of salinity and linoleic or α-linolenic acid-based diets on ontogenetic development and metabolism of unsaturated fatty acids in pike perch larvae (Sander lucioperca). Aquaculture 500, 550–561.

Miest, J.J., Politis, S.N., Ademeka, M., Tomkiewicz, J., 2019. Molecular ontogeny of larval immunity in European eel at increasing temperatures. Fish Shellfish Immunol. 87, 105–119.

Mordenti, O., Casalini, A., Mandelli, M., Di Biase, A., 2013. A closed recirculating aquaculture system for artificial seed production of the European eel (Anguilla anguilla): technology development for spontaneous spawning and eggs incubation. Aquac. Eng. 58, 88–94.

Müller, A.V., McEvoy, F.J., Tomkiewicz, J., Politis, S.N., Amigo, J.M., 2016. Ultrasonographic predictors of response of European eels (Anguilla anguilla) to hormonal treatment for induction of ovarian development. Am. J. Vet. Res. 77 (5), 478–486. https://doi.org/10.2460/ajvr.77.5.478.

Norambuena, F., Estévez, A., Mañanós, E., Bell, J.G., Carazo, I., Duncan, N., 2013. Effects of graded levels of arachidonic acid on the reproductive physiology of Senegalese sole (Solea senegalensis): fatty acid composition, prostaglandins and steroid levels in the blood of broodstock bred in captivity. Gen. Comp. Endocrinol. 191, 92–101.

Olsen, Y., 2009. Biogeochemistry of Inland Waters. Eds. Likens, G.E., p. 744.

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.

Peñaranda, D.S., Pérez, L., Gallego, V., Barrera, R., Jover, M., Asturiano, J.F., 2010. European eel sperm diluent for short-term storage. Reprod. Domest. Anim. 45, 407–415.

Politis, S.N., Butts, I.A.E., Tomkiewicz, J., 2014. Light impacts embryonic and early larval development of the European eel. J. Exp. Mar. Biol. Ecol. 461, 407–415.

Politis, S.N., Mazurais, D., Servili, A., Zabonino-Infante, J.-L., Miest, J.J., Sørensen, S.R., Tomkiewicz, J., Butts, I.A.E., 2017. Temperature effects on gene expression and morphological development of European eel, Anguilla anguilla larvae. PLoS One 12 (8), e0182726.

Politis, S.N., Mazurais, D., Servili, A., Zambonino-Infante, J.L., Miest, J., Tomkiewicz, J., Butts, I.A.E., 2018a. Salinity reduction benefits European eel larvae: insights at the morphological and molecular level. PLoS One 13 (6), e0198294. https://doi.org/10. 1371/journal.pone.0198294.

Politis, S.N., Sørensen, S.N., Mazurais, D., Servili, A., Zambonino-Infante, J.-L., Miest, J., Clemmesen, C., Tomkiewicz, J., Butts, I.A.E., 2018b. Molecular ontogeny of firstfeeding European eel larvae. Front. Physiol. 9, 1477. https://doi.org/10.3389/fphys. 2018.01477.

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 14C-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 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 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., Lund, I., Perez, J.A., Acosta, N.G., Abdul-Jalbar, B., Bolaños, A., Rodríguez, C., 2020. Esterification and modification of [1-14C] n-3 and n-6 polyunsaturated fatty acids in pikeperch (Sander lucioperca) larvae reared under linoleic or α-linolenic acidbased diets and variable environmental salinities. Comp. Biochem. Physiol. B. https://doi.org/10.1016/j.cbpb.2020.110449. CBPB-D-20-00023R2.

Riemann, L., Alfredsson, H., Hansen, M.M., Als, T.D., Nielsen, T.G., Munk, P., Aarestrup, K., Maes, G.E., Sparholt, H., Petersen, M.I., Bachler, M., Castonguay, M., 2010. Qualitative assessment of the diet of European eel larvae in the Sargasso Sea resolved by DNA barcoding. Biol. Lett. 6, 819–822. https://doi.org/10.1098/rsbl.2010.0411.

Righton, D., Westerberg, H., Feunteun, E., Økland, F., Gargan, F., et al., 2016. Empirical observations of the spawning migration of European eels: the long and dangerous road to the Sargasso Sea. Sci. Adv. 2, e1501694.

Saito, H., Kurogi, H., Chow, S., Mochioka, N., 2015. Variation of lipids and fatty acids of the japanese freshwater eel, Anguilla japonica, during spawning migration. J. Oleo Sci. 64, 603–616. https://doi.org/10.5650/jos.ess14293.

Rodríguez, Covadonga, Pérez, José 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). Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology 132 (3), 559–570.

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, 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.

Silvius, J.R., Brown, P.M., O'Leary, T.J., 1986. Role of head group structure in the phase behavior of amino phospholipids. 1F. Hydrated and dehydrated lamellar phases of saturated phosphatidylethanolamine analogues. Biochemistry 25, 4249–4258.

Simopoulos, A.P., Cleland, L.G., 2003. Omega-6/Omega-3 Essential Fatty Acid Ratio: The Scientific Evidence. Basel Karger Medical and Scientific Publishers, Basel. https:// doi.org/10.1159/000073789.

Sørensen, S.R., Skov, P.V., Lauesen, P., Tomkiewicz, J., Bossier, P., Schryver, D.S., 2014. Microbial interference and potential control in culture of European eel (Anguilla anguilla) embryos and larvae. Aquaculture 426, 1–8.

Sørensen, S.R., Tomkiewicz, J., Munk, P., Butts, I.A., Nielsen, A., Lauesen, P., Graver, C., 2016a. Ontogeny and growth of early life stages of captive-bred European eel. I.Aquaculture 456, 50–61.

Sørensen, S.R., Butts, I.A.E., Munk, P., Tomkiewicz, J., 2016b. Effects of salinity and sea salt type on egg activation fertilization, buoyancy and early embryology of European eel, Anguilla anguilla. Zygote 24, 121–138.

Støttrup, J.G., Jacobsen, C., Tomkiewicz, J., Jarlbæk, H., 2013. Modification of essential fatty acids composition in broodstock of cultured European eel Anguilla anguilla L. Aquac. Nutr. 19, 172–185.

Støttrup, J.G., Tomkiewicz, J., Jacobsen, C., Butts, I.A.E., Holst, L.K., Krüger-Johnsen, M., Graver, C., Lauesen, P., Fontagné-Dicharry, S., Heinsbroek, L.T.N., Corraze, G., Kaushik, S., 2016. Development of a broodstock diet to improve developmental competence of embryos in European eel, Anguilla anguilla. Aquac. Nutr. 22 (4), 725–737.

Tanaka, H., 2015. Progression in artificial seedling production of Japanese eel Anguilla japonica. Fish. Sci. 81, 11–19. https://doi.org/10.1007/s12562-014-0821-z.

Tanaka, H., Kagawa, H., Ohta, H., Unuma, T., Nomura, K., 2003. The first production of glass eel in captivity: fish reproductive physiology: facilitates great progress in aquaculture. Fish Physiol. Biochem. 28, 493–497.

Tanaka, H., Nomura, K., Yamamoto, T., Oku, H., 2006. Development of artificial diets and rearing systems for eel larvae, – The first success of production of glass eel in captivity. Bull. Fish. Res. Agency (Suppl. 5), 63–69.

Tesch, F.W., 2003. The Eel. Blackwell Science, Oxford.

Tesch, F.W., Rohlf, N., 2003. Migration from continental waters to the spawning grounds. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer, Tokyo.

Tocher, D.R., 1995. Glycerophospholipid metabolism. In: Biochemistry and Molecular Biology of Fishes. Vol. 4. Elsevier, pp. 119–157.

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, J.G., 2008. The role of phospholipids in nutrition and metabolism of teleost fish. Aquaculture 280, 21–34.

Tomkiewicz, J. (Ed.), 2012. Reproduction of European Eel in Aquaculture (REEL): Consolidation and New Production Methods. National Institute of Aquatic Resources, Technical University of Denmark, pp. 1–47 DTU Aqua Report No 249-2012.

Tomkiewicz, J., Kofoed, T.M.N., Pedersen, J.S., 2011. Assessment of testes development during induced spermatogenesis in European eel Anguilla anguilla. Mar. Coast. Fish. Dyn. Manag. Ecosyst. Sci. 3, 106–118.

Tomkiewicz, J., Politis, S.N., Sørensen, S.R., Butts, I.A.E., Kottmann, J.S., 2019. European eel – an integrated approach to establish eel hatchery technology in Denmark. In: Don, A., Coulson, P. (Eds.), Eels - Biology, Monitoring, Management, Culture and Exploitation: Proceedings of the First International Eel Science Symposium. 5m Publishing, Sheffield, pp. 340–374.

Valenzuela, A., Sanhueza, J., Garrido, A., 1999. Ácidos grasos poliinsaturados de cadena larga omega-3: cuándo y por qué es necesaria la suplementación con estos ácidos grasos. Aceites Grasas 9, 294–299. van Ginneken, V.J.T., Maes, G.E., 2005. The European eel (Anguilla anguilla, Linnaeus), its lifecycle, evolution and reproduction: a literature review. Rev. Fish Biol. Fish. 15, 367–398.

van Meer, G., Voelker, D.R., Feigenson, G.W., 2008. Membrane lipids: where they are andhow they behave. Nat. Rev. Mol. Cell Biol. 9, 112–124.

Wang, S., Monroig, O., Tang, G., Zhang, L., You, C.H., Tocher, D.R., Li, Y., 2014. Investigating long-chain polyunsaturated fatty acid biosynthesis in teleost fish: functional characterization of fatty acyl desaturase (Fads2) and Elovl5 elongase in the catadromous species, Japanese eel Anguilla japonica. Aquaculture 434, 57–65.

Xu, W., Wang, S., You, C., Zang, Y., Monroig, O., Tocher, D.R., Li, Y., 2020. The catadromous teleost Anguilla japonica has a complete enzymatic repertoire for the biosynthesis of docosahexaenoic acid from α-linolenic acid: cloning and functional characterization of an Elovl2 elongase. Comp. Bioch. Physiol. B 240, 110373.