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The n-3 highly unsaturated fatty acid requirement and effect on hepatic composition and histopathology of meagre (*Argyrosomus regius*, Asso, 1801) fingerlings

Marta Ribeiro Carvalho

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Dissertação de Mestrado apresentada à
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Recursos Biológicos Aquáticos

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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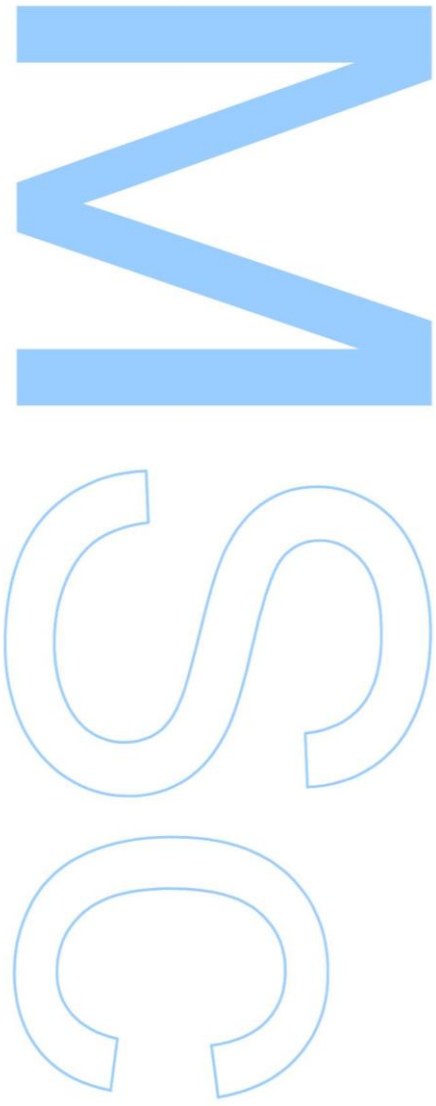

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Publications associated to this Master thesis

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Conferences

Part of this study was presented as oral presentation with the title: “Dietary requirement for essential fatty acid (docosahexanoic acid, DHA, and ecosapentanoic acid, EPA) for meagre (*Argyrosomus regius*) fingerlings”, in IJUP (Investigação Jovem na Universidade do Porto), which took place at Faculty of Medicine of Porto, in Oporto (Portugal), on 8th February 2017.

“Dietary requirement for essential fatty acid (docosahexanoic acid, DHA, and ecosapentanoic acid, EPA) for optimal growth of meagre (*Argyrosomus regius*) fingerlings”, in 2nd AQUAIMPROVE Workshop, which took place at CIIMAR, in Oporto (Portugal), on 17th March 2017.

And it will be presented as oral presentation with the title “Requirement for n-3 HUFA of meagre *Argyrosomus regius* (Asso, 1801) fingerlings” in the 17th International Symposium of Aquaculture Europe, AE2017, which will take place at the VALAMAR Hotel and Resort, in Drubrovnik, Croatia from 17-20 October 2017.

Abstract

The costs associated with aquafeeds, generally, constitute the largest percentage of expenditure in aquaculture production. Suboptimal feeds lead to relevant economic losses, especially due to suboptimal growth of fish, feed waste, and, consequently, water deterioration. Thereby, formulating diets of excellent nutritional quality is mandatory to optimise aquaculture profitability. Moreover, to guarantee the competitiveness, the sustainability and the market expansion of the Mediterranean aquaculture sector, it is also urgent to diversify the aquaculture species produced. One of the most promising species for the diversification is meagre (*Argyrosomus regius*) due to its fast growth, excellent feed conversion ratios, easy adaptation to captivity, high market price, as well as its high nutritional value for consumers. However, scarce information is available on meagre nutritional requirements, and no information about its essential fatty acid requirements exist. Fatty acids play vital biological roles in animals, being involved in several metabolic pathways as membrane structure and function, precursors of eicosanoids synthesis, control of lipid homeostasis and energy production. Marine fish have restricted ability to synthetize *de novo* n-3 and n-6 HUFA from their precursors LNA and LA, due to an impairment or deficiency in Δ5 or Δ6 desaturases and elongases. Thus, DHA, EPA and possibly, ARA are considered essential for marine species. Reduced survival, poor growth and several pathologies are associated to a deficiency of essential fatty acids. Therefore, they must be included in the diet in adequate levels to fulfil species-specific requirements for normal growth, survival and development.

The liver plays a key role in the intermediary metabolism and integrates a large part of nutrient uptake, being involved in a wide range of functions, as lipid metabolism. For instance, an insufficient or an imbalance dietary EFA levels, frequently, leads to steatosis and other pathologies.

The general aim of this thesis was to determine the n-3 HUFA requirements for meagre fingerlings and their effect on hepatic composition and histopathology. For that purpose, a feeding trial testing 5 increasing dietary n-3 HUFA levels (0.9, 1.5, 2.0, 3.0 and 3.9% DM) was performed and growth, feed utilisation and composition of whole-body and liver were determined. Besides, histopathology of liver was also analysed and related to the diets. Results of this study showed one of the highest specific growth

rates (4.1 to 4.6% day $^{-1}$) and lowest feed conversion ratios (0.7 to 0.8) recorded for this species, highlighting its great potential for aquaculture. Fish fed 0.9% n-3 HUFA showed the lowest growth, which was significantly improved by increasing the dietary n-3 HUFA levels up to 3.0%. DHA was selectively retained over EPA in whole fish body. Additionally, the reduction of the dietary n-3 HUFA level below optimum levels led to an accumulation of n-9 FA, as well as an increase in the OLA/n-3 HUFA ratio. Meagre seems to have active Δ6 desaturase and elongase (Elovl5), but their activities being insufficient to produce enough DHA and EPA from PUFA precursors to sustain fast growth. Hereupon, based on a second-order polynomial model, relating dietary n-3 HUFA content and weight gain, the dietary n-3 HUFA level for 95% maximal weight gain was estimated to be 2.6-3.0% DM.

Fish fed the two lowest n-3 HUFA diets (0.9 and 1.5%) showed a higher HSI as well as a higher lipid infiltration in liver. Consequently, these fish showed a more severe steatosis than fish fed 2.0-3.9% n-3 HUFA. The liver FA composition followed the same trend as dietary FA profile, similarly to whole-body, although more markedly. However, meagre FA composition of liver polar lipids was less affected by the diets, preserving n-3 HUFA in phospholipids. Furthermore, the lowest dietary content in n-3 HUFA (0.9%) also led to a higher incidence of hepatic granulomas, suggesting a possible relation between EFA-deficiency and hepatic granulomatosis in meagre. These results suggest that a minimum of 3.0% DM n-3 HUFA is required to maintain the essential fatty acid profile and normal histomorphology of liver, corroborating the requirement value estimated for growth.

Keywords: *Argyrosomus regius*; docosahexaenoic acid; eicosapentaenoic acid; essential fatty acids; granuloma; nutritional requirements; steatosis

Resumo

Os custos associados às rações, geralmente, constituem a maior percentagem de gastos na produção em aquacultura. Dietas sub-ótimas conduzem a perdas económicas relevantes, especialmente devido ao crescimento também sub-ótimo dos peixes, ao desperdício de alimento, e, consequentemente, à deterioração da água. Deste modo, a formulação de dietas de excelente qualidade nutricional é peremptória para otimizar a rentabilidade das indústrias de aquacultura. Além disso, para garantir a competitividade, sustentabilidade e expansão do sector da aquacultura Mediterrânea, surgiu uma necessidade urgente de encontrar novas espécies para a produção em aquacultura. Uma das espécies mais promissoras para a diversificação da aquacultura é a corvina (*Argyrosomus regius*) devido ao seu rápido crescimento, excelentes taxas de conversão de alimento, fácil adaptação às condições de cativeiro, alto valor de mercado, bem como excelente valor nutricional para o consumidor. No entanto, encontra-se disponível pouca informação sobre as exigências nutricionais de esta espécie, e, em particular, não existe ainda informação sobre as suas necessidades em ácidos gordos essenciais. Os ácidos gordos desempenham papéis biológicos vitais nos animais, estando envolvidos em várias vias metabólicas, dentro das quais são o controle da estrutura e função da membrana celular, precursores da síntese de eicosanóides, controle da homeostasia lipídica e produção de energia. Os peixes marinhos têm uma capacidade restrita para sintetizar *de novo* n-3 e n-6 HUFA a partir dos seus precursores: o LNA e o LA, devido a uma deficiência enzimática nas Δ5 e Δ6 desaturases e elongases. Assim, o DHA, EPA e, possivelmente, o ARA são considerados essenciais para espécies marinhas. Sobrevivência reduzida, crescimento pobre e diversas patologias, estão associados a uma deficiência de ácidos gordos essenciais. Deste modo, os mesmos devem ser incluídos na dieta em níveis adequados para atender aos requisitos específicos de cada espécie para o normal crescimento, sobrevivência e desenvolvimento.

O fígado desempenha um papel fundamental no metabolismo intermediário e integra uma grande parte da absorção de nutrientes, estando envolvido numa ampla gama de funções, como o metabolismo lipídico. Por exemplo, uma insuficiência ou um

desequilíbrio dos níveis de EFA da dieta, frequentemente, conduz a esteatose e outras patologias.

O objetivo geral desta tese foi determinar os requisitos de n-3 HUFA para juvenis de corvina e o seu efeito na composição e histopatologia hepática. Por esse motivo, um ensaio de crescimento foi conduzido, testando 5 níveis crescentes de n-3 HUFA na dieta (0,9, 1,5, 2,0, 3,0 e 3,9% MS). Os efeitos do aumento dos níveis de n-3 HUFA na dieta foram relacionados com o crescimento, a utilização do alimento, e a composição bioquímica de corpo inteiro e do fígado do peixes. Além disso, a histopatologia do fígado também foi analisada e relacionada com as dietas. Os resultados deste estudo refletiram uma das maiores taxas de crescimento específica (4,1 a 4,6% dia^{-1}) e das menores taxas de conversão de alimento (0,7 a 0,8) registadas para esta espécie, destacando assim o seu grande potencial para a aquacultura. Os peixes alimentados com 0,9% de n-3 HUFA apresentaram o menor crescimento, o qual foi aumentado pela elevação dos níveis de n-3 HUFA na dieta até 3,0%. O DHA foi seletivamente retido sobre o EPA. Além disso, a redução de n-3 HUFA na dieta conduziu a uma acumulação de ácidos gordos da série n-9, bem como um aumento do índex OLA/n-3 HUFA. A corvina parece ter ativas a Δ6 desaturases e a Elovl5, embora as suas actividades sejam insuficientes para produzir suficiente DHA e EPA para manter um rápido crescimento, a partir dos seus precursores. Deste modo, foi estimado que as necessidades ótimas de n-3 HUFA para um ótimo crescimento de juvenis de corvina é de cerca de 2,6-3,0% MS n-3 HUFA na dieta.

Menores níveis de n-3 HUFA na dieta (0,9 e 1,5%) conduziram a um maior índice hepatossomático, bem como uma maior infiltração lipídica no fígado. Consequentemente, os mesmos níveis causaram esteatose hepática com maior severidade do que nos peixes alimentados com níveis superiores. O conteúdo lipídico do fígado foi menor nos peixes alimentados com 3,0% n-3 HUFA do que nos peixes alimentados com 0,9% e 1,5% n-3 HUFA. A composição de ácidos gordos do fígado seguiu as mesmas tendências das dietas, semelhantemente ao corpo inteiro, embora de forma mais acentuada. No entanto, o perfil de ácidos gordos dos lípidos polares do fígado foi menos afectado pela composição da dieta, preservando n-3 HUFA nos fosfolípidos. Além disso, o menor conteúdo dietético em n-3 HUFA (0,9%) também causou significativamente mais granulomas hepáticos, sugerindo uma possível relação entre a deficiência de EFA e a granulomatose hepática em corvina. Estes resultados sugerem que um mínimo de 3,0% MS n-3 HUFA é necessário para manter o normal perfil de ácidos gordos essenciais e

a normal histomorfologia do fígado, corroborando o valor da necessidade estimada para o crescimento.

Palavras-chave: ácido docosahexaenóico; ácido eicosapentaenóico; ácidos gordos essenciais; *Argyrosomus regius*; esteatose*;* exigências nutricionais; granuloma

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Abbreviations

ARA- arachidonic acid

CFU- Colony forming unit

EFA- essential fatty acid

EPA- eicosapentaenoic acid

DHA- docosahexaenoic acid

DM- dry matter

EU- European Union

FA- fatty acid(s)

FAMES- fatty acid methyl esters

FCR- feed conversion ratio

FI- feed intake

H&E- haematoxylin and eosin

HSI- hepatosomatic index

HUFA- highly unsaturated fatty acids

LcPUFA- long-chain PUFA

LNA- linolenic acid

LA- linoleic acid

MUFA- monounsaturated fatty acids

OLA- oleic acid

PUFA- polyunsaturated fatty acids

SFA- saturated fatty acids

SGR- specific growth rate

WG- weight gain

ZN- Ziehl-Neelsen

Chapter 1. General introduction

1.1 Aquaculture sector development

1.1.1 Global availability of aquatic products

Aquaculture and fisheries are two activities that have taken place together in the face of the challenge of increasing global demand for healthy and nutritious aquatic products. In 2014, world aquatic production was 195.8 million tons, considering the sum of fisheries and aquaculture production (Figure 1; FAO, 2016). This production has grown steadily over the last five decades at an average rate of almost 3%, exceeding the growth rate of the world population, which has been 1.6%. Recently, world per capita consumption of aquatic products has increased from 9.9 kg in 1960 to 19.2 kg due to the incessant increase in aquatic food production (APROMAR, 2015).

Aquatic products are one of the most important sources of animal protein in the world. According to FAO (2016), in recent years fish has accounted for 16.7% of the world's animal protein intake and 6.5% of all protein consumed, accounting for 20% of total protein consumed in developing countries and 15% in Europe and North America. This proportion may exceed 50% in some countries. A 150 g serving of fish provides between 50% and 60% of the daily protein needs of an adult, in addition to the value of their omega-3 oils.

The intensification of fisheries exploitation and new technologies applied to fishing fleets has led to maximum exploitation of wild fish resources. Consequently, the world catches from fisheries have stabilized in the last 20 years at around 90 million tons per year, never exceeding the 95 million tons. In 2014, total catches amounted to 94.6 million tonnes (Figure 1; FAO, 2016). The stabilization of fisheries at practically impossible levels to overcome, added to the steady increase in the demand for aquatic products have driven the development of aquaculture for the global supply of these foods. Also, it is precisely in the moment that fishing activity stagnates, when aquaculture sector, producing 101.1 million tons in 2014, has exceeded fishing output by 6.5 million tons (FAO, 2016).

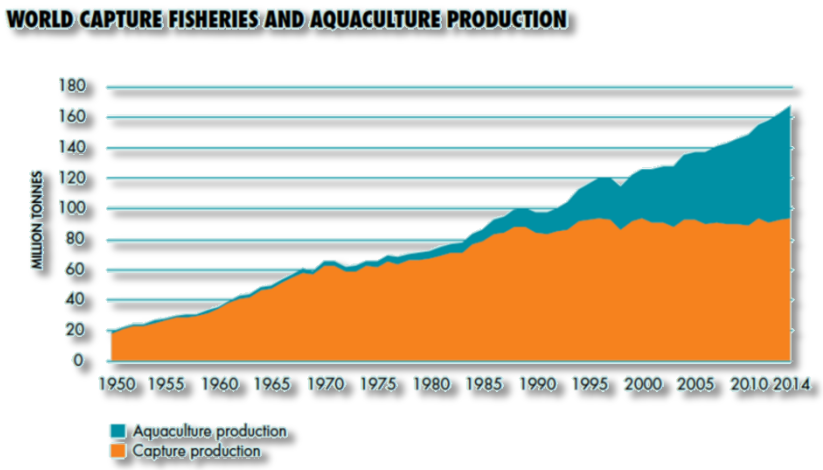

Figure 1: Evolution of world aquaculture and fisheries production between 1950 and 2014 (FAO, 2016).

1.1.2 European and Mediterranean aquaculture

Aquaculture is one of the fastest growing food-producing activities in the world representing, nowadays, 50 percent of the world's food fish and contributing to release pressure on wild stocks (Monfort, 2010; APROMAR, 2016).

Although, in Europe, aquaculture production has increased in comparison with previous decades, also the consumption of aquatic products in the EU has increasing in the past years, and the insufficient annual increase of aquaculture production added to the reduction of the fisheries have caused an import of 7 million of tons, which represents 56.8% of the total consumption (APROMAR, 2015). For that reason, European seafood market is facing a dramatic deficit depending extremely on external supplies (Monfort, 2010).

Mediterranean aquaculture started centuries ago and during the past three decades, it has expanded, diversified and intensified (Abellán and Basurco, 1999; Mente and Smaal, 2016). Aquaculture in European Union (EU) has highly grown during the '80s and '90s, also increasing by 25% from 1992. Nevertheless, from 2002 to 2011 the production had been stagnant (FAO, 2013). In 2012, the situation reversed and EU aquaculture production started to increase afresh. In 2014, EU aquaculture production reached 1.3 million of tons in volume which represented 4.178 million of Euros (APROMAR, 2016).

The most produced species are the mussel (*Mytilus galloprovincialis*) with 493 192 tons, rainbow trout (*Oncorhynchus mykiss*) with 194 081 tons, and the Atlantic salmon with 175 090 tons, in 2014 (APROMAR, 2016). Although Mediterranean aquaculture used to be more important in mollusc production, since the last decades fish production is increasing constantly, similarly to the global aquaculture trend (Abellán and Basurco, 1999). For that reason, farmed fish consumption has increased in recent years, mainly salmon and shrimp even though farmed seabream and seabass also have gained a high reputation in the market (Monfort, 2010). The gilthead seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax)* aquaculture production was estimated in 181 442 tons and 176 970 tons, respectively, for 2015, which represents a considerable portion of the total production (APROMAR, 2016). Nevertheless, driven by the saturation of traditional markets of seabass and seabream, their commercial value has decreased. In order to improve the competitiveness of the Mediterranean aquaculture sector and to expand the market, an urgent need to look for new aquaculture species that guarantee the competitiveness and sustainability of Mediterranean aquaculture emerged. Therefore, European aquaculture not only increased the total fish supply in volume, but also in the offer of farmed species (Abellán and Basurco, 1999). This diversification allows the growth of the Mediterranean aquaculture sector. In this context, meagre is considered one of the best candidates for the marine aquaculture diversification and so its production is expected to further increase.

1.1.3. Aquaculture development in Portugal

Aquaculture production in Portugal has never reached the economic importance of the fisheries sector. However, due to the overexploitation of fish wild stocks, Portuguese fisheries declined more than 65% since 1986 (FAO, 2013). Consequently, the interest in aquaculture has increased. Although in the first years very scarce knowledge limited the survival of many commercial productions, since the late ‘80s, the improvement of the knowledge on the area allowed growth of the sector in ‘90s (INE, 1998).

Meanwhile, between 2000 and 2008, national productions experienced a stagnation due to the importation of big amounts of fish from other countries, especially from Greece, affecting fish prices and competitiveness production costs, which decreased significantly (FAO, 2013).

In 2013, national aquaculture production was 9 955 tons, generating a revenue of 54 million Euros. These results translated into a reduction in quantity (-9.0%) but an increase in value (+3.1%), compared to 2012. The reduction of production was mainly due to reduction in turbot production. However, the aquaculture product sold was more valued in relation to previous year. Production in brackish and marine waters seems to be the most important, accounting for about 92% of total production in 2013 (DGRM, 2015).

According to DRGM (2016), Portuguese aquaculture, in 2014, reached 10 791 tons which represents a revenue of 50.3 million Euros. These values reflected an increase in quantity (+7.2%) but a decrease in value (-8.3%), compared to 2013. This result was justified by the increase of turbot production but with lower value compared to the previous year. The production of finfish in brackish and marine waters accounted for 47.7% of the total production, within which gilthead sea bream and turbot represented 91.0%. Bivalve molluscs represented 45.0% of total production, with clams remaining the most relevant species (2 251 tons), followed by mussels (1 547 tons).

1.2 Biology and aquaculture production of meagre *(Argyrosomus regius,* Asso, 1801*)*

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Percomorphi

Suborder: Percoidei

Family: Sciaenidae

Genus: *Argyrosomus*

Species: *Argyrosomus regius* (Asso, 1801)

Meagre is a marine fish belonging to Sciaenidae family, within the Percomorphi Order, Suborder Percoidei. This family includes about 70 genera and 270 species: marine, freshwater and brackish water, distributed all over the world (Nelson, 2006). The genus *Argyrosomus* is composed of 9 species (Table 1).

Table 1: Species belonging to genus *Argyrosomus* (Froese and Pauly, 2014)

Species	Author	Common name
Argyrosomus amoyensis	Bleeker, 1863	Amoy croaker
Argyrosomus beccus	Sasaki, 1994	Pugnose kob
Argyrosomus coronus	Griffiths y Heemstra, 1995	Dusky kob
Argyrosomus heinii	Steindachner, 1902	Arabian sea meagre
Argyrosomus hololepidotus	Lacépède, 1801	Southern meagre
Argyrosomus inodorus	Griffiths y Heemstra, 1995	Mild meagre
Argyrosomus japonicus	Temminck y Schlegel, 1843	Japanese meagre
Argyrosomus regius	Asso, 1801	Meagre
Argyrosomus thorpei	Smith, 1977	Squaretail kob

1.2.1 Morphological characteristics

Meagre (Figure 2) has a big head with an elongated and slender body, almost fusiform and slightly compressed. Its body colour is silver-grey, with dorsally bronze traits, whitish belly and reddish-brown fin base (Quéméner, 2002; FAO, 2017). Mouth is positioned at the terminal portion of the body without barbells. The buccal cavity is orange yellow, wide and slightly oblique, with small teeth arranged in several series. In the upper jaw the larger teeth are arranged at the anterior area, while in the lower jaw they are placed posteriorly or between the small teeth. It has large ctenoids scales throughout the body, with exception of some parts of the head, nose and below the eyes, where they are small and cycloid (Whitehead et al., 1984, 1986). The dorsal fin is unique and divided in two through a deep cleft: the anterior part is formed by 9 to 10 hard rays and the posterior is the longest one formed by 1 hard and 26 to 29 soft rays (Muus y Nielsen, 1999; FAO, 2017). The eyes are small and the lateral line is perfectly visible, black and it extends up to the caudal fin (Quéméner, 2002; Monfort, 2010). The caudal peduncle is strong and the caudal fin is truncated in the young fish, or s-shaped in adults. The anal fin has a first short and spiny ray, and a second one very thin (FAO, 2017). Several branched appendices are present in the gas bladder, which is associated with the lateral muscles of the body, and whose vibration produces a typical

sound (Tower, 1908; Takemura et al., 1978; FAO, 2017). It is also characterised for its large otoliths and, after death, its coloration turns brown (FAO, 2017).

Meagre is a large, corpulent and very agile fish (Piccolo et al., 2008) which can reach a total length of 2.3 meters (Maigret and Ly, 1986), weigh 103 kilograms (Quéro and Vayne, 1987) and reach 42 years old. However, the most common is to capture individuals between 0.5 and 1 m and 55 kg (Quéro y Vayne, 1987; Quéméner, 2002; González-Quirós *et al.*, 2011).

The digestive tract is relatively short, typical of carnivorous species and represents about 70% of its body length. It has a short, large-diameter oesophagus with muscular walls followed by a sac-shaped stomach, which allows the ingestion of large preys. It has developed glands with secretory function and muscular layers. Both the oesophagus and the intestine are inserted into the anterior portion of the stomach. The intestine is short and walls vary in thickness. At its anterior portion, near the pyloric area of the stomach, there are 8 to 10 pyloric caeca which have secretory and absorptive functions (Oliva et al., 2005; Gil et al., 2009).

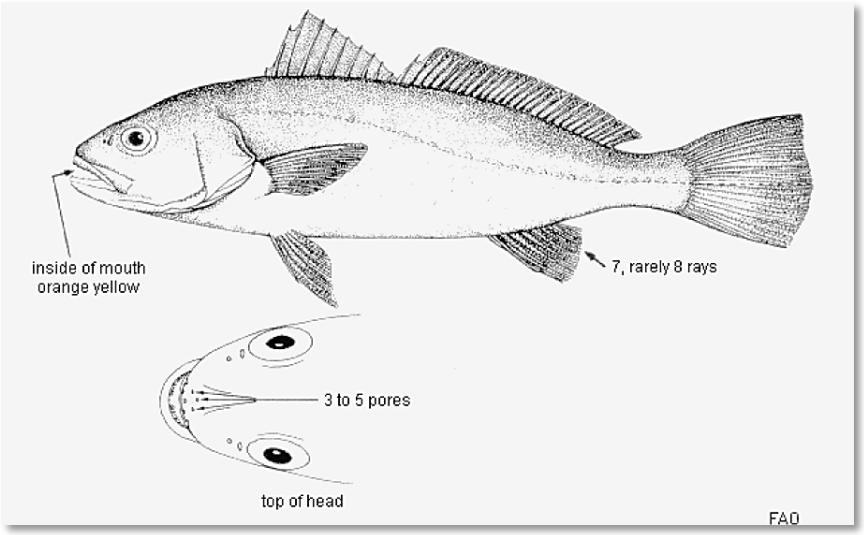

Figure 2: Morphological characteristics of *Argyrosomus regius* (Schneider, 1990).

1.2.2 Habitat and biological features

This species is widely distributed along the eastern coast of the Atlantic, from southern Sweden and Norway, to bay of Dakar, in Senegal, which seems to be the southern limit of its distribution (Quéro and Vayne, 1987). It is also present in the Mediterranean, Black Seas and Iceland Islands (Floeter et al., 2008; FAO, 2013). It is found throughout the Marmara Sea and in the Red Sea, where it reaches the Suez Canal in its migrations (Figure 3; Quéro, 1989; Chao 1990; Morales-Nin et al., 2012; Gil et al., 2013).

Meagre is a nectobenthic species establishing on rocky or sandy bottoms, from surface areas of 15 m to almost 300 m depth (Schneider, 1990; Quéméner, 2002). In addition, it is an euryhaline and eurytherm species, which allows its adaptation to very diverse environments (Lavié et al., 2008; Cárdenas, 2010). Growth is mainly achieved during summer and its feeding activity is reduced when sea temperatures drop below 13-15 ºC.

Meagre spawning season is between March and August, with a peek between April and June. It is usually located at the mouths of rivers and estuarine lagoons during the breeding season (Quéro and Vayne, 1987). This species performs what is known as anadromous migration that is related to its reproductive cycle (González-Quirós et al., 2011; Morales-Nin et al., 2012). The main places for its spawning in the North Atlantic and Mediterranean Sea are the Nile delta (Egypt), the Lévrier Bay (Mauritania) and the Gironde estuary (France) (Queró, 1989). At end of May, adults approach the coastal zones forming banks and enter the estuaries to spawn (González-Quirós et al., 2011; Morales-Nin et al., 2012). During this time, males produce a typical deep sound used to form spawning aggregations (Lagardère and Mariani, 2006). From mid-June until the end of July they leave estuaries to feed along the coast. They stay in shallow water until the beginning of autumn and during winter, meagre return to deeper water.

Apparently, the most determining factor for these reproductive migrations seems to be water temperature (Cárdenas, 2013). The arrival of adults and the departure of juveniles occur when water temperature is close to 17 and 20 ºC, respectively (Quéro and Vayne, 1987).

This species is a gonochoric with an asynchronous (García-Pacheco and Bruzón, 2009; González-Quirós et al., 2011; Morales-Nin et al., 2012; Mylonas et al., 2013) or in synchronic groups ovarian development (Abou Shabana et al., 2012; Schiavone et al., 2012). The fertilization of the eggs is external (Chao, 1990) and they remain sexually undifferentiated up to 9 months of age, with sexual differentiation occurring at 10-12 months of life (Schiavone et al., 2012). The first sexual maturity is reached between 4 and 5 years of age and usually occurs first in females than in males (García-Pacheco and Bruzón, 2009).

In captivity, natural spawns are not recorded and viable eggs are only produced by artificial induction with hormones treatments. After the hormones administration, the spawning occurs spontaneously without being required stripping or artificially fertilization of the eggs (Duncan et al., 2008; Mañanós et al., 2008).

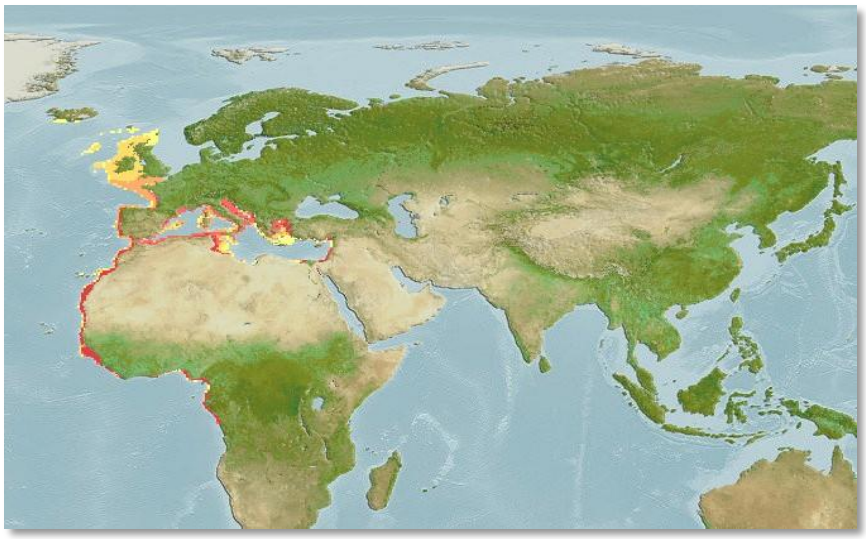

Figure 3: Geographical distribution of meagre (Froese and Pauly, 2014).

Regarding its feed habits, meagre is a carnivorous species. In the wild, it feeds on Mysidacea, Decapode and Teleostei (Cabral and Ohmert, 2001). The proportion of these preys in the diet varies according to the age. Thus, juveniles feed essentially on Mysidacea and Copepods (Baldó and Drake, 2002; Jiménez et al., 2005; Cárdenas, 2010) and as they grow, the diet becomes more piscivorous, based on demersal and benthic fish (Jiménez et al., 2005; Pasquaud et al., 2008, 2010; Gil et al., 2013).

1.2.3 Meagre aquaculture production

The world aquaculture production has increased from a few tons in 2000 to more than 10 000 tons in 2010, evidencing the appearance of new aquaculture species on the market, such as meagre (Monfort, 2010). Therefore, in recent years, a great interest for meagre production has grown and this species constitutes one of the most promising species for the diversification of aquaculture, considering it as a priority species in the research and development programs of the Mediterranean countries (Poli et al., 2003; Mateos, 2007; Chatzifotis et al., 2010). Meagre has several characteristics that support it as a suitable candidate for commercial production, such as easy adaptation to captivity (Cárdenas, 2010; Monfort, 2010), fast growth (around 1 kg per year) and excellent feed conversion rates, thus increasing the profitability of its production (Calderón et al., 1997; Jiménez et al., 2005; El-Shebly et al., 2007). In addition, it possesses a high flesh nutritional quality, with high protein levels and low mesenteric and muscular fat which is very appreciated by consumers. Total lipids are characterised by high levels of polyunsaturated fatty acids (PUFA), and especially a high proportion of n-3 PUFA and low n-6/n-3 ratio values (Table 2; Poli et al., 2003; Piccolo et al., 2008; Monfort, 2010).

Table 2: Chemical-nutritional composition of cultured meagre from Italy productions units (adapted from Orban et al., 2008)

	Min	**Max**
Weight (kg)	0.65	4.83
Lenght (cm)	43	84
Values referred to 100g tissue		
Water (g)	72.69	76.1
Protein (g)	19.14	21.71
Total lipid (g)	1.68	4.18
Ash (g)	1.12	0.35
Energy value (kcal)	97	124
Fatty acids (% of total FA)		
Saturated	29.29	30.41
Monounsaturated	26.28	30.82
Polyunsaturated	34.48	39.16
n-3	20.7	26.72
n-6	11.71	13.79
n-3/n-6	1.5	2.15

Meagre capture from fisheries in Europe is low (few hundred tons) although in some countries, such as Spain and Portugal, this amount increased considerably since it is highly appreciated product (Monfort, 2010). As aforementioned, due to the need for species diversification in the Mediterranean aquaculture sector, Italian and French producers centred efforts to start meagre production in the late '90s. France registered the first commercial production of this species, and ever since its production has slowly expanded to nearby regions, especially in Spain, Italy, Egypt and other Mediterranean countries. Using adapted rearing and feeding protocols of sea bream (*Sparus aurata*), once both species have similarities, meagre aquaculture reached a production output of 11 770 tons in 2014 (Figure 4; Monfort, 2010; FAO, 2017).

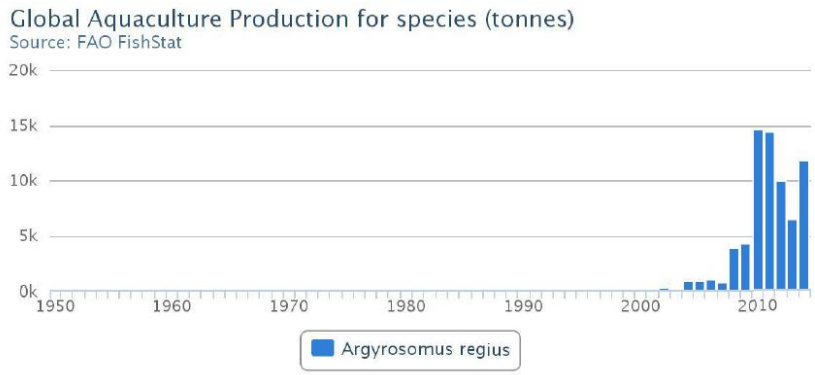

Figure 4: Meagre (Argyrosomus regius) production from aquaculture (FAO, 2017).

The commercial scale production of this species takes place in several Mediterranean countries (Monfort, 2010). According to FAO (2013), meagre production in Europe in 2010 registered 2350 tons. The main producing country is Spain with approximately 60.9% of the total production, followed by France and Italy (Figure 5).

Figure 5: Meagre aquaculture top producers (Portugal, Spain, France and Italy; FAO, 2017).

The increase of meagre aquaculture production has contributed to the creation of small niches in the European market, especially in those countries where it has been consumed traditionally, as in Portugal, Spain and part of France and Italy (Monfort, 2010). However, despite the good quality of both fillets and whole fish, this species is not very well known in Europe, and its current average price in the European market is 7 to 12 € /kg, depending on the areas of consumption (FAO, 2017). Since 2002, the commercialization of meagre is carried out according to size, and there is already a certain degree of processing of the product. Thus, the 600 g to 1 kg specimens are sold whole or filleted, while the large specimens are commercially filleted and smoked (Monfort, 2010). Besides, more than 50% of the fish sold is 1-2 kg, 30% is more than 2 kg and the remaining is below 1 kg (Duncan et al., 2013).

Intensive production of this species is mainly made in land-based tanks or in sea cages (nowadays, the most used). Spain, France, Greece, Italy and Egypt are the major producers of juveniles (Suquet et al., 2009). Since the number of meagre production units is low, cost comparisons with other species are difficult to estimate. In land-based systems the costs depend mainly on the size of the farm. However, in sea

cage culture the main expense is the cost of juveniles (FAO, 2017). According to Duncan et al. (2013), the cost of production of the meagre is the same of the seabream and is estimated to be approximately 3.9 € /kg (Table 3). Usually, aquafeeds represent another of the highest costs during the growing, but for meagre is lower than for other marine fish species (FAO, 2017), since the feed conversion ratio (FCR) for this species is generally better (Jimenez et al., 2005; El-Shebly et al., 2007).

Table 3: Comparison of costs between meagre and seabream productions

	Meagre[a]		**Seabream[b]**	
	Cost kg^{-1}	**% of costs**	**Cost kg^{-1}**	**% of costs**
Juveniles	0.8 €	21 %	0.83 €	21 %
Feed	1.6 €	41 %	1.51 €	39 %
Workhand	0.5 €	13 %	0.87 €	22 %
Others	1.0 €	26%	0.70 €	18%
Total	3.9 €	100 %	3.91 €	100 %

[a] Duncan *et al.* (2013), [b] Merinero *et al.* (2005).

Due to the gap in the knowledge on meagre nutritional requirements, diets for bream have been used. Initially, optimal protein of 45-48% and 20-24% of lipids were suggested (Duncan et al., 2013). However, Chatzifotis et al. (2012) proved that highest growth was obtained with 40-50% protein and meagre lipid requirement was lower than the initially recommended, with a dietary lipid of 17% being sufficient to satisfied the requirement (Chatzifotis et al., 2010, 2012).

Despite the known great potential of meagre for large-scale production, there still are several questions that need to be addressed to achieve the mass scale production of this species. One of most important aspect is to establish the optimal rearing conditions and optimal diets for larvae and juvenile production to guarantee the quantity and quality of fish necessary for intensive production of the species, as well as the nutritional requirements, especially for early stages.

1.3 Lipids and fatty acids

1.3.1 Importance of the study of lipids and fatty acids

For the development and intensification aquaculture production is necessary to provide adequate feed. Fish nutrition has become a very important area of research, since aquafeed costs generally constitute a substantial percentage (up to 50%) of the total operating costs of an aquaculture enterprise. To optimise the aquaculture production of fish and crustaceans, one of the most important aspects is to characterize the nutritional requirements of the species, allowing the development of diets that cover the basic metabolic and physiological needs at the lowest cost. In the last two decades, there was a great interest in understanding the lipid nutritional requirements of aquaculture species for optimize their production (Tocher, 2003). Even tough, nowadays, little information is available regarding the nutritional requirements of lipids and fatty acids (FA), compared to other nutrients, since lipids have a certain chemical and metabolic complexity (Tocher, 2003).

1.3.2 Functions of fatty acids

Lipids are one of the main nutrients presented in fish as well in their diets, as they play an important role as metabolic energy substrate, having the highest total energy per unit weight (9.5 Kcal / g), being source of essential fatty acids (EFA) and, also, a carrier of vitamins and certain micronutrients of liposoluble character (Tocher, 2003, 2010; Glencross, 2009).

FA are the main and the most important components of lipids. According to the International Union of Physical and Applied Chemists (IUPAC), a FA is defined based on: the number of carbon atoms, the number of unsaturated bonds in its chain, and the position of those bounds relative to the methyl terminus (Glencross, 2009; Tocher and Glencross, 2015). Nowadays, many FA are known (Table 4). Briefly, there are basically three important groups of fatty acids based on their number of unsaturated bounds: fatty acids with no unsaturated bounds designated as saturated fatty acids (SFA); with only one unsaturated bound known as monounsaturated fatty acids (MUFA); and those with more than one unsaturated bound designated polyunsaturated fatty acids (PUFA). In aquaculture, usually the terms highly unsaturated fatty acids (HUFA) or long-chain PUFA (lcPUFA) are used to define PUFA with more than 20 carbon atoms (Nichols, 2004). In this dissertation, the term HUFA was adopted.

Table 4: Nomenclature of principal fatty acids (adapted from Glencross, 2009)

Shorthand	Trivial name	Abbreviations
8:0	Caprylic	-
10:0	Capric	-
12:0	Lauric	-
14:0	Myristic	-
14:1n-5	Myristoleic	-
16:0	Palmitic	-
16:1n-7	Palmitoleic	-
17:0	Margaric	-
18:0	Steric	-
18:1n-9	Oleic	OLA
18:2n-6	Linoleic	LA
18:3n-3	Alpha linolenic	αLNA
18:3n-6	Gamma linolenic	ɣLNA
18:4n-3	Stearidonic	SDA
20:0	Arachidic	-
20:1n-9	Gondoic	-
20:3n-6	Homo-g-linolenic	-
20:4n-6	Arachidonic	ARA
20:5n-3	Eicosapentaenoic	EPA
22:0	Behenic	-
22:1n-9	Erucic	-
22:4n-6	Adrenic	-
22:5n-3	Docosopentaenoic	DPA
22:6n-3	Docosohexaenoic	DHA
24:0	Lignoceric	-
24:1n-9	Nervonic	-

-, no trivial name or abbreviation.

FA play key biological roles in animals, including fish. HUFA are the most biologically active group of FA, which are involved in several metabolic pathways in all vertebrates, such as energy production, membrane structure and function, control of lipid homeostasis and are precursors of eicosanoids. The latter are short-life autocrine molecules produced by cells of every tissue, with a broad range of physiological actions as blood clotting, immune and inflammatory response, cardiovascular, neural and renal function, and reproduction (Tocher, 2003). N-3 and n-6 HUFA are of pivotal importance within which can be highlighted the eicosapentaenoic acid (20:5n-3, EPA), the docosahexaenoic acid (22:6n-3, DHA) and the arachidonic acid (20:4n-6, ARA).

DHA is the major component in cell membranes, maintaining their integrity and being component of phospholipids (Sargent et al., 1995; Izquierdo, 2005). It is necessary for reproduction, growth, survival, flat fish metamorphosis and disease prevention. It is present in great quantity in the membrane of the rods of the retina, reason why it is associated to the development of the vision and in the neural membranes or the central nervous system (Sargent et al., 1995; Mourente et al., 1999; Izquierdo, 2005). Due to the lower cell oxidation rates than other fatty acids, DHA is more retained in starved or low-EFA fed fish (Madsen et al., 1999; Izquierdo, 2005). Besides, it functions as a substrate for some lipoxygenases (Asturiano, 1999) and is known to have a greater potential as a growth promoter, being more effective for growth and survival than the other n-3 HUFA (Watanabe et al., 1989a; Watanabe et al., 1993; Izquierdo, 1996, 2005).

EPA is particularly important for growth although more important in early life stages (Watanabe et al., 1989a) and for broodstock fertility (Fernández-Palacios et al., 1995). It is required as substrate for cyclooxygenases and lipoxygenases, being precursor of proteinoids and leukotrienes, respectively, and competing with ARA for these enzymes regulating eicosanoids synthesis (Izquierdo, 2005). In marine fish, EPA is a main component of polar lipids, with important function in the regulation of membrane integrity and function, although less important than DHA (Izquierdo, 2005).

Finally, ARA requirements for fish are less studied, due to its lower direct impact on growth when compared to DHA and EPA, although ARA also plays important roles in fish metabolism (Izquierdo and Koven, 2010). ARA is the main component of the phosphatidyl inositol (a cell membrane component), is the preferred substrate for most cyclooxygenases and is the main precursor of prostaglandin synthesis (Izquierdo, 2005). Despite the increase of dietary ARA level does not affect fish growth, in general, it increases fish survival and stress resistance (Harel et al., 2001; Koven et al., 2001). However, an excess of dietary ARA can influence negatively marine fish, reducing survival, growth and pigmentation. The negative effect of an excess of ARA may be related to the competition of ARA and EPA for the enzymes inducing an eicosanoid imbalance (Izquierdo and Koven, 2010).

1.3.3 Fatty acid requirements in teleost fish

EFA is the term used when animals are unable to synthesize it *de novo* (endogenous synthesis) in the required amounts. Moreover, its presence is necessary for the maintenance of vital cellular functions (Sargent et al., 1989). The environment in which fish evolved has conditioned the type and availability of food which has led to the determination of essential nutrients in these organisms, in particular that of FA (Sargent et al., 2002). In marine fish, there is an uptake of HUFA associated with the phyto and zooplankton consumption of this environment. Therefore, marine fish had no evolutionary pressure to produce n-3 HUFA by endogenous pathways, whereas freshwater species maintained this pressure and being able to produce them (Tocher, 2010).

All vertebrates can desaturate the palmitic acid (16:0) and the steric acid (18:0) to palmitoleic acid (16:1n-7) and oleic acid (18:1n-9, OLA), respectively. This process occurs in the endoplasmic reticulum of cells of certain tissues through an aerobic via using CoA units and requiring NAD(P)H and the Δ9 fatty acid desaturase enzyme (Tocher, 2003). However, vertebrates have a dietary requirement for specific PUFA, namely members of both n-3 and n-6 series since these FA cannot be synthetized *de novo.* This is because most animals lack the Δ12 and Δ15 (n-3) desaturases so they are not able to synthesise linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA) from their precursor OLA (Tocher, 2006, 2010). LNA and LA can be further desaturated and elongated to yield more biologically active forms of PUFA, including the DHA, EPA and ARA (Figure 6). The inability of most marine fish to synthesize these FA *de novo* in sufficient quantities has been proven and several negative effects have been observed associated with their deficiency in the diet (Watanabe, 1987; Webster and Lowell, 1990). It is well recognized that this incapacity is due to an impairment or deficiency in the enzymes required for the desaturation and elongation processes, specifically the Δ5 and Δ6 desaturases and the elongases (Figure 6). Therefore, the EFA requirements between freshwater and marine fish, vary qualitatively and quantitatively. While in freshwater fish, the EFA requirements can be provided mainly by LNA and LA, in marine fish the requirements are provided by EPA, DHA and, possibly, ARA (Watanabe, 1993; Sargent et al., 2002; Bell and Sargent, 2003; Tocher, 2003).

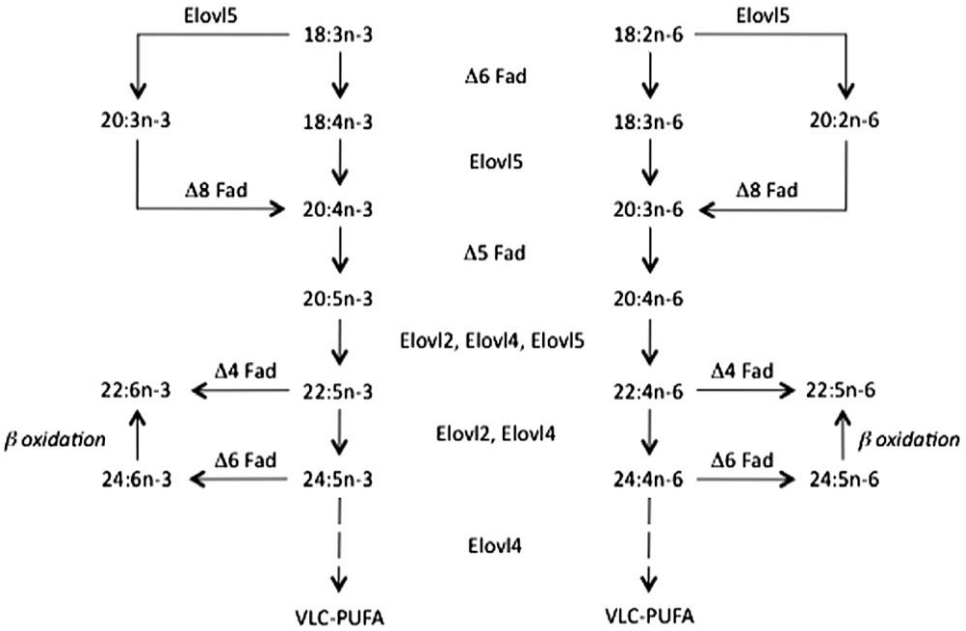

Figure 6: Biosynthesis pathways of highly unsaturated fatty acids (HUFA) from n-3 and n-6 precursors. Δ4 Fad, Δ5 Fad andΔ6 Fad, fatty acyl desaturases; Elovl2, Elovl4 and Elovl5, fatty acid elongases. All activities have been demonstrated in teleost fish species but not all species express all activities (Tocher, 2015).

The EFA requirements can also vary during the different life stages (Table 5). In general, fish in early life stages have higher requirements compared with juveniles and sub-adults (Tocher, 2010). Besides, the EFA requirement in broodstock is also important since dietary EFA level may affect reproduction, egg quality, rate of fertilization, hatching and survival of the new larvae (Tocher, 2010).

Table 5: n-3 HUFA requirements (% diets) of marine fish species

	Broodstock	Larvae	Juveniles
S. aurata	2.2[12]	>3[12]	1.0-2.5[12,13]
S. maximus	-	3.2[2]	0.8[3]
D. labrax	-	-	0.7-1.0[4]
S. dumerilii	-	4[1]	2.5[1]
P. pagrus	-	<3.4[5]	2.7-3.2[11]
C. carpio	-	0.05	-
S. raidneri	0.64[7]	nd	1[8]
D. dentex	-	4[9]	-
A. regius	nd	>3[10]	nd

[1]Izquierdo (own data); [2]Le Milinaire, 1984; [3]Gatesoupe *et al.*, 1977; [4]Coutteau et al., 1996 and Skalli and Robin, 2004; [5]Hernández-Cruz *et al.*, 1999; [6]Radunz-Neto *et al.*, 1993; [7]Corraze *et al.*, 1933; [8]Takeuchi and Watanabe, 1976; [9]Mourente *et al.*, 1999; [10] El-Kertaoui et al., 2015; [11]Takeuchi et al., 1992[a]; [12]Izquierdo, 2005; [13]Ibeas et al., 1996; nd: not detemined.

Other factors can influence the EFA requirement in fish. The trophic level is one of those factors since in the wild, types and contents of EFA differ among the trophic chain (Takeuchi, 1997). Planktivorous fish have higher intakes of EFA than icthyvorous species, so the higher the trophic level the higher requirement for n-3 HUFA (Izquierdo, 2005). Moreover, EFA requirements may also be influenced by environmental conditions such as temperature, salinity and light since these factors affect lipid composition of fish. It has been observed that the higher salinity the higher requirements for n-3 HUFA. Additionally, an increase in the number of double bonds and carbons in the FA enhances the fluidity of the membranes which would explain the increase of HUFA in fish that inhabit colder waters (Izquierdo, 2005). In fact, it has been experimentally observed that decreasing the water temperature increase the HUFA retention (Izquierdo, 2005).

1.3.4 Metabolic effects of essential fatty acid deficiency

It is well known the importance of EFA for the normal development of a live organism. As mentioned, marine fish are unable to synthesize *de novo* DHA, EPA, ARA, so it is necessary to incorporate them in the diet for the normal development and growth (Webster y Lovell, 1990).

In fish, the most obvious clinical signs of EFA-deficiency are poor growth and increased mortality. However, other pathologies are frequently observed, as myocarditis, fins erosion, shock syndrome, fatty liver and steatosis, lordosis, bleeding and disaggregation of gill epithelia, as well as increased sensibility to stressful situations, immune-deficiency, increased cortisol levels, and decreased reproductive capacity (Izquierdo, 2005; Glencross, 2009; Tocher, 2010). In addition, a few biochemical markers can be helpful to identify EFA-deficiency. When the level of dietary EFA is insufficient, the production of mead acid (20:3n-9) in freshwater fish, and its ratio to DHA increase. Values of 20:3n-9/DHA higher than 0.4 are considered indicator of EFA-deficiency (Castell et al., 1972; Watanabe et al., 1974; Watanabe et al., 1989a; Glencross, 2009). However, in marine fish, the production of mead acid is not a suitable indicator of EFA-deficiency, since they do not have the enzymes necessary to produced it from its precursor (OLA), as these enzymes are the same involved in the production of EPA and ARA (∆5 and ∆6 desaturases and elongases), absents in marine species (Tocher, 2010). Nevertheless, 18:2n-9 and 20:2n-9 have been reported in tissues of seabream and turbot fed low EFA diets (Tocher et al., 1988; Kalogeropulos et al., 1992). Indeed, using cell lines from marine or freshwater species origin, Tocher et al. (1989) reported that n-9 FA are accumulated in cell lines grown in the absence of EFA and were reduced when they are supplemented. Therefore, for marine fish species, the ratios 18:1n/n-3 HUFA or OLA/n-3 HUFA have been considered as biochemical indicators of EFA-deficiency (Fujii et al., 1976; Kalogeropoulos et al., 1992).

1.3.5 Importance of EFA provided from fish consumption to human nutrition

Of all dietary nutrients, FA have the greatest direct impact on the fish quality for human consumption. Fish represent the major or even the unique source of n-3 HUFA in human diet, the so-called ‘omega-3’, and several benefits to human health are associated to their consumption (Tocher, 2010). The n-3-HUFA benefits in human health and well-being include babyhood vision development, and prevention of cancer, cardiovascular and brain diseases, as well depression (Kris-Etherton et al., 2002). Furthermore, n-3 HUFA controls satiety, triglycerides blood levels, and inflammatory response (Calder, 2006). High levels of n-3 HUFA are ensured in aquaculture fish from the dietary inclusion of fish meal and fish oil. For all fish species, even for freshwater species, the dietary increase of vegetable oils will reduce the fillet n-3 HUFA content,

reducing the nutritional benefit to consumer (Naylor et al., 2009; Tocher, 2010; Tocher and Glencross, 2015). Thus, this issue has also highlighted the need for further research on EFA nutritional requirements and metabolism of aquaculture species.

1.4 Objectives of this thesis

1.4.1 General objective

- To assess the effect of increasing levels of n-3 HUFA on growth, feed utilisation, whole-body and liver composition and histopathology of meagre (*Argyrosomus regius)* fingerlings.

1.4.2 Specific objectives

- To evaluate the effect of 5 increasing dietary levels of n-3 HUFA (0.9%, 1.5%, 2.0%, 3.0% and 3.9% of dry matter) on fish growth performance, feed utilisation and whole-body composition, and to estimate the n-3 HUFA requirement of meagre fingerlings.

- To evaluate the effect of 5 increasing dietary levels of n-3 HUFA, below and above the requirement, on hepatosomatic index, liver composition, and liver histopathology and bacteriology of meagre fingerlings.

Chapter 2. Dietary requirement for n-3 highly unsaturated fatty acids for fast growth of meagre (*Argyrosomus regius,* Asso, 1801) fingerlings*

*****The following chapter was submitted to Aquaculture Journal (IF: 2.57) as: Carvalho, M., Peres, H., Saleh, R., Fontanillas, R., Rosenlund, G., Oliva-Teles, A., Izquierdo, M., 2017. Dietary requirement for n-3 highly unsaturated fatty acids for fast growth of meagre (*Argyrosomus regius*, Asso, 1801) fingerlings.

2.1 Abstract

The establishment of well balance diets that meet nutrient requirements is important to optimize a large-scale production of new aquaculture species. This is the case of meagre (*Argyrosomus regius*), a promising new aquaculture species, with great potential owing to its high growth rate, feed efficiency and easy adaptation to captivity. Knowledge on the nutritional requirements of this species is still scarce, namely regarding essential fatty acids, which are required to sustain growth, development, immune status and survival. A feeding trial was performed testing 5 increasing dietary n-3 HUFA levels (0.9, 1.5, 2.0, 3.0 and 3.9% DM) with the purpose of evaluating the n-3 HUFA requirements for fast growth of meagre fingerlings. Meagre reflected very high specific growth rates (4.1 to 4.6%) and low feed conversion ratios (0.7 to 0.8), thus highlighting its great potential for aquaculture production. Fish fed 0.9% n-3 HUFA showed the lowest growth, which was significantly improved by increasing the dietary n-3 HUFA levels up to 3.0%. DHA was selectively retained over EPA in whole fish body. Additionally, the reduction of the dietary n-3 HUFA level below optimum levels led to an accumulation of n-9 FA, as well as an increase in the OLA/n-3 HUFA ratio. Meagre seems to have active Δ6 desaturases and Elovl5, but their activities being insufficient to produce enough DHA and EPA from PUFA precursors to sustain fast growth. Thus, young meagre shows a typical marine requirement for n-3 HUFA,

particularly EPA and DHA, estimated to be circa 2.6-3.0% DM n-3 HUFA based on a second-order polynomial model.

2.2 Introduction

Aiming the competitiveness and sustainability of the Mediterranean aquaculture sector and the expansion of the market, an urgent need to look for new aquaculture species emerged (Abellán and Basurco, 1999). In recent years, a great interest for meagre (*Argyrosomus regius*) production has grown and this species constitutes one of the most promising species for the diversification of Mediterranean aquaculture, being considered as a priority species in research and development programs of the Mediterranean countries (Poli et al., 2003; Mateos, 2007; Chatzifotis et al., 2010). Meagre is a carnivorous fish belonging to the *Scianidae* family and inhabiting eastern coast of the Atlantic and Mediterranean and Black Seas (Quéro and Vayne, 1987; Floeter et al., 2008; FAO, 2013). The species has several characteristics that support it as a suitable candidate for commercial production, such as easy adaptation to captivity (Cárdenas, 2010; Monfort, 2010), fast growth (reaching more than 1kg in cages in less than 2 years) (Quèmèner, 2002; Suquet et al., 2009), and excellent feed conversion ratios (Calderón et al., 1997; Jiménez et al., 2005; El-Shebly et al., 2007). In addition, it has a high nutritional flesh quality, with high protein and n-3 polyunsaturated fatty acids (PUFA) as well with low n-6/n-3 ratio values (Poli et al., 2003; Piccolo et al., 2008; Monfort, 2010). In the wild, meagre feeds on Mysidacea, Decapode and Teleostei (Cabral and Ohmert, 2001). In captivity, commercial diets available for sea bream have been used. Commercial small-scale production of this species already takes place in several Mediterranean countries, mostly in Spain, France and Italy (FAO, 2017). However, to achieve successful large-scale, the development of commercial high quality species specific diets is required (Saavedra et al., 2016), but knowledge on the nutritional requirements of emerging aquaculture species, such as meagre, is still scarce (Chatzifotis et al., 2010; El-Kertaoui et al., 2015).

Fatty acids (FA) play key biological roles in animals, with highly unsaturated fatty acids (HUFA) being involved in several metabolic pathways including energy production, membrane structure and function, eicosanoids production and control of lipid homeostasis (Watanabe, 1982; Tocher, 2003). The n-3 HUFA, such as docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA), are the main n-3 HUFA of marine origin and are of pivotal importance for fish, particularly

marine species (Tocher, 2003; Izquierdo and Koven, 2010). Unlike freshwater species, marine fish have restricted ability or are unable to synthetize de novo n-3 and n-6 HUFA from their precursors, linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA), respectively (Tocher, 2003; Izquierdo and Koven, 2010; Oliva-Teles, 2012). This is due to a low expression or deficiency of the enzymes required for the desaturation and elongation processes, specifically the $\Delta 5$ or $\Delta 6$ desaturases and the elongases (Sargent, 1995; Izquierdo et al., 2005; Tocher, 2010). Therefore, n-3 HUFA are considered essential fatty acids (EFA) for marine fish and must be included in the diet in adequate levels to fulfil fish requirements for growth, survival and development (Sargent et al., 1995; Glencross, 2009). The n-3 HUFA requirements have been studied for juveniles of many marine fish species (Tocher, 2010; NRC, 2011). However, specific EFA requirements for meagre are still undetermined. Thus, the present study aims to evaluate n-3 HUFA dietary levels for fast growth of meagre fingerlings, based on growth performance, feed utilization and body composition.

2.3 Material and Methods

This experiment was conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at Fundación Canaria Parque Científico Tecnológico (FCPCT), University of Las Palmas de Gran Canaria (Canary Islands, Spain).

2.3.1 Experimental fish and rearing conditions

The feeding trial was conducted with meagre (*Argyrosomus regius*) fingerlings with an initial body weight of 2.80±0.23 g and an initial total length of 6.37±0.20 cm. Triplicate groups of meagre fingerlings, produced at Grupo de Investigación en Acuicultura (GIA) facilities, were randomly distributed in 15 experimental tanks (200 L fibreglass cylinder tanks with conical bottom and painted with light grey colour) at a density of 45 fish per tank and fed manually one of the experimental diets until visual apparent satiety, three times a day, 6 days per week, during 30 days. Daily feed intake was calculated by recording diet uptake and subtracting uneaten pellets. The tanks were installed in open system and supplied with filtered seawater (37 mg L^{-1} salinity). Water was continuously aerated and dissolved oxygen was maintained above 6.0± 0.2

mg L^{-1} during the trial. Average water temperature along the trial was 23.0±0.2 ºC. The experiment was run under natural photoperiod between September and October 2016.

At the beginning and end of the trial, all fish were individually weighed and sized (total length) after being unfed for 24 hours. At the beginning of the feeding trial ten fish were euthanized with excess of clove oil, and frozen at -80ºC to determine the initial whole-body composition as well as initial FA composition. At the end of the experiment, five fish per tank were collected for the same purposes.

2.3.2 Experimental diets

Five isoproteic and isolipidic experimental diets were formulated containing fish oil and vegetable oils (linseed, palm and rapeseed oils) as lipid sources. Five dietary increasing levels of n-3 HUFA, namely eicosatrienoic acid (20:3n-3, ETE), eicosatetraenoic acid (20:4n-3, ETA) docosapentaenoic acid (22:5n-3, DPA), EPA and DHA were defined: 0.9, 1.5, 2.0, 3.0 and 3.9% of dry matter (DM), where the last two accounted for 93% of total n-3 HUFA. The n-3 HUFA of each experimental diet was achieved by successively replacing vegetable oils by fish oil. Diet composition and proximate analysis are shown in Table 6 and dietary fatty acid composition in Table 7. The experimental diets were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) with a pellet size of 2 mm, analysed for proximate and fatty acid composition at GIA laboratories (Ecoaqua Institute, University of Las Palmas de Gran Canaria, Spain) and kept in a cold room at 10ºC until use.

Table 6: Composition and proximate analysis of the experimental diets for meagre fingerlings

	Dietary n-3 HUFA level (% DM)				
	0.9	1.5	2.0	3.0	3.9
Ingredients (%)					
Fish meal, N. Atlantic[1]	15.0	15.0	15.0	15.0	15.0
Corn gluten[2]	10.0	10.0	10.0	10.0	10.0
Faba beans[1]	10.0	10.0	10.0	10.0	10.0
Wheat[1]	8.0	8.0	8.0	8.0	8.0
Wheat gluten[1]	18.4	18.4	18.4	18.4	18.4
Soy protein concentrate[1]	25.0	25.0	25.0	25.0	25.0
Fish oil, S. American[1]	0.0	2.7	5.4	8.2	10.9
Linseed oil[3]	1.6	1.2	0.8	0.4	0.0
Palm oil[3]	3.3	2.5	1.7	0.8	0.0
Rapeseed oil[1]	6.0	4.5	3.0	1.5	0.0
Premix[4]	2.8	2.8	2.8	2.8	2.8
Proximate analysis (% DM)					
Protein	56.5	54.5	54.5	56.0	54.3
Lipids	16.2	17.0	16.5	16.9	16.2
Ash	4.9	5.0	5.1	5.2	5.0
Moisture	8.7	8.5	8.5	8.2	7.9

1: Skretting, Stavanger, Norway

2: Cargill Nordic AS, Charlottenlund, Denmark

3: AAK AB, Karlshamn, Sweden

4: Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins and minerals. Vitamin and mineral supplementation as estimated to cover requirements according NRC (2011).

Table 7: Fatty acid composition of the experimental diets for meagre fingerlings (% total identified fatty acids) (to be continued in the next page)

Fatty acid	Dietary n-3 HUFA level (% DM*)				
	0.9	1.5	2.0	3.0	3.9
14:0	1.5	2.2	3.6	4.6	5.9
14:1n-5	0.1	0.1	0.1	0.2	0.3
15:0	0.1	0.2	0.3	0.4	0.5
16:0	16.5	17.0	18.1	18.4	18.6
16:1n-7	1.4	2.2	3.6	5.0	6.2
16:1n-5	0.1	0.1	0.2	0.2	0.3
16:2n-4	0.2	0.3	0.4	0.6	0.8
16:3n-4	0.1	0.1	0.2	0.2	0.2
16:3n-3	0.1	0.1	0.1	0.2	0.3
16:4n-3	0.2	0.4	0.6	0.9	1.2
17:0	0.1	0.7	0.4	0.6	0.8
18:0	3.1	3.2	3.3	3.5	3.5
18:1n-9	34.3	30.8	25.6	18.3	11.1
18:1n-7	1.9	2.0	2.3	2.4	2.6
18:2n-9	0.0	0.0	0.0	0.1	0.1
18:2n-6	20.4	18.8	16.4	14.2	12.2
18:2n-4	0.1	0.1	0.1	0.2	0.3
18:3n-6	0.0	0.1	0.1	0.2	0.2
18:3n-4	0.1	0.1	0.1	0.1	0.2
18:3n-3	9.1	7.9	5.7	3.7	1.5
18:3n-1	0.0	0.0	0.0	0.0	0.0
18:4n-3	0.5	0.7	1.1	1.6	2.2
18:4n-1	0.0	0.1	0.1	0.1	0.1
20:0	0.4	0.4	0.4	0.4	0.3
20:1n-9	0.1	0.1	0.2	0.2	0.2
20:1n-7	1.6	1.6	1.9	1.9	2.0
20:1n-5	0.1	0.1	0.2	0.2	0.3
20:2n-9	0.0	0.0	0.0	0.1	0.1
20:2n-6	0.1	0.1	0.1	0.1	0.2
20:3n-9	0.0	0.0	0.0	0.1	0.1
20:3n-6	0.0	0.0	0.1	0.1	0.1
20:3n-3	0.0	0.0	0.0	0.0	0.1
20:4n-6	0.2	0.3	0.4	0.6	0.8
20:4n-3	0.1	0.2	0.3	0.4	0.5
20:5n-3	2.4	3.7	5.3	8.0	10.7

22:1n-11	1.6	1.7	2.1	2.3	2.5
22:1n-9	0.3	0.3	0.3	0.3	0.3
22:4n-6	0.0	0.0	0.1	0.1	0.1
22:5n-6	0.0	0.0	0.0	0.1	0.1
22:5n-3	0.3	0.4	0.6	0.9	1.2
22:6n-3	2.8	4.2	5.6	8.5	11.3
Σ SFA	21.8	23.3	26.1	27.9	29.6
Σ MUFA	41.4	39.1	36.4	31.3	25.9
Σ n-3	15.5	17.6	19.2	24.0	28.6
Σ n-3 HUFA	5.6	8.5	11.8	17.8	23.8
Σ n-6	20.7	19.4	17.2	15.3	13.7
Σ n-6 HUFA	0.3	0.5	0.7	0.9	1.3
Σ n-9	34.7	31.3	26.1	19.9	11.9
OLA/n-3 HUFA	6.1	3.6	2.2	1.0	0.5
EPA/DHA	0.9	0.9	0.9	0.9	1.0
EPA/ARA	14.2	13.9	13.1	13.5	13.6
EPA+DHA	5.2	7.9	10.9	16.5	22.0

* n-3 HUFA (% total FA) x dietary lipids (%DM).

2.3.3 Analytical methods

Prior to biochemical analysis, samples were homogenized (T25 Digital Ultra-turrax, IKA®, Germany) to obtain one pooled sample from each tank, which was analysed in triplicate. The same method was applied for initial fish sample. Moisture, ash and protein were determined according to A.O.A.C. (2000). Total lipid content was extracted with chloroform/methanol (2:1 v/v) (Folch et al., 1957). Fatty acid methyl esters (FAMES) were obtained by transmethylation of total lipids as suggested by Christie et al. (1989). FAMES were separated by gas liquid chromatography under the conditions described by Izquierdo et al. (1990), quantified by a flame ionizator detector (Finnigan Focus SG, Thermo electron Corporation, Milan, Italy) and identified by comparison with previous characterized standards. Retention of the most relevant fatty acids was calculated.

2.3.4 Statistical analysis

Data are presented as means ± standard error (SE). All data were tested for normality and homogeneity of variances using Shapiro–Wilk and Levene's tests, respectively, and analysed by one-way ANOVA. When p-values were significant ($P<0.05$), means were compared with Tukey´s multiple range test (Tukey, 1949). Different regression models were used for estimation of EFA requirements, but only a second-order polynomial model provided adequate fitting to the data. All statistical analyses were done using the SPSS 21.0 software package for Windows.

2.4 Results

2.4.1 Growth performance

During the feeding trial, no external damage or abnormal behaviour were observed. Survival was high in all treatments and was not affected by diet composition (Table 8). After 30 days of feeding, fish growth was significantly affected by the dietary n-3 HUFA levels. Fish fed the 0.9% n-3 HUFA diet showed the lowest final total length and weight ($P<0.05$). Final length significantly increased with the increase of dietary n-3 HUFA levels up to 2.0-3.0%, while final weight was significantly lower for the 0.9% n-3 HUFA diet than for the other diets, among which no significant differences were observed. There was a trend for WG and SGR to increase as dietary n-3 HUFA increased up to 3.0%. Feed intake, FCR, PER and LER were not affected by the dietary treatment.

A second-order polynomial model best fitted dietary n-3 HUFA level to WG (Figure 7). Based on this model, the dietary n-3 HUFA for 95% maximal WG was estimated to be 3.0% DM.

Table 8: Growth performance of meagre fingerlings fed the experimental diets for 30 days[1]

	Dietary n-3 HUFA level (% DM)				
	0.9	1.5	2.0	3.0	3.9
Survival (%)[2]	93.3±0.74	97.8±1.28	99.3±0.74	94.8±1.48	97.8±2.22
Initial total length (cm)	6.4±0.04	6.3±0.05	6.3±0.04	6.4±0.04	6.2±0.04
Final total length (cm)	9.0^{c}±0.11	9.4^{b}±0.12	9.3^{ab}±0.09	9.6^{a}±0.08	9.3^{bc}±0.11
Initial body weight (g)	2.8±0.05	2.8±0.06	2.7±0.05	2.7±0.05	2.6±0.05
Final body weight (g)	9.5^{b}±0.29	10.4^{a}±0.34	10.2^{ab}±0.36	10.7^{a}±0.30	10.4^{a}±0.32
Weight gain (g)[3]	6.7±0.43	7.6±0.39	7.5±0.34	8.0±0.28	7.8±0.21
Specific growth rate (% day^{-1})[4]	4.1±0.14	4.3±0.21	4.5±0.10	4.5±0.11	4.6±0.06
Feed intake (g feed $fish^{-1}$ day^{-1})[5]	0.2±0.01	0.2±0.00	0.2±0.01	0.2±0.00	0.2±0.00
Feed conversion ratio[6]	0.8±0.06	0.7±0.03	0.7±0.02	0.7±0.03	0.7±0.04
Condition factor[7]	1.3±0.02	1.3±0.01	1.3±0.05	1.2±0.05	1.3±0.04
Protein efficiency ratio[8]	2.38±0.17	2.57±0.11	2.54±0.07	2.63±0.12	2.58±0.05
Lipid efficiency ratio[9]	8.29±0.59	8.40±0.62	7.91±0.51	8.24±0.35	8.20±0.31

[1]Values (mean ± SE) with different superscript letters in the same row are significantly different (P<0.05);

[2]Survival: (number of dead fish/ total fish) x 100;

[3]WG= final weight- initial weight;

[4]SGR: (((ln (final body weight)– ln (initial body weight)) / days) x 100;

[5]FI: feed intake/ days/ number of fish;

[6] FCR: dry feed intake/ weight gain;

[7] CF: (body weight/ total length^3) x100;

[8]PER: weight gain/protein consumed;

[9]LER: weight gain/lipid consumed.

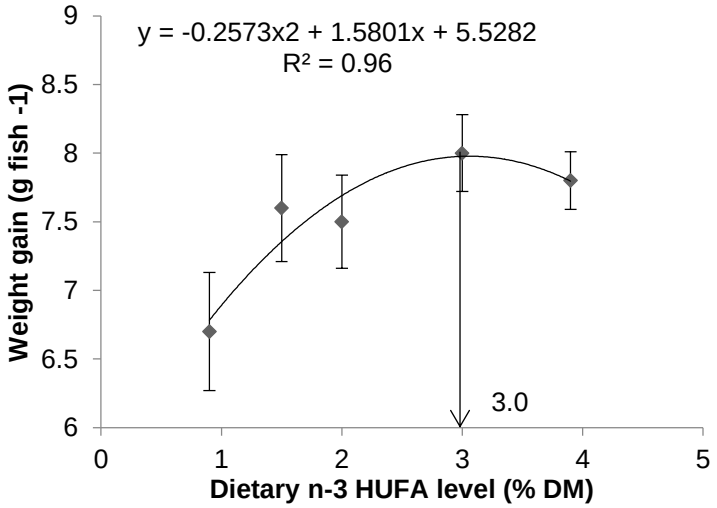

Figure 7: Second-order polynomial model fitting dietary n-3 HUFA levels to weight gain of meagre fingerlings fed the experimental diets for 30 days; the arrow indicates the requirement for dietary n-3 HUFA (% DM).

2.4.2 Biochemical and fatty acid composition

Compared to the initial values, final body composition of meagre was higher in lipid and lower in ash contents (Table 9). At the end of the trial, no significant differences were observed in protein, lipids, ash, and water content in whole-body composition.

Table 9: Whole-body composition (% wet weight) of meagre fingerlings fed the experimental diets for 30 days[1]

		Dietary n-3 HUFA level (% DM)				
	Initial	0.9	1.5	2.0	3.0	3.9
Protein	15.6±0.49	15.8±0.17	16.1±0.12	15.6±0.28	16.3±0.50	16.0±0.61
Lipid	2.2±0.12	4.1±0.47	4.1±0.25	3.8±0.24	3.8±0.28	3.9±0.25
Ash	3.9±0.26	2.7±0.22	2.7±0.09	2.8±0.14	2.8±0.04	2.6±0.29
Water	78.6±0.28	78.1±0.82	77.7±0.03	78.0±0.60	78.5±0.13	78.7±0.20

[1]Values (mean ± SE) with different superscript letters, in the same row, are significantly different (P<0.05).

Regarding the whole-body fatty acid composition (Table 10), the most abundant saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), n-3 and n-6 PUFA were palmitic acid (16:0), oleic acid (18:1n-9, OLA), DHA and LA, respectively. SFA linearly increased and MUFA linearly decreased (r^2=0.96 for both) with the increase of dietary n-3 HUFA content, both following the dietary pattern. LA and LNA also reflected the dietary trend, decreasing linearly (r^2=0.86 and 0.93, respectively) with the increase of dietary n-3 HUFA levels. DHA content linearly increased (r^2=0.82) while total n-3 HUFA, EPA and ARA exponentially increased (r^2=0.89, 0.92 and 0.92, respectively) with the increase of dietary n-3 HUFA levels, also similarly to the tendency observed in diets. Total n-3 HUFA and EPA levels of fish fed the 3.9% n-3 HUFA diet was two times higher than in fish fed the 3.0% n-3 HUFA diet ($P<0.05$). Additionally, fish fed the 0.9% and the 1.5% n-3 HUFA diets presented the highest content in eicosadienoic acid (20:2n-6) and ETE (20:3n-3), despite their lowest inclusion levels in the diets ($P<0.05$). The increase of dietary n-3 HUFA levels led to a reduction of whole-body OLA content ($P<0.05$). Consequently, the ratio of whole-body OLA/n-3 HUFA decreased as dietary n-3 HUFA increased, with fish fed the 3.9% n-3 HUFA diet showing the lowest value. Moreover, a second-order polynomial relation adjusted OLA/n-3 HUFA ratio and WG (r^2=0.94), whereupon WG was decreasing with the increase of OLA/n-3 HUFA ratio from 2.55 (fish fed 3.0% n-3 HUFA) to 5.54 (fish fed 0.9% n-3 HUFA, Figure 8).

Table 10: Fatty acid composition (% total identified fatty acids) of whole-body total lipids of meagre fingerlings fed the experimental diets for 30 days[1] (to be continued in the next page)

Fatty acid		Dietary n-3 HUFA level (% DM)				
	Initial	0.9	1.5	2.0	3.0	3.9
14:0	2.66±0.18	1.23±0.08	1.36±0.06	2.46±0.11	3.37±0.19	3.82±0.20
14:1n-5	0.09±0.00	0.06±0.00	0.06±0.00	0.12±±0.01	0.16±0.01	0.18±0.01
14:1n-7	0.02±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00
15:0	0.39±0.01	0.20±0.01	0.22±0.00	0.35±0.02	0.44±0.03	0.48±0.02
15:1n-5	0.03±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.03±0.00	0.05±0.00
16:0	18.40±0.54	16.58±0.91	15.87±0.25	20.24±0.78	21.38±1.57	19.83±0.86
16:1n-7	4.77±0.18	2.32±0.11	2.76±0.06	4.00±0.16	5.29±0.07	6.64±0.10
16:1n-5	0.18±0.04	0.07±0.01	0.10±0.00	0.15±0.01	0.24±0.01	0.26±0.02
16:2n-6	0.01±0.00	0.01±0.00	0.00±0.00	0.08±0.03	0.08±0.01	0.02±0.01
16:2n-4	0.25±0.04	0.21±0.02	0.20±0.01	0.32±0.05	0.41±0.03	0.60±0.01
16:3n-4	0.29±0.01	0.16±0.01	0.16±0.01	0.21±0.00	0.24±0.02	0.27±0.01
16:3n-3	0.12±0.01	0.05±0.00	0.07±0.00	0.14±0.01	0.17±0.01	0.20±0.01
16:3n-1	0.33±0.06	0.31±0.03	0.28±0.03	0.32±0.01	0.33±0.01	0.33±0.03
16:4n-3	0.18±0.04	0.15±0.05	0.11±0.02	0.22±0.06	0.20±0.04	0.27±0.04
16:4n-1	0.06±0.01	0.04±0.02	0.03±0.01	0.03±0.01	0.04±0.00	0.05±0.00
17:0	0.16±0.00	0.16±0.01	0.13±0.01	0.15±0.03	0.21±0.03	0.38±0.02
18:0	5.88±0.47	6.34±0.68	5.91±0.10	6.44±0.47	6.31±0.68	5.29±0.13
18:1n-9	28.46±0.72	35.2^{a}±0.15	32.58^{b}±0.22	28.26^{c}±0.91	23.70^{d}±0.54	16.99^{e}±0.39
18:1n-5	0.20±0.02	0.10±0.01	0.10±0.01	0.11±0.01	0.13±0.00	0.15±0.00
18:1n-7	3.49±0.12	2.03±0.11	2.46±0.01	2.83±0.05	3.11±0.12	3.15±0.06
18:2n-9	0.09±0.00	0.08±0.01	0.05±0.00	0.05±0.01	0.06±0.01	0.07±0.01
18:2n-6	11.41±0.28	17.28^{ab}±0.63	18.42^{a}±0.09	14.56^{bc}±0.90	14.01^{c}±0.80	13.42^{c}±0.06
18:2n-4	0.16±0.01	0.09±0.00	0.08±0.02	0.15±0.01	0.20±0.01	0.18±0.07
18:3n-6	0.10±0.01	0.09^{b}±0.01	0.12^{ab}±0.01	0.09^{b}±0.02	0.11^{ab}±0.02	0.20^{a}±0.04
18:3n-4	0.12±0.01	0.07±0.06	0.09±0.10	0.08±0.01	0.11±0.01	0.16±0.01
18:3n-3	1.98±0.36	4.88^{a}±0.26	5.59^{a}±0.00	3.06^{b}±0.36	2.35^{bc}±0.39	1.59^{c}±0.05
18:3n-1	0.01±0.00	0.02±0.01	0.01±0.03	0.02±0.00	0.01±0.00	0.01±0.00
18:4n-3	0.48±0.04	0.31^{b}±0.02	0.44^{b}±0.00	0.42^{b}±0.09	0.59^{b}±0.13	1.20^{a}±0.09
18:4n-1	0.07±0.01	0.12±0.04	0.06±0.00	0.19±0.03	0.23±0.05	0.16±0.03
20:0	0.31±0.00	0.38±0.01	0.31±0.01	0.35±0.03	0.35±0.02	0.29±0.01
20:1n-9	0.36±0.01	0.17±0.02	0.17±0.02	0.22±0.01	0.26±0.01	0.27±0.01
20:1n-7	3.03±0.09	2.10±0.04	1.95±0.00	2.15±0.06	2.21±0.07	2.06±0.07
20:1n-5	0.21±0.01	0.12±0.01	0.12±0.00	0.19±0.01	0.23±0.00	0.26±0.01
20:2n-9	0.11±0.01	0.04±0.00	0.09±0.00	0.05±0.01	0.06±0.01	0.07±0.00
20:2n-6	0.63±0.02	0.39^{a}±0.02	0.32^{ab}±0.01	0.31^{b}±0.01	0.31^{b}±0.02	0.34^{ab}±0.02
20:3n-9	0.02±0.00	0.02±0.01	0.02±0.00	0.02±0.01	0.03±0.01	0.05±0.00
20:3n-6	0.14±0.00	0.06^{b}±0.01	0.06^{b}±0.00	0.06^{b}±0.01	0.07^{b}±0.01	0.11^{a}±0.01
20:3n-3	0.21±0.01	0.16^{a}±0.02	0.14^{a}±0.00	0.11^{ab}±0.01	0.08^{b}±0.01	0.08^{b}±0.01

20:4n-6	0.76±0.05	0.27^{b}±0.02	0.37^{b}±0.02	0.34^{b}±0.06	0.43^{b}±0.06	0.82^{a}±0.04
20:4n-3	0.39±0.02	0.18^{b}±0.01	0.25^{b}±0.00	0.21^{b}±0.04	0.27^{b}±0.06	0.49^{a}±0.05
20:5n-3	2.72±0.55	1.50^{b}±0.14	2.44^{b}±0.08	1.92^{b}±0.50	2.74^{b}±0.75	6.19^{a}±0.68
22:1n-11	2.09±0.08	1.31±0.06	1.30±0.04	1.63±0.07	1.95±0.04	1.96±0.11
22:1n-9	0.75±0.05	0.53±0.01	0.48±0.00	0.52±0.03	0.51±0.03	0.45±0.02
22:4n-6	0.10±0.01	0.05^{c}±0.00	0.06^{bc}±0.00	0.06^{bc}±0.01	0.08^{b}±0.01	0.12^{a}±0.01
22:5n-6	0.03±0.00	0.05±0.02	0.03±0.00	0.09±0.02	0.09±0.03	0.06±0.00
22:5n-3	0.98±0.07	0.45^{b}±0.04	0.69^{b}±0.01	0.51^{b}±0.12	0.67^{b}±0.18	1.37^{a}±0.17
22:6n-3	5.60±0.48	4.16^{b}±0.50	3.91^{b}±0.11	6.09^{ab}±0.83	6.02^{ab}±0.68	8.93^{a}±0.76
Σ SFA	27.16±0.65	24.75^{cb}±0.72	23.79^{c}±0.25	29.98^{abc}±1.20	32.06^{b}±2.38	30.09^{ab}±1.14
Σ MUFA	40.23±0.83	44.04^{a}±0.07	42.11^{ab}±0.24	40.21^{bc}±0.81	37.82^{c}±0.82	32.43^{d}±0.7
Σ n-3	13.58±1.02	11.83^{b}±0.50	13.64^{b}±0.11	12.67^{b}±1.11	13.08^{b}±2.10	20.33^{a}±1.76
Σ n-3 HUFA	10.42±0.98	6.44^{b}±0.51	7.43^{b}±0.05	8.84^{b}±0.91	9.78^{b}±1.58	17.07^{a}±1.61
Σ n-6	13.08±0.30	18.11^{ab}±0.66	19.27^{a}±0.12	15.52^{bc}±0.93	15.11^{bc}±0.90	14.90^{c}±0.10
Σ n-6 HUFA	1.67±0.07	0.82^{b}±0.03	0.85^{b}±0.04	0.87^{b}±0.07	1.02^{b}±0.11	1.46^{a}±0.06
Σ n-9	29.33±0.68	35.79^{a}±0.14	33.13^{b}±0.22	28.85^{c}±0.89	24.30^{d}±0.55	17.56^{e}±0.41
OLA/n-3 HUFA	2.83±0.07	5.54^{a}±0.48	4.39^{ab}±0.02	3.29^{bc}±0.44	2.55^{cd}±0.39	1.02^{d}±0.11
EPA/DHA	0.58±0.57	0.37^{b}±0.06	0.63^{ab}±0.04	0.33^{b}±0.09	0.45^{ab}±0.09	0.69^{a}±0.04
EPA/ARA	4.28±0.10	5.61±0.25	6.67±0.56	5.55±0.48	6.06±0.81	7.49±0.41
EPA+DHA	8.84±0.87	5.66^{b}±0.52	5.24^{b}±1.30	8.01^{b}±0.87	8.75^{b}±1.36	14.98^{a}±1.26

[1]Values (mean ± SE) with different superscript letters in the same row are significantly different (P<0.05).

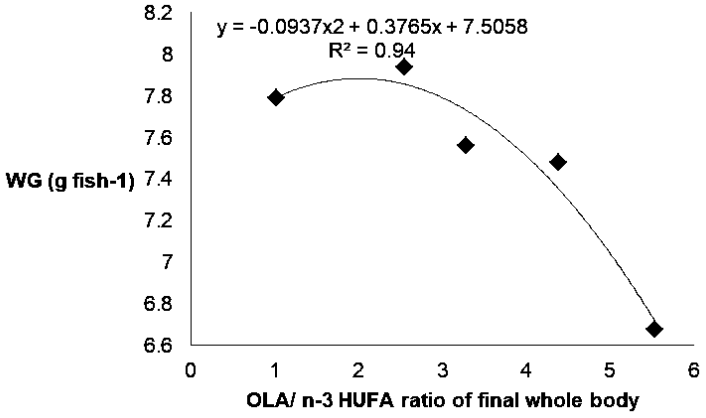

Figure 8: Relation of the whole-body OLA/n-3 HUFA ratio and weight gain.

Whole-body FA retention efficiency, expressed as percentage of FA intake, is presented in Table 11. In general, fish presented retentions below 100% for most FA. However, retention of 18:2n-9, 20:2n-9, 20:2n-6, 20:3n-6 and 20:3n-3 was over 100% in fish fed the 0.9% n-3 HUFA diet ($P<0.05$). Retention of EPA was unaffected by diet composition while that of DHA was higher in fish fed the 0.9% n-3 HUFA diet than the 3.0% n-3 HUFA diet ($P<0.05$), no further differences being observed among groups. Additionally, retention of LNA was higher in fish fed 3.9% n-3 HUFA than in fish fed the 0.9% and 2.0% ($P<0.05$).

Table 11: Retention[2] efficiency (% FA intake) of specific fatty acids in whole body of meagre fingerlings fed the experimental diets for 30 days[1]

Fatty acid	Dietary n-3 HUFA level (% DM)				
	0.9	1.5	2.0	3.0	3.9
14:0	25.9±4.28	22.8±4.10	26.8±0.72	29.3±5.35	30.4±5.10
17:0	18.9±3.74	21.6±3.73	14.4±3.38	15.8±0.6	23.0±2.26
18:0	84.0±19.77	81.7±6.81	78.1±7.28	63.1±17.54	66.6±10.63
18:1n-9	42.5±6.97	47.6±4.67	43.7±3.36	45.9±6.55	59.6±8.33
18:2n-9	405.2^{a}±69.19	117.6^{b}±15.43	63.0^{b}±18.51	38.4^{b}±0.94	34.5^{b}±1.27
18:2n-6	36.9±7.02	46.4±4.22	37.4±4.59	40.4±1.50	50.4±6.00
18:3n-6	83.9^{a}±19.70	73.2^{ab}±9.99	31.8^{ab}±9.01	24.1^{b}±1.44	44.4^{ab}±8.65
18:3n-3	24.2^{b}±4.83	35.0^{ab}±3.82	23.1^{b}±4.10	27.3^{ab}±2.74	45.0^{a}±4.57
18:4n-3	19.9±3.11	24.8±4.33	14.6±4.31	16.0±2.79	27.0±2.92
20:0	28.3±3.82	31.1±3.03	34.5±1.63	36.5±7.75	38.6±7.01
20:2n-9	213.0^{a}±50.01	70.1^{b}±11.76	45.9^{b}±11.02	39.0^{b}±1.21	44.9^{b}±5.47
20:2n-6	173.8^{a}±29.30	117.2^{ab}±8.09	88.6^{b}±9.96	66.7^{b}±8.71	60.6^{b}±12.38
20:3n-9	49.4±15.34	32.06±3.96	24.0±12.88	29.4±2.51	35.0±2.89
20:3n-3	188.5^{a}±40.70	181.3^{a}±17.41	92.7^{b}±8.78	47.2^{b}±0.56	48.6^{b}±7.60
20:3n-6	126.7^{a}±19.28	74.6^{b}±6.80	35.3^{b}±9.73	34.2^{b}±1.28	49.6^{b}±6.65
20:4n-6	47.7±15.79	52.7±2.55	27.8±8.67	27.7±2.52	47.5±6.02
20:4n-3	49.3±12.35	56.6±7.13	27.4±8.17	26.5±4.59	43.1±5.82
20:5n-3	21.4±5.75	28.5±4.04	13.4±4.78	15.3±3.48	27.8±3.99
22:4n-6	80.3±19.12	71.8±3.53	43.5±9.32	46.5±3.09	58.8±7.09
22:5n-6	68.7±19.77	52.2±0.94	124.0±29.81	111.1±14.45	44.7±3.29
22:5n-3	61.1±17.67	73.6±6.22	32.1±11.00	30.5±7.64	53.1±8.10
22:6n-3	55.5^{a}±5.78	39.6^{ab}±2.99	44.0^{ab}±4.94	31.5^{b}±0.57	37.3^{ab}±3.48

[1]Values (mean ± SE) with different superscript letters, in the same row, are significantly different ($P<0.05$).

[2]Retention: (final weight x % specific FA in final body composition x % lipids wet weight final body composition) – (initial weight x % specific FA in initial body composition x % lipids wet weight in final body composition)/ (feed intake x dietary % lipids wet weight x dietary % specific FA) x 100.

2.5 Discussion

Meagre is one of the best candidates for Mediterranean aquaculture production. Nevertheless, little information is available regarding its specific nutritional requirements. To our knowledge, this is the first study aiming to determine the n-3 HUFA requirements for meagre juveniles. To successfully determine fish nutritional requirements, it is of pivotal importance that fish grow fast and in this study meagre presented very high growth rates (SGR of 4.1 to 4.6%) and low feed conversion ratio (0.7 to 0.8) values that are within the highest values recorded to date for this species. The SGR values of meagre in the present study were markedly higher than that recorded recently for 15 g meagre fingerlings grown at similar temperature (1.43% day^{-1}, Güroy et al., 2017), despite the slight larger fish size of the latter study. Furthermore, FCR values in the present study were quite good, as FCR previously recorded for meagre juveniles were in the range of 0.9-1.7 (FAO, 2017; Monfort, 2010).

Reduced survival, bacterial diseases, fins erosion or shock syndrome are some of the usual effects observed in fish with EFA-deficiency (Izquierdo, 2005; Glencross, 2009; Tocher, 2010). For instance, red sea bream fingerlings with 3.1 g, showed poor appetite and high mortality after 1 week of feeding a diet with only 0.03% n-3 HUFA (Takeuchi et al., 1990). However, in the present study none of the above mentioned EFA deficiency signs was detected, possibly as the EFA deficiency was not very extreme. This agrees with the lack of high mortalities also observed in juveniles and fingerlings of other species fed low n-3 HUFA diets (Watanabe et al., 1989a; Takeuchi et al., 1992a, b; Lochman and Gatlin, 1993; Ibeas et al., 1994; Lee et al., 2003; Kim and Lee, 2004; Skalli and Robin, 2004).

In the present trial, growth was lowest in juveniles fed the 0.9% n-3 HUFA diet, and this is in line with results observed in meagre larvae, where a dietary n-3 HUFA inclusion level of 0.4% was not sufficient to cover EFA requirements (El-Kertaoui et al., 2015). In this trial, growth increased as dietary n-3 HUFA levels increased up to 3.0%. However, no further improvement of growth occurred by the inclusion of higher dietary levels. Based on these results and the second-order polynomial model, the n-3 HUFA requirement for best growth performance of meagre fingerlings was satisfied with a dietary inclusion level of 3.0% n-3 HUFA (1:1 of EPA/DHA). A broken-line model was also applied to our data (r^2=0.86 for WG, data not shown) and based on this model a lower n-3 HUFA requirement (2.6%) was estimated. However, it is argued that broken-

line model frequently underestimate nutrient requirements and that curvilinear models provide more accurate responses for population (Baker, 1986; Shearer, 2000).

The n-3 HUFA requirement for meagre fingerlings determined in the present study is within the requirement values observed for other fingerlings of marine species such as red sea bream (2.7-3.2% n-3 HUFA, Takeuchi et al., 1992a) or yellowtail (2.1-3.1%, Takeuchi et al., 1992c), but higher than that of other marine fish fingerlings such as red drum (0.5% DM n-3 HUFA, Lochman and Gatlin, 1993), starry flounder (0.9% DM n-3 HUFA, Lee et al., 2003), sea bass (0.7-1.0% DM, Coutteau et. al., 1996; Skalli and Robin, 2004), gilthead sea bream (1.0% DM n-3 HUFA, Ibeas et al., 1996) and striped jack (1.7% DM n-3 HUFA, Watanabe et al., 1989c). EFA requirements depend on fish stage of development, but may also vary with dietary lipid content and EPA/DHA ratio (Gatesoupe and Le Millinaire, 1985; Takeuchi et al., 1992a; Oliva-Teles, 2012). Thus, the requirement estimated in this study should consider the experimental conditions, namely that the diets contained 17% DM lipids and a dietary EPA/DHA ratio of 1:1.

Diet is the most important factor influencing fish fatty acid composition (Cowey and Sargent, 1972). In general, the whole-body FA composition of meagre reflected the FA composition of the diets. The most abundant SFA, MUFA, n-3 and n-6 PUFA in final whole-body composition were the palmitic acid, OLA, DHA and LA. These results agree with those reported for other Sciaenids, such as *Cynoscion xanthulus* (García and Pacheco, 2003) and *Totoaba macdonaldi (*López et al., 2006). However, in *Cynoscion reticulatus,* steric acid (18:0) together with palmitic acid and OLA, were the main FA (García and Pacheco, 2003). The increase of SFA and of marine origin FA (ARA, EPA and DHA), and the decrease of MUFA, LA and LNA with the increase of dietary n-3 HUFA, is expected due the decreasing dietary substitution of vegetable oils by fish oil.

Marine fish have a restricted physiologically capacity to produce HUFA from C_{18}-PUFA precursors due to a limited enzymatic capacity for elongation and desaturation processes. Therefore, ARA, EPA and DHA are EFA for marine species (Kanazawa, 1997). In the present study, the high retention of 18:2n-9 and 20:3n-6 in fish fed the diet with the lowest n-3 HUFA level (0.9%) suggests an endogenous synthesis of these PUFA through Δ6 desaturases. The higher endogenous production of n-9 or n-6 PUFA rather than n-3 PUFA was possibly due to the higher availability of dietary OLA and LA than LNA, all substrates for Δ6 desaturases.

Similarly, the high retention of chain-elongation products of OLA, LA and LNA, namely 20:2n-9, 20:2n-6 and 20:3n-3, in meagre fed the lowest n-3 HUFA levels (0.9%) also suggest an endogenous biosynthesis of these PUFA through fatty acid elongase 5 (Elovl5). These results indicate that meagre has some *in vivo* capacity to elongate and desaturate C_{18}-PUFA. The present results agree with an *in vitro* study also performed in meagre, where a functional characterisation of the desaturases and elongases activities concluded that this species possess at least one elongase (Elovl5) capable of elongating C_{18}-$_{20}$ PUFA, and a dual Δ6/Δ8 desaturase (Fads2) (Monroing et al., 2013). The same ability to biosynthesize longer chain-PUFA from C_{18}-PUFA precursors was reported in another Sciaenid fish, the red drum (Lochman and Gatlin, 1993), as well as in marine fish from other families, such as gilthead sea bream (Mourente and Tocher, 1993), flounder (Izquierdo et al., 1992; Lee et al., 2003; Kim and Lee, 2004) or turbot (Owen et al., 1975). Further studies are being conducted to confirm the *in vivo* ability of meagre fingerlings to express *elovl5* and *fads2* genes. Synthesis of DHA and EPA also requires Δ5 and, possibly, Δ4 desaturases, besides Δ6 desaturase and Elovl5. Thus, the insufficient capacity of meagre to satisfy EFA requirements from PUFA precursors may be due to the lack of these enzymes or to the insufficient activity of one or several elongases or desaturases.

For instance, it is accepted that DHA requirement is higher than that of EPA as it has greater biological value as EFA (Watanabe et al., 1989b) since it is the major component in cell membranes therefore being necessary to sustain their structure and functionality (Izquierdo, 2005). Accordingly, retention of DHA in fish fed 0.9% n-3 HUFA was the highest, and DHA retention efficiency in all groups was higher than that of EPA, suggesting that EPA was preferably catabolised, for energy or for eicosanoids production rather than DHA (Mourente and Bell, 2006). These results suggest a selective retention of DHA. In agreement, previous studies also reported a selective retention of DHA in Atlantic salmon (Bell et al., 2001, 2002, 2003), rainbow trout (Caballero et al., 2002), European sea bass (Mourente and Bell, 2006), gilthead sea bream (Fountoulaki et al., 2009), Senegalese sole (Borges et al., 2014) or turbot (Bell et al., 1994; Regost et al., 2003). The mechanisms justifying this selective retention of DHA comprise the higher specificity of fatty acyl transferases and the lower functionality as substrate for β-oxidation of DHA, in such way that EPA and other FA are preferably catabolised for energy production and DHA conserved in membrane phospholipids (FrØyland et al., 1997; Bell et al., 2001; Sargent et al., 2002).

The increased production of n-9 FA and the ratio of OLA to n-3 HUFA are biochemical indicators of EFA-deficiency (Watanabe, 1982). In the present study, the highest retentions of n-9 PUFA, particularly 18:2n-9 and 20:2n-9, was observed in fish fed the 0.9% n-3 HUFA diet, suggesting that these fish are EFA-deficient. Similarly, the production of 18:2n-9 was reported in tissues of seabream and turbot fed insufficient EFA levels (Tocher et al., 1988; Kalogeropoulos et al., 1992; Tocher, 2010). Increasing dietary n-3 HUFA levels led to a decrease of OLA and, consequently, of the OLA/n-3 HUFA ratio. Similar results were also found in liver phospholipids of gilthead seabream (Kalogeropoulos et al., 1992), red seabream (Fujii et al., 1976), striped jack (Watanabe et al., 1989c), and rockfish (Lee, 2001). Furthermore, at the optimum dietary EFA concentration, the OLA/n-3 HUFA ratio was 2.55 and therefore values of this order of magnitude or lower indicate an EFA sufficiency for meagre juveniles. Lower ratios were found in total lipids of gilthead sea bream and sea bass fillets (1.0 and 0.8, respectively, Yildiz et al., 2006) and in whole-body polar lipids of starry flounder (1.6, Lee et al., 2003), suggesting species differences in whole-body fatty acid profiles or in the ability to elongate and desaturate C_{18} precursors.

2.6 Conclusions

In conclusion, meagre is a competitive candidate for Mediterranean aquaculture production and this fact was highlighted by its high growth rates and excellent conversion ratios in the present study. Meagre showed the ability to selectively conserve key FA, particularly DHA in response to EFA-deficiency. Furthermore, meagre seems to have active Δ6 desaturases and Elovl5, but their activities being insufficient to produce enough DHA and EPA from PUFA precursors to sustain fast growth. Therefore, meagre fingerlings have n-3 HUFA requirement for fast growth of around 2.6-3.0% DM.

Chapter 3. Effect of increasing dietary levels of n-3 highly unsaturated fatty acids on liver composition and histopathology of meagre (*Argyrosomus regius*, Asso, 1801) fingerlings*

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3.1 Abstract

Meagre (*Argyrosomus regius*) is a promising new aquaculture species, with great potential due to its high growth rate, feed efficiency and easy adaptation to captivity. Essential fatty acids (EFA) are required to sustain growth, development, immune status and survival. EFA requirement of meagre fingerlings were previously estimated. A feeding trial was performed testing 5 increasing dietary n-3 HUFA levels, below and above the requirement levels (0.9, 1.5, 2.0, 3.0 and 3.9% DM), on liver biochemical, FA composition, and histopathology of meagre fingerlings. Fish fed the two lowest n-3 HUFA diets (0.9 and 1.5%) showed a higher hepatosomatic index as well as a higher hepatic lipid infiltration. Consequently, these fish showed a more severe steatosis than fish fed 2.0-3.9% n-3 HUFA. FA composition of liver total lipids reflected the dietary composition with an increase of SFA, n-3 HUFA (EPA, DHA) and n-6 HUFA (ARA), and a decrease on MUFA, LA and LNA, with the increase of dietary n-3 HUFA. However, meagre FA composition of liver polar lipids was less affected by the diets, preserving n-3 HUFA in phospholipids. Furthermore, the lowest dietary content in n-3 HUFA (0.9%) also led to a higher incidence of hepatic granulomas, suggesting a possible relation between EFA deficiency and hepatic granulomatosis in meagre.

These results suggest that a minimum of 3.0 to DM n-3 HUFA is required to maintain the EFA profile of hepatic total lipids and normal histomorphology of liver, corroborating the requirement value estimated for growth.

3.2 Introduction

Meagre (*Argyrosomus regius*) aquaculture production is relatively recent. Mediterranean countries, particularly Spain, France and Italy are currently producing this species, but still in commercial small-scale (FAO, 2017). Nowadays, meagre is considered one of the most promissory species for the diversification of Mediterranean aquaculture sector. Meagre is a fast-growing species, achieving magnificent feed conversion ratios and adapting easily to culture conditions (Calderón et al., 1997; Jiménez et al., 2005; El-Shebly et al., 2007; Cárdenas, 2010; Monfort, 2010). Moreover, it has good market characteristics, including high nutritional value, low fat, excellent taste and firm texture (Monfort, 2010). Despite the interest on its intensive aquaculture production, knowledge on nutritional requirements of meagre is impairing the production of specific feeds and feeding protocols (Roo et al., 2010). Chatzifotis et al. (2010) estimated that lipid requirement of meagre juveniles is 17% in dry weight. Moreover, requirements of essential fatty acids (EFA) to maximize growth rate of meagre fingerlings were recently estimated to be circa 2.6-3.0 % n-3 HUFA (dry matter basis, DM; Carvalho et al., unpublished data).

Fatty acids (FA) play vital functions in organisms, including fish. Highly unsaturated fatty acids (HUFA), particularly n-3 HUFA, are involved in several metabolic pathways namely energy production, membrane structure and function, eicosanoids synthesis (mediators of inflammatory and immune response) and control of lipid homeostasis (Watanabe, 1982; Tocher, 2003). Docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) are the main marine origin n-3 HUFA and are considered EFA for marine fish due to their restricted endogenous ability to *de novo* synthetize them (Tocher, 2003; Izquierdo and Koven, 2010).

EFA-deficiency is characterised by different symptomatology as reduced growth and survival, swimming disorders, fin erosion, and severe lipid infiltration, particularly in lipid storage tissue, as liver (Tacon, 1996). Liver is recognized as key organ in intermediary metabolism, playing a central role in the regulation of lipid metabolism, particularly in the synthesis and β-oxidation of FA (Segner and Juario, 1986; Caballero

et al., 1999; Caballero et al., 2004). Since the regulatory enzymes of lipid metabolism pathways present different affinities for different FA, an imbalance of dietary FA could affect liver physiology and functionality (Henderson, 1996; Caballero et al., 2004). Moreover, in many fish species, the liver is the main organ for lipid storage, which is the case of gilthead sea bream and European sea bass (Peres and Oliva-Teles, 1999; Roberts, 2002). Therefore, when lipid infiltration in liver surpasses its capacity to store them, liver physiology is affected (Spisni et al., 1998; Roberts, 2002).

Granulomatosis has been considered a constraint of meagre aquaculture production (Ghittino et al., 2004; Kružić et al., 2016). This disease is characterised by an extensive development of granulomas in internal organs, including in liver (Paperna et al., 1980; Paperna, 1987; Elkesh et al., 2012). Meagre granulomatosis aetiology is unknown, being hypothesised to be an immunological response to different stimuli, including pathogenic infections (especially *Mycobacterium spp.* and *Nocardia sp.*; Elkesh et al., 2012), heavy metals or nutritional deficiencies (Good et al., 2016). The specific causes of non-infectious granulomatosis remain unclear, but water quality and nutritional imbalances such as calcium, phosphorus, magnesium, ascorbic acid or nutritional inadequacy (plant ingredients, tyrosine, etc.) have been pointed out (Herman, 1996).

Present study aimed to improve the understanding of the modulation action of dietary n-3 HUFA on hepatic lipid profile and it possible role on the development of liver steatosis and granulomatosis in meagre. For that purpose, the effect of increasing dietary n-3 HUFA levels on biochemical and histological alterations in liver of meagre fingerlings were studied.

3.3 Material and Methods

This experiment was conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at Fundación Canaria Parque Científico Tecnológico (FCPCT), University of Las Palmas de Gran Canaria (Canary Islands, Spain).

3.3.1 Experimental fish, diets and rearing conditions

Experimental fish, diets and rearing conditions of the experiment were the same described in detail in Chapter 2. However, since the diet formulation and composition are also relevant to the current study it is presented in Table 12 and 13.

Table 12: Composition and proximate analysis of the experimental diets for meagre fingerlings

	Dietary n-3 HUFA level (% DM)				
	0.9	1.5	2.0	3.0	3.9
Ingredients (%)					
Fish meal, N. Atlantic[1]	15.0	15.0	15.0	15.0	15.0
Corn gluten[2]	10.0	10.0	10.0	10.0	10.0
Faba beans[1]	10.0	10.0	10.0	10.0	10.0
Wheat[1]	8.0	8.0	8.0	8.0	8.0
Wheat gluten[1]	18.4	18.4	18.4	18.4	18.4
Soy protein concentrate[1]	25.0	25.0	25.0	25.0	25.0
Fish oil, S. American[1]	0.0	2.7	5.4	8.2	10.9
Linseed oil[3]	1.6	1.2	0.8	0.4	0.0
Palm oil[3]	3.3	2.5	1.7	0.8	0.0
Rapeseed oil[1]	6.0	4.5	3.0	1.5	0.0
Premix[4]	2.8	2.8	2.8	2.8	2.8
Proximate analysis (% DM)					
Protein	56.5	54.5	54.5	56.0	54.3
Lipids	16.2	17.0	16.5	16.9	16.2
Ash	4.9	5.0	5.1	5.2	5.0
Moisture	8.7	8.5	8.5	8.2	7.9

1: Skretting, Stavanger, Norway;

2: Cargill Nordic AS, Charlottenlund, Denmark;

3: AAK AB, Karlshamn, Sweden;

4: Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins and minerals;

Vitamin and mineral supplementation as estimated to cover requirements according NRC (2011).

Table 13: Fatty acid composition of the experimental diets for meagre fingerlings (% total identified fatty acids) (to be continued in the next page)

Fatty acid	Dietary n-3 HUFA level (% DM*)				
	0.9	1.5	2.0	3.0	3.9
14:0	1.5	2.2	3.6	4.6	5.9
14:1n-5	0.1	0.1	0.1	0.2	0.3
15:0	0.1	0.2	0.3	0.4	0.5
16:0	16.5	17.0	18.1	18.4	18.6
16:1n-7	1.4	2.2	3.6	5.0	6.2
16:1n-5	0.1	0.1	0.2	0.2	0.3
16:2n-4	0.2	0.3	0.4	0.6	0.8
16:3n-4	0.1	0.1	0.2	0.2	0.2
16:3n-3	0.1	0.1	0.1	0.2	0.3
16:4n-3	0.2	0.4	0.6	0.9	1.2
17:0	0.1	0.7	0.4	0.6	0.8
18:0	3.1	3.2	3.3	3.5	3.5
18:1n-9	34.3	30.8	25.6	18.3	11.1
18:1n-7	1.9	2.0	2.3	2.4	2.6
18:2n-9	0.0	0.0	0.0	0.1	0.1
18:2n-6	20.4	18.8	16.4	14.2	12.2
18:2n-4	0.1	0.1	0.1	0.2	0.3
18:3n-6	0.0	0.1	0.1	0.2	0.2
18:3n-4	0.1	0.1	0.1	0.1	0.2
18:3n-3	9.1	7.9	5.7	3.7	1.5
18:3n-1	0.0	0.0	0.0	0.0	0.0
18:4n-3	0.5	0.7	1.1	1.6	2.2
18:4n-1	0.0	0.1	0.1	0.1	0.1
20:0	0.4	0.4	0.4	0.4	0.3
20:1n-9	0.1	0.1	0.2	0.2	0.2
20:1n-7	1.6	1.6	1.9	1.9	2.0
20:1n-5	0.1	0.1	0.2	0.2	0.3
20:2n-9	0.0	0.0	0.0	0.1	0.1
20:2n-6	0.1	0.1	0.1	0.1	0.2
20:3n-9	0.0	0.0	0.0	0.1	0.1
20:3n-6	0.0	0.0	0.1	0.1	0.1
20:3n-3	0.0	0.0	0.0	0.0	0.1
20:4n-6	0.2	0.3	0.4	0.6	0.8
20:4n-3	0.1	0.2	0.3	0.4	0.5
20:5n-3	2.4	3.7	5.3	8.0	10.7
22:1n-11	1.6	1.7	2.1	2.3	2.5
22:1n-9	0.3	0.3	0.3	0.3	0.3

22:4n-6	0.0	0.0	0.1	0.1	0.1
22:5n-6	0.0	0.0	0.0	0.1	0.1
22:5n-3	0.3	0.4	0.6	0.9	1.2
22:6n-3	2.8	4.2	5.6	8.5	11.3
Σ SFA	21.8	23.3	26.1	27.9	29.6
Σ MUFA	41.4	39.1	36.4	31.3	25.9
Σ n-3	15.5	17.6	19.2	24.0	28.6
Σ n-3 HUFA	5.6	8.5	11.8	17.8	23.8
Σ n-6	20.7	19.4	17.2	15.3	13.7
Σ n-6 HUFA	0.3	0.5	0.7	0.9	1.3
Σ n-9	34.7	31.3	26.1	19.9	11.9
OLA/n-3 HUFA	6.1	3.6	2.2	1.0	0.5
EPA/DHA	0.9	0.9	0.9	0.9	1.0
EPA/ARA	14.2	13.9	13.1	13.5	13.6
EPA+DHA	5.2	7.9	10.9	16.5	22.0

* n-3 HUFA (% total FA) x dietary lipids (%DM).

At the end of the feeding trial, thirteen fish per tank were randomly sampled, individually weighed, euthanized with an excess of clove oil, and livers were collected and weighed to calculate hepatosomatic index (HSI). Five of those livers were used for biochemical and FA composition analysis, other five for histological studies, and three for bacteriological analysis.

3.3.2 Biochemical analysis

All samples were kept at -80ºC until analysis. Prior to analysis, liver samples were homogenized (T25 Digital Ultra-turrax, IKA®, Germany) to obtain one pooled sample per tank and analysed in triplicate. Due to the small size of livers only moisture and lipid analysis could be performed. Moisture were determined according to A.O.A.C. (2000) and total lipid content was extracted with chloroform/methanol (2:1 v/v) (Folch et al., 1957). Besides, neutral and polar lipid fractions from liver lipid content were separated according to Juaneda and Rocquelin (1985). Polar lipids were eluted with methanol whereas neutral lipids by chloroform and chloroform:methanol (49:1 v/v). Fatty acid methyl esters (FAMES) from liver total lipid and polar fraction were obtained by transmethylation according to Christie et al. (1989). FAMES were separated by gas liquid chromatography (GLC) under the conditions described by Izquierdo et al. (1990), quantified by a flame ionizator detector (FID) (Finnigan Focus SG, Thermo electron

Corporation, Milan, Italy) and identified by comparison with previous characterized standards.

3.3.3. Histological studies

Immediately after collection, livers were fixed in 4% buffered formalin, dehydrated in a graded ethanol series and embedded in paraffin wax. Samples were processed to obtain paraffin blocks which were cut with a Leyca microtome to form sections of 4µm. The sections were placed in slides, dried, stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) and mounted with xylol. Additional sections of liver tissue were stained with acid-fast (Ziehl-Neelsen) for searching the presence of *Mycobacterium sp.* and *Nocardia sp.* All slides were examined under light microscopy (BX51TF, Olympus, Tokyo, Japan) and blinded evaluated by three different investigators to define visual differences among treatments. Sections were evaluated for checking the overall integrity of hepatocytes, lipid infiltration level, and presence and number of granulomas in hepatic tissue. A semi-quantitative score evaluation of lipid infiltration level was used, ranging from 0 to 3. Score 0-1 was defined as normal liver histomorphology, score 1-2 was considered as moderate steatotic alterations in hepatic tissue with moderate lipid infiltration and score 2-3 severe steatotic alterations in hepatic tissue with high lipid infiltration. For granulomas, the number and stage of development of granulomas in each section was counted.

3.3.4 Bacteriological analysis

At the end of the trial, three liver samples from each experimental treatment were seeded using blood (sheep) agar, supplemented with 1.5% sodium chloride (AS-1.5% NaCl), brain-heart infusion agar supplemented with salt (BHIA-1.5% NaCl) and YEME as culture media to discard the presence of *Nocardia spp.* and *Streptomyces sp*. Cultures were incubated at 25ºC for 4 weeks with daily check for bacteria growth.

3.3.5 Statistical analysis

Numeric data are presented in tables, as means ± standard error (SE), and were tested for normality and homogeneity using Shapiro–Wilk and Levene's tests, respectively, and analysed by one-way ANOVA. When p-values were significant ($P<0.05$), means were compared with Tukey´s multiple range test (Tukey, 1949). When appropriated, response data were also subjected to regression analysis (linear or exponential) where dietary FA level (analysed) served as the independent variable. All statistical analyses were done using the SPSS 21.0 software package for Windows.

3.4 Results

3.4.1 Liver indexes and composition

At the end of the feeding trial, HSI decreased linearly with the increase of dietary n-3 HUFA levels ($r^2=0.96$, $p=0.01$; Table 14). Similarly, hepatic lipid content decreased linearly with the increase of dietary n-3 HUFA levels ($r^2=0.93$, $p=0.02$; Table 14). Consequently, the inverse was true for hepatic moisture content ($r^2=0.91$, $p=0.03$; Table 14).

In general, FA composition of liver total lipids reflected the dietary composition (Table 14). Saturated fatty acids (SFA) increased linearly ($r^2=0.88$, $p=0.02$), while monounsaturated fatty acids (MUFA, $r^2=0.99$, $p=0.00$), linolenic acid (18:3n-3, LNA, $r^2=0.99$, $p=0.00$) and linoleic acid (18:2n-6, LA, $r^2=0.96$, $p=0.00$) decreased linearly with the increase of dietary n-3 HUFA. N-3 HUFA and n-6 HUFA increased exponentially ($r^2=0.99$, $p=0.00$ and 0.96, $p=0.00$ respectively), as well as DHA ($r^2=0.99$, $p=0.00$), EPA ($r^2=0.99$, $p=0.01$) and ARA ($r^2=0.98$; $p=0.00$) with the increase of dietary n-3 HUFA levels. N-9 fatty acids decreased linearly ($r^2=0.99$; $p=0.00$) with the increase of dietary n-3 HUFA, reflecting the dietary pattern. Consequently, the ratio OLA/n-3 HUFA also decreased linearly ($r^2=0.90$; $p=0.00$), being significantly higher in fish fed 0.9 and 1.5% n-3 HUFA than in fish fed levels above 2.0% ($P<0.05$). In contrast, fish fed the two lowest n-3 HUFA diets (0.9 and 1.5%) presented the highest content in eicosadienoic acid (20:2n-6) and eicosatrienoic acid (20:3n-3, ETE), despite their lowest inclusion levels in the diets ($P<0.05$).

Table 14: Hepatosomatic index (%), biochemical (% wet weight) and fatty acid composition (% total identified fatty acid) of liver total lipids of meagre fed the experimental diets for 30 days[1] (to be continued in the next page)

	Dietary n-3 HUFA level (% DM)				
	0.9	1.5	2.0	3.0	3.9
Hepatosomatic index (%)[2]	3.08^{a}±0.22	2.97^{ab}±0.19	2.57^{bc}±0.18	2.23^{cd}±0.17	2.14^{d}±0.16
Liver composition (% wet weight)					
Lipids	16.67^{a}±1.08	16.34^{a}±1.06	11.67^{ab}±1.94	10.14^{b}±1.02	8.93^{b}±1.21
Moisture	60.91^{c}±2.04	62.45^{bc}±1.24	68.24^{ab}±0.90	68.03^{ab}±1.30	70.98^{a}±1.50
Fatty acid composition (% total identified FA)					
14:0	0.64±0.06	0.87±0.02	1.39±0.28	1.95±0.40	2.58±0.16
14:1n-5	0.04±0.00	0.05±0.00	0.09±0.01	0.01±0.01	0.15±0.00
14:1n-7	0.00±0.00	0.00±0.00	0.01±0.00	0.12±0.00	0.01±0.00
15:0	0.13±0.02	0.15±0.00	0.27±0.03	0.32±0.04	0.41±0.01
15:1n-5	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.01	0.03±0.00
16:0	13.28±0.22	14.64±0.62	17.36±1.03	19.66±0.62	19.71±0.11
16:1n-7	2.25±0.08	2.95±0.06	4.10±0.14	5.91±0.35	7.45±0.29
16:1n-5	0.07±0.01	0.12±0.00	0.17±0.00	0.21±0.01	0.29±0.01
16:2n-6	0.00±0.00	0.00±0.00	0.02±0.02	0.01±0.02	0.01±0.00
16:2n-4	0.07±0.01	0.11±0.01	0.24±0.03	0.30±0.03	0.42±0.03
16:3n-4	0.16±0.01	0.19±0.01	0.21±0.00	0.20±0.01	0.31±0.01
16:3n-3	0.06±0.01	0.08±0.00	0.11±0.00	0.27±0.03	0.21±0.00
16:3n-1	0.02±0.00	0.02±0.01	0.13±0.10	0.21±0.10	0.06±0.02
16:4n-3	0.04±0.01	0.05±0.01	0.12±0.06	0.03±0.04	0.14±0.01
16:4n-1	0.00±0.00	0.01±0.00	0.02±0.01	0.10±0.01	0.02±0.00
17:0	0.05±0.01	0.07±0.00	0.13±0.01	0.01±0.01	0.27±0.01
18:0	9.28±1.05	9.71±0.53	7.61±0.44	8.45±0.69	7.47±0.18
18:1n-9	37.78^{a}±0.63	34.89^{a}±0.13	29.68^{b}±0.11	22.56^{c}±1.33	15.98^{d}±0.04
18:1n-5	0.08±0.01	0.09±0.00	0.11±0.00	0.13±0.00	0.15±0.00
18:1n-7	2.24±0.08	2.44±0.04	2.85±0.03	3.08±0.06	3.45±0.06
18:2n-9	0.13±0.02	0.11±0.02	0.07±0.01	0.08±0.01	0.06±0.00
18:2n-6	18.62^{a}±0.62	16.91^{a}±0.79	16.32^{a}±0.35	13.60^{b}±0.50	12.48^{b}±0.46
18:2n-4	0.07±0.11	0.11±0.00	0.17±0.01	0.16±0.01	0.31±0.01
18:3n-6	0.21±0.02	0.18±0.01	0.15±0.02	0.22±0.02	0.21±0.01
18:3n-4	0.06±0.01	0.10±0.00	0.10±0.02	0.17±0.02	0.20±0.00
18:3n-3	5.72^{a}±0.13	5.04^{a}± 0.27	3.92^{b}±0.25	2.33^{c}±0.16	1.04^{d}±0.05
18:3n-1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
18:4n-3	0.20±0.02	0.27±0.01	0.45±0.03	0.69±0.07	0.93±0.05
18:4n-1	0.03±0.00	0.04±0.00	0.09±0.02	0.10±0.02	0.12±0.01
20:0	0.23±0.01	0.25±0.01	0.26±0.03	0.23±0.04	0.22±0.00

20:1n-9	0.13±0.00	0.15±0.01	0.21±0.01	0.25±0.02	0.28±0.01
20:1n-7	2.03±0.03	2.16±0.08	2.04±0.04	1.94±0.09	1.78±0.01
20:1n-5	0.09±0.01	0.12±0.00	0.16±0.01	0.18±0.01	0.24±0.01
20:2n-9	0.05±0.01	0.05±0.01	0.05±0.00	0.06±0.00	0.07±0.01
20:2n-6	0.52^{a}±0.00	0.50^{a}±0.00	0.41^{b}±0.04	0.38^{b}±0.02	0.41^{b}±0.01
20:3n-9	0.01±0.00	0.02±0.00	0.03±0.00	0.04±0.01	0.06±0.00
20:3n-6	0.08^{b}±0.01	0.08^{b}±0.01	0.08^{b}±0.01	0.10^{ab}±0.01	0.13^{a}±0.00
20:3n-3	0.23^{a}±0.01	0.21^{a}±0.01	0.15^{b}±0.01	0.11^{bc}±0.00	0.08^{c}±0.01
20:4n-6	0.18^{c}±0.04	0.26^{c}±0.02	0.42^{bc}±0.03	0.68^{b}±0.11	0.99^{a}±0.07
20:4n-3	0.18^{c}±0.01	0.25^{bc}±0.01	0.31^{bc}±0.04	0.43^{ab}±0.08	0.62^{a}±0.02
20:5n-3	1.08^{c} ±0.17	1.62^{c}± 0.08	2.46^{bc}±0.27	4.23^{b}±0.75	6.04^{a}±0.03
22:1n-11	0.67±0.01	0.69±0.02	1.05±0.24	10.93±0.31	1.02±0.03
22:1n-9	0.47±0.01	0.47±0.03	0.45±0.01	0.42v0.02	0.44±0.01
22:4n-6	0.04^{c}±0.00	0.04^{c}±0.00	0.06^{c}±0.00	0.10^{b}±0.01	0.14^{a}±0.01
22:5n-6	0.02^{b}±0.00	0.02^{b}±0.00	0.04^{ab}±0.01	0.05^{ab}±0.01	0.07^{a}±0.00
22:5n-3	0.39^{c}±0.05	0.60^{bc}±0.03	0.81^{bc}±0.13	1.42^{b}±0.30	2.01^{a}±0.08
22:6n-3	2.31^{c} ±0.38	3.25^{bc}± 0.12	5.19^{bc}±0.12	7.43^{b}±1.22	10.80^{a}±0.98
Σ SFA	23.61^{c}±0.90	25.70^{c}± 1.16	27.33^{bc}±0.96	30.81^{a}±0.58	30.66^{ab}±0.19
Σ MUFA	45.87^{a}±0.46	44.15^{a}± 0.13	40.94^{b}±0.11	35.77^{c}±1.28	31.27^{d}±0.46
Σ n-3	10.22^{b}±0.63	11.37^{b}± 0.48	13.03^{b}±0.78	17.01^{b}±2.29	21.89^{a}±0.93
Σ n-3 HUFA	4.19^{c}±0.60	5.93^{c}±0.21	8.43^{bc}±0.57	13.67^{b}±2.32	19.57^{a}±1.03
Σ n-6	19.46^{a}±0.64	17.82^{a}± 0.79	17.36^{a}±0.39	14.86^{b}±0.39	14.23^{b}±0.38
Σ n-6 HUFA	0.83^{c}±0.04	0.91^{bc}±0.01	1.01^{bc}±0.05	1.31^{b}±0.13	1.73^{a}±0.09
Σ n-9	38.32^{a}±0.63	35.42^{a}± 0.13	30.21^{b}±0.10	23.08^{c}±1.34	16.54^{d}±0.04
OLA/n-3 HUFA	7.67^{a}±0.99	5.07^{ab}± 0.15	3.15^{bc}±0.21	1.54^{c}±0.42	0.75^{d}±0.04
EPA/DHA	0.47±0.02	0.50±0.02	0.52±0.05	0.57±0.03	0.59±0.05
EPA/ARA	6.19±0.57	6.30±0.69	5.84±0.31	6.28±0.29	6.17±0.43
EPA+DHA	3.39^{c}±0.54	4.88^{c}± 0.17	7.16^{bc}±0.39	11.66^{b}±1.97	16.85^{a}±0.95

[1]Values (mean ±SE) with different superscript letters in the same row are significantly different (P<0.05);

[2]HSI: (liver weight/body weight) x100.

In contrast, the FA composition of liver polar lipids was less affected by the n 3-HUFA level of the experimental diets (Table 15). Although for few FA the same tend was observed as in liver total lipids, for most FA no significant differences were observed among treatments. MUFA content of polar lipids was higher in meagre fed the lowest n-3 HUFA level (0.9%) than in those fed 2.0-3.9% n-3 HUFA (P<0.05), reflecting the dietary composition. Also, meagre fed 0.9% n-3 HUFA showed significant higher content in LA and LNA than fish fed n-3 HUFA levels above 2.0% (P<0.05). Meagre fed the 0.9% n-3 HUFA also showed the highest content in OLA in polar lipid

fraction of liver (P<0.05); consequently, a tendency to decrease the OLA/n-3 HUFA ratio in meagre fed the two lowest n-3 HUFA levels (0.9 and 1.5%) was observed.

Table 15: Fatty acid composition (% total identified fatty acids) of liver polar lipids of meagre fed the experimental diets for 30 days[1] (to be continued in the next page)

	Dietary n-3 HUFA level (% DM)				
	0.9	1.5	2.0	3.0	3.9
Fatty acid composition (% total identified FA)					
14:0	0.85±0.23	0.87±0.16	1.20±0.33	1.40±0.21	1.83±0.27
14:1n-7	0.08±0.04	0.10±0.07	0.06±0.03	0.06±0.03	0.14±0.06
14:1n-5	0.09±0.05	0.09±0.05	0.10±0.04	0.09±0.01	0.18±0.05
15:0	0.27±0.07	0.28±0.04	0.38±0.05	0.47±0.07	0.60±0.06
15:1n-5	0.02±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.02±0.00
16:0	22.32±2.28	23.49±2.49	28.71±4.27	30.64±4.41	33.02±1.97
16:1n-7	1.25±0.23	1.14±0.02	1.34±0.04	1.66±0.13	2.25±0.27
16:1n-5	0.09±0.02	0.12±0.02	0.20±0.04	0.23±0.04	0.31±0.01
16:2n-6	0.10±0.05	0.04±0.02	0.06±0.02	0.09±0.04	0.14±0.07
16:2n-4	0.10±0.06	0.03±0.00	0.03±0.02	0.14±0.08	0.07±0.05
16:3n-4	0.12±0.01	0.12±0.01	0.16±0.03	0.16±0.01	0.21±0.00
16:3n-3	0.10±0.02	0.10±0.01	0.21±0.06	0.26±0.04	0.33±0.02
16:3n-1	0.30±0.08	0.36±0.03	0.25±0.07	0.42±0.07	0.49±0.18
16:4n-3	0.49±0.07	0.77±0.04	0.54±0.15	0.77±0.10	0.48±0.05
16:4n-1	0.04±0.02	0.06±0.02	0.05±0.02	0.09±0.02	0.10±0.04
17:0	0.09±0.02	0.08±0.01	0.10±0.01	0.05±0.03	0.09±0.01
18:0	16.73±0.93	15.79±1.58	14.05±1.11	16.15±1.65	15.18±0.42
18:1n-9	21.94^{a}±1.32	17.09^{b}±0.16	15.78^{b}±0.81	13.31^{b}±0.63	13.32^{b}±0.86
18:1n-7	2.64±0.16	2.52±0.05	2.98±0.16	2.95±0.07	3.32±0.09
18:1n-5	0.08±0.01	0.08±0.01	0.09±0.01	0.10±0.01	0.11±0.01
18:2n-9	0.04±0.00	0.03±0.00	0.05±0.01	0.03±0.01	0.04±0.00
18:2n-6	16.81^{a}±1.75	14.48^{ab}±1.65	11.60^{abc}±1.70	9.20^{bc}±1.13	7.89^{c}±0.19
18:2n-4	0.22±0.06	0.34±0.15	0.36±0.05	0.27±0.04	0.28±0.06
18:3n-6	0.07^{c}±0.01	0.08^{bc}±0.02	0.13^{abc}±0.01	0.14^{ab}±0.01	0.17^{a}±0.03
18:3n-4	0.04±0.00	0.04±0.01	0.06±0.01	0.07±0.02	0.12±0.06
18:3n-3	2.29^{a}±0.44	1.86^{ab}±0.18	1.45^{ab}±0.24	0.81^{b}±0.13	0.96^{b}±0.28
18:3n-1	0.02±0.01	0.03±0.02	0.02±0.00	0.02±0.00	0.04±0.03
18:4n-3	0.11±0.04	0.09±0.01	0.13±0.02	0.10±0.03	0.13±0.01
18:4n-1	0.20±0.08	0.32±0.21	0.26±0.05	0.21±0.03	0.28±0.10
20:0	0.39±0.03	0.37±0.04	0.36±0.03	0.40±0.03	0.40±0.03
20:1n-9	0.10±0.03	0.07±0.02	0.09±0.03	0.08±0.02	0.12±0.02
20:1n-7	1.85±0.12	1.62±0.04	1.67±0.11	1.67±0.04	1.58±0.04
20:1n-5	0.10±0.01	0.08±0.01	0.12±0.02	0.12±0.01	0.18±0.02
20:2n-9	0.03±0.01	0.01±0.00	0.02±0.00	0.01±0.00	0.03±0.00

20:2n-6	0.68±0.12	0.63±0.08	0.64±0.07	0.60±0.08	0.58±0.04
20:3n-9	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
20:3n-6	0.16±0.05	0.16±0.02	0.13±0.02	0.12±0.01	0.13±0.02
20:4n-6	0.60±0.13	1.26±0.07	1.21±0.19	1.45±0.25	1.44±0.41
20:3n-3	0.21±0.04	0.23±0.01	0.22±0.03	0.17±0.02	0.15±0.02
20:4n-3	0.13±0.02	0.14±0.02	0.18±0.03	0.15±0.03	0.16±0.04
20:5n-3	1.53±0.40	2.36±0.67	2.47±0.54	2.33±0.65	2.12±0.59
22:1n-11	0.55±0.09	0.45±0.04	0.49±0.09	0.58±0.04	0.64±0.04
22:1n-9	0.33±0.02	0.28±0.02	0.26±0.00	0.28±0.01	0.26±0.02
22:4n-6	0.05±0.01	0.09±0.02	0.10±0.02	0.11±0.03	0.11±0.04
22:5n-6	0.20±0.04	0.33±0.03	0.24±0.09	0.31±0.09	0.22±0.08
22:5n-3	0.57±0.18	0.96±0.29	0.98±0.30	0.90±0.31	0.84±0.34
22:6n-3	4.98±1.26	10.46±1.91	9.83±2.48	10.66±3.17	8.77±2.61
Σ SFA	41.83±3.93	40.89±4.27	44.80±5.59	49.32±5.97	51.12±2.67
Σ MUFA	28.95^{a}±1.82	24.94^{ab}±0.73	23.20^{b}±0.48	21.18^{b}±0.60	22.42^{b}±1.17
Σ n-3	10.40±2.24	10.10±4.46	16.00±3.66	16.13±4.36	13.93±3.33
Σ n-3 HUFA	7.42±1.88	7.28±4.52	13.68±3.38	14.20±4.16	12.03±3.56
Σ n-6	14.36±5.52	16.99±1.88	13.98±2.02	8.60±2.56	10.52±0.54
Σ n-6 HUFA	2.33±0.69	2.47±0.20	2.32±0.34	2.71±0.55	2.49±0.57
Σ n-9	16.37±6.13	17.49±0.19	16.07±0.80	9.99±3.01	13.62±0.88
OLA/n-3 HUFA	3.42±0.92	4.74±2.10	1.31±0.33	1.20±0.45	1.33±0.39
EPA/DHA	0.31^{a}±0.02	0.19^{b}±0.04	0.26^{ab}±0.01	0.22^{ab}±0.01	0.25^{ab}±0.02
EPA/ARA	3.77^{a}±0.59	1.19^{b}±0.44	2.04^{ab}±0.36	1.90^{ab}±0.51	1.47^{b}±0.05
EPA+DHA	6.51±1.65	12.82±2.56	12.31±3.02	12.99±3.80	10.88±3.20

[1]Values (mean ± SE) with different superscript letters in the same row are significantly different (P<0.05).

3.4.2 Liver histopathology

Histological examination of cross-section of hepatic tissue showed that no necrotic tissue was found in meagre fed different dietary n-3 HUFA levels. However, hepatic steatotic alterations decreased linearly with the increase of the dietary n-3 HUFA levels (r^2=0.88, p=0.19; Table 16). Liver of fish fed 0.9 and 1.5% n-3 HUFA presented a severe steatosis, reflected by the hypertrophy of the hepatocytes and consequently nuclei were displaced from central position in the cell to the periphery (Figure 9A & 9B). Contrarily, liver of fish fed 2.0-3.9% n-3 HUFA showed smaller hepatocytes, with spherical nuclei and, mostly, located at a central position of the cell although some lipid infiltration was also observed at a lower extension (Figure 9C, 9D & 9E).

Table 16: Histomorphological evaluation of hepatic tissue of meagre fed the experimental diets for 30 days[1]

	Dietary n-3 HUFA level (% DM)				
	0.9	1.5	2.0	3.0	3.9
Steatosis[2]	2.6^{a}±0.2	2.4^{ab}±0.2	1.7^{ab}±0.2	1.2^{b}±0.0	1.2^{b}±0.2
Granulomas[3]	5.3^{a}±1.6	2.1^{ab}±1.1	1.4^{b}±1.0	0.1^{b}±0.1	0.7^{b}±0.4

[1]Means with different superscript letters in the same row are significantly different (P<0.05);

[2]Mean score value: score 0-1: normal liver histomorphology, score 1-2: moderate lipid infiltration; and score 2-3 high lipid infiltration;

[3]Granulomas: measured in number of granulomas observed in each sample.

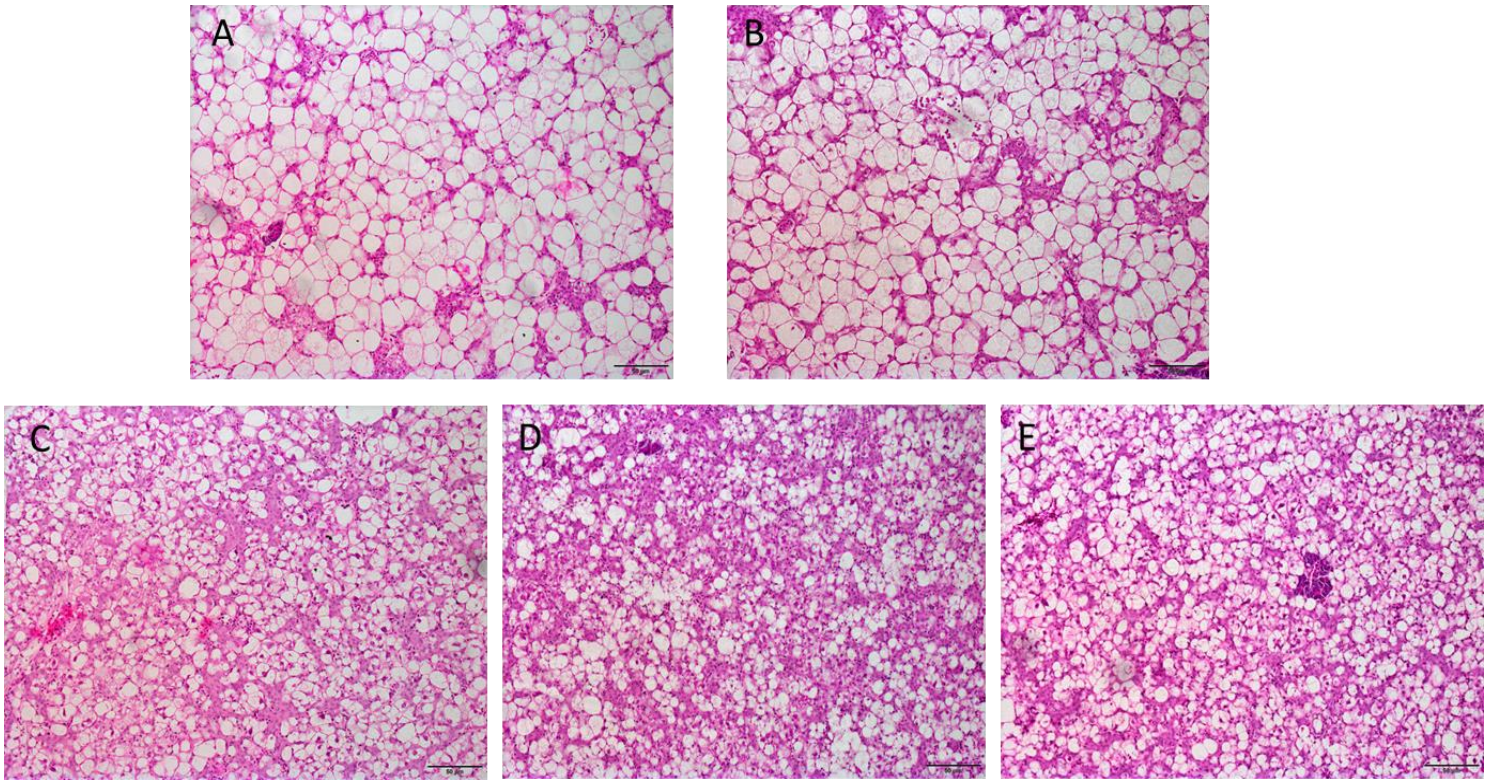

Figure 9: Liver sections from meagre fed different n-3 HUFA levels stained with H&E: A, 0.9% n-3 HUFA; B, 1.5% n-3 HUFA; C, 2.0% n-3 HUFA; D, 3.0% n-3 HUFA); E, 3.9% n-3 HUFA (Bars 50µm).

Additionally, fish fed the lowest dietary n-3 HUFA level (0.9%) presented higher ($P<0.05$) number of hepatic granulomas than fish fed 2.0-3.9% n-3 HUFA (Table 16). However, no relation was found between the development stage of granulomas and the dietary n-3 HUFA level. Even though, two main development stages were observed, irrespectively of the diet: an early stage characterized by concentric layers of macrophages and inflammatory cells around (Figure 10A & 10B); and a more developed stage characterized by necrotic center with external fibroblast layer and inflammatory cells (Figure 10C & 10D).

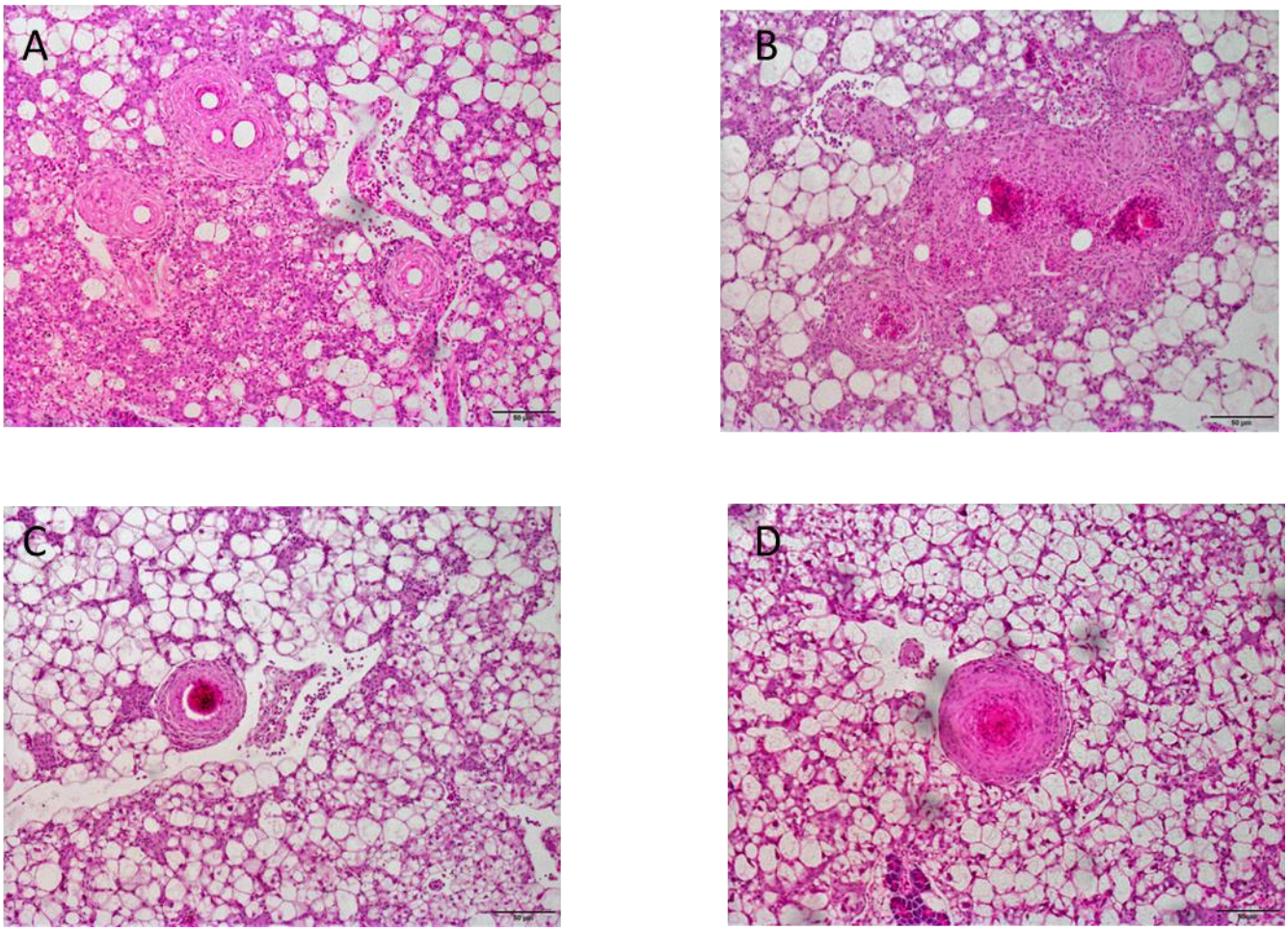

Figure 10: Liver sections of meagre with presence of granulomas at different stages of development: A and B showing concentric layers of macrophages and inflammatory cells around and C and D showing necrotic center with external fibroblast layer and inflammatory cells (Bars 50 µm).

3.4.3 Bacteriological results

Liver sections stained with acid-fast (Ziehl-Neelsen) led to a negative result for the presence of *Mycobacterium sp.* and *Nocardia spp.* (Figure 11) and no colony forming units (CFU) of *Nocardia spp.* and *Streptomyces sp.* grown in any culture media utilised.

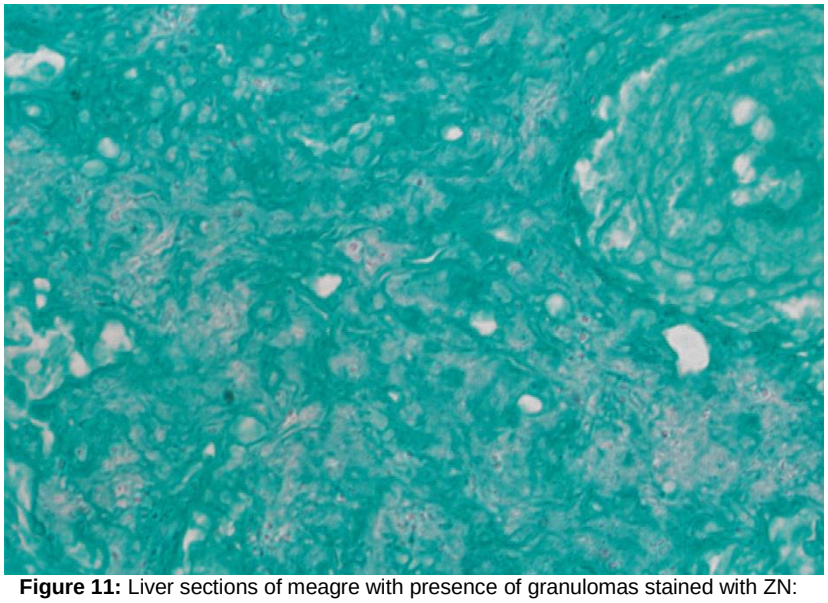

Figure 11: Liver sections of meagre with presence of granulomas stained with ZN: Negative result (Bars 100µm).

3.5 Discussion

Several studies have been using organ level biomarkers, as the HSI, to evaluate the effect of dietary components, such as lipids and carbohydrates, on hepatomegaly (Shearer, 1994; Peres and Oliva-Teles, 1999; Montero et al., 2001; Bolla et al., 2011; Castro et al., 2015). Generally, under normal conditions, each organ represents a fixed percentage of the total weight of the fish, irrespective of its size. However, depending on the species and the nutritional characteristics of the feed, high levels of both carbohydrates and lipids, or an imbalance in dietary FA may influence liver composition and, consequently, liver weight. These changes could be due to a deposition or reduction in glycogen or lipid content in the hepatic tissue (Shearer, 1994; Peres and Oliva-Teles, 1999; Castro et al., 2015). N-3 HUFA play an important role in avoiding hepatic lipid accumulation (Fukazawa et al.,1971; Watanabe 1982; Montero et al., 2001). This accumulation may be due to an impaired FA metabolism (Watanabe, 1982). Deficiency n-3-HUFA may affect the adequate phospholipids content of cell membranes, prejudicing FA transport from liver to other tissues, reducing lipid oxidation (Sargent et al., 1989) and the adequate synthesis of the lipoproteins to guarantee its transportation (Fukuzawa, et al., 1971), resulting in abnormal lipid accumulation in liver. Similarly, in the present study, meagre fed EFA-deficient diets (0.9 and 1.5% n-3 HUFA diets), below the EFA requirements previously established for meagre fingerlings (Carvalho et al., unpublished data), showed the highest HSI and liver lipid content, suggesting an imbalance in dietary FA due to an insufficient content of n-3 HUFA. A similar increasing lipid deposition in hepatic tissue and, consequently, in HSI was also found in gilthead seabream *Sparus aurata* juveniles (Kalogeropoulos et al., 1992; Ibeas et al., 1994; Montero et al., 2001) and turbot *Scophthalmus maximus L.* (Peng et al., 2014) fed deficient EFA diets, or in consequence of the replacement of fish oil by vegetable oils (Fernandes et al., 2012; Borges et al., 2014).

Generally, fish FA composition reflected the dietary FA composition (Cowey and Sargent, 1972). In the present study, the decrease of dietary substitution of vegetable oils by fish oil increased the dietary n-3 HUFA, which was followed by an increase of liver SFA, n-3 HUFA, particularly EPA and DHA, and n-6 HUFA, as ARA, and by a decrease of MUFA, LNA, LA and n-9 FA, mainly OLA. Previously, the same trend was also observed in whole-body FA composition of meagre fingerlings fed increased dietary n-3 HUFA levels (Carvalho et al., unpublished data). Moreover, in the present study, most FA of liver total lipids reflected the dietary pattern. However, liver content in 20:2n-6 and 20:3n-3 was contrary to dietary FA pattern, since meagre fed

the two lowest n-3 HUFA diets showed the highest content in these FA. These results suggest that possibly meagre is capable of elongating LA and LNA to yield longer-chain HUFA. Indeed, recent studies found that meagre seemed to have active the fatty acyl elongase 5 (Elovl5) and the ∆6 fatty acyl desaturase, although their activities were insufficient to cover meagre requirement for n-3 HUFA (Monroing et al., 2013; Carvalho et al., unpublished data). Similarly, an up-regulation of longer-chain HUFA biosynthesis has also been reported in fish fed vegetal oil but being insufficient to maintain EPA and DHA tissue levels similar to those fed fish oil based diets (Tocher, 2015).

The accumulation of n-9 FA, such as OLA and the increase of the ratio OLA/n-3 HUFA are recognized as biochemical indicators of EFA-deficiency (Watanabe, 1982; Sargent et al., 2002; Baweja and Babbar, 2015; Torrecillas et al., 2017). In the present study, meagre fed n-3 HUFA levels below the EFA requirement showed significantly higher values of this ratio than fish fed levels around or above the EFA requirement. Similarly, OLA/ n-3 HUFA ratio of whole body total lipids of meagre fed the same n-3 HUFA levels followed the same trend, although being lower in liver than in whole-body lipid (Carvalho et al., unpublished data). Thus, values of OLA/n-3 HUFA of 1.54 or lower could be expected in meagre livers fed diets covering its EFA requirements.

Contrarily to the FA profile of level total lipids, the FA profile of liver polar lipids was less affected by diet composition, with most of the FA levels being constant among meagre fish fed different n-3 HUFA levels. Since n-3 HUFA are required for sustaining the structure and functionality of cell membranes, the FA profile of polar lipid is more constant than neutral or total lipid (Sargent et al., 1989). Thus, the marked decrease of n-3 HUFA of liver total lipid of meagre fed n-3 HUFA levels below the requirement compared to the smaller decrease in liver polar lipid fraction suggests that meagre preserved n-3 HUFA in phospholipids in response to a dietary EFA-deficiency. Similar results were reported when EFA-deficient diets were fed to gilthead seabream fed (Koven et al., 1989; Ibeas et al., 1996; Montero et al., 2001), golden grey mullet *Liza aurata (*Vegner et al., 2014), turbot (Regost et al., 2003) and Senegalese sole *Solea senegalensis* (Borges et al., 2014). Nevertheless, although no significant differences were observed in OLA/n-3 HUFA ratio this trended to increase in fish fed deficient n-3 HUFA diets (2.6-3.0% according to Carvalho et al., unpublished data) in accordance to what was observed in liver total lipids. Similarly, it was observed that OLA/n-3 HUFA ratio decreased with increasing dietary n-3 HUFA level in hepatic phospholipids of gilthead seabream (Kalogeropoulos et al., 1992; Ibeas et al., 1994; Ibeas et al., 1996),

red seabream *Pragus major* (Takeuchi et al., 1990) and flounder *Paralichthys olivaceus* (Kim and Lee, 2004).

In mammals, hepatic steatosis was described as a metabolic pathology associated to an excessive infiltration of FA into the hepatic tissue. Consequently, an increase of β-oxidation occurs, leading to the production of free radicals, release of inflammatory cytokines and causing aggression on the hepatic tissue (Caballero et al., 2004; Benedito-Palos et al., 2008). The same disorder is known to appear in fish and has been observed particularly in marine aquaculture fish species associated to high dietary lipid levels, use of artificial diets, quality of dietary oils used (Caballero et al., 2004) and increase of dietary inclusion of vegetable oils (Caballero et al., 2004; Ribeiro et al., 2015). All these possible steatotic agents are related to an imbalance in FA metabolism and are frequently associated to deficient levels of EFA (Verreth et al., 1994; Montero et al., 2001). In present study, meagre fed the two lowest n-3 HUFA levels (0.9 and 1.5%), deficient in EFA according to Carvalho et al., unpublished data, showed a more severe steatosis, with high HIS and lipid infiltration, which was consistent with the lower levels of n-3 HUFA (EPA and DHA) and higher levels of monounsaturated FA, OLA, LNA and LA. Moreover, meagre liver presenting higher degree of steatosis also showed the highest OLA/n-3 HUFA ratio in liver total lipids as well as in phospholipids. These results may be due to insufficient levels of EPA and DHA as well as to an excessive level of OLA since these conditions have been reported to induced liver lipid. Indeed, EPA is known to reduce lipid vacuoles in liver since it increases the area surface of mitochondria and peroxisomes (FrØyland et al., 1996; Totland et al., 2000; Caballero et al., 2004) and decreases the FA availability for triglycerides synthesis (Berge et al, 1999). Hepatic steatosis is also associated to low DHA contents in diets and high content of OLA, so maintaining a normal OLA/DHA or OLA/n-3 HUFA ratio is important (Spisni et al., 1998). Contrary to what happens in mammals and freshwater species, where OLA is well tolerated, in marine species an excessive content in this FA is detrimental (Spisni et al., 1998). Also for meagre juveniles, it has been reported that high inclusion levels of vegetable oils induced hepatic steatosis (Ribeiro et al., 2015). For other fish species, steatosis has been observed in gilthead seabream (Montero et al., 2001; Caballero et al., 2004) and Japanese sea bass *Lateolabrax japonicus* (Xu et al., 2016) juveniles in response to a reduction of dietary EFA. Nevertheless, in the present research, meagre fed the n-3 HUFA levels around or above the requirement (2.0-3.9%) also presented hepatic steatosis, suggesting that the high dietary lipid used in this study, might induce lipid

infiltration in the hepatic tissue, although at a much lower extension. Similarly, Ribeiro et al. (2015) also observed signs of hepatic steatosis in meagre, even in when fed with reference diets including only fish meal and fish oil. Indeed, meagre fed fish oil based commercial diets, in a commercial context, showed an increasing lipid deposition (steatosis) in hepatocytes when lipid content of the diets increased from 15 to 20% (Fountoulaki et al., 2017).

In the present study, hepatic granulomas were observed in meagre, with higher incidence in those fed the lowest n-3 HUFA level (0.9%) than in those fed levels around or above the requirement (2.0-3.9%) n-3 HUFA. This condition is most likely of metabolic origin rather than to infection agent. Bacterial cause by infection with *Nocardia sp., Streptomyces sp.* or *Mycobacterium spp.* was discarded. Thus, these results suggest that hepatic granulomas of meagre fingerlings are probably associated to the dietary n-3 HUFA deficiency, as higher incidence of this disease was observed in fish fed n-3 HUFA levels below the requirement estimated for the species. To date, no other studies reported a relation between EFA-deficiency and granulomatosis in fish, but in mammals, fatty livers diseases are associated to higher incidence of hepatic lipogranulomas (Coash et al., 2012). In seabream, granulomatosis has been associated to a hypertyrosinaemia (Paperna et al., 1980; Paperna et al., 1987) and, in turbot to a deficiency of vitamin C (Tixerant et al., 1984). The development stage of such granulomas was variable among the dietary treatment, and a relation between the granuloma development stage and dietary n-3 HUFA is difficult to affirm.

3.6 Conclusion

Based on the present results, a minimum n-3 HUFA dietary inclusion level of 3.0% DM is recommended to maintain a normal hepatic biochemical composition, as well as a normal hepatic histomorphology in meagre fingerlings, corroborating the requirement value estimated for growth (2.6-3.0% DM n-3 HUFA; Carvalho et al., unpublished data). Low n-3 HUFA diets led to an increase of HSI and liver lipid infiltration inducing severe steatosis. Furthermore, a higher incidence of hepatic granulomas was observed in fish fed the lowest n-3 HUFA diet, suggesting a possible relation between EFA-deficiency and incidence of hepatic granulomatosis in meagre. FA composition of liver total lipids was more influenced by dietary composition than that of phospholipids.

Due to the high incidence of granulomatosis in meagre aquaculture production, further research is need to investigate the relation between this pathology and dietary FA composition in meagre. Increase the knowledge of the possible causes of this disease is an important step to prevent its appearance.

Chapter 4. General conclusions and future perspectives

Meagre is a promising aquaculture species due to its high growth rate and excellent conversion rate, being expected an increase in its production in the near future. To optimised large-scale production of meagre, several questions need to be addressed. One of most important issue is to establish the optimal rearing conditions and nutritional requirements to maximize production and to ensure adequate diet formulation to guarantee high performances and final flesh quality for human consumption. Since it is a marine species, meagre has an essential requirement for n-3 HUFA, especially DHA and EPA. For meagre this requirement had not been studied so far. The Chapter 2 of the present study was the first study aiming to determine the n-3 HUFA requirement for meagre fingerlings, which was estimated to be circa 2.6-3.0% DM. Dietary FA profile could influence fish nutritional and health status, so the study of histomorphology of key organs, such the liver, is crucial to understand the mechanisms beyond FA metabolism and its implication in fish health status. Therefore, Chapter 3 aimed to evaluate the effect of different levels of EFA, below and above the requirement levels estimated in Chapter 2, on liver composition and histopathology. Corroborating the results obtained in Chapter 2, it was observed that a minimum of 3.0% DM of n-3 HUFA in the diet is required to avoid excessive liver lipid infiltration, steatosis and impaired FA profile of hepatic total lipid and in less degree of polar lipid. Besides, meagre production is facing a growing incidence of granulomatosis, which aetiology remains unclear. In the Chapter 3, a possible cause/effect between EFA deficiency and hepatic granulomatosis could emerged.

Further research need to be conducted to deeply study FA metabolism in meagre. Since EFA requirement varies with many factors, including fish size, further similar nutritional studies need to be done to determine the requirement for larger meagre. Furthermore, following steps could lead to understand the consequences of EFA deficiency on the development of nutritional related diseases, namely granulomatosis. It is expected that decreasing the gap on knowledge of meagre nutrition could enable to a new step on formulating specific diets for this species and, consequently, maximize the production and reduce losses.

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