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Faculdade de Ciências e Tecnologias

STUDY OF THE EXPRESSION OF DIFFERENT GENES OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS (LC-PUFA) METABOLISM DURING THE EARLY PARALARVAL DEVELOPMENT OF THE COMMON OCTOPUS (*Octopus vulgaris*)

Joana Maria Teixeira de Moura

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Study of the expression of different genes of long-chain polyunsaturated fatty acids (LC-PUFA) metabolism during the early paralarval development of the common octopus (*Octopus vulgaris*)

Joana Maria Teixeira de Moura

Dissertation

Master in Aquaculture and Fisheries

Master's thesis co-ordinate by:

- Elsa Cabrita, PhD, Faculty of Sciences and Technology (FCT) and Centre of Marine Sciences (CCMAR), University of Algarve, Faro, Portugal
- Francisco Hontoria Danés, PhD, Instituto de Torre de la Sal (IATS-CSIC), Castellón, Spain

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- **Declaração da autoria do trabalho**

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

Joana Maria Teixeira de Moura

Abstract

The common octopus, *Octopus vulgaris,* presents characteristics as high market price, high fecundity, short life cycle, rapid growth and high food conversion rates, which make this species a potential target for future aquaculture. However, two main problems have been pointed as the main causes for the high mortalities observed in the culture of this cephalopod: (i) the lack of standardized culture conditions and (ii) the absence of an appropriate diet that fulfil all the nutritional requirements of paralarvae. Particularly, the lack of a balance in lipid and fatty acid composition such as a deficiency in long-chain polyunsaturated fatty acid (LC-PUFA) has been pointed out as one of the main problems. Additionally, it has been proved that the early life stages of octopus paralarvae have high PUFA requirements. LC-PUFA are considered key factors for a suitable growth during planktonic life of octopus and are biosynthesized by enzymes called desaturases and elongases. In the present study, our main objective was to study the expression of the genes encoding for the Stearoyl-CoA Desaturase with Δ9 activity (Scd), the Fatty acyl desaturase with Δ5 activity (Fad) and the Elongases of Very Long-Chain fatty acids (Elovl5 and Elovl4), all them involved in the LC-PUFA metabolism. The expression of these genes has been analyzed through quantitative PCR (qPCR) from hatching on (day 0) and at days 5, 10, 15 and 20 of paralarval development. These specific genes are already characterized for the adult octopus. Our results revealed the presence of all these enzymes in paralarvae during all the studied period. Moreover, despite some exceptions, a tendency of an increase of the expression of these genes from day 0 to day 20 is observed. This could suggest that each enzyme is incorporated/biosynthesized when the development becomes more complex and/or when this endogenous biosynthesis capacity appears as a response to a poor diet, in order to fulfill the nutritional requirements. The high expression at day 0 observed in *scd* and *elovl5* could also indicate that the activation of the zygote genome can occurs during the embryonic development of the common octopus*,* although further research will be necessary. In conclusion, this study elucidates and clarifies how these genes are expressed in octopus paralarvae during the first days of development. It will allow to fill the knowledge gaps that still exist on the culture of *Octopus vulgaris,* possibly contributing to reduce the high mortalities observed in the early paralarval

development, and, at the same time, to help to identify the essential fatty acids for paralarvae stages.

Keywords: *Octopus vulgaris* paralarvae, LC-PUFA, s*cd*, *fad*, *elovl4*, *elovl5*, qPCR.

Sumário

O polvo comum, *Octopus vulgaris*, apresenta características como o alto preço no mercado, altas taxas de fecundidade, curto ciclo de vida, crescimento rápido e altas taxas de conversão de alimento, que fazem desta espécie um grande potencial para a aquacultura. Contudo, dois grandes problemas têm sido apontados como as principais causas para as altas mortalidades observadas no cultivo deste cefalópode: (i) a falta de condições padrão no seu cultivo e (ii) a ausência de uma dieta que complete todas as necessidades nutricionais desta paralarva. Particularmente, é observado uma falta de equilíbrio na composição de lípidos e ácidos gordos na dieta, como a deficiência em ácidos gordos polinsaturados de cadeia longa (AGP-CL). Para além disso, tem vindo a ser provado que os estádios primários das paralarvas de polvo apresentam grandes necessidades em ácidos gordos polinsaturados (AGP). Os ácidos gordos polinsaturados de cadeia longa são considerados fatores chave para um bom crescimento durante a vida plantónica do polvo e são biosintetizados por enzimas designadas de desaturases e elongases. O presente estudo teve como principal objectivos o estuda da expressão dos genes que codificam a Estearoil-CoA Dessaturase com actividade Δ9 (Scd), a dessaturase de ácido gordo com actividade Δ5 (Fad) e as Elongases de ácidos gordos de cadeia longa Elovl5 e Elovl4, envolvidos no metabolismo dos AGP-CL. A expressão destes genes foi analisada através de PCR quantitativo (qPCR) a partir da eclosão (dia 0) e aos dias 5, 10, 15 e 20 do desenvolvimento das paralarvas. Estes genes em específico já foram anteriormente caraterizados em polvo adulto. Os resultados revelaram a presença de todas as enzimas nas paralarvas durante o período de tempo estudado. Além disso, e salvo algumas exceções, é observado um aumento da expressão destes genes desde o dia 0 até ao dia 20. Sugere-se que esta tendência pode ser explicada pelo facto das enzimas serem incorporadas/biosintetizadas à medida que o desenvolvimento se torna mais complexo e/ou quando a capacidade endogena de biosintetizar estas enzimas pode aparecer como resposta a uma dieta nutricionalmente pobre, de forma a preencher as necesidades nutricionais destas paralarvas. Foi também verificada uma elevada expressão dos genes *scd* e *elovl5* ao dia 0, podendo indicar a ativação do genoma do zigoto durante o periodo embrionário do polvo comum, embora seja necessária mais pesquisa nesta linha de investigação. Concluíndo, este estudo permitiu

elucidar e clarificar como estes genes se expressam nas paralarvas de polvo durante os primeiros dia de desenvolvimento, podendo ajudar na identificação dos ácidos gordos essenciais de cada estádio das paralarvas. Ao mesmo tempo, veio aumentar o conhecimento do cultivo da espécie de polvo, *Octopus vulgaris*, contribuindo para reduzir as elevadas mortalidades observadas nos estádio iniciais de desenvolvimento das paralarvaras.

Palavras-chave: paralarvas de *Octopus vulgaris*, *scd*, *fad*, *elovl4* e *elovl5***,** PCRq**.**

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Index

Abstract IV
Keywords V
Sumário VI
Palavras-chave VII
Acknowledgements VIII
1. Introduction 1
1.1 Biology of the common octopus, *Octopus vulgaris* 1
1.2 Importance of *O. vulgaris* for the market and fisheries. Potential for aquaculture. 2
1.3 Culture of *Octopus vulgaris* 3
1.4 Biosynthesis of Polyunsaturated Fatty Acids (PUFA) 5
1.5 Nutritional requirements of *O. vulgaris* paralarvae and juveniles 11
1.6 Future research perspectives on octopus aquaculture 12
1.7 Main objectives 12
2. Material and Methods 14
2.1 *Octopus vulgaris* broodstock (IEO) 14
2.2 Rearing conditions of *Octopus vulgaris* paralarvae (IEO) 15
2.3 Sampling (IEO) 16
2.4 Principles of animal welfare used in this experiment 16
2.5 Sample preparation and production of standards (IATS-CSIC) 17
2.6 Expression of the genes *scd*, *fad*, *elovl5* and *elovl4* (IATS-CSIC) 20
2.7 Statistical analysis 21
3. Results 22
4. Discussion 24
5. Conclusion 28
6. References 29

1. Introduction

1.1 Biology of the common octopus, *Octopus vulgaris*

The common octopus belongs to the kingdom Animalia, phylum Mollusca, class Cephalopoda, order Octopoda, family Octopodidae, subfamily Octopodinae, genus *Octopus* and species *Octopus vulgaris*, Cuvier 1797 (Felley *et al.*, *2001*).

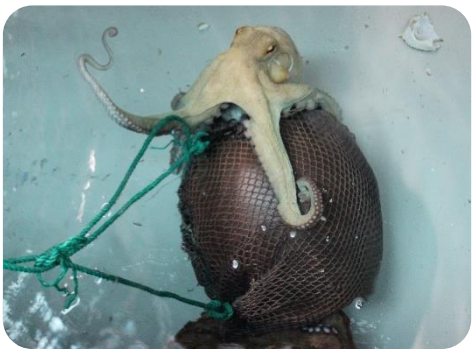


Figure 1.1 Adult common octopus, *Octopus vulgaris*, cultivated in IEO facilities (Tenerife).

It is a species with a worldwide distribution in temperate and tropical waters. It is benthic, occurring from 0 to 200 m depth in habitats as rocks, coral reefs and grass beds. Octopus can reach a maximum total length of 1.2 m in females and 1.3 m in males and the common weight is around 3 kg. The reproductive season of the common octopus depends on the geographical zone: a peak is observed in April-May most important in the Mediterranean and other peak in October most significant in Japan (Roper *et al.,* 1984).

Sexual dimorphism is not obvious, however males can have a few enlarged suckers on the arms used in a "sucker display" meant possibly for sexual identification. Males possess a modification of one arm, the hectocotylus, which helps the transportation of spermatophores to the female. Octopus are mostly solitary and thus little courtship behavior is observed before mating. This species copulates in one of two ways: a male leap upon a female, mounting her mantle, or a male sit near the female and extend the hectocotylus towards her. The fertilization occurs internally and the species is oviparous. When eggs are released, can be deposited by two methods: cemented individually to a hard substrate in the den, or individual eggs are intertwined with other eggs in clusters

of many tens and then cemented to a substrate (Halon and Messenger, 1998). This species lays as many as 100 000 – 500 000 eggs, each about 1.0 × 2.0 mm (Boyle and Roadhouse, 2005). After egg laying, the female never leaves the den and does not feed. She guards and cares for the eggs, investing amounts of time and energy cleaning and aerating them. This process can be as long as one to three months, perhaps a quarter of her life span. After hatching occurs, the female become emaciated and die soon (Halon and Messenger, 1997).

Octopus hatchlings have around 3 mm of total length and 2 mm of mantle length (see Figure 1.2). Contrarily to sub-adults and adults forms, in this phase octopus present a body size twice longer than the arms with 3 relatively large suckers in each arm. A relatively small size is a common characteristic of young Octopodidae that live for some time in the plankton (its first 5-12 weeks as an active predator) (Mangold, 1983). Thus, the term "paralarvae" was suggested designating an early development stage that resides in the near-surface plankton and that differs from later stages in habit, habitat and morphology (Young and Harman, 1988). After this period, it is possible to observe a gradual change to benthic life which means an attachment to a substrate and also crawling or walking around in search of prey (Boletzky, 1977), a similar behavior to the adult form.

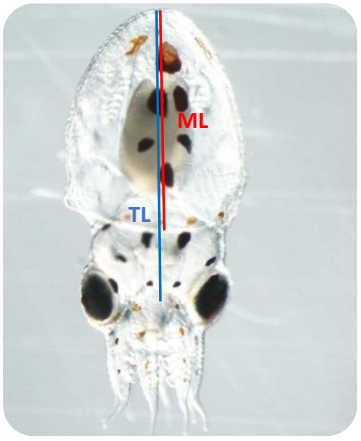


Figure 1.2 Newly-hatched *Octopus vulgaris* paralarvae. **ML** – mantle length, **TL** – total length (photo by Martim Magro).

1.2 Importance of *O. vulgaris* for the market and fisheries. Potential for aquaculture.

Different cephalopods have been used in several research areas, such as medicine and biology, due to their nervous system and sense organs, physiological, neurological, immunological, molecular biology and nutritional biochemistry (Lee, 1994; Oestmann, 1997). Furthermore, *Octopus vulgaris* is very appreciated and frequently consumed in Asia, Mediterranean and Latin-America, supporting industrial and artisanal fisheries. In markets, this species is found fresh, frozen, and whole or sliced. As the edible part is over 90% of its body size, makes this cephalopod a very interesting product (Iglesias and

Fuentes, 2014). Thus, the global captures of this species has been around 40 000 tones since 2009 to 2012 (FAO, 2013), being specifically identified in Spain, Portugal, Italy and Greece (Pierce *et al.*, 2010). However, as many other fish species, the octopus fishery has been overexploited in the past decades, which force the research on aquaculture techniques as an alternative source (Iglesias and Fuentes, 2014).

1.3 Culture of *Octopus vulgaris*

In addition to the high market price of the common octopus, the culture of this species is an interesting subject due to its high fecundity (Iglesias *et al*., 1997; Mangold, 1983), high protein content with around 70-90% dry weight of its body composition (Lee, 1994; O'Dor and Wells, 1987), short life cycle (12-18 months), rapid growth (>13% body weight per day) and high food conversion rates of 30% to 60%, depending on temperature and diet (García and Giménez, 2002; Mangold, 1983; Mangold and Boletzky, 1973).

The main problem associated to the culture of octopus is the high mortality rates that occur during the first 2 months of paralarval rearing. The lack of a standardized culture system and the absence of an appropriate diet that fulfil all the nutritional requirements of paralarvae have been pointed as the possible causes for this mortality (Iglesias *et al.*, 2007), specially the lack of a balance in lipid and fatty acid composition such as a deficiency in polyunsaturated fatty acids (PUFA) (Navarro and Villanueva, 2003, 2000). Despite some companies in the north-west of Spain (Galicia) have made attempts to culture the *O. vulgaris*, these efforts were limited to ongrowing subadult wild individuals, and the majority of them are now closed. The reasons are: (i) the impossibility to complete the biological life cycle in captivity at an industrial level; (ii) it is not possible to provide a mass production of subadults for ongrowing and the fisheries supply is not always guaranteed; (iii) there is no commercial diet available (García *et al.,* 2014).

Several years ago in Japan, a pioneer group had succeed in culturing octopus paralarvae using *Palaemon serrifer* zoeae as their main live prey. They obtained benthic juveniles at 33 days with a temperature of 24.7°C, reaching a survival rate of 5% at 60

days (Itami *et al.,* 1963). Iglesias *et al.,* (2004) achieved for the first time in Spain the complete culture cycle of *Octopus vulgaris* at an experimental level, using *Artemia* and spider crab zoeae as live preys.

Enriched *Artemia* nauplii have been recommended as live prey for the culture of octopus paralarvae due to its easy manipulation and availability together with copepods, crustacean zoeae or microdiets (Iglesias *et al*., 2007). *Artemia* nauplii are well accepted by octopus paralarvae and if they are enriched, they produce positive results until one month after hatching. However, *Artemia* is far from the adequate fatty acid requirements for *O. vulgaris* paralarvae, even enriched with microalgae (Reis, 2011), because of the lack of long-chain polyunsaturated fatty acids (LC-PUFA). Particularly very low DHA content (Seixas *et al.,* 2008) and low amounts of EPA (Navarro *et al*., 1992, 1993) are present in this type of food.

Zooplankton is naturally rich in phospholipids, cholesterol and n-3 LC-PUFA, such as DHA and EPA and ARA (Navarro and Villanueva, 2000; Bell *et al.,* 2003). Decapod crustacean zoeae are a primary prey used for the small-sized mouth of the octopus paralarvae, with which the most successful rearing results at laboratory scale are reached (Reis, 2011; Villanueva and Norman, 2008). However, this method is not applicable in aquaculture facilities due to its limited availability and its difficulty to culture at a commercial level (Iglesias *et al*., 2007) as well as, due to the risks of associated diseases (Bell *et al*., 2003).

Lately, the research lines have shifted to the use of artificial preys. Thus, the determination of the feeding requirements of the octopus paralarvae, comparing the biochemical profile of the cultured paralarvae with wild juveniles, and both natural and artificial preys are being done. Also the enzymatic and digestive capabilities of the paralarvae are being determined (Iglesias *et al.,* 2007). Paralarval rearing using enriched *Artemia* nauplii or *Artemia* biomass with dry or encapsulated microdiets revealed poor growth and high mortality, probably caused by a nutritional imbalance in the fatty acid profile, for example in DHA/EPA ratio (Navarro and Villanueva, 2003; Villanueva *et al.,* 2002).

In this way, zoeae cannot be produced at a commercial level and efforts to obtain a balanced microdiet with appropriate palatability and acceptance by the octopus paralarvae are being made. At the moment, *Artemia* seems to be the best alternative as

a live prey for the *O. vulgaris* paralarvae. Accordingly to this, it is necessary a continuous search for an ideal live prey or an *Artemia* enrichment that covers more efficiently the basic nutritional requirements and a formulation of suitable and digestive microdiet (Iglesias *et al.,* 2007).

1.4 Biosynthesis of Polyunsaturated Fatty Acids (PUFA)

Nutrition is a crucial factor in captive conditions both for research studies and aquaculture, allowing a proper growth and survival under mass conditions. Thus, it is necessary to know and understand the main pathways of the essential elements, the enzymes that are involved and which are the specific requirements for each species.

In the case of lipids, they are considered a key factor for a suitable growth during the planktonic life of octopus and thus, the knowledge of their biosynthetic pathways can help to reduce the high mortality rates observed in this species (Navarro *et al*., 2014).

One component of lipids are the fatty acids, which are defined as aliphatic chains containing a carboxylic (-COOH) and a methyl group ($-CH_3$) at their two ends. The fatty acids can be classified as saturated (no double bonds present), unsaturated (one double bond) and polyunsaturated (two or more double bonds). The designation of these compounds is based on their chain lengths, degree of unsaturation (number of double bonds) and the position of the double bonds in the aliphatic chain. For example, 14:0 and 16:0 are fatty acids with 14 and 16 carbon atoms, respectively, with no double bonds. The case of 18:1n-9 and 18:1n-7 designated as fatty acids with 18 carbon atoms whose single double bonds are 9 and 7, respectively, from the methyl end of the molecule. The terminology $18:1^{\Delta 9}$ and $18:1^{\Delta 11}$ is identical to 18:1n-9 and 18:1n-7, respectively. The Δ means the position of the double bond from the carboxyl end of the molecule. The trivial names such as palmitic acid 16:0, oleic acid 18:1n-9 and linolenic acid 18:3n-3 often reflect their common origin as in palm, olive and linseed oils, respectively. The more formalized name such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6N-3) reflect the number of carbon atoms (20 and 22) and the double bonds (5 and 6) they contain (Sargent *et al*., 2002). Moreover, the saturated and the unsaturated fatty acids can be endogenous synthesize by the living organisms and those which organisms cannot synthesize are called essential fatty acids,

for example some PUFA (polyunsaturated fatty acids) and thus, need to be obtained by the diet. The fatty acids are converted by the action of desaturase and elongase enzymes located in the endoplasmatic reticulum. Desaturases catalyze the introduction of a double bond into the acyl chain and elongases add two carbon atoms at the carboxyl group, i.e., elongating a pre-existing fatty acyl chain, as their name indicate (Monroig *et al.*, 2011; Sargent *et al*., 2002). The long-chain PUFA (LC-PUFA) have been pointed as essential nutrients for cephalopods, with relevant importance for the early-life stages (Navarro and Villanueva, 2000). They present two or more double bonds and 20 or more carbons (Monroig *et al*., 2011). The key-enzymes involved in the biosynthesis of this LC-PUFA are responsible for desaturation, that occurs by the action of Stearoyl-CoA desaturase (Scd) or by Fatty acyl desaturases (Fad) and elongations, that occurs by the action of Elongase of very long-chain fatty acid (Elovl) (Monroig *et al*., 2013c).

The fatty acid synthase (FAS), a cytosolic multifunctional enzyme complex present in all living organisms, is responsible for *de novo* biosynthesis of saturated fatty acids such as 16:0 (Palmitic acid) and 18:0 (Stearic acid). Thus, in fish as in cephalopods, saturated fatty acids can be desaturated by the action of the enzyme Stearoyl-CoA desaturase (Scd), which exhibits a Δ9-desaturation activity responsible to convert the 16:0 and 18:0 to 16:1n-7 (Palmitoleic acid) and 18:1n-9 (Oleic acid), respectively (see Figure 1.3). In contrast, vertebrates, including fish and invertebrates, as cephalopods cannot produce PUFA *de novo* from saturated and monounsaturated fatty acids (Monroig *et al*., 2011). Consequently, PUFA are considered essential nutrients that they must obtain through their diet. The ability for PUFA biosynthesis appears to vary among species depending upon on the enzymatic complement of desaturase and elongase enzymes involved in these metabolic reactions (Monroig *et al*., 2013c). In the case of cephalopods, they lack the Δ12 and Δ15 desaturases required to desaturate the oleic acid (18:1n-9) to 18:2n-6 (LA, Linoleic acid) and then to 18:3n-3 (LNA, Linolenic acid) (see Figure 1.3). Enzymes with either Δ6- or Δ8-desaturation activities are required for the production of 20:3n-6 and 20:4n-3 from the C18 precursors LA and LNA, respectively. However, there is no evidence of the existence of neither Δ6- nor Δ8-desaturase activities among cephalopods. The cephalopod elongase (Elovl5) efficiently elongate C18 and C20 PUFA substrates, at this point of the pathway. After this, a further desaturase with Δ5-desaturation activity is required for the introduction of a double bond at the Δ5 position,

converting 20:3n-6 and 20:4n-3 to 20:4n-6 (ARA, Arachidonic acid) and 20:5n-3 (EPA, Eicosapentaenoic acid), respectively. This desaturase is already described in some marine fishes and appear to be widely distributed among mollusks, including for the common octopus, *O. vulgaris* (Monroig *et al*., 2012b). The 22:6n-3 (DHA, Docosahexaenoic Acid) is also considered an essential fatty acid for cephalopods, since there is no detected activity of Δ4 desaturase in this group of animals, enabling the direct conversion of 22:5n-3 to 22:6n-3. Also in cephalopods, there is no evidence of the alternative, *Sprencher shunt* (Sprecher, 2000), pathway involving elongation of 22:5n-3 to 24:5n-3, Δ6 desaturation to 24:6n-3, and chain-shortening to DHA, as they do not have Δ6-activity (see Figure 1.3). The absence of key desaturation activities required in different steps of the pathways, can concluded that EPA and ARA cannot be biosynthesized endogenously at physiologically significant rates and therefore are essential dietary nutrients for cephalopods (Monroig *et al*., 2011, 2013c; Sargent *et al*., 2002).

The main long-chain PUFA show essential roles in physiological processes such as inflammation, reproduction and hemostasis, which are regulated and modulated by ARA and EPA (Funk, 2001). DHA is crucial for cell membrane lipids of neuronal tissues (Salem *et al*., 2001). In the case of mollusks, they are considered potential sources of PUFA and, consequently, an important source of n-3 long-chain PUFA, as EPA and DHA, which are vital nutrients for the human diet (Tur *et al*., 2012). It has also been proved to afford health benefits in neural development and, in a range of human diseases and pathologies as cardiovascular and inflammatory neurological disorders (Brouwer *et al*., 2006; Eilander *et al*., 2007; Torrejon *et al*., 2007). Like other marine invertebrates, mollusks also possess a particular group of lipids called non-methylene-interrupted (NMI) fatty acids that can be biosynthesized endogenously and thus, are a possible source of unusual PUFA. The NMI fatty acids are opposed to the common methylene-interrupted PUFA, as they have unusual desaturation features where their double (ethylenic) bonds are separated by more than one methylene group. They were discovered in mollusks, although in minor components, the most often encountered structures were the $20{:}2^{\Delta5,11}$ and $20{:}2^{\Delta5,13}$ and their chain elongation products $22{:}2^{\Delta7,13}$ $22{:}2^{\Delta7,15}$. Although their biological role and function is not totally understood, it has

been suggested to play an important role in structural and protection in cell membranes (Barnathan, 2009).

Thus, there are key enzymes pointed to mediate the production and metabolism of important fatty acids in mollusks. They are elongases and two distinct desaturases (with Δ9- and Δ5-desaturase activity) (Barnathan 2009; Kornprobst and Barnathan 2010; Zhukova 2007).

The Stearoyl-CoA desaturase with Δ9 activity (Scd) is an enzyme responsible for the production of the palmitoleic acid (16:1n-7) and oleic acid (18:1n-9), from palmitic acid (16:0) and stearic acid (18:0), respectively. It is universally distributed in all living organisms, for example in zebrafish, *Danio rerio*, (Hsieh *et al*., 2003), in oysters, *Crassostrea gigas*, (David *et al*., 2005, 2007 and 2012), in the chinese mitten crab, *Eriocheir sinensis,* (Guo *et al*., 2013) and also confirmed to exist in the adult common octopus (Monroig *et al*., 2013a). It was demonstrated that the adult common octopus expresses a fad-like gene that encodes an enzyme with Δ5-desaturation activity towards saturated fatty acids and PUFA substrates (Monroig *et al*., 2012b and 2013a). In other words, this enzyme has the ability to introduce a double bond into both saturated fatty acids and PUFA and thus, efficiently catalyzes the conversion of 16:0 and 18:0 to 16:1n-11 and 18:1n-13, respectively, as well as that of 20:3n-6 and 20:4n-3 to ARA and EPA, respectively. The Fad also could participate in the endogenous production of EPA and, especially, ARA. However, this synthesis might be limited to the availability of its precursors 20:4n-3 and 20:3n-6 which could indicate that are essential fatty acids required in diet. In addition, the octopus Δ5 Fad might participate in the biosynthesis of non-methylene-interrupted (NMI) fatty acids (Monroig *et al*., 2011). This enzyme was previously characterized in other animals, as zebrafish, *Danio rerio* (Hasting *et al*., 2001) and Atlantic salmon (*Salmo salar*) (Hasting *et al*., 2005), in the abalone *Haliotis discus hannai* Ino (Li *et al*., 2013) and in cuttlefish, *Sepia officinalis* (Monroig *et al*., 2013b). Another study revealed that adult common octopus possesses an Elovl-like with high homology to vertebrate Elovl5 and Elovl2 enzymes, which are involved in the production of long-chain PUFA. Results showed its ability to elongate C18 and C20 PUFAs, but no activity towards C22. Moreover, the octopus Elovl showed higher elongation efficiency towards n-6 than to n-3 PUFA substrates, suggesting that this compounds (mainly ARA) might play pivotal roles in this cephalopod (Monroig *et al*., 2012a). This pattern of

substrate specificity of octopus elongase is consistent with that of vertebrate Elovl5 proteins and also observed in scallop, *Chlamys nobilis* (Liu *et al*., 2013). Consequently, Elovl5/2 is not capable to elongate 22:5n-3 to 24:5n-3 required for the DHA synthesis by the Sprecher pathway, which emphasizes the importance of this fatty acid in diet. Furthermore, the Elovl might be also involved in the biosynthesis of NMI fatty acids (Monroig *et al*., 2012a). This elongase, Elovl5, has been already studied in many other aquatic organisms as zebrafish (Agaba *et al*., 2004), green abalone, *Haliotis fulgens* (Durazo-Beltran *et al*., 2003), cuttlefish (Monroig *et al*., 2013b), yellow clam *Mesodesma mactroides* (De Moreno *et al*., 1976) and oyster *Crassostrea gigas* (Waldock and Holland, 1984). Recently, it has been cloned an elongase with high homology with Elovl4 proteins of vertebrates, which demonstrate roles in the biosynthesis of very long-chain PUFA (C > 24). These studies have been developed in the sponge *Amphimedon queenslandica* (Srivastava *et al*., 2010), zebrafish (Monroig *et al*. 2010) and Atlantic salmon (Carmona-Antoñanzas *et al*., 2011). Functional characterization of this enzyme in adult octopus suggests that Elovl4-like protein has an apparent activity in the elongation of C22 PUFA substrates (Monroig, O., personal communication). The presence of four NMI fatty acids has been identified from adult octopus samples from nephridium, male gonad, eye and caecum. They have been recognized as $20{:}2^{\Delta5,11}$, $20{:}2^{\Delta7,13}$, $20{:}3^{\Delta5,11,14}$ and $22{:}2^{\Delta7,13}$ (Monroig *et al*., 2012a).

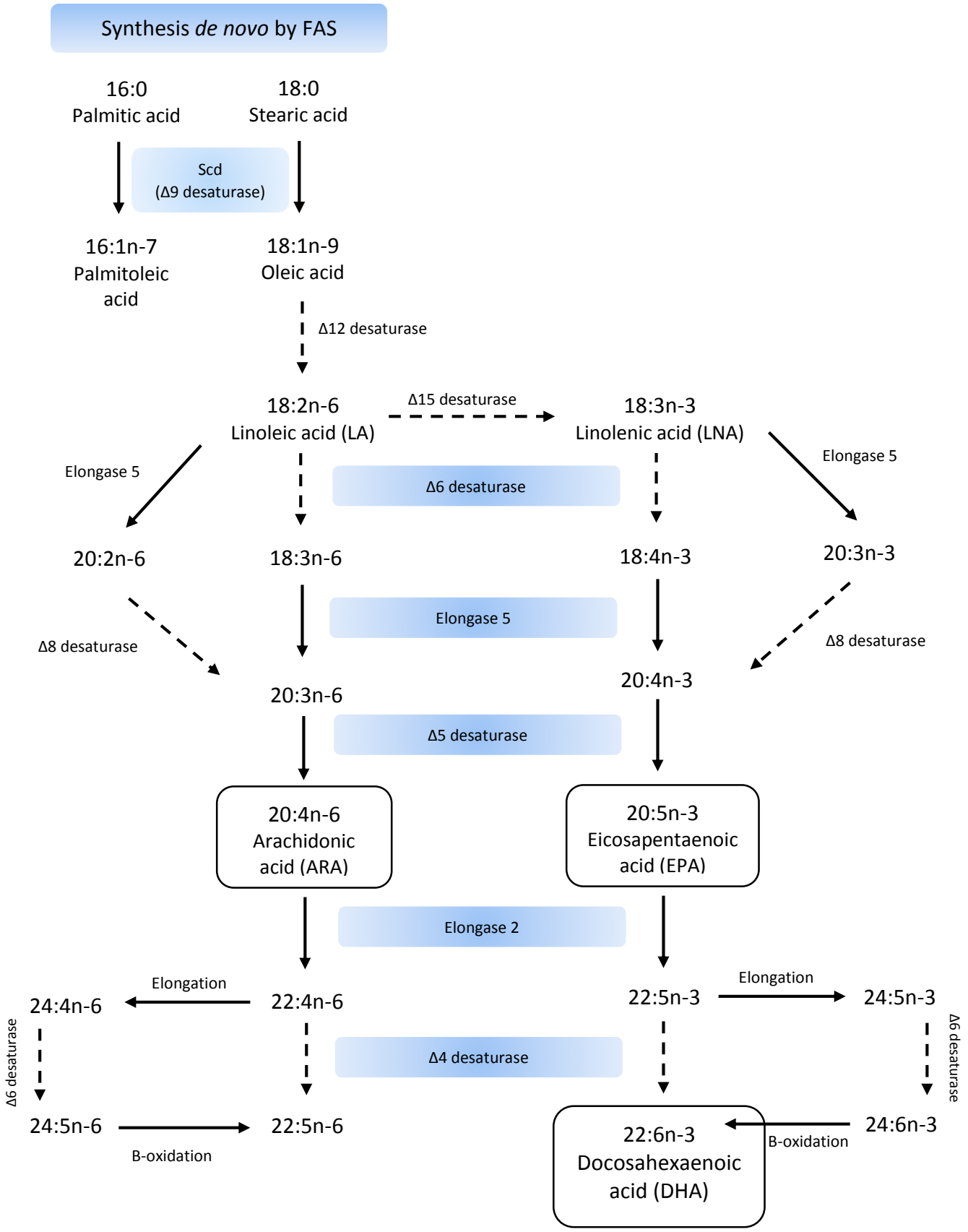


Figure 1.3 PUFA biosynthesis pathways in cephalopods from 18:0 (Stearic acid). Solid arrows indicate demonstrated activity and dashed arrows show activities not determined in cephalopods.

1.5 Nutritional requirements of *O. vulgaris* paralarvae and juveniles

It was already demonstrated that the total lipids in the primordial stages of octopus are relatively low. In general, the *Octopus vulgaris* hatchlings are rich in cholesterol (24%), phosphatidylcholine (21%), phosphatidylethanolamine (16%), sterol esters (14%) and triacylglycerides (6%). Fatty acids represent 4.6% of lipids with 27% of saturated, 14% are unsaturated and the higher percentage are PUFA with 49%. The n-3 PUFA reveal higher percentage (36%) than n-6 PUFA (13%) (Navarro and Villanueva, 2000). It was demonstrated that DHA is especially critical in the early development stages of fishes and crustaceans due to its high demand for membrane synthesis where n-3 PUFA are incorporated (Sargent *et al.*, 2002) and the same is expected for octopus. Moreover, due to the fast growth of these carnivore animals (Villanueva, 1995) it is expected that they have high requirements for DHA and EPA and other structural lipids (Seixas *et al*., 2010). These facts emphasized the importance of a diet rich in PUFA, mainly in n-3 as DHA.

On the other hand, in wild juveniles around 50% of fatty acids are PUFA, with n-3 six times more abundant than n-6. DHA and 16:0 are the single fatty acids more abundant (30-40% of total fatty acids), followed by EPA (10.3-16.7%), producing DHA/EPA ratios from 1:1 to 1:6. Next are 18:0 (7.9-10.6%) and ARA varying from 3.8 to 6.9%. These percentages are significantly higher than those found in the analysis of fatty acid composition of mature ovary and late eggs. It was also observed that wild juveniles tend to lose lipids as they increase in weight (Navarro and Villanueva, 2003). This fact can be explained due to the morphometric changes that occur on the arm growth of octopus (Villanueva, 1995).

Studies on the lipid composition of the different diets used in octopus paralarvae culture, revealed that the natural food (mysids and zoeae) are very rich in PUFA, mainly in n-3 and the content of DHA and EPA are also very high with a 1:1 ratio. *Artemia* seems to be the less adequate diet for these paralarvae, since the higher contents are in monounsaturated fatty acids and demonstrate low levels of DHA and DHA/EPA ratio. The pellets show higher PUFA levels than *Artemia*, but low success are achieved in the ingestion of this type of diet (Navarro and Villanueva, 2000, 2003).

According to several studies, the effect of food fatty acid composition on octopus paralarvae culture is evident and critical for the early development, since their levels

are associated with health and normal growth (Moxica *et al.*, 2002; Navarro and Villanueva, 2000, 2003; Okumura *et al*., 2005). Thus, the nutritional requirements for paralarvae of *Octopus vulgaris* are low-lipid prey, rich in polar lipids (PL), polyunsaturated fatty acids (PUFA) and possibly cholesterol (Navarro and Villanueva, 2000, 2003; Okumura *et al*., 2005; Seixas *et al*., 2008).

1.6 Future research perspectives on octopus aquaculture

Despite all efforts to improve the knowledge of *Octopus vulgaris* culture, aspects as rearing techniques, reproduction, growth, development, hatchling, settlement and nutrition are worthy of further investigation, since several aspects still represents a bottleneck on this cultivation. Regarding the feeding requirements, it is still necessary to explore new live preys, analyze the biochemical composition of the diets, know the exact paralarvae energetic demands and develop formulated diets (Iglesias *et al.*, 2007; Villanueva and Norman, 2008).

Although there is still a lot of work to be done, the developments reached in the last decades contributed to the possibility of including this species in the list of farmed species in a future vision. Due to the overexploitation, the octopus aquaculture could have an appropriate market position. In this way, could be ensured a constant availability of this product in the market, when all the rearing problems are solved (Vaz-Pires *et al.*, 2004).

1.7 Main objectives

The aim of the present work was to study the expression of specific genes that are involved in the metabolism of long-chain polyunsaturated fatty acids (LC-PUFA), particularly the Stearoyl-CoA desaturase with Δ9 activity (Scd), Fatty acyl desaturase with Δ5 activity (Fad) and the elongases of very long-chain 4 and 5 (Elovl4 and Elovl5). Analysis will be done through quantitative PCR (qPCR) from hatching on (day 0) and at days 5, 10, 15 and 20 of paralarval development. This study pretends to identify the essential fatty acids for paralarvae stages, in order to contribute for a better

understanding of the LC-PUFA mechanisms. In this way, it would be possible to develop a more adequate diet for the first days of octopus paralarvae.

2. Material and Methods

2.1 *Octopus vulgaris* broodstock (IEO)

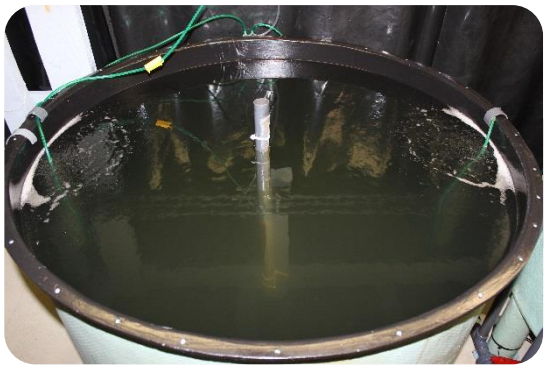
Figure 2.1 Adult octopus broodstock, *O. vulgaris*, maintained in IEO facilities.

Wild adult *Octopus vulgaris* individuals were captured by professional artisanal fishermen near the Tenerife island coast (Canary Islands, Spain). Then, transported to the Instituto Español de Oceanografía (IEO) of Canary Islands (Spain) facilities where the sex determination was performed by verifying the existence of the hectocotilized arm in males. Individuals of similar weight (2.5 kg – 3 kg, approximately) were maintained in a fiberglass white tank of 1000 L, with a sex ratio of 2 females per male (2:1). The tanks were maintained in natural photoperiod (12L:12D), with a natural water temperature (around 23-24°C) and salinity (35 ‰). The surface was covered with a shady net (50 % of tank surface). An open seawater system was used with a flow of 6 L/min entering on the top of the tank and exiting through a filter mesh (1 cm) located at the bottom. Water quality parameters as oxygen levels, ammonia, nitrites and pH were monitored. PVC pipes and clay pots were placed inside the tanks to provide dens. The food was supplied to satiety and consisted of a mixture of frozen squid (*Loligo opalescens*), mussels (*Mytilus edulis*) and prawns (*Parapenaeus longirostris*).

The presence of eggs was verified once a week to avoid disturbing the breeders. When an egg mass was observed, the remaining broodstock individuals were removed and placed in a different tank, leaving the female alone with the egg mass. When the paralarvae were detected, the tank filter was changed to a 363 µm mesh and paralarvae with 0 days (hatchlings) were removed and placed in the experimental tanks.

2.2 Rearing conditions of *Octopus vulgaris* paralarvae (IEO)

The experiment started counting and placing 2500 freshly hatched *Octopus vulgaris* paralarvae to each tank (a total of two tanks, duplicate). Fiberglass 500 L cylinder-conical black tanks, with a flow-through seawater system and a central filter 150 µm mesh were utilized. The salinity of water was 35 ‰ and was supplied through the top of the tanks, with 1 L/min flow rate, ensuring at least 100% water renovation per day. The water quality was promoted through the use of a filtration system, consisting of three inline mesh filters (with a porosity of 20, 5 and 1 µm) and of a UV filter, prior to the entering to each tank of the culture system. Dissolved oxygen was provided through the use of two moderate aeration (porous plastic aeration stones 3 cm in length) placed in each tank. The tanks were under a light regime of 700 lux of intensity and a photoperiod of 12L:12D. These rearing conditions were adapted from Iglesias and Fuentes, (2014). Parameters as water temperature and dissolved oxygen were measured weekly and maintained at 23-24°C and approximately 100 %, respectively. Ammonia (NH_3), nitrites (NO_2) and pH were also controlled weekly using a TETRA test (Tetra GmbH, Melle, Germany) and a pH meter (Hanna Instruments, Rhode Islands, USA).



Figures 2.2 and 2.3 Fiberglass 500 L tanks used for the *O. vulgaris* paralarvae experiment in IEO.

The green-water technique was applied using 10^6 cells/ml of *Nannochloropsis* sp. microalgae, supplied every morning before the light being turned on. The live food used was 24 hours-old metanauplii of *Artemia franciscana* enriched with *Isochrysis* affinity *galbana* (T-ISO) reaching a total concentration of 0.5 *Artemia*/mL divided in two meals (one in the morning and the other one in the afternoon).

2.3 Sampling (IEO)

The sampling days occurred when paralarvae were 0 (hatching), 5, 10, 15 and 20 days-old. During these days, three samples of 20 paralarvae each were collected from each tank and sacrificed using chilled water to ensure the lowest level of suffering for animals. Then, samples were washed with distilled water and kept in a centrifuge tube with RNA later (five times the volume of each paralarvae sample) and maintained at 4°C overnight. After this period, samples were stored at -20°C until further analysis. This experiment was designed to use and sacrifice as few as possible animals considering the objectives to be achieved.

2.4 Principles of animal welfare used in this experiment

Octopus broodstock and paralarvae were used in this experiment taking into account the principles of animal welfare. Optimum conditions for captive maintenance, maturation and spawning of breeding individuals were particularly necessary for the proper development of this experience. The animals were kept in tanks of appropriate capacity, supplying food to satiety in the amounts and times that have been tested as suitable on recent research on the species. Water quality parameters (sufficiency, oxygen levels, ammonia, pH) were ensured. The paralarvae used were born in captivity. They were sacrificed using chilled water. The sacrifice was made by experienced personnel with appropriate material considering the paralarvae size to ensure the lowest level of suffering for the animals. The experiments have been designed to use and sacrifice as few replicates as possible considering the objectives to be achieved. The type of assays performed in this study involved minimum pain or injury to the octopuses. Stress was reduced to a minimum, being associated only with processes which are essential to carry out, in particular, cleanup operations of the holding tank. All staff of the IEO culture facilities are suitably trained and experienced for the design and performance of experimental studies with aquatic animals and to avoid or minimize animal pain and suffering. Under the third transitional provision of Royal Decree RD53/2013 of 1 of February, on the protection of animals used for experimental and

other scientific purposes, the researchers involved in the experience hold the C degree and research assistants category B.

This experimentation was performed according to the Spanish legislation (Royal Decree RD53/2013) and the European Directive 2010/63/EU for the protection of animals used for experimentation and other scientific purposes.

2.5 Sample preparation and production of standards (IATS-CSIC)

Firstly, 30 mg of each sample was used for RNA extraction (Maxwell 16 LEV Simply RNA Tissue Kit, Promega) treated with DNase I. RNA purity was assessed spectrophotometrically (NanoDrop 2000C, Thermo Scientific) by measuring the A260/A280 ratio and the 280/230 ratio, in which > 1.8 indicates good quality RNA. RNA integrity was checked by gel electrophoresis on a TAE buffer (Tris base, acetic acid and EDTA) agarose gel in the presence of the ethidium bromide fluorescent dye. Immediately after the quality control assessment, a conversion of RNA to cDNA was performed to avoid the risk of RNA degradation. The cDNA synthesis consisted in using 2 µg of RNA from each sample, random primers and oligo_dT (3:1). A reverse transcription (M-MLV (Promega) and RNAsin) reaction was then performed, in a total volume of 25 µl, at 37°C during 1 hour. cDNA samples were stored frozen at -20°C until used.

To produce quantitative Polymerase Chain Reaction (qPCR) standards, PCR reactions using specific primers (see Figure 2.7) with flanking fragments of 300-500 base pairs long and constructs of the ORF of the genes *scd*, *fad*, *elovl5* and *elovl4,* already cloned into plasmids in previous studies as templates, were used. Primers were designed using PrimerPlus3 program and optimization was assessed by choosing primers with melting temperature (T_m) around 60°C and GC (Guanine-Cytosine) content around 50%. The PCR conditions consisted in an initial denaturation step at 95°C for 2 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 40 s; and a final extension at 72°C for 5 min (GoTaq® Green Master Mix, Promega). The PCR products were subsequently separated using 1 % agarose gel electrophoresis, purified (GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare) and ligated into

pGEM-T Easy Vector System I (Promega). These plasmids were then used to transform *Escherichia coli* bacteria that were grown in agar plates with LB Broth medium and ampicillin. Positive clones were cultivated in liquid medium with LB Broth and ampicillin after PCR screening to confirm the insert contained in the colony. Then, plasmids were extracted from bacteria (Gen Elute™ Plasmid Miniprep Kit, Sigma-Aldrich™) and linearized with restriction enzymes. Finally, a new purification was performed and quantified spectrophotometrically (NanoDrop 2000C, Thermo Scientific).

Figures 2.4 and 2.5 Polymerase Chain Reaction (PCR) thermocycler.

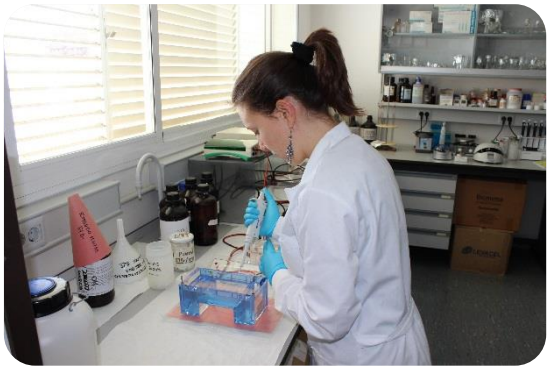


Figure 2.6 Agarose gel electrophoresis.

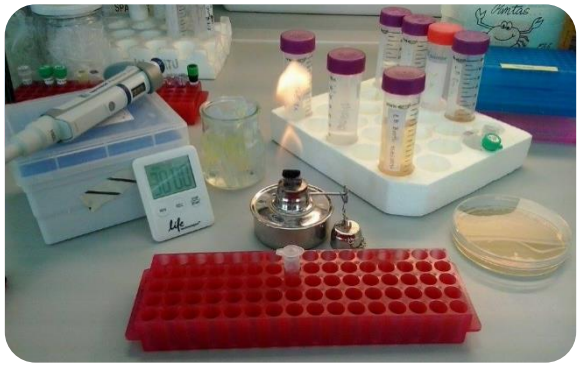


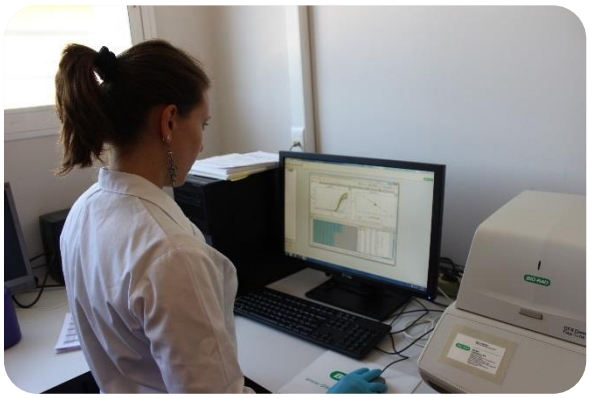
Figure 2.7 Material used to proceed to the *E. coli* transformation.

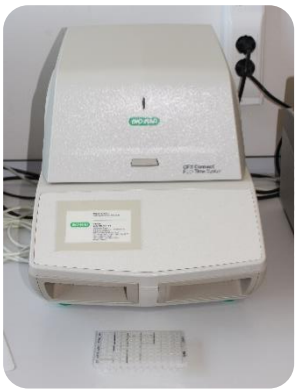
Figure 2.8 Primer name, sequences of primers used (for standard production and qPCR) and number of base pairs (bp) of each sequence used as reference for the primer design.

Aim	Primer name	Primer sequence	Frag (bp)
β-*actin* standard	OVstActF1	TCCAGGCTGTGTTGTCTCTG	409
	OVstActR1	GCAGATTCCATACCCAGGAA	
β-*actin* qPCR	OVqActF1	TTCTTGACTCCGGAGATGGT	100
	OVqActR1	AGATCACGACCAGCCAAGTC	
scd standard	OVstD9F1	CGAGACCACAGAGTTCACCA	498
	OVstD9R1	AGTGAGATTGATCCGCCAAC	
scd qPCR	OVqD9F2	TGGGGTTATCGACCCTATGA	150
	OVqD9R2	AGTGAGATTGATCCGCCAAC	
fad standard	OVstDESF1	GCCACATGCATTACCAACAC	459
	OVstDESR1	CAATATCACAGGTCGCATGG	
fad qPCR	OVqDESF1	GCCACTCCTGTTTCCTGTGT	145
	OVqDESR1	CACTCCCCAGAATCCAAGAA	
elovl5 standard	OVstELO5F1	CAACTGCAGATCCAAGGACA	477
	OVstELO5R1	AACAAGCACCGAACCAAGTC	
elovl5 qPCR	OVqELO5F1	TGCTCGAGTTCTTTGGTGGT	117
	OVqELO5R1	GCATGATGGAAGACATGCAG	
elovl4 standard	OVstELO4F2	CGGCTACTCGTGGGTTTACT	317
	OVstELO4R2	TGTAGCCAGCACGGTAGGAT	
elovl4 qPCR	OVstELO4F3	CCAAGAGTTACTGATTGGCCCT	115
	OVstELO4R3	ATGGCTTTCGTTTGTGCATGT	

2.6 Expression of the genes *scd*, *fad*, *elovl5* and *elovl4* (IATS-CSIC)

Expression of target genes that encode for the Stearoyl-CoA desaturase (Scd) with Δ9 activity, Fatty acyl desaturase with Δ5 activity (Fad) and elongases of very long-chain 4 and 5 (Elovl4 and Elovl5) were measured by quantitative real-time PCR (qPCR) (adapted from Pfaffl, 2001). To determine the qPCR reaction efficiency, a standard curve was generated from the linearized constructs (pGEM-T Easy + ORF gene fragment) through a serial dilution of known concentration, which was used to quantify the unknown samples. Specific primers were designed to flank 100-150 base pairs fragments (see Figure 2.7) into the standards and optimized by choosing pairs of primers with T_m and GC content of around 60°C and 50%, respectively . The qPCR amplifications were carried out in duplicate using a Bio-Rad CFX Real-Time PCR machine, in a final volume of 20 µl containing 5 µl of diluted (1/20) cDNA in the unknown samples or 5 µl of each serially diluted linearized plasmid in the standard measures as templates, 0.5 µl of each primer (forward and reverse), 10 µl of bi-distillated and sterilized water (ddH_2O) and 4 µl of 5x PyroTaq EvaGreen® Mix (Cultek Molecular Bioline). Single, transparent plates were used and sealed with adhesive. A systematic negative control (NTC, no template control) containing no cDNA was always included with quantification cycles (Cq) less than 38. The qPCR runs consisted of an initial activation step at 95°C for 15 min, followed by 40 cycles of 15 s at 95°C, 20 s at the specific primer pair annealing temperature, and 15 s at 72°C. After the amplification phase, a dissociation curve at 0.5°C increments from 60 to 90°C was performed, enabling confirmation of the amplification of a single product in each reaction. The specificity of the PCR products were checked by the efficiency (>90% - <110%), coefficient of determination (r^2 > 0.980) and a melt curve displaying a single sharp peak. The β-actin gene was also quantified as reference as described by Monroig *et al*., (2012a,b).





Figures 2.9 and 2.10 Quantitative PCR procedure and Bio Rad CFX Real-Time PCR machine.

2.7 Statistical analysis

A two-way ANOVA (tank and sampling time as factors) proved that there were no significant differences in the expression of the genes (β-actin relative index = number of copies of each gene / number of copies of β-actin gene) between the two tanks. Consequently, results are expressed as means (mean ± SD) of the 6 replicates obtained from the two tanks and analyzed by a one-way ANOVA, followed by the Tukey HSD *a posteriori* mean comparison test ($p \leq 0.05$). In case of variance heteroscedasty, the Games-Howell robust test for mean comparisons was utilized instead. All the statistical analyses were performed with the SPSS 22 statistical packages and according to Zar, (1999).

3. Results

The study of the expression of the genes Stearoyl-CoA Desaturase with Δ9 activity (*scd*), Fatty acyl desaturase with Δ5 activity (*fad*) and Elongases of very long chain fatty acid 5 (*elovl5*) and 4 (*elovl4*) in *Octopus vulgaris* paralarvae at days 0 (hatching), 5, 10, 15 and 20 is represented in Figure 3.1.

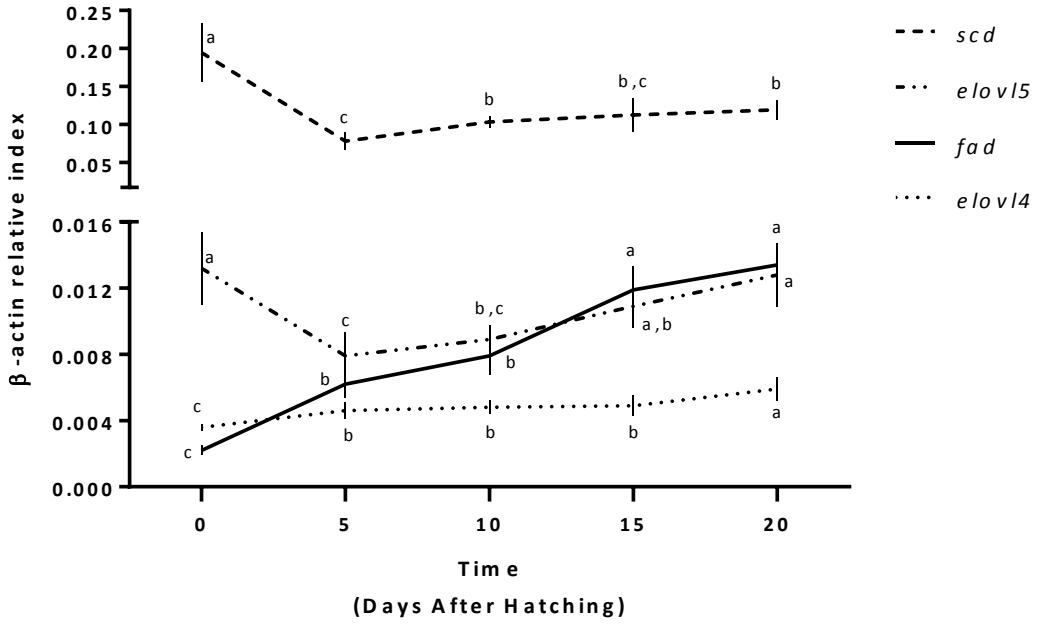


Figure 3.1 Expression (mean and standard deviation) of the genes *scd*, *fad*, *elovl5* and *elovl4* relatively to β-actin. Actin relative index is the number of copies of the target gene / number of copies of the β-actin gene. Means with the same letter do not present significant differences (one-way ANOVA followed by *a posteriori* mean comparison tests, Tukey HSD and Games-Howell tests, when appropriate, p≤0.05) in the same gene.

These results showed the presence of the genes *scd*, *fad*, *elovl5* and *elovl4* in the RNA of octopus paralarvae in all age samples (0, 5, 10, 15 and 20 days-old paralarvae). In general, a significant increase of the expression of all genes after day 0 (hatching) until day 20 is observed, with an exception in the expression of *scd* and *elovl5* at day 0, where the expression means of the paralarvae sampled were higher.

The *scd* gene revealed the higher levels of expression compared with the remaining genes. At day 0, the expression of this gene is higher than that of the older paralarvae (5, 10, 15 and 20). From day 0 to day 5, a decrease was observed followed by an increase

until day 20. No significant differences were detected among means from days 10, 15 and 20, as well as means between the samples from days 5 and 15. Mean of day 0 (hatching) was significantly different from those of all the other days.

The expression of *elovl5* followed a similar pattern to that of *scd* gene. This means that the highest level of *elovl5* expression is observed at day 0 (hatching), followed by a decrease until day 5, starting to increase from this point until day 20. Expression means among the samples from days 0, 15 and 20 did not present significant differences and the same was observed in the means of the paralarvae sampled between days 10 and 15. The expression in the paralarvae from day 5 presented a mean significantly lower than that of the remaining samples (0, 10, 15 and 20 days).

The *fad* gene showed the lowest expression at day 0 (hatching) compared with the other genes at the same day. The mean of the expression from the paralarvae sampled on day 0 was significantly lower when compared with the mean of the expression measured for the samples on the remaining days (5, 10, 15 and 20). After this, an increase in the expression levels measured for the sampled paralarvae was observed until day 20. Expression means of the paralarvae sampled on days 5 and 10 revealed no significant differences, as well as expression means among days 15 and 20.

Finally, the *elovl4* gene presented the lowest and more constant levels of expression compared with the other genes. A slight increase in the expression of the means of the paralarvae sampled from day 0 (hatching) until day 5 was observed and after this point, the values remain statistically similar. Thus, expression means measured on sampled paralarvae among days 5, 10 and 15 had no significant differences. The expression levels measured on samples at day 0 and 20 were significantly lower and higher, respectively, than the means measured on samples at the other days (5, 10 and 15).

4. Discussion

The expression of the genes Stearoyl-CoA Desaturase with Δ9 activity (*scd*), Fatty acyl desaturase with Δ5 activity (*fad*) and Elongases of very long chain fatty acid 5 (*elovl5*) and 4 (*elovl4*) from *Octopus vulgaris* paralarvae at days 0 (hatching), 5, 10, 15 and 20 of development was successfully studied using quantitative PCR (qPCR).

The expression of these genes was previously studied in adult octopus (Monroig *et al.*, 2012a,b and 2013a). As far as we know, this is the first time that these genes are proved to be present in the first days of development of this species paralarvae.

From the statistical test analysis, in general, all genes studied (*scd, fad, elovl5* and *elovl4*) showed an increase in the expression from day 0 until day 20, although with an exception on the genes *scd* and *elovl5*, where the expression measured on the sampled paralarvae were higher at day 0. From this general tendency, we could suggest that paralarvae increase their capacity of biosynthesizing the enzymes responsible for the LC-PUFA metabolism, while the development and complexity associated to the growth also increase. Due to this endogenous biosynthesis capacity of paralarvae during their development process, the need for PUFA and LC-PUFA supplementation during the first days of the octopus is again strengthened. A similar gene expression pattern was observed in common carp (*Cyprinus carpio*), where the *Elovl5-a* and *Elovl5-b* mRNA showed an increase in expression as larvae developed from newly hatched to 20 DAH (Days After Hatching), indicating the capacity of endogenous synthesis of highly unsaturated fatty acids (HUFA) to support larvae development process (Ren *et al*., 2015). In zebrafish, *Danio rerio*, a gradual increase in fatty acyl elongase gene expression was also observed. A study in spatial-temporal expression of *elovl5*, *elov2* and *fad* during embryogenesis revealed the presence of all three genes in the brain as early as 24 hours post fertilization (hpf), which implies LC-PUFA synthesis in the embryonic brain. Also a significant increase in both *elovl2* and *fad* expression from 96 hpf onwards parallels the increase of expression in liver and intestine (major organs sites for LC-PUFA biosynthesis), which presumably contributes to the overall increase in embryonic mRNA levels of both genes (Tan *et al*., 2010). Previously to this study, Monroig *et al.,* (2009) demonstrated the same developmental expression of *elovl2*, *elovl5* and *fad* in 0-72 hpf zebrafish embryos that, combined with the dynamic of fatty acids composition of

embryos, denoted endogenous production of LC-PUFA. Another possibility for this increase of activity could also be a response to a poor diet, since in this experimental work we fed paralarvae *Artemia franciscana*, already proved to be inefficient for the first days of octopus paralarvae (Navarro *et al*., 1992, 1993; Reis, 2011; Seixas *et al*., 2008). Perhaps, octopus paralarvae are able to endogenously produce LC-PUFA to compensate their nutritional needs to some extent. Obviously, this compensatory mechanism is not enough to most of the octopus paralarvae fed *A. franciscana*, since they die during their first weeks of life, also due to their limited activity on important desaturases (see Figure 1.3). In Atlantic salmon (*Salmo salar*) studies, the expression of desaturases and elongases genes, as well as, the activity of the highly unsaturated fatty acids (HUFA) biosynthetic pathway, were proved increase in response to the inclusion of vegetable oil-substituted diets compared to fish fed fish oil (Zheng *et al*., 2004, 2005). Despite the phylogenetic distance with octopus, a similar response also happens with birds, where the LC-PUFA deposited by the hen are not sufficient to fulfill the requirements of the embryo, and consequently the chicken embryo biosynthesize very actively the LC-PUFA in order to compensate such deficiency (Cherin and Sim, 2001).

The gene *scd* presented the higher level of expression in the paralarvae from all sampling periods, when compared with the remaining genes studied. This higher expression could be explained by the fact that this enzyme (Scd) is the primary precursor of the LC-PUFA biosynthesis pathways. In other words, a first desaturation occurs by the action of Scd, from the 16:0 (Palmitic acid) and 18:0 (Stearic acid) to 16:1n-7 (Palmitoleic acid) and 18:1n-9 (Oleic acid), respectively (see Figure 1.3). This desaturation is in the base of LC-PUFA formation (Monroig *et al.*, 2011 and 2013c). Our results agree with those of a previous study (Monroig *et al*., 2013a), where the gene that codifies for the enzyme Scd was cloned in adult octopus. These results were consistent with the function that is described for other living organisms, participating in the biosynthesis of fatty acids monounsaturated and reveal potential in the production of some NMI fatty acids. In the case of adult common octopus, the Scd is responsible for the production of the oleic acid (18:1n-9) from the 18:0. This enzyme also revealed a function in the production of Δ5,9 NMI fatty acids, characteristic in mollusks.

At hatching, the genes *scd* and *elovl5* presented a higher expression when compared with those samples collected at the remaining ages (days 5, 10, 15 and 20 of paralarval

development), but also when compared with the expression of the other genes (*fad* and *elovl4*) at this same development stage. These results are tentatively explained by the activation of the zygote genome that can occurs during the embryonic development of the *Octopus vulgaris,* although further research would be necessary to confirm this hypothesis. After the beginning of embryogenesis, activated by the egg, all animal embryos pass through a stage during development that is controlled from maternally provided gene products (as mRNAs and proteins) to those synthesized from the zygotic genome. A process called maternal-to-zygotic transition (MZT) consists in a first step of elimination of maternal transcripts, and a second step, where the transcription of the zygotic genome begins, named Zygote Genome Activation (ZGA) (Tadros and Lipshitz, 2009). In fish, the MZT occurs during development in an event called 'mid-blastula transition' (MBT), where the synchronous divisions are followed by asynchronous cleavages, with the introduction of gap phases. Examples of this are the model organisms as zebrafish, *Danio rerio* (Mathavan *et al*., 2005), nematodes, *Caenorhabditis elegans* (Guven-Ozkan *et al*., 2008), insects, *Drosophila laevis* (De Renzis *et al*., 2007), amphibians, *Xenopus laevis* (Stancheva and Meehan, 2000), mammals, *Mus musculus* (Hamatani *et al.,* 2004) and echinoderms, where the MZT has been extensively studied. In the case of sea urchin, *Strongylocentrotus purpuratus*, is observed that the embryos begin ZGA the earliest, with the first wave of transcription commencing at the one-cell stage (Tadros and Lipshitz, 2009), at the two-cell stage, the early blastula stage, early gastrula stage and beyond (Wei *et al.,* 2006). From here, it is possible to suppose that the MZT also occurs in the first stages of embryonic development of the *O. vulgaris*. Probably near stage I – III, where an equivalent to the MBT with asynchronous cleavage occurs (Naef, 1928). The embryonic development of octopus presents different displays when compared with the embryonic development of fish. For example, the octopus development takes a longer period of time (Caverivière *et al*., 1999; Hamasaki and Morioka, 2002; Katsanevakis and Verriopoulus, 2006) and the yolk sac formation (stage XV) and absorption by the larvae, happens before hatching (Naef, 1928). This facts support the idea that in the moment of hatching, the octopus paralarvae is so developed that already expresses their own genome. In this way, it is possible to understand that the high levels in the expression of the genes *elovl5* and *scd* observed in the sampled paralarvae at hatching (day 0) are due to paralarval genome machinery. However, to

ascertain this theory, a study of the expression of these genes during the all embryonic development of *O. vulgaris* would be important to complement the present work and crucial to determine the moment of transition from the maternal genes effect to the ZGA. In other hand, to explain the observed decrease in the expression of the genes *elovl5* and *scd* of sampled paralarvae from day 0 to day 5, another hypothesis is proposed. It is well known the existence of isoforms of the same genes that are expressed differentially during different phases of individual development. This could be the case of these genes having different unknown isoforms in octopus. The existence of two Elovl5-like elongase gene in common carp, *Cyprinus carpio* (Ren *et al*., 2012) and in Atlantic salmon, *Salmo salar* (Xue *et al*., 2015) supports this idea. Each isoform could present different roles for each phase of the development: embryogenesis and paralarval development. In other words, could be suggested that an isoform of *elovl5* and *scd* could be involved in the embryonic development and contrary, another isoform have a role from hatching and onwards. Thus, the decrease in the expression of the genes observed in the results could be understood by the expression of one isoform and suppression of the other. Despite the differences from *elovl5* and *scd* to *vasa* genes, Pacchiarini *et al*., (2013) also found four isoforms of the *Ssvasa* with different expression during *Solea senegalensis* development. *Ssvasa1* and *Ssvasa2* expression was restricted to the 2-cell up to somitogenesis stage and no vasa1-2 expression was found in Senegalese sole late embryos, larvae or early juveniles. Contrarily, *Ssvasa3* and *Ssvasa4* showed very low expression during embryonic development, increasing after hatching and during larvae life, remaining constant during metamorphosis and exhibiting the highest expression values in juveniles. Again, to confirm this theory, it will be necessary to proceed to an analysis of the expression of the genes during embryogenesis to understand if these isoforms exists and if so they have different moments of expression during each stage of *O. vulgaris* development.

5. Conclusion

In conclusion, the present study demonstrates the expression of the genes Stearoyl-CoA Desaturase with Δ9 activity (*scd*), Fatty acyl desaturase with Δ5 activity (*fad*) and Elongases of very long chain fatty acid 5 (*elovl5*) and 4 (*elovl4*), involved in the biosynthesis of LC-PUFA during the early octopus (*Octopus vulgaris*) paralavae development. Despite the exception in the expression of the genes *scd* and *elovl5* measured from sampled paralarvae at day 0, in general, all genes studied revealed an increase in the expression from day 0 (hatching) until day 20, suggesting that paralarvae could increase their capacity to biosynthesize enzymes involved in the LC-PUFA metabolism, as growth also increase, but also, this endogenous capacity could happen in response to a poor diet, in order to fulfill the nutritional requirements. Finally, a higher expression of *scd* and *elovl5* in the paralarvae samples is observed at day 0 and could be a sign of zygote genome activation during the embryonic development, although further research is necessary. Through this study, we can demonstrate the activity of key enzymes involved in the biosynthesis of LC-PUFA in the early *Octopus vulgaris* paralarvae development. Consequently, we contribute to determine the essential fatty acids for the first days of octopus paralarvae development, knowledge that could be used to find a more appropriate diet, helping to solve the high mortalities observed in its culture.

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