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

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

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46 **Keywords** *European eel larvae - PUFA metabolism - Phospholipids - Radiolabelled substrates -*
47 *Arachidonic acid*

48

49 ***Abstract***

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

70

71 **1. Introduction**

72 Nutritional requirements of first-feeding European eel larvae (*Anguilla anguilla*) are largely unknown and
73 research within this area is highly necessary for obtaining viable leptocephalus larvae and a future sustainable
74 production of glass eels in aquaculture, which until now relies entirely on wild caught specimens. Research in
75 hatchery technology has raised European eel breeding from a state of reproductive failure to a stable production
76 of viable eggs and yolk-sac larvae from hormonally induced wild caught and farmed eel broodstock
77 (Tomkiewicz, 2012; Tomkiewicz et al., 2019; Butts et al., 2014). Reproductive method improvements imply
78 progress in hormonal induction of eel maturation (Tomkiewicz et al., 2011; Mordenti et al., 2013; Müller et
79 al., 2016; da Silva et al., 2018a, 2018b; Kottmann et al., 2020a); optimization and standardization of spawning,
80 and fertilization and incubation protocols (Butts et al., 2014), including reduction of egg microbial activity
81 (Sørensen et al., 2014) especially during the early life history stages that seem to be immunocompromised
82 (Miest et al., 2019). Further achievements include improved larval culture technology in terms of light (Politis
83 et al., 2014), temperature (Politis et al., 2017) and salinity (Sørensen et al., 2016b; Politis et al., 2018a),
84 enhancing larval survival until the first feeding stage, i.e. the end of yolk-sac and lipid droplet depletion
85 (Sørensen et al., 2016a). T To promote development of pre-leptocephalus larvae into the leptocephalus larval
86 stage and ultimately transformation into glass eels, studies on DNA gut content of wild caught European eel
87 leptocephalus larvae from the Sargasso Sea (Riemann et al., 2010; Ayala et al., 2018) have suggested potential
88 preys but remain inconclusive. Inert diets based on shark egg powder, krill hydrolysate and soybean peptide
89 as main components have been successful in generating glass eels of the Japanese eel (*Anguilla japonica*)
90 (Tanaka, 2015; Tanaka et al., 2006). Feeding trials have been conducted on European eel larvae defining the
91 first-feeding "window" (Butts et al., 2016; Politis et al., 2018b). However, the development into leptocephalus
92 larvae and ultimately glass eels has still not been achieved, which complicates research on dietary nutrient
93 requirements. Most strict marine carnivorous fish larvae have high and specific dietary requirements for lipids
94 and long chain polyunsaturated fatty acids (LC-PUFA) during endogenous development and first-feeding
95 phases, while euryhaline and freshwater species may display lower dietary FA requirements and higher
96 metabolic capacities. European eel is catadromous with a complex life cycle and a unique migratory ability
97 between sea- and freshwater and although spending most of its juvenility and adult life in freshwater, it
98 suggests a high plasticity for physiological and metabolic adaptation. During its lifespan in freshwater, eels

99 build up high stores of fat and lipids in tissues. Upon returning to the Sargasso Sea spawning area, silver eels
100 likely cease feeding and undergo maturation while using freshwater obtained fat resources, as was reported for
101 migrating Japanese silver eels (Saito et al., 2015). Not only tissue levels of fat seems of importance, but muscle,
102 visceral fat and ovary of wild female silver eels have revealed significantly higher ARA content than farmed
103 eels fed standard commercial fish meal based diet (Støttrup et al., 2013) and evidence of the significance of
104 supplemented ARA in the reproductive success of farmed European and Japanese eels has been documented
105 (Furuita et al., 2006; Støttrup et al., 2016; Kottmann et al., 2020b). These findings moreover suggest a
106 significant importance of ARA for first-feeding of European eel larvae, although this has been questioned in
107 Japanese pre-leptocephalus larvae for which a low lipid diet (i.e. defatted shark eggs) low in n-6:n-3 (0.22);
108 low in ARA:EPA (0.32) and high in DHA:EPA (1.61) promoted better survival and growth compared with a
109 similar not defatted diet (Furuita et al., 2014). Freshwater food webs are generally characterized by a higher
110 n-6:n3 ratio, including higher abundance of ARA than marine food webs (Jobling, 2001; Olsen, 2009), and
111 18:2n-6 and 20:4n-6 are characteristically contained in freshwater fishes. This may likely explain the high
112 ARA tissue content in wild silver eels (Saito et al., 2015), but may also reflect a certain ability to modify
113 dietary FA precursors as elongation/ desaturation of dietary [1-14C] linoleic acid to ARA has been confirmed
114 in forced-fed European glass eels (Kissil et al., 1987), and enzymatic expression of all key desaturase and
115 elongase enzymes revealed the capacity to elongate C18 PUFA substrates in adult Japanese eels (Xu et al.,
116 2020). Although a similar capacity for European eel adults cannot be precluded, it is also known that prior to
117 migration to the Sargasso Sea area for spawning, silver eel females seem to carry out a particularly selective
118 feeding on LC-PUFA in coastal lagoons habitats (Capoccioni et al., 2018), with preys such as the blue crab
119 (*Callinectes sapidus*), which provides high levels of n-3 and n-6 LC-PUFA including EPA, DHA and ARA
120 (Çelik et al., 2004). The extent to which European eel larvae can convert C18 PUFA to C20-C22 LC-PUFA,
121 mainly EPA, DHA and ARA is at present unknown and complicated by the ability to manipulate and test this
122 in experimental diets. In vivo incubation of larvae with radiolabelled nutrient markers has proven a reliable
123 methodology for obtaining useful knowledge on nutrient metabolism during early ontogeny of both marine, -
124 freshwater fishes as well as invertebrate species for which dietary nutritional requirements are limited (Reis et
125 al., 2014; Reis et al., 2016a, 2016b, 2020; Lund et al., 2019). Thus, the present experiment studied the in vivo
126 larval capability of European eel larvae to incorporate and metabolize a range of [1-14C] n-3 and n-6 PUFAs

127 to provide first insights on eel larval FA requirements. Larvae at several ontogenetic stages without (4 and 8
128 days post hatch (DPH)) or with a functional feeding apparatus (12 DPH) were sampled from different parental
129 combinations. Larval capacity for de-acylation and re-acylation of [1-14C] ARA, initially bound to
130 phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was also investigated. Results are discussed
131 related to the control larvae lipid class composition and the fatty acid profiles of total lipids, and individual
132 PC, PE, phosphatidylinositol (PI) and phosphatidylserine (PS).

133 **2. Materials and methods**

134 *2.1. Broodstock maturation and husbandry*

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

155 2.2. *Gamete production and embryonic rearing*

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

175

176 *Table 1 Reproductive outcome of Anguilla anguilla broodstock according to female ID, hormonal treatment*
177 *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

178

179 *Results represent means ± SD; n = 3.*

180

181 2.3. Larval rearing and ontogenic development

182 Directly after hatch, larvae of each batch were stocked in separate 250 L rearing units connected to a
183 recirculation system containing filtered seawater (FSW) at a salinity of 36 PSU. The system comprised a sump
184 reservoir of ~1 m³ from where water passed to a 320 L combined bio-trickling filter (RK bioelements) and
185 thereafter re-entered the sump. A protein skimmer (Turboflotor 5000 single 6.0, Aqua Medic GmbH.,
186 Bissendorf, Germany) was included for removal of waste protein. In each rearing unit, flow rate was kept at
187 ~10 L min⁻¹ and temperature at 18 °C (Politis et al., 2017), while light regime was set to a low intensity 12
188 light/12 dark photoperiod (Politis et al., 2014). Non-fed larvae at 4, 8 and 12 DPH were used for the subsequent
189 incubations. Fig. 1 illustrates larval ontogenetic development at 18 °C. At this temperature the yolk is usually
190 depleted at around 10 DPH, while the oil droplet may remain visible until 15–17 DPH (Politis et al., 2017).

191 2.4. Ethics statement

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

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

200 *2.5. In vivo incubation with labelled [1- ^{14}C] fatty acids*

201

202 Larval capability to incorporate and metabolize unsaturated FAs was studied by in vivo radio tracing of [1-
203 ^{14}C] FAs (Reis et al., 2014, 2016a, 2020). Larvae at different stages of ontogeny (4, 8, 12 DPH) were incubated
204 in 10 mL flat-bottom tissue culture plates (SARSTEDT AG & Co., Nümbrecht, Germany) at a density of 150
205 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
206 (free fatty acid molecules labelled with ^{14}C in its first carbon counting from the carboxyl head) including either
207 18:3n-3 (LA) and 18:2n-6 (ALA; PerkinElmer Inc., Waltham, Massachusetts, USA), as well as [1- ^{14}C] ARA
208 molecule esterified into the sn-2 position of phosphatidylcholine (^{14}C -PC; L- α -1-palmitoyl-2-arachidonyl-
209 [arachidonyl-1- ^{14}C]) and of phosphatidylethanolamine (^{14}C -PE; L- α -1-palmitoyl-2-arachidonyl-arachidonyl-
210 1- ^{14}C); American Radiolabelled Chemicals, Inc.). Additionally, 12 DPH larvae were also incubated under the
211 same conditions with [1- ^{14}C] 20:5n-3 (EPA), and 20:4n-6 (ARA; American Radiolabelled Chemicals Inc., St.
212 Louis, Missouri, USA). The selection of 12 DPH to perform those incubations, was mainly based on mouth
213 opening and functionality, likely ensuring the incorporation of the substrates by ingestion. All larvae were
214 incubated in triplicates under similar water conditions as mentioned above and the radiolabelled substrates
215 were directly added to the medium. Each age group of larvae was also incubated ($n = 3$) without radiolabelled
216 substrate as a control group for larval lipid composition determination. After incubation, larvae were
217 thoroughly washed twice with water to remove radiolabelled substrate excess. Subsequently, euthanized larvae
218 were transferred to Eppendorf vials and stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

219 *Fig. 1. European eel larvae 0, 4, 8 and 12 days post hatch (DPH) illustrating developmental characteristics*
220 *from hatch to first-feeding stage (reared at 18 $^{\circ}\text{C}$ and 36 PSU).*



221

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

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

241 PS and PI fractions, were obtained by acid-catalysed transmethylation. FAME were purified by thin-layer
242 chromatography (TLC) (Christie, 2003), separated and quantified using a TRACE-GC Ultra gas
243 chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) equipped with an on-column
244 injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (30 m × 0.32 mm
245 I.D. × 0.25 µm; Sigma-Aldrich Co., St. Louis, Missouri, USA). Helium was used as carrier gas and temperature
246 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
247 min⁻¹ and, finally, to 230 °C at 40 °C min⁻¹. Identity of individual FAME was confirmed by GC–MS
248 chromatography (DSQ II, Thermo Fisher Scientific Inc.).

249 *2.7. Metabolism of radiolabelled substrates*

250 An aliquot of 0.1 mg of TL extracts from each [1-¹⁴C] incubated sample was transferred into scintillation
251 vials, to determine radioactivity incorporated into eel larvae. Radioactivity was quantified on an LKB Wallac
252 1214 RackBeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, USA) following
253 Reis et al. (2019). To determine the esterification pattern of each [1-¹⁴C] FA into the different LC and the
254 capacity of larvae to remodel the metabolic fate of [1-¹⁴C] ARA when bound to PC or PE, another aliquot of
255 0.1 mg of eel TL extract was applied to HPTLC plates and separated in discrete lipid bands as mentioned in
256 Reis et al. (2016a, 2019). The developed HPTLC plates were placed in closed exposure cassettes (Exposure
257 Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Image Screen-
258 K, BioRad) for 1 week. The screens were then scanned with an image acquisition system (Molecular Imager
259 FX, BioRad) and the bands quantified in percentage by an image analysis software (Quantity One, BioRad).
260 Finally, transformation of incubated [1-¹⁴C] FA substrates by desaturation/elongation processes were
261 determined using pre-coated TLC plates G-25 (20 cm × 20 cm; MachereyNagel GmbH & Co. KG) pre-
262 impregnated with a solution of 2 g silver nitrate in 20 mL of acetonitrile (Reis et al., 2019). Developed TLC
263 plates were then placed in BioRad closed exposure cassettes in contact with Image Screen-K for 2 weeks. The
264 screens were then scanned with a Molecular Imager FX and bands quantified by Quantity One software. The
265 identification of labelled bands was confirmed by radiolabelled standards run on the same plate (Rodríguez et
266 al., 2002).

267 *2.8. Statistical analysis*

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

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

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

280
 281 *Results represent means \pm SD;*
 282 *n = 3.*
 283 *Mean values with unlike superscript letters are significantly different ($p < 0.05$).*
 284

285 3. Results

286 3.1. Larvae lipid and FA composition

287 Average wet weight (w.w.) of eel larvae at 4–12 DPH ranged between 0.32 and 0.46 mg, with TL contents
 288 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
 289 100 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).
 290 Regardless of stage, eel larvae contained higher levels of neutral lipids (NL, 68.9–75.1%) than polar lipids
 291 (PL), with sterol esters (SE) being the most abundant LC (20.6–30.6%; Table 2). Assessment of the PL fraction
 292 profiles showed that PC and PE were the major lipid classes representing around 14 and 8%, respectively. A

293 decrease in SE and triacylglycerol (TAG) contents was observed during larval development, while relative
294 levels of cholesterol (CHO) increased significantly ($p < 0.05$; Table 2). At 8 DPH, there also seemed to be a
295 relative prominence of other structural lipids including a significant increment of PI ($p < 0.05$; Table 2).
296 Similarly, to the lipid content, total FA content of eel larvae decreased with age from $12.1 \pm 0.5 \mu\text{g larvae}^{-1}$
297 at 4 DPH to $4.4 \pm 0.0 \mu\text{g larvae}^{-1}$ at 12 DPH (Table 3). Larvae TL FA profile was mainly composed by
298 monounsaturated fatty acids (MUFA, 36.1–48.4%) primarily 18:1n-9, followed by saturated fatty acids (SFA,
299 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
300 (5.6–9.6%) was also observed with DHA and EPA being the main n-3 PUFA, and ARA representing between
301 44 and 61% of total n-6 PUFA. A relative decrease of MUFA (from $48.4 \pm 0.5\%$ at 4 DPH to $36.1 \pm 1.6\%$ at
302 12 DPH), together with a relative increase of SFA and n-6 LC-PUFA proportions were evident with age ($p <$
303 0.05 ; Table 3). Both ARA/EPA and DHA/EPA ratios reached the highest values at 12 DPH. Regarding the FA
304 profile of individual glycerophospholipids (Table 4), 18:1n-9 was also a relevant fatty acid together with 16:0,
305 particularly in PC, whereas 18:0 became the most abundant saturate in PS and PI. DHA was by far the most
306 prominent fatty acid by all lipid classes, with particular emphasis in PE. Interestingly, ARA was also a relevant
307 fatty acid not only in PI, but also in PC and PE (Table 4).

308 *3.2. Incorporation of radiolabelled substrates into larvae total lipids and its distribution among lipid classes*

309 $[1-^{14}\text{C}]$ ALA was incorporated to a higher extent into eel larval lipids at 12 DPH ($31.0 \pm 10.5 \text{ pmol mg prot}^{-1}$
310 h^{-1}) than at 4 and 8 DPH (8.8 ± 2.5 and $8.9 \pm 3.5 \text{ pmol mg prot}^{-1} \text{ h}^{-1}$, respectively) whereas incorporation
311 of $[1-^{14}\text{C}]$ LA remained unchanged with respect to the initial value (15.0 ± 5.0 and $18.9 \pm 0.9 \text{ pmol mg prot}^{-1}$
312 h^{-1} , for 4 and 12 DPH, respectively) (Table 5). At 12 DPH, not only ALA but also $[1-^{14}\text{C}]$ EPA presented
313 higher incorporation rates into larval tissues than both n-6 $[1-^{14}\text{C}]$ PUFAs (LA and ARA). The incorporation
314 of $[1-^{14}\text{C}]$ ARA, when bounded to both PC and PE (^{14}C -PC and ^{14}C -PE, respectively), was the lowest at 12
315 DPH (1.5 ± 0.2 and $2.5 \pm 0.7 \text{ pmol mg prot}^{-1} \text{ h}^{-1}$, respectively; $p < 0.05$) and much lower at this stage than
316 that displayed, when the incubation was performed as free fatty acid (Table 5). Independently of larval stage,
317 all $[1-^{14}\text{C}]$ FAs were highly esterified into TL (only 5.8–17.0% of radioactivity present as FFA) with PL (62.0–
318 83.1%), and more precisely PC (46.1–63.2%) and PE (6.6–10.7%) being the main metabolic target for
319 esterification (Table 6). $[1-^{14}\text{C}]$ ARA was the most incorporated substrate into phosphatidylinositol (PI) of 12
320 DPH larvae (9.2% vs 2.7–4.1% for the other $[1-^{14}\text{C}]$ FAs; $p < 0.05$). Regardless of stage, the esterification

321 pattern of all [^{14}C] FAs into NL was: Partial acylglycerols (PAG; monoacylglycerols + diacylglycerols) >
322 TAG \geq SE. Minor differences existed in the esterification of LA between developmental stages, whereas that
323 of ALA remained unchanged (Table 6). Important differences were observed in the esterification pattern of
324 [^{14}C] ARA, when this fatty acid was not provided to the larvae in its free form but bound to PC or PE. This
325 was especially evident at 4 DPH, while these differences were less prominent at 12 DPH (Table 7). For
326 instance, and regardless of stage, over 40% of [^{14}C] ARA was recovered into larval PC, when it was added
327 to the culture media bound to PC, whereas when incubated as PE, [^{14}C] ARA was more equitably distributed
328 into both PC and PE. Interestingly, PI was again a secondary target for the esterification of ARA (Table 7). In
329 spite of the high incorporation and esterification rates, no elongation/desaturation products were obtained from
330 incubated [^{14}C]FA. Only the incubation with [^{14}C]EPA displayed a reduced proportion of total
331 radioactivity ($4.5 \pm 0.6\%$) as an unknown but shorter product detected further above the [^{14}C]EPA band
332 (Table 8). The position of this product on the plate did not correspond to any known product, and its accurate
333 identification was not possible through the available standards.

334

335 **Table 3** Total fatty acid content ($\mu\text{g larvae}^{-1}$) and main fatty acid composition (% of total FA) of *Anguilla*
336 *anguilla* larvae at 4, 8 and 12 DPH.

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

337

338 Results represent means \pm SD; n = 3. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids;
339 PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; DMA,
340 dimethylacetals. LA, linoleic acid; ARA, arachidonic acid; ALA, alpha-linolenic acid; EPA,
341 eicosapentaenoic acid; DHA, docosahexaenoic acid. Σ include some minor components not shown. Mean
342 values with unlike superscript letters are significantly different ($p < 0.05$).

343 **Table 4** Main fatty acid composition (% of total FA) of individual phospholipids from *Anguilla anguilla*
344 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
DHA:EPA	2.97	7.44	7.34	9.62

345

346 $n = 1$; PC, Phosphatidylcholine; PS, Phosphatidylserine; PI, Phosphatidylinositol; PE,
 347 Phosphatidylethanolamine.nd, not detected.

348 ^a Mainly n-7 isomer.

349 ^b Mainly n-9 isomer.

350 **Table 5** Incorporation of radiolabelled substrates into total lipid (pmol mg prot⁻¹ h⁻¹) of *Anguilla anguilla*
 351 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

352

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

356 3.3. Transformation of radiolabelled fatty acids by elongation/desaturation processes

357 **Table 6** Esterification pattern (%) of [¹⁴C]FA substrates into the different lipid classes of *Anguilla*
 358 *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

359

360 Results represent means ± SD;

361 $n = 3$. Nd, not detected.

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

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

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

367

368 Table 7 Re-esterification pattern (%) of [1-¹⁴C]ARA into different lipid classes of *Anguilla anguilla* larvae
 369 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 [○]	57.4 ± 2.7	38.5 ± 6.1 ^{*Δ}	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 ^{*○Δ}	11.9 ± 0.1	15.7 ± 1.8 ^{*Δ}	5.0 ± 0.3●	8.0 ± 2.0 ^{*○}
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 ^{*○Δ}	86.0 ± 2.4	74.9 ± 5.5 ^{*Δ}	62.1 ± 15.9	55.7 ± 7.1 [○]
Partial Acylglycerols	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 ^{*○Δ}	14.0 ± 2.4	25.1 ± 5.5 ^{*○}	37.9 ± 15.9	44.3 ± 7.1Δ

370

371 Results represent means ± SD; $n = 3$.

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

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

376

377 Table 8 Distribution of recovered radioactivity (%) from [1-¹⁴C]FA substrates as FA metabolites in 12 DPH
 378 *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

379

380 Results represent means ± SD. UK, unknown product.

381

382 **4. Discussion**

383 Understanding the dietary requirements of lipids and FA in early eel larval stages is of fundamental importance
384 and a first step in the development of artificial diets. So far, no studies on the European or the Japanese eel
385 pre-leptocephalus larvae have determined requirements of macronutrients including lipids and FA. While pre-
386 leptocephalus larvae of the European eel have never been found in the wild, the natural diet of wild caught
387 leptocephalus larvae seems to be mainly composed of a variety of planktonic organisms with gelatinous
388 zooplankton being highly important (Riemann et al., 2010; Ayala et al., 2018). To date, the success in the
389 transformation of Japanese first-feeding eel larvae to glass eels has been obtained by use of shark-egg based
390 diets (Tanaka et al., 2003; Furuita et al., 2014). Eel eggs are characterized by large oil droplets and an unusually
391 high amount of lipids (Heinsbroek et al., 2013). At hatch, the yolk sac extends along the entire abdomen of the
392 larva and generally lasts up to 2 weeks, coinciding with the full development of a functional feeding apparatus
393 (Sørensen et al., 2016a). During the eel pre-leptocephalus stages examined here (until 12 DPH) an accentuated
394 progressive decrease in the TL content occurred, indicating the exhaustion of the yolk sac. The high amount
395 of NL (up to 75%) during this period, and the reduction of SE and TAG contents indicates the importance of
396 these lipid classes as energy reserves during early growth as well as their likely role as FA reservoir for the
397 processes of lipid synthesis in neural tissues, and digestive and osmoregulatory systems, which are under
398 development (Sargent et al., 1999). Cholesterol also represented a high percentage of eel larval TL, increasing
399 its preservation proportion up to 31% in 12 DPH larvae. Cholesterol is known to be fundamental during both
400 temperature and salinity changes, due to its decisive role in the lipid-bilayer fluidity fluctuations (Kamat and
401 Roy, 2016; Arashiki and Takakuwa, 2019; Biederman et al., 2019; Farhat et al., 2019). While migrating from
402 the continental shelves of Europe to oceanic spawning areas (Tesch and Rohlf, 2003), the European eel
403 undergoes drastic changes in salinity, temperature and feeding habitats with a surprisingly high adaptability
404 (Righton et al., 2016) suggesting lipid restructuring to preserve membrane structure and function, where
405 cholesterol levels play a pivotal role. An increase in cholesterol levels hardens the membrane by decreasing
406 fluidity (Simopoulos and Cleland, 2003). Among PL, preservation of phosphoglycerides such as PC, PE and
407 specifically PI seem to be also relevant during early development. Indeed, young fish contain abundant
408 phospholipids received during embryonic and larval development either from endogenous yolk sac or
409 exogenous lipids and are particularly important in fish larvae as necessary components for cellular bio-
410 membranes and organelles formation among other functions. Additionally PI is involved in a complex

411 signaling system controlling a wide range of biological processes such as, cytoskeleton regulation and motility
412 or regulation of intracellular membrane traffic in early development in vertebrates (Tocher et al., 2008). The
413 present study showed an accentuated decrease of all MUFA proportions with age, mainly 18:1n-9, indicating
414 a preferred oxidation of this FA as source for metabolic energy during the pre-leptocephalus stage.
415 Interestingly, eel larvae presented low n-3:n-6 ratios ranging between 1.2 (at 4 and 12 DPH) and 1.9 (at 8
416 DPH), similarly to values reported for freshwater fish species by Tocher (1995). The high n-6 content was
417 mainly due to a high amount of ARA (up to $7.5 \pm 0.8\%$ at 12 DPH), which is not usually found in such ranges
418 of abundance in strict stenohaline, marine species denoting the importance of this FA for eel larval
419 development. Interestingly, this fatty acid was highly esterified not only in PI as widely described for fish and
420 other vertebrates, but also in PC and PE (PC > PE > PI, Table 6). During the early nonfeeding pre-leptocephalus
421 stage, larval development is fully supported by the yolk (Sørensen et al., 2016a), denoting that the high ARA
422 content must have been exclusively provided by the parent broodstock. An adequate amount of dietary ARA
423 is known to improve marine fish larval survival and growth rates (Atalah et al., 2011), fish adaptive
424 physiological response to hypersalinity stress and hypo-osmoregulatory ability (Carrier III et al., 2011),
425 recovery from infections (Khozin-Goldberg et al., 2006) and also to improve fish reproductive performance
426 (Furuita et al., 2003; Norambuena et al., 2013). In this regard, ARA content and eicosanoids derivatives seem
427 also important in the reproductive success of the European eel. *A. anguilla* females seem to have the ability to
428 accumulate ARA into tissues, that can be later transferred to the ovaries during induced vitellogenesis (Støttrup
429 et al., 2013, 2016). Moreover, an increase in the ARA content of broodstock, induces a higher production of
430 fertilized eggs and a successful embryonic development and production of eel larvae (Støttrup et al., 2016;
431 Kottmann et al., 2020b). During *A. anguilla* catadromous migration to the Sargasso Sea area of the North
432 Atlantic Ocean, mature silver eels do not feed, thus energy and nutrients required for reproduction need to be
433 built in before this period. In captivity, as performed in the present study, wild caught female silver eels are
434 relocated and accustomed as broodstock for subsequent hormonal induction of ovarian development, and then
435 transferred to seawater, where the fish are no longer fed. Thus, modifications into eel broodstock lipid
436 composition should take place during the period prior to seawater and induction of vitellogenesis, being critical
437 to supply an adequate ARA dietary content during this stage. A strategy not used here, however, may involve
438 continuous feeding during seawater transfer until observation of a cease in voluntary feed intake to ensure

439 adequate FA tissue levels during vitellogenesis. The high DHA:EPA ratios (3.0–4.7) observed here are
440 common in marine fish eggs and larvae that have not yet started exogenous feeding and also denote the
441 relevance of DHA during early life stages (Sargent et al., 1999; Olsen et al., 2014). Interestingly, DHA is
442 provided by eel broodstock females which live for a long period (10–18 years) in a freshwater environment
443 (van Ginneken and Maes, 2005; Bruijs and Durif, 2009). In this sense, the high DHA content of European eel
444 broodstock tissues (Støttrup et al., 2013), eggs (Støttrup et al., 2016, Kottmann et al., 2020b) and larvae
445 (Kottmann et al., 2020b) suggest that this species may have the enzymatic battery to endogenously
446 biosynthesize EPA and DHA from ALA and in the same way of ARA from LA. *A. anguilla* glass eels have
447 shown the capacity to biosynthesize ARA after being force-fed with [1-14C]18:2n-6, nonetheless, seven days
448 after administration only 4% of radioactivity was recovered as ARA, showing a relatively slow conversion
449 rate (Kissil et al., 1987). Recent studies on the functional characterization of fatty acyl desaturases and
450 elongases in adults of Japanese eel (*Anguilla japonica*) revealed that this species has the complete enzymatic
451 repertoire required for the biosynthesis of LC-PUFA from C18 PUFA (Wang et al., 2014; Xu et al., 2020).
452 Interestingly, the LC-PUFA metabolism of this species presented neither a strict marine nor a freshwater
453 pattern; while its FA requirements can be satisfied by C18 PUFA (like freshwater species), a higher enzymatic
454 expression was detected in brain and eyes (similar to marine species), indicating the importance of LC-PUFA
455 in eel neural tissues (Wang et al., 2014; Xu et al., 2020). The incorporation of the radiolabelled FA substrates
456 by European eel larvae could be the result of the continuous intake of the surrounding incubation water within
457 an osmoregulatory process typical of marine fish. Even though images at 0 and 4 DPH (Fig. 1) indicate an
458 apparent lack of mouth formation and passage to the esophagus, studies on the Japanese eel larvae by use of
459 scanning electron microscopy and fluorescent dextran have revealed that the mouth appears as a slit with larvae
460 being capable of drinking already at the day of hatching (Ahn et al., 2015*). This suggests a similar capacity
461 for the European eel larvae examined here at 4 DPH. Although drinking or uptake through premature gills
462 cannot totally be ruled out, images at DPH 4 indicate lack of mouth formation and no passage to esophagus.
463 However, it cannot be completely ruled out, thus, that part of the lipid uptake observed is most likely explained
464 by a high permeability of the naked integument of the *A. anguilla*, similar to cephalopod species (Boucaud-
465 Camou and Roper, 1995; de Eguileor et al., 2000). Therefore, the experimental design developed in our study
466 helps to elucidate some aspects of lipid metabolism during the first stages of development. Thus, radiolabelled

467 substrates either as free FA, or bound to PC and PE, may also have entered the larvae via skin at 4 and at 8
468 DPH, encountering presumably a completely different set of enzymes for lipid metabolism to that of gut, while
469 at 12 DPH a major component of ingestion by intake of the surrounding water is the most likely. Authors,
470 however, consider it important to highlight the consistent results obtained here in comparison to previous
471 studies applying the present methodology (e.g. FA composition of the different phospholipids and in vivo free
472 FA esterification patterns; Reis et al., 2014, 2016a, 2016b, 2020). At 12 DPH, larvae did not show any elongase
473 or desaturase activity towards incubated substrates (ALA, LA, ARA and EPA). These results indicate the
474 absence or inactivity of elongase/desaturase enzymes during eel preleptocephalus stage, which might be related
475 to the high endowment of newly hatched eel larvae with ARA, EPA and DHA necessary for their proper
476 development. Moreover, it has been suggested that the natural diet of leptocephalus eel is mainly based on
477 gelatinous zooplankton (Hydrozoa, Thaliacea and Ctenophora) fed on phytoplankton, especially
478 photosynthetic, capable of synthesizing EPA, DHA and ARA very efficiently (Valenzuela et al., 1999). Eel
479 larvae, besides likely being carnivorous, are born in marine environment with high salinity, which has been
480 linked to the inactivation of desaturases enzymes especially in migratory species such as salmonids just after
481 smoltification and adaptation to the marine environment has been performed (Tocher, 2015). In any case, the
482 present data undoubtedly suggest that LC-PUFA (EPA, DHA and ARA) are essential FA not just during the
483 *A. anguilla* preleptocephalus stage, but also at the time prior to and during mouth opening, indicating these
484 FAs must be provided firstly through female broodstock and subsequently through diet at least during first-
485 feeding stage. The notably higher incorporation of n-3 FA (LA and EPA) over n6 FA (ALA and ARA) in 12
486 DPH larvae evidenced here, agrees well with what has been described for marine species (Sargent et al., 1999)
487 and must be also taken into account in dietary formulations for European eel larvae. Despite the absence of
488 elongase/desaturase enzymatic activity during the pre-leptocephalus stage, eel larvae do present the capacity
489 to re-modulate dietary phospholipids through de-acylation/re-acylation processes. Phospholipids catalytic
490 hydrolysis is under the control of phospholipase A1 and A2 enzymes (Tocher, 1995). The [1-14C]ARA
491 molecule esterified to phospholipids used for our experimental design was bound to the sn-2 position of PC
492 and PE molecules. Therefore, the radioactivity detected into new lipid classes such as PS, PI, PAG or TAG
493 evidences the presence of phospholipase A2 (PLA2) in larval tissues, which is generally believed to be the
494 active digestive enzyme that hydrolyses phospholipids at the sn-2 position (for details see Olsen et al., 2014).

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

518 **5. Conclusion**

519 The results of the present study showed that similarly to other marine organisms (Reis et al., 2014,
520 2016a, 2016b, 2017, 2020; Lund et al., 2019), direct incubation with ¹⁴C-labelled FA is a feasible method to
521 investigate in vivo FA and phospholipids metabolism during the preleptocephalus stage of the European eel.
522 As European pre-leptocephalus eel larvae have no desaturation/elongation capacity over PUFA, it is mandatory

523 to provide diets with LC-PUFA such as ARA, DHA and EPA, as these are essential FA for this species at first-
524 feeding stage. Although the capacity of eel larvae to metabolize TAG was not determined in the present study,
525 larvae possess the ability to metabolize phospholipids such as PC and PE. In this sense, and considering that
526 LC-PUFA esterified into phospholipids seems to improve its availability to larvae (Cahu et al., 2009), these
527 FA, and particularly DHA and ARA, should be provided through phospholipids rather than TAG for optimal
528 eel larval nutrition.

529 **Declaration of competing interest**

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

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538 **Author's contribution**

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

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