***In vivo* metabolism of unsaturated fatty acids in *Octopus vulgaris*
hatchlings determined by incubation with ¹⁴C-labelled fatty acids
added directly to seawater as protein complexes**

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Abstract

The high mortalities observed during *Octopus vulgaris* paralarvae culture have been associated with a nutritional imbalance, with long-chain polyunsaturated fatty acids (LC-PUFA) appearing to have a critical role. In order to determine the *in vivo* capability of *O. vulgaris* hatchlings to incorporate and metabolise unsaturated fatty acids (FA), hatchlings were incubated in flat-bottom 6-well tissue culture plates at a density of 90 hatchlings/well in 10 mL of seawater (36 ‰). Incubations were performed with gentle stirring at 21 °C for 6 h with 0.2 µCi (0.3 µM) of [1-¹⁴C]-labelled FA including 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6 (ARA), 20:5n-3 (EPA) or 22:6n-3 (DHA), which were added directly to the seawater as their potassium salts bound to bovine serum albumin (BSA). A control treatment without [1-¹⁴C]FA was also assessed. *O. vulgaris* hatchlings not only possessed the ability to incorporate FA bound to BSA, but also to esterify them into phospholipid, with marked specificity. [1-¹⁴C]DHA and [1-¹⁴C]C18 FA substrates were mainly esterified into phosphatidylcholine, while [1-¹⁴C]ARA and [1-¹⁴C]EPA were esterified into phosphatidylethanolamine. The majority of radioactivity from [1-¹⁴C]FA incorporated into hatchling total lipid was recovered as unmodified FA with elongation being the only metabolism detected. Of the FA investigated, [1-¹⁴C]ARA was the most efficiently incorporated into hatchling lipids, but it was also the least modified FA. The fact that no desaturation activity was recorded towards the FA tested in this experiment may indicate that the nutritional requirements of *O. vulgaris* hatchlings in terms of FA are highly specific and LC-PUFA must be considered essential dietary nutrients.

Keywords: Common octopus; Lipid metabolism; *Octopus vulgaris* hatchlings; radiolabeled substrates; unsaturated fatty acids.

1. Introduction

The common octopus (*Octopus vulgaris*) is a cephalopod species that has been suggested as a candidate for large scale culture (Vaz-Pires et al., 2004), not only due to its high nutritional value and demand in several countries, but also due to biological characteristics such as short life cycle, rapid growth, high fecundity rate, easy adaptability to rearing conditions, high food conversion rates and acceptance of non-living natural foods during the juvenile and adult stages (Estefanell et al., 2011; Iglesias et al., 2007; Vaz-Pires et al., 2004).

Currently this species is being cultured in NW Spain by ongrowing wild-captured sub-adults in land-based tanks or sea cages until they reach 2-3 Kg (Iglesias et al., 2007).

However, *O. vulgaris* culture is limited by low profitability since no adequate artificial diet has been developed (Domingues et al., 2010), and due to the dependence on captured wild juvenile specimens as a result of the low survival rates registered during the rearing of planktonic stages (Iglesias et al., 2013, 2007; Moxica et al., 2002; Viciano et al., 2011; Villanueva and Norman, 2008).

The high mortalities registered during paralarvae rearing have been associated with potential nutritional imbalances (Iglesias et al., 2007; Navarro and Villanueva 2003, 2000; Villanueva et al., 2004; Seixas 2010a, 2010b). However, studies of nutritional requirements are complex, as paralarvae display both external and internal digestion, and identification of prey by analysis of stomach contents is therefore challenging (Villanueva and Norman, 2008). Moreover, performing feeding trials with octopus paralarvae is highly demanding given the low survival rates obtained during its rearing (Iglesias et al., 2007; Moxica et al., 2002; Viciano et al., 2011; Villanueva and Norman, 2008). A recent study was able to determine a predominance of crustacean zoeae in wild *O. vulgaris* paralarvae diet (Roura et al., 2012). However, when crustacean zoeae were provided as prey in paralarvae rearing, high mortalities and low growth rates were still obtained, despite the higher growth registers compared to *Artemia* sp. (Iglesias et al., 2007). In addition according to those same authors, the use of crustacean zoeae is demanding regarding the logistics of provision of zooplankton for large scale culture. Several studies have reported that fatty acids (FA) have a critical role in *O. vulgaris* development (Miliou et al., 2006; Navarro and Villanueva 2003; Okumura et al., 2005). Among those, polyunsaturated fatty acids (PUFA), and particularly 20:5n-3 (EPA) and 22:6n-3 (DHA), were suggested as essential fatty acids (EFA) for normal development of *O. vulgaris* paralarvae (Navarro and Villanueva, 2003). In contrast, whether 20:4n-6

(ARA) was also an EFA remains an open question. Miliou et al. (2006) determined that ARA levels in octopuses were not related to dietary input, suggesting a possible production of ARA in this species. It is interesting that a similar statement was made by Almansa et al. (2006) for another cephalopod, *Sepia officinalis*. More recently, Monroig et al. (2012a) reported that *O. vulgaris* tissues possess an enzyme with $\Delta 5$ -desaturation activity that participates in the endogenous production not only of ARA from 20:3n-6 but also of EPA from 20:4n-3. However, 20:4n-3 and especially, 20:3n-6 are not readily available in the natural diets of *O. vulgaris*, and their production depends on the elongation and desaturation of precursors (18:3n-3 and 18:2n-6, respectively), and the relevant fatty acyl elongase and desaturase ($\Delta 6$) activities have not been reported in *O. vulgaris* tissues.

On the other hand, not only the dietary FA profile seems crucial for the normal development of early life-cycle stages of *O. vulgaris*. In fact, the way FA are presented to paralarvae may also be important, as this may affect specific lipid class biosynthesis or FA exchange between and/or within phospholipids (PL) and triacylglycerols (TAG) (Sargent et al., 1999). According to Navarro and Villanueva (2003, 2000), *O. vulgaris* paralarvae require prey rich, not only in PUFA, but also in PL and cholesterol. Viciano et al. (2011) determined that *O. vulgaris* PL present a distinct FA profile compared to neutral lipids (NL), with the former presenting a higher content of PUFA and the latter of monounsaturated FA. However, octopus nutritional requirements for specific lipid classes have not been determined.

The aim of the present study was to contribute to determine the endogenous ability of *O. vulgaris* to biosynthesise lipid classes containing essential FA that would likely ensure normal growth and development of paralarvae. To this end, the *in vivo* capability of *O. vulgaris* hatchlings to incorporate, esterify into different lipid classes and metabolise unsaturated FA was investigated.

2. Materials & Methods

2.1. Experimental animals

O. vulgaris broodstock (30 individuals) with an average wet weight of 1309 ± 503 g caught by professional artisanal fishermen on the Tenerife coast (Canary Islands, Spain), were maintained in 1000 L circular fiberglass tanks in a flow-through seawater system under natural photoperiod (from 10L:14D to 11L:13D), water temperature of 21.01 ± 0.69 °C and salinity of 36.8 ± 0.14 ‰. Three octopuses with similar size and

with a sex ratio of 2 females per male were placed in each tank. Individuals were fed *ad libitum* with frozen squid (*Loligo opalescens*). The presence of eggs was checked once a week and when egg masses were observed, the ovate female was left alone in the tank by removing the other individuals. Approximately one month later octopus began to hatch. Hatchlings were removed daily from the female/rearing tank, so newly hatched octopus (less than 24 h old) would be obtained for experiments.

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

In order to investigate the ability of *O. vulgaris* to incorporate and metabolise unsaturated FA, 1890 hatchlings were incubated in flat-bottom 6-wells tissue culture plates (SARSTEDT AG & CO., Germany) at a density of 90 hatchlings/well in 10 mL of seawater (36 ‰). Incubations were performed with gentle stirring at 21 °C for 6 h, with 0.2 μCi (0.3 μM) of [$1\text{-}^{14}\text{C}$]FA including 18:1n-9, 18:2n-6, 18:3n-3, ARA, EPA and DHA (n=4), which were added individually to separated incubation chambers as potassium salts bound to bovine serum albumin (BSA) (Ghioni et al., 1997). The incubation conditions were previously optimised through several density and time-course assays. Hatchlings incubated without [$1\text{-}^{14}\text{C}$]FA (n=3) were used as control treatment. A survival rate of 100% was obtained in all incubations. After incubation, *O. vulgaris* hatchlings were washed thoroughly with seawater to remove excess [$1\text{-}^{14}\text{C}$]FA. Extraction of total lipids (TL) was performed according to the Folch method as described by Christie (2003). The organic solvent was evaporated under a stream of nitrogen and the lipid content determined gravimetrically. The TL extracts were stored at -20 °C in chloroform/methanol (2:1) with 0.01% butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10 mg/mL and under an inert atmosphere of nitrogen until analysis.

The experiments of this work were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.3. Lipid classes and fatty acids composition of non-radioactive samples

The control group incubations were analysed for lipid class and FA compositions. Lipid classes were separated by double development high-performance thin-layer chromatography (HPTLC; Olsen and Henderson, 1989) and quantified by charring followed by calibrated densitometry using a dual-wavelength flying spot scanner CS-9001PC (Shimadzu). FA were obtained by acid-catalysed transmethylation of 1 mg of

lipid extracts. The fatty acid methyl esters (FAME) were purified by thin-layer chromatography (TLC) (Christie, 2003), separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Scientific) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (Sigma-Aldrich, Madrid, Spain). Identity of individual FAME was confirmed by GC-MS chromatography (DSQ II, Thermo Scientific).

2.4. Incorporation of radioactivity into total lipids

To determine the radioactivity incorporated into hatchlings, an aliquot of 0.1 mg of TL was transferred to a scintillation vial. Radioactivity was quantified in a RackBeta 1214 liquid scintillation β -counter (LKB, Wallac, USA). Results in dpm were transformed into pmol/mg protein/hour ($\text{pmol mg}^{-1} \text{h}^{-1}$) taking into account specific activity of each substrate, and hatchlings TL and protein contents (Lowry et al., 1951).

2.5. Esterification of radioactivity into lipid classes

To define the esterification pattern of $[1-^{14}\text{C}]\text{FA}$ in lipid classes, a sample of 0.1 mg of TL was applied to HPTLC plates. Lipid classes were separated by single-dimensional double-development HPTLC as previously described by Tocher and Harvie (1988). The developed HPTLC plates were placed in closed exposure cassettes (Exposure Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Image Screen-K, BioRad, Madrid, Spain) for one week. The screens were then scanned with an image acquisition system (Molecular Imager FX, BioRad) and the bands quantified in percentage by image analysis software (Quantity One, BioRad).

2.6. Transformation of radiolabelled fatty acids

To determine metabolism patterns of radiolabelled substrates in terms of desaturation/elongation processes, a sample of 1.7 mg of TL was subjected to acid-catalysed transmethylation to prepare FAME as detailed above (Christie, 2003). FAME were separated by TLC using plates impregnated with a solution of 2 g silver nitrate in 20 mL acetonitrile followed by activation at 110 °C for 30 min. The plates were fully developed in toluene/acetonitrile (95:5, v/v), which resolves the FAME into discrete bands based on both degree of unsaturation and chain length (Wilson and Sargent, 1992). Developed TLC plates were placed in BioRad closed exposure cassettes in contact with Imagen Screen-K for two weeks. The screens were then scanned with

Molecular Imager FX and bands quantified by Quantity One software. Identification of labelled bands was confirmed by radiolabelled standards run on the same plate (Rodríguez et al., 2002).

2.7. Materials

[1-¹⁴C]C18 FA were purchased from PerkinElmer, Inc. (USA). [1-¹⁴C] LC-PUFA were purchased from American Radiolabeled Chemicals, Inc. (USA). BSA was purchased from Sigma-Aldrich (USA). TLC plates (20 X 20 cm X 0.25 mm) were purchased from Macherey-Nagel GmbH & Co.KG (Germany). HPTLC plates, (10 X 10 cm X 0.15 mm) pre-coated with silica gel 60 (without fluorescent indicator), were purchased from Merck KGaA (Germany). OptiPhase “HiSafe” 2 scintillant liquid was purchased from PerkinElmer, Inc. (USA). Organic solvents used were of reagent grade and were purchased from Merck KGaA (Germany), Sigma-Aldrich (USA) and Panreac Química S.L.U. (Spain).

2.8. Statistical analysis

Results are present as means $\pm$ SD. For all statistical tests, *p* values of less than 0.05 were considered statistically different. Data were checked for normal distribution with the one-sample Kolmogorov–Smirnov 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). Comparisons between the six FA means and within [1-¹⁴C]C18 FA (18:1n-9, 18:2n-6, 18:3n-3) and [1-¹⁴C] LC-PUFA (ARA, EPA, DHA) were analysed by 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 Kruskal-Wallis non-parametric test, followed by a Games-Howell non-parametric multiple comparison test (Zar, 1999). The statistical analysis was performed using the IBM SPSS statistics 20.0 (IBM Corp., Armonk, NY).

3. Results

3.1. Lipid class and fatty acid composition of *O. vulgaris* hatchlings (non-radioactive samples)

The TL content of control *O. vulgaris* hatchlings was 384.9 ± 57.5 μ g lipid/mg protein, which consisted mainly of PL (64.5 ± 3.2 %), with phosphatidylethanolamine (PE, 25.5

± 1.5 %) and phosphatidylcholine (PC, 19.6 ± 0.6 %) as the main PL classes (Table 1). Cholesterol was the most abundant lipid class of octopus hatchlings, corresponding to 30.1 ± 2.9 %, and TAG and sterol esters (SE) accounted for 1.2 ± 0.4 and 2.1 ± 0.8 % of TL, respectively (Table 1).

The *O. vulgaris* hatchlings were particularly rich in the n-3 LC-PUFA, DHA and EPA, and the saturated fatty acids (SFA) 16:0 and 18:0 (Table 2). The n-3 LC-PUFA content was 42.4 ± 0.4 % of total FA, while total LC-PUFA accounted for 46.4 ± 0.5 %. The DHA/EPA ratio of *O. vulgaris* hatchling TL was 1.8 ± 0.0 and the EPA/ARA ratio was 3.8 ± 0.2 (Table 2).

3.2. Incorporation of radioactivity into total lipids and its distribution into lipid classes

Table 3 shows the incorporation of radiolabelled FA into TL of *O. vulgaris* hatchlings and its distribution among lipid classes. Incorporation of [1-¹⁴C]ARA into TL was significantly higher than that of all other radiolabelled FA ($p < 0.05$). No differences were detected between incorporation of [1-¹⁴C]C18 FA and [1-¹⁴C]LC-PUFA (EPA and DHA). All ¹⁴C-labelled FA incorporated were esterified into lipid classes, with less than 6 % of radioactivity being recovered as free fatty acids (FFA) (Table 3). The majority of the [1-¹⁴C]FA were recovered in PL. The esterification pattern of C18 FA was: PC > TAG > PI for [1-¹⁴C]18:1n-9, PC > PE > PS for [1-¹⁴C]18:2n-6 and PC > PS > PE for [1-¹⁴C]18:3n-3. Interestingly, the highest incorporation into PC was obtained with [1-¹⁴C]DHA, with 78.4 ± 3.9 % of radioactivity being esterified into this PL ($p < 0.05$). In contrast, [1-¹⁴C]ARA and [1-¹⁴C]EPA were predominantly esterified into PE ($p < 0.05$), followed by PS > PI ≥ PC for ARA and PC > PS ≥ PI for EPA (Table 3). The proportion of radioactivity recovered as TAG was higher for the [1-¹⁴C]C18 FA than for LC-PUFA ($p < 0.05$).

3.3. Transformation of radiolabelled fatty acids

The majority of radioactivity from [1-¹⁴C]FA incorporated into *O. vulgaris* hatchlings TL was recovered as unmodified substrate (Table 4). Nonetheless, small proportions of the radiolabelled substrate FA were transformed into other FA products. [1-¹⁴C]18:1n-9 and [1-¹⁴C]18:2n-6 were the FA showing most transformation, as well as the only substrates where two different metabolic products were detected. The radioactivity incorporated into hatchling TL as [1-¹⁴C]18:1n-9 was converted to 20:1n-9 (19.2 ± 1.6 %) and 22:1n-9 (9.2 ± 1.3 %). Radioactivity from [1-¹⁴C]18:2n-6 was also recovered

as 20:2n-6 (31.6 ± 1.5 %) and 22:2n-6 (3.2 ± 0.4 %). With [1- 14 C]LC-PUFA substrates, DHA was the most transformed with approximately 4 % of radioactivity being recovered as 24:6n-3. In contrast, with ARA only 1.2 ± 0.4 % of radioactivity was recovered as 22:4n-6, the only metabolic product. It was noteworthy that only elongation products were detected for all added substrates (Table 4).

4. Discussion

The present results demonstrate the feasibility of investigating the *in vivo* FA metabolism of *O. vulgaris* hatchlings, by following the incorporation of [1- 14 C]FA bound to BSA added to seawater. The capacity of *O. vulgaris* paralarvae to incorporate nutrients dissolved in seawater was previously recorded by Villanueva et al. (2004), by incubating octopus paralarvae with radiolabelled amino acids. Nonetheless, the way those nutrients are incorporated is not yet completely understood. Fluids within the digestive tract are isosmotic in relation to seawater (Wells and Wells, 1989) and starved adult octopuses drink only 4 ml kg $^{-1}$.h $^{-1}$ (Wells and Wells, 1988). This probably suggests that in paralarvae, water uptake by passive drinking might be insignificant. Alternatively, de Eguileor et al. (2000) determined that *S. officinalis* epithelial tissues possess microvilli-like absorptive epithelia, with the capacity for uptake of radiolabelled amino acids. Moreover, Boucaud-Camou and Roper (1995) revealed the existence of alkaline phosphatase activity in *O. vulgaris* paralarvae skin, indicating the possibility for active absorption of nutrients from seawater during the planktonic stage. These studies may suggest that skin could play an important role in cephalopod nutrition. For instance, Villanueva et al. (2004) investigated the effects of seawater dissolved amino acids in *O. vulgaris* paralarvae culture, finding that survival rate was three-fold higher in the supplemented group compared to that of the control group. Therefore, future research using dissolved organic matter might be considered as a way to improve paralarvae rearing.

As previously reported, *O. vulgaris* hatchlings presented high contents of PC, PE and cholesterol among their lipid classes (Navarro and Villanueva, 2003, 2000; Seixas et al., 2010a), with 16:0, 18:0, EPA and DHA being the most abundant FA (Navarro and Villanueva, 2000; Okumura et al., 2005; Seixas et al., 2010a, 2010b, 2008). The consistent high contents of those lipid classes and FA in the lipid profiles of *O. vulgaris* hatchlings may suggest their importance in the development of this species. Accordingly, Navarro and Villanueva (2003, 2000) have suggested that octopus

288 paralarvae require a prey rich in PUFA, PL and cholesterol, with a moderate content of
 289 NL.
 290 Of all incubated radiolabelled FA, ARA was the most efficiently incorporated into
 291 hatchling lipids. As the same quantity of radiolabelled substrates was added to each
 292 treatment, the amount of ARA incorporated into *O. vulgaris* hatchlings appears to be
 293 unrelated to its abundance in the surrounding water. Preferential incorporation of dietary
 294 ARA into TL was previously reported by Atalah et al. (2011a) in *Sparus aurata* larvae.
 295 However, incorporation of ARA into larval TL of this species increased, and that of
 296 EPA decreased, when dietary ARA was increased, and vice-versa (Atalah et al., 2011a).
 297 A similar inverse relation between ARA and EPA incorporation was also reported for
 298 *Paralichthys olivaceus* eggs polar lipids (Furuita et al., 2003), as well as in *O. vulgaris*
 299 tissues (Miliou et al., 2006). This inverse relation could possibly be related competition
 300 between these two LC-PUFA for acylases and transacylases in FA esterification
 301 (Sargent et al., 1999). As these enzymes do not have tight specificity for particular FA,
 302 tissue compositions are partly determined by dietary FA levels and ratios. In the present
 303 study and although in different proportions, the general esterification pattern of ARA
 304 and EPA into lipid classes was similar, with the majority of radioactivity being
 305 recovered in PE. This could contribute to the competition described above, and
 306 therefore, adequate dietary inputs of both FA might be crucial in *O. vulgaris*
 307 development. In previous studies investigating dietary lipid in *O. vulgaris* paralarvae
 308 rearing, the EPA/ARA ratio was usually not considered (Kurihara et al., 2006; Navarro
 309 and Villanueva, 2003, 2000; Okumura et al., 2005; Seixas et al., 2010a, 2010b; Viciano
 310 et al., 2011). Nonetheless, calculating the EPA/ARA ratio of octopus hatchlings in those
 311 studies, great variability was observed, ranging from 1.7 (Navarro and Villanueva,
 312 2000) to 4.5 (Seixas et al., 2008). Additionally, the ideal EPA/ARA ratio that promotes
 313 normal development of this species is difficult to discern, due to the inconsistent results
 314 obtained, in terms of growth and survival, during paralarvae rearing (Kurihara et al.,
 315 2006; Navarro and Villanueva, 2003, 2000; Okumura et al., 2005; Seixas et al., 2010a,
 316 2010b; Viciano et al., 2011).
 317 Similar to Viciano et al. (2011), who detected a higher content of MUFA in the NL
 318 fraction of *O. vulgaris*, in the present study the proportion of radioactivity recovered as
 319 TAG was more specifically higher for [1-¹⁴C]18:1n-9. This preferential esterification
 320 might be related to the energy-generating FA oxidation systems (Sargent et al., 1989).
 321 In marine fish larvae, NL and more specifically TAG are presumably used to satisfy

energy demands (Rainuzzo et al., 1997, Sargent et al., 1989), with SFA and MUFA being the main substrates. In contrast, LC-PUFA are preferentially esterified into PL, revealing their important structural role in cell membranes (Sargent et al., 1989). Following Linares and Henderson (1991), the esterification of LC-PUFA into lipid classes is usually associated to their abundance in that class. Therefore, it would be expected that in *O. vulgaris* hatchlings a higher content of LC-PUFA would be found in PL, as reported by Viciano et al. (2011) and more specifically, that DHA would be relatively abundant in PC, while EPA and ARA would be in PE.

A higher esterification rate of DHA, 18:1n-9, 18:2n-6 and 18:3n-3 and their products into PC was observed in the present study. *Artemia* sp., one of the most commonly used live preys for octopus paralarvae rearing, have high levels of 18:1n-9, 18:2n-6 and 18:3n-3, but very limited levels of DHA, often even after enrichment (Fuentes et al., 2011; Navarro and Villanueva, 2000; Seixas et al., 2010a, 2010b; Viciano et al., 2011). Preferential esterification of PUFA into TAG was already shown in *Artemia* sp. (Guinot et al., 2013; Navarro et al., 1999). If, as reported for marine fish (Sargent et al., 1999), *O. vulgaris* have only limited FA exchange between PL and TAG, the enrichment of *Artemia* sp. with DHA or other LC-PUFA may be worthless. In fact, Seixas et al. (2010a), suggested that high DHA content in *Artemia* sp. was not necessarily related to improved development of paralarvae. This should be taken into account in octopus nutrition since it may influence rearing success, particularly if endogenous metabolism of *Artemia* sp. or other live prey compromises the availability of EFA for paralarvae.

While DHA and EPA have been regarded as EFA for *O. vulgaris*, the essentiality of ARA is still under debate. Following Milliou et al. (2006), ARA levels in octopus tissues were not related to dietary input, and possible endogenous biosynthesis of this FA was suggested (García-Garrido et al., 2010; Milliou et al., 2006). In fact, the existence of an enzyme with $\Delta 5$ -desaturation activity that participates in the endogenous production of ARA from 20:3n-6 and also of EPA from 20:4n-3 has been shown (Monroig et al., 2012a). In the present study, when [1- 14 C]18:2n-6 and [1- 14 C]18:3n-3 were incubated, it was not possible to detect any trace of ARA and EPA synthesis. These data do not invalidate possible $\Delta 5$ fatty acyl desaturase enzyme activity in ARA or EPA *in vivo* biosynthesis, since the FA substrates that this enzyme is known to act upon were not tested (Monroig et al., 2012a). The fact that no desaturation activity was observed towards the FA substrates used in this *in vivo* experiment may indicate that the

nutritional requirements of *O. vulgaris* hatchlings in terms of FA are highly specific and that LC-PUFA must be considered as EFA and so provided in the diet.

Of the substrates incubated, [1-¹⁴C]ARA was not only the most efficiently incorporated into hatchling lipid, it was also the most unmodified FA. The importance of ARA in *O. vulgaris* physiology was previously suggested by several studies (Estefanell et al., 2011; García-Garrido et al., 2010; Millioui et al., 2006; Monroig et al., 2012a, 2012b). The positive effects of increased dietary ARA in fish development were previously reported regarding reproductive performance (Furuita et al., 2003; Norambuena et al., 2013), larvae survival and growth rates (Atalah et al., 2011b), adaptive physiological response to hypersalinity stress and hypo-osmoregulatory ability (Carrier III et al., 2011) and recovery from infections (Khozin-Goldberg et al., 2006). Nonetheless, further increased levels of this FA in the diet appears to contradict these positive effects (Atalah et al., 2011a; Furuita et al., 2003; Khozin-Goldberg et al., 2006), possibly due to a potential inhibitory effect on EPA bioconversion (Furuita et al., 2003) or incorporation (Atalah et al., 2011a, 2011b; Villalta et al., 2005).

The existence of an elongase of very long-chain FA (Elovl) in *O. vulgaris* tissues that transforms C18 and C20 PUFA was previously detected through a molecular approach (Monroig et al., 2012b). However, C22 PUFA substrates tested in that study remained unmodified. In the present study, elongation of DHA to 24:6n-3 was verified. This may possibly indicate the presence of an Elovl in *O. vulgaris* hatchlings not otherwise identified. Interestingly, this possible Elovl did not act on 22:4n-6 or 22:5n-3, products of ARA and EPA elongations.

Of the FA substrates used in the present study, [1-¹⁴C]18:2n-6 and [1-¹⁴C]18:1n-9 were the most effectively transformed, with two different metabolic products being detected. In contrast, from the radioactivity incorporated into hatchlings TL as [1-¹⁴C]18:3n-3, only 8.5 % was recovered as 20:3n-3. A previous study reported preferential elongation activity towards n-6 FA substrates in octopus (Monroig et al. 2012b). This was similar here, at least for 18:2n-6 versus 18:3n-3, since [1-¹⁴C]ARA was the least transformed substrate. Possibly the Elovl activity towards C18 substrates was different from that acting on higher chain FA like ARA or EPA, with the latter not showing preferential activity towards n-6 substrates.

The present study confirmed that it was possible to perform *in vivo* studies with *O. vulgaris* hatchlings, using direct incubation with [1-¹⁴C]FA bound to BSA added to the seawater. The esterification pattern of radiolabelled FA substrates was highly specific,

with [1-¹⁴C]DHA and [1-¹⁴C]C18FA being preferably esterified into PC and [1-¹⁴C]ARA and [1-¹⁴C]EPA into PE. Of the FA incubated, ARA was the most efficiently incorporated, as well as the least transformed. No desaturation activity was recorded towards the FA used during this experiment with only elongations being detected. The nutritional requirements of *O. vulgaris* hatchlings in terms of FA seems to be highly specific and so LC-PUFA must be considered as EFA and provided by dietary sources at least during crucial early life stages.

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Table 1 – Total lipid content and lipid class composition of *Octopus vulgaris* hatchlings

TL content (µg lipid.mg protein ⁻¹)	384.9 ± 57.5
<i>Lipid Class (%)</i>	
Σ Polar lipids	64.5 ± 3.2
Lysophosphatidylcholine	0.3 ± 0.1
Sphingomyelin	0.6 ± 0.3
Phosphatidylcholine	19.6 ± 0.6
Phosphatidylserine	10.7 ± 0.4
Phosphatidylinositol	3.9 ± 0.9
Phosphatidylglycerol	4.0 ± 0.5
Phosphotidylethanolamine	25.5 ± 1.5
Σ Neutral lipids	35.5 ± 3.2
Diacylglycerols	0.7 ± 0.2
Cholesterol	30.1 ± 2.9
Free fatty acids	1.4 ± 0.2
Triacylglycerols	1.2 ± 0.4
Sterol esters	2.1 ± 0.8

^a Results represent means ± SD of three replicates.

Table 2 – Fatty acid composition (% of total fatty acids) of *O. vulgaris* hatchlings

C16:0	21.5 ± 0.5
C18:0	10.0 ± 0.1
Total saturated	39.9 ± 0.8
C16:1 ^c	0.7 ± 0.0
C18:1(n-13)	2.1 ± 0.0
C18:1(n-9)	2.5 ± 0.1
C20:1(n-9)	3.7 ± 0.1
Total monoenes	12.3 ± 0.1
C18:2(n-6)	0.5 ± 0.1
C20:2(n-6)	0.4 ± 0.1
C20:4(n-6)	3.7 ± 0.2
Total n-6 FA	5.0 ± 0.4
C20:3(n-3)	1.0 ± 0.7
C20:5(n-3)	14.3 ± 0.2
C22:5(n-3)	1.0 ± 0.0
C22:6(n-3)	26.0 ± 0.7
Total n-3 FA	42.4 ± 0.4
Total PUFA	47.7 ± 0.7
Total LC-PUFA	46.4 ± 0.5
n-3/n-6	8.5 ± 0.6
DHA/EPA	1.8 ± 0.0
EPA/ARA	3.8 ± 0.2

^a Results represent means ± SD of three replicates

^b Totals include some minor components not shown

^c Contain n-9, n-7 and n-5 isomers

^d PUFA – Polyunsaturated fatty acids

^e LC-PUFA – Long-chain polyunsaturated fatty acids

^f ARA – 20:4n-6; EPA – 20:5n-3; DHA – 22:6n-3

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

Substrate	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Incorporation	7.0 ± 0.5 ^{c•}	10.5 ± 0.9 ^{b▲}	11.3 ± 1.6 ^{bc▲•}	33.8 ± 1.2 ^{aΔ}	24.2 ± 2.1 ^{bc◊}	18.7 ± 1.4 ^{bo}
<i>Lipid Class</i>						
Triacylglycerols	13.4 ± 2.9 ^{a▲}	9.2 ± 0.9 ^{b▲•}	7.5 ± 0.8 ^{b•}	1.1 ± 0.6 ^{d◊}	3.6 ± 0.8 ^{cΔ}	2.7 ± 0.7 ^{ca}
Free Fatty Acids	5.4 ± 0.7 ^{a▲}	3.6 ± 0.7 ^{b•}	3.1 ± 0.4 ^{bc•}	2.0 ± 0.7 ^{c◊}	4.1 ± 0.4 ^{abΔ}	2.2 ± 0.5 ^{co}
Partial Acylglycerols	8.2 ± 1.0 ^{a▲}	6.7 ± 0.5 ^{ab•}	5.4 ± 0.5 ^{b■}	1.9 ± 1.5 ^{bo}	6.4 ± 1.0 ^{abΔ}	3.6 ± 1.2 ^{ba◊}
Phosphatidylethanolamine	9.1 ± 0.5 ^{cd}	11.0 ± 2.3 ^c	8.7 ± 0.2 ^{cd}	57.3 ± 2.8 ^{aΔ}	48.4 ± 3.0 ^{bo}	7.7 ± 0.8 ^{d□}
Phosphatidylglycerol	0.0 ± 0.0 ^{b•}	0.0 ± 0.0 ^{b•}	2.6 ± 0.2 ^{a▲}	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b	0.0 ± 0.0 ^b
Phosphatidylinositol	10.8 ± 0.6 ^{a▲}	7.2 ± 0.5 ^{b•}	2.2 ± 0.4 ^{c■}	11.6 ± 1.3 ^{aΔ}	11.0 ± 1.1 ^{aΔ}	2.6 ± 0.5 ^{co}
Phosphatidylserine	6.1 ± 2.3 ^{c•}	10.8 ± 1.9 ^{ab▲}	10.0 ± 1.3 ^{b▲}	14.0 ± 0.6 ^{aΔ}	11.3 ± 0.9 ^{ab◊}	2.7 ± 0.6 ^{d□}
Phosphatidylcholine	47.0 ± 0.9 ^{d■}	51.5 ± 1.8 ^{c•}	60.5 ± 2.1 ^{b▲}	11.1 ± 0.7 ^{co}	12.4 ± 0.7 ^{co}	78.4 ± 3.9 ^{aΔ}
Unknown	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	0.0 ± 0.0 ^c	0.9 ± 0.4 ^{bo}	2.7 ± 0.2 ^{aΔ}	0.0 ± 0.0 ^{c□}
Total neutral lipids	27.1 ± 1.3 ^{a▲}	19.5 ± 1.5 ^{b•}	16.1 ± 1.4 ^{b■}	5.0 ± 2.7 ^{c◊}	14.1 ± 2.2 ^{ba}	8.5 ± 2.4 ^{ca◊}
Total polar lipids	72.9 ± 1.3 ^{c■}	80.5 ± 1.5 ^{b•}	84.9 ± 1.4 ^{b▲}	95.0 ± 2.7 ^{aΔ}	85.9 ± 2.2 ^{bo}	91.5 ± 2.4 ^{aΔ◊}

^a Results represent means ± SD; n=4

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

^c Data of esterification are given in percentage

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

^e Different full symbols in superscript (▲••) within the same row represent significant differences within C18 FA ($p < 0.05$)

^f Different hollow symbols in superscript (Δ◊□) within the same row represent significant differences within LC-PUFA ($p < 0.05$).

Table 4 – Recovery of radioactivity
from [1-¹⁴C]FA substrates in FA
metabolites in *Octopus vulgaris*
hatchlings

Fatty acids	% recovery
[1- ¹⁴ C]18:1n-9	
18:1n-9	71.5 ± 0.5
20:1n-9	19.2 ± 1.6
22:1n-9	9.2 ± 1.3
[1- ¹⁴ C]18:2n-6	
18:2n-6	65.2 ± 1.5
20:2n-6	31.6 ± 1.5
22:2n-6	3.2 ± 0.4
[1- ¹⁴ C]18:3n-3	
18:3n-3	91.5 ± 0.9
20:3n-3	8.5 ± 0.9
[1- ¹⁴ C]20:4n-6	
20:4n-6	98.8 ± 0.1
22:4n-6	1.2 ± 0.1
[1- ¹⁴ C]20:5n-3	
20:5n-3	97.9 ± 0.4
22:5n-3	2.1 ± 0.4
[1- ¹⁴ C]22:6n-3	
22:6n-3	96.0 ± 0.4
24:6n-3	4.0 ± 0.4

^a Results represent means ± SD; n=4