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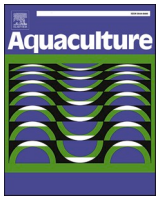

In vivo biosynthesis of long-chain polyunsaturated fatty acids by the euryhaline rotifer (*Brachionus plicatilis*)

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ABSTRACT

In order to increase omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) availability and to improve the design of rotifer's enrichment protocols for marine larvae production, the endogenous fatty acid (FA) metabolism of the marine rotifer, *Brachionus plicatilis* was determined. To this purpose, the *in vivo* ability of this species to incorporate, esterify into the different lipid classes and to elongate/desaturate unsaturated FAs was investigated. Rotifers were incubated in 75 cm^2 tissue culture flasks, at a density of 75,000 rotifers per incubation, for 4 h. Incubations ($n = 4$) were performed at 21 °C, with 0.3 μCi of [1-^{14}C]FAs including C18 FAs (18:1n-9, OA; 18:2n-6, LA; 18:3n-3, ALA) and LC-PUFA (20:4n-6, ARA; 20:5n-3, EPA; 22:6n-3, DHA). The present study demonstrates that rotifers possess an active metabolism over dietary FAs, being this species able to produce LC-PUFA from its C18 FA precursors. Interestingly, the action of a Δ12 desaturase that converts OA to LA and of a Δ15 desaturase over LA to produce ALA, seems to be also present, in agreement with previously reported functional characterization of ωx desaturases genes in this species. However, a globally low transformation rate was obtained, in addition to a lower incorporation of DHA into rotifer tissues compared to the other incubated substrates. The aforementioned factors, together with a competitive esterification pattern of DHA, LA and ALA into phosphatidylcholine (PC) or of EPA and ARA into phosphatidylinositol (PI), may give rise to rotifers with inadequate essential FA ratios for marine larvae nutrition, if ignored or incorrectly handled in the lipid enrichment process of this live prey species.

1. Introduction

Every year, the industrial culture of several fish species is established, and the progress of larval rearing methodology for these new species requires appropriate feeding protocols adjusted to their nutritional needs. The majority of freshwater fish larvae can be produced on a commercial scale using exclusively formulated diets from the start of exogenous feeding (Mæhre et al., 2013). Contrarily, and despite recent advances in the production of inert diets for marine fish larvae (Hamre et al., 2013), most cultured marine species still depends on the use of live feeds during their early life stages, mostly due to their reduced size, undeveloped digestive system and poor acceptance of inert inputs (Conceição et al., 2010; Tocher, 2010). Under natural conditions, fish larvae feed on a wide spectrum of phyto- and zooplankton species which provide them a complete and balanced diet. Nonetheless, the mass production of plankton species in large-scale aquaculture facilities still remains challenging, particularly due to arduous and cost-ineffective protocols (Conceição et al., 2010; Iglesias et al., 2007; Sarkisian et al., 2019).

Rotifers and *Artemia* are the most commonly used live preys in aquaculture (Conceição et al., 2010). The marine rotifer *Brachionus plicatilis* has been mass cultured from the 60s, when its acclimatization to seawater was achieved (Ito, 1960). Since then, rotifers have been widely provided as first feed for the early life stages of many marine fishes and crustaceans (Lubzens et al., 1989; Lubzens and Zmora, 2003), with the length of this period depending on the species. Several characteristics

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favour the use of rotifers as live prey in hatcheries, including their small size (150–250 μm), high population growth rates at high culture densities, good tolerance to culture conditions (*e.g.* changes in temperature and/or salinity), and their passive filtering nature. Although the nutritional value of rotifers is poor, they can be fed/enriched with different types of particles in suspension making it easy to balance their lacking nutrients and consequently, improving their nutritional value to fulfil larval requirements prior to be added to the culture media (Conceição et al., 2010; Izquierdo, 1996; Kotani, 2017).

One of the most significant factors influencing fish larval development is the dietary amount and adequate balance of n-3 and n-6 polyunsaturated fatty acids (PUFAs). A deficiency/imbalance of these FAs, normally give rise to reduced growth and increased mortality (Rodríguez et al., 1998a; Tocher, 2015). The lack of Δ12 and Δ15 desaturases prevents fish to *de novo* synthesize linoleic acid (18:2n-6; LA) from oleic acid (18:1n-9; OA) or α-linolenic acid (18:3n-3; ALA) from LA, respectively. Similarly, most fish possess reduced ability to convert C18 FAs into LC-PUFA ($\geq C_{20}$), such as arachidonic acid (20:4n-6; ARA), eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) (Tocher, 2010). As a consequence, these FAs are considered essential and must be supplied thought the diet (Sargent et al., 2002). In this sense, the FA composition of fish depends on the interaction between dietary FA and their endogenous metabolism (Tocher, 2003), leading to differences in dietary LC-PUFA requirements between species.

The availability of LC-PUFA to fish larvae seems to be positively correlated with their presence into dietary phospholipids (Gisbert et al., 2005; Olsen et al., 2014; Wold et al., 2009), with higher levels improving larval growth and development in Atlantic cod (*Gadus morhua;* Olsen et al., 2014), European seabass (*Dicentrarchus labrax*; Gisbert et al., 2005), and gilthead seabream (*Sparus aurata*; Lund et al., 2018).

Current rotifer's enrichment protocols to tailor its LC-PUFA profile towards fish larval nutritional needs frequently includes microalgae, lipid emulsions, microparticles or microcapsules containing LC-PUFA-rich lipids (Thépot et al., 2016; Ghaderpour and Estevez, 2020). However, commercial lipid emulsions rich in DHA and EPA consist mainly of TAG but not phospholipids. The phospholipid content of live preys is hard to modify, since they are mainly associated with membrane structural and functional roles (McEvoy et al., 1996), and live prey tend to store these lipids as TAG (Reis et al., 2017, 2019). Nonetheless, and within limits, phospholipid FA profile might be handled to increase the bioavailability of specific FAs for larvae. In this sense, there is an increasing need to extend the current knowledge about dietary FA esterification pattern into live preys' tissues and on how phospholipids can be enriched with LC-PUFA.

In order to assist in designing effective and appropriate nutritional enrichment protocols of rotifers adapted to specific fish larvae needs, it is especially valuable to determine its endogenous FA metabolism capacities. Hence, the aim of the present research was to elucidate the metabolic fate of dietary FA into rotifers by investigating the *in vivo* capability of *B. plicatilis* to incorporate, esterify into different lipid classes, and elongate/desaturate unsaturated FAs.

2. Material and methods

2.1. Experimental rotifers

Rotifers *B. plicatilis*, strain S1, were cultured at the Instituto Español de Oceanografía (IEO-CSIC) – Centro Oceanográfico de Canarias (Tenerife, Spain) at a density of 200 rotifers mL^{-1} in 20 psu filtered water under continuous illumination (2000 lx) and moderate aeration. Rotifers were fed 1 g baker's yeast (*Saccharomyces cerevisiae*) per million $rotifer^{-1}$ day^{-1} displayed four times a day (09:00, 12:00, 15:00 and 18:00 h).

2.2. Lipid class and fatty acid composition of rotifers

The total lipid (TL) content of rotifers was extracted by homogenization with chloroform/methanol (2:1, *v*/v) according to the Folch method as described by Christie (2003). The organic solvent was evaporated under a stream of nitrogen and the lipid content determined gravimetrically. The TL extracts were stored at −20 °C in chloroform/methanol (2:1) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10 mg mL^{-1} under an inert atmosphere of nitrogen until further analysis. Lipid classes (LC) were separated by high-performance thin-layer chromatography (HPTLC) in a one-dimensional double-development on 10 cm × 10 cm HPTLC plates (Merck KGaA, Darmstadt, Germany) using propanol/chloroform/methyl acetate/methanol/0.25% potassium chloride (5:5:5:2:1.8, *v*/v) as developing solvent for polar lipid classes, and hexane/diethyl ether/acetic acid (22.5:2.5:0.25, v/v) for the neutral fractions. The LC were visualized by charring at 160 °C after spraying with 3% (*w/v*) aqueous cupric acetate containing 8% (*v*/v) phosphoric acid, and quantified by means of a CAMAG TLC Visualizer (CAMAG Chemie-Erzeugnisse und Adsorptionstechnik AG, Muttenz, Switzerland) at a wavelength of 370 nm. Identification of the different LC was performed by running a known standard (cod roe lipid extract) on the same plates, and the results expressed as percentage of TL.

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

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

A total of 75,000 rotifers were placed in each 75 cm^2 cell culture flask (Sarstedt AG & Co., Nümbrecht, Germany) containing 50 mL of filtered 20 psu water (1500 rotifers mL^{-1}). Incubations ($n = 4$) were performed for 4 h and gentle stirring at 21 °C with 0.3 μCi (0.45 μM) of [1-^{14}C]FAs (free FA molecule labelled with ^{14}C in its first carbon from the carboxyl head), including C18 FAs (18:1n-9, 18:2n-6, 18:3n-3) and LC-PUFA (20:4n-6, 20:5n-3, 22:6n-3). The [1-^{14}C]FAs were individually added to separate flasks as their potassium salts bound to bovine serum albumin (BSA), as described in Ghioni et al. (1997). After incubation, rotifers were filtered with a 60 μm mesh and thoroughly washed with filtered brackish water to remove excess radiolabelled FA. Rotifers incubated without [1-^{14}C]FAs were also taken to assess LC and FA profiles.

2.4. Incorporation of radiolabelled fatty acids into total lipids

An aliquot of 0.1 mg of radiolabelled lipid extract from [1-^{14}C]-incubated rotifers was taken to determine total radioactivity incorporated. Extracts were transferred to scintillation vials and radioactivity read in a LKB Wallac 1214 Rackbeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, USA). Results in disintegration per minute (dpm) were converted into pmol per mg of protein per hour of incubation (pmol mg $protein^{-1}$ h^{-1}) taking into account the specific activity of each substrate and rotifer total lipid and protein contents. The protein content was determined according to Lowry et al. (1951).

2.5. Esterification of radiolabelled fatty acids into lipid classes

A second 0.1 mg of TL extract from radioactive samples was applied to HPTLC plates to determine the esterification pattern of [1-^{14}C]FA into LC. Lipid classes were separated as described in section 2.2 and then, the developed HPTLC plates were placed for 1 week in closed exposure cassettes (Exposure Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Imagen Screen-K, Biorad). The screens were then scanned with an image acquisition system (Molecular Imager FX, BioRad), and the bands quantified by an image analysis software (Quantity One, BioRad).

2.6. Transformation of radiolabelled fatty acids

Transformation of incubated [1-^{14}C]FAs by elongation/desaturation processes was assessed by acid-catalysed transmethylation of 1 mg of TL extract from radioactive samples to obtain FAME, as previously detailed (Christie, 2003). FAME were separated by TLC using plates impregnated with a solution of 2 g silver nitrate in 20 mL acetonitrile followed by activation at 110 °C for 30 min. Plates were fully developed in toluene/acetonitrile (95:5, *v*/v), which resolves the FAME into discrete bands based on both, degree of unsaturation and chain length (Wilson and Sargent, 1992). Bands were revealed and quantified as mentioned in section 2.5. Identification of labelled bands was confirmed by radiolabelled standards formulated with a blend of commercially available ^{14}C-FA substrates (including 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, 20:4n-6, 20:5n-3 and 22:6n-3) at a concentration of 1 μCi mL^{-1} hexane each, simultaneously run on the same plate (Rodríguez et al., 2002). Radioactivity of the standard mixture was previously validated by means of the scintillation β-counter.

2.7. Materials

[1-^{14}C]C18 FA were purchased from PerkinElmer, Inc. (Waltham, Massachusetts, USA) and [1-^{14}C] LC-PUFA from American Radiolabelled Chemicals, Inc. (St. Louis, Missouri, USA). BSA was acquired from Sigma-Aldrich Co. (St. Louis, Missouri, USA). TLC plates (20 × 20 cm × 0.25 mm) were from Macherey-Nagel GmbH & Co. KG (Düren, Germany) and HPTLC plates (10 × 10 cm × 0.15 mm) pre-coated with silica gel 60 (without fluorescent indicator) from Merck KGaA (Düsseldorf, Germany). OptiPhase "HiSafe" 2 scintillant liquid was purchased from PerkinElmer, Inc. All organic solvents used were of reagent grade and were purchased from Merck KGaA, Sigma-Aldrich Co. and Panreac Química S.L.U. (Barcelona, Spain).

2.8. Statistical analysis

Results are presented as means ± SD ($n = 4$). Comparisons between incorporation of substrates into TL, esterification into LC and recovery of substrate after transformation processes of the six [1-^{14}C]FA, and within [1-^{14}C]C18 FAs (OA, LA, ALA) and [1-^{14}C]LC-PUFA (ARA, EPA, DHA) were analysed by one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test. Data were checked for normal distribution with the Kolmogorov-Smirnov test and the one-sample Shapiro-Wilk test, as well as for the homogeneity of variances with the Levene test. When normal distribution of the data and/or homogeneity of the variances were not achieved, data were subjected to the Welch and the Brown-Forsythe robust tests, followed by a Games-Howell non-parametric multiple comparison test. For all statistical tests, $p < 0.05$ was considered statistically different. The statistical analysis was performed using the IBM SPSS statistics 19.0 (IBM Co., USA).

Table 1
Total lipid content (μg lipid mg protein^{-1}) and lipid class composition (% total lipid) of the rotifer *Brachionus plicatilis*.

Total lipid content	189.9 ± 31.7
Lipid class	
Lysophosphatidylcholine	0.6 ± 0.1
Sphingomyelin	0.9 ± 0.3
Phosphatidylcholine	13.2 ± 0.4
Phosphatidylserine	3.5 ± 0.3
Phosphatidylinositol	6.0 ± 0.6
Phosphatidylglycerol	5.4 ± 0.5
Phosphatidylethanolamine	18.1 ± 0.6
Unknown	4.2 ± 0.5
Total polar lipids	51.5 ± 1.5
Monoacylglycerols	6.6 ± 0.8
Cholesterol	9.9 ± 1.1
Free fatty acids	8.8 ± 0.8
Triacylglycerols	9.3 ± 1.1
Sterol esters	13.9 ± 0.7
Total neutral lipids	48.5 ± 1.5

Results are given as means ± SD; $n = 4$.

Table 2
Main fatty acid composition (% of total FA) of the rotifer *Brachionus plicatilis*.

Fatty acids	
14:0	2.0 ± 0.0
16:0	8.3 ± 0.2
18:0	4.5 ± 0.1
Σ SFA	16.5 ± 0.2
16:1n-9	1.3 ± 0.2
16:1n-7	18.2 ± 0.2
18:1n-11	3.3 ± 0.0
18:1n-9 (OA)	24.3 ± 0.3
18:1n-7	5.1 ± 0.2
20:1n-11	0.9 ± 0.0
20:1n-9	3.7 ± 0.1
Σ MUFA	64.5 ± 0.2
18:2n-6 (LA)	6.7 ± 0.2
20:2n-6	2.0 ± 0.1
20:4n-6 (ARA)	0.5 ± 0.0
Σ n-6 LC-PUFA	2.5 ± 0.1
20:3n-3	1.1 ± 0.1
20:5n-3 (EPA)	0.8 ± 0.0
22:5n-3	0.7 ± 0.0
22:6n-3 (DHA)	1.3 ± 0.1
Σ n-3 LC-PUFA	3.9 ± 0.3
Σ PUFA	17.1 ± 0.5
Σ LC-PUFA	6.4 ± 0.3
n-3/n-6	1.4 ± 0.0
DHA/EPA	1.6 ± 0.1
EPA/ARA	1.7 ± 0.1

Results are given as means ± SD; $n = 4$. Σ include some minor components not shown. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; PUFA, polyunsaturated fatty acids; OA, oleic acid; LA, linoleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

3. Results

3.1. Lipid and fatty acid composition

Rotifers TL presented a similar proportion of total polar and total neutral lipids (Table 1). Within phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the main LC whereas sterol ester (SE) was the major neutral lipid, followed by triacylglycerols (TAG) and cholesterol (CHO) (Table 1).

Rotifers were particularly rich in monounsaturated fatty acids (MUFA) which represented near two-thirds of total FAs with 16:1n-7

Table 3
Incorporation of radioactivity into total lipid (pmol mg protein^{-1} h^{-1}) and its esterification (% of incorporated radioactivity) into lipid classes of the rotifer *Brachionus plicatilis* incubated with [1-^{14}C]FA substrates.

Substrate	18:1n-9 (OA)	18:2n-6 (LA)	18:3n-3 (ALA)	20:4n-6 (ARA)	20:5n-3 (EPA)	22:6n-3 (DHA)
Incorporation	16.6 ± 3.0$^{cd●}$	29.6 ± 2.7$^{a▲}$	26.8 ± 3.5$^{ab▲}$	26.6 ± 4.2$^{ab\Delta}$	19.9 ± 4.2$^{bc\Delta}$	10.7 ± 3.3$^{d\circ}$
Lipid Class						
Lysophosphatidylcholine	0.5 ± 0.2$^{b●}$	0.7 ± 0.2$^{b▲●}$	1.0 ± 0.4$^{b▲}$	0.7 ± 0.2$^{b\circ}$	0.8 ± 0.5$^{b\circ}$	2.3 ± 0.3$^{a\Delta}$
Phosphatidylcholine	23.8 ± 1.1$^{d■}$	38.4 ± 0.8$^{a▲}$	34.8 ± 1.0$^{b●}$	8.4 ± 0.4$^{f\square}$	14.0 ± 0.8$^{e\circ}$	30.8 ± 1.5$^{c\Delta}$
Phosphatidylserine	2.5 ± 0.4^{b}	2.3 ± 0.3^{b}	2.8 ± 0.0^{b}	7.7 ± 1.4^{a}	7.0 ± 3.7^{ab}	5.6 ± 0.7^{a}
Phosphatidylinositol	5.7 ± 0.3$^{d▲}$	3.0 ± 0.2$^{e●}$	2.5 ± 0.2 $^{e■}$	46.5 ± 3.4$^{a\Delta}$	34.8 ± 2.5$^{b\circ}$	10.8 ± 1.5$^{c\square}$
Phosphatidylglycerol	6.7 ± 0.6$^{c■}$	11.3 ± 0.8$^{b●}$	18.2 ± 1.4$^{a▲}$	4.1 ± 0.3$^{d\circ}$	4.7 ± 0.6$^{d\circ}$	7.5 ± 0.5$^{c\Delta}$
Phosphatidylethanolamine	35.5 ± 1.5$^{a▲}$	29.4 ± 0.7$^{b●}$	27.7 ± 0.3$^{b■}$	10.1 ± 0.7$^{e\square}$	12.8 ± 0.5$^{d\circ}$	19.7 ± 0.2$^{c\Delta}$
Unknown	1.8 ± 0.1$^{▲}$	1.3 ± 0.1$^{●}$	1.3 ± 0.0$^{●}$	nd	nd	nd
∑ Polar lipids	76.6 ± 2.2$^{bd●}$	86.4 ± 0.8$^{a▲}$	88.4 ± 0.7$^{a▲}$	77.5 ± 1.1$^{b\Delta}$	74.1 ± 1.0$^{cd\circ}$	76.7 ± 2.5$^{bc\Delta\circ}$
Partial acylglycerols	10.2 ± 1.4$^{b▲}$	6.7 ± 1.6$^{c●}$	7.9 ± 0.7$^{bc▲●}$	18.3 ± 1.5$^{a\circ}$	21.4 ± 0.9$^{a\Delta}$	19.5 ± 1.8$^{a\Delta\circ}$
Free fatty acids	4.2 ± 0.9$^{a▲}$	2.6 ± 0.4$^{ab▲●}$	2.0 ± 0.2$^{ab●}$	1.5 ± 0.3^{b}	1.8 ± 0.6^{ab}	1.6 ± 0.8^{b}
Triacylglycerols	6.5 ± 1.0$^{a▲}$	3.6 ± 0.5$^{b●}$	1.5 ± 0.3$^{c■}$	1.8 ± 0.1^{c}	1.7 ± 0.4^{c}	1.8 ± 0.5^{c}
Sterol esters	2.5 ± 0.7$^{a▲}$	0.7 ± 0.4$^{ab●}$	0.3 ± 0.2$^{b●}$	0.8 ± 0.2^{abc}	1.0 ± 0.8^{ab}	0.4 ± 0.3^{bc}
∑ Neutral lipids	23.4 ± 2.2$^{ab▲}$	13.6 ± 0.8$^{d●}$	11.6 ± 0.7$^{d●}$	22.5 ± 1.1$^{bc\circ}$	25.9 ± 1.0$^{a\Delta}$	23.3 ± 2.5$^{ac\Delta\circ}$

Results are given as means ± SD; $n = 4$. Different letters in superscript within the same row denote significant differences between radiolabelled fatty acids ($p < 0.05$). Different full symbols in superscript (■●▲) within the same row denote significant differences between C18 FA ($p < 0.05$). Different hollow symbols in superscript (□○Δ) within the same row represent significant differences between LC-PUFA ($p < 0.05$). nd – not detected.

and 18:1n-9 being the most abundant components (Table 2). Total PUFA content was less than one fifth of total fatty acids of which LC-PUFA only represented 6.4%, with values of 0.5, 0.8 and 1.3% for ARA, EPA and DHA, respectively. As a result, DHA/EPA and EPA/ARA ratios were 1.6 and 1.7, respectively (Table 2).

3.2. Incorporation of radiolabelled fatty acids into total lipids and its esterification into lipid classes

Radiolabelled OA was the least incorporated substrate of the three [1-^{14}C]C18 FAs assayed (Table 3). Within LC-PUFA, [1-^{14}C]ARA showed the highest incorporation rate, and DHA the lowest one ($p < 0.05$). Overall, rotifers tend to incorporate n-6 FAs (LA and ARA) rather than n-3 (ALA and EPA) (Table 3) although these differences were not significant ($p > 0.05$).

All radiolabelled substrates were extensively esterified into rotifer tissues, with less than 5% of the incorporated radioactivity recovered as free fatty acids (FFA; Table 3). After 4 h of incubation, most radiolabelled substrates were mainly esterified into rotifer polar lipids (74.1–88.4%). The esterification pattern of both [1-^{14}C18] PUFA was PC > PE > phosphatidylglycerol (PG) > partial acylglycerols (PAG), significantly differing from that of [1-^{14}C]OA (PE > PC > PAG). Similarly, the esterification sequence of [1-^{14}C]LC-PUFA into rotifer LC also varied between substrates ($p < 0.05$; Table 3). Thus, whilst [1-^{14}C]ARA and [1-^{14}C]EPA were mainly esterified into PI > PAG > PE ≥ PC, [1-^{14}C] DHA was into PC > PE = PAG.

3.3. Transformation of radiolabelled fatty acids

Over 78% of all [1-^{14}C]FA were incorporated into rotifers as unmodified substrate (Table 4). [1-^{14}C]OA was the most transformed substrate ($p < 0.05$), also showing the highest *de novo* synthesis rate of shorter chain-length (<18C) FAs. The *de novo* < 18C FAs obtained from the rest of incubated substrates ranged between 1.9 and 4.5%, except that of [1-^{14}C]DHA where no *de novo* FA production was detected. In addition, with the exception of DHA, all radiolabelled substrates were elongated and desaturated (Table 4). However, DHA was obtained by elongation/desaturation processes over both [1-^{14}C]LNA and [1-^{14}C] EPA. Similarly, incubation with [1-^{14}C]LA, displayed a band corresponding to [1-^{14}C]ARA. Surprisingly, radioactive bands corresponding to EPA and DHA standard positions were also obtained after incubations with [1-^{14}C]OA and [1-^{14}C]LA, while ARA seemed to be also synthesized from [1-^{14}C]OA (Table 4).

4. Discussion

4.1. Lipid and fatty acid composition

Rotifers analysed contained a five-fold lower lipid than protein content, similar proportions of polar and neutral lipids, particularly high MUFA contents, and moderate-low LC-PUFA levels. This biochemical profile matches well to previously reported composition of non-enriched rotifers, maintained with baker's yeast (devoid of LC-PUFA), showing an eminently structural lipid profile characterized by low fat levels and reduced lipid reserves such as TAG (Imada et al., 1979; Watanabe et al., 1978), highly abundant in rotifers enriched with commercial lipid emulsions (Olsen et al., 2014; Rainuzzo et al., 1994; Rodríguez et al., 1998a, 1998b). In this sense, rotifers must be nutritionally enriched with n-3 LC-PUFA, chiefly EPA and DHA, previously to be added to the culture media since these FA are essential for the successful development and survival of larvae from marine organisms (Izquierdo et al., 2000; Sargent et al., 1999; Watanabe et al., 2016).

4.2. Incorporation of [1- ^{14}C] fatty acids into lipids

DHA was the least incorporated [1-^{14}C]FAs into rotifer TL (9.6 ± 3.4 pmol mg protein^{-1} h^{-1}). Reduced incorporation of DHA in *Artemia sp.* has been related to the preferential oxidation of this FA (Estévez et al., 1998; Reis et al., 2017, 2019) and to the capacity of *Artemia* to retroconvert DHA into EPA (Navarro et al., 1999). Nonetheless, neither *de novo* synthesis of shorter chain-length FAs (*i.e.* obtained by the recycling of the labelled acetyl-CoA produced from [1-^{14}C]DHA beta-oxidation) nor evidences for retroconversion of DHA into EPA were registered in the present study. By contrast, the incubation with the other five [1-^{14}C] FAs suggested a direct and inverse correlation between the FA incorporation into rotifer tissues and the amount of *de novo* FA obtained from their catabolism (Table 4), as observed in previous studies with other marine invertebrates using a similar *in vivo* protocol (Reis et al., 2017, 2019). Interestingly, the lower incorporation of [1-^{14}C]DHA among all LC-PUFA has been also described in common octopus (*Octopus vulgaris*; Reis et al., 2014), European cuttlefish (*Sepia officinalis*; Reis et al., 2016), and pikeperch larvae (*Sander lucioperca*; Lund et al., 2019). Hence, the observed metabolic feature of DHA in *B. plicatilis* might not be related to its oxidation, as observed in *Artemia sp.*, but to a poorer ability of C22 highly unsaturated FAs to be retained into tissue lipids.

Table 4
Recovery (% of incorporated substrate) of [1-^{14}C]FA substrates and their elongation/desaturation metabolites in the rotifer *Brachionus plicatilis*.

Substrate	Products	% recovered	
[1-^{14}C]18:1n-9 (OA)			
	18:1n-9 (OA)	78.0 ± 2.3	d■
	20:1n-9	5.4 ± 0.6	
	22:1n-9	3.9 ± 0.7	
	18:2n-6 (LA)	1.8 ± 0.7	
	18:3n-6^{s}	1.0 ± 0.1	
	20:3n-6^{s}	0.3 ± 0.1	
	20:4n-6 (ARA)	0.6 ± 0.1	
	20:5n-3 (EPA)	0.3 ± 0.1	
	22:5n-3^{s}	0.3 ± 0.1	
	22:6n-3 (DHA)	0.2 ± 0.1	
	de novo	8.2 ± 0.6	
[1-^{14}C]18:2n-6 (LA)			
	18:2n-6 (LA)	90.4 ± 2.7	abc●
	20:2n-6	0.7 ± 2.5	
	22:2n-6	0.5 ± 0.3	
	18:3n-6^{s}	2.6 ± 0.4	
	20:3n-6^{s}	0.8 ± 0.2	
	20:4n-6 (ARA)	1.0 ± 0.2	
	22:4n-6	0.3 ± 0.2	
	20:5n-3 (EPA)	0.5 ± 0.1	
	22:5n-6^{s}	0.5 ± 0.2	
	22:6n-3 (DHA)	0.3 ± 0.2	
	de novo	2.3 ± 0.7	
[1-^{14}C]18:3n-3 (ALA)			
	18:3n-3 (ALA)	92.5 ± 0.5	ab●
	20:3n-3	0.8 ± 0.1	
	22:3n-3	0.5 ± 0.1	
	18:4n-3	2.0 ± 0.9	
	20:4n-3	0.9 ± 0.1	
	20:5n-3 (EPA)	0.7 ± 0.1	
	22:5n-3	0.1 ± 0.1	
	22:6n-3 (DHA)	0.3 ± 0.1	
	24:6n-3	0.1 ± 0.0	
	de novo	1.9 ± 0.4	
[1-^{14}C]20:4n-6 (ARA)			
	20:4n-6	86.1 ± 2.7	c□
	22:4n-6	3.2 ± 0.7	
	20:5n-3 (EPA)	2.9 ± 0.2	
	22:5n-6^{s}	1.7 ± 0.4	
	22:6n-3 (DHA)	1.5 ± 0.3	
	de novo	4.5 ± 1.8	
[1-^{14}C]20:5n-3 (EPA)			
	20:5n-3	89.6 ± 4.2	bc□
	22:5n-3	2.7 ± 1.3	
	22:6n-3 (DHA)	4.1 ± 0.6	
	de novo	3.6 ± 2.3	
[1-^{14}C]22:6n-3 (DHA)			
	22:6n-3	95.8 ± 1.2	a○
	24:6n-3	4.2 ± 1.2	

Results are given as means ± SD; n = 4. Different letters denote significant differences between unmodified substrates recovered ($p < 0.05$). Different full symbols in superscript (■●) denote significant differences between C18 FA unmodified substrates recovered ($p < 0.05$). Different hollow symbols in superscript (□○) represent significant differences between LC-PUFA ($p < 0.05$). *de novo*, synthesis of fatty acids with shorter chain-length (less than 18 carbons). ^{s} may also correspond to a fatty acid with the same chain length and number of unsaturation but from ω3 series.

4.3. Esterification of [1-^{14}C] fatty acids into lipid classes

One novel outcome of the present study was to establish the esterification pattern of unsaturated FAs into rotifer lipids. It has been previously reported that the most suitable route to provide LC-PUFA to larval stages of marine fish species is in the form of phospholipids (Gisbert et al., 2005; Lund et al., 2018; Olsen et al., 2014; Wold et al., 2009). Our findings demonstrate a preferential esterification of [1-^{14}C] LC-PUFA into rotifer phospholipids, supporting their use as LC-PUFA vehicle to fish larvae. Differently, *Artemia sp.* show not just a preferential catabolism over dietary LC-PUFA, and more precisely over DHA, but also a higher esterification of this FA into TAG (Reis et al., 2017, 2019), even when provided as phospholipid (Guinot et al., 2013). Despite the reduced incorporation of DHA into rotifer TL, it was preferentially esterified into PC and PE, which is aligned with the needs recently described for fishes and cephalopods larvae (Li and Olsen, 2015; Li et al., 2014; Olsen et al., 2014; Reis et al., 2014, 2016, 2020). Nonetheless, a high esterification rate of [1-^{14}C]LA and [1-^{14}C]ALA into rotifers PC was also noted. Considering the low specificity of acylases and transacylases enzymes for dietary FAs (Sargent et al., 1999), the DHA content in rotifers might be partly determined by a competition with dietary 18C PUFAs, which in addition to the low incorporation of [1-^{14}C]DHA, may lead to an inadequate balance of this FA for marine fish larvae.

A similar consideration might be also done regarding the enrichment of rotifers with ARA and EPA, due to the preferential esterification of both FAs into PI. ARA is known to have a high affinity for PI in vertebrates, including fish (Sargent et al., 2002; Tocher et al., 2008). Therefore, the preferential esterification of ARA into PI observed in *B. plicatilis*, encourages the suitability of rotifers for the daily supply of this essential FA to fish larvae. A higher proportion of EPA over ARA is common in marine organisms, where EPA is mostly esterified into PE or into PC (Reis et al., 2014, 2017, 2020). Contrarily, EPA was preferentially esterified into PI (34.8%) rather than into PC or PE (14.0% and 12.8%, respectively) in the present study. A dietary imbalance of ARA/EPA or n-6/n-3 PUFA ratios has been reported to be critical for fish larvae proper development (Carrier III et al., 2011; Schmitz and Ecker, 2008). An increase in the dietary ARA content showed beneficial effects on larval performance (Atalah et al., 2011), recovery from infection (Khozin-Goldberg et al., 2006) and adaptive physiological response to hypersalinity stress and hypo-osmoregulatory ability (Carrier III et al., 2011). However, a further increase in ARA content and concomitantly ARA/EPA ratio, seems to contradict the described positive effects (Atalah et al., 2011; Furuita et al., 2003; Khozin-Goldberg et al., 2006) being also associated with flatfish malpigmentation and impaired eye migration (Hamre and Harboe, 2008a, 2008b). This might be possibly related to a potential inhibitory effect of ARA on EPA incorporation as stated for *S. aurata* larvae TL (Atalah et al., 2011), *Paralichthys olivaceus* eggs polar lipids (Furuita et al., 2003) or adult *O. vulgaris* tissues (Miliou et al., 2006), where an inverse relationship between ARA and EPA incorporation was reported. Taking into account the similar incorporation and esterification pattern of ARA and EPA into rotifers TL, and the previously mentioned low specificity of acylases and transacylases enzymes for dietary FAs, the ARA/EPA and n-6/n-3 ratios in rotifers should be carefully considered in live prey enrichment protocols, in order to meet specific fish larvae requirements.

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

Rotifers are passive, non-selective filtering organisms, which allows an easy manipulation of their nutritional value through enrichment techniques (Conceição et al., 2010). Nonetheless, the results of the present study showed the capacity of rotifers to elongate and desaturate dietary FA, being able to biosynthesize LC-PUFA like EPA, DHA and ARA from their C18 FA precursors. The biosynthesis of PUFA by *B. plicatilis* was initially suggested by Lubzens et al. (1985) through their incubation with [1-^{14}C] acetic acid. More recently, functional characterization of ωx desaturases genes revealed the existence of a Δ12 and ω3 (Δ15, Δ17 and Δ19) desaturases, in a wide range of invertebrate species including the rotifer *Adineta vaga* (Kabeya et al., 2018), the polychaeta *Hediste diversicolor* (Kabeya et al., 2020) and the cephalopod *O. vulgaris* (Garrido et al., 2019), indicating that invertebrates can be one of the major contributors to FA trophic upgrading in aquatic food webs. To our knowledge, this is the first *in vivo* study evidencing the ability of *B. plicatilis* to elongate/desaturate [1-^{14}C]C18 FAs to yield LC-PUFAs, and even to desaturate OA to form LA. According to Kabeya et al. (2018), the incubated OA might be converted into LA by means of a Δ12

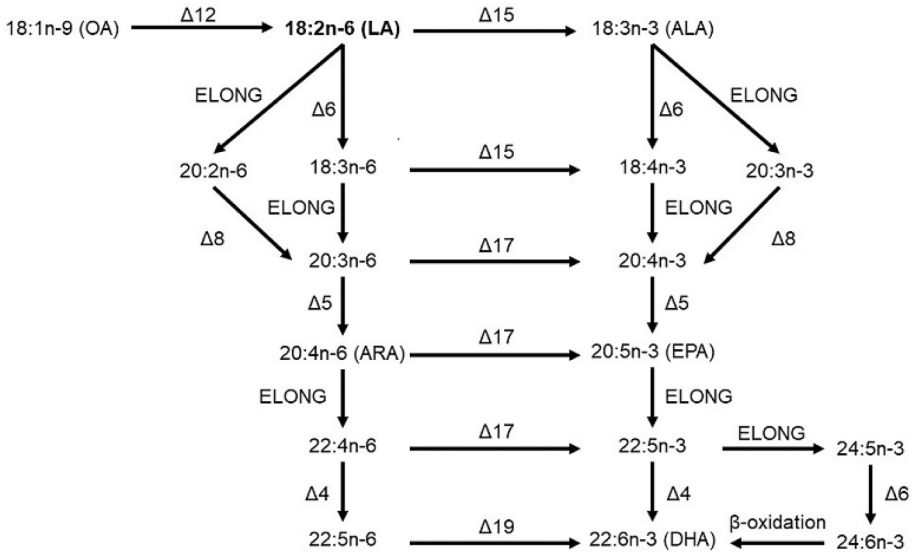

Fig. 1.. Possible biosynthetic pathway of long-chain polyunsaturated fatty acids in the rotifer *Brachionus plicatilis*. ELONG, elongases.

desaturase, while ω3 desaturase enzyme activities would enable the conversion of n-6 PUFA into n-3 PUFA (*e.g.* conversion of LA into ALA or ARA into EPA). In the present study, the radioactive bands corresponding to both LA and ALA positions found when *B. plicatilis* were incubated with [1-^{14}C]OA, confirm the activity of Δ12 and Δ15 desaturases, supporting the wide distribution of both enzymes across aquatic invertebrates (Garrido et al., 2019; Kabeya et al., 2018, 2020).

Interestingly, the position of the radioactive bands of the standard mixture suggests that ARA, EPA and DHA also seemed to be synthesized not only from LA or LNA, but also when incubations were performed with [1-^{14}C]OA, indicating the presence of active Δ6 and Δ5 desaturases enzymes. However, the *in vivo* activity of Δ4 and Δ8 desaturases or other ω3 desaturases, such as Δ17 or Δ19 reported for *A. vaga* (Kabeya et al., 2018) cannot be completely discarded (Fig. 1). The detected activities in *B. plicatilis* would theoretically favour the production of n-3 LC-PUFA in rotifer tissues, and consequently, the attainment of marine fish larvae DHA and EPA requirements, even when enriched with vegetable oils (rich in LA and ALA). Although this would boost the use of terrestrial oils to partially reduce the utilization of fish oils in larviculture contributing to aquaculture sustainability (Hardy, 2010), the biosynthesis rate detected was extremely low with only 0.7% and 0.3% of incorporated [1-^{14}C]ALA being recovered as EPA and DHA, respectively.

5. Conclusions

Rotifers present an active metabolism over dietary FA, being able to produce LC-PUFA from their C18 FA precursors. However, the low transformation rates obtained in addition to the poor incorporation of DHA, and the competitiveness between DHA, LA and ALA for its esterification into PC, or between EPA and ARA into PI, could produce rotifers with unbalanced essential fatty acid ratios for marine fish larval nutritional needs. The lipid metabolic ability of *B. plicatilis* must be considered in order to improve the efficiency of lipid enrichment protocols for this species. Thus, it is advisable to supply *B. plicatilis* with food not only rich in DHA but also in OA and ALA, as valuable omega-3 LC-PUFA precursors, to enhance rotifer's nutritional value for marine fish larvae.

CRediT authorship contribution statement

José A. Pérez: Formal analysis, Investigation, Writing – review & editing. **Diana B. Reis:** Formal analysis, Investigation, Writing – original draft. **Daniel Ramírez:** Investigation. **Nieves G. Acosta:** Methodology, Resources. **Roberto Dorta-Guerra:** Formal analysis, Writing – review & editing. **Salvador Jerez:** Methodology, Resources, Writing – review & editing. **Covadonga Rodríguez:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

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

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