UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

Fatty acid and lipid class metabolism of common octopus (*Octopus vulgaris*) and European cuttlefish (*Sepia officinalis*) at early life stages.

Diana Filipa Botelho Reis

Tese para obtenção do grau de Doutor
em Ciências do Mar, da Terra e do Ambiente,
ramo Aquacultura, especialidade Nutrição

Trabalho efectuado sob a orientação de:
Professor Doutor José Pedro Andrade, Universidade do Algarve
Doutor António V. Sykes, Centro de Ciências do Mar
Doutora Covadonga Rodríguez, Universidad de La Laguna

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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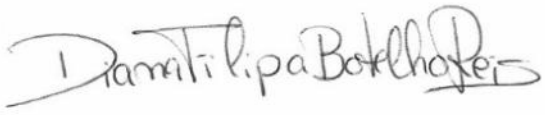

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Resumo

O polvo-comum (*Octopus vulgaris*) e o choco-comum (*Sepia officinalis*) são consideradas duas espécies de cefalópodes com elevado potencial para produção em aquacultura. No entanto, o reduzido conhecimento acerca da fisiologia nutricional destas espécies durante as primeiras fases da sua vida tem dificultado o desenvolvimento de uma dieta adequada para o seu cultivo à escala industrial. Neste sentido, o principal objectivo da presente tese foi ampliar o conhecimento dos requerimentos lipídicos do polvo (*O. vulgaris*) e do choco (*S. officinalis*) durante as fases iniciais de desenvolvimento pós-embrionário e, deste modo, contribuir para o aperfeiçoamento dos protocolos de enriquecimento de presas vivas e/ou a formulação de rações para o cultivo destas espécies. Para tal, foi definido um conjunto de três sub-objectivos: (1) desenvolver um protocolo que permitisse o estudo *in vivo* do metabolismo lipídico em cefalópodes, utilizando ácidos gordos (AG) marcados radioactivamente com ^{14}C (Capítulo 2); (2) investigar a capacidade natural destas espécies para metabolizar ácidos gordos de cadeia larga (Capítulos 3, 4 e 5); e (3) investigar o efeito do metabolismo lipídico das presas vivas na biodisponibilidade de ácidos gordos essenciais (AGE) para o crescimento das paralarvas de polvo.

De modo a desenvolver um protocolo viável para o estudo do metabolismo lipídico dos cefalópodes durante as primeiras fases de vida, foi estudada a capacidade dos "hatchlings" de polvo (usados como espécie modelo) para incorporar, esterificar e transformar o ácido gordo 18:1n-9 marcado radioactivamente com ^{14}C (Capítulo 2). Nesse sentido, foram testadas diferentes condições de cultivo (dispositivos, densidades e períodos de incubação). Os resultados demostraram a viabilidade da metodologia desenvolvida para o estudo do metabolismo lipídico no polvo. Posteriormente, esta metodologia foi adaptada segundo a espécie e o tipo de substrato (molécula radioactivamente marcada) estudado, permitindo, deste modo, não só determinar a capacidade *in vivo* dos "hatchlings" de polvo e choco para esterificar AGs nas diferentes classes lipídicas, mas também para transformar AG através de processos de desnaturação/elongação (Capítulos 3 e 4), e para remodelar fosfolípidos (Capítulo 5). Este protocolo permitiu igualmente estudar o metabolismo lipídico de metanáuplios de *Artemia* sp. e zoeas de *Grapsus adscensionis*, presas vivas utilizadas no cultivo de paralarvas de polvo (Capítulo 6), e o efeito desse metabolismo na biodisponibilidade de AGE para o desenvolvimento das mesmas (Capítulo 7).

Os resultados dos Capítulos 3 e 4 demonstram que os "hatchlings" de polvo apenas apresentam capacidade para alongar os diversos AGs incubados (18:1n-9,18:2n-6 e 18:3n-3, 20:4n-6 (ácido araquidónico; ARA), 20:5n-3 (ácido eicosapentaenóico; EPA), 22:6n-3 (ácido docosahexaenóico; DHA)), enquanto os "hatchlings" de choco apresentam a capacidade de alongar estes AGs e de desnaturar AGs de 18 carbonos (nomeadamente: 18:1n-9, 18:2n-6 e 18:3n-3). Contudo, ambas as espécies foram incapazes de sintetizar ARA ou EPA a partir dos seus percussores de 18C (18:2n-6 e 18:3n-3, respectivamente), ou DHA a partir do EPA. Assim, o ARA o EPA e o DHA devem ser considerados como AGE para o normal crescimento de ambas espécies, pelo menos durante a primeira fase de desenvolvimento. Tanto o polvo como o choco apresentaram um padrão de esterificação de AGs similar, onde o ARA e o EPA foram preferencialmente esterificados na fosfatidiletanolamina (FE) e o DHA e os AGs de 18C na fosfatidilcolina (FC). Esta similitude nos padrões de esterificação dos diferentes AGs em determinadas classes lipídicas deve ser considerada aquando da formulação de uma dieta adequada para o cultivo destas espécies já que, neste caso, uma relação ARA/EPA ou DHA/AG de 18C inadequada poderia levar a uma maior incorporação de um determinado AG em relação ao outro e, consequentemente, à síntese de lípidos com uma estrutura provavelmente desadequada às suas funções.

No Capítulo 5 foi observada a capacidade do polvo e do choco para remodelar fosfolípidos, incubando "hatchlings" de ambas espécies com moléculas de FC e FE, nas quais estava esterificado na posição sn-2 destes fosfolípidos uma molécula de ARA marcado com ^{14}C. Enquanto no polvo o padrão de esterificação do ARA após o período de incubação foi relativamente semelhante independentemente do fosfolípido de origem, e onde foi detectada uma esterificação preferente na FE; no choco, o ARA foi recuperado na sua maioria esterificado no fosfolípido de origem. Estes resultados indicam que os "hatchlings" de polvo e de choco possuem diferentes capacidades para remodelar fosfolípidos, sugerindo a existência de diferenças na actividade das enzimas envolvidas neste processo (fosfolipases).

De maneira a ampliar o conhecimento das necessidades lipídicas do polvo e do choco durante as fases iniciais do seu desenvolvimento, para além dos estudos metabólicos foram também analisados os perfis lipídicos destas espécies (Capítulos 3, 4, 5 e 7). Tanto o polvo como o choco, depois de eclodir, apresentaram um elevado conteúdo em 16:0, 20:5n-3 (EPA) e 22:6n-3 (DHA). Contudo, enquanto o ARA foi um dos principais AGs no polvo (Capítulo 3), o valor deste AG no choco foi relativamente reduzido (Capítulo

4). Além disso, nas várias determinações realizadas foi observada uma maior incorporação do ARA ao lípido total (LT) do polvo (Capítulos 3, 5 e 7), o que poderá significar um requerimento diferente entre ambas espécies para este AG em particular. Os principais fosfolípidos dos "hatchlings" de ambas espécies de cefalópodes exibiram um perfil de AGs específico, onde a FC apresentou um alto conteúdo em 16:0 e DHA; a fosfatidilserina (FS) em 18:0, DHA e EPA; o fosfatidilinositol (FI) em AGs saturados; e a FE em DHA e EPA. É importante destacar a semelhança encontrada entre o padrão de esterificação dos AGs marcados e o perfil de AGs dos fosfolípidos, nos quais se verificou um elevado conteúdo de ARA em FE e não em FI, como registrado em peixes e outras espécies marinhas; bem como um elevado conteúdo de EPA em FE e de DHA em PC. No entanto, foi também observado um elevado valor de DHA em FE. Por essa razão, o rácio DHA/EPA/ARA deve ser igualmente considerado aquando da formulação de uma dieta para ambas espécies.

Finalmente, de modo a ajustar o enriquecimento da *Artemia* sp. e assim promover o desenvolvimento normal das paralarvas de polvo, foi analisado o metabolismo lipídico de duas presas vivas usadas no cultivo desta fase de vida desta espécie: metanáuplios de *Artemia* sp. e zoeas de *Grapsus adscensionis* (Capítulo 6). Além disso determinou-se com o efeito do metabolismo lipídico dos metanáuplios afecta a biodisponibilidade dos AGE para o desenvolvimento das paralarvas de polvo (Capítulo7). Os metanáuplios e as zoeae exibiram um perfil lipídico diferente, no qual os primeiros apresentaram um maior conteúdo em triglicéridos e AGs como o 18:1n-9 e o 18:3n-3, e os segundos em lípidos polares e AGs de cadeia larga. O perfil de esterificação dos diferentes AGs nas diversas classes lipídicas foi também diferente entre presas. Nas zoeas o perfil de esterificação foi equivalente ao observado no Capitulo 3 para os "hatchlings" de polvo. Em contrapartida, apesar de todos os substratos incubados terem sido preferencialmente esterificados nos lípidos polares da *Artemia* sp., entre os AGs de cadeia larga foi detectada uma maior esterificação do DHA em TAG (Capítulo 6). Além disso, foi detectada uma menor incorporação do DHA ao lípido total da *Artemia* sp., que parece estar associada com um maior catabolismo deste AG, já que com este substrato foram alcançados valores de síntese *de novo* de AG com cadeia carbónica inferior a 18 carbonos de 30 % (Capítulos 6 e 7). Os resultados deste estudo demonstraram a existência de modelos distintos de metabolismo lipídico entre os metanáuplios de *Artemia* sp. e o as zoeas de *Grapsus*, ilustrando a dificuldade no enriquecimento da *Artemia* sp. com AGE para os organismos marinhos. A quantidade de DHA e 18:3n-3 incorporados pelas paralarvas de polvo após

a sua alimentação com metanáuplios de *Artemia* sp. marcados radioactivamente com ^{14}C, foi extremamente reduzido, o que dificultou a analise dos resultados obtidos. Contudo, e embora não tenha sido possível tirar conclusões com elevada exactidão, foram formuladas algumas hipóteses em relação ao efeito do metabolismo da *Artemia* sp. na biodisponibilidade de AGE para as paralarvas de polvo. Aquando da alimentação das paralarvae com metanáuplios de *Artemia* sp. marcados, foi detectada uma maior incorporação de ARA e EPA ao lípido total das paralarvas (Capítulo 7). Por outro lado, a incorporação do DHA e do 18:3n-3 foi quase vestigial. A *Artemia* sp. apresentou uma maior taxa de esterificação do DHA e do 18:3n-3 aos lípidos neutros, e principalmente aos TAG, quando comparada com o EPA ou o ARA. Considerando uma analogia entre larvas de peixes marinhos e cefalópodes, os reduzidos valores de incorporação do DHA e do 18:3n-3 aos lípidos do polvo podem estar associados com a presença destes AGs nos TAG da *Artemia* sp.. Por outro lado, tanto ARA como o EPA foram preferencialmente esterificados nos fosfolípidos, o que pode ter aumentado a sua biodisponibilidade para as paralarvas. No entanto, estes resultados podem estar igualmente relacionados com o catabolismo da *Artemia* sp. durante o período de 24 h de cultivo das paralarvas. De modo a comprovar as hipóteses aqui apresentadas, sugere-se a realização de estudos complementares que considerem diferentes tempos de incubação e de cultivo, por forma a aumentar a quantidade de substrato radioactivo incorporado pelas paralarvas.

Através do método desenvolvido foi possível determinar alguns dos destinos metabólicos dos diferentes AGs, elucidando alguns dos requerimentos lípidos do polvo e do choco durante as fases iniciais de desenvolvimento. Contudo, a investigação futura deverá complementar a informação adquirida. Estudos *in vitro* e *in vivo* da actividade das enzimas digestivas sobre fosfolípidos e triglicéridos serão de grande relevância. Considerando a capacidade dos cefalópodes para incorporar nutrientes (ácidos gordos e amino ácidos) directamente a partir da água, será interessante testar a possibilidade de utilizar matéria orgânica dissolvida em alternativa à *Artemia* sp. como fonte de DHA e fosfolípidos. Finalmente, o método desenvolvido para este estudo poderá ser também uma ferramenta útil para a determinação do efeito das condições de cultivo (ex. dieta, temperatura, salinidade, etc.) no metabolismo lipídico *in vivo* de cefalópodes, presas vivas (*Artemia* sp., rotíferos) e outras espécies marinhas ou de água doce.

Palavras-chave: Metabolismo lipídico; Nutrição; Presas vivas; *Octopus vulgaris*; *Sepia officinalis*

Abstract

Octopus vulgaris and *Sepia officinalis* are two species of cephalopods that have been recognised with a great potential for aquaculture. However, the limited knowledge regarding the nutritional physiology of these species during their early life stages has been hampering its industrial large scale culture. In this sense, the aim of this thesis was to provide a better knowledge of *Octopus vulgaris* and *Sepia officinalis* lipid requirements during early live stages, which should contribute to the improvement of live preys' enrichment protocols and/or formulated diets for these species culture. *O. vulgaris* and *S. officinalis* hatchlings presented a slightly different fatty acid (FA) metabolism (Chapters 3, 4 and 5). Nonetheless, ARA, EPA and DHA, must be considered as essential fatty acids (EFA) for these species and DHA/ARA/EPA and LC-PUFA/C18 FA ratios should be also be taken into account while designing a suitable diet for these species (Chapters 3 and 4). Moreover, despite the different remodelling results obtained, hatchlings from both species seem to possess the enzymatic pathway necessary for phospholipids remodelling (Chapter 5). Live preys endogenous metabolism seems to have some influence in the availability of EFA for *O. vulgaris* paralarvae (Chapters 6 and 7), particularly due to the *Artemia* sp. metanaplii DHA preferential catabolism and/or esterification into neutral lipids. The enrichment of this species with this FA seems to be of limited value, and so other alternatives of providing DHA to paralarvae must be studied. The present thesis allowed unveiling some of the fates of dietary unsaturated FAs in *O. vulgaris* and *S. officinalis* hatchlings, providing a better knowledge on both species lipid metabolism and requirements during their early life stages. Prospects for future research are suggested.

Keywords: Live preys; Lipid metabolism; Nutrition; *Octopus vulgaris*; *Sepia officinalis*

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List of Abbreviations

ARA – Arachidonic acid; 20:4n-6

BHT - Butylated hydroxytoluene

BSA – Bovine serum albumin

CHO - Cholesterol

C18 FA – Fatty acids with a carbon chain of 18 carbons

C20 FA - Fatty acids with a carbon chain of 20 carbons

C22 FA - Fatty acids with a carbon chain of 22 carbons

DAG – Diacylglycerol

DHA – Docosahexaenoic acid; 22:6n-3

DPM - Disintegrations per minute

EFA - Essential fatty acids

Elovl – Elongase of very long chain fatty acids

EPA – Eicosapentaenoic acid; 20:5n-3

FA - Fatty acids

FAME – Fatty acids methyl esters

FFA – Free fatty acids

HPTLC – High-performance thin-layer chromatography plates

HUFA – Highly unsaturated fatty acids

LC – Lipid classes

LC-PUFA – Long chain polyunsaturated fatty acids

MAG – Monoacylglycerol

NL – Neutral lipids

PAG – Partial acylglycerols

PC – Phosphatidylcholine

PE – Phosphatidylethanolamine

PG – Phosphatidylglycerol

PI – Phosphatidylinositol

PL – Polar lipids

PLA1 – Phospholipase A1

PLA2 – Phospholipase A2

PS – Phosphatidylserine

PUFA – Polyunsaturated fatty acids

SE – Sterol esters

TAG – Triacylglycerol

TL – Total lipid

TLC – Thin-layer chromatography plates

UK – Unknown

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Chapter 1.

General Introduction

1.1 Fish production and market demands

From 1950, world human population has increased 4.8 billions, reaching 7.3 billion in the present year, and it is expected to keep increasing, reaching 9.5 billion in 2050. With a further population growth, exists the necessity to ensure a global food supply. In 2011 fish protein corresponded to 16.7 % of global human population animal protein intake, with a raise in the per capita fish consumption from an average of 9.9 kg in the 1960s, to 19.2 kg in 2012 (FAO, 2014a). This increase in fish consumption is mainly explained by the growth on global fish production during the last decades, which was attained by aquaculture production since fishery captures have met their limit in 1996, with 93.8 million tonnes and are being relatively stable since then (Fig. 1.1). Aquaculture production has been growing 6.1 % per year, reaching 66.6 million tonnes in 2012, which corresponded to 42.2 % of the global fish production (FAO, 2014b).

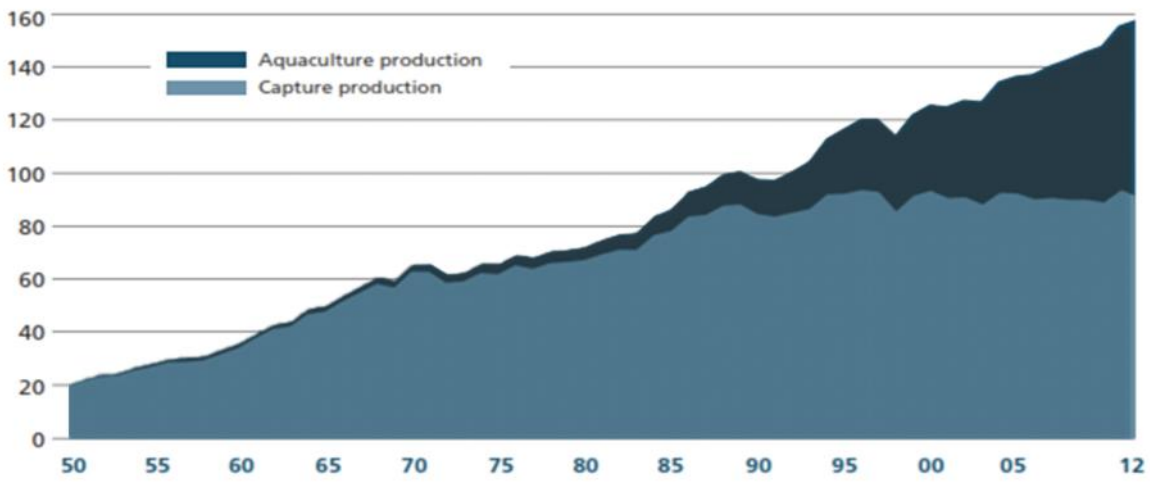


Figure 1.1 - World captured fisheries and aquaculture production from 1950 until 2012 (adapted from FAO, 2014b).

However, in Europe aquaculture only represented 18 % of the total fish production of 2012. Moreover, Europe was the continent with the lowest annual growth rate from 2000 to 2012, with an average of only 2.9 % (FAO, 2014b). Considering the raise in the European per capita fish consumption up to 22 kg per year in 2012, the decrease in fish captures in this continent (see FAO, 2014b), and the mentioned low growth of aquaculture production, fish consumption in Europe is highly dependent on imports. In fact, the European Union is the largest single market for imported fish and fisheries products, with about 23 % of world imports and showing an increasing tendency (FAO, 2014b).

European aquaculture is being mainly focused on the culture of finfish species such as the Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Common Carp (*Cyprinus carpio*), European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and the turbot (*Psetta maxima*). However, with the marked differences in consumers' tastes and demands between countries, and the decline of domestic fisheries production and fish stocks, there is a necessity to offer new products, which includes a diversification on farmed species. Candidate new species for aquaculture production should be chosen considering a widespread consumption and high commercial value.

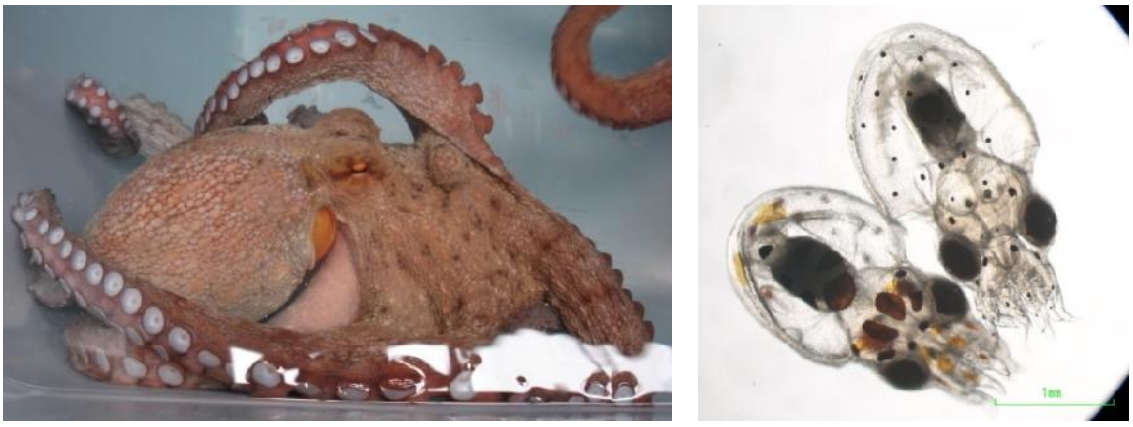
Cephalopods are highly appreciated and consumed in the Asian and Mediterranean markets, with Spain, Italy and Japan being the largest consumers and importers (FAO, 2014b). Moreover, most of the European countries are net importers of cephalopods (Pierce and Portela, 2014). In this sense, it seems to exist a niche for aquaculture production for human consumption of some cephalopod species in Europe (Iglesias et al., 2007). Nonetheless, cephalopods culture could go beyond their use as food, as by-products of the food industry could be used in aqua-feeds and pharmacological industries (Koueta et al., 2014), and as these animals might be used as a model for several research fields and for public exhibition in aquariums (see Sykes et al., 2014). Moreover, these species commercial culture could have high impact on fisheries by reducing illegal catches of small individuals from the natural environment (Sykes et al., 2006a). For example, small cuttlefish individuals (of approximately 50 g) are considered a delicacy and attain the highest commercial value in countries like Portugal. These small individuals might be produced in only 45 - 60 days, leading to a small rearing period and high income (Sykes et al., 2014). Considering that, studies on the development of cephalopods aquaculture production were performed (see Iglesias and Fuentes, 2014), and from a wide range of available species, the common octopus (*Octopus vulgaris*) and the European cuttlefish (*Sepia officinalis*) were identified as the most probable for large-scale commercial culture in Europe (Bouchaud-Camou 1989; Coelho et al. 1989; Villanueva et al., 2014).

1.2 Species biology

1.2.1 *Octopus vulgaris*

O. vulgaris (Fig 1.2 A) belongs to the Class Cephalopoda, Order Octopoda, Family Octopodidae and Genus *Octopus*, which includes around 200 species. This species is found in the Eastern Atlantic Ocean and Mediterranean Sea (Jereb et al., 2014).

Nonetheless, the name *O. vulgaris* is currently applied to several morphological similar but unresolved taxa with a separate distribution across subtropical and temperate waters worldwide (see Jereb et al., 2015). It is found in warm tropical and subtropical waters from the coastal line to the outer edge of the continental shelf, at depths up to 200 - 250 m (Guerra, 1992; Jereb et al., 2015; Mangold, 1983). The lifespan of the common octopus, is between 12 - 15 months (Hernández-López et al., 2001), and individuals normally reach between 3 - 5 kg. Even so, higher weights have been recorded.



A B

Figure 1.2 - Individuals of *O. vulgaris* species A- adult individual, B- paralarvae (Photos by D.B. Reis).

Octopus spawns throughout the year, but there are two reproductive peaks, one during spring, and another during autumn (Gonçalves, 1991; Guerra, 1992; Hernández-García et al., 2002). Before reproduction, octopus migrates to coastal waters, mainly to depths between 30 and 60 m (Guerra, 1992; Mangold, 1983; Mangold-Wirz, 1963). The average fecundity of this species is 70 000 - 600 000 eggs, depending on female weight. Females lay the eggs in grape-like clusters in lairs on sandy to muddy bottoms, under rocks or abandoned mollusc shells. After spawning, females remain inside the den cleaning, ventilating, and caring the eggs (Guerra, 1992; Mangold, 1983). They rarely leave the egg mass, and usually do not feed during the whole period, dying immediately after the eggs hatch, while males usually die after mating. The embryonic development depends on temperature taking between 22 and 30 days at 25 ºC or 100 - 120 days at 13 ºC (Mangold, 1983). Octopuses hatch with a total length of 2 - 3 mm. Despite being a benthic species during most of its life span, during the first stage of life (1 to 2 months after hatching depending on temperature) young octopuses are planktonic and named “paralarvae” (Fig. 1.2 B). This term was defined by Young and Harman (1988) since the morphological,

physiological and ecological transformations that octopus undergo during that stage cannot be classified as a full metamorphosis. After this planktonic period, octopus become juveniles, settling and adopting a benthic stage of life.

1.2.2 *Sepia officinalis*

S. officinalis belong to Class Cephalopoda, Order Sepiida, Family Sepiidae, and Genus *Sepia* that comprises around 100 species. This species is mostly abundant in the Northeast Atlantic and the Mediterranean Sea; however, its distribution extends from the North Sea to Northeast Africa (Jereb et al., 2015). This species occurs in coastal waters and on the continental shelf at depth lower than 150m (Boletzky, 1983).

S. officinalis life cycle is closely related to environment factors. In colder waters (English Channel), cuttlefish reproduce during the second year of life and during a short breeding period (2 - 3 months) during latter spring and yearly summer (Boucaud-Camou et al., 1991; Önsoy and Salman, 2005). In contrast, in the Mediterranean the majority of cuttlefish reproduces during the first year and over a longer period (Guerra and Castro, 1988). *S. officinalis* attains sexual maturity at very different sizes/weights (Sykes et al., 2006a) and has an estimated potential fecundity of a maximum of 8 000 eggs in nature (Laptikhovsky et al., 2003). This species presents an intermittent spawning and female dies shortly after spawning (Rocha et al., 2001), with no parental care of the eggs being recorded. Female lay eggs on hydrodynamic locations attached to wild flora, fauna, or human-related structures (Boletzky, 1983). Eggs are usually black with a flask shape and a diameter ranging from 1.2 to 1.4 cm (Sykes et al., 2006a). Embryonic development is also dependent on temperature and may range from 25 days at 25 ºC (Sykes et al., 2006a) to 80 - 90 days at 15 ºC (Boletzky et al., 2006). *S. officinalis* hatch with a mantle length of 6 - 9 mm, and an adult-like morphology (Fig. 1.3). After hatchling, individuals immediately adopt a benthic lifestyle and adults' basic behaviour (Hanlon and Messenger, 1996; Warnke, 1994).

Figure 1.3 – *S. officinalis* hatchlings (Photo by A.V. Sykes).

1.3 State of the art of *O. vulgaris* and *S. officinalis* culture

1.3.1 Species potential and bottlenecks

These species have a large set of features that favour their large-scale culture, namely their nutritional value for human consumption, being a good source of protein, essential lipids, mineral salts and vitamins (Sinanoglou and Miniadis-Meimaroglou, 2000); and biological characteristics like short life cycle, fast growth rates (Aguado-Giménez and García García, 2002; Almansa et al., 2006; Domingues et al., 2003, 2004; Ferreira et al., 2010; García García and Cerezo Valverde, 2004) and high food conversion ratios (Almansa et al., 2006; Castro and Lee, 1994; Delgado et al., 2011; Domingues et al., 2004, 2009; García García and Cerezo Valverde, 2004; Tuñón et al., 2001).

The culture of *O. vulgaris* and *S. officinalis* are of particular interest in Mediterranean countries, with several research centres and companies being dedicated to the development of these species culture (García García et al., 2004, Sykes et al., 2014). Industrial on-growing of octopus juveniles have been carried out by private companies in Galicia (northeast region of Spain) since 1996 (García García et al., 2014). Despite the promising potential of *O. vulgaris* for commercial culture (with a maximum production of 49.4 tonnes in 2000), this activity is slowing down. This deceleration is mostly related with the current dependence on sub-adults collected from the wild and the high prices of natural preys, used in feeding (García García et al., 2014). Consequently, this was considered a high-risk activity with low profits. On the other hand, *S. officinalis* has been successfully reared in extensive aquaculture in Portugal, France and Italy (Sykes et al., 2014). However, similarly to *O. vulgaris* rearing, this activity is also dependent on the natural environment, as caught eggs are left on earth ponds, where cuttlefish grow by feeding on natural occurring preys, until reaching a marketable size (Sykes et al., 2014).

Therefore, to increase the culture scale to intensive aquaculture and reduce the natural environment dependence, numerous laboratory studies have been performed, analysing aspects like biology (Nixon and Young 2003; Önsoy and Salman, 2005; Otero et al., 2007; Wodinsky, 1972), behaviour (Adamo et al., 2000, Cole and Adamo, 2005; Fiorito and Gherardi, 1999; Villanueva et al., 1996), physiology (Repollo et al, 2014; Iwakoshi et al., 2002; Kanda et al., 2003), feeding (Anraku et al., 2005; Darmaillacq et al., 2004; Hormiga et al., 2010; Koueta and Bouchaud-Camou, 2003; Márquez et al., 2007), nutrition (Almansa et al., 2006; Bouchaud and Galois, 1990; Guinot et al., 2013a; Seixas et al., 2010a, 2010b; Estefanell et al., 2011; Domingues et al., 2010; Iglesias et al., 2004, 2014; Navarro and Villanueva, 2003; Sykes et al., 2009; Cerezo Valverde et al., 2008; Reis et

al., 2015), reproduction (Di Cristo et al., 2003; Forsythe, 1994; Gauvrit et al., 1997; Lourenço et al., 2012; Neves et al., 2009; Silva et al., 2002), and also rearing systems (Estefanell et al., 2012; Koueta and Bouchaud-Camou, 2001, 2003; Rodríguez et al., 2006; Sánchez et al., 2013; Sykes et al., 2010), density (Domingues et al., 2008; Sykes et al., 2003), abiotic factors influence (Cerezo Valverde and García García, 2004, 2005; Domingues et al., 2001a, 2002; Grigoriou and Richardson, 2010; Mázon et al., 2007; Melzner et al., 2006; Raimundo et al., 2010; Cerezo Valverde and García García, 2004, 2005), within other factors.

From those studies, it was possible to identify the major bottlenecks limiting the transition of these species culture to an industrial level. According to Sykes et al., (2014), the three major bottlenecks delaying *S. officinalis* large-scale culture are (1) the dependence on natural prey during the first part of the life cycle, (2) the lack of an adequate artificial diet for all life stages of this species and (3) full control of reproduction in captivity. On the other hand, the main factors hampering *O. vulgaris* industrial culture are (1) the lack of a standardised culture system of paralarvae (2) the absence of appropriated food sources that fulfil nutritional requirements of this species during its early life stage and (3) the lack of a commercial diet that allows the development of this species during the growth-out stage (Iglesias and Fuentes, 2014; García García et al., 2014). Identified bottlenecks, show that the limited knowledge on nutritional physiology of both species is one of the main factors hampering the commercial culture of these species.

1.3.2 Diet

1.3.2.1 On-growing stage

S. officinalis and *O. vulgaris* are carnivorous species with high metabolic rates and fast growth, which require substantial amounts of food (O'Dor and Wells, 1987). In the natural environment, these species display a preference of predating a high variety of crustaceans, fishes, and molluscs (Castro and Guerra, 1990; Jereb et al., 2015; Nixon, 1985). For this reason, on-growing experiments have been mostly based on the use of similar diets. While the on-growing culture of *O. vulgaris* is mainly composed by fishery discards (e.g. Estefanell et al., 2011, 2012; García García and Giménez, 2002; García García and Cerezo Valverde 2006; Mazón et al., 2007; Prato et al., 2010); *S. officinalis* on-growing experiments have been mainly performed with live or frozen diets based on prawn, shrimps and crabs (Sykes et al., 2014). However, as previously mentioned, in order to develop an on-growing large-scale industry of these species, and so avoid the

dependence on fishing, a commercial diet, designed to achieve proper survival and growth must be developed. In this sense, several studies have been performed using formulated diets on on-growing trials (Castro, 1991; Castro et al., 1993; Castro and Lee 1994; Cerezo Valverde et al., 2008, 2013; Domingues et al., 2005, 2008; Ferreira et al., 2010; Lee et al., 1991; Morillo-Velarde et al., 2012). However, all these attempts failed to achieve proper growth and survival, displaying poor results when compared with natural diets. These results were attributed to formulated diets poor palatability or/and deficient nutritional content.

1.3.2.2 Paralarvae and hatchling stage

O. vulgaris and *S. officinalis* have two distinct life-cycles. While cuttlefish hatch with an adult-like morphology and a benthic behaviour, during the 1st to the 2nd month after hatching young octopus are planktonic and undergo morphological, physiological and ecological transformations. Despite this difference, both octopus and cuttlefish early life stages are characterised by the highest growth, food consumption and feeding rates (Villanueva, 1995; Sykes et al., 2014). Nonetheless, this is also the phase hampering the industrialization of these species culture, due to the need of proper food.

At hatching cephalopods already possess complex sensory organs and an elaborate nervous system (Nixon and Young, 2003), and normally start to feed within the first few hours after hatching (Domingues et al., 2001b; Hanlon and Messenger, 1988; Iglesias et al., 2006; Reis et al., 2015). Preys are visually located and must be provided at sufficient density and frequency to facilitate intake (Mather and Scheel, 2014). Even though cuttlefish and octopus inner yolk reserves could last for a few days after hatching (Boletzky and Villanueva, 2014; Sykes et al., 2004), hatchlings may feed on size-appropriate prey from day 1 (Hanlon and Messenger, 1988), where a mixed feeding overlaps between inner yolk reserves and external food consumption (Boletzky, 1974). Therefore, an external diet must be given during this period or a point of no return might be reached and cuttlefish will die (Sykes et al., 2004).

In nature, young octopus and cuttlefish prey preferably on crustaceans (Boletzky, 1983; Roura et al., 2012) and, under rearing conditions, both species depend on the use of natural food. Several crustacean species such as mysids (*Mesodopsis slabberi*, *Mysidopsis almyra*, *Paramysis nouveli*, *Ashistomysis* sp.), crabs (*Carcinus maenas*) and shrimps (*Cragon crangon*, *Gammarus* sp., *Palaemonetes varians*) have been tested as food for cuttlefish (Darmaillacq et al., 2004; Domingues et al., 2001a, 2003, 2004; Grigoriou and

Richardson, 2004; Koueta and Boucaud-Camou, 1999; Koueta et al., 2002; Sykes et al., 2006b, 2012). Within those, *P. varians* was the prey with the best results in terms of survival, and is currently used to attain the culture of consecutive cuttlefish generations in captivity (Sykes et al., 2006b). On the other hand, crustacean species such as *Maja brachydactyla*, *Maja squinado*, *Palaemon elegans*, *Palaemon serrifer* and *Grapsus adscensionis*, were tested as live prey in octopus paralarvae rearing (Carrasco et al., 2006; Iglesias et al., 2002, 2004, 2014; Moxica et al., 2002; Reis et al., 2015; Villanueva, 1994, 1995). Nonetheless, the high cost associated with prey production, makes them not economically viable. Therefore, the use of alternative preys was proposed. Due to its availability and acceptance by paralarvae, *Artemia* sp. has been used as alternative live prey in *O. vulgaris* rearing. Despite the promising results obtained by Hamasaki et al. (1991) and Imamura (1990) with *Artemia* sp. as sole prey, in Europe, paralarvae settlement was only achieved when *Artemia* sp. was provided in co-feeding with crustacean zoeae (Carrasco et al., 2006; Iglesias et al., 2004; Villanueva, 1994, 1995). *Artemia* sp. has also been tested as live prey during the first days after cuttlefish hatch (Domingues et al., 2001a, 2001b, 2002; Koueta et al., 2002; Perrin et al., 2004). However, results were poor in cuttlefish. Recently, Sykes et al., (2013) fed *S. officinalis* with frozen grass shrimp from the first day after hatching, showing the potential of using an inert diet from birth. But once again, lower growth (~1/3 of that achieved with live food) and higher mortality rates (~20 % more with frozen grass shrimp) were obtained when compared to live preys outcomes. However, this opens the possibility of using a prepared diet from hatching. The lower rearing results obtained when alternative food is used were suggested to be related to an imbalance on prey nutritional content and/or cephalopod digestive system maturation (Perrin et al., 2004; Sykes et al., 2013). Therefore, to improve the rearing results and design an artificial diet that might effectively replace live preys or improve alternative preys' nutritional composition, and allow the large-scale culture of these species, it is important to understand their nutritional requirements at this early life stage.

1.3.3 Nutrition

Nutrition is a key factor for proper growth and survival under captive conditions and large-scale culture. Nonetheless, the lack of knowledge regarding octopus and cuttlefish physiology and metabolism remains as one of the bottlenecks to overcome reaching an economical viable aquaculture production of these species (Villanueva et al., 2014).

Studies on the nutritional requirements of both species during early life stages are complex, as they have both external and internal digestion, which represents a challenge to prey identification by analysis of stomach content (Nixon, 1985; Villanueva and Norman, 2008). Moreover, since formulated diets have been largely not accepted, or, when ingested, have resulted in poor growth and survival rates (Castro, 1991; Castro and Lee, 1994; Castro et al., 1993; Lee et al., 1991; Navarro and Villanueva, 2003; Seixas et al., 2010b; Sykes et al., 2006a), the design of a diet to satisfy the nutritional requirements of these species cannot be performed only through doses-response studies. In this sense, feeding studies using preys with well characterized composition (Almansa et al., 2006; Domingues et al., 2003; Navarro and Villanueva, 2003; Villanueva et al., 2004; Reis et al., 2015), studies of egg embryonic metabolism (Bouchaud and Galois, 1990; Navarro and Villanueva, 2003; Sykes et al., 2009; Villanueva et al., 2004, 2009) and on nutrient composition during starvation conditions, have been performed (Castro et al., 1992; Sykes et al., unpublished data).

Proteins are the most abundant macronutrient in cephalopods corresponding to approximately 15.5 % of octopus and cuttlefish wet weight, while lipids only attain approximately 1.5 % (Zlatanos et al., 2006). Cephalopods efficiently absorb, digest, and utilize dietary proteins that are further used for locomotion, structural support, energy source, oxygen transport, and osmoregulation (Boucher-Rodoni et al., 1987; Domingues et al., 2005; Lee, 1994). Moreover, octopus growth is primarily based in body muscle mass increased by protein synthesis and deposition (Houlihan et al., 1990). Therefore, it is believed that these species have a high amino acid requirement for optimal growth (Navarro et al., 2014). Nonetheless, there are two different theories in relation to what cephalopods use as energy substrate. The first considers that under normal feeding conditions, both growth and energy use protein as fuel (Boucher-Rodoni and Mangold 1994; Lee, 1994); the second considers that carbohydrates are used as energy source and the protein fraction is exclusive used for growth (Hochachka, 1994; Storey and Storey, 1983). These discrepant theories might have been originated from species physiological adaptation to a given geographical location, with populations from subtropical regions using carbohydrates as energy, while populations from lower temperature regions would prefer proteins and lipids as energy source (see Navarro et al., 2014). Although, the carbohydrate theory needs to be proven, it has been reported that these species are able to rapidly catabolize dietary carbohydrates to account for energy demands in explosive

activities such as prey capture and fleeing from predators, and that there is a carbohydrates metabolism (Morillo-Velarde et al., 2011; Navarro et al., 2014).

Contrasting with the protein content, lipids are one of the minor components in octopus and cuttlefish body composition (Zlatanos et al., 2006), and both species display poor capacity for mitochondrial lipid oxidation (O'Dor et al., 1984; Hochachaka, 1994). Nonetheless, lipids like cholesterol, phospholipids and long-chain polyunsaturated fatty acids have been suggested as critical dietary components for the early life stages of these species (Almansa et al., 2006; Navarro and Villanueva, 2000, 2003; Sykes et al., 2009). Apart from that, little is known about these species lipid requirements, with assumption being made mostly from their body composition and feeding habits.

Given the importance of lipids in animal nutrition and physiology, and that lipids are the main objective in the studies performed in *O. vulgaris* and *S. officinalis* throughout the present study, it seems vital to first briefly explain some functional and structural characteristics as well as biosynthesis pathways of the most important lipids.

1.3.3.1 Lipids function and structure

Lipids as a class of molecules, display a wide diversity in structure and biological functions, playing a vital role in maintaining cell structure and providing essential nutrients for the organism function. Moreover, they also serve as carriers of fat-soluble vitamins and pigments, and are components of hormones and precursors for the synthesis of intra- and extra-cellular messengers, such as prostaglandins and other eicosanoids (Izquierdo et al., 2000; Sargent et al., 2002). Within lipids, fatty acids (FA) like, 20:5n-3 (EPA), 22:6n-3 (DHA) and 20:4n-6 (ARA) are physiologically essential nutrients, playing an important role in structural, functional, and signalling functions. These FAs are major components of biological membranes, particularly neural tissues and immune cells (Bell and Tocher, 1989; Gil et al., 2003; Koletzko et al., 2008), being also involved in a large range of metabolic and immune pathways, either via direct activation of transcription of multiple genes, by functioning as secondary messengers, or acting as potent bioactive molecules and precursors of eicosanoids with pro- or anti-inflammatory properties. Therefore, despite the important role of these FAs in the normal development and health, they are also related to several diseases processes (Gil et al., 2003; Koletzko et al., 2008; Riediger et al., 2009).

There are two major groups within lipids, the polar lipids (PL), and the neutral lipids (NL). While PL have a predominant structural role, within NL, triacylglycerols (TAG)

display a high energetic function, whereas cholesterol, a lipid that does not contain FAs in its structure, plays an important structural role in cell membranes being also important hormones and other bio-molecules precursor (Sargent et al., 2002). The phospholipids are named considering the alcohol or the amino group associated with the phosphatidic acid. In this sense, the major phospholipids of animals are named: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE; Sargent et al., 2002). Phospholipids are constituted by two molecules of FAs esterified at position sn-1 and sn-2 of the L-glycerol and in which an alcohol or an amino group is associated to the phosphate of sn-3 position (Fig. 1.4). On the other hand, TAG (Fig.1.5) consists of tree molecules of FAs esterified into sn-1, sn-2 and sn-3 position of L-glycerol. Monoacylglycerols (MAG) and diacylglycerols (DAG) also NL, consist in one and two FAs esterified into the L-glycerol, respectively.

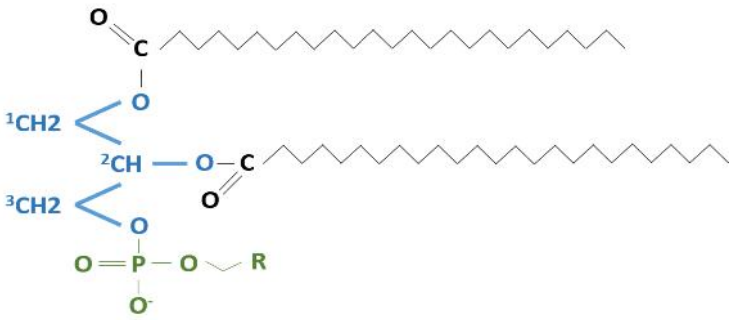


Figure 1.4 - Phospholipid structure

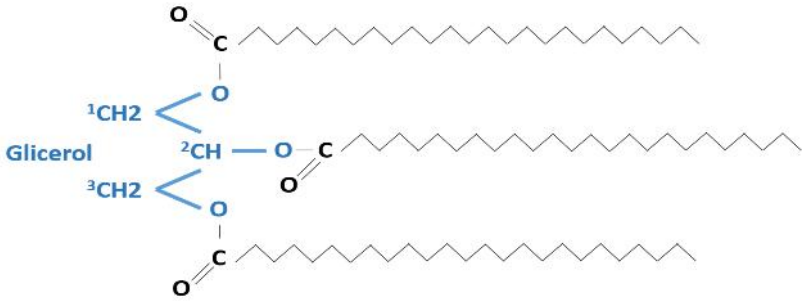


Figure 1.5 - Triacylglycerol structure

The majority of lipid classes (LC) are composed of FAs, which are organic acids formed by a hydrophobic hydrocarbon chain with variable length. These molecules possess a carboxyl terminus in one extreme and a methyl end in the other (Fig.1.6). FAs are designated in accordance to their chain length (number of carbons), their degree of unsaturation (number of double bounds) and position of the double bound on the carbon

chain. For example, 18:2n-6 is a fatty acid with 18 carbon atoms and 2 double bounds. A single methylene group generally interrupts these bounds. Therefore, its structure is normally defined by specifying the position of the first double bound. In 18:2n-6 the first double bound is thus situated at the six-carbon atom from the methyl end of the FA molecule (Fig. 1.6). The n- or omega (ω) nomenclature is the most commonly used when defining FA structure, however this is not the only. Δ nomenclature is more precise as indicates the exact position of each double bound from the carboxyl end, and is generally used to specify FA desaturase activities (Tocher, 2003). Thus 18:2n-6 can also be defined as 18:2Δ9,12 (Fig. 1.6). FAs can also be referred by their common English name, in the case of 18:2n-6, as linoleic acid, mentioning their first isolation from linseeds oils; or their Greek-Latin name such as docosahexaenoic acid for 22:6n-3 that mentioning the numbers for carbon atoms and double bonds (Tocher, 2003).

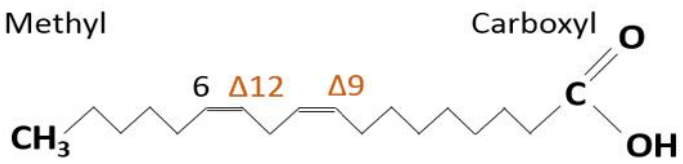


Figure 1.6 – 18:2n-6 fatty acid structure.

FAs can be saturated, when there are no double bonds (e.g. 16:0, 18:0) or unsaturated, when they possess one (monounsaturated, e.g. 18:1n-9) or more double bonds (polyunsaturated, e.g. 18:3n-3) on the carbon chain. Within polyunsaturated fatty acids (PUFA), the ones having a carbon chain length of 20 or more carbons, and three or more double bonds, are named highly-unsaturated FA (HUFA) or referred as long-chain polyunsaturated fatty acids (LC-PUFA, e.g. 22:6n-3).

1.3.3.2 Lipid metabolism

In order to incorporate dietary lipids into animals' tissues, it is necessary to occur a chain of processes that go from lipid digestion to absorption, re-esterification and transport. In vertebrates', lipid digestion occurs mainly in the intestine by the action of several lipase enzymes, like: pancreatic lipases, 1-3 specific lipase, phospholipase A2 (PLA2) and phospholipase A1 (PLA1), within others (Tocher, 2003). Lipid digestion products, which might include free fatty acids (FFA), produced by lipolytic action on all the major LC, partial acylglycerols (that can include 2-monoacylglycerols, DAG and glycerol) from

digestion of TAG, 1-acyl-lyso-glycerophospholipids, from phosphoglycerides digestion among others; will be solubilized or emulsified in bile salt micelles that will later be uptaken into the enterocytes probably by passive diffusion (Tocher, 2003). In the endoplasmic reticulum of the enterocyte, the majority of the FFAs are re-esterified into partial acylglycerols and 1-acyl-lyso-glycerophospholipids to produce TAG and phosphoglycerols with a new structure. Nonetheless, dietary FAs could follow other metabolic fates being β-oxidase for energy production or transformed, undergoing elongation and desaturation processes (Sargent et al., 2002).

All organisms can synthetize *de novo* saturated FAs, and yield 16:1n-7 and 18:1n-9 by the action of Δ9 fatty acids desaturase (Fad) enzyme towards 16:0 and 18:0, respectively. Nonetheless, only plants can produce 18:2n-6 or 18:3n-3 from 18:1n-9, through the action of Δ12 and Δ15 Fads over this FA. Therefore, both 18:2n-6 and 18:3n-3, are considered essential fatty acids (EFA) for all animals and must be supplied through diet (Tocher, 2003). These FAs can be further desaturated (introducing a double bound to the FA molecule through a desaturase enzyme activity) and elongated (adding two new carbons to the hydrocarbon chain by elongase enzyme activity) to produce C20 and C22 PUFA like 20:4n-6, 20:5n-3 and 22:6n-3 (Fig. 1.7). These conversions depend (1) on the relative activity of elongase and desaturase enzymes, like the Δ5 or Δ6 Fads in animals' tissue, and (2) on the extent to which animals can or cannot readily obtain these final products from the diet (Tocher, 2003). For instance, 18:2n-6 and 18:3n-3, appear to satisfy the EFA requirements of freshwater fishes with these species being able to synthesise ARA, EPA and DHA from these FAs. On the other hand, marine fishes do not possess the ability to produce LC-PUFA from C18 FAs and so ARA, EPA and DHA must be provided through diet, being those considered EFA for those species (see Sargent et al., 2002).

Until recently, the only known DHA biosynthesis pathway in vertebrates was the "Sprecher pathway", which compromises two sequential elongations steps of EPA to 24:5n-3, follow by Δ6 desaturation and one round of peroxisomal β-oxidation (Fig. 1.7; Sprecher, 2000). In 2010, Li et al. found a more direct route involving elongation of EPA to 22:5n-3 followed by a Δ4 desaturation to be active in the marine herbivorous fish, *Siganus canaliculatus*. Further studies have later shown that other fish species, such as the marine carnivore *Solea senegalensis* present a similar gene encoding a protein with Δ4 Fad activity in *in vitro* heterologous expression assays (Morais et al., 2012, 2015; Fonseca-Madrigal et al., 2014).

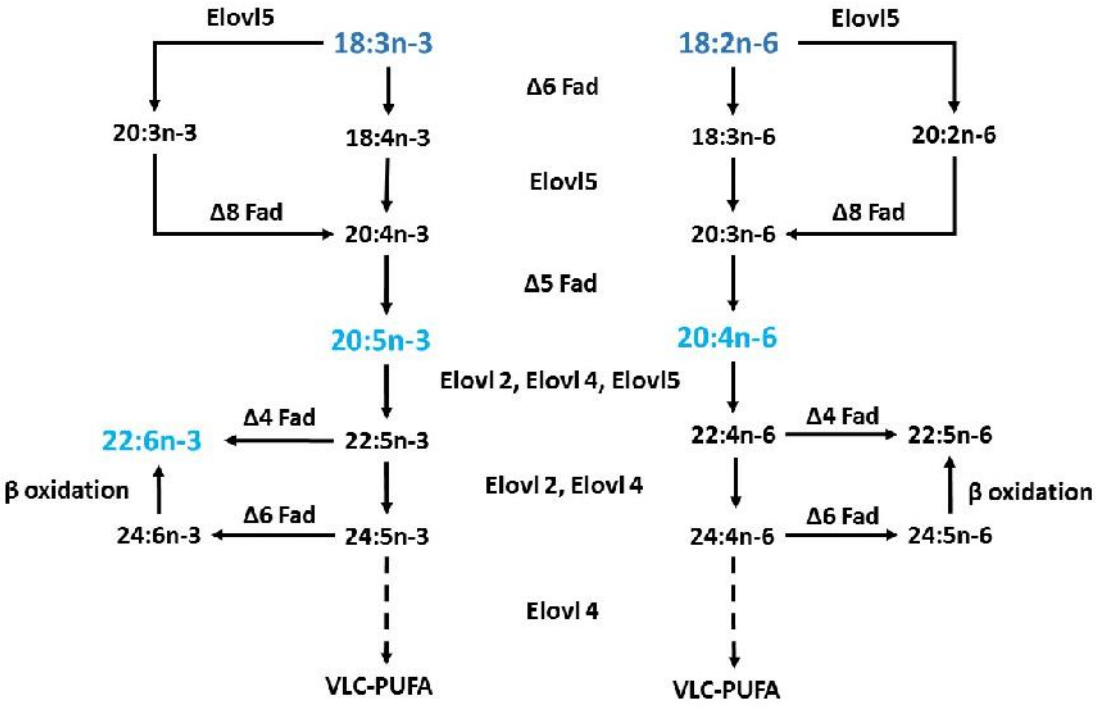


Figure 1.7 – Overview on pathways of long-chain polyunsaturated fatty acids in fish (Adapted from Tocher, 2015).

1.3.3.3 *O. vulgaris* and *S. officinalis* lipids

The first approaches concerning the lipid metabolism on cephalopods were performed during the 90's by Bouchand and Galois (1990) and Castro et al. (1992), who determined the effect of water temperature in egg-yolk lipid utilization during the embryonic development of *S. officilanis* and the changes in cuttlefish digestive gland and mantle muscle composition during starvation, respectively. The first experiments regarding the lipid requirements of cephalopods during early live stages under rearing conditions were latter performed by Navarro and Villanueva (2000, 2003). These authors pinpointed that nutritional imbalances in the lipid and FA profile of alternative diets could be the main reasons for high mortality. After, several nutritional studies have been performed, in order to improve the knowledge regarding *O. vulgaris* and *S. officinalis* lipid requirements, and the improvement of the lipid profile of alternative diets towards both species rearing (Almansa et al., 2006; Guinot et al., 2013a; Iglesias et al., 2014; Miliou et al., 2006; Monroig et al., 2012a, 2012b; Özyurt et al., 2006; Reis et al., 2015; Seixas et al., 2008, 2010a, 2010b; Sykes et al., 2009; Cerezo Valverde et al., 2013). Those studies determined 16:0, 18:0, DHA and the EPA, as well as, phosphoglycerides (mainly PC and PE) and

cholesterol as the most significant FA and LC in *O. vulgaris* and *S. officinalis* lipid composition. On the other hand, these species normally present a small TAG content, not just during the hatchling or paralarvae stage (Bouchaud and Galois, 1990; Navarro and Villanueva, 2000; Reis et al., 2015), but also in eggs (Bouchaud and Galois, 1990; Sykes et al., 2009; Quintana et al., 2015), juveniles or adult tissues (Almansa et al., 2006; Cerezo Valverde et al., 2013). The low TAG and high phospholipids contents indicate that in these species, lipids may have a predominant structural function (Sykes et al., 2009). Nonetheless, this may differ between populations with individuals from subtropical regions using carbohydrates as energy, while at lower temperatures they would prefer lipids and proteins as energy source (see Navarro et al., 2014).

EPA seems to be particularly important in hatchlings (it may play an important role for the brain and visual system), displaying a 1:1 EPA:DHA ratio in octopus tissues (Quintana, 2009) and 2:1 EPA:DHA in cuttlefish (Sykes, 2007); as opposed to the 1:2 proportion generally reported for marine fish larvae. Apart from DHA and EPA, ARA is one of the most abundant FA in *O. vulgaris* paralarvae FA profile. Nonetheless, the essentially of this FA for *O. vulgaris* or *S. officinalis* development remains an open question, with several studies postulating a certain ability of these species to biosynthesise ARA from adequate precursors (Almansa et al., 2006; Miliou et al., 2006). Recent studies on molecular cloning and functional characterisation of fatty acyl desaturase and elongase in *O. vulgaris* and *S. officinalis* adult tissues (Monroig et al., 2012a, 2012b, 2016), have shown that both species possess an enzyme with Δ5 Fad activity and an elongase of very long chain fatty acids (Elovl) that participates in the endogenous production not only of ARA from 20:3n-6 but also of EPA from 20:4n-3. Nonetheless, no Δ4, Δ6 or Δ8 Fad activity was recorded, and both 20:4n-3 and 20:3n-6 are not readily available in the natural diets. Therefore, EPA, ARA and DHA were defined as EFA for these species (Monroig et al., 2012a, 2016). Nevertheless, these findings require confirmation through *in vivo* studies, since it is not certain that enzymes activity will be expressed in live animals as they do in molecular studies.

An adequate dietary input of EFA, considering not just their amount and ratios but also their lipid form (mainly phospholipids and TAG), might be important as these factors could affect the availability of those FAs (Guinot et al., 2013a). For instance, it has been reported that marine fish larvae have limited capacity for FA exchange between TAG and phospholipids (Olsen et al., 2014; Sargent et al., 1999). Considering an approach of possible resemblance between cephalopods hatchlings and marine fish larvae

metabolism, it is likely that an association of EFA with the NL fraction, and more precisely with TAG of live preys and/or feeds, could influence cephalopods development. In fact, Seixas et al. (2010a) showed no relation between higher DHA content in *Artemia* sp. juveniles, and an improvement of paralarvae performance, which could be partly explained by the association of this FA with *Artemia* sp. TAG (Guinot et al., 2013b; Navarro et al., 1999). On the other hand, dietary phospholipids are considered a better vehicle to provide EFA to fish larvae, not just due to their high content on those EFA compared to NLs but also as they tend to improve lipid digestibility (Sargent et al., 1999; Taylor et al., 2015; Tocher et al., 2008).

As previously mentioned, the rearing of *O. vulgaris* paralarvae, currently depends on the use of *Artemia* sp. (Iglesias et al., 2007). Also, it is known that *Artemia* sp. naturally presents a low LC-PUFA and phospholipids contents (Soorgelos et al., 2001), which are considered essential in paralarvae development. In order to tailor *Artemia* sp. lipid composition towards octopus paralarvae nutritional needs, numerous types of enrichment have been tested. Those included using lipid emulsions (Guinot et al., 2013a; Navarro and Villanueva, 2003; Seixas et al., 2010a, 2010b; Viciano et al., 2011; Villanueva et al., 2002), microalgae (Carrasco et al., 2006; Fuentes et al., 2011; Iglesias et al., 2006; Seixas et al., 2008; 2010a, 2010b; Viciano et al., 2011) and/or microdiets (Fuentes et al., 2011). Nonetheless, and despite the many efforts, all of these works share common results of poor growth and survival rates. In order to improve *Artemia* sp. enrichment protocols towards *O. vulgaris* rearing it seems important to consider that *Artemia* sp. will not be a passive carrier of FA (Navarro et al., 1999), and contemplate that not only the EFA content but also their presence in specific lipid classes, such as PL and the total or individual LC-PUFA and ratios, might be essential factors to attain a normal development in this species. In this respect, it also seems necessary to determine the fate of FAs and to also discern lipid pathways of live prey.

1.4 Objectives

The major aim of this thesis was to provide a better knowledge of *Octopus vulgaris* and *Sepia officinalis* lipid requirements during early live stages, which should contribute to the improvement of live preys' enrichment protocols and/or formulated diets for these species culture. In order to achieve this goal, the following set of objectives, were established:

1. To develop a correct method to perform *in vivo* metabolism studies in cephalopods;

2. To investigate the natural capability of these cephalopods to metabolize PUFA;

3. To investigate the effect of live *Artemia* sp. endogenous lipid metabolism on the availability of essential fatty acids.

Chapter 2.

Development of a new methodology to perform *in vivo* fatty acid metabolism studies in cephalopods

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2.1 Abstract

The aim of the present study was to develop a method to study the *in vivo* metabolism of FAs in newly hatched cephalopods, through an adaptation of the methodology employed in fish cell FA metabolism studies. In this sense, the survival of *O. vulgaris* hatchlings (used as a model) to different experimental conditions and their capability to uptake and metabolized radiolabelled 18:1n-9 directly added to the water, as their potassium salt bound to bovine serum albumin (BSA) was analysed. In order to determine the best conditions to perform further *in vivo* FA radio-tracing assays, three different experiments were performed in triplicate. In the first trial, the survival of 60 hatchlings, placed in 10 mL of filtrated seawater (36 ‰) at 21 ºC, was evaluated during two different incubation periods (2 and 6 h), and in two different incubation devices (50 mL Falcon or flat-bottom 6 wells tissue culture plates). In the second trial, both density and incubation periods were raised up to 90 hatchlings and 12 h of incubation, respectively, to test the possibility of increasing the availability of total lipids, and the amount of incorporated radiolabelled FA substrate. In the third experiment, the ideal incubation period, and the capability of hatchlings to incorporate, esterify, and metabolise labelled 18:1n-9, used as a model directly from seawater, were checked. *O. vulgaris* hatchlings were capable to incorporate [1-^{14}C]18:1n-9 bound to BSA into their tissues. Since β-oxidation rate was not evaluated, the total amount of incorporated FA might be under-estimated, as the label present in the first carbon can be lost during FA catabolism. Nonetheless, the results demonstrated the feasibility of the adapted methodology to investigate the *in vivo* fate of the incorporated [1-^{14}C]FA, not only by determining its transformation through elongation and desaturation processes, but also by its esterification pattern into the different lipid classes. The final protocol for *in vivo* FA metabolism studies of *O. vulgaris* hatchlings was set as: 90 hatchlings incubated in 10 mL of filtrated seawater (36 ‰), during 6 h at 21 ºC and gentle stirring, in flat-bottom 6 wells cell culture plates, with 0.3 µM of [1-^{14}C]FAs added to water as their potassium salts bound to BSA. An adaptation of the suggested methodology might be applied according to the studied species or radiolabelled product.

Keywords: Cephalopods; Fatty acids; Hatchlings; *In vivo*; Metabolism

2.2 Introduction

Octopus vulgaris and *Sepia officinalis* are two cephalopods species with a great potential for aquaculture production (Villanueva et al., 2014). Nonetheless, the commercial culture of these species has been hampered mainly by the scarce existing knowledge on nutrition requirements (Villanueva et al., 2014). Within nutrients, and according to their tissue composition in wild, lipids like cholesterol, phospholipids and long-chain polyunsaturated fatty acids (LC-PUFA) have been suggested as critical dietary components for cephalopods development (Almansa et al., 2006; Navarro and Villanueva, 2000, 2003; Sykes et al., 2009), whith fatty acids (FA), like 20:5n-3 (EPA) and 22:6n-3 (DHA) being defined as essential fatty acids (EFA; Navarro and Villanueva (2003). On the other hand, 20:4n-6 (ARA) has been suggested as a non-essential FA for cephalopods, since the high levels of this FA in cephalopods tissues seems not be related to a dietary input (Almansa et al., 2006; Miliou et al., 2006), which may suggest certain capacity for endogenous production from shorter n-6 precursors. Therefore, in order to improve the knowledge on lipid requirements of these species, it seems very important to elucidate their dynamic aspects of lipid nutrition.

The most common methodology employed in the study of fish lipid requirements is based in the analysis of survival and growth of fish fed with diets that present controlled concentrations of determine FAs (doses-response studies), as they normally reflect dietary imbalances (Conceição et al., 2007; Sargent et al., 2002). Nonetheless, since formulated diets have been largely not accepted by cephalopods, 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; Navarro and Villanueva, 2003; Seixas et al., 2010b), the lipid requirements of these species cannot be accurately performed through doses-response studies. In addition, it is extremely difficult to adapt these studies to early life stages, given their dependence on life preys like *Artemia* sp., which presents a variable and is extremely difficult to control regarding its lipid composition (Guinot et al., 2013a, 2013b; Morais and Conceição, 2009). Consequently, this approach might not be the most adequate to determine the exact EFA requirements (Conceição et al., 2007; Morais and Conceição, 2009).

To overcome these difficulties, other methodologies have been developed. For instance, the ability of fish to convert C18 to C20 FA through desaturation/elongation pathways, have been intensively studied through cultured cell lines methods (Almaida-Pagán et al., 2007; Bell et al., 2001, 2002, 2006; Díaz-Lopéz et al., 2010; Fonseca-Madrigal et al.,

2006; Mourente et al., 2005; Rodríguez et al., 2002; Stubhaug et al., 2005; Tocher and Ghioni, 1999; Tocher et al., 1997, 2002, 2004). More recently, characterization of genes encoding desaturases and elongases involved in LC-PUFA biosynthesis pathways have also been performed in fish and also in cephalopods (Agaba, et al., 2005; Li et al., 2010, 2013; Liu et al., 2013, 2014; Monroig et al., 2011a, 2011b, 2012a, 2012b, 2013; Zheng et al., 2005, 2009). Within those, Monroig et al. (2012a, 2012b, 2016) studies were focused on the identification of EFA for *Octopus vulgaris* and *Sepia officinalis* normal development. Overall, those studies identified EPA, ARA and DHA as EFA for both species, since the endogenous production of these FAs from its precursors appears to be limited. Even though, these findings require to be confirmed and completed by *in vivo* studies in the species in question, because there is no certainty that enzymes involved in the PUFA elongation/desaturation pathways continue to be expressed *in vivo* exactly as they do *in vitro*.

In vivo metabolic studies on fish larvae have been extensively performed using both stable and radioactive isotopes, determining aspects like feed intake, digestion, absorption and nutrient utilization (see Conceição et al., 2007). Since, stable isotope tracers do not present physiological risk (Wolfe, 1992), their use has been replacing radioactive tracers. Nonetheless, among other desadvantages, stable isotopes tend to be more expensive and time consuming, when compared to radioactive tracers' methodologies, in addition to its lower detection sensitivity (Conceição et al., 2007).

Currently, there are four basic methods described to deliver tracers to fish larvae: micro-diet labelling, live food labelling, tube feeding and uptake from water (Conceição et al., 2007). As previously mentioned, formulated diets are largely not accepted nor ingested by thse two cephalopods species. On the other hand, the study of FA desaturation/elongation pathways and specific FA requirements using labelled live preys like *Artemia* sp., which is accepted by both octopus and cuttlefish individuals, would be extremely difficult given the active *Artemia* sp. FA metabolism even during small rearing periods (Estevéz et al., 1998; Guinot et al., 2013b; Navarro et al., 1999). The tube feeding methodology has been adapted to a large number of fish larvae species (see Conceição et al., 2007). However, its use in cephalopods, would be constrained by the existence of strong beaks in the buccal mass (Messenger and Young, 1999), which could break the feeding tube, and the lack of appropriate anaesthesia agents and methods for the younger stages (Fiorito et al., 2015; Sykes and Gestal, 2014). In this sense, radiolabelled uptake

from water might be an alternative to study the *in vivo* basal FA metabolism of cephalopods during their early life stages.

The capacity of *O. vulgaris* and *S. officinalis* to uptake amino acids directly from seawater, has been previously reported by Villanueva et al. (2004) and de Eguileor et al. (2000), respectively. However, the uptake of tracers from seawater methods has been mostly used on *in vivo* amino acids metabolism studies (Conceição et al., 2007). In this sense, FA metabolic studies should be performed by providing these molecules with vehicles such as albumin (McLaren et al., 2011), similarly to the methodology described in fish cell FA metabolism studies (Díaz-López et al., 2010; Rodriguez et al., 2002). Therefore, the aim of the present study was to develop a method to study the *in vivo* metabolism of FAs on newly hatched cephalopods, through an adaptation of the methodology employed in fish cell FA metabolism studies (Díaz-López et al., 2010; Rodriguez et al., 2002). Both the survival of *O. vulgaris* hatchlings (used as a model) to different experimental conditions, as well as their capability to uptake and metabolized radiolabelled 18:1n-9 directly added to the water, as their potassium salt bound to bovine serum albumin (BSA) were determined to assess the viability of the new method.

2.3 Material and Methods

2.3.1 Experimental animals

O. vulgaris broodstock (30 individuals) with an average wet weight of 1309 ± 503 g, were maintained at the Instituto Español de Oceanografia de Canarias (IEO de Canarias), in a sex ratio of two females per male (with similar weight), in 1000 L circular fiberglass tanks, with a flow-through seawater system under natural photoperiod (11L:13D), water temperature of 21.01 ± 0.69 °C and salinity of 36.8 ± 0.14 ‰. Octopuses were fed *ad libitum* with frozen squid (*Loligo opalescens*). Eggs presence was checked once a week and when a mass was detected, the ovate female was left alone. Approximately one-month later octopus began to hatch. Hatchlings were removed daily from the female/rearing tank, so that newly hatched octopus (less than 24 h old) would be available for experiments. A total of 1890 hatchlings were used during the present study.

2.3.2 Experimental conditions

To determine the best conditions to perform *in vivo* FA radio-tracing assays, a set of different experiments were performed. These experiments were based on an adaptation of *in vitro* FA metabolism studies in fish (Díaz-López et al., 2010; Rodriguez et al., 2002)

into an *in vivo* study in cephalopods. Firstly, the best incubation conditions (first and second experiments), and secondly, the capability of this species to incorporate and metabolize FA bound to BSA added directed to seawater were determined. After the incubation, octopus hatchlings were sacrificed in iced seawater (3 ºC) and stored at -80ºC (Fiorito et al., 2015). The experiments of this work were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.3.2.1 First Experiment

During this trial, the survival rate of octopus hatchlings in two different incubation devices was determined, considering a higher water column using 50 mL Falcons (SARSTEDT AG & CO., Germany) *vs* a higher bottom surface using flat-bottom 6-wells tissue culture plates (SARSTEDT AG & CO., Germany). A total of 720 hatchlings were used during this experiment. Sixty *O. vulgaris* hatchlings were placed in each well or Falcon with 10 mL of filtered seawater (36 ‰). Incubations were performed during two different incubation periods (2 and 6 h) in a Unicronic Reciprocal Shaking Bath (J.P. SELECTA S.A., Barcelona, Spain) at 21 ºC and gentle stirring (30 horizontal oscilations per minute). Incubations were performed in triplicate.

2.3.2.2 Second Experiment

Considering the results obtained duing the first experiment, both density and incubation period were raised up during the second trial. A total of 1170 hatchlings were used during this experiment. Firstly, the survival rate of hatchlings to a higer incubation period (12 h) was determined, using the same incubation device and hatchlings of the first experiment (n = 3). Secondly, in order to increase the avaibale amount of TL to perform futher analysis, a density of 90 hatchlings in 10 mL of seawater was also tested, at the three different incubation periods (2, 6 and 12 h) but only in the flat-bottom 6-wells tissue culture plates (n = 3). Incubation conditions were similar to those of the first experiment.

2.3.2.3 Third experiment

In the third trial, the capacity of octopus hatchlings to incorporate 18:1n-9 directly from seawater, and to metabolise this FA was determined. In order to stablish the ideal incubation time, two of the previous different incubation periods (2 and 6 h) were tested again. [1-^{14}C]18:1n-9 was selected as FA substrate due to is much lower price compared to other radiolabelled unsaturated FAs. Ninety hatchlings were incubated in flat-bottom

6-wells tissue culture, at similar conditions described in experiment 1, with 0.3 µCi (0.5 µM) of [1-^{14}C]18:1n-9 (n = 3), which was added as its potassium salt bound to bovine serum albumin (BSA; Ghioni et al., 1997). After incubation, hatchlings were sacrificed in iced seawater (3 ºC) and thoroughly washed with filtered seawater to remove excess [1-^{14}C]FA. Samples were stored at -80 ºC until analysis.

An aliquot of 0.5 mL of incubated water from each incubation well, was transferred to scintillation vials, in order to determine the amount of radioactivity not incorporated by hatchlings. Radioactivity was quantified in an LKB Wallac 1214 Rackbeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, U.S.A.). Results are presented in disintegrations per minute (dpm).

2.3.3 Evaluation of substrate intake and metabolism

2.3.3.1 Lipid extraction

Hatchings TL was extracted with chloroform/methanol (2:1, v/v) 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, v/v) with 0.01 % butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10 mg mL^{-1} and under an inert atmosphere of nitrogen.

2.3.3.2 Incorporation of radioactivity into hatchlings TL

In order to determine the radioactivity incorporated into hatchlings TL, an aliquot of 0.1 mg of TL extract was transferred into scintillation vials and radioactivity quantified in an LKB Wallac 1214 Rackbeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, U.S.A.).

2.3.3.3 Transformation of [1-^{14}C]18:1n-9 into longer/desaturated FA

To study the capability of hatchlings to elongate or desaturate radiolabelled FA, a sample of 1 mg of TL was subjected to acid-catalysed transmethylation to prepare fatty acids methyl esters (FAME; Christie, 2003). FAME were separated by thin-layer chromatography (TLC) using 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 resolves the FAME into discrete bands based on both degrees of unsaturation and chain length (Wilson and Sargent, 1992). For visualisation of radiolabelled FAs bands, developed TLC 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) for one week. After that period, the screens were scanned with an image acquisition system (Molecular Imager FX, BioRad) and visualised in an image analysis software (Quantity One, BioRad). Identification of labelled bands was confirmed by radiolabelled standards run on the same plate (Rodríguez et al., 2002).

2.3.3.4 Esterification of [1-^{14}C]18:1n-9 into lipid classes

The capability of *O. vulgaris* hatchlings to esterify the incorporated [1-^{14}C]18:1n-9 into the different lipid classes was also determined. For that 0.1 mg of TL extracts were applied to high-performance thin-layer chromatography (HPTLC) plates. Lipid classes (LC) were separated by single-dimensional double-development HPTLC as previously described by Tocher and Harvie (1988). The LC were visualized by brief exposure to iodine vapour and bands position labelled with radioactive ink spots. For visualisation of labelled LC bands (a specific LC containing esterified molecules of [1-^{14}C]18:1n-9), 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 Molecular Imager FX (BioRad, Madrid, Spain) and bands quantified by Quantity One software (BioRad, Madrid, Spain).

2.3.4 Materials

[1-^{14}C]18:1n-9 was purchased as free FA form, dissolved in ethanol, from PerkinElmer, Inc. (Waltham, Massachusetts, U.S.A.). BSA was purchased from Sigma-Aldrich (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 (St. Louis, Missouri, U.S.A.) and Panreac Química S.L.U. (Barcelona, Spain).

2.3.5 Statistical analysis

Results are presented as means ± SD. For all statistical tests, $p < 0.05$ was considered statistically different. Data were checked for normal distribution with the one-sample Shapiro-Wilk test, as well as for homogeneity of the variances with the Levene test (Zar, 1999). Arcsine square root transformation was applied to all data expressed as percentage (Fowler et al., 1998). Differences between octopus hatchlings survival rate to different incubation devices and incubation periods, as well as the amount of radioactivity recovered from incubated seawater and incorporated into octopus TL, were tested using Student´s *t*-test (Zar, 1999). Differences between octopus hatchlings survival rate to different incubation periods at a density of 90 hatchlings/well (second experiment) were analysed by one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test (Zar, 1999). The statistical analysis was performed using the IBM SPSS statistics 22.0 (IBM Co., USA).

2.4 Results

2.4.1 First experiment

Table 2.1 shows the survival rate of *O. vulgaris* hatchlings for the different incubations devices (50 mL Falcon and flat-bottom 6 wells tissue culture plates) after 2 and 6 h of incubation. After the 2 h incubation period, a survival rate of 100 % in all incubations from either Falcons or plates/wells was achieved. On the other hand, and after 6 h of incubation, only 70 % survival was obtained in Falcons, while in tissue culture plates the survival rate was 100 % (Table 2.1).

Table 2.1 – Experiment 1. Survival rate (%) of *O. vulgaris* hatchlings at the different experimental incubation conditions.

Incubation period/incubation device	**Falcons**	**Wells**
2 h	100 ± 0	100 ± 0
6 h	70 ± 10*	100 ± 0[1]

Results represent means ± SD (n=3). Data are presented in percentage. * Represents significant differences between 2 and 6 h of incubation within the same incubation device ($p < 0.05$). [1] Represents significant differences between incubation devices, within the same incubation period ($p < 0.05$).

2.4.2 Second Experiment

After the 12 h incubation period tested in wells, a null survival rate at both densities (60 and 90 hatchlings per well) was attained. Nonetheless, incubation of 90 hatchlings during 2 and 6 h displayed a survival rate of 100 % (Table 2.2).

Table 2.2 – Experiment 2. Survival rate (%) of *O. vulgaris* hatchlings at the different experimental incubation conditions in wells.

Incubation period/hatchlings number	60	90
2 h	-	100 ± 0^{a}
6 h	-	100 ± 0^{a}
12h	0 ± 0	0 ± 0^{b}

Results represent means ± SD (n = 3). Data are presented in percentage; Different letters in superscript within the same column represent significant differences between incubation periods for the same incubation density ($p < 0.05$)

2.4.3 Third Experiment

Table 2.3 shows the amount of radioactivity recovered from the incubation water after the incubation period and the incorporation rate of [1-^{14}C]18:1n-9 into hatchlings TL. Similar to that obtained at the 1st and 2nd experiments with wells a survival rate of 100 % was obtained in the present experiment. The 0.3 µCi of [1-^{14}C]18:1n-9 added to each incubation well corresponded to 666000 dpm. The amount of radioactivity recovered from the incubation water was not significantly different between the different incubation periods, mostly due to the high values of standard deviation recorded (638512.0 ± 37021.9 dpm after 2 h of incubation and 523971.3 ± 35194.3 dpm after 6 h). On the hand, the amount of [1-^{14}C]18:1n-9 incorporated into octopus hatchlings TL was higher after 6 h of incubation ($p < 0.05$).

Table 2.3 –Radioactivity (dpm) present in the incubation water and radioactivity (dpm) incorporated from [1-^{14}C]18:1n-9 into *O. vulgaris* hatchlings TL.

	2h	6h
Radioactivity recovered in water	638512.0 ± 107021.9	523971.3 ± 35194.3
Radioactivity incorporated into TL	615.6 ± 49.1	1340.4 ± 34.4*

Results are presented as dpm (desintegrations per minute), and represent means ± SD (n = 3). * Within the same row, represent significant differences between incubation periods ($p < 0.05$).

The capacity of hatchlings to transform incorporated 18:1n-9 into another FA by elongation/desaturation steps was proven by the methodology employed (Fig. 2.1). The darkest band observed corresponded to the original incubated FA, while the other bands, corresponded to products of its metabolism. It was possible to observe three different bands above the band of the original incorporated FA (better visualization at 6 h incubation). The uppermost slightest one could be a speck, since it was also observed in the multi-standard simultaneously spotted at the right hand size of each TLC plate. Nonetheless, the other two bands above 18:1n-9 represent elongation products of this FA (20:2n-9 and 22:2n-9). No bands were detected below 18:1n-9 band, which means that no desaturation products such as 18:2n-9 were obtained (Fig. 2.1).

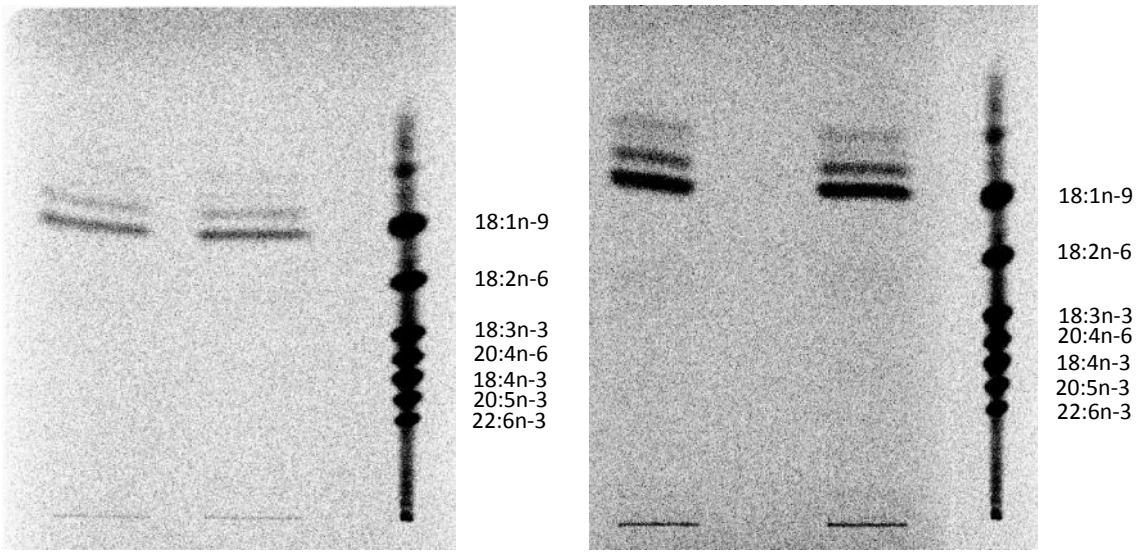

Figure 2.1 – Transformation of 18:1n-9 by elongation/desaturation processes after 2 and 6 h of incubation. Image obtained by a Molecular imager FX and Quantity One software.

Figure 2.2 shows the esterification pattern of [1-^{14}C]18:1n-9 into LC of *O. vulgaris* hatchlings. The round darkest marks on HPTLC plates (Fig. 2.2) correspond to radioactive ink spots used to identify specific LC position, while the wider bands correspond to the labelled lipid classes. The darker and wider the LC band, the higher the amount of [1-^{14}C]18:1n-9 esterified into that lipid class. As observed in figure 2.2, even after only a 2 h incubation period, it was possible to observe the esterification pattern of [1-^{14}C]18:1n-9. Nonetheless, the darkest bands were obtained after the 6 h incubation period. Interestingly, [1-^{14}C]18:1n-9 was mainly recovered esterified into the different lipid classes rather than as free fatty acid (FFA), its original molecular form.

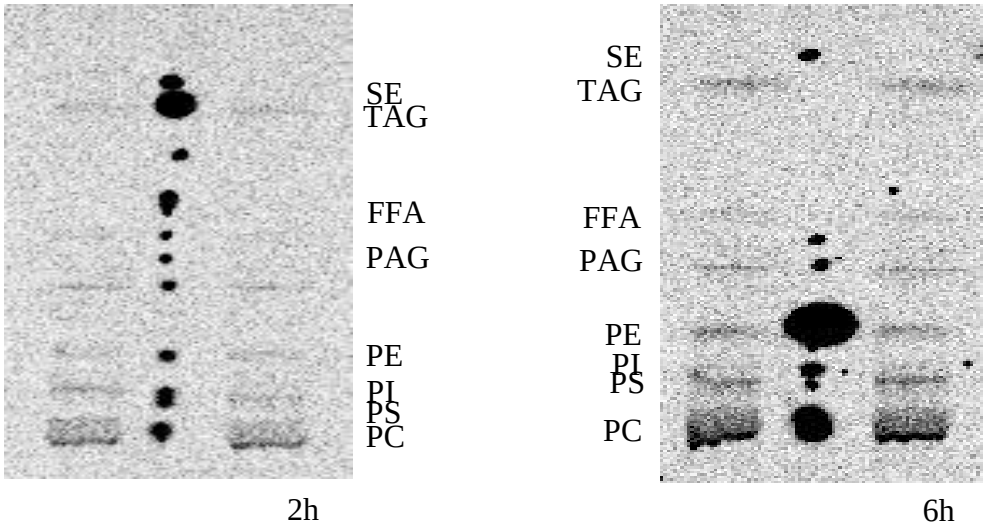

Figure 2.2 – Esterification pattern of [1-^{14}C]18:1n-9 into the lipid classes of *O. vulgaris* hatchlings after 2 and 6 h of incubation; SE- Sterol esters; TAG– Triacylglycerols; FFA- Free fatty acids; PAG- Partial acylglycerols, which include monoacylglycerols and diacylglycerols; PE- Phosphatidylethanolamine; PG- Phosphatidylglycerol; PI- Phosphatidylinositol; PS- Phosphatidylserine; PC- Phosphatidylcholine. Image obtained by a Molecular imager FX and Quantity One software.

2.5 Discussion

Considering the survival rate of octopus hatchlings to the different experimental conditions, *in vivo* incubations might be performed during 2 or 6 h, at a density of 90 hatchlings per 10 mL of filtered seawater (90 hatchlings/well), in flat-bottom 6 wells cell culture plates. An incubation period of 9 h was not tested during the time course assay, since this was less feasible in terms of laboratory timetable.

Similarly to that previously reported by Villanueva et al. (2004) for amino acids, *O. vulgaris* hatchlings were capable to incorporate labelled 18:1n-9 bound to BSA into their tissues, when this FA was added directly to seawater. The way nutrients are incorporated is not yet completely understood, as fluids within the digestive tract are isosmotic in relation to seawater (Wells and Wells, 1989) and starved adult octopuses merely drink 4 ml kg^{-1} h^{-1} (Wells and Wells, 1988), which may suggest that 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 an active absorption of nutrients from seawater during the planktonic stage, which suggests that skin could play an

important role in octopus nutrition during early life stages. Even though, since BSA used in the present study to bound the FA substrate is a big molecule so as to easily cross skin surface, further experiments are needed to elucidate the precise mechanism by which the radiolabelled FA was incorporated from seawater into octopus tissues.

It has been reported that cephalopods may preferentially use protein as energy source (Boucher-Rodoni and Mangold, 1994; Lee, 1994). Nonetheless, other theories, considered that carbohydrates and lipids might be also used as energy fuel (Hochachka, 1994; Morillo-Velarde et al., 2011; Navarro et al., 2014; Storey and Storey, 1983). The oxidation rate of incubated substrate was not determined during the present study. Therefore, it was not possible to determine the amount of incorporated FA used as energy substrate. This may also have influenced, by under-estimation, the incorporation values obtained when using this method (Conceição et al., 2007; Tocher et al., 2003), since the labelled carbon (acetyl-CoA) could have been lost during the first step of FA catabolism. Nonetheless, the results obtained during this study demonstrate the feasibility of the adapted methodology to investigate the *in vivo* fate of the incorporated [1-^{14}C]FA bound to BSA and added to seawater, not only by determining its transformation though elongation/desaturation processes, but also its esterification pattern into the different lipid classes, allowing to determine the basal FA metabolism in cephalopods hatchlings without the interference of live prey endogenous metabolism.

In vitro incubations with radiolabelled FAs are normally performed during 2 to 3 h (Bell et al., 2001; Díaz-López et al., 2010; Rodríguez et al., 2002). During this period the amount of labelled FA incorporation into cell TL, corresponds to approximatly 200 pmoles per mg of protein per hour (pmoles mg pp^{-1}h^{-1}). In the present *in vivo* study the amount of [1-^{14}C]18:1n-9 incorporated into octopus hatchlings TL was of aproximatly 8 pmoles mg pp^{-1}h^{-1} (data not shown). Despite the different incorporation rates of radiolabelled FAs into TL between *in vitro* and *in vivo* studies, after a period of only 2 h of incubation, it was possible to obtain a suitable amount of radioactivity to evaluate the 18:1n-9 metabolism. Nonetheless, the incubation period of 6 h, which also displayed 100 % survival, provided a higher incorporation value, which lead to a better visualization of the radiolabelled bands (Fig. 2.1 and 2.2) and so to a greater validation of the results. Therefore, an incubation period of 6 h should by applied at *in vivo* FA metabolism experiments.

Similarly to the *in vitro* methodology, the adapted method allowed to investigate the *in vivo* fate of the incorporated [1-^{14}C]18:1n-9 bound to BSA and added to seawater, by

determining its transformation though elongation/desaturation processes. In the present study hatchlings were incubated with [1-^{14}C]18:1n-9 (as FA model), which is not normally tested in *in vitro* or molecular metabolic studies. Despite that, the developed *in vivo* method, in similarity to the results reported by Monroig et al. (2012b) through a molecular approach in *O. vulgaris* tissues, allowed to detect the action of an elongase towards C18 and C20 FAs in octopus hatchlings. On the other hand, no desaturation activity was recorded towards the incubated FA. Until now, Δ5 FA desaturase (Δ5 Fad) is the only reported Fad to act on *O. vulgaris* tissues (Monroig et al., 2012a). Nonetheless, the FA substrates over which this enzyme is known to act upon (20:4n-3 and 20:3n-6) were not tested during the present study. Moreover, as reported in *in vitro* studies, the developed method also allowed to determine the esterification pattern of a FA substrate into the different LCs. Interestingly, [1-^{14}C]18:1n-9 was mainly recovered in its esterified form into the different lipid classes, rather than in its original form (as FFA).

The present results show the proof of concept and the applicability of the adaptation of the original *in vitro* methodology to a novel *in vivo* approach that uses whole animals, for FA metabolic studies using radiolabelled substrates. The conditions proposed for a good protocol in *O. vulgaris* hatchlings studies are as follows: 90 hatchlings incubated in 10 mL of filtrated seawater (36 ‰), during 6 hours at 21 ºC and gentle stirring, in flat-bottom 6 wells cell culture plates and with 0.3 µM of [1-^{14}C]FA added to the water as their potassium salts bound to BSA. Adaptations of the suggested methodology might also be applied according to the species or the radiolabelled marker under study.

Chapter 3.

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

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3.1 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 ºC for 6 h with 0.2 µCi (0.3 µM) of [1-^{14}C]-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-^{14}C]FAs was also assessed. *O. vulgaris* hatchlings not only possessed the ability to incorporate FAs bound to BSA, but also to esterify them into phospholipid, with marked specificity. [1-^{14}C]DHA and [1-^{14}C]C18 FAs substrates were mainly esterified into phosphatidylcholine, while [1-^{14}C]ARA and [1-^{14}C]EPA were esterified into phosphatidylethanolamine. The majority of radioactivity from [1-^{14}C]FAs incorporated into hatchling total lipid was recovered as unmodified FAs with elongation being the only metabolism detected. Of the FAs investigated, [1-^{14}C]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 FAs tested in this experiment may indicate that the nutritional requirements of *O. vulgaris* hatchlings in terms of FAs are highly specific and that LC-PUFAs must be considered essential dietary nutrients.

Keywords: Common octopus; Lipid metabolism; *Octopus vulgaris* hatchlings; Radiolabelled substrates; Unsaturated fatty acids.

3.2 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., 2007, 2014; 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 2000, 2003; 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 Δ5-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 (Δ6) 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 and triacylglycerols (TAG) (Sargent et al., 1999). According to Navarro and Villanueva (2000, 2003), *O. vulgaris* paralarvae require prey rich, not only in PUFAs, but also in phospholipids and cholesterol. Viciano et al. (2011) determined that *O. vulgaris* phospholipids present a distinct FA profile compared to neutral lipids (NL), with the former presenting a higher content of PUFAs and the latter of monounsaturated FAs. 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 FAs 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 FAs was investigated.

3.3 Materials and Methods

3.3.1 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.

3.3.2 *In vivo* incubation with labelled [1-^{14}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 ºC for 6 h, with 0.2 µCi (0.3 µM) of [1-^{14}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-^{14}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-^{14}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 ºC in chloroform/methanol (2:1, v/v) 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.

3.3.3 Lipid classes and fatty acids composition of non-radioactive samples

The control group incubations were analysed for lipid class (LC) 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).

3.3.4 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 (pmol mg pp^{-1} h^{-1}) taking into account specific activity of each substrate, and hatchlings TL and protein contents (Lowry et al., 1951).

3.3.5 Esterification of radioactivity into lipid classes

To define the esterification pattern of [1-^{14}C]FA in LC, a sample of 0.1 mg of TL was applied to HPTLC plates. LC 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, Madrid, Spain) and the bands quantified in percentage by image analysis software (Quantity One, BioRad, Madrid, Spain).

3.3.6 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 ºC 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 degrees 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).

3.3.7 Materials

[1-^{14}C]C18 FAs 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 purchase from Sigma-Aldrich (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 (St. Louis, Missouri, U.S.A.) and Panreac Química S.L.U. (Barcelona, Spain).

3.3.8 Statistical analysis

Results are present as means ± 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–Smirnoff 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-^{14}C]C18 FA (18:1n-9, 18:2n-6, 18:3n-3) and [1-^{14}C] 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 Kruskall-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).

3.4 Results

3.4.1 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 µg lipid mg protein^{-1}, which consisted mainly of polar lipids (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 3.1). Cholesterol was the most abundant LC 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 3.1).

Table 3.1 – Total lipid (µg lipid mg protein^{-1}) content and lipid class composition (%) of *Octopus vulgaris* hatchlings

TL content	384.9 ± 57.5
Lipid Class	
∑ 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
∑ 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

Results represent means ± SD (n=3).

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 3.2). The n-3 LC-PUFA content was 42.4 ± 0.4 % of total FAs, 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 3.2).

Table 3.2 – Fatty acid composition (% of total fatty acids) of *Octopus vulgaris* hatchlings

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

Results represent means ± SD (n=3); [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

3.4.2 Incorporation of radioactivity into total lipids and its distribution into lipid classes

Table 3.3 shows the incorporation of radiolabelled FAs into TL of *O. vulgaris* hatchlings and its distribution among LC. Incorporation of [1-^{14}C]ARA into TL was significantly higher than that of all other radiolabelled FA ($p < 0.05$). No differences were detected between incorporation of [1-^{14}C]C18 FAs and [1-^{14}C]LC-PUFA (EPA and DHA).

All ^{14}C-labelled FAs incorporated were esterified into lipid classes, with less than 6 % of radioactivity being recovered as free fatty acids (FFA; Table 3.3). The majority of the [1-^{14}C]FA were recovered in PL. The esterification pattern of C18 FAs was: PC > TAG > PI for [1-^{14}C]18:1n-9, PC > PE > PS for [1-^{14}C]18:2n-6 and PC > PS > PE for [1-^{14}C]18:3n-3. Interestingly, the highest incorporation into PC was obtained with

[1-^{14}C]DHA, with 78.4 ± 3.9 % of radioactivity being esterified into this PL ($p < 0.05$). In contrast, [1-^{14}C]ARA and [1-^{14}C]EPA were predominantly esterified into PE ($p < 0.05$), followed by PS > PI ≥ PC for ARA and PC > PS ≥ PI for EPA (Table 3.3). The proportion of radioactivity recovered as TAG was higher for the [1-^{14}C]C18 FAs than for LC-PUFA ($p < 0.05$).

Table 3.3 – 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-^{14}C]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$^{c\bullet}$	10.5 ± 0.9$^{b\blacktriangle}$	11.3 ± 1.6$^{bc\blacktriangle\bullet}$	33.8 ± 1.2$^{a\Delta}$	24.2 ± 2.1$^{bc\circ}$	18.7 ± 1.4$^{b\circ}$
Lipid Class						
Phosphatidylcholine	47.0 ± 0.9$^{d\blacksquare}$	51.5 ± 1.8$^{c\bullet}$	60.5 ± 2.1$^{b\blacktriangle}$	11.1 ± 0.7$^{e\circ}$	12.4 ± 0.7$^{e\circ}$	78.4 ± 3.9$^{a\Delta}$
Phosphatidylserine	6.1 ± 2.3$^{c\bullet}$	10.8 ± 1.9$^{ab\blacktriangle}$	10.0 ± 1.3$^{b\blacktriangle}$	14.0 ± 0.6$^{a\Delta}$	11.3 ± 0.9$^{ab\circ}$	2.7 ± 0.6$^{d\square}$
Phosphatidylinositol	10.8 ± 0.6$^{a\blacktriangle}$	7.2 ± 0.5$^{b\bullet}$	2.2 ± 0.4$^{c\blacksquare}$	11.6 ± 1.3$^{a\Delta}$	11.0 ± 1.1$^{a\Delta}$	2.6 ± 0.5$^{c\circ}$
Phosphatidylglycerol	0.0 ± 0.0$^{b\bullet}$	0.0 ± 0.0$^{b\bullet}$	2.6 ± 0.2$^{a\blacktriangle}$	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Phosphatidylethanolamine	9.1 ± 0.5^{cd}	11.0 ± 2.3^{c}	8.7 ± 0.2^{cd}	57.3 ± 2.8$^{a\Delta}$	48.4 ± 3.0$^{b\circ}$	7.7 ± 0.8$^{d\square}$
Unknown	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.9 ± 0.4$^{b\circ}$	2.7 ± 0.2$^{a\Delta}$	0.0 ± 0.0$^{c\square}$
∑ Polar lipids	72.9 ± 1.3$^{c\blacksquare}$	80.5 ± 1.5$^{b\bullet}$	84.9 ± 1.4$^{b\blacktriangle}$	95.0 ± 2.7$^{a\Delta}$	85.9 ± 2.2$^{b\circ}$	91.5 ± 2.4$^{a\Delta\circ}$
Partial acylglycerols	8.2 ± 1.0$^{a\blacktriangle}$	6.7 ± 0.5$^{ab\bullet}$	5.4 ± 0.5$^{b\blacksquare}$	1.9 ± 1.5$^{b\circ}$	6.4 ± 1.0$^{ab\Delta}$	3.6 ± 1.2$^{b\Delta\circ}$
Free fatty acids	5.4 ± 0.7$^{a\blacktriangle}$	3.6 ± 0.7$^{b\bullet}$	3.1 ± 0.4$^{bc\bullet}$	2.0 ± 0.7$^{c\circ}$	4.1 ± 0.4$^{ab\Delta}$	2.2 ± 0.5
Triacylglycerols	13.4 ± 2.9$^{a\blacktriangle}$	9.2 ± 0.9$^{b\blacktriangle\bullet}$	7.5 ± 0.8$^{b\bullet}$	1.1 ± 0.6$^{d\circ}$	3.6 ± 0.8$^{c\Delta}$	2.7 ± 0.7$^{c\Delta}$
∑ Neutral lipids	27.1 ± 1.3$^{a\blacktriangle}$	19.5 ± 1.5$^{b\bullet}$	16.1 ± 1.4$^{b\blacksquare}$	5.0 ± 2.7$^{c\circ}$	14.1 ± 2.2$^{b\Delta}$	8.5 ± 2.4$^{c\Delta\circ}$

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 ($p < 0.05$).

3.4.3 Transformation of radiolabelled fatty acids

The majority of radioactivity from [1-^{14}C]FAs incorporated into *O. vulgaris* hatchlings TL was recovered as unmodified substrate (Table 3.4). Nonetheless, small proportions of the radiolabelled substrate FA were transformed into other FA products. [1-^{14}C]18:1n-9 and [1-^{14}C]18:2n-6 were the FAs showing most transformation, as well as the only substrates where two different metabolic products were detected. The radioactivity incorporated into hatchling TL as [1-^{14}C]18:1n-9 was converted to 20:1n-9 (19.2 ± 1.6 %) and 22:1n-9 (9.2 ± 1.3 %). Radioactivity from [1-^{14}C]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 3.4).

Table 3.4 – Recovery of radioactivity (%) from [1-^{14}C]FA substrates in FA metabolites in *Octopus vulgaris* hatchlings

Substrates	Products	%
[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

Results represent means ± SD (n=4).

3.5 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]FAs 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, 2000, 2003; Seixas et al., 2010b), with 16:0, 18:0, EPA and DHA being the most abundant FA (Navarro and Villanueva, 2000; Okumura et al., 2005; Seixas et al., 2008; 2010a, 2010b). The consistent high contents of those LC and FA in the lipid profiles of *O. vulgaris* hatchlings may suggest their importance in the development of this species. Accordingly, Navarro and Villanueva (2000, 2003) have suggested that octopus paralarvae require a prey rich in PUFA, phospholipids and cholesterol, with a moderate content of NL.

Of all incubated radiolabelled FAs, ARA was the most efficiently incorporated into hatchling lipids. As the same quantity of radiolabelled substrates was added to each treatment, the amount of ARA incorporated into *O. vulgaris* hatchlings appears to be unrelated to its abundance in the surrounding water. Preferential incorporation of dietary ARA into TL was previously reported by Atalah et al. (2011a) in *Sparus aurata* larvae. However, incorporation of ARA into larval TL of this species increased, and that of EPA decreased, when dietary ARA was increased, and vice-versa (Atalah et al., 2011a). A similar inverse relation between ARA and EPA incorporation was also reported for *Paralichthys olivaceus* eggs polar lipids (Furuita et al., 2003), as well as in *O. vulgaris* tissues (Miliou et al., 2006). This inverse relation could possibly be related competition between these two LC-PUFA for acylases and transacylases in FA esterification (Sargent et al., 1999). As these enzymes do not have tight specificity for particular FA, tissue compositions are partly determined by dietary FA levels and ratios. In the present study and although in different proportions, the general esterification pattern of ARA and EPA

into lipid classes was similar, with the majority of radioactivity being recovered in PE. This could contribute to the competition described above, and therefore, adequate dietary inputs of both FA might be crucial in *O. vulgaris* development. In previous studies investigating dietary lipid in *O. vulgaris* paralarvae rearing, the EPA/ARA ratio was usually not considered (Kurihara et al., 2006; Navarro and Villanueva, 2000, 2003; Okumura et al., 2005; Seixas et al., 2010a, 2010b; Viciano et al., 2011). Nonetheless, calculating the EPA/ARA ratio of octopus hatchlings in those studies, great variability was observed, ranging from 1.7 (Navarro and Villanueva, 2000) to 4.5 (Seixas et al., 2008). Additionally, the ideal EPA/ARA ratio that promotes normal development of this species is difficult to discern, due to the inconsistent results obtained, in terms of growth and survival, during paralarvae rearing (Kurihara et al., 2006; Navarro and Villanueva, 2000, 2003; Okumura et al., 2005; Seixas et al., 2010a, 2010b; Viciano et al., 2011).

Similar to Viciano et al. (2011), who detected a higher content of MUFA in the NL fraction of *O. vulgaris*, in the present study the proportion of radioactivity recovered as TAG was more specifically higher for [1-^{14}C]18:1n-9. This preferential esterification might be related to the energy-generating FA oxidation systems (Sargent et al., 1989). 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 phospholipids, revealing their important structural role in cell membranes (Sargent et al., 1989). Following Linares and Henderson (1991), the esterification of LC-PUFA into LC 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., 2013b; 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 Δ5-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 we re incubated, it was not possible to detect any trace of ARA and EPA synthesis. These data do not invalidate possible Δ5 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 EFAs and so provided in the diet.

Of the substrates incubated, [1-^{14}C]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; Milliou 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 (Monriog 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-^{14}C]18:2n-6 and [1-^{14}C]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-^{14}C]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-^{14}C]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 is was possible to perform *in vivo* studies with *O. vulgaris* hatchlings, using direct incubation with [1-^{14}C]FAs bound to BSA added to the seawater. The esterification pattern of radiolabelled FA substrates was highly specific, with [1-^{14}C]DHA and [1-^{14}C]C18 FA being preferably esterified into PC and [1-^{14}C]ARA and [1-^{14}C]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 EFAs and provided by dietary sources at least during crucial early life stages.

Chapter 4.

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

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4.1 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 FAs, as well as lipid classes (LC) containing essential fatty acids (EFA). In the present study, cuttlefish hatchlings were incubated with [1-^{14}C]FAs including C18 FAs (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 FAs 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 FAs were the most transformed FAs 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-PUFAs 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 EFAs 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 FAs and LC-PUFA levels and ratios should be considered, since the esterification pattern detected in the present study suggested competition between these FAs for esterification into specific LC. Moreover, considering the observed esterification pattern of LC-PUFA into different LC,

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.

4.2 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 natural 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., 2003, 2004; 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. (Chapter 3; 2014) recently reported the *in vivo* lipid metabolism in *O. vulgaris* hatchlings by incubating paralarvae with several ^{14}C-labelled fatty acids (FA), where LC-PUFA were determined as essential for the normal development of the species. Moreover, considering the specific esterification patterns of the incubated FAs, 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 FAs and lipid classes containing those FAs was determined by investigating the *in vivo* capability of the species to incorporate, esterify into different lipid classes, and modify unsaturated FA.

4.3 Materials and Methods

4.3.1 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 %.

4.3.2 *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, v/v) 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, v/v) with 0.01 % butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10 mg.mL^{-1} and under an inert atmosphere of nitrogen. The experiments of this work were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

4.3.3 Lipid classes and fatty acids composition of control samples

Aliquots of 20 µg 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.).

4.3.4 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).

4.3.5 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. (2014).

4.3.6 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. (Chapter 3; 2014).

4.3.7 Materials

[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).

4.3.8 Statistical analysis

Results are presented as means ± SD. 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 FAs (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.).

4.4 Results

4.4.1 Lipid composition of hatchlings

The TL content and LC composition of *S. officinalis* hatchlings are presented in Table 4.1. 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 4.1 – Total lipid content (µg lipid mg protein^{-1}) and lipid class composition (%) of *Sepia officinalis* hatchlings

TL content	384.9 ± 57.5
Lipid Class	
Sphingomyelin	0.8 ± 0.4
Phosphatidylcholine	24.0 ± 1.9
Phosphatidylserine	7.0 ± 0.6
Phosphatidylinositol	5.0 ± 0.2
Phosphatidylglycerol	2.4 ± 0.2
Phosphotidylethanolamine	18.9 ± 1.0
∑ Polar lipids	58.1 ± 1.0
Cholesterol	32.2 ± 2.1
Free fatty acids	0.8 ± 0.2
Triacylglycerols	6.3 ± 1.1
Sterol esters	2.6 ± 0.8
∑ Neutral lipids	41.9 ± 1.0

Results represent means ± SD (n = 4).

Table 4.2 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.

Table 4.2 – Main fatty acid composition (% of total FA) of *Sepia officinalis* hatchlings

14:0	2.0 ± 0.4
15:0	1.1 ± 0.3
16:0	20.4 ± 1.6
18:0 DMA	3.8 ± 0.2
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[a,d]	1.3 ± 0.4
18:3n-3	0.4 ± 0.1
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[a,d]	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
DHA/EPA/ARA[e]	17 / 13 / 1

Results represent means ± SD (n = 4). Data are presented in percentage of total FA 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.

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 FAs. The DHA/EPA ratio of hatchlings was 1.3 ± 0.0, while the EPA/ARA ratio was 13.4 ± 3.9.

4.4.2 Incorporation of radiolabelled FA into TL and its distribution among LC

The incorporation of radiolabelled FAs into *S. officinalis* hatchling TL and its distribution among LC is presented in Table 4.3. 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).

Table 4.3 – Incorporation (pmoles mg pp^{-1} h^{-1}) and lipid classes distribution (%) of [1-^{14}C]FA substrates into *Sepia officinalis* hatchlings total lipids

Substrate	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Incorporation	$8.2 \pm 1.4^{a\bullet}$	$15.4 \pm 3.4^{bc\blacktriangle}$	$18.0 \pm 3.7^{c\blacktriangle}$	$18.0 \pm 1.3^{c\Delta}$	$14.6 \pm 2.9^{bc\circ\Delta}$	$11.4 \pm 1.1^{ab\circ}$
Lipid Class						
Phosphatidylcholine	$38.3 \pm 3.0^{c\bullet}$	$39.2 \pm 1.7^{bc\bullet}$	$44.7 \pm 2.1^{b\blacktriangle}$	$26.9 \pm 1.1^{d\circ}$	$26.7 \pm 0.5^{d\circ}$	$70.6 \pm 4.8^{a\Delta}$
Phosphatidylserine	$25.2 \pm 1.4^{a\blacktriangle}$	$22.2 \pm 1.4^{b\bullet}$	$21.5 \pm 0.5^{bc\bullet}$	$19.3 \pm 1.4^{cd\Delta}$	$17.2 \pm 1.4^{d\Delta}$	$2.9 \pm 0.3^{e\circ}$
Phosphatidylinositol	4.1 ± 0.9^{c}	3.2 ± 0.5^{c}	3.4 ± 0.1^{c}	$11.1 \pm 1.4^{a\Delta}$	$11.5 \pm 0.9^{a\Delta}$	$6.1 \pm 1.4^{b\circ}$
Phosphatidylglycerol	$0.0 \pm 0.0^{c\blacksquare}$	$3.9 \pm 0.3^{a\blacktriangle}$	$3.3 \pm 0.2^{b\bullet}$	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
Phosphatidylethanolamine	$12.9 \pm 0.8^{b\bullet}$	$15.0 \pm 0.8^{b\blacktriangle}$	$14.2 \pm 1.1^{b\blacktriangle\bullet}$	$36.6 \pm 2.2^{a\Delta}$	$37.5 \pm 1.9^{a\Delta}$	$12.6 \pm 1.0^{b\circ}$
∑ Polar lipids	$80.6 \pm 3.4^{d\bullet}$	$83.5 \pm 1.9^{cd\blacktriangle\bullet}$	$87.1 \pm 3.1^{bc\blacktriangle}$	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	$11.7 \pm 1.3^{a\blacktriangle}$	$9.3 \pm 1.5^{ab\blacktriangle\bullet}$	$7.7 \pm 1.2^{b\bullet}$	3.3 ± 1.1^{c}	4.1 ± 1.3^{c}	3.4 ± 0.6^{c}
∑ Neutral lipids	$19.4 \pm 3.4^{a\blacktriangle}$	$16.5 \pm 1.9^{ab\blacktriangle\bullet}$	$12.9 \pm 3.1^{bc\bullet}$	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 per mg protein per hour. 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 ($p < 0.05$).

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.

4.4.3 Transformation of radiolabelled fatty acids

The transformation of incorporated [1-^{14}C]FA substrates into other FA metabolites is presented in Table 4.4. [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).

Table 4.4 – Distribution of radioactivity (% of total radioactivity incorporated) from [1-^{14}C]FA substrates in *Sepia officinalis* hatchlings

Substrates	Product	%
[1-^{14}C]18:1n-9		
	18:1n-9	43.2 ± 7.3^{d}
	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^{d}
	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

Results represent means ± SD (n = 4). Different letters in superscript represent differences within unmodified FA ($p < 0.05$).

Table 4.4 – Distribution of radioactivity (% of total radioactivity incorporated) from [1-^{14}C]FA substrates in *Sepia officinalis* hatchlings (cont.)

[1-^{14}C]18:3n-3		
	18:3n-3	58.5 ± 6.3^{c}
	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^{ab}
	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^{a}
	22:5n-3	1.7 ± 0.3
[1-^{14}C]22:6n-3		
	22:6n-3	94.5 ± 1.3^{b}
	24:6n-3	5.5 ± 1.3

Results represent means ± SD (n = 4). Different letters in superscript represent differences within unmodified FA ($p < 0.05$).

4.5 Discussion

In the present study, similar to that previously reported for *O. vulgaris* (Chapter 3; Reis et al., 2014), 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, 1998, 2000). 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, phospholipids 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 (Chapter 3; Reis et al., 2014), [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; Chapter 3; Reis et al., 2014, 2015; 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; Chapter 3; Reis et al., 2014, 2015).

Interestingly, and similar to that described by Reis et al. (Chapter 3; 2014) 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* (Chapter 3; Reis et al., 2014), 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 (Chapter 3; Reis et al., 2014), 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* (Chapter 3; Reis et al., 2014), 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 FAs 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 FAs. 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 EFAs within phospholipids and between TAG and phospholipids.

When analysing the variation of FAs 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 FAs. 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 FAs to C20 FAs. 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 characterization of an elongase of very long-chain FA (Elovl) gene in *O. vulgaris*, which showed the capacity of this species to convert C18 and C20 PUFA substrates to their corresponding 2C elongated products, although no activity towards C22 PUFA was detected with this Elovl. More recently, Monroig et al. (2013) reported the functional characterization of a second Elovl, which suggested octopus had the capability to elongate C22 PUFA. The present results, and those obtained in octopus (Chapter 3; Reis et al., 2014), showed elongation activity towards DHA. Interestingly, this activity was not

detected in any C22 FA products obtained from C18 or C20 FA substrates. Considering, the low amount of radioactivity recovered as C22 FAs, and the apparent low elongation activity on these FAs, (less than 6 % of [1-^{14}C]DHA incorporated was recovered as 24:6n-3), it is possible that the band obtained would be unnoticed on a TLC plate. Additionally, as mentioned above, in some replicates of [1-^{14}C]18:1n-9, the bands corresponding to C22 FA appeared to be split. However, an unequivocal and accurate confirmation of its existence and identity was not possible.

In contrast to fish, where more efficient elongation of n-3 is reported (Agaba et al., 2005; Monroig et al., 2012c; Morais et al., 2011), in the present study higher activity towards n-6 FA substrates was detected. Thus, 18:2n-6 and 20:4n-6 were transformed at higher rates than the corresponding n-3 FA, namely 18:3n-3 and 20:5n-3, respectively. Preferential elongation activity towards n-6 FA rather than n-3 FA was also previously reported in *O. vulgaris* (Monroig et al., 2012b; Chapter 3; Reis et al., 2014). Nonetheless, considering the incorporated radioactivity into *S. officinalis* hatchlings TL, the absolute value of elongated FA was similar between n-3 and n-6 FAs (7.2 ± 1.1 and 7.6 ± 0.3 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 pmoles mg pp^{-1} h^{-1} for EPA and ARA, respectively).

Almansa et al. (2006) suggested the existence of active n-6 LC-PUFA metabolism in juvenile and maturing cuttlefish as biosynthesis of 22:5n-6 from ARA was implied. In animals, 22:5n-6 has been considered as the final end product of the desaturation pathways of n-6 FAs (Tocher et al., 1998). The synthesis of this FA from ARA involves one of two possible metabolic pathways: the "Sprecher pathway", which comprises two sequential elongations of ARA to 24:4n-6, followed by a Δ6 desaturation and one round of peroxisomal β-oxidation (Δ4-independent pathway; Sprecher, 2000); or a direct route involving elongation of ARA to 22:4n-6 followed by a Δ4 desaturation (Li et al., 2010). For many years the activity of Δ4 fatty acyl desaturase enzyme (Fad) was not demonstrated in vertebrates, and it was assumed that DHA was synthesised by the Δ4-independent pathway (Tocher et al., 1998). However, recent studies have identified the activity of a Δ4 Fad, not just in lower eukaryotes (Pereira et al., 2003), but also in some teleost fish species (Li et al., 2010; Morais et al., 2012, 2015; Fonseca-Madrigal et al., 2014). As previously mentioned, the present study confirmed the elongation of [1-^{14}C]ARA to 22:4n-6. Moreover, another unidentified metabolic product from [1-^{14}C]ARA was observed, which could support the previous suggestion of Almansa et al. (2006). However, a similar band detected in *O. vulgaris* hatchling [1-^{14}C]ARA

metabolism was determined as a metabolic product other than a FA (Chapter 5; Reis et al., unpublished data). Furthermore, no similar band was obtained from [1-^{14}C]EPA. Interestingly, a similar metabolic product to that detected from [1-^{14}C]ARA, was also obtained from [1-^{14}C]18:2n-6. Since there was no evidence for ARA production from [1-^{14}C]18:2n-6, it would be unexpected that this band corresponds to 22:5n-6. Although it was not possible to identify the identity of this band, it is important to mention its appearance when n-6 FAs were incubated, since it may possibly indicate a different requirement of cephalopods for n-6 FAs.

The synthesis of EPA and ARA from 18:3n-3 and 18:2n-6, respectively, requires the activity of a Δ6 Fad, followed by elongation, and then a second Fad introducing a double bond at the Δ5 position of the elongated FA (Cook, 1996). However, in some marine fish species, the Δ6 enzyme also presents a Δ8 activity and, so the first two steps could be reversed in order (Monroig et al., 2011a). In the present study, according to the position of co-running standards, the potential resultant desaturation products from [1-^{14}C]C18 FA substrates might involve a Δ6 desaturation. Nonetheless, unequivocal identification of the desaturation products obtained from C18 FA (18:2n-9, 18:3n-6 and 18:4n-3) would be difficult, since the route involving an elongation followed by Δ8 desaturation (producing 20:2n-9, 20:3n-6 and 20:4n-3) cannot be eliminated. In this sense, the characterization of the enzymes involved in PUFA biosynthesis of cuttlefish would be an invaluable tool.

Functional characterization in recombinant yeast showed that *O. vulgaris* possessed a Fad that exhibited Δ5 desaturation activity towards PUFA substrates (Monroig et al., 2012a). Recently, similar results were also obtained regarding Fad genes of *S. officinalis* hatchlings (Monroig et al., 2013). However, no Δ5 Fad activity was detected in the present study. Despite this, the present data do not rule out a possible Δ5 Fad activity in 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* FAs 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.

Chapter 5.

Characterisation of *Sepia officinalis* and *Octopus vulgaris* hatchlings main phospholipid fatty acid composition and metabolism

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5.1 Abstract

Recent studies have highlighted the importance of an adequate dietary input of essential fatty acids (EFA) for *Octopus vulgaris* and *Sepia officinalis* early live stages development. These consider not only EFA amount and ratios, but also the lipid class in which they are esterified, as this could affect the availability of those fatty acids (FA). The objective of the present study was to characterise the FA profiles of the major phospholipids, of *O. vulgaris* and *S. officinalis* hatchlings, namely phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE), and to evaluate if the way EFA are supplied to hatchlings influences their bioavailability. Thus, *O. vulgaris* and *S. officinalis* hatchlings were *in vivo* incubated with 0.3 µM of L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PC or L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PE dissolved in ethanol and added directly to seawater. The major phospholipids of octopus and cuttlefish hatchlings showed characteristic FA profiles with PC presenting high contents of 16:0 and 22:6n-3 (DHA); PS having high 18:0, DHA and 20:5n-3 (EPA); PI a high content of saturated FA; and PE showing high contents of DHA and EPA. Interestingly, the highest content of 20:4n-6 (ARA) was found in PE rather than PI. Radiolabelled ARA bound at the sn-2 positions of PC or PE was recovered in several lipid classes of cephalopods tissues. The later showing that hatchlings of both species possess the enzymatic activities necessary for the de-acylation of dietary phospholipids and re-acylation of free fatty acids (FFA) into lyso-phospholipids to produce new phospholipids. However, irrespective of the phospholipid class in which [1-^{14}C]ARA was initially bound (either PC or PE), the esterification pattern of labelled ARA in *O. vulgaris* lipids was highly specific and similar to that found in their tissues with high esterification of this FA into PE. In contrast, when [1-^{14}C]ARA was provided to *S. officinalis* hatchlings bound in PC, up to 73.3 ± 5.7 % of the incorporated ARA was recovered in PC; and, when [1-^{14}C]ARA was provided bound in PE, 46.3 ± 3.3 % of this FA was recovered in PE. These results show a contrasting capability of the two species to remodel dietary phospholipids, which may suggest a difference in phospholipase activities. Further studies are necessary in order to determined specific phospholipase activities in *O. vulgaris* and *S. officinalis*.

Keywords: Hatchlings; Metabolism; *Octopus vulgaris*; Phospholipids; *Sepia officinalis*.

5.2 Introduction

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

Cephalopods have a high protein level and low lipid content (Lee, 1994) but, despite that, lipids were defined as crucial in cephalopod nutrition, with long-chain polyunsaturated fatty acids (LC-PUFA), cholesterol and phospholipids being suggested to play critical roles in their development (Almansa et al., 2006; Navarro and Villanueva, 2000, 2003). Within LC-PUFA, 22:6n-3 (DHA), 20:5n-3 (EPA) and also 20:4n-6 (ARA) were recently defined as essential fatty acids (EFA; Monroig et al., 2012a, 2016; Chapters 3 and 4; Reis et al., 2014, 2016). The latest studies have highlighted the importance of an adequate dietary input of those EFAs, considering not only their amount and ratios (Chapters 3 and 4; Reis et al., 2014, 2016) but also their lipid form (Guinot et al., 2013a), as this could affect the availability of the EFA for specific functions and tissue structures. According to Sargent et al. (1999) and Olsen et al. (2014) marine fish larvae show limited capacity to metabolise FA from triacylglycerols (TAG). If an analogy is considered for cephalopod hatchling and paralarval stages, then it would be likely that an association of EFA with TAG of live preys and/or feeds could negatively influence the EFA availability for normal development of cephalopods. In fact, Seixas et al. (2010a) showed that there was no relationship between DHA content in *Artemia* sp. juveniles and *O. vulgaris* paralarvae performance, which could be partially explained by the association of this FA with *Artemia* sp. TAG (Guinot et al., 2013b; Navarro et al., 1999).

On the other hand, dietary phospholipids are considered a better vehicle to provide EFA to fish larvae, not only due to the higher content of those FA found in polar lipids compared to neutral lipids, but also as they tend to improve lipid digestibility (see Sargent et al., 1999 and Tocher et al., 2008). It is assumed that fish phospholipid digestion is similar to that of mammals. Phospholipids are digested by intestinal phospholipase A_2 to produce lyso-phospholipids and free fatty acids (FFA; Sargent et al., 1989) that are then taken up by enterocytes, where re-acylation of FFA with lyso-phospholipids to form phospholipids occurs (Tocher et al., 2008). Although phospholipid digestion has not yet been determined in cephalopods, recent *in vivo* studies on fatty acid metabolism of

O. vulgaris (Chapter 3; Reis et al., 2014) and *S. officinalis* hatchlings (Chapter 4; Reis et al., 2016) revealed a high specificity in the esterification of LC-PUFA into polar lipids (PL), with DHA being preferentially esterified into phosphatidylcholine (PC), and ARA and EPA into phosphatidylethanolamine (PE). This, in addition to the potential process for phospholipid synthesis mentioned above, suggested that the FA composition and lipid class molecular species of live prey and/or feeds might be very important to determine the final composition and configuration of new tissue phospholipids and, therefore, for proper development of hatchlings.

In this respect, to ensure a high availability of EFA, dietary FA contents and lipid class molecular species should reflect larval requirements, which may be extrapolated from egg yolk lipid profile (Sargent et al., 1999) or the hatchlings PL composition (Olsen et al., 2014; Sargent et al., 2009). Eggs of *O. vulgaris* and *S. officinalis* show low lipid contents with high proportions of PL with PC being the main lipid class (Quintana et al., 2015; Sykes et al., 2009). Interestingly, no lipid consumption was recorded during embryonic development (Navarro and Villanueva, 2003; Quintana et al., 2015; Sykes et al., 2009), other than under stressful conditions, such as starvation (Sykes, 2007). Similarly to eggs, *O. vulgaris* and *S. officinalis* hatchlings generally have higher PL than neutral lipids (NL) content (Bouchaud and Galois, 1990; Navarro and Villanueva, 2000), with the former presenting a high proportion of polyunsaturated fatty acids (PUFA) and the latter a high percentage of monounsaturated fatty acids (Viciano et al., 2011; Sinanoglou and Miniadis-Meimaroglou, 1998). However, the specific FA profiles of the major phospholipid classes have not been determined for the hatchlings of these two species.

Therefore, in order to improve live prey and inert feed lipid profiles to enhance *O. vulgaris* and *S. officinalis* culture, the aims of the present study were to determine the FA profiles of the major phospholipid classes, and to evaluate if the molecular species in which EFA are supplied to cephalopod hatchlings influence their availability. To this end, the capability of these species for de-acylation and re-acylation of ARA (as a commercial available EFA model), initially bound to PC or PE, was investigated.

5.3 Material and Methods

5.3.1 Experimental animals

An *O. vulgaris* broodstock (30 individuals) was caught by professional artisanal fishermen on the Tenerife coast (Canary Islands, Spain) and maintained at the Instituto

Español de Oceanografia (IEO) de Canarias (Santa Cruz de Tenerife - 28º29'59.1''N; 16º11'44.54''W). The broodstock rearing conditions were similar to those described by Reis et al. (Chapter 3; 2014). The presence of eggs was checked once a week and when egg masses were observed, the ovate female was isolated in the tank by removing the other individuals. After approximately one month of embryonic development the eggs began to hatch and hatchlings were removed daily from the female rearing tank, to provide newly hatched octopus (less than 24 h old) for experiments.

All cuttlefish used in the present study were from a single brood obtained from F4 cultured females reproducing at the Ramalhete Aquaculture Station (Ria Formosa, South of Portugal - 37º00'22.39''N; 7º58'02.69''W). Twenty-four hour laid eggs were transported to IEO facilities in Tenerife and maintained in a 100 L circular fiberglass tank in a flow-through seawater system. The embryonic development of eggs and the broodstock rearing followed procedures described by Sykes et al. (2014). Eggs were maintained under rearing conditions similar to those described in Reis et al. (Chapter 4; 2016).

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

A total of 550 *O. vulgaris* and 11 *S. officinalis* hatchlings were incubated following an adaptation of the method of Reis et al. (Chapter 2; 2014, 2016) to determine the *in vivo* [1-^{14}C]FA metabolism in these species. Incubations (n = 4) were performed in flat-bottom 6-well tissue culture plates (SARSTEDT AG & CO., Nümbrecht, Germany), at a density of 50 or 1 hatchling/well for *O. vulgaris* and *S. officinalis*, respectively, in 10 ml of filtered seawater (36 ‰) with gentle stirring at 21 ºC for 5 h, supplemented with 0.2 μCi (0.3 μM) of L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PC or L-∝-1-palmitoyl-2-[1-^{14}C]arachidonyl-PE dissolved in ethanol. Labelled lipid classes were individually added to separate wells. Control treatments (n = 3) of hatchlings without addition of labelled phospholipid were also included. A 100 % survival rate was registered in all incubations.

After incubation, hatchlings were immediately euthanized in iced seawater (3 ºC) and thoroughly washed with filtered seawater to remove excess [1-^{14}C] phospholipid. Samples were stored at -80 ºC until analysis. Extraction of total lipids was performed according to the Folch method as described by Christie (2003) and lipid content was determined gravimetrically. The total lipid (TL) extracts were stored at -20 ºC in

chloroform/methanol (2:1, v/v) with 0.01 % butylated hydroxytoluene (BHT) as antioxidant, at a concentration of 10 mg/mL and under nitrogen until analysis.

5.3.3 Lipid composition of control samples

Aliquots of 20 µg of TL extracts of hatchling control groups were used to determine lipid class composition (n = 3). Lipid classes (LC) were separated by double development high-performance thin-layer chromatography (HPTLC), and quantified by charring followed by calibrated densitometry using a dual-wavelength flying spot scanner CS-9001PC (Shimadzu Co., Kyoto, Japan; Tocher and Harvie, 1988). Identification of individual LC was performed by running known standards (cod roe lipid extract and a mixture of single standards from BIOSIGMA S.r.l., Venice, Italy) on the same plates.

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 identity were confirmed by GC-MS chromatography (DSQ II, Thermo Fisher Scientific Inc., Waltham, Massachusetts, U.S.A.).

5.3.4 FA composition of phospholipids

Approximately 300 mg of *S. officinalis* and 500 mg of *O. vulgaris* hatchling samples, previously stored at -80 ºC, were used for lipid extraction (n = 3). Extraction of total lipid (TL) was performed as described above and polar lipid classes were separated by single-dimensional development on thin-layer chromatography (TLC) silica plates. The lipid classes were visualized under UV light after brief exposure to dichlorofluorescein. Each phospholipid class band was scraped from the TLC plates and subjected to direct acid-catalysed transmethylation on silica to obtain fatty acid methyl esters (FAME). FAME were purified by TLC (Christie, 2003) and then separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (Sigma-Aldrich Co., St. Louis, Missouri, USA). Identification of individual FAME was confirmed by GC-MS chromatography (DSQ II, Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA).

5.3.5 Incorporation of radioactivity into total lipids

In order to determine 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, USA). Results in dpm were converted into pmoles per mg of protein per h of incubation (pmol mg protein^{-1} h^{-1}) taking into account the specific activity of each substrate, and the hatchlings lipid and protein contents. Protein content of hatchlings (n = 4) was determined according to Lowry et al. (1951).

5.3.6 Remodelling of radiolabelled FA into lipid classes

An aliquot of 0.1 mg of the TL was taken for measurement of total radioactivity present in hatchlings from each incubation. Lipid classes were separated from other TL aliquots by single-dimensional double-development HPTLC as previously described by Tocher and Harvie (1988). Developed HPTLC plates were placed for 2 weeks in closed exposure cassettes (Exposure Cassete-K, BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Imagen Screen-K, Biorad, Madrid, Spain). The screens were then scanned with an image acquisition system (Molecular Imager FX, BioRad), and bands quantified by an image analysis software (Quantity One, BioRad). Identification of labelled bands was confirmed by radiolabelled standards simultaneously run on the same plate (Rodríguez et al., 2002).

5.3.7 Materials

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

5.3.8 Statistical analysis

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

5.4 Results

5.4.1 Hatchling lipid compositions

The TL contents and LC compositions of *O. vulgaris* and *S. officinalis* hatchlings are presented in Table 5.1. Both species presented a similar profile with no statistical differences detected ($p > 0.05$). Within polar lipids, PC (21.6 ± 1.2 % in octopus and 22.6 ± 2.7 % in cuttlefish) and PE (23.4 ± 1.1 % in octopus and 22.5 ± 1.7 % in cuttlefish) were the major classes, whereas cholesterol (31.6 ± 2.5 % in octopus and 33.1 ± 1.8 % in cuttlefish) was the major neutral lipid class and the most abundant lipid class.

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

Table 5.1 – Total lipid content (µg lipid mg protein^{-1}) and lipid class composition (%) of *Octopus vulgaris* and *Sepia officinalis* hatchlings

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

Results represent means ± SD (n = 3).

5.4.2 FA compositions of phospholipids

The main FAs detected in PC of octopus and cuttlefish hatchlings were 16:0 (33.4 ± 0.6 % of total FAs in octopus and 30.3 ± 0.3 % in cuttlefish) and DHA (29.5 ± 0.4 % in octopus and 21.5 ± 0.0 % in cuttlefish). In phosphatidylserine (PS), 18:0 followed by DHA and EPA were the main FAs in both species (Table 5.3). Compared to the other classes analysed, phosphatidylinositol (PI) presented a higher content of saturated FAs, representing over 60 % of total FAs (66.4 ± 0.1 % in octopus and 63.9 ± 12.4 % in cuttlefish), with only 5.0 ± 0.6 % and 2.9 ± 1.5 % of DHA being detected in PI of *O. vulgaris* and *S. officinalis* hatchlings, respectively. High contents of n-3 FA, LC-PUFA and 16:0 DMA and 18:0 DMA were detected in PE of both species (Table 5.3). While in octopus hatchlings ARA was mainly found in PE followed by PS, in cuttlefish ARA showed similar percentages in both PE and PS. EPA was mainly found in PE in both species. High n-3/n-6 PUFA ratios were detected in PC of *O. vulgaris* hatchlings (12.3 ± 0.1) and in PC and PE of *S. officinalis* hatchlings (13.2 ± 1.0 and 12.4 ± 1.4, respectively), while the lower values were found in PI of both species (2.7 ± 0.9 in octopus and 1.9 ± 2.3 in cuttlefish). The EPA/ARA ratio in phospholipids was generally higher in *S. officinalis*, especially in PC (10.5 ± 0.8) and PE (9.5 ± 1.1). In *O. vulgaris*, the lowest EPA/ARA ratios were detected in PS (3.1 ± 0.2) and PE (3.3 ± 0.2). With the exception

of octopus PC, where DHA/EPA ratio was clearly higher (3.7 ± 0.0), this ratio in the different classes of both species ranged between 0.6 and 1.8 (Table 5.3).

Table 5.2 – Main fatty acid composition (% of total FA) of *Octopus vulgaris* and *Sepia officinalis* hatchlings total lipids.

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

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

Table 5.3- Fatty acid composition (% total FA) of PC, PS, PI and PE of *O. vulgaris* and *S. officinalis* hatchlings

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

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

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

The incorporation of the radiolabelled ARA, originally esterified into PC or PE, was higher in *O. vulgaris* hatchlings ($p < 0.05$). The esterification pattern of [1-^{14}C]ARA supplied as PC or PE into the different lipid classes of *O. vulgaris* hatchlings was generally similar, with differences only being detected for free fatty acids (FFA), partial acylglycerols and PC ($p < 0.05$; Table 5.4). Despite being provided in two different forms (esterified into PC or PE), [1-^{14}C]ARA in octopus was preferentially re-esterified into PE (54.7 ± 3.2 % when added as PC and 56.2 ± 2.7 % when added as PE). On the other hand, in *S. officinalis* hatchlings, when [1-^{14}C]ARA was provided esterified into PC or PE a higher amount of the radioactivity incorporated was recovered re-esterified in the same class of origin: 73.3 ± 5.7 % of [1-^{14}C]ARA was recovered esterified into PC when added as PC, and 46.3 ± 3.3 % was recovered as PE when added as PE.These results show different esterification patterns of [1-^{14}C]ARA when incubated as PC orPE into LC of both species ($p < 0.05$).

Table 5.4 – Incorporation of [1-^{14}C]ARA into total lipid (pmoles mg pp^{-1} h^{-1}) and its re-esterification pattern (%) into lipid classes of *Octopus vulgaris* and *Sepia officinalis* hatchlings when provided bounded to PC or PE

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

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

5.5 Discussion

O. vulgaris and *S. officinalis* presented similar lipid classes profiles, with PC and PE being the major phospholipids. In contrast, the FA profile of both species presented

several differences, with the major one being a higher ARA content in octopus, which influenced similar differences in total n-6 FA content and EPA/ARA ratio. It was previously shown, that different to *S. officinalis* (Chapter 4; Reis et al., 2016), *O. vulgaris* hatchlings show a preferential incorporation of ARA (Chapter 3; Reis et al., 2014), which could reflect different requirements for this FA between species. ARA appears to have an important physiological role in *O. vulgaris* development (Estefanell et al., 2011; García-Garrido et al., 2010; Milliou et al., 2006; Monroig et al., 2012a, 2012b), with a high content of this FA being found in *O. vulgaris* brain, corresponding to 15.2 % of brain total FA composition (the third most significant FA), while EPA accounted for 11.8 % and DHA for 18.7 % (Monroig et al., 2012a). In contrast, n-6 PUFA accounted for less than 1 % in the central nervous system of *S. officinalis* (Dumont et al., 1992), indicating that these species possibly have different requirements for ARA. Like EPA, ARA is a precursor of eicosanoids, which are hormone-like compounds that are known to regulate many physiological processes, including immune and inflammatory responses, cardiovascular tone, renal and neural function including that related to camouflage behaviour and reproduction (Sargent et al., 2002). While EPA produces eicosanoids of lower biological activity, ARA is the preferred substrate and produces eicosanoids of higher biological activity in fish (Bell et al., 1994). In addition, increased dietary ARA appears to have a positive effect on development of fish larvae (Atalah et al., 2011b). Nonetheless, dietary ARA levels must be controlled, as it can influence EPA incorporation (Atalah et al., 2011a, 2011b; Chapter 3; Reis et al., 2014, 2015; Villalta et al., 2005) and bioconversion (Furuita et al., 2003; Sargent et al., 2002). Furthermore, competition between these PUFA for eicosanoid production can also influence fish development (Sargent et al., 2002). In a similar way, it is likely essential to provide a specific dietary EPA/ARA ratio for cephalopod development (Chapters 3 and 4; Reis et al., 2014, 2015, 2016).

The present results also showed characteristic FA profiles for individual phospholipids of octopus and cuttlefish that were generally similar in both species. According to Tocher et al. (2008), this pattern is related to the roles of specific phospholipid classes in membrane structure and function. Fish phospholipid FA profiles are characterised by a high proportion of 16:0 and a relatively lower LC-PUFA content in PC; an intermediate level of saturated FA and monounsaturated FA and high levels of C20 and C22 PUFA in PE; PS is characterised by high 18:0 and C22 LC- PUFA; and PI also present a high 18:0 and relatively lower LC-PUFA but with a particularly high content of ARA (Tocher, 1995).

In the present study, all phospholipid classes showed a generally high LC-PUFA content, except for PI that showed a higher content of saturated FA in both octopus and cuttlefish hatchlings. In the latter, these FA represented over 60 % of the total FA content with 16:0 being predominant. Several authors previously reported selective location/retention of ARA in PI in fish tissues (Bell and Dick, 1990; Bell and Tocher, 1989; Bell et al., 1997; Sargent et al., 2002; Tocher et al., 1995). Interestingly, *O. vulgaris* showed a higher content of ARA in PE, while *S. officinalis* hatchlings had a similar percentage of this FA in PE and PS. Nonetheless, considering the high level of PE in *S. officinalis* lipid, the absolute amount of ARA in PE would be much higher than in PS. The preferential esterification of ARA into cephalopod PE has already been observed in studies of *in vivo* FA metabolism (Chapters 3 and 4; Reis et al., 2014, 2016). This may indicate an important role of ARA in PE in these species, which could be associated with the above-mentioned higher content of ARA in octopus brain. However, further studies are necessary in order to elucidate the specific role of ARA in cephalopods, and more precisely in *O. vulgaris* development.

Similar to fish (Tocher, 1995), PE was the phospholipid with highest LC-PUFA content and a DHA/EPA ratio of around 1. Moreover, this LC had the highest EPA content, which was consistent with the preferential esterification of [1-^{14}C]EPA into octopus and cuttlefish PE as reported by Reis et al. (Chapters 3 and 4; 2014, 2016). Sargent et al. (2002) reported that the highest DHA levels in fish tissues were usually found in PE, similar to that found in the present study in cephalopod species. However, a similar high level of DHA was also found in PC (29.5 ± 0.4 % and 21.5 ± 0.0 % for *O. vulgaris* and *S. officinalis* hatchlings, respectively). These data are also in agreement with the results obtained from *in vivo* esterification studies on the two cephalopod species, where preferential esterification of [1-^{14}C]DHA into PC was detected (Chapters 3 and 4; Reis et al., 2014, 2016).

As previoulsy mentioned, he present results showed a higher content of EFA in PE. Considering the apparent low specificity of acylases and transacylases, enzymes responsible for FA esterification into phospholipids (Sargent et al., 1999), and the pattern of FA distribution among phospholipids detected in the present and previous *in vivo* FA metabolism studies (Chapters 3 and 4; Reis et al., 2014, 2016), the dietary ratio and molecular form of EFA might be crucial in cephalopods. It is assumed that de-acylation/re-acylation turnover processes have an important role in maintaining the characteristic FA esterification pattern among lipid classes (Tocher et al., 2003). This

turnover process is highly influenced by dietary FA profile and by the endogenous capability of the organism to complete this process (Olsen et al., 2014). By using a similar method, following the incorporation of specific [1-^{14}C]FA, to that employed by Reis et al. (Chapters 3 and 4; 2014, 2016) to determine the *in vivo* FA metabolism of *O. vulgaris* and *S. officinalis* hatchlings, it was possible to investigate the *in vivo* capability of these species for phospholipid turnover and remodelling. It is assumed that phospholipid digestion in fish occurs by the action of phospholipase A_2 (PLA_2) over phosphlipids which results in the production of 1-acyl lyso-phospholipids and FFA that are later re-esterified (Tocher et al., 2008). In the present study, ARA bound at the sn-2 position of PC or PE was recovered, not just esterified into these specific phospholipids, but also into other LC. This shows that both *O. vulgaris* and *S. officinalis* hatchlings may possess the enzymatic activities necessary for the de-acylation of dietary phospholipids and re-acylation of FFA into lyso-phospholipids to produce new molecular species of phospholipids.

Interestingly, regardless of their molecular form, higher incorporation of radioactivity was observed into lipids in *O. vulgaris* hatchlings. As the label was located in the first (carboxyl) carbon of the ARA molecule, these results might be a consequence of the preferential incorporation of ARA into octopus total lipid, as reported by Reis et al. (Chapter 3; 2014). Despite the lipid class to which [1-^{14}C]ARA was bound (PC or PE), the esterification pattern obtained after re-acylation in *O. vulgaris* lipids was similar to that reported by Reis et al. (Chapter 3; 2014), who detected higher esterification of [1-^{14}C]ARA into PE. In contrast, in *S. officinalis* hatchlings, ARA was mainly recovered in the same LC that was provided, that is when [1-^{14}C]ARA was provided bound to PC, up to 73.3 ± 5.7 % of the incorporated ARA was recovered as PC, and when [1-^{14}C]ARA was provided bound to PE 46.3 ± 3.3 % of this FA was recovered as PE.

Although further studies are necessary in order to determined specific phospholipase activities, the present results suggest a different mechanism in *O. vulgaris* and *S. officinalis* biosynthesis of new phospholipids. It is generally believed that PLA_2 is the digestive enzyme lysing FA from the sn-2 position of phospholipids (see Olsen et al., 2014) and that LC-PUFA are esterified at the sn-2 position of phospholipid molecules (Sargent et al., 1999). In *O. vulgaris* hatchlings, after the ARA re-acylation process the esterification pattern obtained was highly similar, with the majority of ARA being recovered as PE, suggesting the existance of a high PLA_2 activity. Therefore, the action of PLA_2 would cleave the LC-PUFA esterified at sn-2 acyl chain of dietary lipids, and so

these FAs would be part of the pool of FFA available for later re-esterification. Similar to phospholipids, it is assumed that LC-PUFA are mainly esterified into the sn-2 position of TAG in fish (Sargent et al., 1999). Nonetheless, TAG digestive enzymes (1-3 lipases) normally remove the FAs from sn-1 and sn-3 position, with LC-PUFA being retained in monoacylglycerols (Olsen et al., 2014; Tocher et al., 2003). Therefore, after dietary lipid de-acylation, the FFA pool would contain LC-PUFA from phospholipids, but also saturated, monounsaturated and polyunsaturated FAs from TAG digestion. *Artemia* sp., used as prey in *O. vulgaris* paralarvae rearing (Navarro et al., 2014), normally have high levels of TAG, 18:1n-9, 18:2n-6 and 18:3n-3, and lower levels of phospholipids and LC-PUFA (Fuentes et al., 2011; Reis et al., 2015; Seixas et al., 2010a, 2010b; Viciano et al., 2011). Consequently, the dietary FFA pool when *Artemia* sp. is used, would contain high C18 FAs and low LC-PUFA. In this sense, the probability for re-acylation of C18 FAs into phospholipids would be higher than that for DHA or other LC-PUFA. This could be a possible explanation for the lower levels of DHA found in *O. vulgaris* paralarvae reared with enriched *Artemia* sp.

Interestingly, the *S. officinalis* turnover data indicate an apparent lower PLA_2 enzyme activity when compared to *O. vulgaris* hatchlings. In fish, PLA_2 activity tends to increase during development, with seabream and sea bass larvae showing no PLA_2 activity during very early larval stages (Izquierdo and Henderson, 1998; Zambonino Infante and Cahu, 2001). Both octopus and cuttlefish possess inner yolk reserves that could last for a few days after hatching (Boletzky and Villanueva, 2014; Sykes et al., 2004) and it is known that it can take up to 30 days for the digestive system to fully mature in cuttlefish (Boucaud-Camou and Yim 1980; Yim and Boucaud-Camou 1980; Boucaud-Camou 1982). Nonetheless, these species may feed on size-appropriate prey from day 1 (Hanlon and Messenger, 1988), when mixed feeding overlaps inner yolk reserves and external food consumption (Boletzky, 1974; Sykes et al., 2013). It has been suggested that diet may influence digestive enzymes activity (Koven et al., 2001; Perrin et al., 2004; Villanueva et al., 2002), and it is known that PLA_2 activity can be stimulated by increased dietary phospholipids in fish (Zambonino Infante and Cahu, 2001). In the present study, cuttlefish individuals were unfed, which could explain the low turnover rate recorded. On the other hand, octopus hatchlings under similar feeding conditions displayed a high turnover rate. The differences regarding both species phospholipid metabolism detected here might be related with the different lifestyles of these species during their first live

stages (Halon and Messenger, 1996; Young and Harman, 1988) and/or different eggs origins (see Bouchaud and Galois et al., 1990).

Since ARA was mainly recovered in the same LC that was provided, there is also the possibility that phospholipid de-acylation in cuttlefish hatchlings might be at least partially performed by alternative enzymes like, phospholipase A_1 (PLA_1) and 1-3 lipase digestive enzymes that cleavage the sn-1 acyl chain of dietary lipids. Further studies are required to determine possible activities and roles of these enzymes in phospholipid digestion of *S. officinalis*. Nonetheless, the action of these enzymes towards the labelled phospholipids would preserve the radiolabelled ARA in sn-2, and so re-acylated phospholipid would still contain the label, as observed in the present study. Due to the preservation of LC-PUFA into phospholipids skeleton, the interference of dietary TAG content and de-acylation in the re-acylation process in *S. officinalis* might be less critical than that suggested to *O. vulgaris*.

A small proportion of the incorporated [1-^{14}C]ARA was also recovered esterified into monoacylglycerols and diacylglycerols, that are involved in *de novo* synthesis of phospholipids and TAG (see Olsen et al., 2014 and Tocher et al., 2008). Interestingly, no [1-^{14}C]ARA was found re-esterified into TAG, the main class for lipid storage and energy provision in fish (Tocher et al., 2003). Nonetheless, *O. vulgaris* and *S. officinalis* hatchlings normally show only low TAG levels representing 1.8 ± 0.5 % of total lipid in *O. vulgaris* and 3.3 ± 2.6 % in *S. officinalis*. Low TAG in these species was also previously recorded not just in hatchlings (Chapters 3 and 4; Navarro and Villanueva, 2000; Reis et al., 2014, 2015, 2016), but also in eggs (Boucaud and Galois, 1990; Sykes et al., 2009; Quintana et al., 2015), juveniles and adult tissues (Almansa et al., 2006; Valverde et al., 2012).

The present study showed that, despite the general similarity between *O. vulgaris* and *S. officinalis* lipid compositions, these species may have different ARA requirements. Both octopus and cuttlefish hatchlings showed characteristic FA profiles for the major phospholipids, with PC presenting a high content of 16:0 and DHA; PS of 18:0, DHA and EPA; PI a high content of saturated FA; and PE a high content of DHA and EPA. The highest content of ARA was found in PE rather than PI. Although further studies are necessary in order to determined specific phospholipase activities, the present results suggest a different capacity of both species for phospholipids remodelling. While dietary phospholipid/TAG ratio is likely being of great importance in *O. vulgaris* nutrition, in

S. officinalis this TAG interference might be less critical due to the apparent preservation of LC-PUFA association with the phospholipid skeleton.

Chapter 6.

Comparative study on fatty acid metabolism of two crustacean species: *Artemia* sp. metanauplii and *Grapsus adscensionis* zoeae, as potential live prey for *Octopus vulgaris* paralarvae

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6.1 Abstract

In order to tailor a suitable diet for rearing *Octopus vulgaris* paralarvae, the *in vivo* metabolism of unsaturated fatty acids (FA) of two potential live preys, *Artemia* sp. metanauplii and *Grapsus adscencionis* zoeae was determined. Incubations were performed over 5 h in 6-well flat-bottom tissue culture plates at a density of 10 000 metanauplii or 1 000 zoeae per well with gentle stirring at 24 ºC and 21 ºC, respectively. The zooplankton were supplemented with 0.3 µM of [1-^{14}C]FA including, 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6 (ARA), 20:5n-3 (EPA) and 22:6n-3 (DHA), which were added individually to separate wells as their potassium salts bound to bovine serum albumin. Control treatments of metanauplii and zoeae without the addition of any [1-^{14}C]FA were also assessed. Compared to metanauplii, zoeae contained twice the content of polar lipids (PL) and eight fold the content of long-chain polyunsaturated fatty acids (LC-PUFA). Most [1-^{14}C]FA presented a similar esterification pattern into zoeae lipid classes to that found in *O. vulgaris* hatchlings, in addition to a higher elongation activity over LC-PUFA. On the other hand, an increased catabolism over LC-PUFA and, more importantly, over DHA was detected in *Artemia* sp. metanauplii metabolism, confirming the difficulties to obtain proper enrichment of metanauplii with DHA. Overall, considering the lipid composition and metabolism of both prey, zoeae seems to be more suitable than *Artemia* sp. metanauplii for *O. vulgaris* paralarvae rearing. Nonetheless, since the provision of decapod crustacean zoeae for large scale culture of octopus paralarvae is logistically difficult and economically unviable, the rearing of *O. vulgaris* paralarvae currently depends on the use of live *Artemia* sp. as prey. In this sense, a feeding scheme utilizing a co-feeding with low value decapod crustacean zoeae (rich in PL and EFA, mainly DHA), plus *Artemia* sp. enriched with ARA and EPA might be an alternative to the enrichment of *Artemia* sp. with DHA or to the use of an expensive live prey with an EPA/ARA ratio of 1, such as that present in *Grapsus* zoeae which might not be suitable for extended rearing periods of paralarvae.

Keywords: *Artemia* sp. metanauplii; *Grapsus adscensionis* zoeae; Lipid metabolism; *Octopus vulgaris* paralarvae; Unsaturated fatty acids.

6.2 Introduction

The common octopus (*Octopus vulgaris*) has some biological features, including short life cycle, rapid growth and high feed conversion ratio, (Vaz-Pires et al., 2004) that makes it a promising candidate for large-scale culture. Nonetheless, the rearing of its planktonic stage remains the main bottleneck in the culture of this species, mainly due to high mortality rates verified during this life stage that have been related to potential nutritional imbalances (Navarro et al., 2014).

Artemia sp. is a live prey widely used in marine larvae rearing, as a result of its high availability, acceptance by a large number of species, and since no appropriate artificial feed formulations are yet available for most species (Sorgeloos et al., 2001). Currently, the rearing of *O. vulgaris* paralarvae depends on the use of *Artemia* sp. (Iglesias et al., 2007). However, its use has resulted in low growth and survival rates (Fuentes et al., 2011; Reis et al., 2015; Viciano et al., 2011). These have been associated with a nutritional imbalance in the lipid profile of *Artemia* sp. (Navarro and Villanueva, 2003, 2000; Reis et al., 2015; Seixas et al., 2010a, 2010b). It is known that *Artemia* sp. present a low content of long-chain polyunsaturated fatty acids (LC-PUFA), such as 20:5n-3 (EPA), and especially 22:6n-3 (DHA) (Sorgeloos et al., 2001). These fatty acids, in addition to 20:4n-6 (ARA), were defined as essential fatty acids (EFA) for *O. vulgaris* paralarvae development (Monroig et al., 2012a; Chapter 3; Reis et al., 2014). Furthermore, *Artemia* sp. also presents a low content of phospholipids, which are also important in paralarvae development (Navarro and Villanueva, 2000, 2003).

In order to tailor *Artemia* sp. lipid composition towards octopus paralarvae nutritional needs, numerous types of enrichment using lipid emulsions (Guinot et al., 2013a; Navarro and Villanueva, 2003; Seixas et al., 2010a, 2010b; Viciano et al., 2011; Villanueva et al., 2002), microalgae (Carrasco et al., 2006; Fuentes et al., 2011; Iglesias et al., 2006; Seixas et al., 2008, 2010a, 2010b; Viciano et al., 2011) and/or microdiets (Fuentes et al., 2011) have been tested. Nonetheless, and despite many efforts, all of these works share common results of poor growth and survival rates. In order to overcome this nutritional problem and improve paralarval rearing results, *Artemia* sp. have been used in co-feeding with inert diets (Navarro and Villanueva, 2003; Seixas et al., 2010b; Villanueva et al., 2002) or other live prey, mainly decapod crustacean zoeae (Carrasco et al., 2006; Iglesias et al., 2014; Moxica et al., 2002; Reis et al., 2015; Villanueva, 1994, 1995) which were defined as the main natural prey of wild *O. vulgaris* paralarvae (Roura et al., 2012). While inert diets were ignored (Seixas et al., 2010b) or inefficiently digested and/or assimilated by

paralarvae (Navarro and Villanueva, 2003), decapod crustacean zoeae were highly accepted and growth rates were increased when compared to the use of *Artemia* sp. as sole prey (Iglesias et al., 2007; Reis et al., 2015).

Compared to *Artemia* sp., decapod crustacean zoeae had higher contents of phospholipids and EFA for octopus paralarvae (Iglesias et al., 2014; Navarro and Villanueva, 2000; Reis et al., 2015), which could at least partly explain the better results obtained. However, large-scale culture of zoeae is difficult logistically and is not economically viable (Iglesias et al., 2007). For that reason, economical alternative protocols to improve the nutritional value of *Artemia* sp. metanauplii are required (Guinot et al., 2013a). One way to improve the use of *Artemia* sp. metanauplii is not to consider this organism as a passive carrier of fatty acids (FA; Navarro et al., 1999) but to ponder that not only the EFA content but also their presence in specific lipid classes, such as the polar lipids and the total or individual LC-PUFA and ratios are essential factors for marine larvae performance (Olsen et al., 2014; Sargent et al., 1999). In this respect, it is necessary to determine the fate of dietary FA and discern the lipid pathways of *O. vulgaris* paralarvae potential prey. Therefore, it makes the whole sense to compare the most used prey *Artemia* sp. with other that has recently promoted higher growth and survival rates, e.g. *Grapsus adscensionis* zoeae (Reis et al., 2015).

In this sense, the aim of the present study was to compare the *in vivo* capability of *Artemia* sp. metanauplii and *G. adscensionis* zoeae to incorporate, esterify into different lipid classes, and metabolise unsaturated FAs.

6.3 Material and Methods

6.3.1 Experimental animals

Artemia sp. metanauplii were obtained by hatching EG *Artemia* Cysts (INVE Aquaculture, Belgium). Following the protocol of Sorgeloos et al. (2001), 2 g of *Artemia* sp. cysts were decapsulated with bleach, followed by deactivation with $Na_2S_2O_3$ dissolved in filtered seawater (0.02% w/v). Incubation of cysts was performed over 24 h in a 3 L cylinder-conical fiberglass tank containing filtered seawater (36‰) at 28 ºC, with continuous light and vigorous aeration. After hatching, nauplii were separed by hatching wastes and place in similar tanks with newly fresh filtrated sewater at 24 ºC for a 8 h period, until instar II stage (metanauplii stage - mouth and anus opening) was reached. Prior to incubation with radiolabelled FA substrates, metanauplii were filtered and concentrated in 400 mL of filtered seawater and metanauplii density was determined.

G. adscensionis broodstock (40 individuals) was caught off the NE and N coast of Tenerife (Canary Islands, Spain) and reared in 3 000 L cylinder-conical fibreglass tanks in a flow-through system, under natural photoperiod (13L:11D) with a natural water temperature of 21.0 ± 0.7 °C and salinity of 36.8 ± 0.1 ‰. The tank water column was ≈10 cm in height and the water flow was 6 L min^{-1}. Crabs were fed daily *ad libitum* on a diet consisting of 50 % (w/w) of frozen mackerel (*Scomber scombrus*) and squid (*Loligo opalescens*). Newly hatched crab zoeae were collected with a 500 µm mesh placed at the end of the flow-water system. Prior to incubation with radiolabelled fatty acids substrates, zoeae were thoroughly sorted, from possible algae and other organisms found in the broodstock rearing tank and placed into filtered seawater.

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

Artemia sp. metanauplii and *G. adscensionis* zoeae were incubated in 6-well flat-bottom tissue culture plates (Sarstedt AG & Co., Nümbrecht, Germany) with 10 mL of filtered seawater. The incubation protocol was adapted from Reis et al. (Chapter 2; 2014). Incubations were performed for 5 h at a density of 10 000 metanauplii or 1 000 zoeae per incubation well with gentle stirring at 24 ºC and 21 ºC, respectively, with 0.2 µCi (0.3 µM) of [1-^{14}C]FA including 18:1n-9, 18:2n-6, 18:3n-3, ARA, EPA or DHA (n = 4). The [1-^{14}C]FA were added individually to separate wells as their potassium salts bound to bovine serum albumin (BSA), as described in Ghioni et al. (1997). Control treatments of metanauplii and zoeae, without addition of [1-^{14}C]FA, were also assessed. A survival rate of 92 ± 4 % was obtained in all incubations.

After incubation, *Artemia* sp. metanauplii and *Grapsus* zoeae were filtered with a 100 µm mesh and washed thoroughly with filtered seawater to remove excess radiolabelled 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 until analysis at a concentration of 10 mg mL^{-1} in chloroform/methanol (2:1, v/v) with 0.01 % butylated hydroxytoluene (BHT) as antioxidant at -20 ºC under an inert atmosphere of nitrogen.

6.3.3 Lipid class and fatty acid composition of *Artemia* sp. metanauplii and *G. adscensionis* zoeae

Aliquots of 20 µg of TL extract of metanauplii and zoeae control groups were used to determine lipid classes (LC) compositions (n = 4). LC were separated by single-

dimensional double-development high-performance thin-layer chromatography (HPTLC) and quantified by charring followed by calibrated densitometry using dual-wavelength flying spot scanner CS-90001PC (Shimadzu Co., Japan; Tocher and Harvie, 1988). LC identification was performed by running known LC standards (cod roe lipid extract) on the same plates.

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

6.3.4 Incorporation of radiolabelled fatty acids into total lipids

An aliquot of 0.1 mg of *Artemia* sp. metanauplii and *G. adscensionis* zoeae TL extract was taken to determine total radioactivity incorporated. Extracts were transferred to scintillation vials and radioactivity determined on a LKB Wallac 1214 Rackbeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, USA). Results in dpm were converted into pmoles per mg protein per h of incubation (pmol mg pp^{-1} h^{-1}), considering specific activity of each substrate and metanauplii and zoeae total lipid and protein contents. Protein was determined according to Lowry et al. (1951) in metanauplii and zoeae (n = 4).

6.3.5 Esterification of radiolabelled fatty acids into lipid classes

An aliquot of 0.1 mg of TL extract from radioactive samples was also applied to HPTLC plates to determine the esterification of [1-^{14}C]FA into LC. Lipid classes were separated by single-dimensional double-development HPTLC, as previously described by Tocher and Harvie (1988). Esterification pattern of each [1-^{14}C]FA into LC was determined by image analysis following Reis et al. (Chapter 2; 2014).

6.3.6 Transformation of radiolabelled fatty acids

An aliquot of 0.9 to 1.1 mg of TL extract from radioactive samples was subjected to acid-catalysed transmethylation to obtain FAME as previously detailed (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 ºC 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). FAME identification was performed by image analysis following the method described in Reis et al. (Chapter 2; 2014).

6.3.7 Materials

[1-^{14}C]C18 FA were purchased from PerkinElmer, Inc. (Waltham, Massachusetts, USA). [1-^{14}C] LC-PUFA were purchased from American Radiolabelled Chemicals, Inc. (St. Louis, Missouri, USA). BSA was purchase from Sigma-Aldrich Co. (St. Louis, Missouri, USA). TLC plates (20 × 20 cm × 0.25 mm) were purchased form Macherey-Nagel GmbH & Co. KG (Düren, Germany). HPTLC plates, (10 × 10 cm × 0.15 mm) pre-coated with silica gel 60 (without fluorescent indicator), were purchased from Merck KGaA (Düsseldorf, Germany). OptiPhase "HiSafe" 2 scintillant liquid was purchased from PerkinElmer, Inc. (Waltham, Massachusetts, USA). Organic solvents used were of reagent grade and were purchased from Merck KGaA (Düsseldorf, Germany), Sigma-Aldrich Co. (St. Louis, Missouri, USA) and Panreac Química S.L.U. (Barcelona, Spain).

6.3.8 Statistical analysis

Results are presented as means ± SD (n = 4). For all statistical tests, $p < 0.05$ was considered statistically different. Data were checked for normal distribution with the one-sample Shapiro-Wilk test, as well as for homogeneity of 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-^{14}C]C18 FAs (18:1n-9, 18:2n-6, 18:3n-3) and [1-^{14}C] 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 Welch robust test, followed by a Games-Howell non-parametric multiple comparison test (Zar, 1999). Differences between LC and FA compositions of *Artemia* sp. metanauplii and *G. adscensionis* zoeae control groups and [1-^{14}C]FAs incorporation into TL and its transformation rate between both species, were tested using Student´s *t*-test (Zar, 1999). The statistical analysis was performed using the IBM SPSS statistics 22.0 (IBM Co., USA).

6.4 Results

6.4.1 Lipid composition of *Artemia* sp. metanauplii and *G. adscensionis* zoeae

Artemia sp. metanauplii TL was particularly rich in neutral lipids (NL), with triacylglycerols (TAG) being the main lipid component (51.9 ± 2.3 %), followed by cholesterol (14.4 ± 0.5 %; Table 6.1). *G. adscensionis* zoeae also presented a high proportion of NL, although lower than that of metanauplii ($p < 0.05$), and TAG and cholesterol were similarly the most abundant lipid classes. Within the polar lipid (PL) fraction, zoeae presented 17.9 ± 0.7 % of phosphatidylcholine (PC) and 12.0 ± 1.1 % of phosphatidylethanolamine (PE) whereas metanauplii contained 7.8 ± 1.1 % and 6.8 ± 0.8 % PC and PE, respectively (Table 6.1). Zoeae presented twice the PL amount than metanauplii ($p < 0.05$).

Table 6.1 – Total lipid content (µg lipid mg protein^{-1}) and lipid class composition (%) of *Artemia* sp. metanauplii and *G. adscensionis* zoeae

	Artemia sp.	*G. adscensionis*
TL content	624.6 ± 77.8	453.0 ± 150.4
Lipid classes		
Sphingomyelin	0.0 ± 0.0	0.5 ± 0.3*
Phosphatidylcholine	7.8 ± 1.1	17.9 ± 0.7*
Phosphatidylserine	1.2 ± 0.3	5.2 ± 0.1*
Phosphatidylinositol	1.4 ± 0.4	2.3 ± 0.2*
Phosphatidylglycerol	2.2 ± 0.2	3.5 ± 0.7*
Phosphatidylethanolamine	6.8 ± 0.8	12.0 ± 1.1*
∑ Polar lipids	19.4 ± 1.7	41.4 ± 2.1*
Diacylglycerols	1.5 ± 0.6	0.0 ± 0.0*
Cholesterol	14.4 ± 0.5	18.5 ± 1.9*
Free Fatty Acids	5.3 ± 2.3	2.6 ± 0.3
Triacylglycerols	51.9 ± 2.3	31.3 ± 1.0*
Sterol Esters	7.6 ± 0.9	6.2 ± 1.0
∑ Neutral lipids	80.6 ± 1.7	58.6 ± 2.1*

Results represent means ± SD (n = 4). LC data are presented in percentage of total lipid content.
* Represent significant differences between *Artemia* sp. and *G. adscensionis* ($p < 0.05$).

The FA compositions of *Artemia* sp. metanauplii and *G. adscensionis* zoeae were also substantially different (Table 6.2). *Artemia* sp. metanauplii were particularly rich in 18:3n-3, followed by 18:1n-9 and 16:0, while *G. adscensionis* zoeae were rich in 18:1n-9, 16:0, ARA, EPA and DHA.

Table 6.2 – Fatty acid composition (% total FA) of *Artemia* sp. metanauplii and *G. adscensionis* zoeae

	Artemia sp.	*G. adscensionis*
C16:0	13.5 ± 0.6	19.1 ± 0.2*
C18:0	7.6 ± 0.5	7.2 ± 0.2
∑ Saturated[a]	24.6 ± 0.8	31.3 ± 0.2*
C16:1n-9	0.8 ± 0.1	0.4 ± 0.0*
C16:1n-7	2.8 ± 0.0	4.1 ± 0.1*
C18:1n-9	20.5 ± 0.5	24.0 ± 0.4*
C18:1n-7	7.3 ± 0.3	5.5 ± 0.1*
C20:1n-9	0.6 ± 0.1	1.1 ± 0.1*
∑ Monoenes[a]	35.5 ± 0.5	38.3 ± 0.9*
C18:2n-6	5.9 ± 0.4	2.0 ± 0.1*
C20:2n-6	0.2 ± 0.0	1.5 ± 0.0*
C20:4n-6	0.3 ± 0.0	7.1 ± 0.2*
∑ n-6 FA[a]	6.8 ± 0.4	11.1 ± 0.2*
C18:3n-3	25.4 ± 0.5	0.3 ± 0.0*
C18:4n-3	3.9 ± 0.1	0.0 ± 0.0*
C20:3n-3	0.8 ± 0.0	0.3 ± 0.0*
C20:4n-3	0.6 ± 0.0	0.3 ± 0.0*
C20:5n-3	1.0 ± 0.1	7.4 ± 0.3*
C22:6n-3	0.1 ± 0.0	10.0 ± 0.3*
∑ n-3 FA[a]	32.8 ± 0.5	18.6 ± 0.6*
UK[b]	0.0 ± 0.0	0.8 ± 0.2*
∑ PUFA[c]	39.9 ± 0.3	29.7 ± 0.7*
∑ LC-PUFA [d]	3.0 ± 0.4	25.8 ± 0.7*
DHA/EPA[e]	0.1 ± 0.0	1.4 ± 0.0*
EPA/ARA[e]	2.9 ± 0.1	1.0 ± 0.0*
DHA/ARA	0.2 ± 0.0	1.4 ± 0.0*

Results represent means ± SD (n = 4). Data are presented in percentage of total fatty acids content. * Represent significant differences between groups ($p < 0.05$). [a] Totals include some minor components not shown. [b] UK – unknown. [c] PUFA – polyunsaturated fatty acids. [d] LC-PUFA – long-chain polyunsaturated fatty acids (≥ 20C and ≥ 3 double bands). [e] ARA – 20:4n-6; EPA – 20:5n-3; DHA – 22:6n-3.

The total PUFA content was higher in *Artemia* sp. metanauplii ($p < 0.05$), but LC-PUFA represented only 3.0 ± 0.4 % of total FA in metanauplii, while *G. adscensionis* zoeae contained 25.8 ± 0.7 % LC-PUFA, with up to seven-fold of ARA, EPA and DHA content (Table 6.2).

6.4.2 Incorporation of radiolabelled fatty acids into total lipids

Table 6.3 shows the incorporation of radiolabelled fatty acids into the total lipid of *Artemia* sp. metanauplii and *G. adscensionis* zoeae. Most notably, the incorporation of [1-^{14}C]DHA into metanauplii TL was approximately only half the incorporation of all other radiolabelled FA substrates. All [1-^{14}C]C18 FAs were incorporated into metanauplii TL at similar levels. In contrast, within LC-PUFA, [1-^{14}C]ARA showed highest incorporation, whereas DHA was the lowest incorporated ($p < 0.05$). Compared to *Artemia* sp. metanauplii, the incorporation of [1-^{14}C]FA into zoeae TL was generally lower, although only statistically different from metanauplii for 18:1n-9, 18:2n-6 and ARA (Table 6.3). [1-^{14}C]18:1n-9 and [1-^{14}C]DHA were the FA least incorporated into zoeae TL ($p < 0.05$).

Table 6.3 - Incorporation of radioactivity into total lipid (pmoles mg pp^{-1} h^{-1}) of *Artemia* sp. metanauplii and *G. adscensionis* zoeae

	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Artemia sp.	15.6 ± 2.8^{a}	18.3 ± 2.5^{a}	13.6 ± 3.9^{ab}	$19.4 \pm 2.6^{\Delta a}$	$13.4 \pm 2.7^{\circ ab}$	$6.8 \pm 0.8^{\square b}$
G. adscensionis	$3.8 \pm 1.8^{\bullet b*}$	$8.6 \pm 1.4^{\blacktriangle a*}$	$10.1 \pm 2.7^{\blacktriangle a}$	$10.1 \pm 0.9^{\Delta a*}$	$10.5 \pm 2.2^{\Delta a}$	$4.5 \pm 1.5^{\circ b}$

Results represent means ± SD (n = 4). Data are presented in pmoles of ^{14}C fatty acid incorporated/mg of protein per hour of incubation. Different letters in superscript within the same row represent significant differences between all fatty acids ($p < 0.05$). Different full symbols in superscript ($^{\blacktriangle\bullet\blacksquare}$) within the same row represent significant differences between C18 fatty acids ($p < 0.05$). Different hollow symbols in superscript ($^{\Delta\circ\square}$) within the same row represent significant differences between LC-PUFA ($p < 0.05$).* within the same column represent significant differences for an specific fatty acid, between *Artemia* sp. metanauplii and *G. adscensionis* zoeae ($p < 0.05$).

6.4.3 Esterification of radiolabelled fatty acids into lipid classes

The distribution of incorporated radioactivity into lipid classes of *Artemia* sp. metanauplii is presented in Table 6.4. All radiolabelled FA were extensively esterified by metanauplii, with less than 10 % of the incorporated radioactivity being recovered as free fatty acids (FFA). After 5 hours, the majority of the radiolabelled substrates were esterified into PL, mainly in PE and PC, except for [1-^{14}C]ARA that showed much higher esterification into PI (26.3 ± 2.6 % of radioactivity incorporated). Some differences were detected between [1-^{14}C]C18 FAs and [1-^{14}C]LC-PUFA substrates, with higher (almost two-fold) esterification of [1-^{14}C]C18 FAs into TAG, and of [1-^{14}C]LC-PUFA into PI and PS. The esterification patterns of [1-^{14}C]18:1n-9 and [1-^{14}C]18:2n-6 were similar (PE > TAG > PC > SE > FFA ≥ PAG > PI > PS). In contrast, the esterification pattern of [1-^{14}C]18:3n-3, was different to the other [1-^{14}C]C18 FAs, being predominantly esterified

into PE, PC and TAG. The esterification pattern of [1-^{14}C]LC-PUFA into metanauplii LC also varied between FA ($p < 0.05$; Table 6.4). [1-^{14}C]ARA was esterified into PI > PC > PE, [1-^{14}C]EPA into PC > PE > PI and [1-^{14}C]DHA into PC ≥ PE > TAG.

Table 6.4 – Esterification (%) of [1-^{14}C]FA substrates into *Artemia* sp. metanauplii lipid classes

	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Phosphatidylcholine	19.2 ± 0.9 $^{▲c}$	19.3 ± 1.2 $^{▲c}$	26.1 ± 0.8 $^{●b}$	24.1 ± 0.4 ^{b}	31.5 ± 3.6 ^{a}	24.7 ± 1.6 ^{b}
Phosphatidylserine	3.8 ± 1.0 ^{b}	3.5 ± 0.5 ^{b}	2.4 ± 1.0 ^{b}	6.7 ± 0.4 ^{Δa}	8.4 ± 0.2 $^{○a}$	6.1 ± 0.6 ^{Δa}
Phosphatidylinositol	4.1 ± 0.9 $^{▲c}$	5.5 ± 0.5 $^{●c}$	4.9 ± 0.5 $^{▲●c}$	26.3 ± 2.6 ^{Δa}	11.2 ± 1.0 $^{○b}$	9.3 ± 0.7 $^{○b}$
Phosphatidylethanolamine	29.8 ± 4.8 ^{a}	29.0 ± 5.5 ^{a}	28.0 ± 4.9 ^{a}	20.3 ± 1.3 ^{b}	23.6 ± 5.2 ^{ab}	24.0 ± 2.0 ^{ab}
∑ Polar Lipids	56.9 ± 2.5 ^{a}	57.2 ± 4.7 ^{a}	61.4 ± 3.3 ^{a}	77.5 ± 3.7 ^{c}	74.7 ± 8.8 ^{bc}	64.1 ± 2.5 ^{ab}
Partial Acylglycerols	5.2 ± 3.9	7.4 ± 2.2	4.6 ± 1.1	5.7 ± 0.9	6.0 ± 2.8	7.3 ± 0.8
Free Fatty Acids	7.4 ± 0.9 $^{▲ab}$	6.7 ± 1.0 $^{▲ab}$	4.7 ± 0.3 $^{●b}$	4.8 ± 1.4 ^{ab}	5.9 ± 2.6 ^{ab}	8.6 ± 0.6 ^{a}
Triacylglycerols	21.7 ± 2.2 $^{▲ab}$	20.7 ± 1.9 $^{▲b}$	25.5 ± 0.8 $^{●a}$	9.1 ± 0.5 ^{Δd}	9.2 ± 1.5 ^{Δd}	14.4 ± 0.6 $^{○c}$
Sterol Esters	8.8 ± 0.9 $^{▲a}$	8.1 ± 1.2 $^{▲a}$	3.8 ± 1.6 $^{●b}$	2.9 ± 0.9 ^{b}	4.2 ± 2.0 ^{b}	5.6 ± 0.9 ^{ab}
∑ Neutral Lipids	43.1 ± 2.5 ^{a}	42.8 ± 4.7 ^{a}	38.6 ± 3.3 ^{a}	22.5 ± 3.7 ^{c}	25.3 ± 8.8 ^{bc}	35.9 ± 2.5 ^{ab}

Results represent means ± SD (n = 4). 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 ($^{▲●■}$) within the same row represent significant differences between C18 FA ($p < 0.05$). Different hollow symbols in superscript ($^{Δ○□}$) within the same row represent significant differences between LC-PUFA ($p < 0.05$).

Table 6.5 shows the esterification pattern of FA into the TL of *G. adscensionis* zoeae. A higher percentage of [1-^{14}C] FA substrates were recovered as FFA when compared with *Artemia* sp.. [1-^{14}C]DHA was the most esterified substrate, with only 8.4 ± 1.3 % of radioactivity recovered as FFA ($p < 0.05$), and it was predominantly esterified into PL (78.5 ± 3.9 % of incorporated radioactivity; $p < 0.05$). While [1-^{14}C]C18 FA showed a tendency for a similar esterification into PL and NL fractions, LC-PUFA were mostly esterified into PL. [1-^{14}C]C18 FA were mainly esterified into TAG, PE and PC, while [1-^{14}C]LC-PUFA were predominantly esterified into PC and PE. Within [1-^{14}C]C18 FA, [1-^{14}C]18:3n-3 presented a slightly different pattern from [1-^{14}C]18:1n-9 and [1-^{14}C]18:2n-6 with higher esterification into PE and a lower esterification rate into TAG ($p < 0.05$). The esterification patterns of the LC-PUFA were generally similar. The only difference was found in the esterification of [1-^{14}C]DHA into PC, which was translated in differences regarding esterification into total PL and NL, compared to the other two LC-PUFA ($p < 0.05$; Table 6.5).

Table 6.5 – Esterification (%) of [1-^{14}C]FA substrates into *G. adscensionis* zoeae lipid classes

	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Phosphatidylcholine	16.1 ± 2.7 ^{c}	16.6 ± 2.1 ^{c}	18.3 ± 0.5 ^{c}	28.9 ± 6.2 $^{\circ b}$	28.5 ± 1.2 $^{\circ b}$	37.4 ± 0.7 $^{\Delta a}$
Phosphatidylserine	3.7 ± 2.1	5.0 ± 0.8	4.2 ± 0.3	4.3 ± 1.3	3.8 ± 0.4	4.6 ± 0.7
Phosphatidylinositol	3.8 ± 2.2	5.3 ± 1.3	5.1 ± 1.1	5.3 ± 1.5	7.1 ± 1.1	4.8 ± 0.4
Phosphatidylethanolamine	18.2 ± 2.6 $^{\bullet d}$	21.0 ± 2.2 $^{\bullet cd}$	25.5 ± 1.3 $^{\blacktriangle bc}$	29.9 ± 1.1 ^{ab}	26.9 ± 0.9 ^{ab}	31.8 ± 4.6 ^{a}
∑ Polar Lipids	41.8 ± 5.9 $^{\bullet d}$	47.8 ± 3.2 $^{\blacktriangle\bullet cd}$	53.1 ± 2.2 $^{\blacktriangle c}$	66.1 ± 7.3 $^{\Delta\circ b}$	64.8 ± 3.0 $^{\circ b}$	78.5 ± 3.9 $^{\Delta a}$
Partial Acylglycerols	9.1 ± 1.5 $^{\blacktriangle a}$	8.7 ± 2.1 $^{\blacktriangle a}$	5.2 ± 1.2 $^{\bullet b}$	5.7 ± 1.2 ^{ab}	7.9 ± 0.6 ^{ab}	5.7 ± 0.9 ^{ab}
Free Fatty Acids	19.1 ± 1.6 ^{a}	20.3 ± 5.2 ^{a}	19.8 ± 0.7 ^{a}	14.1 ± 4.6 $^{\Delta ab}$	14.5 ± 0.2 $^{\Delta a}$	8.4 ± 1.3 $^{\circ b}$
Triacylglycerols	30.1 ± 5.2 $^{\blacktriangle a}$	23.1 ± 3.8 $^{\blacktriangle\bullet a}$	21.9 ± 2.6 $^{\bullet a}$	11.7 ± 3.7 ^{b}	11.2 ± 0.9 ^{b}	7.4 ± 2.0 ^{b}
∑ Neutral Lipids	58.2 ± 5.9 $^{\blacktriangle a}$	52.2 ± 3.2 $^{\blacktriangle\bullet ab}$	46.9 ± 2.2 $^{\bullet b}$	33.9 ± 7.3 $^{\Delta\circ c}$	35.2 ± 3.0 $^{\Delta c}$	21.4 ± 3.9 $^{\circ d}$

Results represent means ± SD (n = 4). Data of esterification are given in percentage. Different letters in superscript within the same row represent significant differences between all fatty acids ($p < 0.05$). Different full symbols in superscript ($^{\blacktriangle\bullet\blacksquare}$) within the same row represent significant differences between C18 FA ($p < 0.05$). Different hollow symbols in superscript ($^{\Delta\circ\square}$) within the same row represent significant differences between LC-PUFA ($p < 0.05$).

6.4.4 Transformation of radiolabelled fatty acids

The majority of radioactivity incorporated into *Artemia* sp. metanauplii and *G. adscencionis* zoeae TL was present as the unmodified FA substrate (Table 6.6). Nonetheless, with the exception of [1-^{14}C]18:1n-9, the FA metabolic fate that was similar for both species, but showing higher percentages of the substrate FA transformed into shorter FAs in *Artemia* sp. metanauplii ($p < 0.05$). Based on the recycling of labelled acetyl-CoA produced from oxidation of the [1-^{14}C]FA, recovery of radioactivity in FA with shorter chain-lengths (fatty acids with a chain-length of 14, 16 and 18 carbons) was particularly higher in *Artemia* sp. metanauplii for all the FA substrates incubated, although it was also evident for C18 FAs in *Grapsus* zoeae. The higher catabolism of DHA (almost 30 % of incorporated radioactivity) by metanauplii was also noteworthy. Interestingly, in metanauplii this was the only fate of the incubated FAs, since no elongated or desaturated FA products were detected for any of the FA substrates. In contrast, some elongation of LC-PUFA incorporated into zoeae was observed, although no desaturation products were detected from any FA substrate.

Table 6.6 – Recovery of radioactivity (%) from [1-^{14}C]FA substrates in FA metabolites

Substrates	Products	*Artemia* sp.	*G. adscensionis*
[1-^{14}C]18:1n-9			
	18:1n-9	89.1 ± 3.3	91.3 ± 3.0
	de novo[a]	10.9 ± 3.3	8.7 ± 3.0
[1-^{14}C]18:2n-6			
	18:2n-6	84.0 ± 3.6	94.0 ± 1.2[*]
	de novo	16.0 ± 3.6	6.0 ± 1.2
[1-^{14}C]18:3n-3			
	18:3n-3	79.9 ± 6.6	91.7 ± 0.9[*]
	de novo	20.1 ± 6.6	8.3 ± 0.9
[1-^{14}C]20:4n-6			
	20:4n-6	92.8 ± 3.8	97.4 ± 1.8
	22:4n-6	-	2.4 ± 2.1
	de novo	7.2 ± 3.8	0.2 ± 0.3
[1-^{14}C]20:5n-3			
	20:5n-3	88.3 ± 2.4	96.7 ± 1.2[*]
	22:5n-3	-	1.7 ± 1.5
	de novo	11.7 ± 2.4	1.6 ± 0.3
[1-^{14}C]22:6n-3			
	22:6n-3	70.6 ± 6.8	93.4 ± 6.9[*]
	24:6n-3	-	3.4 ± 5.9
	de novo	29.4 ± 6.8	3.1 ± 3.0

Results represent means ± SD (n = 4). Data of transformation are given in percentage. [*] represent significant differences between groups ($p < 0.05$). [a] *de novo* synthesis of fatty acids with shorter chain-length (less than 18 carbons).

6.5 Discussion

Phospholipid/TAG ratio of life preys seems highly important in *O. vulgaris* paralarvae nutrition, as it could influence paralarvae phospholipids structure and function (Chapter 5). In the present study, TAG represented over 50 % of *Artemia* sp. total lipid content, while phospholipids accounted for less than 20 % of this species total lipids. Moreover, this species presented a high content on C18 FAs and low levels of LC-PUFA (Table 6.2). As a consequence, after lipids de-acylation, the dietary FFA pool available for re-acylation in paralarvae phospholipids when *Artemia* sp. is provided as prey, would present a high C18 FAs and a low LC-PUFA content, and so, the probability for re-acylation of a C18 FAs into paralarvae phospholipids over LC-PUFA would be higher

(see Chapter 5 for details in de-acylation/re-acylation processes). On the other hand, and similarly to that previously reported (Navarro and Villanueva, 2000; Iglesias et al., 2014; Reis et al., 2015) decapod crustacean zoeae, possess a much higher content of PL and LC-PUFA compared to *Artemia* sp.. This could possibly explain the better rearing results obtained when decapod crustacean zoeae are provided as prey to octopus paralarvae (Iglesias et al., 2004, 2007, 2014; Reis et al., 2015). However, the rearing of *O. vulgaris* paralarvae currently depends on the use of live *Artemia* sp. as prey, since the provision of decapod crustacean zoeae for large scale culture of octopus paralarvae is logistically difficult and economically unviable (Iglesias et al., 2007).

The main features that theoretically favour the use of *Artemia* sp. are its high availability and the possibility to be enriched with nutrients such as LC-PUFA, which can be essentially provided through a bioencapsulation process (Van Stappen, 1996). *Artemia* sp. are defined as continuous non-selective filter feeders (Reeve, 1963), where the amount of incorporated FA is directly related to its abundance in the enrichment medium (Navarro et al., 1999). Nonetheless, the results of the present study showed a lower incorporation of DHA into metanauplii TL compared to the other substrates. A substantial reduction in DHA incorporation by *Artemia* sp. has been reported to occur under starvation conditions, possibly due to a preferential oxidation of this FA (Estévez et al., 1998; Navarro et al., 1999). In the present study, as radiolabelled FAs were incubated without the addition of other FA or nutrients, the lower values of [1-^{14}C]DHA detected in metanauplii TL, might be related to the preferential oxidation of this FA, which commits well with the almost 30 % of shorter labelled FA obtained when using DHA. It is known that *Artemia* sp. has the capacity to retro-convert DHA into EPA (Navarro et al., 1999). However, in the present study [1-^{14}C]DHA was labelled only at the C1 position, so any chain shortening of this FA would remove the labelled carbon (acetyl-CoA) and the shorter FA obtained from the original labelled DHA would be undetectable.

Despite the higher esterification of LC-PUFA into *Artemi*a sp. metanauplii PL, a higher content of [1-^{14}C]DHA was also recovered esterified into TAG, as previously observed by Navarro et al. (1999). Moreover, Guinot et al. (2013b) has recently shown the capacity of metanauplii to translocate DHA from PL of enrichment diets to NL. After 24 h of starvation and despite higher DHA incorporation into PL of *Artemia* sp., this FA was still highly esterified into NL and, more specifically, into TAG (Navarro et al., 1999). If, as reported for marine fish larvae (Olsen et al., 2014; Sargent et al., 1999), *O. vulgaris* have a limited capacity for PL *de novo* biosynthesis from TAG or for the exchange of

LC-PUFA between TAG and PL, the enrichment of *Artemia* sp. with DHA may be of limited value. Further studies are necessary to elucidate *O. vulgaris* dietary TAG metabolism. In addition, the high C18 FAs and low DHA levels of *Artemia* sp. lipids, their similar esterification pattern into *O. vulgaris* hatchlings lipid classes (Chapter 3; Reis et al., 2014), as well as the preferential oxidation of DHA by *Artemia* sp. possibly explains the lower levels of this FA found in *O. vulgaris* paralarvae reared with enriched *Artemia* sp. (Fuentes et al., 2011; Navarro and Villanueva, 2000; Seixas et al., 2010a, 2010b; Viciano et al., 2011).

Decapod crustacean zoeae, on the other hand, normally possess a much higher content of DHA compared to *Artemia* sp. (Navarro and Villanueva, 2000; Iglesias et al., 2014; Reis et al., 2015). Nonetheless, the DHA content of *G. adscensionis* and *Maja brachydactila* zoeae, other decapod crustacean zoeae that has shown better results in the rearing of *O. vulgaris* paralarvae (Iglesias et al., 2004, 2014; Carrasco et al., 2006), are still lower than the values reported in *O. vulgaris* eggs (Quintana et al., 2015), hatchlings (Iglesias et al., 2014; Navarro and Villanueva, 2000; Chapter 3; Reis et al., 2014, 2015; Seixas et al., 2010a, 2010b). A decrease in DHA contents of marine fish larvae during the first days of feeding, similar to that observed in *O. vulgaris* paralarvae even when decapod crustacean zoeae are provided (Iglesias et al., 2014; Reis et al., 2015), does not necessarily affect fish larval performance (Sargent et al., 1999, 2002). However, a diet poor in DHA can potentially impair neural and visual development, with consequences for physiological and behavioural processes of the larvae (Sargent et al., 1999, 2002). The [1-^{14}C]DHA provided to *Grapsus* zoeae was the PUFA least incorporated (Table 6.3), but the most esterified one (Table 6.5), as well as the [1-^{14}C]LC-PUFA with the highest transformation rate into larger chain-length FA (Table 6.6). In addition, there was a preferential esterification of this FA into PL, similarly to what was recently verified in *O. vulgaris* hatchlings (Chapter 3; Reis et al., 2014), which would favour the use of zoeae as prey for octopus paralarvae.

A *de novo* synthesis of shorter chain-length FAs, as previously mentioned regarding DHA, was also verified from all incubated substrates. This indicates the catabolism of all incubated FA, similarly to that reported to occur for other LC-PUFA during starvation conditions (Estevéz et al., 1998). Likewise, a *de novo* synthesis of shorter chain-length FA was also observed in *G. adscensionis* zoeae metabolism, which may indicate that zoeae were also experiencing starvation. Nonetheless, the catabolism of labelled FA was significantly higher in *Artemia* sp. metanauplii and for [1-^{14}C]C18 FA substrates

incorporated into zoeae TL. Other metabolic particularity, which might favour the use of zoeae over *Artemia* sp. as prey for *O. vulgaris* paralarvae, was the esterification pattern of [1-^{14}C]LC-PUFA. Although both preys presented a higher esterification of all FA into PL, the esterification of ARA into zoeae PL was similar to the esterification of this FA into octopus hatchlings TL (see Chapter 3; Reis et al., 2014). Interestingly, in both octopus hatchings and crab zoeae, and differently from the esterification pattern of ARA observed in *Artemia* sp. and fish larvae (Bell and Sargent, 2003), this FA was not preferentially esterified into PI, but into PE. In zoea, there was also a higher esterification of ARA into PC. However, this may not compromise the esterification of ARA into octopus PE (Chapter 5).

An inverse relationship between EPA and ARA incorporation into *O. vulgaris* TL was reported by Miliou et al. (2006) and Reis et al. (2015). Additionally, Reis et al. (Chapter 3; 2014) detected a similar esterification pattern of these two FA into specific lipid classes of *O. vulgaris* hatchlings, along with a higher incorporation of ARA into octopus TL, indicating the necessity to provide an adequate dietary balance of both FA. The ideal EPA/ARA ratio for *O. vulgaris* paralarvae normal development is difficult to determine due to the inconsistent results obtained in terms of growth and survival of paralarvae during rearing (see Reis et al., 2015). Moreover, hatchlings EPA/ARA ratios also presented some variation, ranging from 2.0 to 4.3, with differences being observed mainly due to different contents of ARA rather than EPA (Iglesias et al., 2014; Chapter 3; Reis et al., 2014, 2015; Seixas et al., 2010a, 2010b). Quintana et al., (2015) detected an EPA/ARA ratio of 0.9 in *O. vulgaris* eggs when the broodstock were fed with crab (*Carcinus maenas*) and an EPA/ARA ratio of 4.9 and 6.7 when the broodstock were fed with *Illex* sp. and *Sardina pilchardus*, respectively. However, spawning quality was negatively affected when the broodstock were fed with *S. pilchardllus*, where a high content on TAG was detected and identified as a possible cause to this lower quality (Quintana et al., 2015). Reis et al. (2015) verified that the highest IGR was achieved when *O. vulgaris* paralarvae were fed with *G. adscensionis* zoeae, which displays a high ARA content, reaching an EPA/ARA ratio of ≈1 (present study and Reis et al. (2015)). Nevertheless, the latter study only covered a rearing period of 9 days. Therefore, it is possible that, in an extended rearing period, the high content of ARA in *G. adscensionis* zoeae might render an unbalanced EPA/ARA ratio, possibly through a potential inhibitory effect on EPA bioconversion (Furuita et al., 2003) or incorporation (Atalah et al., 2011b; Villalta et al., 2005). On the other hand, *M. brachydactila* zoeae has an EPA/ARA ratio

of 2.9, similar to the values observed in hatchlings (Iglesias et al., 2014), and a higher DHA content (Andrés et al., 2010; Iglesias et al., 2014), when compared to *Grapsus* zoeae, which seems to favour its use as prey in *O. vulgaris* rearing. Nonetheless, it is important to highlight that *Maja* zoeae are hard to rear, highly expensive to obtain and that adults have a high commercial value, which turns the large-scale culture of *O. vulgaris* paralarvae economically unviable (Iglesias et al., 2007).

Carrasco et al. (2006) showed that an ideal diet for the culture of paralarvae, at least for the first 5 to 6 weeks after hatching, would be a co-feeding of *Artemia* sp. with crab zoeae on alternate days, leading to a lower demand for zoeae biomass. The present results suggest that it may be difficult to obtain proper enrichment of metanauplii with DHA and inert diets have shown low acceptance by paralarvae (Navarro and Villanueva, 2003; Seixas et al., 2010b). In this sense, a feeding scheme utilizing a co-feeding with low value decapod crustacean zoeae (rich in EFA, mainly DHA), plus *Artemia* sp. enriched with ARA and EPA might be an alternative to the enrichment of *Artemia* sp. with DHA. Since *O. vulgaris* paralarvae are able to preserve its lipid content despite the lipid content of preys (Reis et al., 2015), present low digestibility of lipid (O'Dor et al., 1984) and appears to do not possess a lipid energetic metabolism (Giménez and García García, 2002; Lee, 1994), it seems important to provide a prey with low lipid content. On the other hand, considering the recent results obtained in *O. vulgaris* nutritional requirements (Fuentes et al., 2011; Iglesias et al., 2014; Monroig et al., 2012a, 2012b; Chapter 3; Reis et al., 2014, 2015; Roura et al., 2012; Seixas et al., 2010a, 2010b; Viciano et al., 2011), it seems that the dietary lipid must have a high PL and LC-PUFA content, with LC-PUFA being preferentially esterified into PL. Shorter *Artemia* sp. enrichment protocols could be a way of avoiding translocation of EFA from PL to TAG but this needs confirmation (Guinot et al., 2013b).

Several features favour the use of decapod crustacean zoeae as live prey for *O. vulgaris* paralarvae rearing compared to *Artemia* sp. directly related to their original lipid and fatty acid composition. Besides the changes taking place after the lipid enrichment of both preys point at two very distinct models of lipid metabolism *Artemia* sp. metanauplii show lower content of PL and LC-PUFA, lower content of FA recovered as FFA, higher FA catabolism rates, and a preferential esterification of EFA into TAG. The opposite is true for *Grapsus* zoeae, illustrated for example in a preferential esterification of EFA and especially of ARA into PE over PI. Besides their inherent better nutritional value due to their lipid composition, these evidences may possibly make zoeae a more suitable prey

than *Artemia* sp., but essentially further illustrate the inherent difficulties for an efficient EFA enrichment of *Artemia* sp. as food for marine organisms.

Chapter 7.

Effect of *Artemia* sp. endogenous fatty acid metabolism on the bioavailability of essential fatty acids for *Octopus vulgaris* paralarvae development

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7.1 Abstract

The aim of the present study was to determine the effect of *Artemia* sp. metanauplii endogenous fatty acid (FA) metabolism in the availability of dietary essential fatty acids (EFA) for *Octopus vulgaris* paralarvae development. To this end, both *Artemia* sp. metanauplii endogenous FA metabolism and the *Octopus vulgaris* paralarvae metabolism after being fed with *Artemia* sp. metanauplii enriched with radiolabelled FAs were determined. Ten thousand metanauplii were incubated in 6-well flat-bottom tissue culture plates during 12 h, at 24 ºC, with 0.3 µM of a [1-^{14}C]FA including either 18:3n-3, 20:4n-6 (ARA), 20:5n-3 (EPA) or 22:6n-3 (DHA), added individually to separate wells as their potassium salts bound to bovine serum albumin. Four *Artemia* sp. baths replicates were used to determine metanauplii endogenous metabolism and another eight were used as live prey to feed *O. vulgaris* paralarvae. A total of 3 600 one-day-old paralarvae were reared during 24 h in order to ensure the ingestion of a minimum amount of the labelled *Artemia* sp.. Paralarvae rearing was performed in 4 L cylinder conical tanks of a closed seawater system, at a density of 25 paralarvae L^{-1} (100 paralarvae per tank). Labelled *Artemia* sp. metanauplii were added to each paralarvae rearing tank according to a specific treatment. The present results showed a preferential catabolism of DHA, which was translated into (1) the lower incorporation of this FA into *Artemia* sp. TL; (2) the highest amount of *de novo* synthesis of shorter chain-lenght FAs, as a result of the β-oxidation of the original substrate. Nonetheless, *de novo* synthesis of shorter FAs, was also verified from all incubated substrates indicating a FA catabolism, which could be related to *Artemia* sp. starvation conditions. The registered amounts of radiolabelled substrates incorporated into *O. vulgaris* paralarvae TL while fed with labelled *Artemia* sp. metanauplii were extremely low, which hampered the analysis of the results obtained during the present study. Nonetheless, and although no deep conclusions can be made from these preliminary results, some considerations can be taken regarding the suitability to the novel method used and the effect of *Artemia* sp. endogenous FA metabolism in the availability of EFA for *O. vulgaris* paralarvae. Certain amount of intact [1-^{14}C]ARA and [1-^{14}C]EPA was recovered into octopus paralarvae TL and lipid classes, and suggest the possibility of using *Artemia* sp. as a vehicle to provide ARA and EPA to octopus paralarvae without interfering their bioavailability for the *de novo* synthesis of phospholipids. On the other hand, and despite of the high amount of [1-^{14}C]18:3n-3 incorporated into *Artemia* sp. TL, the FAs with the highest esterification rate into *Artemia* sp. TAG (18:3n-3 and DHA) were also the lowest incorporated into paralarvae TL.

Therefore, the present results suggest that *O. vulgaris* paralarvae may have a potentially low capacity to metabolise dietary TAG. Nonetheless, considering the paralarvae rearing time of 24 h assayed during the present study, it should also be considered some further catabolism of FA substrates in *Artemia* sp., which could also explain the different incorporation rates of the different FAs substrates in octopus paralarvae. Future studies should test different incubations/predation times in order to increase radioactivity incorporation in paralarvae tissues and confirm the presented hypothesis. Furthermore, different *Artemia* sp. incubation conditions considering different incubations periods and the use of enrichment products might be performed in order to avoid *Artemia* sp. possible starvation during labelling process.

Keywords: *Artemia* sp. metanauplii; *In vivo*; Lipid metabolism; *Octopus vulgaris* paralarvae; Unsaturated fatty acids.

7.2 Introduction

In nature, marine organisms feed on a wide spectrum of zoo- and phytoplankton during their early life stages, which provides them a complete and balanced diet. Nonetheless, mass production of zooplankton for large-scale aquaculture industry is still a challenge due to the difficult logistics and cost-effective protocols (Iglesias et al., 2014; Støttrup and Norsker, 1997). For that reason, economical alternative protocols for marine larval production are required.

Despite the recent progress in the development of inert diets, the rearing of early live stages of marine organisms still depends on the use of live feeds. According to Conceição et al. (2010), this dependence was associated with several factors, such as (1) the development and maturation of the larvae digestive system, which is, in most cases, incapable of processing formulated diets from onset; (2) the constant availability of feed on the water column for the larvae due to the swimming capacity of preys; (3) stimulation of larval feeding response by prey movement; (4) higher palatability of live preys due to its high water content.

Within live feeds, *Artemia* sp. is widely used in marine larvae rearing because of its high availability and acceptance by a large number of species (Sorgeloos et al., 2001). However, it is known that *Artemia* sp. is not a perfect feed in aquaculture and naturally presents a low content of long-chain polyunsaturated fatty acids (LC-PUFA), such as 20:5n-3 (EPA), and especially 22:6n-3 (DHA), which are essential fatty acids (EFA) for marine fish larvae normal development (Sargent et al., 1999). In this sense, bioencapsulation is used to tailor *Artemia* sp. lipid composition towards marine larval nutritional needs (Van Stappen, 1996). A typical *Artemia* sp. enrichment protocol includes the incubation of newly hatched *Artemia* sp. with lipid emulsions added every 12 h at 25 -28 ºC during 24h at a maximum density of 2g L^{-1}, oxygen saturation near to saturation and strong illumination (Sorgeloos et al., 2001; Van Stappen, 1996). Nonetheless, lower temperatures and enrichment times (e.g., 23 - 24 ºC for 16 - 20 h) may be used in order to reduce *Artemia* sp. lipids metabolism (Conceição et al., 2010; Evjemo et al., 1997). Navarro et al. (1999) showed DHA retro-conversion after 24h of enrichment, whereas Guinot et al. (2013b) verified that even after an enrichment period of only 4h, *Artemia* sp. metanauplii actively translocate ingested DHA provided as polar lipids (PL) to neutral lipids (NL) classes including triacylglycerols (TAG). Therefore, *Artemia* sp. should not only be considered as a mere passive carrier of fatty acids (FA), as it is able to digest, incorporate and metabolise these nutrients (Navarro et al., 1999); fact that may

compromise the use of enriched *Artemia* sp. as a suitable prey for marine larvae species rearing.

Similarly to other marine fish species, the best growth and survival rates of *Octopus vulgaris* during paralarvae phase rearing is currently achieved when crustacean zoeae are used in co-feeding with *Artemia* sp. (Iglesias and Fuentes, 2014). Nonetheless, this method is not transferable to a commercial scale due to the difficult logistics and cost-effective protocols on zoeae production. For that reason, in order to reduce logistic and costs, octopus paralarvae rearing is being adapted to the use of *Artemia* sp. as sole prey (Villanueva et al., 2014). Despite the efforts, poor growth and survival rates have been obtained (De Wolf et al., 2011; Moxica et al., 2006; Navarro and Villanueva, 2000; Seixas et al., 2008, 2010b), being those suggested to be mainly related with *Artemia* sp. FA profile (Navarro e al., 2014; Seixas et al., 2010a). Nonetheless, not only EFA content seems to be important in *O. vulgaris* development but also the presence of these FAs into specific lipid classes, such as PL might be an essential factor to attain a normal development in this species. Octopus hatchlings present a high capacity to remodel tissues phospholipids, which suggests the importance of phospholipid/triacylglycerols ratio in this species nutrition during early life stages (see Chapter 5). Therefore, in theory, the high TAG and low PL contents in *Artemia* sp. lipids would likely affect octopus phospholipids structure (Chapter 5), and their specific role (Tocher et al., 2008). At the light of these facts, and in order to improve *Artemia* sp. enrichment protocols, it seems important to better understand *Artemia* sp. metanauplii lipid metabolism and how metanauplii inherent metabolism affects the availability of EFA for octopus paralarvae normal development. To this end, the *Artemia* sp. metanauplii endogenous FA metabolism, as well as the incorporation, esterification pattern and transformation of FAs by *O. vulgaris* paralarvae fed with *Artemia* sp. metanuplii enriched with radiolabelled FAs were studied in the present work.

7.3 Material and Methods

7.3.1 Experimental animals

Artemia sp. metanauplii were obtained by hatching EG *Artemia* Cysts (INVE Aquaculture, Belgium). Following the protocol of Sorgeloos et al. (2001), 4 g of *Artemia* sp. cysts were decapsulated with bleach, followed by deactivation with $Na_2S_2O_3$ dissolved in filtered seawater (0.02 % w/v). Incubation of cysts was performed over 24 h in a 3 L fiberglass cylinder conical tanks containing clean filtered seawater (36 ‰) at 28 ºC, with

continuous light and vigorous aeration. After hatching, nauplii were placed in similar tanks with clean seawater under similar conditions for an 8 h period, until instar II stage (metanauplii stage - mouth and anus opening) was reached.

One day old *O. vulgaris* paralarvae were obtained from eggs of a broodstock of 30 individuals, caught by professional artisanal fishermen on the Tenerife coast (Canary Islands, Spain) and maintained at Instituto Español de Oceanografia (IEO) de Canarias (Santa Cruz de Tenerife - 28º29'59.1''N; 16º11'44.54''W). The broodstock rearing conditions were similar to those described by Reis et al. (Chapter 3; 2014). The presence of eggs was checked once a week and when egg masses were observed, the ovate female and posture were left alone in the tank by removing the other individuals. After approximately one month of embryonic development octopus began to hatch. Six thousand hatchlings were removed from the female/rearing tank, and transferred to 100 L fiberglass cylinder conical tanks (52 cm of diameter and 56 cm from top to bottom) of a flow-through seawater system with 60 mL min^{-1} water flow at a density of 30 hatchlings L^{-1}. A light intensity of 200 lx (provided by an incandescent 40 W bulb) and a photoperiod of 12L:12D was maintained during 24 hours. The water temperature was 21 ºC, salinity was 36 ‰ and dissolved oxygen was near saturation.

7.3.2 *In vivo* incubations of *Artemia* sp. with labelled [1- ^{14}C] fatty acids

Prior to incubation with radiolabelled FA substrates, metanauplii were filtered and concentrated in 800 mL of filtered seawater and density was determined. *Artemia* sp. metanauplii were incubated in 6-well flat-bottom tissue culture plates (Sarstedt AG & Co., Nümbrecht, Germany) with 10 mL of filtered seawater. Incubations were performed for 12 h at a density of 10 000 metanauplii per incubation well, with gentle stirring at 24 ºC, and 0.2 µCi (0.3 µM) of [1-^{14}C]FA including either 18:3n-3, ARA, EPA or DHA. The specific [1-^{14}C]FA was added individually to separate wells as their potassium salts bound to bovine serum albumin (BSA), as described in Ghioni et al. (1997). A total of 12 incubations per treatment ([1-^{14}C]FA) were performed: 4 replicates were used to determine *Artemia* sp. metanauplii endogenous FA metabolism and 8 replicates were used as live prey to *O. vulgaris* paralarvae, so that the effect of metanauplii endogenous metabolism in FA availability would be determined. Control treatments of metanauplii, without addition of [1-^{14}C]FAs were also assessed (n = 8; 4 replicates for metanauplii lipid composition determination and 4 replicates to provide to control *O. vulgaris* paralarvae as live prey). A survival rate of 92 ± 4 % was obtained in all incubations. After

incubation, *Artemia* sp. metanauplii were filtered in a 100 µm mesh and thoroughly washed with filtered seawater to remove radiolabelled FA excess. Control and radiolabelled replicates for metanauplii metabolism determination (n = 4), were stored at -80 ºC until analysis; the other replicates (n = 8) were provided to *O. vulgaris* paralarvae as live prey.

7.3.3 Rearing of *Octopus vulgaris* paralarvae fed with radiolabelled *Artemia* sp.

A total of 3 600 paralarvae were reared during 24 h in 4 L cylinder conical tanks at a density of 25 paralarvae L^{-1} (100 paralarvae per tank). A light intensity of 200 lx (provided by an incandescent 40 W bulb) and a photoperiod of 12L:12D were maintained throughout the experiment. The water temperature was 21 ºC, salinity was 36 ‰ and dissolved oxygen was near saturation. Ten thousand labelled *Artemia* sp. metanauplii from a specific treatment (see 7.3.2) were added to each paralarvae rearing tank. After the rearing period, the whole tank water volume, containing metanauplii and paralarvae, was filtered with a 500 µm mesh to separate both species. Paralarvae were put back into their respective rearing tanks, with newly fresh filtrated seawater, and left during 1 h to allow the digestion of ingested metanauplii. A paralarvae survival rate of 98.5 ± 1.5 % was obtained in all treatments. After this period, paralarvae were euthanized in iced seawater (3 ºC). Samples were stored at -80 ºC until analysis.

7.3.4 Total lipid extraction

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 until analysis at -20 ºC, at a concentration of 10 mg mL^{-1} in chloroform/methanol (2:1, v/v) with 0.01 % butylated hydroxytoluene (BHT) as antioxidant, and under an inert atmosphere of nitrogen.

7.3.5 Lipid class and fatty acid composition of control samples

Aliquots of 0.02 mg of TL extracts from metanauplii and paralarvae control groups were used to determine lipid classes (LC) and fatty acid compositions (n = 4). LC were separated by single-dimensional double-development high-performance thin-layer chromatography (HPTLC) and quantified by charring followed by calibrated densitometry, using dual-wavelength flying spot scanner CS-90001PC (Shimadzu Co.,

Japan; Tocher and Harvie, 1988). LC identification was performed by running known LC standards (cod roe lipid extract) on the same plates.

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

7.3.6 Incorporation of radioactivity into total lipids

An aliquot of 0.1 mg of radiolabelled *Artemia* sp. metanauplii and *O. vulgaris* paralarvae TL extract was taken to determine total radioactivity incorporated. Extracts were transferred to scintillation vials and radioactivity determined on a LKB Wallac 1214 Rackbeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, USA). Results in dpm were converted into pmoles per mg protein per h of incubation (pmol mg pp^{-1} h^{-1}), considering specific activity of each substrate and metanauplii and paralarvae TL and protein contents. Protein was determined in metanauplii (n = 4) and paralarvae (n = 8) according to Lowry et al. (1951).

7.3.7 Esterification of radioactivity into lipid classes

An aliquot of 0.1 mg of TL extract from radioactive samples was also applied to HPTLC plates to determine the esterification of [1-^{14}C]FA into LC. Lipid classes were separated by single-dimensional double-development HPTLC, as previously described by Tocher and Harvie (1988). Esterification pattern of [1-^{14}C]FA into LC was determined by image analysis following Reis et al. (Chapter 2; 2014).

7.3.8 Transformation of radiolabelled fatty acids

An aliquot of 1.2 mg of TL extract from radioactive samples was subjected to acid-catalysed transmethylation to obtain FAME as previously detailed (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 ºC 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). FAME identification was performed by image analysis following the method described in Reis et al. (Chapter 2; 2014).

7.3.9 Materials

[1-^{14}C]18:3n-3 was purchased from PerkinElmer, Inc. (Waltham, Massachusetts, USA). [1-^{14}C]ARA, [1-^{14}C]EPA, and [1-^{14}C]DHA were purchased from American Radiolabelled Chemicals, Inc. (St. Louis, Missouri, USA). BSA was purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). TLC plates (20 × 20 cm × 0.25 mm) were purchased form Macherey-Nagel GmbH & Co. KG (Düren, Germany). HPTLC plates, (10 × 10 cm × 0.15 mm) pre-coated with silica gel 60 (without fluorescent indicator), were purchased from Merck KGaA (Düsseldorf, Germany). OptiPhase "HiSafe" 2 scintillant liquid was purchased from PerkinElmer, Inc. (Waltham, Massachusetts, USA). Organic solvents used were of reagent grade and were purchased from Merck KGaA (Düsseldorf, Germany), Sigma-Aldrich Co. (St. Louis, Missouri, USA) and Panreac Química S.L.U. (Barcelona, Spain).

7.3.10 Statistical analysis

Results are presented as means ± SD. For all statistical tests, $p < 0.05$ was considered statistically different. Data were checked for normal distribution with the one-sample Shapiro-Wilk test, as well as for homogeneity of the variances with the Levene test (Zar, 1999). Arcsine square root transformation was applied to all data expressed as percentage (Fowler et al., 1998). Differences between [1-^{14}C]FAs incorporation into TL, and esterification into the different lipid classes within a same species, 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 Welch robust test, followed by a Games-Howell non-parametric multiple comparison test (Zar, 1999). Differences between LC and FA compositions of *Artemia* sp. metanauplii and *O. vulgaris* paralarvae control groups and [1-^{14}C]FA incorporation into TL and its transformation rate between both species, were tested using a Student´s *t*-test (Zar, 1999). The statistical analysis was performed using the IBM SPSS statistics 22.0 (IBM Co., USA).

7.4 Results

7.4.1 *Artemia* sp. metanauplii and *O. vulgaris* paralarvae lipid composition

Artemia sp. metanauplii and *O. vulgaris* paralarvae lipid classes composition was highly different (Table 7.1). Metanauplii TL were particularly rich in neutral lipids (NL), with triacylglycerols (TAG) being the main lipid component (52.5 ± 3.1 %). On the other hand, *O. vulgaris* paralarvae presented a high proportion of polar lipids (PL), with phosphatidylethanolamine (PE; 24.1 ± 1.6 %) and phosphatidylcholine (PC; 20.4 ± 0.9 %) as the main PL classes ($p < 0.05$; Table 7.1). Cholesterol was the most abundant lipid class of octopus paralarvae, corresponding to 31.2 ± 2.3 % of paralarvae TL.

Table 7.1 – Total lipid content (µg lipid mg protein^{-1}) and lipid class composition (%) of *Artemia* sp. metanauplii and *O. vulgaris* paralarvae

	Artemia sp.	*O. vulgaris*
TL content	612.3 ± 65.4	273.9 ± 90.4*
Lipid classes		
Sphingomyelin	0.0 ± 0.0	0.7 ± 0.2*
Phosphatidylcholine	8.1 ± 1.1	20.4 ± 0.9*
Phosphatidylserine	0.9 ± 0.2	11.1 ± 1.2*
Phosphatidylinositol	1.3 ± 0.4	4.2 ± 0.4*
Phosphatidylglycerol	1.9 ± 0.3	3.5 ± 0.2*
Phosphatidylethanolamine	6.7 ± 0.9	24.1 ± 1.6*
∑ Polar lipids	18.9 ± 2.3	64.0 ± 2.9*
Diacylglycerols	1.2 ± 0.6	0.6 ± 0.2*
Cholesterol	16.4 ± 1.1	31.2 ± 2.3*
Free Fatty Acids	3.3 ± 2.4	1.2 ± 0.2*
Triacylglycerols	52.5 ± 3.1	1.3 ± 0.5*
Sterol Esters	7.7 ± 0.9	1.7 ± 0.6*
∑ Neutral lipids	81.1 ± 2.3	36.0 ± 2.9*

Results represent means ± SD (n = 4). Data are presented in percentage of total lipid content.* Represent significant differences between *Artemia* sp. metanauplii and *O. vulgaris* paralarvae ($p < 0.05$).

Table 7.2 shows the FA profile of *Artemia* sp. metanauplii and *O. vulgaris* paralarvae. Metanauplii were particularly rich in 18:1n-9 and 18:3n-3 and paralarvae presented a high content of DHA, 16:0 and EPA ($p < 0.05$). Total PUFA content of metanauplii and paralarvae was 45.1 ± 0.7 % and 48.8 ± 1.3 % of total FAs, respectively, while total LC-PUFA content corresponded to 5.3 ± 0.1 % in metanauplii and 45.3 ± 2.4 % in paralarvae ($p < 0.05$). *Artemia* sp. metanauplii presented a DHA/EPA and an EPA/ARA

ratios of 0.0 ± 0.0 and 1.8 ± 0.0, respectively, whereas in *O. vulgaris* paralarvae these ratios were of 1.4 ± 0.1 and 3.1 ± 0.5 respectively ($p < 0.05$).

Table 7.2 – Main fatty acid composition (% of total FA) of *Artemia* sp. metanauplii and *Octopus vulgaris* paralarvae total lipids.

	Artemia sp.	*Octopus vulgaris*
14:0	0.5 ± 0.0	0.7 ± 0.1*
15:0	0.2 ± 0.0	0.2 ± 0.0
16:0	10.4 ± 0.2	17.0 ± 0.9*
16:0 DMA	0.0 ± 0.0	0.8 ± 0.1*
18:0	6.4 ± 0.1	10.1 ± 0.5*
18:0 DMA	0.0 ± 0.0	4.8 ± 0.2*
Total saturated[a]	18.7 ± 0.3	34.6 ± 1.1*
16:1[b]	3.4 ± 0.2	1.5 ± 0.1*
18:1n-13	0.0 ± 0.0	2.4 ± 0.2*
18:1n-9	19.0 ± 0.3	3.7 ± 0.9*
20:1n-9	0.7 ± 0.0	3.9 ± 0.3*
Total monoenes[a]	33.7 ± 0.4	15.6 ± 0.9*
18:2n-6	5.0 ± 0.1	0.9 ± 0.6*
20:2n-6	0.2 ± 0.0	0.6 ± 0.0*
20:4n-6	1.4 ± 0.0	5.0 ± 0.3*
Total n-6 FA[a]	7.2 ± 0.1	6.9 ± 1.1
18:3n-3	28.1 ± 0.4	1.5 ± 1.0*
20:3n-3	0.9 ± 0.0	2.0 ± 0.1*
20:5n-3	2.4 ± 0.1	15.6 ± 1.7*
22:5n-3	0.0 ± 0.0	1.3 ± 0.0*
22:6n-3	0.0 ± 0.0	21.0 ± 1.2*
Total n-3 FA[a]	37.2 ± 0.6	41.5 ± 1.8*
Total PUFA[a,c]	45.1 ± 0.7	48.8 ± 1.3*
Total LC-PUFA[a,d]	5.3 ± 0.1	45.3 ± 2.4*
n-3/n-6	5.2 ± 0.1	6.1 ± 1.1
DHA/EPA[e]	0.0 ± 0.0	1.4 ± 0.1*
EPA/ARA[e]	1.8 ± 0.0	3.1 ± 0.5*

Results represent means ± SD (n = 4). * represents differences between *Artemia* sp. and *O. vulgaris* 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.

7.4.2 Incorporation of radioactivity into total lipid

Table 7.3 shows the incorporation of radiolabelled substrates into the TL of *Artemia* sp. metanauplii after12 h of incubation (enrichment period) and of *O. vulgaris* paralarvae after ingestion and digestion of radiolabelled metanauplii (end of the experiment). Among incubated FAs [1-^{14}C]DHA was the FA with the lowest incorporation rate (3.61 ± 0.70 pmoles mg pp^{-1} h^{-1}) into metanauplii TL, followed by [1-^{14}C]18:3n-3 (17.80 ± 2.06 pmoles mg pp^{-1} h^{-1}). These substrates were also the ones with the lowest incorporation rate into *O. vulgaris* paralarvae TL (0.03 ± 0.01 pmoles mg pp^{-1} h^{-1}for DHA and 0.04 ± 0.02 pmoles mg pp^{-1} h^{-1}for 18:3n-3).

Table 7.3 - Incorporation of radioactivity into total lipid (pmoles mg pp^{-1} h^{-1}) into *Artemia* sp. metanauplii after the 12 h incubation period and into *O. vulgaris* paralarvae after metanuaplii ingestion and digestion.

[1-^{14}C] Substrate	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Artemia sp.	17.80 ± 2.06^{c}	28.40 ± 2.83^{a}	21.87 ± 1.77^{b}	3.61 ± 0.70^{d}
O. vulgaris	$0.04 \pm 0.02^{bc*}$	$0.19 \pm 0.09^{a*}$	$0.11 \pm 0.06^{ab*}$	$0.03 \pm 0.01^{c*}$

Results represent means ± SD (n = 4 for *Artemia* sp. metanauplii and n = 8 for *O. vulgaris* paralarvae). Data are presented in pmoles of ^{14}C fatty acid incorporated/mg of protein per hour of incubation. Different letters in superscript within the same row represents significant differences between all fatty acids ($p < 0.05$). * within the same column represents significant differences for a specific fatty acid between *Artemia* sp. metanauplii and *O. vulgaris* paralarvae ($p < 0.05$).

7.4.3 Esterification of radioactivity into lipid classes

The distribution of incorporated radioactivity into lipid classes of *Artemia* sp. metanauplii is presented in Table 7.4. All substrates were extensively esterified by metanauplii, with a maximum of 5 % of the incorporated radioactivity being recovered as free fatty acids (FFA; Table 7.4). Substrates were mainly esterified into PL, with PE and PC presenting the highest esterification rates of FAs within these lipid classes, the exception being for [1-^{14}C]ARA that showed much higher esterification into PI (37.5 ± 1.5 % of incorporated radioactivity). [1-^{14}C]DHA and [1-^{14}C]18:3n-3 also presented a high esterification rate into *Artemia* sp. NL and more precisely into TAG (21.2 ± 1.6 % for DHA and 27.6 ± 4.2 % for 18:3n-3). Due to the low incorporation rates of 18:3n-3 and DHA into *O. vulgaris* paralarvae TL, it was not possible to determine the esterification pattern of these FAs into the different lipid classes. [1-^{14}C]ARA and [1-^{14}C]EPA presented a similar esterification pattern in the paralarvae, with the majority of the incorporated FAs being recovered into polar lipids and more precisely into PC and PE (Table 7.5).

Table 7.4 – Esterification (%) of [1-^{14}C]FA substrates into *Artemia* sp. metanauplii lipid classes after incubation period.

[1-^{14}C] Substrate	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Lipid Class				
Phosphatidylcholine	35.5 ± 2.4^{ab}	28.6 ± 0.8^{c}	41.9 ± 4.0^{a}	30.4 ± 1.9^{bc}
Phosphatidylserine	1.2 ± 1.0^{c}	6.2 ± 1.6^{a}	8.6 ± 0.7^{a}	3.8 ± 0.5^{b}
Phosphatidylinositol	4.5 ± 1.0^{d}	37.5 ± 1.5^{a}	9.4 ± 1.8^{b}	6.5 ± 0.9^{c}
Phosphatidylglycerol	3.2 ± 1.4^{bc}	1.8 ± 0.1^{c}	4.1 ± 1.2^{ab}	5.2 ± 0.4^{a}
Phosphatidylethanolamine	18.6 ± 1.5^{ab}	15.8 ± 0.6^{b}	21.8 ± 1.5^{a}	12.9 ± 1.0^{c}
∑ Polar lipids	63.0 ± 5.2^{b}	89.9 ± 0.5^{a}	85.9 ± 4.8^{a}	58.9 ± 1.6^{b}
Partial acylglycerols	1.9 ± 1.0^{b}	2.5 ± 0.5^{b}	2.4 ± 0.6^{b}	7.0 ± 0.8^{a}
Free fatty acids	3.4 ± 1.2^{ab}	1.6 ± 0.2^{c}	2.0 ± 0.5^{bc}	5.0 ± 0.4^{a}
Triacylglycerols	27.6 ± 4.2^{a}	4.3 ± 0.1^{c}	7.6 ± 2.3^{b}	21.2 ± 1.6^{a}
Sterol esters	4.2 ± 3.2^{ab}	1.7 ± 0.3^{b}	2.1 ± 1.4^{b}	8.0 ± 2.3^{a}
∑ Neutral lipids	37.0 ± 5.2^{a}	10.1 ± 0.5^{b}	14.2 ± 4.8^{b}	41.1 ± 1.6^{a}

Results represent means ± SD (n = 4). Different superscript letters within the same row represent significant differences between all fatty acids ($p < 0.05$).

Table 7.5 – Esterification (%) of [1-^{14}C]FA substrates into *O. vulgaris* paralarvae lipid classes after metanauplii ingestion and digestion.

[1-^{14}C] Substrate	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Lipid Class				
Phosphatidylcholine	-	24.0 ± 4.6	35.3 ± 7.3	-
Phosphatidylserine	-	7.2 ± 4.2	7.7 ± 3.6	-
Phosphatidylinositol	-	15.3 ± 4.7	9.0 ± 3.3	-
Phosphatidylglycerol	-	2.6 ± 3.6	1.9 ± 1.1	-
Phosphatidylethanolamine	-	32.4 ± 8.7	27.3 ± 5.0	-
∑ Polar lipids	-	81.5 ± 8.0	81.0 ± 11.5	-
Partial acylglycerols	-	0.0 ± 0.0	0.0 ± 0.0	-
Free fatty acids	-	0.0 ± 0.0	0.0 ± 0.0	-
Triacylglycerols	-	14.3 ± 8.4	16.5 ± 13.0	-
Sterol esters	-	8.3 ± 7.9	4.0 ± 3.1	-
∑ Neutral lipids	-	18.5 ± 8.0	19.0 ± 11.5	-

Results represent means ± SD (n = 8). * within the same row represent significant differences between ARA and EPA esterified into a specific lipid class ($p < 0.05$).

7.4.4 Transformation of radiolabelled fatty acids

The majority of radioactivity incorporated into *Artemia* sp. metanauplii was recovered as unmodified FA substrate (Table 7.6). Nonetheless, based on the recycling of labelled

acetyl-CoA produced from oxidation of the [1-^{14}C]FA, recovery of radioactivity in FAs with shorter chain-lengths (fatty acids with a chain-length of 14, 16 and 18 carbons) wasevident for all the incubated FA substrates. The higher catabolism of DHA (almost 30 % of incorporated radioactivity) by metanauplii was also noteworthy ($p < 0.05$). Interestingly, after radiolabelled metanauplii ingestion, incorporated radioactivity into octopus paralarvae TL was only recovered as the original labelled FAs (18:3n-3, ARA, EPA or DHA), with no elongated, desaturated or *de novo* products being detected (Table 7.6).

Table 7.6 – Recovery of radioactivity (%) from [1-^{14}C]FA substrates after 12h incubation period in *Artemia* sp. and after metanauplii ingestion and digestion in *O. vulgaris.*

Substrates	Products	*Artemia* sp.	*O. vulgaris*
[1-^{14}C]18:3n-3			
	18:3n-3	87.0 ± 5.2^{b}	100.0 ± 0.0*
	de novo	13.0 ± 5.2	-
[1-^{14}C]20:4n-6			
	20:4n-6	96.4 ± 0.7^{a}	100.0 ± 0.0*
	de novo	3.6 ± 0.7	-
[1-^{14}C]20:5n-3			
	20:5n-3	94.8 ± 1.4^{ab}	100.0 ± 0.0*
	de novo	5.2 ± 1.3	-
[1-^{14}C]22:6n-3			
	22:6n-3	66.2 ± 4.3^{c}	100.0 ± 0.0*
	de novo	29.6 ± 2.9	-

Results represent means ± SD (n = 4 for *Artemia* sp. metanauplii and n = 8, for *O. vulgaris* paralarvae). * represent significant differences within unmodified FA between species ($p < 0.05$). Different superscript letters in the same column represent differences between unmodified FA within the same species ($p < 0.05$). *de novo* - synthesis of new shorter chain-length labelled fatty acids (less than 18 carbons).

7.5 Discussion

Artemia sp. is known to possess an active FA metabolism (Evjemo et al., 1997; Estévez et al., 1998; Guinot et al., 2013a; McEvoy et al., 1995; Navarro et al., 1999), presenting the capacity to convert phospholipids into TAG (Rainuzzo et al., 1994; Guinot et al., 2013a) and to modify fatty acids chains (Navarro et al., 1999). Moreover, this species displays a preferential catabolism over DHA compared to other FAs (Chapter 6; Evjemo et al., 1997; Estévez et al., 1998; Navarro et al., 1999), which hampers its enrichment

with this FA. Similarly to those studies, the present results showed a preferential catabolism of DHA, which was translated into (1) the lower incorporation of this FA into *Artemia* sp. TL (Table 7.3); (2) the highest amount of FAs with a chain-length shorter than 18 carbons synthesised *de novo* by the recycling of the labelled carbon (acetyl-CoA) obtained from DHA oxidation (Table 7.6). Nonetheless, a *de novo* synthesis of shorter chain-length FAs was also verified from all incubated substrates (Table 7.6), indicating *Artemia* sp. the catabolism of all labelled FAs, similarly to that reported to occur during starvation conditions (Evjemo et al., 1997; Estevéz et al., 1998). In order to determine *Artemia* sp. basal metabolism of specific FAs, in the present study incubations were performed without the addition of other FAs or nutrients. Therefore, the detected catabolism of incubated substrates could be related to starvation conditions (see Evjemo et al., 1997; Estévez et al., 1998 and Navarro et al., 1999).

Compared to *Artemia* sp., wild zooplankton display a content with lower total lipids but higher contents of phospholipids and EFA (Evjemo et al., 2003; Iglesias et al., 2014; McEvoy et al., 1998; Olsen et al., 2014; Reis et al., 2015),which could at least partly explain the better results obtained in the rearing "new cultured species", including larvae of Atlantic halibut (Evjemo et al., 2003; Næss et al., 1995) and Atlantic cod (Busch et al., 2010; Imsland et al., 2006) and common octopus paralarvae (Iglesias et al., 2014; Reis et al., 2015). Nonetheless, the better growth and survival results obtained when wild zooplankton is provided to larvae might go beyond the amount of EFA or PL, as the presence of EFA in specific lipid classes, such as the PC or PE and the total or individual LC-PUFA and ratios, are also essential factors for marine larvae performance (Olsen et al., 2014; Sargent et al., 1999). Despite the preferential incorporation of all substrates into *Artemia* sp. polar lipid fraction, both 18:3n-3 and DHA presented a significant esterification rate into metanauplii TAG, which corresponded to more than 20% of total incorporated radioactivity of each substrate. Guinot et al. (2013b) and Navarro et al. (1999) also observed a high proportion of DHA into *Artemia* sp. TAG. It has been previously reported that the most adequate form to present DHA to marine fish larvae is through the PL (Gisbert et al., 2005; Wold et al., 2009). Consequently, several attempts to increase the amount of DHA into *Artemia* sp. PL have been made (Guinot et al., 2013a; Harel et al., 1999; McEvoy et al., 1996; Monroig et al., 2006, 2007; Rainuzzo et al., 1994; Seixas et al., 2008; 2010b). Nonetheless, the inherent translocation of DHA from phospholipids of the enrichment diet to the neutral lipid fraction of *Artemia* sp., detected even during the first 4 h of enrichment, comprise an important handicap for its enrichment

with essential lipid compounds and consequently for their use as live prey in larviculture (Guinot et al., 2013b).

The enrichment of *Artemia* sp. with EPA or ARA, seems to be less difficult to that of DHA, since during the enrichment process these FAs tend to be most keenly incorporated than DHA (Estévez et al., 1998; Dhert at el., 1993; McEvoy et al., 1996). In similitude to those studies, the present results showed a higher incorporation rate of [1-^{14}C]ARA and [1-^{14}C]EPA into *Artemia* sp. metanauplii lipids over DHA or 18:3n-3. Moreover, both EPA and ARA were preferentially esterified into PL, which theoretically would favour these FAs bioavailability to the larvae. The amount of radiolabelled substrates incorporated into *O. vulgaris* paralarvae TL while fed with labelled *Artemia* sp. metanauplii were extremely low for 18:3n-3 and DHA, which hampered the analysis of the results obtained during the present study. Nonetheless, and although no deep conclusions can be made through these results, some considerations can be taken regarding the novel assayed and the effect of *Artemia* sp. endogenous FA metabolism in the availability of EFA for *O. vulgaris* paralarvae.

Since *O. vulgaris* shows an incapacity to biosynthesize LC-PUFA from C18 FAs precursors, ARA, EPA and DHA are considered EFA for *O. vulgaris* development (Monroig et al., 2012a; Chapter 3; Reis et al., 2014). During the first days of life, octopus paralarvae lipid profile is highly influenced by prey composition (Reis et al., 2015) consequently, prey lipid composition supplied during the first day after hatching might be crucial to octopus development, defining the future performance of this species culture. In the present study, *Artemia* sp. presented a high incorporation of ARA and EPA. Interestingly, the esterification pattern of [1-^{14}C]ARA between *Artemia* sp. and octopus paralarvae tissues presented some differences, being the most distinguished one the preferential esterification of this FA into octopus PE, rather than PI as detected in metanauplii (Table 7.4) or fish (Bell and Sargent, 2003). On the other hand [1-^{14}C]EPA, presented a similar pattern to that of metanauplii, with this FA being mainly esterified into PC followed by PE. The preferential esterification of ARA and EPA into PE and PC of octopus has been previously reported by Reis et al., (Chapter 3; 2014), although on that study both FAs were mainly esterified into PE, with more that 45 % of incorporated radioactivity being recovered in this LC. The small differences detected between the present study and the ones reported by Reis et al., (Chapter 3; 2014), in ARA and EPA esterification pattern, might be related with a competition between FAs for re-acylation into paralarvae lipids, as reported by Sargent et al. (1999) to occur in fish. Despite the

low amount of [1-^{14}C]ARA and [1-^{14}C]EPA recovered into octopus paralarvae TL, the present results suggest that *Artemia* sp. metabolism may not interfere in ARA and EPA bioavailability for octopus paralarvae, in the same way as it does for DHA.

It was suggested that marine fish larvae have a limited capacity to metabolise FA from TAG (Sargent et al., 1999; Olsen et al., 2014). Consequently, a higher bioavailability of LC-PUFA for fish would be obtained from phospholipids rather than from TAG (Olsen et al., 2014). Considering an analogy between fish larvae and octopus paralarvae, it would be likely that an association of EFA with TAG of live preys could negatively influence the EFA availability for normal development of this species. In the present study, despite the high amount of [1-^{14}C]18:3n-3 incorporated into *Artemia* sp. TL, the FAs with the highest esterification rate into *Artemia* sp. TAG (18:3n-3 and DHA) were also the lowest incorporated into paralarvae TLs. Therefore, the present results may suggest that *O. vulgaris* paralarvae have a potential low capacity to metabolise dietary TAG. However, these results may also be related to *Artemia* sp. own metabolism in the 24h rearing period. Even during the 12 h of incubation, the catabolism of all substrates, with a higher activity over DHA, was detected. Therefore, considering the paralarvae rearing time of 24 h employed during the present study, the further catabolism of these FAs during this period should also be considered. *Artemia* sp. kept under starvation conditions mobilize FAs esterified into NL including 18:3n-3 and DHA, as substrates for energy production, structure maintenance, and growth (Navarro et al., 1997). On the other hand, FAs esterified into *Artemia* sp. PL fraction, like EPA and ARA, tend to be conserved or catabolised in lower rates compared to DHA (Estévez et al., 1998), which could also explain the different FA incorporation rates obtained in octopus paralarvae (Table 7.3).

The capacity of *O. vulgaris* to metabolise dietary FA trough elongation/desaturation processes has been recently confirmed (Monroig et al., 2012a, 2012b; Chapter 3; Reis et al., 2014). Nonetheless, in the present study no enzymatic activity was noticed towards dietary labelled substrates. These data do not totally invalidate octopus capacity to transform dietary FAs, as it is possible that the band obtained would be unnoticed on TLC plate due to the low amount of radioactivity incorporated into paralarvae TL or the low elongation/desaturation rates recorded in this species (Monroig et al., 2012a, 2012b; Chapter 3; Reis et al., 2014). Interestingly, although a high *de novo* synthesis in *Artemia* sp. towards all substrates was detected, the radioactivity incorporated into paralarvae lipids was recovered in their original form (18:3n-3, ARA, EPA and DHA). This was possibly due to the verified low incorporation of labelled FAs into octopus TL.

Future studies considering different incubations/predation times should be performed in order to increase radioactivity incorporation in paralarvae tissues and to better verify the presented hypothesis. Furthermore, different *Artemia* sp. incubation conditions considering different incubation periods and the use of enrichment products, should be also tested in order to avoid possible metanauplii starvation during the labelling and post-rearing processes, and to better define the influence of the *Artemia* sp. endogenous lipid metabolism on EFA bioavailability for octopus paralarvae and other marine species.

Chapter 8.

Discussion and Final Considerations on *Octopus vulgaris* and *Sepia officinalis* lipid metabolism during their early life stages

The main objective of the present thesis was to provide a better knowledge on *Octopus vulgaris* and *Sepia officinalis* lipid requirements during their early life stages. To that purpose, the first approach was to design a suitable method for the *in vivo* study of cephalopods lipid metabolism during their early live stages. The established method (see Chapter 2 for details) was adapted from the *in vitro* FA metabolism studies (Díaz-López et al., 2010; Rodríguez et al., 2002) and consisted in the incubation of hatchlings in 10 mL of filtrated seawater (36 ‰), during 4 - 6 hours at 21 ºC and gentle stirring, in flat-bottom 6 wells cell culture plates and with 0.3 µM of [1-^{14}C]FA added to the water as their potassium salts bound to BSA. An adaptation of this methodology was latter applied according to species and/or radiolabelled substrate studied. The method developed allowed the study not only of the *in vivo* capability of *O. vulgaris* and *S. officinalis* hatchlings to transform the incubated FAs by elongation/desaturation processes (Chapters 3 and 4), but also to determine their capability to remodel dietary phospholipids (Chapter 5). Furthermore, through this methodology it was also possible to determine the esterification pattern of incubated FAs into the different lipid classes (Chapters 3 and 4), being detected a comparable pattern between radiolabelled FAs esterification and the FA composition of the main phospholipids of octopus and cuttlefish hatchlings (Chapter 5). Finally, this method allowed to determine the *in vivo* FA metabolism of *Artemia* sp. metanauplii and *Grapsus adscensionis* zoeae as potential live prey in *O. vulgaris* paralarvae rearing (Chapter 6) and to study the effect of live preys' endogenous FA metabolism in the bioavailability of dietary EFA for octopus paralarvae development (Chapter 7). Considering the suitablility of the developed methodology to determined the *in vivo* FA metabolism of different species, this method might be also a useful tool to determine not only the endogenous FA metabolism, but also the effect of rearing conditions (e.g. diet, temperature, salinity) in the *in vivo* FA metabolism of cephalopods, live preys like *Artemia* sp. or rotifers and other marine and freshwater species.

In order to provide a better knowledge on *Octopus vulgaris* and *Sepia officinalis* lipid requirements during their early life stages, aside from the FA metabolic studies, the body lipid profiles of these species hatchlings were also determined. Similarly to that previously reported, both species total lipids were particular rich in 16:0, EPA and DHA (Bouchaud and Galois, 1990; Navarro and Villanueva, 2000; Navarro et al., 2014; Reis et al., 2015). Interestingly, ARA was also one of the most significant FA in octopus, but not in cuttlefish total lipids (Chapters 3, 4 and 5). Moreover, differently from *S. officinalis*, *O. vulgaris* hatchlings presented a preferential incorporation of ARA into specific lipid

classes (Chapters 3 and 4), which could point out to a different requirement of octopus and cuttlefish for this FA.

Previous studies have suggested that the high levels of ARA on *O. vulgaris* tissues were not related with a dietary intake of this FA, and that this species might have the capability to biosynthesise ARA (García-Garrido et al., 2010; Miliou et al., 2006). Although the capacity of octopus hatchlings to elongate [1-^{14}C]18:2n-6 and [1-^{14}C]20:2n-6 was detected (Chapter 3), no ARA biosynthesis was recorded.. This may indicate that, at least during the first stage of life, neither Δ6 Fad nor Δ8 Fad are active in *O. vulgaris* tissues. Monroig et al. (2012a) recorded the activity of a Δ5 Fad in *O. vulgaris* adult tissues. However, the activity of this enzyme in this species hatchlings was not detected in the present study, since the FA substrates that this enzyme is known to act upon were not tested or biosynthesised by octopus hatchlings (Fig. 8.1). Therefore, the results obtained indicate that *O. vulgaris* hatchlings are not capable to biosynthesis ARA from 18:2n-6, and considering that 20:3n-6 is a FA that is not largely found in octopus prey (Monroig et al., 2012a), ARA must be considered an EFA and so provided through the diet. In this sense, the high contents of ARA detected in octopus tissues, seem to be mostly related to a preferential incorporation of this FA, as observed in Chapters 3, 5 and 7 of the present thesis, rather than to its biosynthesis from shorter and/or less unsaturated FAs.

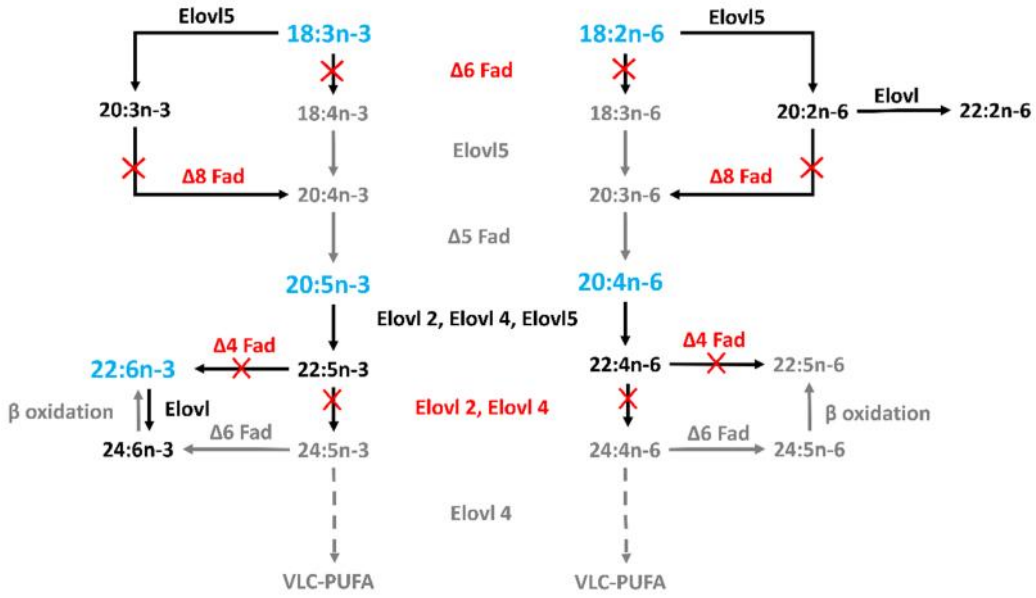

Figure 8.1 – Putative PUFA metabolism by elongation and desaturation pathways in *Octopus vulgaris* hatchlings (Chapter 3). Fatty acids in blue correspond to incubated substrates; Fatty acids and arrows in black correspond to detected desaturation/elongation products and pathways; Fatty acids and arrows in grey correspond to desaturation/elongation products and pathways not detected; Crossed pathways in red correspond to elongation/desaturation processes not verified; Dashed arrows correspond to further elongation/desaturation processes not shown.

S. officinalis hatchlings also presented a null capacity to biosynthesis ARA from 18:2n-6. However, cuttlefish hatchlings showed the capacity to desaturate [1-^{14}C]18:2n-6 (Fig. 8.2). According to the position of co-running standards, the potential resultant desaturation products from 18:2n-6 might involve a Δ6 Fad (Fig. 8.2). Nonetheless, unequivocal identification of the desaturation products obtained would be difficult, since the route involving an elongation to produce 20:2n-6, followed by Δ8 Fad to produce 20:3n-6 cannot be totally excluded (see Chapter 4). The activity of a Δ5 Fad in *S. officinalis* adult tissues was also very recently reported (Monroig et al., 2016). However, despite no Δ5 Fad activity was detected in the present study, the data recorded did not totally exclude a possible Δ5 Fad activity in ARA biosynthesis. Therefore, the absence of [1-^{14}C]ARA, when [1-^{14}C]18:2n-6 was incubated, might be more related with the low desaturation rates detected (Chapter 4; Monroig et al., 2016). Therefore the results of the present thesis indicate the possibility of *S. officinalis* hatchlings to biosynthesize ARA from 18:2n-6, including the existence of a Fad (either Δ6 or Δ8 Fad; Chapter 4). Nonetheless, considering the low desaturation rates detected, this process would not be sufficient to satisfy the demands for normal development of this species, and so ARA must also be considered as an EFA and supplied through the diet at least during the hatchling stage.

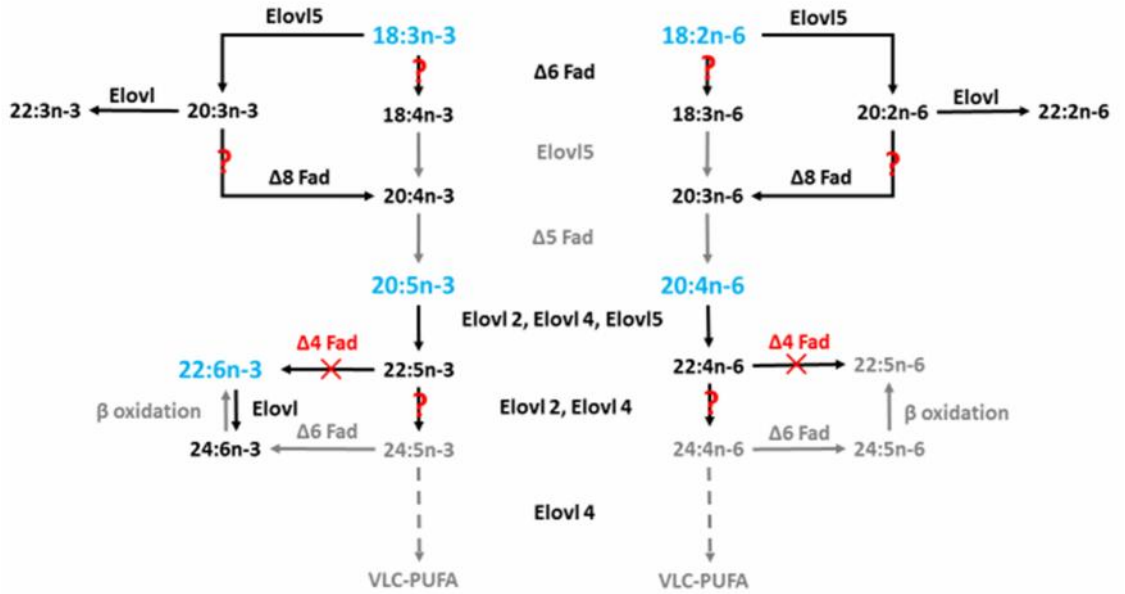

Figure 8.2 – Putative PUFA metabolism by elongation and desaturation pathways in *Sepia officinalis* hatchlings (Chapter 4). Fatty acids in blue correspond to incubated substrates; Fatty acids and arrows in black correspond to detected desaturation/elongation products and pathways; Fatty acids and arrows in grey correspond to desaturation/elongation products and pathways not detected; Interrogation marks in red correspond to pathways not totally confirmed or rejected; Crossed pathways in red correspond to elongation/desaturation processes not verified; Dashed arrows correspond to further elongation/desaturation processes not shown.

The enzymatic pathways necessary to biosynthesise EPA from 18:3n-3 are the same as for the biosynthesis of ARA from 18:2n-6 (Fig. 8.1 and 8.2; Tocher, 2015). Therefore, results obtained in Chapters 3 and 4 regarding *O. vulgaris* and *S. officinalis* hatchlings EPA biosynthesis were highly similar to those of ARA biosynthesis. In this sense, similarly to ARA, EPA must be considered as an EFA for octopus or cuttlefish development, at least during their first life stages.

Regarding the DHA synthesis from EPA, in vertebrates there are two possible metabolic pathways: the "Sprecher pathway", which comprises two sequential elongations of EPA to 24:5n-3, followed by a Δ6 desaturation and one round of peroxisomal β-oxidation (Sprecher, 2000); or a direct route involving the elongation of EPA to 22:5n-3 followed by a Δ4 desaturation (Li et al., 2010). When EPA was incubated, no DHA was obtained, being only recorded a single elongation step in both *O. vulgaris* and *S. officinalis* hatchlings (Chapter 3 and 4). Although a possible Δ6 Fad has been reported for *S. officinalis*, no desaturation product was obtained from [1-^{14}C]EPA in sepia hatchlings. The data of the present thesis do not totally rule out a possible Δ4 or Δ6 Fad activity in DHA biosynthesis, since the absence of this FA could be related with the low EPA elongation rate observed (Chapters 3 and 4), however this activity would not be sufficient to cover the demands for this FA, and so in addition to ARA and EPA, DHA (see Fig. 8.1 and 8.2) must also be considered as EFA for the normal development of *O. vulgaris* and *S. officinalis*. Nonetheless, the expression of desaturase and elongase genes can be regulated in response to dietary FAs composition, being this correlated with LC-PUFA synthesis activity (Tocher et al., 2010). In this sense, further studies, regarding diet effect on LC-PUFA synthesis in *O. vulgaris* and *S. officinalis* during early development stages, using a similar *in vivo* methodology to that of this study, might further clarify these species FAs dietary requirements.

In order to define the lipid requirements of a given species it is important to determine not only the absolute requirements of specific PUFAs, but also the optimal balance between the different PUFAs, and the suitable form to provide those FA as these could affect the availability of EFA (Sargent et al., 1999). In Chapters 3 and 4, the esterification patterns of unsaturated FAs into the different lipid classes of octopus and cuttlefish hatchlings were determined. The general esterification pattern of ARA and EPA within octopus and cuttlefish lipid classes was highly similar, with the majority of radioactivity being recovered as PE. Similarly to that reported in marine fish (Atalah et al., 2011a; Furuita et al., 2003), an inverse relation between ARA and EPA incorporation into

O. vulgaris tissues was also detected by Miliou et al. (2006) and Reis et al. (2015). This inverse relation could possibly be associated with a competition between these two LC-PUFA for acylases and transacylases, enzymes responsible for FA esterification into phospholipids, which do not have tight specificity for a particular dietary FA (Sargent et al., 1999). Therefore, an unbalanced dietary EPA/ARA ratio could negatively affect the proper EPA/ARA ratio in *O. vulgaris* or *S. officinalis* tissues, hampering these species normal development. Both EPA and ARA have unique roles in controlling and regulating cellular metabolism and animals' physiology (Tocher et al., 2010). Moreover, these FAs are known to compete not only for cyclo-oxygenases and lipoxygenases in eicosanoids production, but also for elongase and desaturase enzymes in FAs biosynthesis pathways (Sargent et al., 1999). In addition to those competitions, ARA and EPA presented a similar esterification pattern into octopus hatchlings lipid classes (Chapters 3 and 4), which may lead these FAs to a further competition for acylases and transacylases activity in FAs esterification into phospholipids. Therefore, an unbalance within these may FAs could impair octopus and cuttlefish development. Considering the detected preferential incorporation of ARA into octopus TLs over all the other FAs (Chapter 3, 5 and 7), it is likely that the quantitative requirements of octopus for ARA may not differ markedly from those of cuttlefish, as a low dietary EPA/ARA ratio might lead to a greater incorporation of ARA, promoting therefore an even lower EPA/ARA ratio into octopus tissues, which could impair octopus development.

DHA is normally the main FA of octopus or cuttlefish hatchlings whole tissue lipids (Chapters 3, 4, 5 and 7) and, since no DHA biosynthesis was recorded (Chapters 3 and 4) the dietary requirements of these species for this FA might be relatively high. DHA, similarly to C18 FA, presented a high esterification rate into PC of *O. vulgaris* and *S. officinalis* hatchlings (Chapters 3 and 4), which could lead to the previously mentioned competition between FAs for acylases and transacylases activity. Therefore, the dietary DHA/C18 FAs ratio must also be considered while designing a suitable diet for cuttlefish hatchlings or octopus paralarvae. Despite the observed preferential esterification of DHA into PC of both species (Chapters 3 and 4), the analysis of the FA content of octopus and sepia main phospholipids showed that, with the exception of PI, all phospholipids presented a high content of DHA. Interestingly, alongside with DHA, a high content of ARA and EPA was also detected in PE (Chapter 5). Therefore, the dietary DHA/EPA/ARA ratio would be of greater importance in these species development and so the LC-PUFA/C18 FA ratio might also be crucial. *Artemia* sp., one of the most

commonly used live preys' for octopus paralarvae rearing, often presents very high C18 FAs, particularly 18:3n-3, and low LC-PUFA contents, even after enrichment processes (Fuentes et al., 2011; Navarro and Villanueva, 2000; Seixas et al., 2010a, 2010b; Viciano et al., 2011). As a consequence, the dietary FA pool available for phospholipids re-acylation process would present high C18 FA and low LC-PUFA contents, which could lead to a lower LC-PUFA content in octopus paralarvae lipids and to an inappropriate tissue phospholipids structure. In fact, lower levels of DHA are normally found in *O. vulgaris* paralarvae reared with enriched *Artemia* sp. (Fuentes et al., 2011; Navarro and Villanueva, 2000; Seixas et al., 2010a, 2010b; Viciano et al., 2011), which could be related to the competition between this FA with C18 FAs for phospholipids re-acylation.

The de-acylation/re-acylation turnover process have an important role in maintaining the characteristic FA composition of lipid classes (Tocher et al., 2003), being this highly influenced by dietary FA profile and the endogenous capability of the organism to complete this process (Olsen et al., 2014). Both *O. vulgaris* and *S. officinalis* hatchlings were found to possess the necessary enzymatic chain for the de-acylation of dietary phospholipids and re-acylation of FFA into lyso-phospholipids to produce phospholipids with a new structure (Chapter 5). Despite the lipid class to which [1-^{14}C]ARA was bound (PC or PE), the esterification pattern obtained after re-acylation in *O. vulgaris* lipids was similar to that reported in Chapter 3, where a higher esterification of [1-^{14}C]ARA into PE was detected. In contrast, in *S. officinalis* hatchlings, ARA was mainly recovered in the same LC that the FA was supplied (Chapter 5).

Although further studies are necessary to determine specific phospholipase activities, the present results suggest a different capability of *O. vulgaris* and *S. officinalis* hatchlings for phospholipids remodelling. LC-PUFA are usually esterified at sn-2 position of the phospholipids molecule (Sargent et al., 1999). Therefore, the high PLA_2 activity recorded in *O. vulgaris* hatchlings would cleavage the ester bound of LC-PUFA with dietary phospholipid molecule, with these FAs making part of the pool of FFA available for further re-acylation. Similarly to phospholipids, it is assumed that LC-PUFA are also mainly esterified into the sn-2 position of TAG in fish (Sargent et al., 1999). Nonetheless, TAG digestive enzymes (1-3 lipases) normally remove the FAs from sn-1 and sn-3 position, with LC-PUFA being retained in the monoacylglyceride molecule (Olsen et al., 2014; Tocher et al., 2003). Therefore, after lipids de-acylation, the FFA pool would contain LC-PUFA from phospholipids but also saturated, monounsaturated and

polyunsaturated FA from TAG. If the diet provided to octopus paralarvae presents high TAG and low phospholipids levels, the proportion of saturated, monounsaturated and polyunsaturated FAs from TAG de-acylation available for re-acylation would be higher than the proportion of LC-PUFAs from phospholipids de-acylation. Consequently, the probability for re-acylation of a LC-PUFA into phospholipids would diminish, affecting tissue phospholipids structure, which could influence phospholipids specific role in membrane fluidity and function (Tocher et al., 2008). In this sense, taking into account these considerations and the very low proportion of TAG in octopus tissues, it seems highly important to contemplate the phospholipid/TAG ratio of live prey or formulated diet for *O. vulgaris* paralarvae culture at least during the paralarvae stage. On the other hand, *S. officinalis* turnover data indicate an apparent lower PLA_2 enzyme activity when compared to *O. vulgaris* hatchlings that might be related to the hatchlings digestive system immaturity (Boucaud-Camou and Yim 1980; Yim and Boucaud-Camou 1980; Boucaud-Camou 1982). However, it is also possible that phospholipid de-acylation in cuttlefish hatchlings might be at least partially performed by alternative enzymes like phospholipase A_1 (PLA_1) or 1-3 lipase that cleavage the sn-1 position of phospholipids. Further studies are required to determine possible activities and roles of these enzymes in phospholipid digestion of *S. officinalis*. Nonetheless, the action of these enzymes towards the labelled phospholipids would preserve the radiolabelled ARA in sn-2, and so re-acylated phospholipid would still contain the label, as observed in Chapter 5. Due to the preservation of LC-PUFA into phospholipids skeleton, the interference of dietary TAG content on re-acylation process in *S. officinalis* might be less critical than that suggested to occur in *O. vulgaris*.

Artemia sp. presents a low phospholipids/TAG ratio, with TAG representing over 50 % of *Artemia* sp. total lipid content (Chapter 6 and 7). As previously mentioned, this could hamper phospholipids remodelling process and consequently their structure and function (Tocher et al., 2008), affecting octopus normal development. Decapod crustacean zoeae, on the other hand, normally possess a much higher content of PL and LC-PUFA compared to *Artemia* sp. (Chapter 6; Navarro and Villanueva, 2000; Iglesias et al., 2014; Reis et al., 2015), which could in part explain the better survival and growth rates obtained when decapod crustacean zoeae were provided as prey (Iglesias et al., 2004, 2007, 2014; Moxica et al., 2002; Reis et al., 2015). Similar rearing results have been obtained in "new culture species" like the Atlantic halibut or the Atlantic cod, when fed with wild zooplankton and compared with those obtained with *Artemia* sp. (Busch et al., 2010;

Evjemo et al., 2003; Imsland et al., 2006; Næss et al., 1995). In addition to the higher contents of PL and LC-PUFA, *G. adscensionis* zoeae also displayed a FA metabolism that seemed to favour the use of zoeae over *Artemia* sp. as prey for *O. vulgaris* paralarvae (Chapter 6). *Artemia* sp. is known to process an active FA metabolism presenting the capacity to convert phospholipids into TAG (Rainuzzo et al., 1994; Guinot et al., 2013a) and to retro-convert DHA into EPA (Navarro et al., 1999). Moreover, this species displays a preferential catabolism over DHA compared to other FAs (Chapters 6 and 7; Evjemo et al., 1997; Estévez et al., 1998; Navarro et al., 1999), which hampers its enrichment with this FA. On the other hand, while analysing the esterification pattern of [1-^{14}C]LC-PUFA into zoeae LC, a higher esterification of these FAs into the polar lipid fraction was observed, with 78.5 % of DHA being recovered esterified into these classes. Moreover, the catabolism of labelled FAs was lower in crustacean zoeae when compared to *Artemia* sp., and mainly over C18 FAs (Chapter 6). The results clearly demonstrate the existence of two very distinct models of lipid metabolism between *Artemia* sp. and *Grapsus* zoeae, illustrating the inherent difficulties for an efficient EFA enrichment of *Artemia* sp. as food for marine organisms.

When octopus paralarvae where fed with labelled *Artemia* sp. a higher incorporation of [1-^{14}C]ARA and [1-^{14}C]EPA into *O. vulgaris* paralarvae total lipids was dected, while the incorporation of [1-^{14}C]DHA and [1-^{14}C]18:3n-3 was almost vestigial (Chapter 7). In addition to the preferential catabolism of DHA (Chapters 6 and 7; Evjemo et al., 1997; Estévez et al., 1998; Navarro et al., 1999), this FA and 18:3n-3 were also mainly esterified into *Artemia* sp. TAG (Chapters 6 and 7). It has been previously reported that the most adequate form to present DHA to marine fish larvae is through PL (Gisbert et al., 2005; Wold et al., 2009), as they have a limited capacity to metabolise FAs from dietary TAG. Considering a possible analogy between marine fish larvae and cephalopods, the low values of DHA and 18:3n-3 in octopus lipids may be related with the presence of these FAs in *Artemia* sp. TAG. On the other hand, ARA and EPA were mainly esterified into the polar lipid fraction of *Artemia* sp., which may have increased their bioavailability to paralarvae. However, these results may also be related to *Artemia* sp. FAs metabolism during the 24h of paralarvae rearing period that leaded to a further catabolism of those FAs. Navarro et al. (1997) reported the mobilization of FAs esterified into NL as substrates for energy production, structure maintenance, and growth of *Artemia* sp.; while FAs esterified into polar lipids, like EPA and ARA, tend to be conserved or catabolised in lower rates compared to DHA (Estévez et al., 1998). The latter could also explain the

different FA incorporation rates obtained in octopus (Chapter 7). Nonetheless, future studies considering different incubations/predation times should be performed in order determine the possibility of increasing the radioactivity amount recovered in paralarvae tissues and verify the presented hypothesis.

The method developed during this thesis allowed to unveil some of the metabolic fates of unsaturated FAs, clarifying some aspects of lipid requirements of *O. vulgaris* and *S. officinalis* during their early stages. The results obtained showed that, despite of the different capacity of *O. vulgaris* and *S. officinalis* hatchlings to desaturate/elongate FAs, LC-PUFA such as ARA, EPA and DHA must be considered as EFA for both cephalopod species development. On the other hand, each species presented a different capability to remodel dietary phospholipids that may influence both species dietary requirements. While the phospholipid/TAG ratio is likely to be of great importance for *O. vulgaris* nutrition, TAG interference in *S. officinalis* might be less critical due to the apparent preservation of dietary LC-PUFA association with their phospholipid skeleton. Considering these results, future research in *O. vulgaris* and *S. officinalis* lipid requirements at early live stages, should include the study of specific phospholipase and other digestive enzymes activities in these species. Moreover, further *in vivo* studies using other radiolabelled molecules including TAG and phospholipids with a different molecular structure to those assayed during the present thesis, should be considered in order to complement the *in vitro* and rearing studies. The results of the present thesis suggest an influence of *Artemia* sp. endogenous metabolism in EFA bioavailability, with a highest effect over DHA. Future studies considering different periods of incubation (6 to 8 h) and predation (10 to 14 h) should be performed in order determine the possibility to increase radioactivity amount in paralarvae tissues and avoid metanauplii starvation, allowing this way to verify the presented hypothesis. Considering the capability of *O. vulgaris* paralarvae to incorporate fatty acids and amino acids directly from water, to test the possibility of using dissolved organic matter in alternative to *Artemia* sp. as a way to supply DHA and phospholipids for this species during rearing conditions should be tested.

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