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In vivo metabolism of unsaturated fatty acids in *Sepia officinalis* hatchlings

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Abstract

The transition of *Sepia officinalis* culture to industrial large scale has been hampered due to bottlenecks related to the limited knowledge on nutritional physiology of the species. Determination of the endogenous ability of *S. officinalis* hatchlings to metabolise unsaturated fatty acids (FA) may provide new insight on the capability of hatchlings to biosynthesise different FA, as well as lipid classes containing essential fatty acids (EFA). In the present study, cuttlefish hatchlings were incubated with [1-^{14}C]FA including C18 FA (18:1n-9, 18:2n-6, 18:3n-3) and long-chain polyunsaturated fatty acids (LC-PUFA) (20:4n-6 (ARA), 20:5n-3 (EPA) or 22:6n-3 (DHA)), which were added individually as potassium salts bound to bovine serum albumin. As a result, it was possible to investigate the *in vivo* FA metabolism of *S. officinalis* hatchlings by following the incorporation of specific [1-^{14}C]FA, which points to the suitability of this methodology to study lipid metabolism of newly hatched cephalopods. The majority of radioactivity incorporated was recovered esterified into polar lipids (PL). A pattern was detected, where [1-^{14}C]DHA, [1-^{14}C]C18 FA and their metabolic products were preferentially esterified into phosphatidylcholine, whereas [1-^{14}C]ARA and [1-^{14}C]EPA were mainly esterified into phosphatidylethanolamine. [1-^{14}C]C18 FA were the most transformed FA with several metabolites produced by elongation and possible desaturation being obtained. As a contrary the radioactivity incorporated into hatchling total lipid (TL) from supplemented [1-^{14}C]LC-PUFA only one elongation product was recovered from the three substrates, except for [1-^{14}C]ARA, where an unidentified product was also detected. The present *in vivo* results indicated that *S. officinalis* hatchlings may have capability for the first steps in the biosynthesis of ARA and EPA from 18:2n-6 and 18:3n-3, respectively, including the existence of a desaturase potentially involved. Nonetheless, considering the low desaturation rates detected, this process may not be sufficient to cover EFA demands during development of this species. Therefore, dietary ARA and EPA, as well as DHA, should be supplied during the hatchling stage of *Sepia*. While designing an inert diet, which ensures normal growth and development of this species during the hatchling stage, the C18 FA and LC-PUFA levels and ratios should be considered, since the esterification pattern detected in the present study suggested competition between these FA for esterification into specific lipid classes. Moreover, considering the observed esterification pattern of LC-PUFA into different lipid classes, it is likely that the DHA/EPA/ARA ratio, rather than

DHA/EPA or EPA/ARA ratios, would be of great importance for *S. officinalis* hatchling development.

Keywords: DHA, EPA, ARA; Lipid metabolism; Radiolabelled substrates; *Sepia officinalis* hatchlings; Unsaturated fatty acids.

1. Introduction

The European cuttlefish (*Sepia officinalis*) has been recognized as a species with great potential for aquaculture, due to the short life cycle and high market prices which translate into short investment payback time (Sykes et al., 2014). Nonetheless, its culture could go beyond their use as human food. By-products of the food industry could be utilized in aquafeeds and pharmacological industries (Koueta et al., 2014), in addition to the use of these animals as a model for several research fields and for public exhibition in aquariums (see Sykes et al., 2014).

The rearing of *S. officinalis* has been successfully performed in several countries such as the U.S.A., Italy, France and Portugal. At the National Resource Centre for Cephalopods (U.S.A.) and the Centre of Marine Sciences (Portugal), several consecutive generations have been successfully cultured (Sykes et al., 2014). However, transition of technology to industry has been hampered due to three major bottlenecks: the dependence on live prey during the first part of the life cycle (the hatchling stage); the lack of an adequate artificial diet for all life stages; and the fact that full control of reproduction under rearing conditions has not yet been achieved (Sykes et al., 2006a; Villanueva et al., 2014).

During the hatchling stage, *S. officinalis* were reported to require live food (Sykes et al., 2006a), such as mysids and grass shrimp (Domingues et al., 2004; Sykes et al., 2006b). However, the use of these particular species makes cuttlefish culture not economically feasible (Domingues et al., 2010). Recently, Sykes et al. (2013) was able to feed *S. officinalis* with frozen grass shrimp from the first day after hatching, showing the potential of using an inert diet from birth. However, lower growth (~1/3 of that achieved with live food) and higher mortality rates (~20 % more with frozen grass shrimp) were obtained. The differences in growth and survival were suggested to be associated with the maturation of the digestive system, possible changes in nutritional composition of prey or even the amount of prey captured (Sykes et al., 2013). Nonetheless, in order to design an artificial diet that would effectively replace live prey, it is important to

understand the nutritional requirements and metabolic pathways of the species at this life stage.

Due to the low lipid content of cephalopods (Lee, 1994) and reports of their low capacity to metabolize lipids (O'Dor et al., 1984), lipid nutrition of *S. officinalis* has been neglected and only in recent years have some studies been devoted to this theme. Domingues et al. (2004) showed that either a quantitative or qualitative imbalance of prey lipid content could result in lower growth and survival rates in different life stages. However, as they grow in their natural environment, cuttlefish tend to change their diet with increased consumption of fish and decreased consumption of crustaceans (Castro and Guerra, 1990), possibly reflecting a change in metabolic requirements related to sexual maturity. The lipid composition of *S. officinalis* muscle normally presents high levels of phospholipids, cholesterol and polyunsaturated fatty acids (PUFA). For that reason, several authors suggested those nutrients as important for *S. officinalis* development and the necessity to provide a diet rich in those nutrients (Domingues et al., 2004, 2003; Koueta et al., 2002; Navarro and Villanueva, 2000).

Since formulated diets have been largely not accepted (Sykes et al., 2006a) or, if ingested, have resulted in poor growth and survival rates (Castro 1991, Castro and Lee 1994, Castro et al. 1993; Lee et al., 1991), the design of a diet to satisfy lipid requirements of this species cannot be performed only through doses-response studies. In this sense, studies of egg embryonic metabolism (Bouchaud and Galois, 1990; Sykes et al., 2009), lipid composition during hatchling starvation (Castro et al., 1992; Sykes et al., unpublished data), and feeding using prey with well characterized composition have been performed (Almansa et al., 2006; Domingues et al., 2003). Additionally, Reis et al. (2014a) recently reported the *in vivo* lipid metabolism in *O. vulgaris* hatchlings by incubating paralarvae with several ^{14}C-labelled FA, where LC-PUFA were determined as essential for the normal development of the species. Moreover, considering the specific esterification patterns of the incubated FA, the same authors also suggested the necessity of adapting the dietary input of LC-PUFA in *O. vulgaris* diets. In this sense, a similar approach to that recently employed to determine the lipid requirements of *O. vulgaris*, could contribute to the understanding of lipid metabolism in cuttlefish.

In the present study, the endogenous ability of *S. officinalis* hatchlings to biosynthesise FA and lipid classes containing those FA was determined by investigating the *in vivo* capability of the species to incorporate, esterify into different lipid classes, and modify unsaturated FA.

2. Materials & Methods

2.1. Experimental animals

All cuttlefish hatched on the same day 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) and fed exclusively with frozen grass shrimp (*Palaemonetes varians*). Eggs 24h after being laid were sent to IEO facilities in Tenerife and kept in 100 L circular fiberglass tanks in a flow-through seawater system. The embryonic development of eggs, as well as broodstock rearing, were assured using the technology described by Sykes et al. (2014).

Eggs were kept under a 100 lux incident light intensity and were subjected to a 13h daylight cycle. Water temperature and dissolved oxygen, measured with a Tinytag Plus 2 (TGP-4020; Gemini Data Loggers Ltd., Chichester, West Sussex, UK), and salinity, measured with a Refractometer S/Mill-E (ATAGO, Tokyo, Japan), were determined in the morning on a daily basis. Water temperature was 21 ± 0.69 ºC, salinity was 36.8 ± 0.14 ‰ and dissolved oxygen saturation level was 98.61 ± 1.41 %.

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

Twenty-eight newly hatched cuttlefish were incubated in 6-well flat-bottom tissue culture plates (Sarstedt AG & CO., Nümbrecht, Germany), at a density of 1 hatchling per well, in 10 mL of seawater (36 ‰). Incubations were performed in quadruplicate at 21 ºC for 6 h with gentle stirring and with 0.2 µCi (0.3 µM) of one [1-^{14}C]FA including 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6 (ARA), 20:5n-3 (EPA) or 22:6n-3 (DHA). [1-^{14}C]FA were individually added to separate wells as potassium salts bound to bovine serum albumin (BSA) as described by Ghioni et al. (1997). A control treatment of hatchlings was also assessed without the addition of [1-^{14}C]FA. A 100 % survival rate was obtained in all incubations.

After the incubation period, hatchlings were sacrificed in iced seawater and thoroughly washed with filtered seawater to remove excess [1-^{14}C]FA. Samples were stored at -80 ºC until analysis. Total lipids (TL) were extracted with chloroform/methanol (2:1 by volume) according to the Folch method, as described by Christie (2003), and lipid content determined gravimetrically. The TL extracts were stored until analysis 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^{-1} and under an inert atmosphere of nitrogen.

2.3. Lipid classes and fatty acids composition of control samples

Aliquots of 0.2 µl of TL extract of hatchling control groups were used to determine lipid class (LC) profiles (n = 4). Classes were separated by single-dimensional double-development high-performance thin-layer chromatography (HPTLC) as previously described by Tocher and Harvie (1988) and quantified by charring, followed by calibrated densitometry using dual-wavelength flying spot scanner CS-9001PC (Shimadzu Co., Kyoto, Japan). 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.

Fatty acid methyl esters (FAME) obtained by acid-catalysed transmethylation of 1 mg of TL extracts, were purified by thin-layer chromatography (TLC) (Christie, 2003), and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, U.S.A.) equipped with a fused silica capillary column Supelcowax TM 10 (Sigma-Aldrich Co., St. Louis, Missouri, USA), on-column injector and flame ionization detector. Individual FAME were confirmed by GC-MS chromatography (DSQ II, Thermo Fisher Scientific Inc., Waltham, Massachusetts, U.S.A.).

2.4. 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 an LKB Wallac 1214 Rackbeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, U.S.A.). Results in dpm were converted into pmoles per mg of protein per hour of incubation (pmol·mg pp^{-1}·h^{-1}) taking into account the specific activity of each substrate and hatchling lipid and protein contents. The protein content of hatchlings (n = 4) was determined according to Lowry et al. (1951).

2.5. Esterification of radiolabelled FA into lipid classes

In order to define the esterification pattern of each [1-^{14}C]FA into different LC, 0.1 mg of TL extract was applied to HPTLC plates. LC were separated by single-dimensional double-development HPTLC as described previously (Tocher and Harvie, 1988). The

esterification pattern of each [1-^{14}C]FA into a given LC was determined by image analysis following the method described by Reis et al. (2014a).

2.6. Transformation of radiolabelled FA

To determine the metabolism of radiolabelled substrates by desaturation/elongation, a sample of 1.3 mg of TL was subjected to acid-catalysed transmethylation to prepare FAME as detailed above. FAME separation was achieved using TLC plates impregnated with a solution of 2 g silver nitrate in 20 mL acetonitrile followed by activation at 110 ºC for 30 min. The plates were fully developed in toluene/acetonitrile (95:5, v/v), which resolved the FAME into discrete bands based on both degree of unsaturation and chain length (Wilson and Sargent, 1992). FAME identification and quantification was performed by image analysis following the method described by Reis et al. (2014a).

2.7. Reagents

[1-^{14}C]C18 FA were purchased as free FA form, dissolved in ethanol from PerkinElmer, Inc. (Waltham, Massachusetts, U.S.A.). [1-^{14}C] LC-PUFA were also purchased as free FA from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri, U.S.A.). BSA was purchased from Sigma-Aldrich Co. (St. Louis, Missouri, U.S.A.). 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 scintillant liquid was purchased from PerkinElmer, Inc. (Waltham, Massachusetts, U.S.A.). Organic solvents used were of reagent grade and were purchased from Merck KGaA (Düsseldorf, Germany)**,** Sigma-Aldrich Co. (St. Louis, Missouri, U.S.A.) and Panreac Química S.L.U. (Barcelona, Spain).

2.8. Statistical analysis

Results are presented as means ± SD (n = 4). For all statistical tests, p values < 0.05 were considered statistically different. Data were checked for normal distribution with the one-sample Shapiro-Wilk test (Zar, 1999), 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). Incorporation and

esterification comparisons between the six FA means and within [1-^{14}C]C18 FA (18:1n-9, 18:2n-6, 18:3n-3) and [1-^{14}C] LC-PUFA (ARA, EPA, DHA), as well as data of unmodified FA recovery between the six FA means were performed by one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test (Zar, 1999). When normal distribution was not achieved, data were subjected to a Kruskal-Wallis non-parametric test, and when the homogeneity of variances were not achieved, data were subjected to the Welch robust ANOVA, followed by a Games-Howell non-parametric multiple comparison test (Zar, 1999). The statistical analysis was performed using the IBM SPSS statistics 22.0 (IBM Co., Armonk, New York, U.S.A.).

3. Results

3.1. Lipid composition of hatchlings

The TL content and LC composition of *S. officinalis* hatchlings are presented in Table 1. The TL profile of *S. officinalis* hatchlings consisted mainly of polar lipids (PL; 58.1 ± 1.0 %) and cholesterol (32.2 ± 2.1 %). Within PL, phosphatidylcholine (PC; 24.0 ± 1.9 %) and phosphatidylethanolamine (PE; 18.9 ± 1.0 %) were the main classes. On the other hand, triacylglycerols (TAG; 6.3 ± 1.1 %) were the second major class within neutral lipids (NL; 41.9 ± 1.0 %).

Table 2 shows the FA profile of *S. officinalis* hatchlings. Hatchlings were particularly rich in DHA (21.5 ± 1.7 % of total FA), 16:0 (20.4 ± 1.6 %) and EPA (16.4 ± 1.0 %). ARA, 18:1n-9 and 18:2n-6 only accounted for 1.3 ± 0.4 %, 1.5 ± 0.3 % and 0.6 ± 0.1 % of total FA, respectively. The polyunsaturated FA content of hatchlings was 43.1 ± 2.6 % of total FA, while LC-PUFA accounted for 41.8 ± 2.7 % of total FA. The DHA/EPA ratio of hatchlings was 1.3 ± 0.0, while the EPA/ARA ratio was 13.4 ± 3.9.

3.2. Incorporation of radiolabelled FA into TL and its distribution among LC

The incorporation of radiolabelled FA into *S. officinalis* hatchling TL and its distribution among LC is presented in Table 3. Within the C18 FA, [1-^{14}C]18:1n-9 was the least incorporated FA ($p < 0.05$). [1-^{14}C]18:3n-3 and [1-^{14}C]ARA were the most incorporated FA, although not statistically different from [1-^{14}C]18:2n-6 or [1-^{14}C]EPA. All [1-^{14}C]FA substrates incorporated were highly esterified into LC, with less than 2.5 % of radioactivity being recovered as free fatty acids (FFA). The majority of radioactivity incorporated was recovered esterified into PL, with over 90 % of the LC-PUFA substrates being esterified into this lipid fraction. The proportion of radioactivity

recovered in TAG was higher for the [1-^{14}C]C18 FA than for LC-PUFA ($p < 0.05$). Nonetheless, [1-^{14}C]C18 FA were preferentially esterified into PC and phosphatidylserine (PS). The esterification of [1-^{14}C]ARA and [1-^{14}C]EPA into the different LC was similar, showing a preferential esterification into PE ($p < 0.05$), followed by PC > PS > phosphatidylinositol (PI). On the other hand, [1-^{14}C]DHA was mainly esterified into PC (70.6 ± 4.8 % of radioactivity incorporated; $p < 0.05$). The esterification pattern of all the [1-^{14}C]C18 FA substrates was similar, with preferential esterification into PC > PS > PE > TAG.

3.3. Transformation of radiolabelled fatty acids

The transformation of incorporated [1-^{14}C]FA substrates into other FA metabolites is presented in Table 4. [1-^{14}C]C18 FA were the most transformed FA ($p < 0.05$) with several metabolic products being detected. For instance, [1-^{14}C]18:1n-9 was elongated to 20:1n-9 (42.0 ± 5.6 %) and 22:1n-9 (12.7 ± 1.9 %). Although the band corresponding to 22:1n-9 appeared to be split, unequivocal confirmation of the presence of two single bands was not possible. Similar to [1-^{14}C]18:1n-9, [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 presented two elongation products. In addition, potential desaturation products from the three [1-^{14}C]C18 FA substrates were also detected. A second potential desaturation product from [1-^{14}C]18:2n-6 was also observed, although it was not possible to confirm its identity. Of the radioactivity incorporated into hatchlings TL from supplemented [1-^{14}C]LC-PUFA only one elongation product was recovered, except for [1-^{14}C]ARA where an unidentified product, obtained by a potential desaturation, was also detected (Table 4).

4. Discussion

In the present study, similar to that previously reported for *O. vulgaris* (Reis et al., 2014a), it was possible to investigate the *in vivo* FA metabolism of *S. officinalis* hatchlings by following the incorporation of specific [1-^{14}C]FA. The obvious incorporation of all the substrates, points to the suitability of this methodology to study lipid metabolism of newly hatched cephalopods even though it is not clear yet if the FA are being incorporated through drinking activity and/or the integument. As in other marine invertebrates, the epithelial tissues of cephalopods possess microvilli-like absorptive epithelia with the capacity to uptake organic compounds directly from the environment (de Eguileor et al., 2000).

As previously reported by Navarro and Villanueva (2000), cuttlefish hatchlings presented high contents of PC, PE and cholesterol, with 16:0, 18:0, EPA and DHA being the most abundant FA. High contents of these LC and FA were also detected in cuttlefish eggs (Sykes et al., 2009), and in juvenile and adult mantle (Almansa et al., 2006; Ferreira et al., 2010; Sinanoglou and Miniadis-Meimaroglou, 2000, 1998). The consistent high levels of these LC and FA in lipid profiles of *S. officinalis* may reflect their importance during lifespan of this species. Based on that, Navarro and Villanueva (2000) suggested that *S. officinalis* requires a diet rich in PUFA, PL and cholesterol, with moderate content of NL for normal development.

In cuttlefish hatchings, different from that previously reported for *O. vulgaris* hatchlings where a specifically higher incorporation of ARA into TL was observed (Reis et al., 2014a), [1-^{14}C]ARA was not preferentially incorporated compared to EPA or C18PUFA. The ARA levels found in *S. officinalis* tissues (Almansa et al., 2006; Navarro and Villanueva, 2000; Sykes et al., 2009) are normally lower than those detected in *O. vulgaris* (Monroig et al., 2012a; Navarro and Villanueva, 2000; Reis et al., 2014a, 2014b; Viciano et al., 2011), which could point to different requirements for this FA between these species. In contrast, the EPA content found in the present study was generally similar to that reported for *O. vulgaris* hatchlings (see Navarro and Villanueva, 2000; Reis et al., 2014a, 2014b).

Interestingly, and similar to that described by Reis et al. (2014a) for *O. vulgaris* hatchlings, cuttlefish hatchlings presented a similar pattern of esterification of labelled ARA and EPA into different LC, with the majority of the incorporated LC-PUFA being recovered in PE. However, contrary to the results of the earlier study on octopus, both EPA and ARA also showed high esterification into PC and PS in cuttlefish. Sinanoglou and Miniadis-Meimaroglou (2000) determined the FA content of PE and PC of *S. officinalis* mantle, and observed a high content of EPA and ARA in PE. On the other hand, a high level of DHA was observed in PC by the same authors. Similarly, in the present study, DHA was mainly esterified into PC (70.6 ± 4.8 % of incorporated [1-^{14}C]DHA). When comparing the esterification pattern of [1-^{14}C]LC-PUFA in *S. officinalis* hatchlings with that of *O. vulgaris* (Reis et al., 2014a), there appears to be lower specificity in the esterification of FA into specific LC in cuttlefish. Therefore, the DHA/EPA/ARA ratio would be of greater importance in *S. officinalis* development, rather than the EPA/ARA ratio, which appears to have greater importance for *O. vulgaris* paralarvae.

Sinanoglou and Miniadis-Meimaroglou (1998) detected a high content of monounsaturated FA in the NL fraction of *S. officinalis* mantle. In the present study, the proportion of radioactivity esterified into NL was slightly higher for [1-^{14}C]18:1n-9, (19.4 ± 3.4 % of incorporated [1-^{14}C]18:1n-9). Nonetheless, when compared to [1-^{14}C]LC-PUFA, high proportions of [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 were also recovered in NL. This was similar to the data described for *O. vulgaris* hatchling metabolism (Reis et al., 2014a), where [1-^{14}C]C18 FA were highly esterified into NL, as well as into PC. Moreover, in the present study, there was a significant proportion of [1-^{14}C]C18 FA esterified into PS, when compared to [1-^{14}C]LC-PUFA. Again, similar to esterification pattern of [1-^{14}C]LC-PUFA, when comparing *S. officinalis* hatchlings with *O. vulgaris* (Reis et al., 2014a), lower specificity in the esterification of [1-^{14}C]C18 FA substrates into particular LC was observed in cuttlefish.

It is interesting to note that all substrates were mainly esterified into the major LC of *S. officinalis* hatchlings (PC and PE, followed by PS and TAG). However, a pattern was observed, where [1-^{14}C]DHA, and [1-^{14}C]C18 FA were preferentially esterified into PC, whereas [1-^{14}C]ARA and [1-^{14}C]EPA were mainly esterified into PE. In this respect, and considering the competition between these FA for several enzyme activities (Sargent et al., 1999), it seems important to ensure an adequate dietary input of those FA. For that, while designing a suitable diet for *S. officinalis* hatchlings ARA/EPA/DHA, as well as C18 FA/LC-PUFA ratios must be considered. Moreover, future studies are necessary in a way to elucidate the capacity of this animals to translocate dietary EFA within phospholipids and between TAG and phospholipids.

When analysing the variation of FA composition during embryonic development of *S. officinalis*, Sykes et al. (2009) observed an increase in the n-9 FA fraction, particularly 20:1n-9, suggesting possible *de novo* synthesis of these FA. Moreover, the amount of 20:2n-6 and 20:3n-3 also increased during embryogenesis in wild eggs (Sykes et al., 2009). The results of the present study confirmed a high capability of *S. officinalis* hatchlings to elongate [1-^{14}C]C18 FA to C20 FA. In addition, a second elongation step was also noted, as a C22 FA band was obtained from all the [1-^{14}C]C18 FA substrates. However, only 22:1n-9 was previously reported by Sykes et al. (2009) in cuttlefish eggs and by Dumont et al. (1992) in the central nervous system of this species, albeit in insignificant amounts.

The capacity of cuttlefish hatchlings to elongate FA was further confirmed with the [1-^{14}C]LC-PUFA substrates. Monroig et al. (2012b) have reported the functional

365 characterization of an elongase of very long-chain FA (Elovl) gene in *O. vulgaris*,
366 which showed the capacity of this species to convert C18 and C20 PUFA substrates to
367 their corresponding 2C elongated products, although no activity towards C22 PUFA
368 was detected with this Elovl. More recently, Monroig et al. (2013) reported the
369 functional characterization of a second Elovl, which suggested octopus had the
370 capability to elongate C22 PUFA. The present results, and those obtained in octopus
371 (Reis et al., 2014a), showed elongation activity towards DHA. Interestingly, this
372 activity was not detected in any C22 FA products obtained from C18 or C20 FA
373 substrates. Considering, the low amount of radioactivity recovered as C22 FA, and the
374 apparent low elongation activity on these FA, (less than 6 % of [1-^{14}C]DHA
375 incorporated was recovered as 24:6n-3), it is possible that the band obtained would be
376 unnoticed on a TLC plate. Additionally, as mentioned above, in some replicates of [1-
377 ^{14}C]18:1n-9, the bands corresponding to C22 FA appeared to be split. However, an
378 unequivocal and accurate confirmation of its existence and identity was not possible.
379 In contrast to fish, where more efficient elongation of n-3 is reported (Agaba et al.,
380 2005; Monroig et al., 2012c; Morais et al., 2011), in the present study higher activity
381 towards n-6 FA substrates was detected. Thus, 18:2n-6 and 20:4n-6 were transformed at
382 higher rates than the corresponding n-3 FA, namely 18:3n-3 and 20:5n-3, respectively.
383 Preferential elongation activity towards n-6 FA rather than n-3 FA was also previously
384 reported in *O. vulgaris* (Monroig et al., 2012b; Reis et al., 2014a). Nonetheless,
385 considering the incorporated radioactivity into *S. officinalis* hatchlings TL, the absolute
386 value of elongated FA was similar between n-3 and n-6 FA (7.2 ± 1.1 and 7.6 ± 0.3
387 pmoles.mg pp^{-1}.h^{-1} for 18:3n-3 and 18:2n-6, respectively; and 0.3 ± 0.0 and 0.4 ± 0.2
388 pmoles.mg pp^{-1}.h^{-1} for EPA and ARA, respectively).
389 Almansa et al. (2006) suggested the existence of active n-6 LC-PUFA metabolism in
390 juvenile and maturing cuttlefish as biosynthesis of 22:5n-6 from ARA was implied. In
391 animals, 22:5n-6 has been considered as the final end product of the desaturation
392 pathways of n-6 FAs (Tocher et al., 1998). The synthesis of this FA from ARA involves
393 one of two possible metabolic pathways: the "Sprecher pathway", which comprises two
394 sequential elongations of ARA to 24:4n-6, followed by a Δ6 desaturation and one round
395 of peroxisomal β-oxidation (Δ4-independent pathway; Sprecher, 2000); or a direct route
396 involving elongation of ARA to 22:4n-6 followed by a Δ4 desaturation (Li et al., 2010).
397 For many years the activity of Δ4 fatty acyl desaturase enzyme (Fad) was not
398 demonstrated in vertebrates, and it was assumed that DHA was synthesised by the Δ4-

399 independent pathway (Tocher et al., 1998). However, recent studies have identified the
400 activity of a Δ4 Fad, not just in lower eukaryotes (Pereira et al., 2003), but also in some
401 teleost fish species (Li et al., 2010; Morais et al., 2015, 2012; Fonseca-Madrigal et al.,
402 2014). As previously mentioned, the present study confirmed the elongation of [1-
403 ^{14}C]ARA to 22:4n-6. Moreover, another unidentified metabolic product from [1-
404 ^{14}C]ARA was observed, which could support the previous suggestion of Almansa et al.
405 (2006). However, a similar band detected in *O. vulgaris* hatchling [1-^{14}C]ARA
406 metabolism was determined as a metabolic product other than a FA (Reis et al.,
407 unpublished data). Furthermore, no similar band was obtained from [1-^{14}C]EPA.
408 Interestingly, a similar metabolic product to that detected from [1-^{14}C]ARA, was also
409 obtained from [1-^{14}C]18:2n-6. Since there was no evidence for ARA production from
410 [1-^{14}C]18:2n-6, it would be unexpected that this band corresponds to 22:5n-6. Although
411 it was not possible to identify the identity of this band, it is important to mention its
412 appearance when n-6 FA were incubated, since it may possibly indicate a different
413 requirement of cephalopods for n-6 FA.
414 The synthesis of EPA and ARA from 18:3n-3 and 18:2n-6, respectively, requires the
415 activity of a Δ6 Fad, followed by elongation, and then a second Fad introducing a
416 double bond at the Δ5 position of the elongated FA (Cook, 1996). However, in some
417 marine fish species, the Δ6 enzyme also presents a Δ8 activity and, so the first two steps
418 could be reversed in order (Monroig et al., 2011). In the present study, according to the
419 position of co-running standards, the potential resultant desaturation products from [1-
420 ^{14}C]C18 FA substrates might involve a Δ6 desaturation. Nonetheless, unequivocal
421 identification of the desaturation products obtained from C18 FA (18:2n-9, 18:3n-6 and
422 18:4n-3) would be difficult, since the route involving an elongation followed by Δ8
423 desaturation (producing 20:2n-9, 20:3n-6 and 20:4n-3) cannot be eliminated. In this
424 sense, the characterization of the enzymes involved in PUFA biosynthesis of cuttlefish
425 would be an invaluable tool.
426 Functional characterization in recombinant yeast showed that *O. vulgaris* possessed a
427 Fad that exhibited Δ5 desaturation activity towards PUFA substrates (Monroig et al.,
428 2012a). Recently, similar results were also obtained regarding Fad genes of *S. officinalis*
429 hatchlings (Monroig et al., 2013). However, no Δ5 Fad activity was detected in the
430 present study. Despite this, the present data do not rule out a possible Δ5 Fad activity in
431 ARA or EPA biosynthesis *in vivo*. The low desaturation rates observed in the present

study and the low Δ5 Fad activity on PUFA substrates, reported by Monroig et al. (2012a), could explain the absence of radiolabelled ARA or EPA bands.

In summary, the methodology employed in the present study allows to investigate the *in vivo* FA metabolism of *S. officinalis* hatchlings by following the tissue incorporation of specific [1-^{14}C]FA, and points out to the suitability of this methodology to study lipid metabolism of newly hatched cephalopods. The present results may indicate the possibility that *S. officinalis* hatchlings have *in vivo* capability for the first steps of ARA and EPA biosynthesis from 18:2n-6 and 18:3n-3, respectively, including a possible desaturase. Nonetheless, considering the low desaturation rates detected, this process would not be sufficient to satisfy the demands for normal development of this species. Therefore, both ARA and EPA, along with DHA, must be supplied in the diet at least during the hatchling stage. In addition, the esterification specificity for DHA into PC and of ARA and EPA into PE and PC, detected in the present study, could be hampered by competition between them and also between C18 FA and LC-PUFA. Therefore, in order to design an efficient inert diet that would ensure normal growth and development of this species during early life stages, DHA/EPA/ARA ratio, rather than EPA/ARA or EPA/DHA ratios and also C18 FA and LC-PUFA ratio, must be considered to reflect the EFA requirements for *S. officinalis* hatchlings development. Nonetheless, future studies are necessary in a way to elucidate the capacity of this animals to translocate dietary EFA within phospholipids and between TAG and phospholipids.

5. Aknowledgements

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615 **Table 1** – Total lipid content and lipid class
616 composition of *Sepia officinalis* hatchlings

617 TL content (μg lipid.mg protein^{-1})	384.9 ± 57.5
618 *Lipid Class* (%)	
619 Sphingomyelin	0.8 ± 0.4
620 Phosphatidylcholine	24.0 ± 1.9
Phosphatidylserine	7.0 ± 0.6
621 Phosphatidylinositol	5.0 ± 0.2
622 Phosphatidylglycerol	2.4 ± 0.2
623 Phosphotidylethanolamine	18.9 ± 1.0
624 ∑ Polar lipids	58.1 ± 1.0
625	
626	
627 Cholesterol	32.2 ± 2.1
628 Free fatty acids	0.8 ± 0.2
629 Triacylglycerols	6.3 ± 1.1
630 Sterol esters	2.6 ± 0.8
631 ∑ Neutral lipids	41.9 ± 1.0
632	
633	

634
635 Results represent means ± SD; n = 4.
636 Data are presented in percentage of TL content.

637 **Table 2 –** Fatty acid composition (% of total
638 FA) of *S. officinalis* hatchlings

16:0	20.4 ± 1.6
18:0	10.9 ± 0.3
∑ saturated[a]	42.3 ± 2.7
16:1[b]	0.9 ± 0.0
18:1n-9	1.5 ± 0.3
18:1n-7	3.4 ± 0.3
20:1n-9	3.4 ± 0.1
∑ monoenes[a]	13.8 ± 0.7
18:2n-6	0.6 ± 0.1
20:2n-6	0.3 ± 0.0
20:4n-6	1.3 ± 0.4
∑ n-6 FA[a]	2.2 ± 0.3
∑ n-6 LC-PUFA	1.3 ± 0.4
20:3n-3	1.3 ± 0.4
20:5n-3	16.4 ± 1.0
22:5n-3	1.4 ± 0.1
22:6n-3	21.5 ± 1.7
∑ n-3 FA[a]	40.9 ± 2.9
∑ n-3 LC-PUFA	40.5 ± 3.0
∑ PUFA[a,c]	43.1 ± 2.6
∑ LC-PUFA[a,d]	41.8 ± 2.7
n-3/n-6	18.8 ± 3.4
DHA/EPA[e]	1.3 ± 0.0
EPA/ARA[e]	13.4 ± 3.9

639 Results represent means ± SD; n = 4.
640 Data are presented in percentage of total FA content.
641 [a] Totals include some minor components not shown
642 [b] Contain n-9, n-7 and n-5 isomers
643 [c] PUFA – Polyunsaturated fatty acids
644 [d] LC-PUFA – Long-chain polyunsaturated fatty acids
645 [e] ARA – 20:4n-6; EPA – 20:5n-3; DHA – 22:6n-3
646

Table 3 – Incorporation of radioactivity into total lipid (pmoles.mg pp^{-1}.h^{-1}) and its esterification (%) into lipid classes of *S. officinalis* hatchlings incubated with [1-^{14}C]FA substrates

Substrate	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Incorporation	8.2 ± 1.4 $^{a\bullet}$	15.4 ± 3.4 $^{bc\blacktriangle}$	18.0 ± 3.7 $^{c\blacktriangle}$	18.0 ± 1.3 $^{c\Delta}$	14.6 ± 2.9 $^{bc\circ\Delta}$	11.4 ± 1.1 $^{ab\circ}$
Lipid Class (%)						
Phosphatidylcholine	38.3 ± 3.0 $^{c\bullet}$	39.2 ± 1.7 $^{bc\bullet}$	44.7 ± 2.1 $^{b\blacktriangle}$	26.9 ± 1.1 $^{d\circ}$	26.7 ± 0.5 $^{d\circ}$	70.6 ± 4.8 $^{a\Delta}$
Phosphatidylserine	25.2 ± 1.4 $^{a\blacktriangle}$	22.2 ± 1.4 $^{b\bullet}$	21.5 ± 0.5 $^{bc\bullet}$	19.3 ± 1.4 $^{cd\Delta}$	17.2 ± 1.4 $^{d\Delta}$	2.9 ± 0.3 $^{e\circ}$
Phosphatidylinositol	4.1 ± 0.9 ^{c}	3.2 ± 0.5 ^{c}	3.4 ± 0.1 ^{c}	11.1 ± 1.4 $^{a\Delta}$	11.5 ± 0.9 $^{a\Delta}$	6.1 ± 1.4 $^{b\circ}$
Phosphatidylglycerol	0.0 ± 0.0 $^{c\blacksquare}$	3.9 0.3 $^{a\blacktriangle}$	3.3 0.2 $^{b\bullet}$	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{c}
Phosphatidylethanolamine	12.9 ± 0.8 $^{b\bullet}$	15.0 ± 0.8 $^{b\blacktriangle}$	14.2 ± 1.1 $^{b\blacktriangle\bullet}$	36.6 ± 2.2 $^{a\Delta}$	37.5 ± 1.9 $^{a\Delta}$	12.6 ± 1.0 $^{b\circ}$
∑ Polar lipids	80.6 ± 3.4 $^{d\bullet}$	83.5 ± 1.9 $^{cd\blacktriangle\bullet}$	87.1 ± 3.1 $^{bc\blacktriangle}$	93.9 ± 2.0 ^{a}	92.9 ± 1.5 ^{a}	92.2 ± 2.5 ^{ab}
Partial acylglycerols	5.4 ± 2.2 ^{a}	5.1 ± 0.5 ^{a}	3.3 ± 1.2 ^{ab}	2.1 ± 0.7 ^{b}	2.1 ± 0.7 ^{b}	2.9 ± 1.1 ^{ab}
Free fatty acids	2.3 ± 0.9	2.1 ± 1.0	1.9 ± 0.7	0.7 ± 0.3	0.9 ± 0.3	1.5 ± 1.1
Triacylglycerols	11.7 ± 1.3 $^{a\blacktriangle}$	9.3 ± 1.5 $^{ab\blacktriangle\bullet}$	7.7 ± 1.2 $^{b\bullet}$	3.3 ± 1.1 ^{c}	4.1 ± 1.3 ^{c}	3.4 ± 0.6 ^{c}
∑ Neutral lipids	19.4 ± 3.4 $^{a\blacktriangle}$	16.5 ± 1.9 $^{ab\blacktriangle\bullet}$	12.9 ± 3.1 $^{bc\bullet}$	6.1 ± 2.0 ^{d}	7.1 ± 1.5 ^{d}	7.8 ± 2.5 ^{cd}

Results represent means ± SD; n=4

Data of incorporation are presented in pmoles of ^{14}C fatty acid incorporated /mg protein per hour

Data of esterification are given in percentage

Different letters in superscript within the same row represent significant differences within all fatty acids (p<0.05)

Different full symbols in superscript ($^{\blacktriangle\bullet\blacksquare}$) within the same row represent significant differences within C18 FA (p<0.05)

Different hollow symbols in superscript ($^{\Delta\circ\square}$) within the same row represent significant differences within LC-PUFA (p<0.05).

Table 4 – Recovery of radioactivity from [1-^{14}C]FA substrates in FA metabolites in *S. officinalis* hatchlings

Fatty acids	% recovery
[1-^{14}C]18:1n-9	
18:1n-9	43.2 ± 7.3
20:1n-9	40.0 ± 5.6
22:1n-9	12.1 ± 1.9
18:2n-9	4.7 ± 0.7
[1-^{14}C]18:2n-6	
18:2n-6	48.3 ± 1.7
20:2n-6	43.4 ± 1.8
22:2n-6	5.9 ± 0.6
18:3n-6	1.3 ± 0.2
UK	1.1 ± 0.1
[1-^{14}C]18:3n-3	
18:3n-3	58.5 ± 6.3
20:3n-3	36.2 ± 5.8
22:3n-3	3.6 ± 0.7
18:4n-3	1.7 ± 0.2
[1-^{14}C]20:4n-6	
20:4n-6	96.9 ± 1.2
22:4n-6	2.1 ± 1.2
UK	1.0 ± 0.0
[1-^{14}C]20:5n-3	
20:5n-3	98.3 ± 0.3
22:5n-3	1.7 ± 0.3
[1-^{14}C]22:6n-3	
22:6n-3	94.5 ± 1.3
24:6n-3	5.5 ± 1.3

[a] Results represent means ± SD; n=4