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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-[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.

Key words: Hatchlings; Metabolism; *Octopus vulgaris*; Phospholipids; *Sepia officinalis*.

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 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 - 28º29’59.1’’N; 16º11’44.54’’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 F4 cultured females reproducing at the Ramalhete Aquaculture Station (Ria Formosa, South of Portugal - 37º00'22.39''N; 7º58'02.69''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 ºC. 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, 400 *O. vulgaris* hatchlings (500 mg) and 3 *S. officinalis* hatchling (300 mg), obtained from individuals previously euthanized in iced seawater (-2 ºC) and stored at -80 ºC, 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 ºC 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 x 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 16-18 h at 50 ºC 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 ºC) 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 ºC per minute from 50 to 150 ºC; the 2nd ramp for a linear increase of 2ºC per minute until 200ºC; the 3rd ramp for a linear increase of 1ºC per minute until 214ºC; and the 4th ramp for a linear increase of 40ºC per minute until 230ºC and hold at that temperature for 5 minutes. FAME were identify by comparison with retention times of a standard mixtures with F.A.M.E Mix C4-C24 (Supelco 18919-1AMP) 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 ºC 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 ºC) and thoroughly washed with filtered seawater to remove excess [1-^{14}C] phospholipid. Samples were stored at -80 ºC until analysis. Extraction of total lipids was performed as mentioned in 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 x 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 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 substrate*2.22)$^{-1}$ where Total dpm value was obtained by multiplying dpm of 0.1 mg of TL extract by samples total lipid content and the fact that 1µCi correspond to 2 220 000 dpm. Pmoles were later divided by the protein content of samples (n=4) determined according to Lowry et al., (1951) and hours of incubation. Results of radioactivity incorporated into cephalopods TL are presented in pmol mg protein^{-1} h^{-1}.

2.6. Remodelling of radiolabelled FA into lipid classes

An aliquot of 0.1 mg of hatchlings TL was taken for determine the esterification pattern of [1-^{14}C]ARA into the different LC. Lipid classes were separated by one-dimensional double-development HPTLC as previously described 2.4. Developed HPTLC plates were placed for 2 weeks in closed exposure cassettes (Exposure Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Imagen Screen-K, Biorad, Madrid, Spain). The screens were then scanned with Molecular Imager FX image acquisition system (BioRad, Madrid, Spain), and the bands were quantified using the Quantity One image analysis software (BioRad, Madrid, Spain). Identification of labelled

bands was confirmed by radiolabelled standards simultaneously run on the same plate (Rodríguez et al., 2002).

2.7. Materials

L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PC and L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PE were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri, USA). TLC plates (20 × 20 cm × 0.25 mm) were purchased form Macherey-Nagel GmbH & Co. KG (Düren, Germany). HPTLC plates, (10 × 10 cm × 0.15 mm) pre-coated with silica gel 60 (without fluorescent indicator), were purchased from Merck KGaA (Düsseldorf, Germany). OptiPhase "HiSafe" 2 scintillation cocktail was purchased from PerkinElmer, Inc. (Waltham, Massachusetts, USA). Organic solvents used were of reagent grade and were purchased from Merck KGaA (Düsseldorf, Germany), Sigma-Aldrich Co. (St. Louis, Missouri, USA) and Panreac Química S.L.U. (Barcelona, Spain).

2.8. Statistical analysis

Results are presented as means ± SD. For all statistical tests, $p < 0.05$ was considered as significantly different. Data were checked for normal distribution with the one-sample Shapiro-Wilk test, as well as for homogeneity of the variances with the Levene test (Zar, 1999). Arcsine square root transformation was applied to all data expressed as percentage (Fowler et al., 1998). Differences between FA content in the main phospholipids within a same species, were analysed by a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test (Zar, 1999). When normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Welch robust ANOVA, followed by a Games-Howell non-parametric multiple comparison test (Zar, 1999). Differences between *O. vulgaris* and *S. officinalis* compositions, either for a specific LC (PC, PS, PI or PE) content or FA compositions, as well as for radioactivity incorporation into hatchlings TL, were analysed by Student´s *t*-test (Zar, 1999). Statistical analysis was performed using the IBM SPSS statistics 22.0 (IBM Co., USA).

3. Results

3.1. FA compositions of phospholipids in cephalopods hatchlings

The main FAs detected in PC of octopus and cuttlefish hatchlings were 16:0 (~33% of total FAs in octopus and ~30% in cuttlefish) and DHA (~30% in octopus and 21.5% in

cuttlefish). In phosphatidylserine (PS), 18:0 followed by DHA and EPA were the main FAs in both species (Table 1). Within the analysed phospholipids, phosphatidylinositol (PI) presented a higher content of saturated FAs, representing over 60% of total FAs, with only 5% and 3% of DHA being detected in PI of *O. vulgaris* and *S. officinalis* hatchlings, respectively. High contents of n-3 FA, LC-PUFA and 16:0 DMA and 18:0 DMA were detected in PE of both species (Table 1). While in octopus hatchlings ARA was mainly found in PE followed by PS, in cuttlefish ARA showed similar percentages in both PE and PS. EPA was mainly found in PE in both species. High n-3/n-6 PUFA ratios were detected in PC of *O. vulgaris* hatchlings (~12) and in PC and PE of *S. officinalis* hatchlings (~13 and 12, respectively), while the lower values were found in PI of both species (2.7 in octopus and 1.9 in cuttlefish). The EPA/ARA ratio in phospholipids was generally higher in *S. officinalis*, especially in PC and PE (Table 1). In *O. vulgaris*, the lowest EPA/ARA ratios were detected in PS and PE. With the exception of octopus PC, where DHA/EPA ratio was clearly higher (3.7), this ratio in the different classes of both species ranged between 0.6 and 1.8 (Table 1).

3.2. Hatchling lipid compositions

The TL contents and LC compositions of *O. vulgaris* and *S. officinalis* hatchlings are presented in Table 2. Both species presented a similar profile ($p > 0.05$). Within polar lipids, PC (21.6 ± 1.2% in octopus and 22.6 ± 2.7% in cuttlefish) and PE (23.4 ± 1.1% in octopus and 22.5 ± 1.7% in cuttlefish) were the major classes, whereas cholesterol (31.6 ± 2.5% in octopus and 33.1 ± 1.8% in cuttlefish) was the major neutral lipid class and the most abundant lipid class.

In contrast to the LC composition, the FA profile of total lipids of the two species presented several differences (Table 3). The main differences detected were in the content of ARA (5.2 ± 0.3% in *O. vulgaris* and 1.2 ± 0.2% in *S. officinalis* hatchlings), and as a consequence, in total n-6 PUFA, and n-3/n-6 and EPA/ARA ratios ($p < 0.05$). On the other hand, both species presented similar proportions of total n-3 PUFA, EPA and DHA, and therefore, a similar DHA/EPA ratio.

3.3. Incorporation of radiolabelled ARA into hatchlings total lipids and its distribution among lipid classes

The incorporation of the radiolabelled ARA, originally esterified into PC or PE, was higher in *O. vulgaris* hatchlings ($p < 0.05$). The esterification pattern of [1-^{14}C]ARA

supplied as PC or PE into the different lipid classes of *O. vulgaris* hatchlings was generally similar, with differences only being detected for free fatty acids (FFA), partial acylglycerols and PC ($p < 0.05$; Table 4). Despite being provided in two different forms (esterified into PC or PE), in octopus [1-^{14}C]ARA was preferentially re-esterified into PE (54.7 ± 3.2% when added as PC and 56.2 ± 2.7% when added as PE). On the other hand, in *S. officinalis* hatchlings, when [1-^{14}C]ARA was provided esterified either into PC or PE a higher amount of the radioactivity incorporated was recovered re-esterified in the same class of origin: 73.3 ± 5.7% was recovered esterified into PC when added as PC, and 46.3 ± 3.3% was recovered as PE when added as PE. These results show a clear difference in the esterification patterns of [1-^{14}C]ARA when incubated as PC or PE into LC of both species ($p < 0.05$).

4. Discussion

The FA profiles for individual phospholipids of octopus and cuttlefish were generally similar in both species. According to Tocher et al. (2008), this pattern is related to the roles of specific phospholipid classes in membrane structure and function. Fish phospholipid FA profiles are characterised by a high proportion of 16:0 and a relatively lower LC-PUFA content in PC; an intermediate level of saturated FA and monounsaturated FA and high levels of C20 and C22 PUFA in PE; PS is characterised by high 18:0 and C22 LC- PUFA; and PI also present a high 18:0 and relatively lower LC-PUFA but with a particularly high content of ARA (Tocher et al., 1995). In the present study, all phospholipid classes showed a generally high LC-PUFA content, except for PI that showed a higher content of saturated FA in both octopus and cuttlefish hatchlings. In the latter, these FAs represented over 60% of the total FA content with 16:0 being predominant. Several authors previously reported selective location/retention of ARA in PI in fish tissues (Bell and Tocher, 1989; Bell and Dick, 1990; Tocher et al., 1995; Bell et al., 1997; Sargent et al., 2002). Interestingly, *O. vulgaris* showed a higher content of ARA in PE, while *S. officinalis* hatchlings had a similar percentage of this FA in PE and PS. Nonetheless, considering the high level of PE in *S. officinalis* lipid, the absolute amount of ARA in PE would be much higher than in PS. The preferential esterification of ARA into PE of cephalopods has already been observed in studies of *in vivo* FA metabolism (Reis et al., 2014, 2016), which may indicate an important role of ARA in PE in these species.

Similar to fish (Tocher et al., 1995), PE was the phospholipid with highest LC-PUFA content and showing a DHA/EPA ratio of around 1. Moreover, this phospholipid class had the highest EPA content, which was consistent with the preferential esterification of [1-^{14}C]EPA into octopus and cuttlefish PE as reported by Reis et al. (2014, 2016). Sargent et al. (2002) reported that the highest DHA levels in fish tissues were usually found in PE, similar to what was found in the present study in the two cephalopod species. However, a similar high level of DHA was also found in PC (30% and 22% for *O. vulgaris* and *S. officinalis* hatchlings, respectively). These data are also in agreement with the results obtained from *in vivo* esterification studies on the two cephalopod species, where preferential esterification of [1-^{14}C]DHA into PC was detected (Reis et al., 2014, 2016).

Despite the similarities between *O. vulgaris* and *S. officinalis* phospholipid profile (Table 1) and lipid class composition (Table 2), the total lipid FA profile of these species presented several differences, with the major one being a higher ARA content in octopus, which influenced similar differences in total n-6 FA content and EPA/ARA ratio. It was previously shown, that different to *S. officinalis* (Reis et al., 2016), *O. vulgaris* hatchlings show a preferential incorporation of ARA (Reis et al., 2014), which could reflect different requirements for this FA between species. ARA appears to have an important physiological role in *O. vulgaris* development (Estefanell et al., 2011; García-Garrido et al., 2010; Milliou et al., 2006; Monroig et al., 2012a, 2012b), with a high content of this FA being found in *O. vulgaris* brain, corresponding to 15.2% of brain total FA composition (the third most significant FA), while EPA accounted for 11.8% and DHA for 18.7% (Monroig et al., 2012a). In contrast, n-6 PUFA accounted for less than 1% in the central nervous system of *S. officinalis* (Dumont et al., 1992), indicating that these species possibly have different requirements for ARA.

The present results showed a higher content of EFA in PE. Considering the apparent low specificity of acylases and transacylases, enzymes responsible for FA esterification into phospholipids (Sargent et al., 1999), and the pattern of FA distribution among phospholipids detected in the present and previous *in vivo* FA metabolism studies (Reis et al., 2014, 2016), the dietary ratio of EFA and the lipid class in which they are esterified might be crucial in cephalopods. It is assumed that de-acylation/re-acylation turnover processes have an important role in maintaining the characteristic FA esterification pattern among lipid classes (Tocher et al., 2003). This turnover process is highly

influenced by dietary FA profile and by the endogenous capability of the organism to complete this process (Olsen et al., 2014).

In vivo metabolic studies on fish larvae have been extensively performed using tracers, existing four basic methods to deliver tracers to larva: micro-diet labelling, live food labelling, tube feeding and uptake from water (Conceição et al., 2007). Formulated diets have been mostly neglected by cephalopods and basal metabolic studies using labelled live food would be extremely difficult given preys own metabolism (Guinot et al., 2013a, 2013b). Tube feeding methodology have been adapt to a large number of fish larvae species (see Conceição et al., 2007). However, its use in cephalopods metabolism studies, would be constrained by the existence of a strong beaks in the buccal mass (Messenger and Young, 1999), which could break the feeding tube, and the lack of appropriate anaesthesia agents and methods for the younger stages (Fiorito et al., 2015; Sykes and Gestal, 2014). In this sense, radiolabelled uptake from water might be an alternative to study the *in vivo* capability of these species for phospholipid turnover and remodelling during their early life stages. The way nutrients are incorporated is not completely understood, as cephalopods have the apparent capacity to absorb nutrients through skin during the first stage of life (Boucaud-Camou and Roper, 1995; de Eguileor et al., 2000; Villanueva et al., 2004). Therefore, there is the possibility that the present results are due to incubated phospholipid classes entering via the skin, encountering a completely different set of enzymes for phospholipids de-acylation and re-acylation to that of the gut. Nonetheless, it seems important to highlight the resemblance between the FA composition of the different phospholipids (Table 1) and the results obtained by Reis et al. (2014, 2016) at *in vivo* free FA metabolism studies, which clear show that the method use is consistent in its results.

Regardless of the phospholipid class to which [1-^{14}C]ARA was bound (PC or PE), a higher incorporation of radioactivity was observed into *O. vulgaris* hatchlings lipids. These results might be a consequence of the preferential incorporation of ARA into octopus total lipid, as reported by Reis et al. (2014). The esterification pattern obtained after re-acylation in *O. vulgaris* lipids was similar to that reported by those authors, who detected higher esterification of [1-^{14}C]ARA into PE. In contrast, in *S. officinalis* hatchlings, ARA was mainly recovered in the same phospholipid class that was provided, that is, when [1-^{14}C]ARA was provided bound to PC, up to 70% of the incorporated ARA was recovered as PC, and when [1-^{14}C]ARA was provided bound to PE 45% of this FA

was recovered as PE. The present results suggest a different mechanism in *O. vulgaris* and *S. officinalis* biosynthesis of new phospholipids.

It is generally believed that PLA_2 hydrolyses phospholipids at the sn-2 position forming FFA and lysophospholipids (see Olsen et al., 2014) and that LC-PUFA are esterified at the sn-2 position of phospholipid molecules (Sargent et al., 1999). In *O. vulgaris* hatchlings, after the ARA re-acylation process, the esterification pattern obtained was highly similar, with the majority of ARA being recovered as PE, suggesting the existence of a high PLA_2 activity. The *S. officinalis* turnover data indicate an apparent lower PLA_2 enzyme activity when compared to *O. vulgaris* hatchlings. In fish, PLA_2 activity tends to increase during development, with seabream and sea bass larvae showing no PLA_2 activity during very early larval stages (Izquierdo and Henderson, 1998; Zambonino Infante and Cahu, 2001). Both octopus and cuttlefish possess inner yolk reserves that could last for a few days after hatching (Sykes et al., 2004; Boletzky and Villanueva, 2014) and it is known that it can take up to 30 days for the digestive system to fully mature in cuttlefish (Boucaud-Camou and Yim 1980; Yim and Boucaud-Camou 1980; Boucaud-Camou 1982). Nonetheless, these species may feed on size-appropriate prey from day 1 (Hanlon and Messenger, 1988), when mixed feeding overlaps inner yolk reserves and external food consumption (Boletzky, 1974; Sykes et al., 2013). It has been suggested that diet may influence digestive enzyme activity (Koven et al., 2001; Villanueva et al., 2002; Perrin et al., 2004), and it is known that PLA_2 activity can be stimulated by increased dietary phospholipids in fish (Zambonino Infante and Cahu, 2001). In the present study, cuttlefish individuals were unfed, which could explain the low turnover rate recorded. On the other hand, octopus hatchlings under similar feeding conditions displayed a high turnover rate. The differences regarding both species phospholipid metabolism detected here might be related with the different morphologies at hatching (paralarvae vs. hatchling) derived from different embryonic developments (indirect vs. direct) that translate into different lifestyles of these species (pelagic vs. benthonic) during their first live stages (Halon and Messenger, 1996; Young and Harman, 1988) and/or different eggs origins (see Bouchaud and Galois et al., 1990).

A small proportion of the incorporated [1-^{14}C]ARA was also recovered esterified into monoacylglycerols and diacylglycerols, that are involved in *de novo* synthesis of phospholipids and TAG (see Tocher et al., 2008 and Olsen et al., 2014). Interestingly, no [1-^{14}C]ARA was found re-esterified into TAG, the main class for lipid storage and energy provision in fish (Tocher et al., 2003). Nonetheless, *O. vulgaris* and *S. officinalis*

hatchlings normally show only low TAG levels representing 1.8 ± 0.5% of total lipid in *O. vulgaris* and 3.3 ± 2.6% in *S. officinalis*. Low TAG in these species was also previously recorded not just in hatchlings (Navarro and Villanueva, 2000; Reis et al., 2014, 2015, 2016), but also in eggs (Boucaud and Galois, 1990; Sykes et al., 2009; Quintana et al., 2015), juveniles and adult tissues (Almansa et al., 2006; Valverde et al., 2012).

The present study showed that, despite the general similarity between *O. vulgaris* and *S. officinalis* lipid compositions, these species may have different ARA requirements. Both octopus and cuttlefish hatchlings showed characteristic FA profiles for the major phospholipids, with PC presenting a high content of 16:0 and DHA; PS of 18:0, DHA and EPA; PI a high content of saturated FA; and PE a high content of DHA and EPA. The highest content of ARA was found in PE rather than PI. The present results suggest a different capacity of both species for phospholipids remodelling, which may suggest a difference in phospholipase activities.

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Table 1 - Fatty acid composition (% total FA) of PC, PS, PI and PE of *O. vulgaris* and *S. officinalis* hatchlings

	Octopus vulgaris				*Sepia officinalis*			
	PC	PS	PI	PE	PC	PS	PI	PE
16:0	33.4 ± 0.6 ^{a}	8.2 ± 0.6 ^{b}	47.8 ± 1.3 ^{a}	7.1 ± 0.3 ^{b}	30.3 ± 0.3 ^{A*}	7.2 ± 0.3 ^{B}	32.0 ± 7.5 ^{A*}	5.9 ± 0.7 ^{C}
16:0 DMA	0.0 ± 0.0 ^{c}	0.3 ± 0.0 ^{b}	0.0 ± 0.0 ^{c}	1.7 ± 0.0 ^{a}	0.1 ± 0.0 ^{B}	0.0 ± 0.0 ^{C*}	0.0 ± 0.0 ^{C}	1.9 ± 0.1 ^{A}
18:0	4.4 ± 0.1 ^{c}	27.0 ± 1.0 ^{a}	12.3 ± 0.7 ^{b}	10.4 ± 0.3 ^{b}	5.4 ± 0.1 ^{D*}	25.8 ± 1.8 ^{A}	16.8 ± 5.1 ^{B}	10.0 ± 0.3 ^{C}
18:0 DMA	0.0 ± 0.0 ^{c}	2.6 ± 0.1 ^{b}	0.5 ± 0.1 ^{c}	12.0 ± 0.4 ^{a}	0.1 ± 0.0 ^{C*}	0.7 ± 0.0 ^{B*}	0.0 ± 0.0 ^{C}	11.5 ± 0.5 ^{A}
Total saturated	41.6 ± 0.7 ^{b}	41.4 ± 1.6 ^{bc}	66.4 ± 0.1 ^{a}	33.8 ± 0.8 ^{c}	43.5 ± 0.7 ^{B*}	40.4 ± 2.0 ^{C}	63.9 ± 12.4 ^{A}	34.8 ± 1.4 ^{D}
16:1 ^{1}	1.1 ± 0.2 ^{a}	0.5 ± 0.1 ^{b}	1.8 ± 0.8 ^{a}	0.3 ± 0.0 ^{b}	0.5 ± 0.0 ^{C}	0.7 ± 0.1 ^{B}	5.2 ± 2.4 ^{A}	0.5 ± 0.1 ^{C*}
18:1(n-13)	2.8 ± 0.0 ^{a}	2.6 ± 0.3 ^{a}	0.3 ± 0.1 ^{c}	0.8 ± 0.0 ^{b}	1.7 ± 0.1 ^{A*}	1.8 ± 0.2 ^{A*}	0.0 ± 0.0 ^{C*}	0.6 ± 0.0 ^{B*}
18:1(n-9)	4.0 ± 0.0 ^{a}	1.3 ± 0.1 ^{b}	1.7 ± 0.9 ^{b}	0.8 ± 0.0 ^{b}	5.3 ± 0.1 ^{A*}	3.4 ± 1.2 ^{B*}	7.6 ± 4.1 ^{AB*}	1.0 ± 0.3 ^{C}
20:1(n-9)	3.9 ± 0.1 ^{b}	6.0 ± 0.6 ^{a}	1.4 ± 0.3 ^{c}	3.9 ± 0.0 ^{b}	3.2 ± 0.1 ^{B*}	3.7 ± 0.1 ^{A*}	1.1 ± 0.3 ^{C}	3.7 ± 0.1 ^{A*}
Total monoenes	14.7 ± 0.1 ^{a}	12.7 ± 0.5 ^{a}	18.4 ± 2.4 ^{a}	6.9 ± 0.1 ^{b}	16.5 ± 0.1 ^{A*}	14.4 ± 1.8 ^{A}	21.5 ± 9.5 ^{A}	9.2 ± 0.6 ^{B*}
18:2(n-6)	0.5 ± 0.0	0.3 ± 0.0	0.9 ± 0.5	0.6 ± 0.0	0.6 ± 0.0 ^{B*}	1.6 ± 0.7 ^{A*}	3.7 ± 2.3 ^{A*}	0.7 ± 0.1 ^{A*}
20:2(n-6)	0.5 ± 0.0 ^{ab}	0.4 ± 0.0 ^{b}	0.4 ± 0.0 ^{b}	0.5 ± 0.0 ^{a}	0.3 ± 0.0 ^{B*}	0.3 ± 0.0 ^{B*}	0.1 ± 0.2 ^{B}	0.4 ± 0.0 ^{A*}
20:4(n-6)	1.7 ± 0.0 ^{c}	4.8 ± 0.5 ^{b}	0.9 ± 0.3 ^{c}	6.8 ± 0.4 ^{a}	1.2 ± 0.1 ^{B*}	2.3 ± 0.4 ^{A*}	0.9 ± 0.8 ^{B}	2.4 ± 0.2 ^{A*}
Total n-6 FA	3.2 ± 0.1 ^{c}	6.3 ± 0.6 ^{b}	4.0 ± 0.3 ^{bc}	8.2 ± 0.3 ^{a}	2.8 ± 0.2 ^{B*}	5.1 ± 1.3 ^{A}	5.1 ± 2.8 ^{AB}	4.1 ± 0.3 ^{A*}
18:3(n-3)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.5 ± 0.0 *	1.9 ± 1.6	1.3 ± 1.3	0.5 ± 0.1 *
20:3(n-3)	1.4 ± 0.1 ^{b}	0.9 ± 0.0 ^{b}	0.3 ± 0.1 ^{c}	2.7 ± 0.0 ^{a}	0.9 ± 0.1 ^{B*}	0.7 ± 0.0 ^{C*}	0.0 ± 0.0 ^{D*}	1.8 ± 0.1 ^{A*}
20:5(n-3)	8.0 ± 0.2 ^{c}	14.8 ± 1.4 ^{b}	5.0 ± 2.5 ^{d}	22.5 ± 0.3 ^{a}	12.1 ± 0.4 ^{C*}	16.3 ± 1.2 ^{B}	5.2 ± 2.6 ^{D}	22.6 ± 1.0 ^{A}
22:5(n-3)	1.0 ± 0.0 ^{ab}	0.8 ± 0.0 ^{b}	0.3 ± 0.0 ^{c}	1.2 ± 0.0 ^{a}	1.3 ± 0.1 ^{B*}	1.2 ± 0.1 ^{B*}	0.0 ± 0.0 ^{C*}	2.0 ± 0.1 ^{A*}
22:6(n-3)	29.5 ± 0.4 ^{a}	22.6 ± 0.2 ^{b}	5.0 ± 0.6 ^{c}	24.1 ± 0.5 ^{ab}	21.5 ± 0.0 ^{B*}	18.8 ± 0.3 ^{C*}	2.9 ± 1.5 ^{D}	23.8 ± 1.4 ^{A}
Total n-3 FA	40.0 ± 0.7 ^{b}	39.2 ± 1.6 ^{b}	10.8 ± 3.0 ^{c}	50.7 ± 0.8 ^{a}	36.6 ± 0.5 ^{C*}	39.4 ± 0.5 ^{B}	9.6 ± 3.2 ^{D}	50.9 ± 2.2 ^{A}
Total PUFA	43.7 ± 0.7 ^{b}	45.9 ± 2.0 ^{b}	15.2 ± 2.7 ^{c}	59.2 ± 0.9 ^{a}	39.5 ± 0.7 ^{C*}	44.7 ± 1.5 ^{B}	14.6 ± 3.0 ^{D}	54.6 ± 2.0 ^{A*}
Total LC-PUFA	42.2 ± 0.7 ^{b}	44.7 ± 2.0 ^{b}	11.7 ± 3.6 ^{c}	57.6 ± 0.9 ^{a}	37.6 ± 0.7 ^{C*}	39.9 ± 1.7 ^{B*}	9.0 ± 3.0 ^{D}	52.9 ± 2.2 ^{A*}
n-3/n-6	12.3 ± 0.1 ^{a}	6.3 ± 0.5 ^{b}	2.7 ± 0.9 ^{c}	6.2 ± 0.3 ^{b}	13.2 ± 1.0 ^{A}	7.8 ± 1.9 ^{B}	1.9 ± 2.3 ^{C}	12.4 ± 1.4 ^{A*}
DHA/EPA	3.7 ± 0.0 ^{a}	1.5 ± 0.1 ^{ab}	1.0 ± 0.6 ^{b}	1.1 ± 0.0 ^{b}	1.8 ± 0.1 ^{A*}	1.2 ± 0.1 ^{B*}	0.6 ± 0.6 ^{B}	1.1 ± 0.0 ^{B}
EPA/ARA	4.7 ± 0.1	3.1 ± 0.2	5.7 ± 1.5	3.3 ± 0.2	10.5 ± 0.8 ^{A*}	7.1 ± 0.8 ^{B*}	5.8 ± 2.4 ^{B}	9.5 ± 1.1 ^{A*}

Results represent means ± SD (n = 3). Totals include some minor components not shown. [1] Contains n-9, n-7 and n-5 isomers. PUFA – Polyunsaturated fatty acids. LC-PUFA – Long-chain polyunsaturated fatty acids. ARA – 20:4n-6; EPA – 20:5n-3; DHA – 22:6n-3. DMA – dimethyl acetal; Different superscript letters represent differences between fatty acid content in PC (phosphatidylcholine), PS (phosphatidylserine), PI (phosphatidylinositol) and PE (phosphatidylethanolamine) within the same species; * represents differences between *O. vulgaris* and *S. officinalis* hatchlings fatty acid content for a given lipid class.

Table 2 – Total lipid content (μg lipid mg protein^{-1}) and lipid class composition (% of total lipid) of *Octopus vulgaris* and *Sepia officinalis* hatchlings

	O. vulgaris	*S. officinalis*
Total lipid content	236.2 ± 58.1	299.9 ± 30.7
Lipid class		
Sphingomyelin	0.5 ± 0.3	1.0 ± 0.2
Phosphatidylcholine	21.6 ± 1.2	22.6 ± 2.7
Phosphatidylserine	10.9 ± 2.2	9.2 ± 2.2
Phosphatidylinositol	7.0 ± 2.0	5.5 ± 0.8
Phosphatidylethanolamine	23.4 ± 1.1	22.5 ± 1.7
∑ Polar lipids	63.5 ± 2.5	60.9 ± 1.3
Cholesterol	31.6 ± 2.3	33.1 ± 1.8
Free fatty acids	1.0 ± 0.4	1.0 ± 0.3
Triacylglycerols	1.8 ± 0.5	3.3 ± 2.4
Sterol esters	2.1 ± 0.4	1.6 ± 0.7
∑ Neutral lipids	36.5 ± 2.5	39.1 ± 1.3

Results represent means ± SD (n = 3).

Table 3 – Main fatty acid composition (% of total FA) of *Octopus vulgaris* and *Sepia officinalis* hatchlings total lipids.

	Octopus vulgaris	*Sepia officinalis*
14:0	0.7 ± 0.1	1.8 ± 0.3*
15:0	0.2 ± 0.0	1.0 ± 0.2*
16:0	16.6 ± 0.8	19.7 ± 1.0*
16:0 DMA	0.8 ± 0.2	0.6 ± 0.0
18:0	10.2 ± 0.6	10.9 ± 0.4
18:0 DMA	4.7 ± 0.2	3.7 ± 0.0*
Total saturated[a]	34.3 ± 0.8	41.1 ± 1.4*
16:1[b]	1.5 ± 0.1	0.9 ± 0.1*
18:1n-13	2.3 ± 0.2	0.0 ± 0.0*
18:1n-9	4.1 ± 1.2	1.5 ± 0.4*
20:1n-9	3.8 ± 0.4	3.3 ± 0.1
Total monoenes[a]	16.2 ± 1.1	13.7 ± 0.8*
18:2n-6	1.1 ± 0.6	0.6 ± 0.1
20:2n-6	0.6 ± 0.0	0.3 ± 0.0*
20:4n-6	5.2 ± 0.3	1.2 ± 0.2*
Total n-6 FA[a]	7.2 ± 1.1	2.1 ± 0.1*
18:3n-3	2.1 ± 1.4	0.4 ± 0.0*
20:3n-3	2.0 ± 0.1	1.4 ± 0.4*
20:5n-3	15.0 ± 2.2	16.8 ± 0.2
22:5n-3	1.3 ± 0.0	1.4 ± 0.1
22:6n-3	20.4 ± 1.7	22.4 ± 0.2
Total n-3 FA[a]	41.1 ± 2.4	42.4 ± 0.7
Total PUFA[a,c]	48.6 ± 1.5	44.4 ± 0.6*
Total LC-PUFA[a,d]	44.3 ± 3.4	43.1 ± 0.5
n-3/n-6	5.8 ± 1.3	20.4 ± 1.0*
DHA/EPA[e]	1.4 ± 0.1	1.3 ± 0.0
EPA/ARA[e]	2.9 ± 0.6	15.0 ± 2.6*

Results represent means ± SD (n = 3). * represents differences between *O. vulgaris* and *S. officinalis* fatty acid content. [a] Totals include some minor components not shown. [b] Contain n-9, n-7 and n-5 isomers. [c] PUFA – Polyunsaturated fatty acids. [d] LC-PUFA – Long-chain polyunsaturated fatty acids. [e] ARA – 20:4n-6; EPA – 20:5n-3; DHA – 22:6n-3.

Table 4 – Incorporation of [1-^{14}C]ARA into total lipid (pmoles mg pp^{-1} h^{-1}) and its re-esterification pattern (%) into lipid classes of *Octopus vulgaris* and *Sepia officinalis* hatchlings when provided bounded to PC or PE

	O. vulgaris		*S. officinalis*	
Lipid Class	PC	PE	PC	PE
Incorporation	4.1 ± 0.5	3.2 ± 0.7	2.3 ± 0.7 *	0.6 ± 0.3 $^{\Delta *}$
Free Fatty Acids	1.0 ± 0.6	2.6 ± 0.7 $^{\Delta}$	3.2 ± 1.7 *	0.5 ± 0.6 $^{\Delta *}$
Partial Acylglycerols	1.8 ± 1.1	7.2 ± 0.9 $^{\Delta}$	2.7 ± 1.6	2.7 ± 1.8 *
Phosphatidylethanolamine	54.7 ± 3.2	56.2 ± 2.7	12.4 ± 1.7 *	46.3 ± 3.3 $^{\Delta *}$
Phosphatidylinositol	9.5 ± 1.6	11.5 ± 2.3	3.3 ± 0.8 *	9.3 ± 2.2 $^{\Delta}$
Phosphatidylserine	12.2 ± 0.8	10.6 ± 2.3	5.2 ± 1.2 *	19.2 ± 4.1 $^{\Delta *}$
Phosphatidylcholine	20.9 ± 0.9	12.0 ± 1.4 $^{\Delta}$	73.3 ± 5.7 *	21.9 ± 1.8 $^{\Delta *}$

Results represent means ± SD (n = 4). Data of incorporation are given in pmoles of ^{14}C fatty acid incorporated per mg protein per hour. Data of esterification are given in percentage. $^{\Delta}$ represent significant differences between lipid classes within the same species. * represent significant differences of a specific lipid class (PC or PE) between *O. vulgaris* and *S. officinalis.*