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Esterification and modification of [1-^{14}C] n-3 and n-6 polyunsaturated fatty acids in pikeperch (*Sander lucioperca*) larvae reared under linoleic or α-linolenic acid-based diets and variable environmental salinities.

D.B. Reis[a], J.A. Pérez[a,*], I. Lund[b], N.G. Acosta[a], B. Abdul-Jalbar[c], A. Bolaños[a], C. Rodríguez[a,*]

[a] Departamento de Biología Animal, Edafología y Geología, Universidad de La Laguna, 38206 La Laguna, Santa Cruz de Tenerife, Canary Islands, Spain
[b] Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Center, P.O. Box 101, DK-9850 Hirtshals, Denmark
[c] Departamento de Matemáticas, Estadística e Investigación Operativa. Universidad de La Laguna, 38206 La Laguna, Santa Cruz de Tenerife, Canary Islands, Spain

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ABSTRACT

To elucidate the *in vivo* endogenous ability of pikeperch (*Sander lucioperca*) larvae to deacylate and reacylate phospholipids and to elongate and desaturate PUFAs, 20 days post hatch (DPH) fish were incubated with either [1-^{14}C]20:4n-6 bound to PC and PE, or with free [1-^{14}C]-labelled fatty acids (18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3). The modulation capacity of both low LC-PUFAs but high 18C PUFAs precursors dietary supply and increasing salinity on larval fatty acid metabolic pathways was also investigated. [1-^{14}C]DHA was incorporated into larval tissues to a lower extent than [1-^{14}C]ARA or [1-^{14}C] EPA. [1-^{14}C]ARA was significantly less abundant in larval tissues when provided bound to PE than when esterified into PC, indicating that PC is a better phospholipid source to provide LC-PUFA to pikeperch larvae. Radioactivity was mainly recovered into phospholipids, especially that of the three LC-PUFAs ARA, EPA and DHA. All substrates were primarily incorporated into PC except [1-^{14}C]ARA which significantly did into PI. Both [1-^{14}C]EPA and [1-^{14}C]DHA showed a similar esterification pattern into lipid classes: PC > PE > PI > TAG, with [1-^{14}C]DHA presenting the highest esterification into PE of all radiolabelled compounds (26.3% *vs* 3.6–14.2%). Although higher rearing salinities tended to increase Δ6 desaturase activity, no radioactivity from [1-^{14}C]18:2n-6 or [1-^{14}C]18:3n-3 was detected in ARA or EPA, proving a deficiency of Δ5 activity and the inability of pikeperch to biosynthesize DHA. This work provides novel information on the lipid metabolism of pikeperch at early development necessary for the design of live prey enrichment protocols and dietary formulations adapted to larval metabolic capabilities.

1. Introduction

Fish are the primary source of healthy n-3 long chain polyunsaturated fatty acids (LC-PUFA) for humans, mainly eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Currently, changes to feed formulations especially of carnivorous species in the aquaculture industry are mandatory due to the rapidly outstripping of global supplies of fish meal and fish oil (FO) rich in these essential fatty acids (FA) (Pike, 2005; Tacon and Metian, 2008; Pérez et al., 2014; Turchini et al., 2019). Therefore, the impact that non marine ingredients have on the nutritional quality of aquafeeds has prompted great interest in the investigation of PUFA metabolism in farmed species in order to optimize the endogenous production and retention of n-3 LC-PUFA (Monroig et al., 2018). Sustainable alternatives to FO are vegetable oils (VO), rich in C18 PUFA, mainly linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3), but devoid of n-3 and n-6 LC-PUFA (Sargent et al., 2002; Turchini et al., 2011). The extent to which fish can convert C18 PUFA to C20-C22 LC-PUFA mainly EPA, DHA and arachidonic acid (ARA; 20:4n-6) is rather limited and varies among species (Garrido et al., 2019), with this capacity determining the level of dietary FO replacement without affecting fish survival, growth and health (Zheng et al., 2009).

The biosynthetic route to form LC-PUFA from their C18 precursors generally involves a series of enzymatic steps catalyzed by two fatty acyl desaturases (Δ-6 and Δ-5 desaturases), two FA elongases (Elovl5 and Elovl2) and a Δ-4 desaturation or a peroxisomal β-oxidation for FA chain shortening (Sprecher, 2000; Sargent et al., 2002; Li et al., 2010). All these enzymatic activities have been demonstrated in teleost fish species, but not all species display all activities (Garrido et al., 2019). Since freshwater fish species generally possess certain ability to

* Corresponding authors at: Departamento de Biología Animal, Edafología y Geología, Universidad de La Laguna, Avenida Astrofísico Francisco Sánchez s/n, 38206 La Laguna, Santa Cruz de Tenerife, Canary Islands, Spain.
E-mail address: covarodr@ull.edu.es (C. Rodríguez).

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biosynthesize LC-PUFA and most marine fish species studied so far do not have such capacity it has been hypothesized that trophic level and environmental salinity might directly drive diversification of teleost Fads2 (mainly Δ-6 and Δ-5 desaturases) (Morais et al., 2012, 2015; Monroig et al., 2018; Garrido et al., 2019). However, very recent results suggest that other factors such as the species' phylogeny seem to be even more influential to potentially biosynthesize LC-PUFA (Garrido et al., 2019).

Pikeperch (*Sander lucioperca*) is a freshwater fish species with strong potential for the diversification of European aquaculture (Dalsgaard et al., 2013; Steinberg et al., 2019). Despite being a freshwater species, pikeperch larvae resemble most marine carnivorous fish larvae with a dietary requirement for both phospholipids and LC-PUFA (Lund et al., 2018). In fact, pikeperch eggs contain a high proportion of DHA which could be related to its strictly carnivorous nature and/or to an evolutionary remnant from life adapted to a marine environment (Craig, 2000; Lund et al., 2014). Interestingly, detailed analysis of fillet FA profiles from wild (Jankowska et al., 2003) and captive (Kowalska et al., 2011) individuals revealed that pikeperch tend to increase the dietary proportions of DHA in their tissues. Similar results were obtained by Xu et al. (2001) in another percid fish, Eurasian perch (*Perca fluviatilis*), who found a high accumulation of DHA in muscle and liver compared to the feed used. This fact has led several authors to traditionally postulate that pikeperch is highly capable of transforming native forms of ALA into LC-PUFA, resulting in flesh containing high DHA (Xu et al., 2001; Xu and Kestemont, 2002; Jankowska et al., 2003). Nonetheless, recent studies have shown that diets low in LC-PUFAs, particularly DHA, during first feeding of pikeperch (*i.e.* within 25 days post hatch, DPH), may lead to a suite of negative consequences in its culture, including increased mortality, shock syndromes and short and long-term stress sensitivity and deficiency in neural development, which may affect swimming behavior and learning (Lund and Steenfeldt, 2011; Lund et al., 2014, 2018). This suggests that pikeperch may have very low or no elongation/desaturation capacity in contrast to other omnivorous or carnivorous freshwater fish species.

Some fish species enhance LC-PUFA synthesis when maintained on a diet devoid of n-3 LC-PUFA (Buzzi et al., 1996; Morais et al., 2012, 2015), or under specific salinities (Li et al., 2008; Fonseca-Madrigal et al., 2012). In this sense, nutritionists are targeting to boost the elongation/desaturation capacity of fish through these and other modulatory factors to optimize the effective use of alternative sources to marine ingredients while maintaining the nutritional quality of farmed fish. Despite farming of pikeperch takes place in freshwater, this species shows high osmotic tolerance and hypo-osmoregulatory capacity in a saline environment, which may suggest a growth potential for rearing in low saline waters or in water isoosmotic to plasma (Lund et al., 2019). On the other hand, partial dietary replacement of FO by VO has yielded good results in pikeperch fingerlings survival and growth (Schulz et al., 2005; Molnár et al., 2006). However, little is known about the responses of their FA metabolism pathways under variable dietary and environmental scenarios.

Phospholipids are considered key factors during the early development of fish species (Li et al., 2008; Tocher et al., 2008; Cahu et al., 2009; Olsen et al., 2014; Feng et al., 2017). LC-PUFAs are selectively esterified into cell membrane glycerophospholipids (GPs) contributing to the modulation of signaling pathways (Crowder et al., 2017), membrane stability and fluidity, and, therefore, cell functions (Tocher, 2003; Fernandez and West, 2005; Yaqoob and Calder, 2007). Several studies suggest that the requirements for GPs during the larval stage might be related to the limited capacity to *de novo* synthesize GPs (Tocher et al., 2008; Daprà et al., 2011; Feng et al., 2017).

We hypothesize here that pikeperch, a freshwater fish species with consequently less availability to dietary LC-PUFAs, may have some enzymatic activity and bioconversion capacity to produce LC-PUFA particularly if fed live food enriched with no FO and exposed to an adequate environmental salinity. Thus, the present study investigates the *in vivo* endogenous ability of *S. lucioperca* larvae to metabolise a range of [1-^{14}C] n-3 and n-6 PUFAs, and the influence of diets limited in LC-PUFAs but rich in their shorter chain precursors, and salinity, on these metabolic pathways. To this aim, the ability of pikeperch larvae to incorporate, esterify into lipid classes and convert free PUFAs into longer and more unsaturated compounds is evaluated. Finally, we also study the capacity of this species to remodel dietary phospholipid by deacylation and reacylation of [1-^{14}C] ARA presented as either a free molecule or bound to phosphatidylcholine (PC) or phosphatidylethanolamine (PE).

2. Material and methods

2.1. Experimental set up

Fertilized eggs of pikeperch close to hatching were obtained from Aquapri Innovation, Egtved (Denmark) and transferred to DTU Aqua at the North Sea Research Centre. Incubation of eggs and larval rearing until 20 DPH was performed as described by Lund et al. (2019). Shortly, the experiment was carried out in flow through system in a triplicate set-up of conical transparent 46 L-tanks per feed type at 0 ppt salinity for the control larvae, and at 3 different salinities: 0 ppt; 5–6 ppt; 10–11 ppt for larvae fed the experimental diets, equalling a total of 21 tanks. Each tank was stocked with 43 larvae/L and reared at 24 h dimmed light conditions (*i.e.* 30–40 Lux at water surface) at 17.5 ± 0.6 °C. From 4 to 9 DPH, larvae were fed unenriched *Artemia* (strain MC460), and from 10 DPH, 24 h EG *Artemia* nauplii (both *Artemia* strains from Artemia Systems INVE, Dendermonde, Belgium) enriched with emulsions based on either sunflower oil (SFO) or linseed oil (LO) to differ in their composition of ALA and LA. Each lipid emulsion was supplemented with olive oil for obtaining similar dietary levels of oleic acid (18:1n-9, OA) (Croda Chemicals Europe, Snaith, UK) (Lund et al., 2019). Control larvae were fed EG *Artemia* enriched with a commercial DHA Selco (Artemia Systems INVE). Main fatty acid composition of enriched *Artemia* is presented in Table 1.

All experimental procedures were approved by the ethical

Table 1
Total fatty acid content (TFA, μg g dry matter^{-1}) and main fatty acid composition (% of total FA) of *Artemia* enriched with a commercial DHA Selco as a control, or with sunflower oil (SFO) and linseed oil (LO) experimental emulsions.

	DHA Selco		SFO		LO	
TFA	239 ± 122		400 ± 173		247 ± 107	
16:0	10.8 ± 0.5		9.7 ± 0.2		9.5 ± 0.4	
Σ SFA	18.9 ± 1.5		17.7 ± 0.7		17.5 ± 0.7	
18:1n-9 OA	25.4 ± 0.1		25.3 ± 0.1		25.9 ± 0.5	
Σ MUFA	31.7 ± 1.4		27.7 ± 0.3		28.4 ± 0.8	
18:2n-6 LA	9.5 ± 0.1	b	18.3 ± 0.0	a	8.1 ± 0.2	c
20:2n-6	nd		nd		nd	
20:4n-6 ARA	1.2 ± 0.4		0.5 ± 0.0		0.6 ± 0.1	
Σ n-6 PUFA	11.6 ± 1.0	b	19.5 ± 0.4	a	9.3 ± 0.5	c
18:3n-3 ALA	24.6 ± 1.0	c	32.0 ± 0.8	b	41.5 ± 0.6	a
20:3n-3	1.3 ± 0.1		1.4 ± 0.0		1.6 ± 0.1	
20:5n-3 EPA	5.5 ± 0.1	a	1.7 ± 0.2	b	1.6 ± 0.2	b
22:6n-3 DHA	6.5 ± 0.1	a	nd	b	nd	b
Σ n-3 PUFA	37.9 ± 1.4	b	35.1 ± 1.0	b	44.8 ± 0.9	a
n-3/n-6	3.3 ± 0.2	b	1.8 ± 0.3	c	4.8 ± 0.4	a
DHA/EPA	1.2 ± 0.0	a	0.0 ± 0.0	b	0.0 ± 0.0	b

Results represent means ± SD; $n = 2$. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; OA, oleic acid; LA, linoleic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Σ, include some minor components not shown. nd, not detected. Mean values with unlike letters are significantly different ($P < .05$).

committee at DTU Aqua and were in accordance with the EU Directive 2010/63/EU regarding the protection and humane use of animals for scientific purpose of the European Parliament and Council of the European Union.

2.2. In vivo incubation with labelled [1-^{14}C] substrates

At 20 DPH a total of 1440 pikeperch larvae (12 h of starvation) were sampled from the different rearing conditions (SFO and LO dietary groups; 0, 5 and 10 ppt salinity); in addition to 240 larvae from the control group receiving *Artemia* enriched with DHA Selco and maintained at 0 ppt salinity. Fish were then distributed and incubated in flat-bottom 6-wells tissue culture plates (Sarstedt AG & Co., Nümbrecht, Germany) containing 10 mL of the same water as provided in the different experimental larval rearing conditions (17.5 ± 0.6 °C and 0, 5, or 10 ppt salinity). Incubations were performed in triplicate ($n = 3$) for 5 h in a temperature-controlled laboratory at 18 °C, at a density of 10 larvae per well, with gentle stirring and 0.2 μCi (0.3 μM) of [1-^{14}C] labelled fatty acids (free FA molecule, labelled with ^{14}C in its first carbon from the carboxyl head), including either 18:2n-6 (LA), 18:3n-3 (ALA) (PerkinElmer, Inc., Waltham, Massachusetts, USA), 20:4n-6 (ARA), 20:5n-3 (EPA) or 22:6n-3 (DHA) (American Radiolabelled Chemicals, Inc., St. Louis, Missouri, USA). Additionally, [1-^{14}C]ARA (used as a commercially available essential FA) esterified in the sn-2 position of phosphatidylcholine (PC; L-α-palmitoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine) or phosphatidylethanolamine (PE; L-α-palmitoyl-2-[1-^{14}C]arachidonyl-phosphatidylethanolamine) (American Radiolabelled Chemicals, Inc.) were also used as substrates. Labelled substrates, dissolved in ethanol, were individually added to separate wells. For each dietary and salinity scenario, triplicate samples of 10 larvae were also incubated for 5 h without the addition of [1-^{14}C] substrate to assess their survival and lipid classes (LC) and FA composition. As a result, a total of 168 incubations (10 larvae each) were performed. A survival rate of 100% was obtained in all incubations. After incubation, pikeperch larvae were sedated with benzocaine, thoroughly washed to remove excess of radiolabelled compounds, and immediately frozen at −80 °C.

2.3. Lipid and fatty acid composition

The larval total lipids (TL) were obtained by sample homogenization of each 10-larvae set with chloroform/methanol (2:1, *v/v*) according to Folch et al. (1957). The organic solvent was evaporated under a stream of nitrogen and the lipid content determined gravimetrically. TL extracts were stored at −20 °C in chloroform/methanol (2:1, *v/v*) containing 0.01% butylated hydroxytoluene (BHT) under an inert atmosphere of nitrogen until further analysis.

TL extracts of pikeperch larvae from the different rearing conditions (control group; SFO and LO dietary groups and 0, 5 and 10 ppt salinity), incubated without radiolabelled substrate were used to determine LC and FA composition ($n = 3$). A 30 μg-aliquot of TL was used to separate LC by high-performance thin-layer chromatography (HPTLC, Merck KGaA, Darmstadt, Germany) in a single-dimensional double-development using 1-propanol/chloroform/methyl acetate/methanol/0.25% KCl (5:5:5:2:1.8, *v/v*) as developing solvent for polar lipid classes, and hexane/diethyl ether/acetic acid (22.5:2.5:0.25, *v/v*) for the neutral fractions. Lipid classes: Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), monoacylglycerols + diacylglycerols (PAG), cholesterol (CHO), free fatty acids (FFA), triacylglycerols (TAG) and sterol esters (SE) were visualized by charring at 160 °C after spraying with 3% (*w/v*) aqueous cupric acetate containing 8% (*v/v*) phosphoric acid and quantified by means of a CAMAG TLC Visualizer (Camag, Muttenz, Switzerland).

Fatty acid methyl esters (FAME) were obtained from all incubations by acid-catalyzed transmethylation of 1 mg of TL extract and purified by thin-layer chromatography (Christie, 2003). For un-radiolabelled larvae, the FAME were separated and quantified by means of 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 (Sigma-Aldrich Co., St. Louis, Missouri, USA) under chromatographic conditions described by Reis et al. (2019). When necessary, identification of individual FAME was confirmed by GC–MS chromatography (DSQ II, Thermo Fisher Scientific Inc.).

2.4. Total incorporation, esterification into lipid classes and transformation of radiolabelled substrates by elongation/desaturation

The incorporation of radiolabelled FAs into TL under the different dietary and salinity regimes was determined as described by Lund et al. (2019). Briefly, an aliquot of 0.1 mg of corresponding TL extracts from each sample was transferred to a scintillation vial and radioactivity determined in a LKB Wallac 1214 Rackbeta liquid scintillation ß-counter (PerkinElmer Inc., Waltham, Massachusetts, USA). Results in dpm were transformed into pmol mg protein^{-1} h^{-1} taking into account the specific activity of each substrate, and sample TL and protein contents (Lowry et al., 1951).

To determine the direct esterification pattern of incorporated [1-^{14}C] FFA into pikeperch larval LC and fish capacity to deacylate and reacylate into their LC the [1-^{14}C]ARA initially bound to PC or PE, a second 0.1 mg-aliquot of all TL extracts was applied to HPTLC plates and separated as described in section 2.3. Developed HPTLC plates were placed for 1 week in closed exposure cassettes (Exposure Cassette-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Imagen Screen-K, BioRad). The screens were then scanned with a Molecular Imager FX image acquisition system (BioRad), and the bands were quantified in percentage by Quantity One image analysis software (BioRad) (Reis et al., 2019).

Transformation of incubated [1-^{14}C]FA by desaturation/elongation processes was determined using pre-coated TLC plates G-25 (20 × 20 cm; Macherey-Nagel GmbH & Co. KG, Düren, Germany), pre-impregnated with a solution of 2 g of silver nitrate in 20 mL acetonitrile (Reis et al., 2019). Radioactive FAME were applied and separated in these TLC plates using toluene/acetonitrile (95:5, *v/v*) as solvents, which resolve the FAME into discrete bands based on both degree of unsaturation and chain length. Bands were then revealed and quantified as mentioned for radioactive LC. Identification of labelled bands was confirmed by radiolabelled standards simultaneously run on the same plate (Reis et al., 2019).

2.5. Statistical analysis

Differences in *Artemia* FA composition between dietary groups were assessed by one-way ANOVA followed by the Tukey HSD *post hoc* test. A two-way ANOVA was used to determine the interaction between fixed factors, diet and salinity, in larval TL content, LC and FA composition. This was also the statistical treatment performed to data of total incorporation of radioactivity and transformation, by elongation and desaturation steps, of radiolabelled substrates incorporated into larval lipids. Additionally, a one-way ANOVA and Tukey's HSD test were used to determine any significant differences between dietary treatments at 0 ppt (Control DHA Selco, SFO and LO). Prior to analysis, normality and homogeneity of data were confirmed within groups and, where necessary, appropriate variance stabilizing transformations were performed.

Data of the average incorporation proportion of the 5 radiolabelled free fatty acids (LA, ALA, ARA, EPA and DHA) into the different lipid fractions (PC, PS, PI, PG, PE, PAG, FFA and TAG) and under the seven different experimental conditions (DHA Selco-control at 0 ppt, and SFO or LO diet at 0, 5 or 10 ppt) were included in a principal component analysis (PCA) model. Factor scores were subsequently used in a hierarchical and in a K-means cluster analysis to identify which

combinations of diet, salinity and substrate displayed similar average incorporation percentages into the different lipid classes. The hierarchical cluster analysis was performed with the Ward linkage method and the squared Euclidean distances.

Results are presented as mean ± standard deviation (SD) and the statistical significance was established at $P < .05$. All statistical analyses were performed using IBM® SPSS Statistics 25.0 software package (IBM Corp., New York, USA) for Windows.

3. Results

No significant differences in larval growth and survival at 30 DPH were found among experimental groups (for details, see Lund et al., 2019).

3.1. Lipid content and lipid class composition of non-radioactive larvae

The total lipid content of 20 DPH pikeperch larvae remained stable in all experimental groups ranging between 245 and 315 μg lipid mg larvae^{-1}. Independently of diet or rearing salinity, larvae contained higher levels of neutral lipids than polar lipids (PL) (60 *vs* 40%, respectively), with triacylglycerols (TAG) being the most abundant LC (20.0–24.5%; Table 2). Within phospholipids, PC and PE were the major lipid fractions representing around 18 and 11–12%, respectively (Table 2). Neither diet nor salinity caused a significant effect on 20 DPH pikeperch larval LC main composition, with the exception of monoacylglycerols, which presented the lowest proportions in larvae reared at 10 ppt salinity receiving the LO diet (Table 2).

3.2. Fatty acid composition of non-radioactive larvae

Rearing salinity did not exert any significant effect on the FA profile of 20 DPH pikeperch larvae (Table 3). However, as expected from the *Artemia* fatty acid profiles (Table 1) fish fed the SFO diet presented higher levels of 18:2n-6 (near 2-fold that of the LO-group), 18:3n-6, 20:2n-6, 20:3n-6 and, consequently, total n-6 PUFA ($P < .05$). Lower amounts of 18:1, total MUFA, 18:3n-3, total n-3 PUFA and n-3/n-6 ratios were also present in the SFO-fish compared to those of the LO-larvae ($P < .05$) (Table 3). When comparing the FA composition of the three groups of larvae reared at 0 ppt, those receiving the LO diet contained similar levels of total n-6 PUFA than the control fish (DHA Selco, 0 ppt), but lower than those of the SFO-group, which in turn, presented the lowest n-3/n-6 ratio. Moreover, the control fish had intermediate proportions of 18:3n-3, the highest proportion of 20:5n-3, 22:5n-3 and total n-6 LC-PUFA (ARA and 22:5n-6), and the lowest proportion ARA/EPA. DHA contents remained stable among the three groups of fish, independently of its dietary supply (Table 3).

3.3. Incorporation of [1-14C]ARA into larval tissues

The average incorporation rate of [1-^{14}C]ARA into larval lipids of a total of 7 different combinations of diet and salinity by triplicate (n = 21) was lower when it was provided esterified to PE than when provided as FFA or bound to PC (3.6 ± 2.1, 17.0 ± 6.0 and 15.3 ± 11.1 pmol mg prot^{-1} h^{-1}, respectively) ($P < .05$).

Regardless of dietary treatment (DHA Selco, SFO or LO) the incorporation of [1-^{14}C]ARA remained unchanged in the three groups of larvae reared at 0 ppt, with values of 18.7 ± 3.9, 21.6 ± 3.2 and 22.4 ± 6.8 pmol mg prot^{-1} h^{-1}, respectively (Table 4). Additionally, no effect of diet or rearing salinity was evident in the re-esterification pattern of [1-^{14}C]ARA when incubated bound to PC. However, when incubated as FFA, [1-14C]ARA was incorporated to a higher extent at 0 ppt than at 5 and 10 ppt, whereas when bound to PE the incorporation rate was maximum at 5 ppt (Table 4).

3.4. Influence of dietary limitation of LC-PUFA and salinity on the esterification pattern of radiolabelled substrates into larval lipid classes

No major effects of dietary LC-PUFA limitation and increasing salinity were registered in the esterification pattern of radiolabelled substrates into larval lipids (Fig. 1). Therefore, results of larvae fed *Artemia* enriched with DHA Selco at 0 ppt salinity (control larvae) are described as a reference below.

Both [1-^{14}C]LA and [1-^{14}C]ALA were esterified to a lower extent than [1-^{14}C]C20-C22 LC-PUFAs into larval body lipids (approximately 14% recovered as FFA compared to 3–5%, respectively) ($P < .05$) (Table 5). In addition, the fish preferentially incorporated [1-^{14}C] free substrates into PL, especially the three LC-PUFAs: ARA, EPA and DHA (83–84%), than the C18 PUFAs (53 and 59%) ($P < .05$) (Table 5). All substrates were primarily re-esterified into PC (mainly [1-^{14}C]EPA,

Table 2
Total lipid content (μg lipid mg larvae^{-1}) and main lipid class composition (% total lipid) of 20 DPH pikeperch larvae fed *Artemia* enriched with DHA Selco (control, 0 ppt), or sunflower oil (SFO) and linseed oil (LO) at three different salinities (0, 5 and 10 ppt).

Diet	DHA Selco	SFO			LO			Two-way ANOVA*			
Salinity (‰)	0	0	5	10	0	5	10	Diet	Salinity		
								SFO *vs* LO	0	5	10
TL content	270.6 ± 25.8	270.5 ± 12.0	243.9 ± 59.0	316.8 ± 40.3	312.7 ± 58.0	274.7 ± 54.6	280.9 ± 21.6	NS		NS	
Lipid class											
Phosphatidylcholine	17.7 ± 0.9	18.6 ± 0.8	17.9 ± 1.0	17.9 ± 0.6	17.7 ± 2.6	17.5 ± 0.7	17.6 ± 0.3	NS		NS	
Phosphatidylserine	3.8 ± 0.4	4.5 ± 0.7	4.1 ± 0.4	4.1 ± 0.5	4.1 ± 0.2	3.9 ± 0.5	4.2 ± 0.4	NS		NS	
Phosphatidylinositol	3.4 ± 0.2	3.4 ± 0.6	3.3 ± 0.3	3.5 ± 0.5	3.1 ± 0.8	3.4 ± 0.2	3.4 ± 0.7	NS		NS	
Phosphatidylglycerol	1.9 ± 0.2	2.1 ± 0.2	2.0 ± 0.2	2.1 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	2.2 ± 0.2	NS		NS	
Phosphatidylethanolamine	11.3 ± 0.2	12.2 ± 0.7	11.4 ± 1.1	11.6 ± 0.6	11.2 ± 0.8	12.0 ± 0.4	11.3 ± 0.5	NS		NS	
Σ Polar Lipids	**39.2 ± 1.9**	**42.7 ± 2.3**	**40.3 ± 2.9**	**40.7 ± 0.9**	**39.6 ± 4.5**	**40.5 ± 2.0**	**40.0 ± 2.0**	NS		NS	
Monoacylglycerols	3.8 ± 0.9	5.4 ± 0.7	4.4 ± 1.0	4.1 ± 0.2	4.7 ± 0.3	3.7 ± 0.4	2.9 ± 0.9	0.016	x	xy	y
Diacylglycerols	2.0 ± 0.1	1.9 ± 0.2	1.8 ± 0.1	2.1 ± 0.1	1.5 ± 0.6	2.0 ± 0.0	1.8 ± 0.2	NS		NS	
Cholesterol	14.2 ± 0.6	15.6 ± 1.5	14.2 ± 1.0	14.5 ± 0.8	14.9 ± 0.9	14.4 ± 0.5	14.2 ± 0.4	NS		NS	
Free fatty acids	3.5 ± 0.5	4.3 ± 0.6	4.0 ± 0.4	3.7 ± 0.6	3.6 ± 0.1	4.1 ± 0.3	4.2 ± 0.5	NS		NS	
Triacylglycerols	24.6 ± 1.4	20.8 ± 4.3	20.0 ± 2.7	20.6 ± 2.7	23.5 ± 3.1	21.4 ± 4.5	23.3 ± 3.2	NS		NS	
Sterol esters	12.5 ± 1.4^{a}	8.0 ± 0.7^{b}	14.3 ± 4.3	13.4 ± 4.1	11.0 ± 1.7^{ab}	12.9 ± 5.1	12.5 ± 3.9	NS		NS	
Σ Neutral Lipids	**60.8 ± 1.9**	**57.3 ± 2.3**	**59.7 ± 2.9**	**59.3 ± 0.9**	**60.4 ± 4.5**	**59.5 ± 2.0**	**60.0 ± 2.0**	NS		NS	

Results represent means ± SD; $n = 3$. Σ, include some minor components not shown. Mean values with unlike superscript letters are significantly different between dietary treatments at 0‰ (a, b) ($P < .05$). * Interaction between dietary treatment and salinity was not significant for any lipid class. Lipid classes with a significant effect of diet or salinity and no interaction are indicated with the *P* value or unlike letters (x,y), respectively ($P < .05$). NS, not significant.

Table 3
Main fatty acid composition (% of total FA) of 20 DPH pikeperch larvae fed *Artemia* enriched with DHA Selco (control, 0 ppt), or sunflower oil (SFO) and linseed oil (LO) at three different salinities (0, 5 and 10 ppt).

Diet	DHA Selco	SFO			LO			Two-way ANOVA*	
Salinity (‰)	0	0	5	10	0	5	10	Diet	Salinity
								SFO *vs* LO	0 5 10
16:0	13.6 ± 0.5	13.8 ± 0.5	13.8 ± 0.3	13.9 ± 0.9	13.6 ± 1.8	13.6 ± 0.5	13.9 ± 0.3	NS	NS
18:0	7.7 ± 0.2	8.2 ± 0.2	8.0 ± 0.0	8.3 ± 0.3	8.1 ± 0.8	8.1 ± 0.3	8.3 ± 0.4	NS	NS
Σ SFA	22.7 ± 0.9	23.2 ± 0.8	23.0 ± 0.3	23.3 ± 1.3	23.0 ± 2.6	22.8 ± 1.2	23.3 ± 0.6	NS	NS
16:1	3.1 ± 0.2	2.5 ± 0.6	2.8 ± 0.6	2.6 ± 0.3	3.2 ± 0.6	2.8 ± 0.1	2.9 ± 0.3	NS	NS
18:1	24.5 ± 0.4	24.6 ± 1.0	25.4 ± 0.9	25.2 ± 0.7	27.1 ± 2.1	26.4 ± 0.9	27.0 ± 1.2	0.015	NS
Σ MUFA	29.0 ± 0.5	28.3 ± 1.5	29.4 ± 1.6	28.9 ± 1.1	31.7 ± 2.4	30.3 ± 0.9	31.2 ± 1.7	0.013	NS
18:2n-6 LA	6.1 ± 0.0^{b}	12.8 ± 1.4^{a}	12.7 ± 0.8	12.6 ± 1.2	6.9 ± 0.5^{b}	7.0 ± 0.4	6.9 ± 0.4	0.000	NS
18:3n-6	0.4 ± 0.0^{ab}	0.5 ± 0.1^{a}	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.0^{b}	0.4 ± 0.1	0.4 ± 0.1	0.003	NS
20:2n-6	0.4 ± 0.0^{b}	0.4 ± 0.0^{a}	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.0^{b}	0.3 ± 0.0	0.3 ± 0.0	0.000	NS
20:3n-6	0.2 ± 0.1^{b}	0.4 ± 0.0^{a}	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0^{ab}	0.4 ± 0.0	0.4 ± 0.0	0.026	NS
20:4n-6 ARA	1.6 ± 0.0	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.3	1.2 ± 0.1	1.1 ± 0.0	NS	NS
22:5n-6	0.6 ± 0.0^{a}	0.1 ± 0.2^{b}	0.1 ± 0.2	0.3 ± 0.0	0.2 ± 0.2^{b}	0.3 ± 0.2	0.1 ± 0.2	NS	NS
Σ n-6 PUFA	9.3 ± 0.2^{b}	15.6 ± 1.5^{a}	15.4 ± 1.2	15.5 ± 1.4	9.3 ± 0.7^{b}	9.6 ± 0.4	9.2 ± 0.3	0.000	NS
Σ n-6 LC-PUFA	2.2 ± 0.0^{a}	1.4 ± 0.4^{b}	1.3 ± 0.3	1.5 ± 0.1	1.5 ± 0.2^{b}	1.4 ± 0.3	1.2 ± 0.2	NS	NS
18:3n-3 ALA	12.4 ± 0.9^{ab}	11.0 ± 2.0^{b}	11.2 ± 0.9	11.1 ± 1.4	15.5 ± 1.7^{a}	15.5 ± 1.3	15.6 ± 1.9	0.000	NS
18:4n-3	2.6 ± 0.3	2.2 ± 0.4	2.3 ± 0.2	2.3 ± 0.4	2.0 ± 0.7	2.8 ± 0.3	2.7 ± 0.4	NS	NS
20:3n-3	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	NS	NS
20:4n-3	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.3	1.4 ± 0.1	1.3 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	NS	NS
20:5n-3 EPA	5.3 ± 0.0^{a}	2.7 ± 0.3^{b}	2.6 ± 0.2	2.6 ± 0.1	2.7 ± 0.8^{b}	2.7 ± 0.2	2.6 ± 0.1	NS	NS
22:5n-3	1.9 ± 0.1^{a}	1.2 ± 0.2^{b}	1.3 ± 0.2	1.4 ± 0.0	1.3 ± 0.4^{b}	1.3 ± 0.1	1.3 ± 0.1	NS	NS
22:6n-3 DHA	10.4 ± 0.1	9.3 ± 0.3	7.9 ± 1.1	8.0 ± 1.9	8.4 ± 2.1	7.7 ± 2.8	6.4 ± 2.0	NS	NS
Σ n-3 PUFA	35.0 ± 1.2^{a}	28.8 ± 0.2^{b}	27.8 ± 0.6	27.9 ± 1.3	32.3 ± 4.5^{ab}	32.9 ± 2.0	31.7 ± 1.3	0.002	NS
Σ n-3 LC-PUFA	19.9 ± 0.2	15.6 ± 2.5	14.3 ± 0.6	14.5 ± 1.8	14.7 ± 3.2	14.6 ± 2.9	13.3 ± 1.9	NS	NS
Σ PUFA	44.2 ± 1.3	44.4 ± 1.5	43.3 ± 1.8	43.4 ± 2.4	41.6 ± 5.2	42.5 ± 2.4	40.9 ± 1.4	NS	NS
n-3/n-6	3.8 ± 0.1^{a}	1.9 ± 0.2^{b}	1.8 ± 0.1	1.8 ± 0.1	3.5 ± 0.3^{a}	3.4 ± 0.1	3.4 ± 0.2	0.000	NS
ARA/EPA	0.3 ± 0.0^{b}	0.5 ± 0.0^{a}	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0^{a}	0.4 ± 0.0	0.4 ± 0.0	NS	NS
DHA/EPA	2.0 ± 0.0^{b}	3.5 ± 0.5^{a}	3.1 ± 0.5	3.1 ± 0.6	3.2 ± 0.2^{a}	2.9 ± 0.8	2.4 ± 0.7	NS	NS

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; LA, linoleic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Σ, include some minor components not shown. Mean values with unlike superscript letters are significantly different between dietary treatments at 0‰ (a, b) ($P < .05$). * Interaction between dietary treatment and salinity was not significant for any fatty acid. Fatty acids with a significant effect of diet and no interaction are indicated with the *P* value ($P < .05$). NS, not significant.

Table 4
Incorporation of [1-^{14}C]ARA (pmol mg prot^{-1} h^{-1}) provided as free fatty acid (FFA-ARA) or bound to PC (ARA-PC) or to PE (ARA-PE) into total lipids of 20 DPH pikeperch larvae fed *Artemia* enriched with DHA Selco (control, 0 ppt), or sunflower oil (SFO) and linseed oil (LO) at three different salinities (0, 5 and 10 ppt).

Diet	DHA Selco	SFO			LO			Two-way ANOVA*			
Salinity (‰)	0	0	5	10	0	5	10	Diet	Salinity		
								SFO *vs* LO	0	5	10
FFA-ARA	18.7 ± 3.9	21.6 ± 3.2	10.2 ± 1.1	14.7 ± 6.5	22.4 ± 6.8	16.7 ± 4.0	12.2 ± 5.7	NS	x	y	y
ARA-PC	9.9 ± 1.4	9.7 ± 2.2	31.6 ± 20.4	9.9 ± 3.5	12.6 ± 3.7	19.1 ± 4.8	12.9 ± 6.7	NS		NS	
ARA-PE	2.7 ± 1.0	2.6 ± 0.6	6.1 ± 2.5	1.7 ± 0.8	3.6 ± 2.3	5.6 ± 1.9	2.9 ± 1.2	NS	y	x	y

Results represent means ± SD; n = 3. FFA, free fatty acid; ARA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine. * Interaction between dietary treatment and salinity was not significant for any variable ($P < .05$). Variables with a significant effect of salinity and no interaction are indicated with unlike letters (x,y). NS, not significant.

54.5 ± 0.4%, $P < .05$), with the exception of [1-^{14}C]ARA which did into PI (36.1 ± 2.3%; Table 5). Conversely, C18 PUFAs were incorporated into larval neutral lipids, mainly TAG, to a higher extent than LC-PUFAs (15–27% *vs* < 8.0%, respectively) ($P < .05$). Both [1-^{14}C]EPA and [1-^{14}C]DHA showed a similar esterification pattern into lipid classes: PC > PE > PI > TAG, with [1-^{14}C]DHA presenting the highest incorporation into PE of all compounds assayed (Table 5).

PCA of average radioactivity recovered in the lipid classes of larvae from a total of 35 different combinations of diet, salinity and free radiolabelled substrate revealed three components that had eigenvalues greater than 1 and together accounted for more than 84% of the total variance. Thus, PC1 was positively related with PE ($r = 0.943$) and PS ($r = 0.874$) and accounted for 47% of the variance. PC2, which explained 25% of the variance, had high positive loadings for PG ($r = 0.902$) and PAG ($r = 0.695$) and negative loadings for TAG ($r = -0.640$) and FFA ($r = -0.626$). Finally, PC3 accounted for 12% of the variance and was negatively correlated with PI ($r = -0.901$) and positively with PC ($r = 0.950$).

According to the obtained dendrogram, the larval incorporation patterns of [1-^{14}C] FFA were classified into four clusters (Fig. 1). In addition, the K-means cluster analysis provides the same classification when K = 4. Cluster 1 and Cluster 2 contained ARA and DHA incubations, respectively. Cluster 3 encompassed all LA and ALA incubations, except for ALA larvae fed SFO diet at 0 and 5 ppt salinity which resulted to be more similar to EPA larvae and therefore, were classified into Cluster 4 together with EPA samples. Fig. 1 shows factor scores for each cluster given as mean ± standard deviation. Thus, Cluster 1, containing ARA incubations indicates that ARA presented the

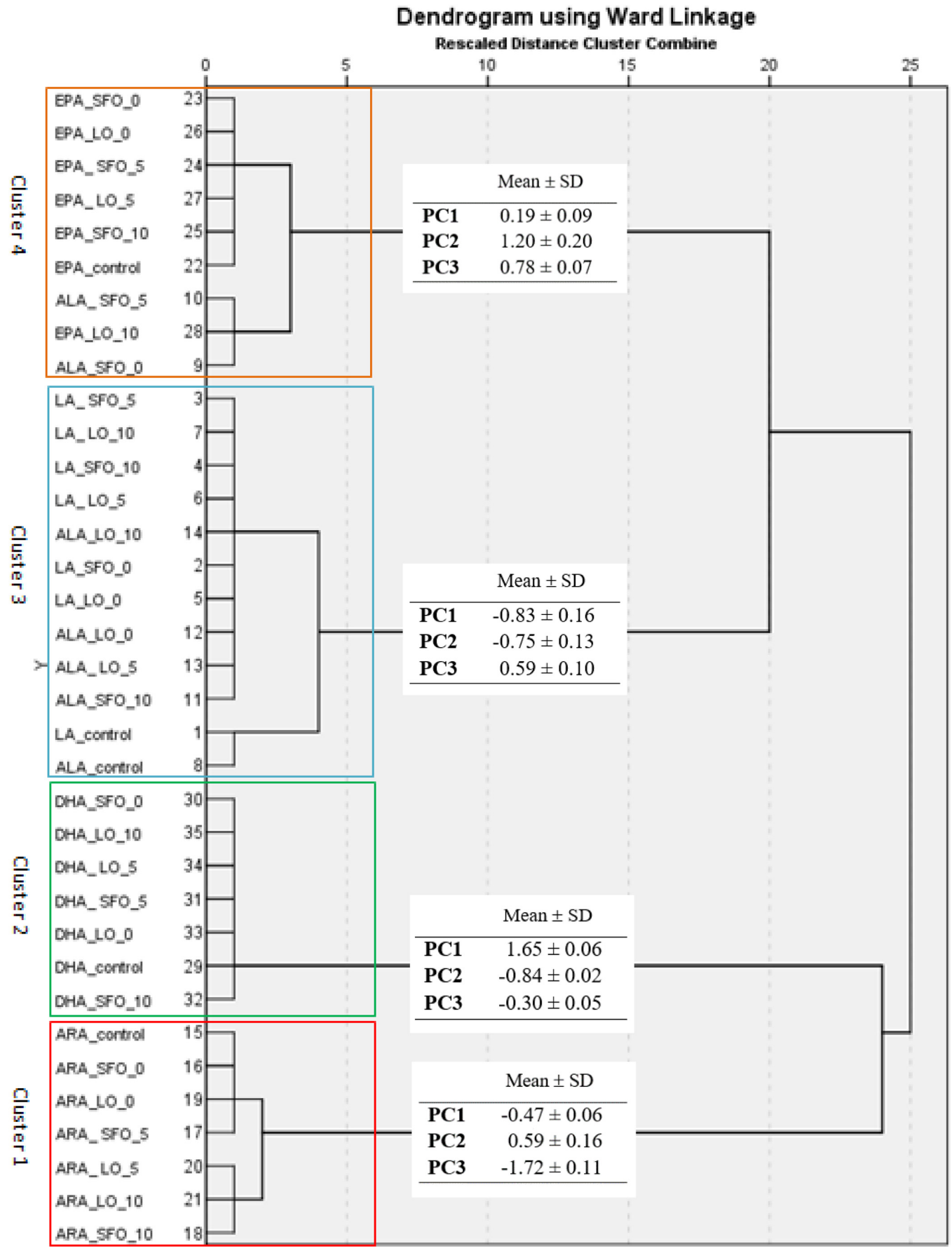

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Fig. 1. Dendrogram showing four clusters of samples according to the average incorporation proportions of [1-^{14}C] radiolabelled FFA (18:2n-6, LA; 18:3n-3, ALA; 20:4n-6, ARA; 20:5n-3, EPA and 22:6n-3, DHA) into the different lipid classes of pikeperch larvae fed *Artemia* enriched with DHA Selco (control, 0 ppt), or sunflower oil (SFO) and linseed oil (LO) at three different salinities (0, 5 and 10 ppt). For each cluster means and standard deviations for PC1, PC2 and PC3 are shown.

lowest average incorporation percentages into PC and the highest into PI. Cluster 2 includes DHA samples, characterized by the highest average incorporation rates into PS and PE, medium-high average incorporation into PI and medium-low into PC. Cluster 3, which encompassed LA and ALA samples except ALA samples under SFO diet with 0 and 5 ppt salinity, reveals that both C18 PUFA presented the lowest average incorporation into PE and PS and medium-high average incorporation into TAG and PC. Finally, Cluster 4, grouping together incubations with EPA and ALA in SFO diet at 0 and 5 ppt salinity, indicates that these larvae presented the highest average incorporation proportions of these FAs into PC, but the lowest ones into TAG and PI. In addition, they also showed medium-high average incorporation percentages into PE and PS.

[1-^{14}C]ARA was better incorporated into larval phospholipids when bound to PC and PE than when provided in its free form. Moreover, either incubated as PC or PE, [1-^{14}C]ARA was similarly re-esterified into larval LC mainly into PI (41–45%) followed by PC (29–36%) (Table 6). When incubated bound to PC, radioactivity recovered into larval PC was higher than when bound to PE (36.1 ± 3.8% *vs* 29.0 ± 0.2%, respectively) whereas the opposite trend was observed when bound to PE (9.2 ± 0.7% *vs* 11.5 ± 0.8%, respectively) ($P < .05$).

3.5. Transformation of radiolabelled fatty acids by elongation/desaturation processes. Influence of dietary fatty acids and environmental salinity

More than 75% of radioactivity from [1-^{14}C]FFA was incorporated into 20 DPH pikeperch larvae as unmodified substrates with a limited proportion transformed into other FA products (Table 7). LA and ALA were the most transformed substrates (12–25%), as well as the only FAs where both elongation and desaturation products were detected. Thus, [1-^{14}C]ALA was partially converted to 18:4n-3, 20:3n-3 and 20:4n-3, while [1-^{14}C]LA was to 18:3n-6, 20:2n-6 and 20:3n-6. Interestingly, only elongation products were detected for [1-^{14}C]LC-PUFA substrates, with [1-^{14}C]EPA being the most transformed one, including an unknown product appearing on the upper half of the plate (above 24:5n-3 band) and which identity was not possible to confirm (Table 7). Both 20:3n-6 and *de novo* products from beta-oxidation and therefore released [1-^{14}C] from original substrates, were significantly more abundant in fish fed the SFO diet, whereas 20:4n-3 content was higher in the LO treatment. Higher rearing salinities tended to increase the rate of modification of radiolabelled LA and ALA compounds.

4. Discussion

The main purpose of the present study was to elucidate the metabolic fate of dietary lipids including phospholipids and free PUFA in pikeperch larvae during the first stages of development and to determine if nutritional and/or environmental conditions may boost/alter the metabolic pathways involved. The trophic level of fish species, along with their habitat (freshwater *vs* marine) and trophic ecology, have all been suggested to influence the biosynthetic capacity of fish to produce LC-PUFA (Castro et al., 2016; Monroig et al., 2018; Garrido et al., 2019) and, consequently, the way essential fatty acids (EFA) requirements are satisfied by the diet. In species with high capacity to convert C18 PUFA to LC-PUFA, EFA needs can be met by blends of VOs such as SFO or LO containing C18 LC-PUFA precursors in their diet. In contrast, species with reduced capacity to produce LC-PUFA from their C18 precursors require a dietary supply of thte former to satisfy their EFA demands, which is typically achieved by including FO (Fonseca-Madrigal et al., 2014; Castro et al., 2016; Monroig et al., 2018). Under our experimental design, changes in dietary FA composition and increasing salinity did not vary the total lipid content nor the main lipid class profile of 20 DPH pikeperch larvae. On the other hand, in agreement with results for many other fish species, the FA composition of larvae reflected dietary FA profile (Olsen et al., 2014; Bonacic et al., 2016; Mozanzadeh et al., 2016; Koven et al., 2018), including the near 2-fold higher content of LA in the SFO-larvae compared to the other two experimental groups, and the almost 1.5-fold higher ALA present in larvae fed *Artemia*-LO. Contrarily, the selective retention of ARA, EPA and, especially DHA in larval tissues compared to their dietary levels confirmed the relevance of these FAs for proper physiological function of this species (Lund et al., 2019).

Both dietary lipid composition and environmental factors, primarily temperature and salinity, may modulate the ability of certain species to biosynthesize FAs in order to adjust and maintain the levels of LC-PUFA in their tissues (Tocher, 1995; Vagner and Santigosa, 2011; Fonseca-Madrigal et al., 2012). Several studies reported increased desaturation/elongation products in marine fish tissues after feeding VO-based diets (Menoyo et al., 2004; Mourente et al., 2005; Díaz-López et al., 2010; Morais et al., 2012; Betancor et al., 2015). The activity of the LC-PUFA route in carp cells was increased by an EFA deficiency (Tocher and Dick, 1999), and modulated by different dietary C18 PUFA (Tocher and Dick, 2000; Díaz-López et al., 2010). Expression of Δ6 FAD mRNA was reduced in salmon fed diets containing FO in comparison to fish receiving diets containing VOs but lacking LC-PUFA (Leaver et al., 2008; Taggart et al., 2008; Zheng et al., 2009). Our present data revealed

Table 5
Esterification pattern (%) of [1-^{14}C] radiolabelled free fatty acid substrates into the different lipid classes of 20 DPH pikeperch larvae fed *Artemia* enriched with DHA Selco (control, 0 ppt).

[1-^{14}C]Substrate	18:2n-6 LA		18:3n-3 ALA		20:4n-6 ARA		20:5n-3 EPA		22:6n-3 DHA	
Phosphatidylcholine	50.4 ± 3.3	ab	43.6 ± 5.9	bc	33.2 ± 1.3	d	54.5 ± 0.4	a	41.3 ± 0.6	c
Phosphatidylserine	2.1 ± 1.2	bc	0.7 ± 0.3	c	3.8 ± 0.4	ab	2.7 ± 0.9	ab	5.0 ± 0.7	a
Phosphatidylinositol	2.6 ± 0.3	c	2.8 ± 1.8	c	36.1 ± 2.3	a	10.0 ± 0.2	b	10.6 ± 1.3	b
Phosphatidylglycerol	0.0 ± 0.00		0.0 ± 0.0		1.0 ± 0.3	b	2.0 ± 0.5	a	0.0 ± 0.0	
Phosphatidylethanolamine	3.6 ± 2.4	c	5.8 ± 1.1	c	10.1 ± 0.3	b	14.2 ± 0.9	b	26.3 ± 0.8	a
Σ Polar Lipids	**58.7 ± 7.5**	**b**	**52.8 ± 5.8**	**b**	**84.1 ± 2.3**	**a**	**83.3 ± 1.8**	**a**	**83.2 ± 1.4**	**a**
Partial acylglycerols	11.9 ± 2.2	a	6.6 ± 1.6	b	11.8 ± 1.0	a	5.5 ± 0.6	b	4.1 ± 1.2	b
Free fatty acids	14.5 ± 6.4	a	14.0 ± 2.4	a	2.8 ± 0.3	b	5.0 ± 0.8	b	4.8 ± 0.2	b
Triacylglycerols	14.9 ± 3.6	b	26.6 ± 3.8	a	1.3 ± 1.5	d	6.1 ± 0.8	c	8.0 ± 0.4	c
Σ Neutral Lipids	**41.3 ± 7.5**	**a**	**47.2 ± 5.8**	**a**	**15.9 ± 2.3**	**b**	**16.7 ± 1.8**	**b**	**16.8 ± 1.4**	**b**

Results represent means ± SD; n = 3. LA, linoleic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Mean values with unlike letters are significantly different ($P < .05$).

Table 6
Esterification pattern (%) of [1-^{14}C]ARA substrates when provided as free fatty acid or bound to PC (ARA-PC) or PE (ARA-PE), into lipid classes of 20 DPH pikeperch larvae fed *Artemia* enriched with DHA Selco (control, 0 ppt).

[1-^{14}C]Substrate	[1-^{14}C]ARA		[1-^{14}C]ARA-PC		[1-^{14}C]ARA-PE	
Phosphatidylcholine	33.2 ± 1.3	ab	36.1 ± 3.8	a	29.0 ± 0.2	b
Phosphatidylserine	3.8 ± 0.4	b	4.3 ± 0.3	b	6.6 ± 1.0	a
Phosphatidylinositol	36.1 ± 2.3	b	41.1 ± 3.6	ab	45.5 ± 2.3	a
Phosphatidylglycerol	1.0 ± 0.3	a	0.0 ± 0.0	b	0.0 ± 0.0	b
Phosphatidylethanolamine	10.1 ± 0.3	ab	9.2 ± 0.7	b	11.5 ± 0.8	a
Σ Polar Lipids	**84.1 ± 2.4**	**b**	**90.7 ± 1.1**	**a**	**92.7 ± 1.9**	**a**
Partial acylglycerols	11.8 ± 1.0	a	5.2 ± 1.3	b	5.0 ± 1.0	b
Free fatty acids	2.8 ± 0.3	b	4.1 ± 0.4	a	2.4 ± 0.3	b
Triacylglycerols	1.3 ± 1.5	a	0.0 ± 0.0	b	0.0 ± 0.0	b
Σ Neutral Lipids	**15.9 ± 2.4**	**a**	**9.3 ± 1.1**	**b**	**7.3 ± 1.9**	**b**

Results represent means ± SD; n = 3. ARA, arachidonic acid, 20:4n-6; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Mean values with unlike letters are significantly different ($P < .05$).

higher proportions of some desaturation/elongation products in the 20 DPH pikeperch larvae. Thus, FAs such as 18:3n-6 (gamma linolenic acid, GLA), 20:2n-6 (eicosadienoic acid, EDA) and 20:3n-6 (dihomo-gamma linolenic acid, DGLA), which were absent or present at extremely low amounts in SFO-enriched *Artemia* were detected in larval tissues. Similarly, contents of 18:4n-3 (stearidonic acid, SA), 20:3n-3 (eicosatrienoic acid, ERA) and 20:4n-3 (eicosatetraenoic acid, ETA) tended to be higher in fish receiving the LO-*Artemia*. The present *in vivo* metabolic fate study confirms that pikeperch larvae are able to elongate both [1-^{14}C]18:2n-6 to yield ^{14}C-20:2n-6 and [1-^{14}C]18:3n-3 to produce ^{14}C-20:3n-3. Furthermore, larvae incubated in the presence of [1-^{14}C]18:2n-6 and [1-^{14}C18]18:3n-3 converted these FAs into ^{14}C-18:3n-6 and ^{14}C-18:4n-3, respectively, through the addition of a double bond to the carboxylic acid chain by a Δ6 desaturase. Under similar feeding conditions, cod exhibited little difference in Δ6 FAD expression and activity indicating that this species is also deficient, both in critical LC-PUFA biosynthetic genes and in the expression levels and regulation of Δ6 FAD compared to salmon (Tocher et al., 2006). Finally, GLA and SA could have been metabolized to ^{14}C-20:3n-6 and ^{14}C-20:4n-3 in pikeperch larvae by a subsequent two-carbon elongation, although their production through the action of a Δ8-desaturase over 20:2n-6 and 20:3n-3, respectively, cannot be ruled out (Monroig et al., 2011).

In this sense, functional analyses performed in yeast suggested that the Δ8 activity of Fads2 fatty acyl desaturases varied markedly among species, with marine fishes generally exhibiting higher Δ8 capabilities than freshwater/diadromous fish (Garrido et al., 2019). It remains

Table 7
Recovery (%) of [1-^{14}C]FA substrates and their elongation/desaturation metabolites in 20 DPH pikeperch larvae fed *Artemia* enriched with DHA Selco (control, 0 ppt), or sunflower oil (SFO) and linseed oil (LO) at three different salinities (0, 5 and 10 ppt).

Recovery								Two-way ANOVA*			
Diet		DHA Selco	SFO		LO			Diet	Salinity		
Salinity (‰)	0	0	5	10	0	5	10	SFO *vs* LO	0	5	10
[1-^{14}C]18:2n-6 LA											
18:2n-6	82.9 ± 4.7	79.9 ± 6.3	74.7 ± 8.7	75.9 ± 9.0	85.5 ± 1.9	81.7 ± 8.3	77.1 ± 5.3	NS		NS	
18:3n-6	4.9 ± 1.3	4.9 ± 2.2	5.3 ± 3.2	5.7 ± 3.1	3.4 ± 0.6	4.8 ± 3.3	6.1 ± 2.0	NS		NS	
20:2n-6	4.3 ± 1.3	5.3 ± 0.8	5.3 ± 0.9	4.6 ± 1.3	6.0 ± 1.6	5.4 ± 2.2	6.1 ± 0.9	NS		NS	
20:3n-6	3.8 ± 0.7	4.2 ± 1.8	4.6 ± 1.8	4.9 ± 1.9	2.0 ± 1.0	1.6 ± 0.9	3.5 ± 0.7	0.005		NS	
de novo	4.1 ± 0.4	5.7 ± 2.0	10.2 ± 3.9	9.0 ± 2.9	3.0 ± 0.5	6.5 ± 3.6	7.3 ± 2.4	0.043	y	x	x
[1-^{14}C]18:3n-3 ALA											
18:3n-3	84.7 ± 6.4	88.6 ± 3.4	85.4 ± 14.8	77.7 ± 8.8	82.1 ± 4.1	83.2 ± 12.2	76.6 ± 4.8	NS		NS	
18:4n-3	2.9 ± 0.1	0.8 ± 0.2	1.2 ± 0.7	4.0 ± 1.5	2.1 ± 0.8	2.9 ± 2.6	4.3 ± 0.6	NS	y	xy	x
20:3n-3	6.9 ± 1.7	7.1 ± 2.0	7.9 ± 4.9	9.2 ± 4.0	9.6 ± 2.9	8.5 ± 1.9	9.9 ± 2.4	NS		NS	
20:4n-3	1.2 ± 0.0	0.8 ± 0.3	0.9 ± 0.0	3.5 ± 1.4	2.2 ± 0.2	2.1 ± 1.2	4.0 ± 0.9	0.027	y	y	x
de novo	4.3 ± 1.2	2.7 ± 1.0	4.6 ± 1.7	5.7 ± 2.3	4.0 ± 0.5	3.3 ± 0.7	5.2 ± 0.4	NS		NS	
[1-^{14}C]20:4n-6 ARA											
20:4n-6	95.0 ± 1.9	93.7 ± 1.1	93.0 ± 2.7	91.0 ± 3.4	94.3 ± 1.2	92.8 ± 3.7	90.5 ± 2.2	NS		NS	
22:4n-6	4.1 ± 1.8	3.5 ± 0.6	3.5 ± 0.8	4.7 ± 1.5	3.3 ± 0.8	4.2 ± 1.8	5.3 ± 1.4	NS		NS	
24:4n-6	1.0 ± 0.5	2.8 ± 0.9	3.5 ± 2.2	4.3 ± 2.1	2.4 ± 0.6	3.0 ± 0.8	4.2 ± 1.7	NS		NS	
[1-^{14}C]20:5n-3 EPA											
20:5n-3	91.0 ± 2.4	92.2 ± 2.7	92.1 ± 3.1	87.6 ± 5.3	92.4 ± 2.7	92.9 ± 1.0	91.4 ± 1.5	NS		NS	
22:5n-3	3.8 ± 1.1	3.4 ± 0.9	3.9 ± 1.9	5.0 ± 1.8	2.5 ± 0.7	2.7 ± 0.3	3.7 ± 0.9	NS		NS	
24:5n-3	1.9 ± 0.4	1.4 ± 0.5	1.3 ± 0.2	2.0 ± 0.7	1.6 ± 0.6	1.4 ± 0.5	1.6 ± 0.4	NS		NS	
UK	2.3 ± 1.2	2.1 ± 1.3	1.8 ± 0.7	3.6 ± 2.1	2.5 ± 1.1	1.9 ± 0.5	2.1 ± 0.4	NS		NS	
de novo	1.1 ± 0.5	0.9 ± 0.4	0.9 ± 0.5	1.9 ± 1.1	1.0 ± 0.7	1.3 ± 0.5	1.1 ± 0.4	NS		NS	
[1-^{14}C]22:6n-3 DHA											
22:6n-3	94.7 ± 2.6	93.0 ± 0.7	96.1 ± 1.8	90.7 ± 4.4	93.0 ± 6.8	95.0 ± 1.3	93.4 ± 0.5	NS		NS	
24:6n-3	5.3 ± 2.6	7.0 ± 0.7	3.9 ± 1.8	9.3 ± 4.4	7.0 ± 6.8	5.1 ± 1.3	6.6 ± 0.5	NS		NS	

Results represent means ± SD; n = 3. LA, linoleic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. * Interaction between dietary treatment and salinity was not significant for any fatty acid. Fatty acids with a significant effect of diet or salinity and no interaction are indicated with the *P* value or unlike letters (x,y), respectively ($P < .05$). NS, not significant; UK, unknown.

unclear the advantage of retaining enhanced Δ8 desaturation capability by marine species, that have natural diets with high availability of preformed EPA and DHA and in which the general absence of a Δ5-desaturase limits the overall activity pathway (Bell and Tocher, 2009; Castro et al., 2012; Monroig et al., 2018; Garrido et al., 2019). Admittedly, the present study was not designed to address this specific concern and complementary metabolic/genomic studies are needed to elucidate the precise pathway to biosynthesize DGLA and ETA from their C18 precursors in this species.

In the present work, neither ARA nor EPA were obtained from [1-^{14}C]18:2n-6 or [1-^{14}C]18:3n-3, respectively, proving a deficiency of Δ5 desaturase activity in pikeperch larvae and the inability of this freshwater species to biosynthesize C20 EFA from their C18 precursors. Moreover, [1-^{14}C]ARA and [1-^{14}C]EPA almost exclusively yielded elongation compounds of 22 and 24-carbons, indicating an extremely low Δ6 desaturase activity over these substrates, and therefore the inability of pikeperch larvae to *de novo* biosynthesize DHA. The substrate specificity and regioselectivity (double bond positioning) of fads largely determined by the interaction between the enzyme and the lipid headgroup may have favoured the activity of this enzyme towards C18 over C22 and C24 substrates (Li et al., 2016) although the higher enzymatic activity over the most consumed/available substrate may not be completely discarded (Chen et al., 2018). Thus, our present results undoubtedly corroborate that ARA, EPA and DHA must be considered essential for pikeperch larvae, and therefore must be supplied adequately through the diet for proper larval development. All above coinciding with results previously reported for several marine species (Izquierdo et al., 2008; Vagner and Santigosa, 2011) providing unequivocal evidences to the similarity of pikeperch lipid metabolism to that of marine teleosts.

Δ6 desaturase activity in pikeperch larvae seemed to be enhanced at higher rearing salinities albeit this effect was not always significant or consistent. Although the effects of salinity on lipid and FA biochemistry and metabolism have been little studied in non-salmonid fish, the expression of Δ6 fatty acyl desaturase in liver of the marine teleosts, rabbitfish (*Siganus canaliculatus*) and red sea bream (*Pagrus major*), was higher in fish maintained at low salinity with respect to fish reared at high salinity (Li et al., 2008; Sarker et al., 2011). However, *Chirostoma estor* showed very low endogenous activity for the LC-PUFA biosynthesis when reared in freshwater but increased this capacity, essentially in hepatocytes, as salinity increased up to 15 ppt (Fonseca-Madrigal et al., 2012). Irrespective to the precise mechanistic links, these results may be related to: (1) the better physiological status of species when the environmental salinity is closer to the internal osmolality; (2) the possible marine origin of this species and a possible evolutionary adaptation to the generally higher levels of DHA in the marine food web (Sargent et al., 1995). Thus, freshwater species might have had higher evolutionary pressure to retain this FA as also shown in the LC-PUFA deficient pikeperch larvae. However, the precise connections between salinity changes and LC-PUFA synthesis in fish requires further investigation.

In a previous recent work, we demonstrated that independently of diet and rearing salinity the preferential incorporation pattern of [1-^{14}C]FFAs into pikeperch larval tissues is EPA > ARA > LA = ALA = DHA (Lund et al., 2019). Interestingly, as a continuation of this work we demonstrate here that the [1-^{14}C] FFAs with shorter chain length synthesized *de novo* were exclusively produced when larvae were incubated with [1-^{14}C]LA, [1-^{14}C]ALA and [1-^{14}C]EPA. These shorter ^{14}C FA metabolites are obtained by β-oxidation of the original substrates at a rate of 3–10% and 1–2% for C18 and C20 FAs, respectively. Therefore, 20 DPH pikeperch larvae can effectively use LA and ALA as energetic substrates, the main fatty acids in SFO and LO, but also EPA, the substrate with higher incorporation and esterification rate into fish lipids, in the present study. Fatty acids are the major energetic compounds in fish (Sargent et al., 1995) and both LA and ALA are widely considered good substrates for β-oxidation and thus, good energy sources (Freemantle et al., 2006; Brown, 2016; Chen et al., 2018). However, the role of EPA as possible energetic source is not well-established. Freemantle et al. (2006) suggested that any beneficial effect of EPA is unlikely to depend exclusively on its conversion to DHA, or to eicosanoids and they speculate that EPA may well facilitate fuel supply to the brain. These authors hypothesized that EPA is an activator of one or more classes of peroxisome proliferator-activated receptors (PPARs). Thus, EPA could promote β-oxidation and ketogenesis through stimulating the activities of enzymes involved in the metabolism of FAs by varying the transcription rates of their genes. In addition, despite their pivotal role as structural components of biomembranes, LC-PUFA, like all FAs, can be also used as energetic substrates when supplied in a high dietary concentration with the exception of DHA, which tends to be conserved irrespective to dietary supply, primarily because of its relatively poor suitability as substrate for β-oxidation in vertebrates (Sargent et al., 2002; Tocher, 2010).

Most fish larvae show a poor development of enterocytes hampering the ability to synthesize phospholipids *de novo* (Coutteau et al., 1997), This was also evidenced in the present study where the metabolic fate of the [1-^{14}C]FFA demonstrates the high and consistent specificity in the esterification pattern of each LC-PUFA. Lund et al. (2018) demonstrated that increasing dietary phospholipid levels up to 8% markedly enhanced pikeperch larval growth, with a possible additional positive effect by the addition of EPA and DHA. Moreover, Senegalese sole (*Solea senegalensis*) larval performance was improved when larvae were fed preys with higher levels of LC-PUFA and n-3 PUFA and this was partly explained by an up-regulation of phospholipid metabolism and apolipoprotein synthesis, which resulted in enhanced lipid transport and mobilization, as well as tissue growth and remodeling (Bonacic et al., 2016). Within essential fatty acids, ARA has been traditionally overlooked in preference to EPA and DHA, but its potential to affect larval growth, survival and stress resistance has been previously acknowledged (Bell and Sargent, 2003) and recent studies have also provided some insight on the molecular mechanisms involved (Alves Martins et al., 2012; Norambuena et al., 2013). One main issue addressed in the present study was to determine if pikeperch larvae may incorporate ARA into tissue phospholipids more efficiently when provided as a free molecule or bound to phospholipids such as PC or PE and, at the same time, to clarify the phospholipid catabolism capacity of this species. PC followed by PE are the main phospholipids in fish eggs and larvae, including those of pikeperch (Craig, 2000; Lund et al., 2014). PC is also the main product of phospholipids synthesis in fish enterocytes (Caballero et al., 2006) and comprises up to 95% of the phospholipids found in very low-density lipoproteins (VLDL) (Lie et al., 1993). Our present results show a significantly higher average incorporation rate of [1-^{14}C]ARA when supplied free or bound to PC than when bound to PE. Dietary supplementation with PC, but not PE, stimulated feeding activity in gilthead seabream larvae, probably through the ingestion of the trimethyl group of the choline base of PC that binds to receptor gustatory cells (Hadas, 1998). When incubated bound to PC, ARA radioactivity recovered into larval PC was higher than when bound to PE whereas the opposite trend was observed when linked to PE denoting that the identity of *de novo* synthesized phospholipid might depend upon availability of structural molecules for the reacylation of the incorporated fatty acids.

Novel insights on the lipid metabolism of pikeperch at early development have been achieved in the present work. The radioactive study reveals that pikeperch larvae shows a low activity of enzymes involved in the biosynthesis of LC-PUFA from exogenously added C18 substrates. Consequently, despite being a freshwater fish, pikeperch seems to be metabolically closer to most marine teleosts than to freshwater species. The identity of *de novo* synthesized phospholipids depends on the biochemical form (free molecule or bound to phospholipids) in which LC-PUFAs are presented to the fish. These new findings would be useful in the design of more specific live prey enrichment protocols and dietary formulation adapted to pikeperch larvae

metabolic capabilities.

Declaration of Competing Interests

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2020.110449.

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