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Composition and metabolism of phospholipids in *Octopus vulgaris* and *Sepia officinalis* hatchlings

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ABSTRACT

The objective of the present study was to characterise the fatty acid (FA) profiles of the major phospholipids, of *Octopus vulgaris* and *Sepia officinalis* hatchlings, namely phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE); and to evaluate the capability of both cephalopod species on dietary phospholipid remodelling. Thus, *O. vulgaris* and *S. officinalis* hatchlings were *in vivo* incubated with 0.3 μM of L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PC or L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PE. Octopus and cuttlefish hatchlings phospholipids showed a characteristic FA profiles with PC presenting high contents of 16:0 and 22:6n-3 (DHA); PS having high 18:0, DHA and 20:5n-3 (EPA); PI a high content of saturated FA; and PE showing high contents of DHA and EPA. Interestingly, the highest content of 20:4n-6 (ARA) was found in PE rather than PI. Irrespective of the phospholipid in which [1-^{14}C]ARA was initially bound (either PC or PE), the esterification pattern of [1-^{14}C]ARA in octopus lipids was similar to that found in their tissues with high esterification of this FA into PE. In contrast, in cuttlefish hatchlings [1-^{14}C]ARA was mainly recovered in the same phospholipid that was provided. These results showed a characteristic FA profiles in the major phospholipids of the two species, as well as a contrasting capability to remodel dietary phospholipids, which may suggest a difference in phospholipase activities.

1. Introduction

The common octopus (*Octopus vulgaris*) and the European cuttlefish (*Sepia officinalis*) are two species of cephalopods that have been recognised with a great potential for aquaculture (Navarro et al., 2014; Pierce and Portela, 2014; Sykes et al., 2014). However, the limited knowledge regarding the nutritional physiology of these species during their early life stages has been hampering their industrial large-scale culture (Iglesias and Fuentes, 2014; Sykes et al., 2014; Villanueva et al., 2014).

Cephalopods have a high protein level and low lipid content (Lee, 1994) but, despite that, lipids were defined as crucial in cephalopod nutrition, with long-chain polyunsaturated fatty acids (LC-PUFA), cholesterol and phospholipids being suggested to play critical roles in their development (Navarro and Villanueva, 2000, 2003; Almansa et al., 2006). Within LC-PUFA, 22:6n-3 (DHA), 20:5n-3 (EPA) and also 20:4n-6 (ARA) were recently defined as essential fatty acids (EFA; Monroig et al., 2012a, 2016; Reis et al., 2014, 2016). The latest studies have highlighted the importance of an adequate dietary input of those EFAs, considering not only their amount and ratios (Reis et al., 2014, 2016) but also their lipid form (Guinot et al., 2013a), as this could affect the availability of the EFA for specific functions and tissue structures.

To ensure a high availability of EFA, dietary fatty acids (FA) contents and lipid class molecular species should reflect larval requirements, which may be extrapolated from egg yolk lipid profile (Sargent et al., 1999) or the hatchlings polar lipids (PL) composition (Sargent et al., 1999; Olsen et al., 2014). Eggs of *O. vulgaris* and *S. officinalis* show low lipid contents with high proportions of PL with phosphatidylcholine (PC) being the main lipid class (Sykes et al., 2009; Quintana et al., 2015). Similarly to eggs, *O. vulgaris* and *S. officinalis* hatchlings generally have higher PL than neutral lipids (NL) content (Bouchaud and Galois, 1990; Navarro and Villanueva, 2000; Quintana et al., 2015), with the former presenting a high proportion of polyunsaturated fatty acids (PUFA) and the latter a high percentage of monounsaturated fatty acids (Sinanoglou and Miniadis-Meimaroglou, 1998; Viciano

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et al., 2011). However, the specific FA profiles of the major phospholipid classes have not been determined for the hatchlings of these two species, which could provide important information regarding the design of a suitable diet for these species development during their first life stages.

In this sense, the aims of the present study were to determine the FA profiles of the major phospholipid classes of *O. vulgaris* and *S. officinalis*, and to evaluate the capability of these species to remodel dietary phospholipids. To this end, the capability of these species for de-acylation and re-acylation of ARA (as a commercial available EFA model), initially bound to PC or phosphatidylethanolamine (PE), was investigated.

2. Material and methods

2.1. Experimental animals

An *O. vulgaris* broodstock (30 individuals) was caught by professional artisanal fishermen on the Tenerife coast (Canary Islands, Spain) and maintained at the Oceanographic Centre of Canary Islands (Spanish Institute of Oceanography; Santa Cruz de Tenerife - 28°29′59.1″N; 16°11′44.54″W). The broodstock rearing conditions were similar to those described by Reis et al. (2014). The presence of eggs was checked once a week and when egg masses were observed, the ovate female was isolated in the tank by removing the other individuals. After approximately one month of embryonic development the eggs began to hatch and hatchlings were removed daily from the female rearing tank, to provide newly hatched octopus (less than 24 h old) for experiments.

All cuttlefish used in the present study were from a single brood obtained from *F*4 cultured females reproducing at the Ramalhete Aquaculture Station (Ria Formosa, South of Portugal - 37°00′22.39″N; 7°58′02.69″W). Twenty-four hour laid eggs were transported to the Spanish Institute of Oceanography facilities in Tenerife and maintained in a 100 L circular fiberglass tank in a flow-through seawater system at 21 ± 0.69 °C. The embryonic development of eggs and the broodstock rearing followed procedures described by Sykes et al. (2014). Eggs were maintained under rearing conditions similar to those described in Reis et al. (2016). The use of animals in the experiments of this work were in accordance with the EU Directive 2010/63/EU for animal experimentation.

2.2. FA composition of phospholipids in cephalopod hatchlings

Approximately, 500 mg of *O. vulgaris* hatchlings and 300 mg of *S. officinalis* hatchlings, obtained from individuals previously euthanized in iced seawater (−2 °C) and stored at −80 °C, were used for lipid extraction (n = 3). Extraction of total lipid (TL) was performed with chloroform/methanol (2:1 by volume) according to the Folch method, as described by Christie (2003) and lipid content was determined gravimetrically. The TL extracts were stored at −20 °C in chloroform/methanol (2:1 by volume) with 0.01% butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10 mg/mL and under a nitrogen atmosphere until analysis. In order to determine the FA composition of PC, PE, phosphatidylserine (PS) and phosphatidylinositol (PI) of *O. vulgaris* and *S. officinalis* hatchlings, aliquots of 5 mg of TL extract were spotted on 20 cm × 20 cm thin-layer chromatography (TLC) silica plates. Polar lipid classes were separated by one-dimension single-development with 1-propanol/chloroform/methyl acetate/methanol/0.25% KCL (25:25:25:10:9 by volume). The phospholipid classes were visualized under UV light after brief exposure to dichlorofluorescein. Each phospholipid class band was scraped from the TLC plates and subjected to direct acid-catalysed transmethylation on silica during 16 h at 50 °C to obtain fatty acid methyl esters (FAME). FAME were purified by TLC (Christie, 2003) with hexane/diethyl ether/acetic acid (90:10:1 by volume) and then separated and analysed using a TRACE-GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) equipped with an on-column injector, a flame ionization detector (240 °C) and a fused silica capillary column (Supelcowax™ 10; Sigma-Aldrich Co., St. Louis, Missouri, USA). The column temperature was programmed for four different ramps of temperature: 1st ramp was programmed for a linear increase of 40 °C per minute from 50 to 150 °C; the 2nd ramp for a linear increase of 2 °C per minute until 200 °C; the 3rd ramp for a linear increase of 1 °C per minute until 214 °C; and the 4th ramp for a linear increase of 40 °C per minute until 230 °C and hold at that temperature for 5 min. FAME were identify by comparison with retention times of a standard mixtures with F.A.M.E Mix C4-C24 (Supelco 18,919-1AMP) diluted to 2 mg/mL in hexane, PUFA No. 3 from menhaden oil (Supelco 47085-U) diluted to 2 mg/mL in hexane; and cod roe FAME. When necessary, identification of individual FAME was confirmed by GC–MS chromatography (DSQ II, Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA).

2.3. In vivo incubations of hatchlings with [1-^{14}C]ARA esterified in the sn-2 position of phosphatidylcholine and phosphatidylethanolamine

A total of 550 *O. vulgaris* and 11 *S. officinalis* hatchlings were incubated following an adaptation of the method of Reis et al. (2014, 2016) to determine the *in vivo* [1-^{14}C]FA metabolism in these species. Incubations (n = 4 for each species) were performed in flat-bottom 6-well tissue culture plates (SARSTEDT AG & CO., Nümbrecht, Germany), at a density of 50 or 1 hatchling/well for *O. vulgaris* and *S. officinalis*, respectively, in 10 mL of filtered seawater (36‰), which was gently stirred at 21 °C for 5 h, supplemented with 0.2 μCi (0.3 μM) of L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PC or L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PE dissolved in 5 μL of ethanol. [1-^{14}C]ARA was used as a commercial available EFA model. Labelled phospholipid classes were individually added to separate wells. Control treatments (n = 3) of hatchlings of either species corresponded to a similar protocol but without the addition of labelled phospholipid. In any case, a 100% survival rate was registered.

After incubation, hatchlings were immediately euthanized in iced seawater (−2 °C) and thoroughly washed with filtered seawater to remove excess [1-^{14}C] phospholipid. Samples were stored at −80 °C until analysis. Extraction of total lipids was performed as mentioned in Section 2.2.

2.4. Lipid composition of control samples

Aliquots of 20 μg of TL extracts of hatchling control groups were used to determine lipid class composition (n = 3). Lipid classes (LC) were separated by one-dimensional double-development with 1-propanol/chloroform/methyl acetate/methanol/0.25% KCL (5:5:5:2:1.8 by volume) for polar lipid classes separation and hexane/diethyl ether/acetic acid (22.5:2.5:0.25 by volume) for neutral lipid classes separation on 10 cm × 10 cm high-performance thin-layer chromatography (HPTLC), and analysed by charring followed by calibrated densitometry using a dual-wavelength flying spot scanner CS-9001PC (Shimadzu Co., Kyoto, Japan; Tocher and Harvie, 1988). Identification of individual LC was performed by running known standards (cod roe lipid extract and a mixture of single standards from BIOSIGMA S.r.l., Venice, Italy) on the same plates.

FAME were obtained by acid-catalysed transmethylation of 1 mg of TL extracts. FAME were purified and analysed as described in Section 2.2.

2.5. Incorporation of radioactivity into total lipids

In order to determine the radioactivity incorporated into hatchling TL, an aliquot of 0.1 mg of TL extract was transferred to scintillation vials and radioactivity quantified in disintegrations per minute (dpm) in a LKB Wallac 1214 Rackbeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, USA). Results in dpm were converted into pmoles by, Total dpm (specific activity of each