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

3

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15

16 **Running title:** *Sepia officinalis* and *Octopus vulgaris* hatchlings main phospholipids.

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27

28 **Abstract**

29 The objective of the present study was to characterise the fatty acid (FA) profiles of the
30 major phospholipids, of *Octopus vulgaris* and *Sepia officinalis* hatchlings, namely
31 phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and
32 phosphatidylethanolamine (PE); and to evaluate the capability of both cephalopod species
33 on dietary phospholipid remodelling. Thus, *O. vulgaris* and *S. officinalis* hatchlings were
34 *in vivo* incubated with 0.3 μ M of L- α -1-palmitoyl-2-[1- 14 C]arachidonyl-PC or L- α -1-
35 palmitoyl-[1- 14 C]arachidonyl-PE. Octopus and cuttlefish hatchlings phospholipids
36 showed a characteristic FA profiles with PC presenting high contents of 16:0 and 22:6n-3
37 (DHA); PS having high 18:0, DHA and 20:5n-3 (EPA); PI a high content of saturated
38 FA; and PE showing high contents of DHA and EPA. Interestingly, the highest content of
39 20:4n-6 (ARA) was found in PE rather than PI. Irrespective of the phospholipid in which
40 [1- 14 C]ARA was initially bound (either PC or PE), the esterification pattern of [1-
41 14 C]ARA in octopus lipids was similar to that found in their tissues with high
42 esterification of this FA into PE. In contrast, in cuttlefish hatchlings [1- 14 C]ARA was
43 mainly recovered in the same phospholipid that was provided. These results showed a
44 characteristic FA profiles in the major phospholipids of the two species, as well as a
45 contrasting capability to remodel dietary phospholipids, which may suggest a difference
46 in phospholipase activities.

47

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

49

50 **1. Introduction**

51

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

58 Cephalopods have a high protein level and low lipid content (Lee, 1994) but, despite that,
59 lipids were defined as crucial in cephalopod nutrition, with long-chain polyunsaturated
60 fatty acids (LC-PUFA), cholesterol and phospholipids being suggested to play critical

61 roles in their development (Navarro and Villanueva, 2000, 2003; Almansa et al., 2006).
62 Within LC-PUFA, 22:6n-3 (DHA), 20:5n-3 (EPA) and also 20:4n-6 (ARA) were recently
63 defined as essential fatty acids (EFA; Monroig et al., 2012a, 2016; Reis et al., 2014,
64 2016). The latest studies have highlighted the importance of an adequate dietary input of
65 those EFAs, considering not only their amount and ratios (Reis et al., 2014, 2016) but
66 also their lipid form (Guinot et al., 2013a), as this could affect the availability of the EFA
67 for specific functions and tissue structures.

68 To ensure a high availability of EFA, dietary fatty acids (FA) contents and lipid class
69 molecular species should reflect larval requirements, which may be extrapolated from
70 egg yolk lipid profile (Sargent et al., 1999) or the hatchlings polar lipids (PL)
71 composition (Sargent et al., 1999; Olsen et al., 2014). Eggs of *O. vulgaris* and *S.*
72 *officinalis* show low lipid contents with high proportions of PL with phosphatidylcholine
73 (PC) being the main lipid class (Sykes et al., 2009; Quintana et al., 2015). Similarly to
74 eggs, *O. vulgaris* and *S. officinalis* hatchlings generally have higher PL than neutral lipids
75 (NL) content (Bouchaud and Galois, 1990; Navarro and Villanueva, 2000; Quintana et
76 al., 2015), with the former presenting a high proportion of polyunsaturated fatty acids
77 (PUFA) and the latter a high percentage of monounsaturated fatty acids (Sinanoglou and
78 Miniadis-Meimaroglou, 1998; Viciano et al., 2011). However, the specific FA profiles of
79 the major phospholipid classes have not been determined for the hatchlings of these two
80 species, which could provide important information regarding the design of a suitable diet
81 for these species development during their first life stages.

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

87

88 **2. Material and Methods**

89

90 *2.1. Experimental animals*

91 An *O. vulgaris* broodstock (30 individuals) was caught by professional artisanal
92 fishermen on the Tenerife coast (Canary Islands, Spain) and maintained at the
93 Oceanographic Centre of Canary Islands (Spanish Institute of Oceanography; Santa Cruz
94 de Tenerife - 28°29'59.1''N; 16°11'44.54''W). The broodstock rearing conditions were

95 similar to those described by Reis et al. (2014). The presence of eggs was checked once a
96 week and when egg masses were observed, the ovate female was isolated in the tank by
97 removing the other individuals. After approximately one month of embryonic
98 development the eggs began to hatch and hatchlings were removed daily from the female
99 rearing tank, to provide newly hatched octopus (less than 24 h old) for experiments.
100 All cuttlefish used in the present study were from a single brood obtained from F4
101 cultured females reproducing at the Ramalhete Aquaculture Station (Ria Formosa, South
102 of Portugal - 37°00'22.39''N; 7°58'02.69''W). Twenty-four hour laid eggs were
103 transported to the Spanish Institute of Oceanography facilities in Tenerife and maintained
104 in a 100 L circular fiberglass tank in a flow-through seawater system at 21 ± 0.69 °C. The
105 embryonic development of eggs and the broodstock rearing followed procedures
106 described by Sykes et al. (2014). Eggs were maintained under rearing conditions similar
107 to those described in Reis et al. (2016). The use of animals in the experiments of this
108 work were in accordance with the EU Directive 2010/63/EU for animal experimentation.
109

110 2.2. FA composition of phospholipids in cephalopod hatchlings

111 Approximately, 400 *O. vulgaris* hatchlings (500 mg) and 3 *S. officinalis* hatchling (300
112 mg), obtained from individuals previously euthanized in iced seawater (-2 °C) and stored
113 at -80 °C, were used for lipid extraction (n=3). Extraction of total lipid (TL) was
114 performed with chloroform/methanol (2:1 by volume) according to the Folch method, as
115 described by Christie (2003) and lipid content was determined gravimetrically. The TL
116 extracts were stored at -20 °C in chloroform/methanol (2:1, by volume) with 0.01%
117 butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10 mg/mL and
118 under a nitrogen atmosphere until analysis. In order to determine the FA composition of
119 PC, PE, phosphatidylserine (PS) and phosphatidylinositol (PI) of *O. vulgaris* and *S.*
120 *officinalis* hatchlings, aliquots of 5 mg of TL extract were spotted on 20 cm x 20 cm thin-
121 layer chromatography (TLC) silica plates. Polar lipid classes were separated by one-
122 dimension single-development with 1-propanol/chloroform/methyl
123 acetate/methanol/0.25% KCL (25:25:25:10:9 by volume). The phospholipid classes were
124 visualized under UV light after brief exposure to dichlorofluorescein. Each phospholipid
125 class band was scraped from the TLC plates and subjected to direct acid-catalysed
126 transmethylation on silica during 16-18 h at 50 °C to obtain fatty acid methyl esters
127 (FAME). FAME were purified by TLC (Christie, 2003) with hexane/diethyl ether/acetic
128 acid (90:10:1 by volume) and then separated and analysed using a TRACE-GC Ultra gas

129 chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) equipped
130 with an on-column injector, a flame ionization detector (240 °C) and a fused silica
131 capillary column (Supelcowax™ 10; Sigma-Aldrich Co., St. Louis, Missouri, USA). The
132 column temperature was programmed for four different ramps of temperature: 1st ramp
133 was programmed for a linear increase of 40 °C per minute from 50 to 150 °C; the 2nd
134 ramp for a linear increase of 2°C per minute until 200°C; the 3rd ramp for a linear increase
135 of 1°C per minute until 214°C; and the 4th ramp for a linear increase of 40°C per minute
136 until 230°C and hold at that temperature for 5 minutes. FAME were identify by
137 comparison with retention times of a standard mixtures with F.A.M.E Mix C4-C24
138 (Supelco 18919-1AMP) diluted to 2 mg/mL in hexane, PUFA N° 3 from menhaden oil
139 (Supelco 47085-U) diluted to 2 mg/mL in hexane; and cod roe FAME. When necessary,
140 identification of individual FAME was confirmed by GC-MS chromatography (DSQ II,
141 Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA).

142

143 2.3. *In vivo* incubations of hatchlings with [1-¹⁴C]ARA esterified in the sn-2 position of 144 phosphatidylcholine and phosphatidylethanolamine

145 A total of 550 *O. vulgaris* and 11 *S. officinalis* hatchlings were incubated following an
146 adaptation of the method of Reis et al. (2014, 2016) to determine the *in vivo* [1-¹⁴C]FA
147 metabolism in these species. Incubations (n=4 for each species) were performed in flat-
148 bottom 6-well tissue culture plates (SARSTEDT AG & CO., Nümbrecht, Germany), at a
149 density of 50 or 1 hatchling/well for *O. vulgaris* and *S. officinalis*, respectively, in 10 mL
150 of filtered seawater (36‰), which was gently stirred at 21 °C for 5 h, supplemented with
151 0.2 µCi (0.3 µM) of L-α-1-palmitoyl-2-[1-¹⁴C]arachidonyl-PC or L-α-1-palmitoyl-2-[1-
152 ¹⁴C]arachidonyl-PE dissolved in 5 µL of ethanol. [1-¹⁴C]ARA was used as a commercial
153 available EFA model. Labelled phospholipid classes were individually added to separate
154 wells. Control treatments (n=3) of hatchlings of either species corresponded to a similar
155 protocol but without the addition of labelled phospholipid. In any case, a 100% survival
156 rate was registered.

157 After incubation, hatchlings were immediately euthanized in iced seawater (-2 °C) and
158 thoroughly washed with filtered seawater to remove excess [1-¹⁴C] phospholipid.

159 Samples were stored at -80 °C until analysis. Extraction of total lipids was performed as
160 mentioned in 2.2.

161

162 2.4. Lipid composition of control samples

163 Aliquots of 20 µg of TL extracts of hatchling control groups were used to determine lipid
164 class composition (n=3). Lipid classes (LC) were separated by one-dimensional double-
165 development with 1-propanol/chloroform/methyl acetate/methanol/0.25% KCL
166 (5:5:5:2:1.8 by volume) for polar lipid classes separation and hexane/diethyl ether/acetic
167 acid (22.5:2.5:0.25 by volume) for neutral lipid classes separation on 10 cm x 10 cm
168 high-performance thin-layer chromatography (HPTLC), and analysed by charring
169 followed by calibrated densitometry using a dual-wavelength flying spot scanner CS-
170 9001PC (Shimadzu Co., Kyoto, Japan; Tocher and Harvie, 1988). Identification of
171 individual LC was performed by running known standards (cod roe lipid extract and a
172 mixture of single standards from BIOSIGMA S.r.l., Venice, Italy) on the same plates.
173 FAME were obtained by acid-catalysed transmethylation of 1 mg of TL extracts. FAME
174 were purified and analysed as described in 2.2.

175

176 *2.5. Incorporation of radioactivity into total lipids*

177 In order to determine the radioactivity incorporated into hatchling TL, an aliquot of 0.1
178 mg of TL extract was transferred to scintillation vials and radioactivity quantified in
179 disintegrations per minute (dpm) in a LKB Wallac 1214 Rackbeta liquid scintillation β-
180 counter (PerkinElmer Inc., Waltham, Massachusetts, USA). Results in dpm were
181 converted into pmoles by, $\text{Total dpm} (\text{specific activity of each substrate} * 2.22)^{-1}$ where
182 Total dpm value was obtained by multiplying dpm of 0.1 mg of TL extract by samples
183 total lipid content and the fact that 1 µCi correspond to 2 220 000 dpm. Pmoles were later
184 divided by the protein content of samples (n=4) determined according to Lowry et al.,
185 (1951) and hours of incubation. Results of radioactivity incorporated into cephalopods
186 TL are presented in $\text{pmol mg protein}^{-1} \text{ h}^{-1}$.

187

188 *2.6. Remodelling of radiolabelled FA into lipid classes*

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

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

199

200 2.7. Materials

201 L- α -1-palmitoyl-2-[1- 14 C]arachidonyl-PC and L- α -1-palmitoyl-2-[1- 14 C]arachidonyl-PE
202 were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri, USA).
203 TLC plates (20 × 20 cm × 0.25 mm) were purchased from Macherey-Nagel GmbH & Co.
204 KG (Düren, Germany). HPTLC plates, (10 × 10 cm × 0.15 mm) pre-coated with silica gel
205 60 (without fluorescent indicator), were purchased from Merck KGaA (Düsseldorf,
206 Germany). OptiPhase “HiSafe” 2 scintillation cocktail was purchased from PerkinElmer,
207 Inc. (Waltham, Massachusetts, USA). Organic solvents used were of reagent grade and
208 were purchased from Merck KGaA (Düsseldorf, Germany), Sigma-Aldrich Co. (St.
209 Louis, Missouri, USA) and Panreac Química S.L.U. (Barcelona, Spain).

210

211 2.8. Statistical analysis

212 Results are presented as means \pm SD. For all statistical tests, $p < 0.05$ was considered as
213 significantly different. Data were checked for normal distribution with the one-sample
214 Shapiro-Wilk test, as well as for homogeneity of the variances with the Levene test (Zar,
215 1999). Arcsine square root transformation was applied to all data expressed as percentage
216 (Fowler et al., 1998). Differences between FA content in the main phospholipids within a
217 same species, were analysed by a one-way analysis of variance (ANOVA) followed by a
218 Tukey’s post hoc test (Zar, 1999). When normal distribution and/or homogeneity of the
219 variances were not achieved, data were subjected to the Welch robust ANOVA, followed
220 by a Games-Howell non-parametric multiple comparison test (Zar, 1999). Differences
221 between *O. vulgaris* and *S. officinalis* compositions, either for a specific LC (PC, PS, PI
222 or PE) content or FA compositions, as well as for radioactivity incorporation into
223 hatchlings TL, were analysed by Student’s *t*-test (Zar, 1999). Statistical analysis was
224 performed using the IBM SPSS statistics 22.0 (IBM Co., USA).

225

226 3. Results

227

228 3.1. FA compositions of phospholipids in cephalopods hatchlings

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

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

246

247 3.2. Hatchling lipid compositions

248 The TL contents and LC compositions of *O. vulgaris* and *S. officinalis* hatchlings are
249 presented in Table 2. Both species presented a similar profile ($p > 0.05$). Within polar
250 lipids, PC ($21.6 \pm 1.2\%$ in octopus and $22.6 \pm 2.7\%$ in cuttlefish) and PE ($23.4 \pm 1.1\%$ in
251 octopus and $22.5 \pm 1.7\%$ in cuttlefish) were the major classes, whereas cholesterol (31.6
252 $\pm 2.5\%$ in octopus and $33.1 \pm 1.8\%$ in cuttlefish) was the major neutral lipid class and the
253 most abundant lipid class.

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

260

261 3.3. Incorporation of radiolabelled ARA into hatchlings total lipids and its distribution 262 among lipid classes

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

265 supplied as PC or PE into the different lipid classes of *O. vulgaris* hatchlings was
266 generally similar, with differences only being detected for free fatty acids (FFA), partial
267 acylglycerols and PC ($p < 0.05$; Table 4). Despite being provided in two different forms
268 (esterified into PC or PE), in octopus [1-¹⁴C]ARA was preferentially re-esterified into PE
269 ($54.7 \pm 3.2\%$ when added as PC and $56.2 \pm 2.7\%$ when added as PE). On the other hand,
270 in *S. officinalis* hatchlings, when [1-¹⁴C]ARA was provided esterified either into PC or
271 PE a higher amount of the radioactivity incorporated was recovered re-esterified in the
272 same class of origin: $73.3 \pm 5.7\%$ was recovered esterified into PC when added as PC,
273 and $46.3 \pm 3.3\%$ was recovered as PE when added as PE. These results show a clear
274 difference in the esterification patterns of [1-¹⁴C]ARA when incubated as PC or PE into
275 LC of both species ($p < 0.05$).

276

277 **4. Discussion**

278

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

299 Similar to fish (Tocher et al., 1995), PE was the phospholipid with highest LC-PUFA
300 content and showing a DHA/EPA ratio of around 1. Moreover, this phospholipid class
301 had the highest EPA content, which was consistent with the preferential esterification of
302 [1-¹⁴C]EPA into octopus and cuttlefish PE as reported by Reis et al. (2014, 2016).
303 Sargent et al. (2002) reported that the highest DHA levels in fish tissues were usually
304 found in PE, similar to what was found in the present study in the two cephalopod
305 species. However, a similar high level of DHA was also found in PC (30% and 22% for
306 *O. vulgaris* and *S. officinalis* hatchlings, respectively). These data are also in agreement
307 with the results obtained from *in vivo* esterification studies on the two cephalopod
308 species, where preferential esterification of [1-¹⁴C]DHA into PC was detected (Reis et al.,
309 2014, 2016).

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

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

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

334 *In vivo* metabolic studies on fish larvae have been extensively performed using tracers,
335 existing four basic methods to deliver tracers to larva: micro-diet labelling, live food
336 labelling, tube feeding and uptake from water (Conceição et al., 2007). Formulated diets
337 have been mostly neglected by cephalopods and basal metabolic studies using labelled
338 live food would be extremely difficult given preys own metabolism (Guinot et al., 2013a,
339 2013b). Tube feeding methodology have been adapt to a large number of fish larvae
340 species (see Conceição et al., 2007). However, its use in cephalopods metabolism studies,
341 would be constrained by the existence of a strong beaks in the buccal mass (Messenger
342 and Young, 1999), which could break the feeding tube, and the lack of appropriate
343 anaesthesia agents and methods for the younger stages (Fiorito et al., 2015; Sykes and
344 Gestal, 2014). In this sense, radiolabelled uptake from water might be an alternative to
345 study the *in vivo* capability of these species for phospholipid turnover and remodelling
346 during their early life stages. The way nutrients are incorporated is not completely
347 understood, as cephalopods have the apparent capacity to absorb nutrients through skin
348 during the first stage of life (Boucaud-Camou and Roper, 1995; de Eguileor et al., 2000;
349 Villanueva et al., 2004). Therefore, there is the possibility that the present results are due
350 to incubated phospholipid classes entering via the skin, encountering a completely
351 different set of enzymes for phospholipids de-acylation and re-acylation to that of the gut.
352 Nonetheless, it seems important to highlight the resemblance between the FA
353 composition of the different phospholipids (Table 1) and the results obtained by Reis et
354 al. (2014, 2016) at *in vivo* free FA metabolism studies, which clear show that the method
355 use is consistent in its results.

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

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

367 It is generally believed that PLA₂ hydrolyses phospholipids at the sn-2 position forming
368 FFA and lysophospholipids (see Olsen et al., 2014) and that LC-PUFA are esterified at
369 the sn-2 position of phospholipid molecules (Sargent et al., 1999). In *O. vulgaris*
370 hatchlings, after the ARA re-acylation process, the esterification pattern obtained was
371 highly similar, with the majority of ARA being recovered as PE, suggesting the existence
372 of a high PLA₂ activity. The *S. officinalis* turnover data indicate an apparent lower PLA₂
373 enzyme activity when compared to *O. vulgaris* hatchlings. In fish, PLA₂ activity tends to
374 increase during development, with seabream and sea bass larvae showing no PLA₂
375 activity during very early larval stages (Izquierdo and Henderson, 1998; Zambonino
376 Infante and Cahu, 2001). Both octopus and cuttlefish possess inner yolk reserves that
377 could last for a few days after hatching (Sykes et al., 2004; Boletzky and Villanueva,
378 2014) and it is known that it can take up to 30 days for the digestive system to fully
379 mature in cuttlefish (Boucaud-Camou and Yim 1980; Yim and Boucaud-Camou 1980;
380 Boucaud-Camou 1982). Nonetheless, these species may feed on size-appropriate prey
381 from day 1 (Hanlon and Messenger, 1988), when mixed feeding overlaps inner yolk
382 reserves and external food consumption (Boletzky, 1974; Sykes et al., 2013). It has been
383 suggested that diet may influence digestive enzyme activity (Koven et al., 2001;
384 Villanueva et al., 2002; Perrin et al., 2004), and it is known that PLA₂ activity can be
385 stimulated by increased dietary phospholipids in fish (Zambonino Infante and Cahu,
386 2001). In the present study, cuttlefish individuals were unfed, which could explain the
387 low turnover rate recorded. On the other hand, octopus hatchlings under similar feeding
388 conditions displayed a high turnover rate. The differences regarding both species
389 phospholipid metabolism detected here might be related with the different morphologies
390 at hatching (paralarvae vs. hatchling) derived from different embryonic developments
391 (indirect vs. direct) that translate into different lifestyles of these species (pelagic vs.
392 benthonic) during their first live stages (Halon and Messenger, 1996; Young and Harman,
393 1988) and/or different eggs origins (see Boucaud and Galois et al., 1990).

394 A small proportion of the incorporated [1-¹⁴C]ARA was also recovered esterified into
395 monoacylglycerols and diacylglycerols, that are involved in *de novo* synthesis of
396 phospholipids and TAG (see Tocher et al., 2008 and Olsen et al., 2014). Interestingly, no
397 [1-¹⁴C]ARA was found re-esterified into TAG, the main class for lipid storage and energy
398 provision in fish (Tocher et al., 2003). Nonetheless, *O. vulgaris* and *S. officinalis*

399 hatchlings normally show only low TAG levels representing $1.8 \pm 0.5\%$ of total lipid in
400 *O. vulgaris* and $3.3 \pm 2.6\%$ in *S. officinalis*. Low TAG in these species was also
401 previously recorded not just in hatchlings (Navarro and Villanueva, 2000; Reis et al.,
402 2014, 2015, 2016), but also in eggs (Boucaud and Galois, 1990; Sykes et al., 2009;
403 Quintana et al., 2015), juveniles and adult tissues (Almansa et al., 2006; Valverde et al.,
404 2012).

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

413

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422

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

	<i>Octopus vulgaris</i>				<i>Sepia officinalis</i>			
	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 ($\mu\text{g lipid mg protein}^{-1}$) and lipid class composition (% of

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

total lipid) of *Octopus vulgaris* and *Sepia officinalis* hatchlings

Results represent means \pm SD (n = 3).

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

	<i>Octopus vulgaris</i>	<i>Sepia officinalis</i>
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 [1-¹⁴C]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

<i>Lipid Class</i>	<i>O. vulgaris</i>		<i>S. officinalis</i>	
	PC	PE	PC	PE
Incorporation	4.1±0.5	3.2±0.7	2.3±0.7*	0.6±0.3 ^{Δ*}
Free Fatty Acids	1.0±0.6	2.6±0.7 ^Δ	3.2±1.7*	0.5±0.6 ^{Δ*}
Partial Acylglycerols	1.8±1.1	7.2±0.9 ^Δ	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 ^{Δ*}
Phosphatidylinositol	9.5±1.6	11.5±2.3	3.3±0.8*	9.3±2.2 ^Δ
Phosphatidylserine	12.2±0.8	10.6±2.3	5.2±1.2*	19.2±4.1 ^{Δ*}
Phosphatidylcholine	20.9±0.9	12.0±1.4 ^Δ	73.3±5.7*	21.9±1.8 ^{Δ*}

Results represent means ± SD (n = 4). Data of incorporation are given in pmoles of ¹⁴C fatty acid incorporated per mg protein per hour. Data of esterification are given in percentage. ^Δ 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*.