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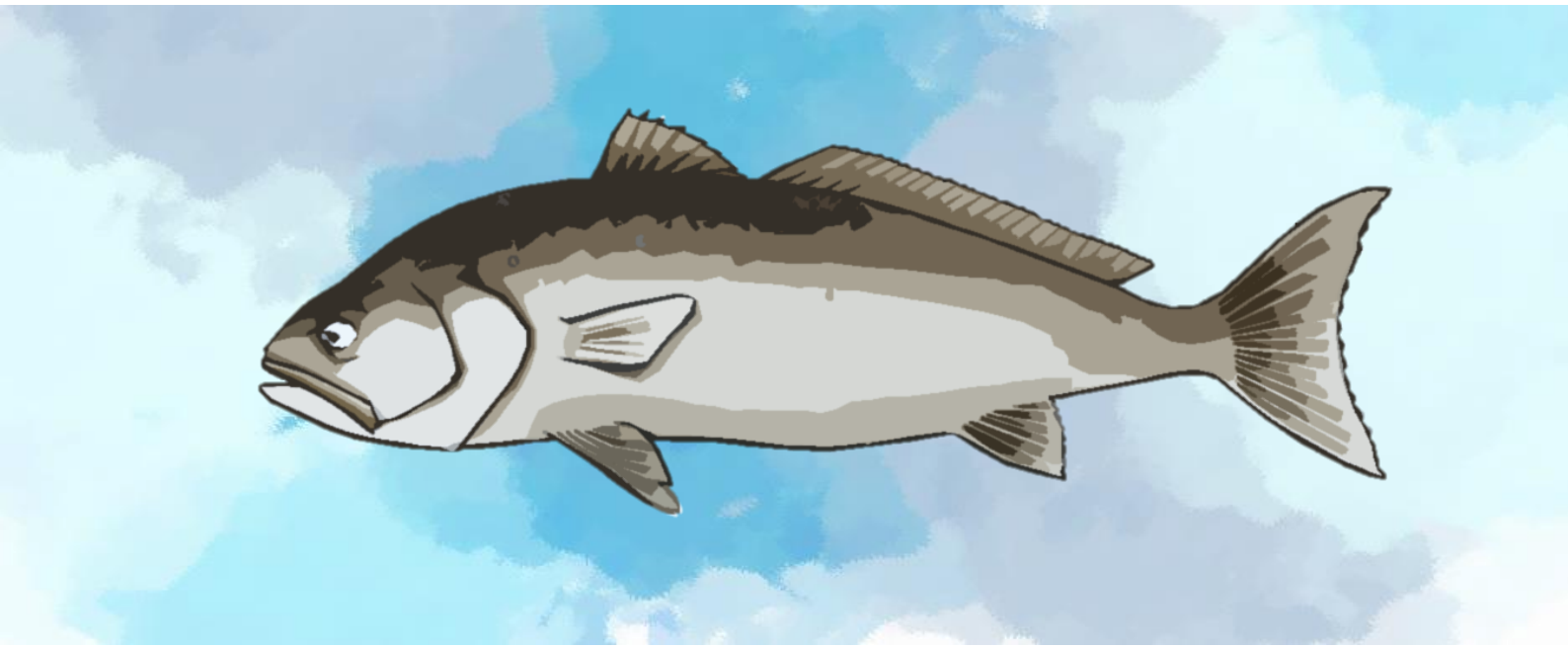

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Master in Aquaculture and Fisheries

(Specialization in Aquaculture)

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Declaração de autoria de trabalho

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

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(Ana Teresa Gomes Viegas)

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Abstract

Meagre (*Argyrosomus regius*) is a species of marine carnivorous fish that have a high demand for protein and lipids, with fish meal (FM) and fish oil (FO) as the main ingredients included in its diet. Currently, replacement of FM by plant proteins can be achieved in diets containing FO as the main lipid source, without affecting growth performance. However, FO has been over-exploited, and krill oil (KO) appeared as an alternative source, since KO is known to be a rich source of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) (namely, eicosapentaenoic acid - EPA and docosahexaenoic acid - DHA), which is crucial for the normal growth of marine fish. The alteration of the dietary fatty acids (FA) profile, for instance, through the replacement of FO by KO in plant-based diets may modify the contents of n-3 LC-PUFA, affecting fish growth. Thus, a 71-days feeding trial was conducted to evaluate the effect of FO replacement by KO in plant-based diets for meagre juveniles. Two experimental diets were formulated with different lipid sources – FO (1.18 and 0.60% of EPA and DHA, respectively) and KO (0.36 and 0.19% of EPA and DHA, respectively). Six homogeneous groups of 60 fish (13.20 ± 0.10 g and 5.28 kg/m^3 of initial weight and density, respectively) were randomly distributed into each tank and the experimental diets were randomly assigned in triplicate. The results showed that, in general, survival and whole-body composition of fish were not significantly ($p<0.05$) affected by the replacement of FO by KO. Nevertheless, reduced levels of EPA and DHA in KO diet affected negatively ($p<0.05$) growth performance, feed utilization, muscle and liver FA profile, and liver structure of fish. In conclusion, although KO could be a good energy source improving the cost effectiveness of the diet, present results suggested that FO cannot be totally replaced by KO without affecting the normal growth of meagre juveniles. Further research is needed to investigate the feasibility of supplementation of EPA and DHA in plant-based diets for this species.

KEYWORDS: *Argyrosomus regius*; nutrition; fish oil; krill oil; EPA; DHA; plant-based diets.

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Resumo

Com o contínuo aumento da população humana mundial, um dos grandes desafios a nível global é a procura por novas formas de suprir as necessidades alimentares, através de uma alimentação saudável e rica em proteínas. Nesse sentido, a aquacultura surge como uma alternativa viável para a produção de proteína de elevada qualidade nutricional, essencial para complementar a indústria pesqueira e garantir a segurança alimentar humana.

A produção da corvina-legítima (*Argyrosomus regius*) tem sido fomentada para a diversificação da aquacultura na Europa. Além de possuir um elevado valor nutritivo, esta espécie de elevado valor comercial apresenta um vasto leque de características produtivas vantajosas, que tornam a sua produção economicamente competitiva.

Existe um interesse crescente em estudar a corvina, não só por ser uma potencial candidata para a diversificação da aquacultura mediterrânea, mas também como forma de repovoar esta espécie e complementar o setor das pescas.

Apesar do interesse na produção de aquacultura intensiva de corvina, a falta de informação sobre as suas necessidades nutricionais, nomeadamente lípidos e ácidos gordos (AG), tem impedido a produção de rações e protocolos alimentares específicos desta espécie.

Os lípidos são uma fonte essencial de ácidos gordos essenciais (AGE), nomeadamente o ácido eicosapentaenóico (EPA), ácido docosa-hexaenóico (DHA) e ácido araquidónico (AA). Esta fonte de energia é principalmente fornecida através da adição de óleo de peixe (OP) às dietas, uma vez que este contém a quantidade necessária de AG. Porém, a contínua expansão da produção aquícola, acompanhada pelo rápido crescimento da produção de rações, levou a uma escassez de fontes de alimentos marinhos, como a farinha de peixe (FP) e o OP. Posto isto, surgiu uma necessidade urgente de explorar fontes lipídicas alternativas e sustentáveis para dietas direcionadas para peixes marinhos. Ingredientes vegetais são atualmente as alternativas mais económicas e ambientalmente sustentáveis. No entanto, enquanto o OP é rico em ácidos gordos polinsaturados de cadeia longa (AGPICL) n-3, os ingredientes vegetais são ricos em AG C_{18} n-6 e n-9. Consequentemente, a substituição da FP e OP por ingredientes de origem vegetal reflete-se, geralmente, na composição dos tecidos dos peixes, com uma diminuição de n-3 e aumento em AG C_{18}, afetando negativamente o normal crescimento dos mesmos. Neste sentido, o óleo de krill (OK) extraído do krill antártico (*Euphausia superba*) tem sido sugerido como uma fonte de lípidos alternativa para as rações, uma vez que é uma fonte rica em AGPICL n-3. No entanto, a alteração do perfil de AG na dieta, por exemplo, através da incorporação do OK

nas rações, pode modificar o conteúdo de AGPICL n-3 e, consequentemente, afetar o crescimento dos peixes.

No âmbito desta necessidade de formular uma dieta rica em ingredientes vegetais tendo como fonte lipídica uma alternativa mais económica e sustentável, o presente estudo, integrado no projeto DIVERSIAQUA II, teve como principal objetivo avaliar o efeito da substituição do OP pelo OK em dietas ricas em ingredientes vegetais no desempenho do crescimento, utilização da ração, composição corporal, perfil de AG do músculo e fígado, e ainda a estrutura do fígado de juvenis de corvina. Para tal, foram estabelecidos dois grupos experimentais: o grupo FO, no qual juvenis de corvina foram alimentados com uma ração baseada em ingredientes vegetais, tendo como principal fonte lipídica o OP (1,18 e 0,60% de EPA e DHA, respetivamente); e o grupo KO, no qual os juvenis foram alimentados com uma dieta rica em ingredientes de origem vegetal, tendo o OK como fonte alternativa de lípidos (0,36 e 0,19% de EPA e DHA, respetivamente). Um total de 360 juvenis de corvina (peso médio inicial de 13,20 ± 0,10 g) foram aleatoriamente distribuídos por 6 tanques retangulares (n = 3, por cada tratamento experimental), numa densidade máxima de 5,28 kg/m^3. O alimento foi fornecido *ad libitum* cinco vezes ao dia, durante os 71 dias de ensaio. Durante o período experimental foram realizadas duas amostragens: uma inicial, para registar o comprimento de cada indivíduo e o peso em biomassa em conjuntos de 10 indivíduos; e uma final, na qual cada peixe foi individualmente pesado e medido. No fim do ensaio, para posterior determinação da composição corporal, foram também recolhidos, aleatoriamente, cinco peixes por tanque (n = 15, por tratamento). Foram igualmente recolhidos seis indivíduos por tanque (n = 18, por tratamento) para a recolha de amostras de tecido. O fígado foi recolhido e pesado para determinação do índice hepatossomático (IHS), e de seguida dissecado em duas porções para análise histológica e do perfil de AG. Porções do músculo dorsal foram também amostradas para análise do perfil de AG. Ao longo do ensaio, foi registada diariamente a ração ingerida para determinação dos parâmetros da utilização das dietas.

Os resultados do presente estudo revelaram que a substituição do OP pelo OK diminuiu significativamente o conteúdo de EPA e DHA na dieta. Relativamente ao desempenho do crescimento, os juvenis de *A. regius* alimentados com a dieta KO apresentaram valores de crescimento significativamente inferiores quando comparados com os juvenis alimentados com a dieta FO. No entanto, tanto a sobrevivência como a composição corporal dos juvenis não foram afetadas significativamente pela substituição da fonte lipídica na dieta. Os resultados da utilização das diferentes dietas experimentais relevaram que os peixes alimentados com níveis dietéticos mais baixos de EPA e DHA (dieta KO) exibiram uma maior taxa de conversão

alimentar (TCA) e consumo diário de ração (CDR) do que aqueles alimentados com níveis mais elevados desses AGE (dieta FO).

Considerando a análise do perfil de AG no músculo, os resultados mostraram que, em todos os indivíduos, todos os AG acumularam neste tecido em menor extensão do que as suas respetivas concentrações na dieta. No entanto, o grupo de peixes alimentados com a dieta KO apresentou valores significativamente inferiores de ácido oleico (AO), EPA, DHA e AA relativamente ao grupo alimentado com a dieta FO. Além disso, em comparação com o EPA, o conteúdo de DHA foi superior no músculo de todos os indivíduos, contrastando com os níveis dietéticos.

Os resultados obtidos no tecido hepático suportam a teoria de que a composição da dieta interfere diretamente na composição corporal do animal, especialmente no fígado, que reflete o conteúdo de AG na dieta. Semelhante ao músculo, todos os AG foram acumulados no fígado em menor extensão do que as suas respetivas concentrações na dieta. Porém, peixes alimentados com a dieta KO exibiram valores reduzidos de 18:0, EPA, DHA e comparativamente aos valores destes AG obtidos em peixes nutridos com a dieta FO.

Relativamente ao IHS, os peixes alimentados com a dieta contendo baixos níveis de EPA e DHA (dieta KO) apresentaram valores mais elevados deste índice do que aqueles alimentados com maiores valores de EPA e DHA (dieta FO). A análise histológica revelou ainda que, independentemente dos níveis dietéticos de EPA e DHA, nenhum indício de necrose foi observado no fígado dos juvenis de corvina. No entanto, sinais evidentes de esteatose foram observados em todos os indivíduos, com aumento da deposição de lípidos nos hepatócitos e consequente deslocamento nuclear. Juvenis de *A. regius* alimentados com a menor percentagem de EPA e DHA na dieta (dieta KO) foram os mais comprometidos, apresentando o fígado com um grau mais elevado de esteatose. A mesma patologia também foi evidente em peixes alimentados com níveis mais elevados de EPA e DHA na dieta (dieta FO), no entanto em menor grau. Da mesma forma, os peixes alimentados com a dieta KO exibiram valores do diâmetro dos hepatócitos significativamente maiores comparativamente aos peixes alimentados com níveis mais elevados de EPA e DHA - dieta FO.

Os resultados obtidos sugerem que, embora o OK possa constituir uma boa fonte de energia, melhorando o custo-benefício da dieta, este pode não substituir totalmente o OP em dietas ricas em ingredientes vegetais para juvenis de corvina. São necessários estudos que permitam determinar o nível adequado de OK nas dietas, de forma a substituir o OP e garantir o normal crescimento, bem como o estado nutricional nesta espécie. Além disso, em futuros ensaios poderá ser analisada a viabilidade da suplementação de AGPICL n-3 em dietas ricas em ingredientes vegetais para juvenis de corvina através da inclusão de uma fonte lipídica

alternativa, sustentável e económica. Desta forma, poderá ser eliminado um dos constrangimentos que o setor aquícola tem enfrentado há mais de 20 anos – o EPA e o DHA como dois dos primeiros AG limitantes em dietas ricas em ingredientes vegetais em peixes marinhos carnívoros.

Palavras-chave: *Argyrosomus regius*; nutrição; óleo de peixe; óleo de krill; EPA; DHA; dietas vegetais.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
ARA	Arachidonic acid
DFI	Daily feed intake
EFA	Essential fatty acid(s)
EPA	Eicosapentaenoic acid
EPPO	Estação Piloto de Piscicultura de Olhão
EU	European Union
FA	Fatty acid(s)
FAO	Food and Agriculture Organization
FBL	Final body length
FBW	Final body weight
FCR	Feed conversion ratio
FI	Feed Intake
FM	Fishmeal
FO	Fish oil(s)
HSI	Hepatosomatic index
H&E	Haematoxylin and eosin
IBL	Initial body length
IBW	Initial body weight
IPMA	Instituto Português do Mar e da Atmosfera
K	Condition factor
KO	Krill oil
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acid(s)
LNA	α-linolenic acid
MUFA	Monounsaturated fatty acid(s)
n	Number
OA	Oleic acid
PL	Phospholipid(s)
SD	Standard deviation
SFA	Saturated fatty acid(s)
SGR	Specific growth rate
TG	Triglyceride(s)
VO	Vegetable oil(s)
WG	Weight Gain

LIST OF UNITS

µm	Micrometre(s)
cm	Centimetre(s)
g	Gram(s)
h	Hour(s)
kg	Kilogram(s)
km	Kilometre(s)
l	Litre(s)
m	Meter(s)
m^3	Cubic meter(s)
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
ºC	Degree(s) Celsius
ppm	Part(s) per million

LIST OF FIGURES AND TABLES

1. INTRODUCTION

1.1. Aquaculture in the world

With the increasing human population, one of the great challenges at a global level is to search for new ways to meet food needs, through a healthy diet rich in proteins. Between 1961 and 2017, the average annual increase in global food consumption of fish for human consumption (3.10%) outpaced population growth (1.60%) and exceeded that of all other animal protein food (e.g., meat, dairy, milk), which increased by 2.10% per year. In *per capita* terms, food fish consumption grew from 9.00 kg in 1961 to 20.50 kg in 2018, at an average rate of about 1.50% per year. In this sense, the aquaculture has become one of the most important ways to complement fisheries in the production of aquatic food, ensuring the food security over the world. Currently, aquaculture continues to grow faster than other major animal production sectors, accounting for 46% of the total production and contributing for 52% of fish for human consumption, expected to reach 60% by 2030 (FAO, 2020). According to FAO (2020), total fish production (excluding aquatic plants) is expected to reach 204 million tonnes in 2030. In 2018, global fish production was estimated to have reached about 179 million tonnes, of which 82 million tonnes came from aquaculture production. Of the overall total, 88% (156 million tonnes) were used for human consumption and the remaining 12% (22 million tonnes) were destined for non-food purposes, of which 82% (18 million tonnes) were used to produce fishmeal (FM), mainly for high-protein feed, and fish oil (FO), as a feed ingredients in aquaculture production (FAO, 2020).

1.2. Aquaculture in Portugal

Portugal has excellent conditions for aquaculture, highlighting the southern region, which makes this country one of the main targets for large investments. Portugal has strong maritime and marine fishing traditions since long ago and mostly because of its approximately 943 km along the coast of mainland. Fishing has always been an activity of great importance to the populations established along the coast, however it is now a high-risk activity given the increasing scarcity of natural marine resources (Bjørndal *et al.*, 2015). In 2018, Portugal was the second most relevant country in the European Union (EU) regarding to the *per capita* consumption of fish, following Malta (EUMOFA, 2020). However, Portugal imports most of the fish it consumes (ca. 2,789.3 million Euros in 2019), since its internal production is not sufficient to satisfy existing needs (INE, 2020). Only recently the production of marine

aquaculture began to show results of the investments made since the beginning of the 90s (Bjørndal *et al.*, 2015). This is mainly due to the introduction of more intensive production systems and an increase in the number of aquaculture units (Bernardino, 2000). The increase in production was mainly due to improvements in infrastructure, greater availability of juveniles, hatcheries and the wider use of appropriate equipment (DGRM, 2014).

Due to the financial support, provided by both the Portuguese Government and European Community funds, a large number of salt ponds were gradually converted into aquaculture facilities. The first data on aquaculture in Portugal emerged in 1965, when the cultivation of marine and brackish species began to be carried out in inland waters, in estuaries and coastal lagoons, using extensive production regimes, and particularly improving infrastructures of the salt industry (Bernardino, 2000).

In the 1970s, the production of aquaculture species focused on species with low commercial value, which represented about 80% of production. After the mid-1980s, when the Community structural policy was applied in Portugal, aquaculture began to be seen as a complement to the fisheries sector and as an alternative source of animal protein production for human consumption (INE, 2020). Beginning in the 1990s, efforts to expand the fishing market in the Mediterranean region stimulated the demand for new species to promote diversification in aquaculture (Castro *et al.*, 2013). Despite the low production compared to the other EU countries, Portugal presents a very diversified aquaculture. However, Portuguese production is led by the production of molluscs and crustaceans, which accounted for 67.20% of total aquatic production in 2018 (increasing 10.50% in 2018), followed by fish production in transitional and marine waters, with only 27.60% of total production (90% of which was constituted by the production of turbot, *Scophthalmus maximus*, and gilthead seabream, *Sparus aurata*), and 5.20% of other species (INE, 2020).

According to the latest INE surveys for sales of Portuguese aquaculture in the national and international markets, the species with the greatest economic value are clams (e.g., *Ruditapes decussates*) and sole (*Solea senegalensis* and *Solea solea*) (12.84 and 12.80 €/kg, respectively), followed by meagre (*Argyrosomus regius*) (11.12 €/kg), European seabass (*Dicentrarchus labrax*) (8.80 €/kg), turbot (8.06 €/kg), and gilthead seabream (5.86 €/kg). However, not all these species have a large contribution to the total national production, being, for instance, the self-enclosed meagre 5.2% in most of the species produced, with a contribution of only 0.14% for total production (INE, 2020).

1.3. Meagre, *Argyrosomus regius* (Asso 1801)

1.3.1. Biology of meagre

Argyrosomus regius (Asso, 1801) is a teleost fish belonging to phylum Chordata, infraphylum Gnathostomata, class Actionopterygii, infraclass Teleostei, order Perciformes and family Sciaenidae, one of the largest families of species in the world, including 68 genera and 311 species worldwide (Mesa, 2012).

Meagre is a species of carnivorous marine fish that reaches large dimensions, reaching up to 200 cm of total length, a maximum age of 42 years and can weigh more than 103 kg (Quéro and Vayne, 1987). However, the most common is to capture individuals between 50 and 100 cm and 55 kg (González-Quirós *et al.*, 2011; Quéméner *et al*., 2002; Quéro and Vayne, 1987). Individuals of this species present a relatively large head and an elongated body (fusiform), large otoliths, small eyes and the mouth situated in the terminal position, with lower jaw teeth in irregular rows, and without barbs (**Figure 1.1**). Since it is a carnivorous species, meagre has large and strong teeth, located in the external zone of the mandible (Cárdenas, 2011).

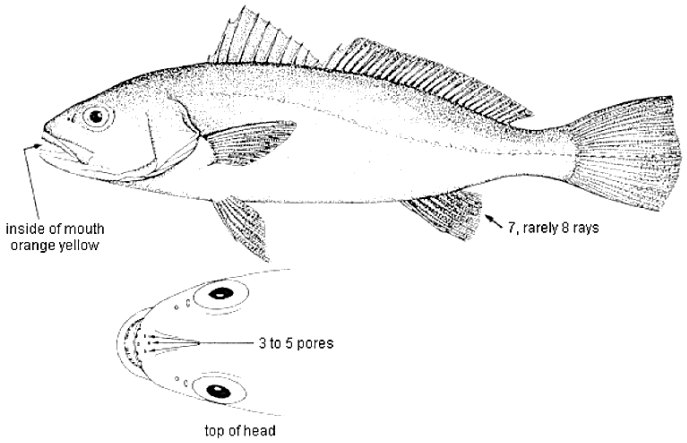

Figure 1.1. Morphological characteristics of meagre (*A. regius*). *In* Monfort (2010).

Meagre has the body silver-grey with bronze features dorsally, covered mostly by ctenoid scales and has particular characteristics, such as the evident lateral line that extends to the caudal fin; the buccal cavity golden-yellow coloured and the base of the fins reddish brown (Cárdenas, 2011; Monfort, 2010). The second dorsal fin is much longer than the first, and the anal fin has a first short spiny ray and a second very thin (Monfort, 2010).

A. regius is an euryhaline and eurythermal species, being able to adapt to a wide range of salinity (5–45 g/l) and temperature (2–38 ºC) values, having an optimal range between 22 and

24 ºC, which contribute for their wide distribution (Cárdenas, 2011). This species occurs in subtropical waters of the Eastern Atlantic ranging from Norway to Congo, including the Mediterranean and the Black Sea (Gil *et al.,* 2013; González-Quirós *et al.*, 2011). Being a semi-benthic fish, meagre generally occupies coastal and shelf waters near the bottom or near the surface (depth range 15 to 300 m), and also estuaries and coastal lagoons, while European seabass and gilthead seabream are semi-pelagic fish species widely cultivated in the Mediterranean Sea (Nathanailides *et al.,* 2013).

Meagre is an anadromous species, which in mid-April approaches the coastline, and migrates to the estuaries at the breeding season at the end of May (FAO, 2020-2021; González-Quirós *et al.,* 2011). After spawning, from mid-June to the end of July, meagre leave the estuaries to feed along the coast, and remain in shallow waters until early October (Cárdenas, 2011; FAO, 2020-2021). In winter, meagre returns to deeper waters to survive cooler conditions, considering that growth is achieved mainly during the summer (FAO, 2020-2021).

The juveniles leave the maternity areas (estuaries) at the end of the summer and migrate to the coastal waters (from 20 to 40 m) to spend the winter. From mid-May, they return to the estuarine areas to feed on small fish and demersal crustaceans. Temperature is crucial at this time, as juveniles need about 20–21 °C to feed. After reaching 30–40 cm, they begin to feed on pelagic fish and cephalopods (FAO, 2020-2021).

1.3.2. Meagre aquaculture

A. regius has several characteristics that makes it a suitable candidate for commercial production: is a medium grower species with growth (over 700 g fish after 12 months and 2–2.5 kg after 24 months) higher than those of most of the common Mediterranean-cultured species, such as gilthead seabream and European seabass (Abou Shabana *et al.,* 2012; Estévez *et al.,* 2011; Jiménez *et al.,* 2005; Quéméner *et al.,* 2002; Ribeiro *et al.,* 2013); has an excellent feed conversion ratio (FCR) of 0.9–1.2 (Duncan *et al.,* 2013; Jiménez *et al.,* 2005; Mañanós *et al.,* 2009; Silberschneider and Gray, 2008); is a highly fertile species and its broodstock are relatively easily to manage, obtaining good quality eggs; larvae are relatively easily to rear with standard industry live feeds and formulated diets, and juveniles do not present reproductive maturation during on-growing phase (Mañanós *et al*., 2009; Pousão-Ferreira *et al*., 2013; Roo *et al*., 2010); and its production technology is similar to other well-established marine fish species, such as seabream (Cárdenas, 2011; Jiménez *et al.,* 2005; Monfort, 2010; Poli *et al.,* 2003; Rojas and Seijo, 2012; Saavedra *et al.,* 2015). From a market perspective, meagre has high nutritional value since it is a lean fish with high nutritional flesh quality, with high protein

and n-3 polyunsaturated fatty acids (PUFA) as well as with low n-6/n-3 ratio values (Monfort, 2010; Piccolo *et al.*, 2008; Poli *et al.*, 2003), which turns this specie well accepted by consumers (Castro *et al.*, 2013; Duncan *et al.*, 2013; Mañanós *et al.*, 2009; Monfort, 2010; Poli *et al.*, 2003). In addition, this species has a long shelf life and a high carcass yield, excellent taste, and firm texture, making it comparable to other available table fishes (Monfort, 2010).

Meagre is farmed in Europe since the late 90s (Monfort, 2010). The activity started in Europe simultaneously in Italy and France, where the first commercial sized fish to be marketed in the late 1997, but since then has spread to Spain, Greece, Egypt, Turkey, Malta and Portugal (Monfort, 2010). The production of this species was only a few tonnes at the beginning of the year 2000 and increased to 14 197 and 23 440 tonnes in 2015 and 2016, respectively (FAO, 2020-2021). According to the last APROMAR's 2020 report, meagre production in the Mediterranean area was estimated at 41 295 tonnes in 2019, representing an increase of 10.5%, compared to 2018. The main producing countries were Egypt (32 000 tonnes), Spain (3 650 tonnes), Turkey (2 600 tonnes), and Greece (1 800 tonnes). In the same report, a production of meagre was estimated to be above 42 000 for 2020 (APROMAR, 2020).

In Portugal, meagre is well known, and most appreciated in the south of the country (Algarve) and is commercialized with a large size (over 5 kg), being also a great candidate for the filleting industry due to its dimensions (Monfort, 2010).

Among the newly cultivated fish species, meagre has been recognized as a species with high potential for the diversification of Mediterranean aquaculture, being considered a priority in research field and development programs of the Mediterranean countries (Chatzifotis *et al.*, 2010; Duncan *et al.*, 2013; Nathanailides *et al.*, 2013; Poli *et al.*, 2003; Saavedra *et al.*, 2020).

Presently, the price of meagre in the market is around 9 €/kg, which can be considered an important factor to continue developing the production protocols on the species. This makes meagre an opponent to European seabass or gilthead seabream, as its price per kg is much more attractive compared to those species.

EPPO (Estação Piloto de Piscicultura de Olhão – from IPMA) began to study this species in 2006, and since then several studies have been carried out, with the aim of improving its production in aquaculture.

Aquaculture produce meagre seems to be less susceptible to diseases than other marine species produced in aquaculture (Soares *et al.*, 2018). Nevertheless, under intensive culture of meagre, the main concern for commercial production is the fish health considering the systemic granulomatosis as the more frequent disease in this species (Ghittino *et al.*, 2004).

Although the natural reproduction of meagre occurs between Spring and Summer with several spawns, it is common to apply hormonal treatments (Cárdenas, 2011) or alteration of the production conditions (e.g., photoperiod and temperature) to guarantee the reproduction of this species in captivity.

Larvae of this species show a good survival rate, about 15-40% at 30 days after hatching and 15% at 60 days after hatching (Cárdenas, 2011). During pre-on-growing, cannibalism can be a problem (up to 15 g) and is recommended feeding frequently, between two and four times a day, to excess. Furthermore, is required approximately bi-monthly size grading in order to maintain populations with low variation in the size frequency distribution, avoiding cannibalism of smaller fish by larger individuals of the group. As the meagre grow, feeding frequency can be reduced to once a day. Meagre feed low in the water column and take time to rise to the surface to eat. This species requires approximately double the ration used for gilthead seabream and can be fed 1–2% body weight in agreement with manufacture's feeding tables (Duncan *et al.*, 2013).

Meagre also shows suitable behaviour during the pre-fattening and fattening stages, suggesting that this species does not present high levels of stress when in captivity and, for this reason, meagre mortality is often nil or absent during this stage of production (Duncan *et al*., 2013).

There is growing interest in studying meagre, not only as a potential candidate to diversify Mediterranean aquaculture but also to repopulate depleted natural fisheries (Duncan *et al.*, 2013; Pastor *et al*., 2013; Saavedra *et al.*, 2015). This species is very vulnerable, due to its geographical distribution is highly explored and it can be easily captured during spawning season, due to sound emission – called "grunt" – produced by males (Cárdenas, 2011; Jiménez *et al.*, 2005; Lagardère and Mariani, 2006). Furthermore, spawning in estuaries is itself a threat to the maintenance of the species due to degradation and pollution of many of these coastal areas (Jiménez *et al.*, 2005; Sadovy and Cheung, 2003). The high adaptability of this species to farming conditions is vital in the sense that can safeguard its growth and reproduction process, in order to protecting them (Millán-Cubillo *et al.*, 2016).

1.3.3. Meagre nutrition

Concerning its feeding habits, meagre is a carnivorous species, which feeds in the wild, on polychaetes, crustaceans, echinoderms, molluscs and other small fish species (*Cupleidae* and *Mugilidae* families) (Jiménez *et al.*, 2005). The proportion of these preys in the diet differs according to the age. Compared to adults, meagre juveniles have a less diversified diet, based

only on Mysidacea and shrimps (Cárdenas, 2011). As they grow, the diet becomes more piscivorous, based on demersal and benthic fish (Jiménez *et al.*, 2005).

Nowadays, commercial diets are available for meagre aquaculture, but also pelleted diets formulated for gilthead seabream and European seabass have been used due to the similarity in feeding requirement between these species (Chatzifotis *et al.*, 2012; Duncan *et al.*, 2013).

Despite the interest in intensive aquaculture production of meagre, there is a lack of information regarding nutritional requirements, that has been impairing the production of specific feeds and feeding protocols (Carvalho *et al.*, 2019).

To achieve a successful large-scale fish production, is critical the development of commercial high quality species-specific diets (Saavedra *et al.*, 2016). Fish nutrition has become a very important topic of investigation, since in aquaculture fish feed costs generally represent a substantial percentage (up to 50%) of the total operating costs of an aquaculture enterprise (Rana *et al.*, 2009). To improve the aquaculture production of fish is crucial to characterize the nutritional requirements of the species, to develop diets that cover the basic metabolic and physiological requirements at the lowest cost.

1.4. Lipids

Lipids are hydrophobic compounds that are relatively insoluble in water but are soluble in organic solvents such as chloroform, ether, hexane, and benzene (Higgs and Dong, 2000; Nelson and Cox, 2004; Turchini *et al.*, 2009).

Fish lipids can be divided into two groups: polar lipids, composed principally by phospholipids (PL); and neutral lipids, composed principally by triacylglycerols, also known as triglycerides (TG) (Khalil *et al.*, 2018; Tocher, 2003). PL make up the integral structure of the unit membranes in cells; it is also why they are often called “structural lipids” (Nelson and Cox, 2004). TG are used for storage of energy in fat depots, usually within special fat cells surrounded by a PL membrane and a rather weak collagen network, being formed by the combining of glycerol with three molecules of fatty acids (FA) (Gunstone *et al.*, 2007).

Lipids represent one of the main dietary energy sources in fish (Watanabe, 1982). According to Craig and Helfrich (2009), this macronutrient is a high-energy nutrient that can be used to partially spare protein in aquaculture feeds since they supply about twice the energy as proteins and carbohydrates. Lipids are also a source of essential fatty acids (EFA), namely: n-3 long-chain polyunsaturated fatty acids (LC-PUFA), particularly docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3); and n-6 series LC-PUFA, mainly arachidonic acid (ARA; 20:4n-6) (Khalil *et al.*, 2018; Parpoura and Alexis, 2001). Thus, lipids

are one of the main nutrients presented in fish and in their diets, making up, in general, about 17% of fish diets (Chatzifotis *et al*., 2010). Marine fish are traditionally fed relatively high-lipid diets using ingredients of marine origin containing high levels of n-3 LC-PUFA, particularly EPA, DHA and ARA (Benedito-Palos *et al.,* 2008; Mourente *et al.,* 2005).

Lipids are mainly supplied through the inclusion of fish oil (FO) that contain the necessary amount of FA and, as for most vertebrates, they are required by fish as a source of available energy, as structural components of membranes, carriers of fat-soluble vitamins, precursors to eicosanoids, hormones and vitamin D, and as enzyme co-factors (Tocher, 2010; Turchini *et al.,* 2009).

A recent trend in fish feeds is to use higher levels of lipids in diet (Craig and Helfrich, 2009). However, fish can use dietary lipids up to a certain level, beyond which growth may be retarded owing to reduced feed consumption (Chatzifotis *et al.,* 2012; Daniels and Robinson, 1986). While increasing dietary lipids can help reduce the high costs of feed by partially sparing protein in the feed, problems such as excessive deposition of lipids in the liver can decrease fish health and market quality of fish (Craig and Helfrich, 2009; Piccolo *et al.,* 2008).

1.5. Fatty acids

Fatty acids are organic compounds containing a hydrophilic group attached to a hydrocarbon chain (varying in the number of carbon atoms), with an additional terminal methyl group, and they are defined based on: number of carbon atoms, number of unsaturated bonds in its chain, and position of those bounds relative to the methyl terminus (Glencross, 2009).

Based on the number of unsaturated bonds, FA can be divided into three groups: saturated fatty acids (SFA) (no double bonds), monounsaturated fatty acids (MUFA) (1 double bond), and polyunsaturated fatty acids (PUFA) (> 1 double bonds) (Glencross, 2009).

In aquaculture, usually the term highly unsaturated fatty acids (HUFA) or LC-PUFA are used to define PUFA with more than 20 carbon atoms, although the distinction between PUFA and HUFA is not clear (Glencross, 2009). In this dissertation, the term LC-PUFA was adopted.

FA play key biological roles in animals, with LC-PUFA being essential in several functions such as controlling and regulating growth performance, survival, lipid metabolism, immune function, cell membrane integrity, nervous system development, and pigmentation (Izquierdo, 2005; Jin *et al.,* 2017; Murray *et al.,* 2015; Tocher, 2003; Torrecillas *et al.,* 2018; Torrecillas *et al.,* 2017). Furthermore, FA modulate immune responses: eicosanoids produced from ARA are recognised as inflammatory agents, while DHA, and especially EPA-derived eicosanoids, exert anti-inflammatory effects (Benedito-Palos *et al.,* 2008).

1.5.1. DHA and EPA

Among the different n-3 LC-PUFA, DHA has been recognized to be partially necessary for reproduction, normal growth, survival, regulation of stress response, normal behaviour, flatfish metamorphosis and disease prevention (Campoverde and Estevez, 2017; Izquierdo, 2005; Sargent *et al.*, 1995, 1997).

DHA is the major component in cell membranes and its incorporation regulates the integrity and function of the membrane, being an important component of PL (Izquierdo, 2005; Sargent *et al.*, 1995; Wassall and Stillwell, 2008). The structure of DHA provides this EFA with several important functions in fish metabolism (Izquierdo, 2005). Therefore, it is expected high DHA requirements in fast growing stages of fish development in order to satisfy the demands of fast forming tissues that accumulate DHA (Jin *et al*., 2017). DHA act as a substrate for some lipoxygenases and is also known to have a greater potential as growth promoter, being more effective for growth and survival than the other n-3 LC-PUFA (Izquierdo, 2005; Watanabe *et al.*, 1989).

DHA is naturally found at very high levels in the neural fish tissue (e.g., brain and eyes), and it is thought to play a particular role in neural membrane structure and function, including visual acuity for optimum hunting success and escape swimming with improved higher speed (Bell and Dick, 1991). According to Pousão-Ferreira (2009), feeding diets deficient in DHA can lead to reduced vision which difficult prey detection and, consequently, retard growth. So that, DHA is a n-3 LC-PUFA commonly used as a feeding supplement for aquatic animals (Wang *et al.*, 2019, 2020).

Regarding EPA, this EFA is also particularly important for growth, mainly in early life stages and for broodstock fertility, playing general and particular roles in fish metabolism (Fernández-Palacios *et al.*, 1995; Trushenski *et al.*, 2012; Watanabe *et al.*, 1989; Wu *et al.*, 2002).

In marine fish, EPA is a major component of polar lipids, with an important role in regulating the integrity and function of the membrane, although less important than DHA (Izquierdo, 2005). While EPA has a major role as a precursor of highly bioactive compounds such as eicosanoids, it can also partly satisfy DHA requirements with adequate elongase and desaturase activities to convert EPA to DHA in species such as European seabass (Castro *et al*., 2016). Recently, *A. regius* proved to have active Δ6 desaturases and *Elovl5*, but their activities are insufficient to produce EPA and DHA from PUFA precursors to sustain fast growth (Carvalho *et al*., 2018).

EPA is a good substrate for some cyclooxygenases and lipoxygenases, being precursor of proteinoids and leukotrienes, respectively. Its competition with ARA for these enzymes allow it to be an essential regulator of eicosanoid synthesis (Izquierdo, 2005).

Several studies have demonstrated a higher biological value (e.g. enhancing growth performance, feed efficiency and immunity) for DHA than for EPA during first feeding in marine fish species such as red seabream (*Pagrus major*) (Watanabe *et al.,* 1989**)**, gilthead seabream (Koven *et al.,* 1993; Rodríguez *et al.,* 1997), turbot (Reitan *et al.,* 1994), striped jack (*Pseudocaranx dentex*) (Takeuchi *et al*., 1992), and meagre (Campoverde and Estevez, 2017; El Kertaoui *et al.,* 2015). Moreover, besides providing adequate levels of EPA and DHA to meet requirements, the ratio of these n-3 LC-PUFA in feed formulations for fast-growing stages of fish has also been demonstrated to exert significant influences on a range of physiological processes in marine fish (Dantagnan *et al.,* 2010; Ibeas *et al.,* 1997; Magalhães *et al.,* 2020; Rodríguez *et al.,* 1997; Trushenski *et al.,* 2012; Wu *et al.,* 2002). Nevertheless, its importance was relatively neglected compared to the total amount of n-3 LC-PUFA (Kim *et al.,* 2002; Lee *et al.,* 2003; Sargent *et al.,* 1999; Skalli and Robin, 2004).

DHA, EPA and ARA are the main LC-PUFA presented in FM and FO, and are of pivotal importance for fish, particularly marine species (Tocher, 2003). As marine ecosystems are naturally rich in LC-PUFA (Sargent, 1995), adaptation to high dietary input of LC-PUFA in marine fish has been postulated as the evolutionary driver accounting for the loss of LC-PUFA biosynthetic capability in these species (NRC, 2011).

1.5.2. Fatty acid requirements in teleost fish

The environment in which fish evolved has conditioned the type and availability of feed which has led to the determination of essential nutrients in these organisms, in particular LC-PUFA (Sargent *et al.,* 2002). Carnivorous marine fish consume smaller fish rich in EPA and DHA, derived from phytoplankton via zooplankton and, consequently, have no evolutionary pressure to convert dietary intake of α-linolenic acid (18:3n-3, LNA) to eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) (Sargent *et al.,* 2002; Tocher, 2010; Torrecillas *et al.,* 2018).

All vertebrates have the ability to desaturate the palmitic acid (16:0) and the steric acid (18:0) to palmitoleic acid (16:1n-7) and oleic acid (OA; 18:1n-9), respectively, through the Δ9 fatty acid desaturase enzyme (Tocher, 2003). However, unlike freshwater species, marine fish, including European seabass, gilthead seabream and meagre, are unable or have restricted ability

to synthetize *de novo* n-3 (20:5n-3 + 22:6n-3) and n-6 (20:4n-6 + 22:5n-6) LC-PUFA from their precursors, α-linolenic acid (LNA; 18:3n-3) and linoleic acid (LA; 18:2n-6), respectively (Glencross, 2009; Izquierdo, 2005; Mourente *et al.,* 2005; Mourente and Tocher, 1994; Sargent *et al.,* 1995; Tocher, 2010) (**Figure 1.2**). This is due to a low expression or deficiency of the enzymes Δ5 or Δ6 desaturases and elongases, required for the elongation and desaturation of the precursors LNA and LA (Izquierdo, 2005; Khalil *et al.,* 2018; Sargent *et al.,* 1995; Tocher, 2010). Therefore, DHA, EPA and ARA are considered essential fatty acids for marine fish and should be included in the diet in adequate levels to fulfil the requirements for normal growth, organ and tissue development and functioning, stress resistance and survival (Glencross, 2009; Izquierdo *et al.,* 2013; Sargent *et al.,* 1995).

EFA requirements change throughout the life cycle of the fish, being, generally, higher in early life stages, compared with juveniles and sub-adults (Tocher, 2010). EFA requirements of fish also vary among species (Watanabe, 1982), being the optimal level around of 0.50–2.00% of dry matter for EPA and DHA for marine fish (Craig and Helfrich, 2009). Furthermore, environmental conditions such as temperature, salinity and light may influence EFA requirements since these factors affect lipid composition of fish tissue (Izquierdo, 2005).

In fact, it has been observed an increase of LC-PUFA retention when water temperature is decreased, and higher contents of LC-PUFA (particularly DHA and ARA) in fish tissues reared at increased salinity. In addition, an increase in the number of double bonds and carbons in the FA enhanced the fluidity of the membranes which would explain the increase of LC-PUFA in fish that inhabit colder waters (Izquierdo, 2005).

n-3 LC-PUFA requirements have been studied for juveniles of many marine species (Tocher, 2010). However, specific EFA requirements in practical diets for meagre juveniles are still undetermined. Only recently, the requirement of n-3 LC-PUFA in practical diets for meagre fingerlings was recommended at 2.10% of the diet (% dry matter basis), for optimal growth performance and feed conversion ratios (Carvalho *et al.,* 2018).

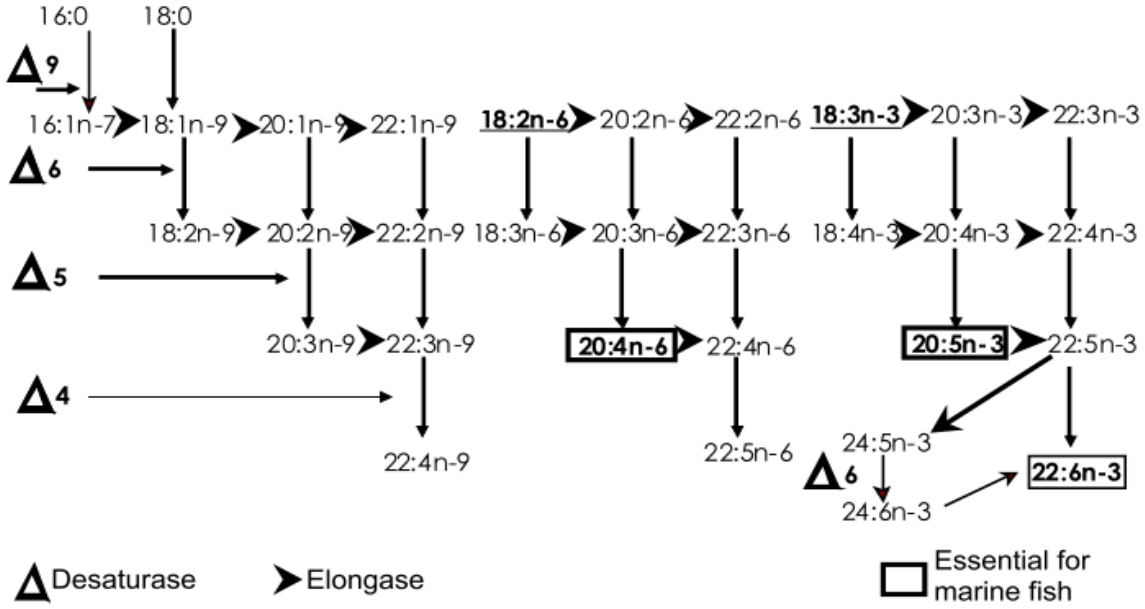

Figure 1.2. Essential fatty acids synthesis pathways present in some fish species. *In* Izquierdo (2005).

1.5.3. Metabolic effects of essential fatty acids deficiency

As previously mentioned, marine fish are unable or have restricted ability to synthesize *de novo* EPA and DHA, so it is crucial incorporate these EFA in the diet for the normal development and growth (Webster and Lovell, 1990).

In marine fish, the ratios 18:1n-9/n-3 LC-PUFA has been considered as a biochemical indicator of EFA-deficiency (Fujii *et al*., 1976; Izquierdo, 1996; Kalogeropoulos *et al*., 1992). The most evident signs of EFA deficiencies in fish are reduced growth and survival (Tacon, 1996). Nevertheless, other pathologies are commonly observed: swimming disorders, skin lesions, fin erosion, shock syndrome, severe lipid infiltration (particularly in lipid storage tissues, for instance the liver), lordosis, bleeding and disaggregation of gill epithelia, increased sensibility to stressful situations, immune-deficiency, increased cortisol levels, and decreased reproductive capacity (Glencross, 2009; Izquierdo, 2005; Tocher, 2010).

Liver is recognized as a key organ in intermediary metabolism, playing a crucial role in the regulation of lipid metabolism, particularly in the synthesis and β-oxidation/degradation of FA (Caballero *et al.,* 1999, 2004). According to Henderson and Sargent (1985), liver is considered as the primary place of lipogenesis in fish, maybe because the activity of lipogenic enzymes is higher in hepatocytes compared with other tissues. In fact, since several regulatory enzymes of lipid metabolism pathways show varying affinities for the different FA, an

imbalance of these nutrients could affect the functionality and morphology of the liver (Caballero *et al.,* 2004). In addition, in certain species such as gilthead seabream, European seabass, and meagre (Estévez *et al*., 2011), liver functions as the main organ for lipid-storage, frequently in the form of TG (Kaushik, 1997). Therefore, excess in dietary lipid or energy, which exceeds liver storage capacity (Castro *et al*., 2015) or inadequate dietary FA composition (Ribeiro *et al*., 2015), particularly an excess of C18:1 (Spisni *et al*., 1998), results in large synthesis and deposition of TG in vacuoles, and pathological alterations may appear, for instance steatosis (Caballero *et al.,* 2004; Montero and Izquierdo, 2010).

Liver steatosis is characterized by an abnormal lipid retention in cells that can, in extreme cases, led to fish death (Caballero *et al.,* 2004), and has been frequently observed associated with nutritional imbalances in cultured fish (Tacon, 1996). In hepatic steatosis, excess TG is usually stored in lipid droplets sized between 50 nm and 1 μm within the hepatocyte (Sahini and Borlak, 2014). Both number and size of lipid droplets, typically present in the cytosol, are well-established histological biomarkers of fatty liver (Willebrords *et al*., 2015).

Interestingly, some n-3 LC-PUFA with anti-inflammatory properties, such as EPA, DHA and LNA, seem to reduce the degree of fat accumulation in liver (Olsvik *et al*., 2019). For example, supplementation of EPA can inhibit hepatic steatosis in obese mice (Inoue-Yamauchi *et al*., 2018). Similar findings have been reported for fish; Liland *et al*. (2013, 2015) proved that high levels of EPA and DHA in the diet protect against nutrient-derived induction of fatty liver in Atlantic salmon (*Salmo salar*).

1.6. Alternative lipid sources to fish oil

Marine FO, rich in LC-PUFA, have traditionally been used as the main lipid constituent in fish feed (Emre *et al.,* 2016; Kjær *et al.,* 2008). However, the continuous expansion of aquaculture production, accompanied by fast growth of aquafeed production led to a shortage of marine sources of feed, such as FM and FO, forcing the industry to explore alternative and sustainable lipid sources for use in marine fish diets (Benedito-Palos *et al.,* 2008; Emre *et al.,* 2016; Gatlin III *et al.,* 2007; Hardy, 2010; Moura *et al.,* 2018; Moura *et al.,* 2019; Mourente *et al.,* 2005; Oliva-Teles *et al.,* 2015). Initially, the response was determined by market forces that drove up prices of FO and FM, which naturally balanced demand to the existing supply (Tocher *et al.,* 2019). Therefore, as aquaculture and the required feed volumes expanded, the limited amount of FO and FM was spread thinner and thinner across the feeds, with FO being increasingly replaced by vegetable oils (VO) (Turchini *et al.,* 2009).

Plant feedstuffs are at present the most economically and environmentally sound alternatives, often containing high levels of n-6 PUFA (Moura *et al.,* 2019; Tocher *et al.,* 2019). However, while FO are rich in n-3 LC-PUFA, particularly EPA and DHA, VO are rich in C_{18} n-6 and n-9 FA (Kjær *et al.,* 2008). Consequently, the replacement of FM and FO by vegetable sources is generally reflected in the composition of the fish tissue, with a decrease in n-3 LC-PUFA and an increase in the C_{18} FA (Carvalho *et al.,* 2019; Kjær *et al.,* 2008). This means that the alteration of the dietary FA profile, for instance through the inclusion of VO in aquafeeds, may modify the contents and proportions between different EFA and, consequently, affect fish growth (Caballero *et al.,* 2004; Carvalho *et al.,* 2019). In this context, EPA and DHA are two of the first limiting FA in plant-based diets, which has been an issue in aquaculture for over 20 years (Tocher *et al.,* 2019).

Nowadays, the aquaculture industry can incorporate considerable amounts of vegetable meals and oils in aquafeeds without compromise growth rates of several target species, including meagre (Carvalho *et al.,* 2018; Ribeiro *et al.,* 2015), being the soybean meal (SBM) the most plant used in fish diets, followed by other oleaginous such as rapeseed and sunflower meal (Couto *et al.,* 2016). In salmonids, the use of VO to replace most of the dietary FO is also currently feasible in practical aquafeeds without loss of growth performance (Bell *et al.,* 2003; Bransden *et al.,* 2003). Nevertheless, EFA requirements differs between species (Benedito-Palos *et al.,* 2008; Mourente *et al.,* 2005). Therefore, LA and α-linolenic acid (LNA) can satisfy the EFA requirements of freshwater fish, whereas marine fish require n-3 and n-6 LC-PUFA for optimal growth and health (Sargent *et al.,* 1995, 1999; Watanabe, 1982). Supporting this, the conversion of LA to ARA, and LNA to EPA and DHA is well established for many freshwater species of fish, but the marine fish species including European seabass (Mourente *et al.,* 2005), gilthead seabream (Mourente and Tocher, 1994) and meagre so far cannot perform the conversions at a significant or appreciable rate (Sargent *et al.,* 2002). In this sense, krill oil (KO) extracted from Antarctic krill (*Euphausia superba*) appeared as an alternative lipid source for aquafeeds, since is a rich source of n-3 LC-PUFA. Moreover, according to Ulven and Holven (2015), krill is by far the most dominant member of the Antarctic zooplankton community, and from Earth, in terms of biomass.

2. THESIS AIMS AND HYPOTHESES

Meagre (*Argyrosomus regius*), being a carnivorous fish, has a high demand for protein and lipids, with fishmeal (FM) and fish oil (FO) as the main ingredients included in its diet. FO that represents the main lipid source of commercial feed for meagre has been over-exploited over the last two decades, hence krill oil (KO) appeared as an alternative source of lipids. KO is known to be a rich source of n-3 LC-PUFA (namely, EPA and DHA), which is crucial for the normal growth of marine fish. In this sense, the present study was designed to test the hypothesis that the replacement of FO by KO in plant-based diets will not influence the normal growth of meagre juveniles. It should provide insights to understand how growth performance, feed utilization, whole-body composition, liver and muscle fatty acid profile, and liver structure are affected by the replacement of lipid source.

Specific objectives:

- Analyse the effect of FO replacement by KO on EPA and DHA levels;
- Assess the impact of EPA and DHA levels on fish growth, feed utilization, whole-body composition, muscle and liver fatty acid composition, and liver morphological alterations;
- Establish suitability of KO as the main lipid source for use in commercial diets for meagre juveniles.

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3. MATERIALS AND METHODS

3.1. Experimental conditions

The experiment was conducted according to the European guidelines on protection of animal used for scientific purposes (Directive 2010/63/EU of European Parliament and of the European Union Council), and all fish manipulations were directed and carried out by trained scientists. The nutrition trial was performed at the Aquaculture Research Station (EPPO) of the Portuguese Institute for the Sea and Atmosphere (IPMA), located in Olhão, Portugal, with meagre (*Argyrosomus regius*) juveniles bred in captivity at the station.

The experiment was carried out in a thermoregulated aquaculture system, with seawater derived from the Ria Formosa's reserve after being filtered and treated and distributed into rectangular fiberglass tanks of 250 l capacity (**Figure 3.1**). Six homogeneous groups of 60 fish (mean initial weight of 13.20 ± 0.10 g; in a density no higher than 5.28 kg/m^3) were randomly distributed into each tank and the experimental diets were randomly assigned in triplicate.

During the nutrition trial, fish were hand-fed to apparent satiation (*ad libitum*), five times a day: three in the morning (9.30 a.m., 10.30 a.m. and 12 a.m.) and two in the afternoon (2.30 p.m. and 5.30 p.m.), for a period of 71 days (from October 1 to December 10, 2018).

Daily all tanks were cleaned twice: in the morning, before the first meal, and in the afternoon, 30 min before the last meal. In addition, 30 min before the first afternoon meal, a purge was made to replace half the amount of water in the tanks.

Over the course of 71 days nutritional study, water-flow was set at about 7.20 l/min, salinity was 35 ± 0.70 ppt and dissolved oxygen was kept near water saturation (5.70 ± 0.20 mg/l) by air stones and constant seawater renewal in each tank. Fish were subjected to a photoperiod of 14 h day and 10 h dark. It was essential to employ a heating system to keep water temperature at 21.50 ± 1.60 °C. Thus, water temperature variation along the experiment was reduced, ensuring normal fish feeding.

Water parameters, feed consumption and mortality were measured and recorded daily, until the end of the trial, with particular concern about the water temperature and salinity, ensuring the equality between replicates.

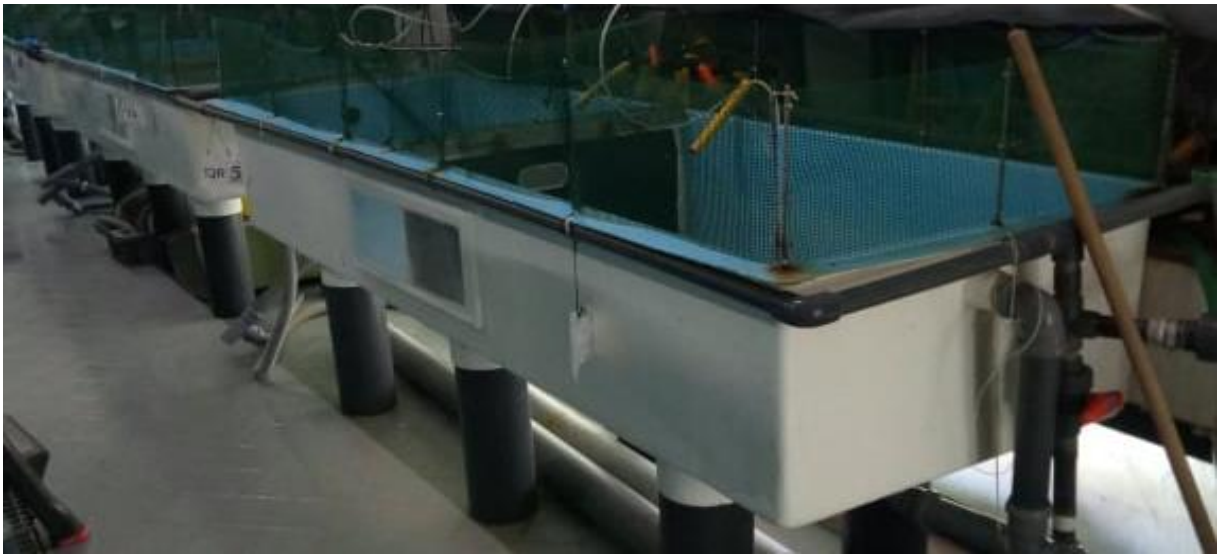

Figure 3.1. Experimental setup composed by six rectangular fiberglass tanks (120 cm side, 70 cm depth and 55 cm water column) equipped with filters and airstones, to hold meagre (*A. regius*) juveniles. **Source:** Aquaculture Research Station (EPPO) of the Portuguese Institute for the Sea and Atmosphere (IPMA), 2018.

3.2. Experimental diets

Two isonitrogenous (61.61 ± 0.07% crude protein), and isocaloric (24.14 ± 0.05 kJ/g gross energy) diets were formulated with fish oil (FO) or krill oil (KO) as the main lipid sources (**Table 3.1**). As protein sources were used: casein, fish gelatin, soy protein concentrate, pea protein concentrate, and wheat gluten.

Carvalho *et al.* (2018) proposed the requirement of n-3 LC-PUFA for young meagre to at least 2.10% in the diet. In this way, FO diet, containing 1.98% n-3 LC-PUFA (dry matter, DM), was considered the control diet, instead of KO diet deprived of FO in its formulation.

Palmitic acid (16:0) and oleic acid (18:1n-9) were the most dominant SFA and MUFA, respectively, in both diets (FO and KO). Moreover, KO diet had higher level of ∑SFA and ∑MUFA, whereas FO diet had higher level of n-3 LC-PUFA. Between experimental diets, FO diet had the highest level of eicosapentaenoic acid (EPA, 1.18% in DM) and moderate levels of docosahexaenoic acid (DHA, 0.36% in DM), whereas KO diet had the lowest levels of EPA (0.60% in DM) and DHA (0.19% in DM) (**Table 3.2**).

During the first three days of the experiment, the fish were fed with a commercial diet – Gemma Ratio 1.8 – for acclimatization. From the 4th day onwards, experimental diets with 2 mm of diameter were offered to fish, and after 30 days of the trial, the transition to a 3 mm diet was started.

Table 3.1. Ingredients (%) and proximal composition (%, dry matter) of the experimental diets (Fish Oil-FO and Krill Oil-KO) for meagre (*A. regius*) juveniles.

	Diet	
	FO	KO
Ingredients (%)		
Casein	5.00	5.00
Fish gelatin	6.00	6.00
Soy protein concentrate	30.00	30.00
Pea protein concentrate	15.00	15.00
Wheat gluten	15.00	15.00
Potato starch gelatinised	5.60	5.60
Fish oil	7.00	
Krill oil		9.12
Rapeseed oil	4.20	2.08
Linseed oil	0.70	0.70
Palm oil	2.10	2.10
Rapeseed lecithin	2.00	2.00
Vitamin and mineral premix	1.00	1.00
Vitamin C 35%	0.10	0.10
Vitamin E 50%	0.10	0.10
Betaine HCl	1.00	1.00
Antioxidant	0.30	0.30
Monoammonium phosphate	2.00	2.00
L-Lysine	0.10	0.10
L-Tryptophan	0.20	0.20
DL-Methionine	0.60	0.60
L-Taurine	2.00	2.00
Proximal composition (% DM)		
Dry matter	91.46	93.75
Protein	61.66	61.56
Lipids	16.61	17.28
Ash	6.20	4.75
Gross energy (kJ/g)	24.10	24.17

Table 3.2. Composition in main fatty acids (mg/g total fatty acids) of the experimental diets (Fish Oil-FO and Krill Oil-KO) for meagre (*A. regius*) juveniles.

Fatty acid (mg/g)	Diet	
	FO	KO
14:0	41	103
16:0	168	205
18:0	31	23
18:1n-9	313	281
18:2n-6	133	104
18:3n-3	53	50
20:4n-6	4	
20:5n-3	71	21
22:6n-3	36	11
Σ SFA	243	335
Σ MUFA	394	428
Σ n-3	184	113
Σ n-3 LC-PUFA[b]	119	35
Σ n-6 LC-PUFA[a]	8	2
DHA/EPA	0.51	0.52
EPA + DHA	107	32
Σ n-3 LC-PUFA (% DM)[c]	1.98	0.60
EPA (% DM)[d]	1.18	0.36
DHA (% DM)[e]	0.60	0.19

Fatty acids with missing values were those whose peak were detected but contents were lower than 0.5 mg/g.

[a] n-3 LC-PUFA: 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3.

[b] n-6 LC-PUFA: 20:2n-6; 20:3n-6; 20:4n-6; 22:2n-6.

[c] *Σ* n-3 LC-PUFA (% total FA) x dietary lipids (% DM).

[d] EPA (% total FA) x dietary lipids (% DM).

[e] DHA (% total FA) x dietary lipids (% DM).

DM dry matter

3.3. Sampling

Initially, a pool of 10 fish from the initial stock was sampled for proximal composition and the remaining fish (n = 180, per treatment) were individually measured and weighed in pools of 10, to obtain a biomass (**Figure 3.2 A** and **B**). At the end of the 71-days trial, all fish were individually measured and weighed (**Figure 3.3 A** and **B**). Five fish per tank (n =15, per treatment) were randomly collected and stored at -20 ºC to determine proximal composition, and six fish per tank (n = 18, per treatment) were collected for tissue sampling. Liver weight was recorded for hepatosomatic index (HSI) determination, before being dissected into two sections, and frozen at -80 ºC for histological and FA profile analysis, respectively (**Figure 3.4 A**). Portions of dorsal muscle (white muscle) were sampled, frozen in liquid nitrogen and stored at -80 ºC for FA profile analysis (**Figure 3.4 B**).

All samplings were performed in the morning, following overnight fasting (24 h after the last daily meal), and all fish were sedated with 100 ppm 2-phenoxyetanol (Barata *et al.*, 2016), whereas for the remaining analytical procedures, fish were sacrificed by sedated with 700 ppm 2-phenoxyetanol and decapitation at the junction of skull and first vertebra with a scalpel.

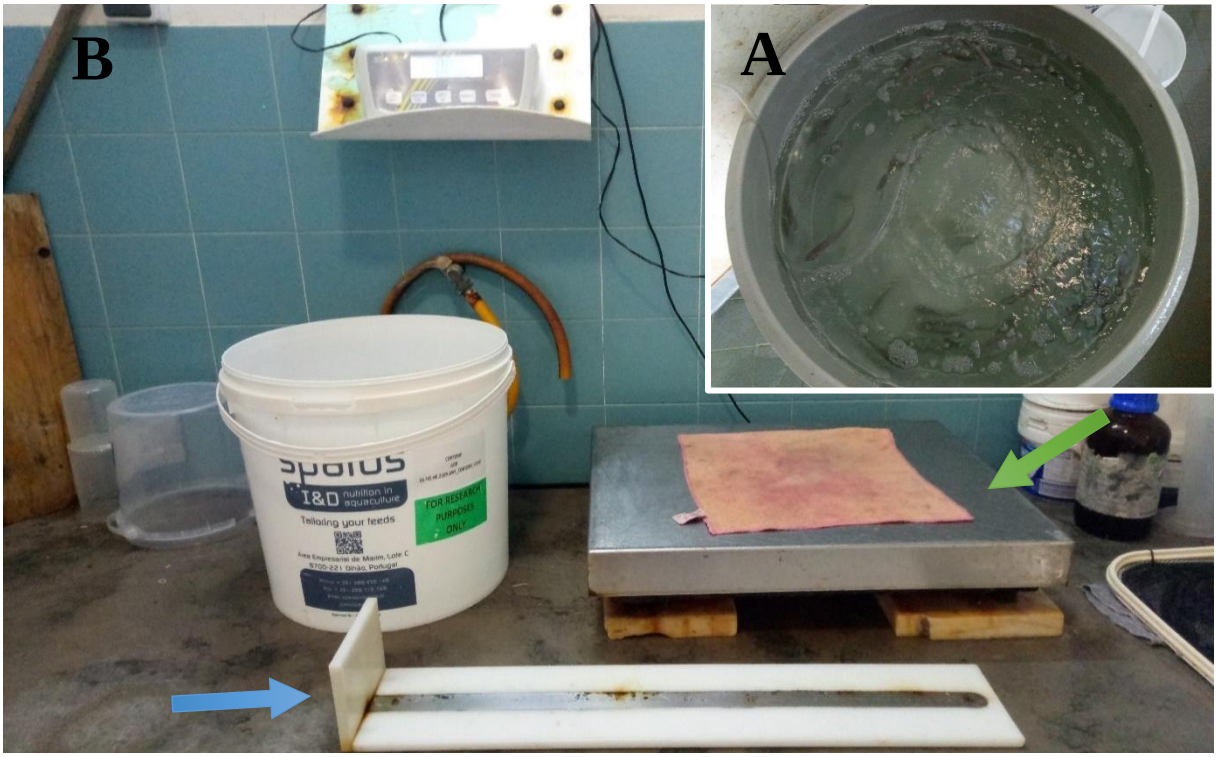

Figure 3.2. Experimental setup to anesthetize meagre (*A. regius*) juveniles, depicting (**A**) one 200 l containers with anesthesia and (**B**) the sampling station for subsequent initial measurement and weight. The length (in cm) was measured with an ichthyometer (indicated by the blue arrow), and weight (in g) was measured in grams using a scale (Ken and Sohn Gmbh, model ITB 35K1IP) with sensitivity of one gram (indicated by the green arrow). **Source:** Aquaculture Research Station (EPPO) of the Portuguese Institute for the Sea and Atmosphere (IPMA), 2018.

Figure 3.3. Sampling setup for (**A**) biometric and (**B**) anthropometric measurements of meagre (*A. regius*) juveniles at the final sampling. **Source:** Aquaculture Research Station (EPPO) of the Portuguese Institute for the Sea and Atmosphere (IPMA), 2018.

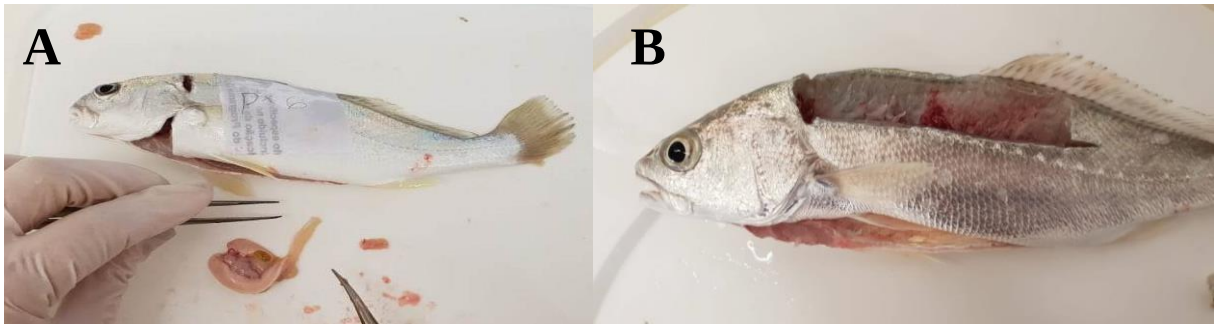

Figure 3.4. Performing of (**A**) liver dissection into two sections for histological and fatty acid profiles analysis and (**B**) sampling of dorsal muscle for fatty acid analysis of meagre (*A. regius*) juveniles. **Source:** Aquaculture Research Station (EPPO) of the Portuguese Institute for the Sea and Atmosphere (IPMA), 2018.

Feed consumption/intake was recorded daily by subtracting uneaten feed from total feed offered. At the end of every meal, all excess feed deposited at the bottom of each tank was collected and weighed. To remove water weight from the wet diets after collecting it from the tanks, it was necessary to calculate the hydration factor that represents how much weight a dry diet gains after being on the tank 21.50 ºC for four hours (**Table 3.3**). For this, was used a known weight of each dry diet. Applying the correction factor to the daily registered values of feed consumption, subtracted by the non-ingested feed, would give the daily feed consumption per tank.

Table 3.3. Hydration factor calculated for each experimental diet (Fish Oil-FO and Krill Oil-KO).

Feed size	2mm		3mm	
Diet	FO	KO	FO	KO
Hydration Factor	2.69 ± 0.04	3.05 ± 0.12	2.48 ± 0.03	2.54 ± 0.10

Values are presented as means ± SD.

3.4. Analytical methods

3.4.1. Proximal composition analysis of diets and fish

All chemical analyses were carried out with analytical pseudo replicates and following the methodology described by AOAC (2006). The collected whole fish were ground and pooled. Dry matter (DM) was determined after drying the samples at 105 ºC for 24 h. Frozen body samples were freeze-dried before carrying the chemical analysis. The ash content of feed and fish whole body were analysed by combustion (550ºC during 5 h) in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany). Crude protein (N × 6.25) was determined by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection with a Leco nitrogen analyser (Model FP-528; Leco Corporation, USA). Crude lipid content was determined by petroleum ether extraction (40–60 ºC) using a Soxtec™ 2055 Fat Extraction System (Foss, Denmark), with prior acid hydrolysis with 8.3M HCl. Gross energy was quantified in an adiabatic bomb calorimeter (Werke C2000; IKA, Germany).

3.4.2. Growth performance and feed utilization

To evaluate the growth performance of meagre juveniles, Condition Factor (K), Specific Growth Rate (SGR), and Weight Gain (WG) were calculated according to the Equations (1)–(3), respectively. K was determined since it is a crude measure of the level of energy reserves (Goede and Barton, 1990) and fish health, and changes in this parameter may indicate alterations in nutritional status of the fish. SGR was based on the natural logarithm of body weight, since it is the most used parameter for fish growth rate expression and can be used to compare growth on a daily basis. This parameter is dependent on the final body weight and allow the comparisons of growth rates among groups with similar initial body weight. WG was calculated based on the initial and final body weight of each individual.

$$K = \frac{Body\ weight\ (g)}{Total\ lenght^3\ (cm)} \times 100 \quad (1)$$

$$SGR\ (\%\ /day) = \frac{\ln[Final\ Body\ Weight\ (g)] - ln\ [Initial\ Body\ Weight\ (g)]}{Experimental\ days\ (n)} \times 100 \quad (2)$$

$$WG\ (\%) = \frac{Final\ Body\ Weight\ (g) - \ Initial\ Body\ Weight\ (g)}{Initial\ Body\ Weight\ (g)} \times 100 \quad (3)$$

To assess the performance of feeds on fish growth, Feed Conversion Ratio (FCR) and Daily Feed Intake (DFI) were determined according to Equations (4) and (5), respectively.

FCR represents the amount of feed (in kg) required to produce 1 kg of farmed animal (round weight) (Ytrestøyl *et al.*, 2015), and was calculated based on feed consumption and weight gain. DFI has to be estimated to reduce production cost, and to avoid loss of feed and the pollution caused by uneaten feed (Sumagaysay, 1999). This parameter was calculated based on feed given, average body weight, and days of experiment.

$$FCR = \frac{Dry\ feed\ given\ (g) - Dry\ remaining\ feed\ recovered\ (g)}{Final\ Body\ Weight\ (g) - Initial\ Body\ weight\ (g)} \quad (4)$$

$$DFI\ (\%\ days^{-1}) = \frac{Dry\ feed\ given\ (g)}{Days \times \frac{Initial\ body\ weight\ (g) + Final\ body\ weight\ (g)}{2}} \times 100 \quad (5)$$

Hepatosomatic index (HSI) represents the percentage of liver mass in relation to body weight. This index allows to quantify the energy (glycogen) stock in the liver (Cyrino *et al*., 2000). Glycogen (one of the several ways to store the energy consumed by fish) is found, in large quantities, in fish liver tissues and muscle. High levels of HSI may indicate excess lipid deposition in the liver, as well as several pathologies associated to this organ. HSI was calculated according to Equation (6), based on liver and body weight.

$$HSI\ (\%) = \frac{Liver\ weight\ (g)}{Body\ weight\ (g)} \times 100 \quad (6)$$

3.4.3. Fatty acid profile analysis on diets, muscle, and liver

The FA composition was determined according to the procedure of Lepage and Roy (1986) modified by Cohen *et al*. (1988), and performed in the IPMA laboratories (Algés, Portugal). This method consists in the transesterification, in acidic medium, of FA methyl esters (FAME). The FA were transesterified using a mixture of acetylchloride and methanol (1:19, v/v) and 10 mg/ml of tricosanoic acid (23:0), as internal standard. FAME were analyzed throw injection of 2 µl into a gas chromatograph (GC; Varian Star CP-3800) equipped with an auto sampler and a flame ionization detector (FID) at 250ºC. The separation was performed in a polyethylene glycol capillary column DB-wax (0.25 mm internal diameter 30 m polar capillary precolumn × 0.25 µM layer thickness) and helium as carrier gas. Helium was used as carrier gas at a flow rate of 1 ml/min, to perform the separation in a capillary column DB-Wax (30 m length × 0.32 mm internal diameter; 0.25 µm film thickness; Hewlett Packard, Albertville, MN, USA) programmed at 180 ºC for 5 min, raised to 220 ºC at 4 ºC/min, and maintained at 220 ºC for 25 min, with the detector and the split injector (100:1) at 250 ºC. FAME identification was

carried out by comparing their retention time with those of Sigma standards. The quantification of the different FA as a function of its peak area, and the peak area of internal standard (23: 0) of the heavy mass of the sample and the total area of FA in the sample using Varian software. FA present in the sample were identified by comparing the retention time obtained for each one and that of the standard pattern Sigma-Aldrich (Supelco Analytical). The accuracy of this methodology was assessed by testing certified reference materials in the same conditions as the samples, and the results obtained in this study showed to agree with the certified values. Complete analysis of FA composition of diets and meagre liver and muscle are exhibited in **Appendix I, II** and **III**, respectively.

3.4.4. Histological analysis

For histological analysis, samples of liver were collected randomly from the coelomic cavity of six fish (n = 18, per each treatment) of almost uniform size, at the end of the feeding trial. Fish were killed by sedated with 700 ppm and sacrificed by decapitation at the junction of skull and first vertebra with a scalpel. Liver samples collected from the upper lobe of the liver were placed into histological cassettes properly identified and fixed with Bouin's solution. Afterwards, all samples were washed with running water for 90 min and stored in 70% ethanol for further histological analysis. Then, the histological cassettes were placed in the tissue processor (Leica Processor TP 1020) to be dehydrated in a graded series of alcohol (80, 96 and 99%). This gradual dehydration was performed to remove the water excess from the tissues maintaining its integrity. Subsequently, successive immersions in xylol allowed the alcohol present in the tissues to be replaced by this solvent, facilitating the impregnation of the tissues with liquid paraffin.

The following step involved the inclusion of samples in liquid paraffin using a paraffin dispenser (Leica EG 1140 H) to provide support to the tissue. Tissues were placed and oriented in a histology mould, covered with liquid paraffin, and then covered with the cassette and placed in a cooling plate. Afterwards, tissues sections of 7 μm thickness were prepared using a Leica® RM-2155 automatic microtome (Leica, Vienna, Austria) and a circular water-bath set at about 35 to 38 °C to stretch the cuts and improve adherence to the microscopy slide. After tissue sectioning, slides were placed in a chamber at 35 ºC, for 24h. Following, slides were stained with haematoxylin and eosin (H&E) according to the embedding process presented in Appendix IV.

This staining procedure is the most widely used in histology and allows localization of nuclei by haematoxylin (blue), and extracellular proteins by eosin (pink) (Alturkistani *et al*.,

2015). This method is of utmost importance as it allows to observe a wide range of normal and abnormal cellular components and tissues (Pichat *et al*., 2018).

Finally, once dried at room temperature, all stained preparations were mounted permanently using DPX for posterior analysis under light microscope (Nikon DS-L3) equipped with a digital camera.

Liver sections were examined for general abnormalities such as the presence of granulomas in hepatic tissue and the overall integrity of hepatocytes. The same sections were also used to measure the diameter of the hepatocyte through the images taken at 100 and 400x magnification, using the software NIS-Elements D. In total, 405 measurements per treatment were used to calculate average values. The criteria used for measurements and observations were based on a combination of the criteria previously reported by Fountoulaki *et al*. (2009) and Wassef *et al*. (2007).

3.5. Statistical analysis

All data are presented as mean ± standard deviation (SD) of treatment replicates (n = 3). Before statistical analysis, all data were checked for normality of distribution (Shapiro-Wilk) and homogeneity of variance (Levene's test). Data were analysed with one-way analysis of variance (ANOVA), to test for significant differences between the experimental diets (FO and KO), considering the lipid source as the independent variable. Significance level was set at 0.05. All statistical analysis were performed using SPSS v21 software package for Windows (IBM Corp., Armonk, NY, USA), and Excel was used to design graphs.

4. RESULTS

4.1. Growth performance and feed utilization

During the feeding trial, no external damage or abnormal behaviour were observed in both fish oil (FO) and krill oil (KO) treatments. All the experimental feeds were well accepted by meagre (*Argyrosomus regius*) juveniles, and all fish promptly adapted to the experimental system, regardless of experimental diet. Survival rate of *A. regius* juveniles was mostly influenced by occasional escapes out of the tanks or intrinsic individual dominance, which is a common behaviour in this species.

The effect of decrease docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) levels as a result of FO replacement by KO became evident on fish growth after 71 days of feeding. At the end of the nutrition trial, fish fed the KO diet had lower ($p = 0.001$) final body weight (FBW) (53.37 ± 1.76 g/fish) and total length (FTL) (16.80 ± 0.23 cm) compared to FO diet fed fish (70.33 ± 2.89 g/fish and 18.85 ± 0.27 cm for FBW and FTL, respectively) (**Tables 4.1**).

The condition factor (K) of fish fed KO diet was higher ($p = 0.003$) than fish fed control diet (FO) (1.13 ± 0.02 and 1.05 ± 0.00, respectively). Both specific growth rate (SGR) and weight gain (WG) were lower ($p = 0.001$ and $p = 0.00$, respectively) in fish fed KO diet compared to those fish fed KO diet (**Table 4.1**).

Feed Conversion Ratio (FCR) showed significant differences between fish fed different diets. The highest ($p = 0.001$) value of FCR (i.e., the worst FCR) was obtained in fish fed KO diet. Regarding daily feed intake (DFI, %/day), fish fed KO diet showed higher ($p = 0$ 0.001) intakes (1.82 ± 0.05 %/day) than fish fed KO diet (1.49 ± 0.04 %/day) (**Table 4.1**).

The hepatosomatic index (HSI) achieved in fish fed KO diet was higher ($p = 0.000$) than those fish fed diet with FO as the alternative source of lipids (**Table 4.1**).

Table 4.1. Growth performance, feed utilization and hepatosomatic index of meagre (*A. regius*) juveniles after 71 days of feeding the experimental diets (Fish Oil-FO or Krill Oil-FO).

Parameters	Diet	
	FO	KO
Initial total length (cm)	10.76 ± 0.01	10.75 ± 0.15
Final total length (cm)	18.85 ± 0.27[a]	16.80 ± 0.23[b]
Initial body weight (g/fish)	13.17 ± 0.02	13.23 ± 0.11
Final body weight (g/fish)	70.33 ± 2.89[a]	53.37 ± 1.76[b]
Condition Factor	1.05 ± 0.00[b]	1.13 ± 0.02[a]
Specific Growth Rate (%/day)	2.36 ± 0.06[a]	1.93 ± 0.05[b]
Weight Gain (%)	433.93 ± 21.94[a]	294.44 ± 13.32[b]
Feed Conversion Ratio	0.77 ± 0.03[b]	1.09 ± 0.05[a]
Daily Feed Intake (%/day)	1.49 ± 0.04[b]	1.82 ± 0.05[a]
HSI (%)	1.64 ± 0.02[b]	2.86 ± 0.19[a]

Data are expressed as mean ± standard deviation (mean ± SD); n = 3, per each treatment. Different letters indicate significant differences ($p < 0.05$) between treatments.

4.2. Biochemical composition analysis

The effect of different levels of EPA and DHA on biochemical composition of the whole-body fish (% DM) are shown in **Table 4.2**. The dry matter, protein, lipid, and gross energy of meagre juveniles showed a growing trend in both dietary treatments – FO and KO diets. There were significant differences ($p = 0.047$) between whole-body dry matter content of fish fed different diets. Contrarily, protein ($p = 0.496$), lipid ($p = 0.365$), ash content ($p = 0.481$), and gross energy ($p = 0.242$) did not show any statistical differences between dietary treatments.

Table 4.2. Biochemical composition (% dry matter) of meagre (*A. regius*) juveniles fed different diets (Fish Oil-FO or Krill Oil-KO) for 71 days.

Body composition (%)	Initial	Diet	
		FO	KO
Dry matter	24.96	26.90 ± 0.69[a]	25.61 ± 0.51[b]
Protein	65.99	67.06 ± 0.39	67.40 ± 0.41
Lipids	14.84	21.86 ± 0.45	22.57 ± 1.36
Ash	16.79	11.53 ± 0.40	10.83 ± 1.09
Gross energy (kJ/g)	20.94	23.84 0.08	24.06 ± 0.33

Data are expressed as mean ± standard deviation (mean ± SD); n = 3, per each treatment. Different letters indicate significant differences ($p < 0.05$) between treatments.

4.3. Analysis of fatty acid profile in muscle and liver

Dietary EPA and DHA levels affected muscle lipid content after 71 days of feeding experimental diets. Muscle of fish fed FO and KO diets presented significantly less content of EPA than DHA ($p = 0.000$ and $p = 0.000$, respectively) in their composition (**Table 4.3**). Muscle of meagre juveniles fed KO diet exhibited lower mean values of EPA and DHA (1.58 ± 0.02 mg/g and 2.87 ± 0.04 mg/g, respectively) compared to muscle of individuals fed FO diet (3.44 ± 0.13 mg/g and 4.87 ± 0.03 mg/g of EPA and DHA, respectively). Similarly, ARA content in muscle of fish fed KO diet was significantly ($p = 0000$; 0.19 ± 0.01 mg/g) lower compared to the levels obtained in the muscle of fish fed FO diet (0.44 ± 0.00 mg/g.

Palmitic acid (16:0) was the most dominant SFA in the muscle of all fish, although there were no significant ($p = 0.578$) differences between treatments. Likewise, total SFA content in muscle was not significantly ($p = 0.947$) affected by the dietary lipid sources in the muscle of fish fed FO or KO diets.

Oleic acid (18:1n-9) was the most dominant MUFA in the muscle from all individuals. Nevertheless, the decrease of EPA and DHA in the KO diet affected the content of OA in muscle of fish fed that diet (9.79 ± 0.79 mg/g). Thus, fish fed FO diet exhibited significantly ($p = 0.036$) higher mean value of OA (11.44 ± 0.47 mg/g) in the muscle. Regarding LC-PUFA, LA (18:2n-6) was the dominant FA, although no significant ($p = 0.094$) differences were found between treatments.

Comparing muscle FA profile of fish fed KO diet with muscle of fish fed FO diet, fish fed KO diet presented significantly ($p = 0.000$; $p = 0.013$, respectively) lower levels of total n-3 and n-6 (7.09 ± 0.35 and 7.23 ± 0.35 mg/g, respectively), compared to those fish fed FO diet (11.46 ± 0.24 and 8.18 ± 0.15 mg/g of total n-3 and n-6, respectively) (**Table 4.3**).

Table 4.3. Composition in main fatty acids (mg/g total fatty acids) in muscle of meagre (*A. regius*) juveniles fed different diets (Fish Oil-FO or Krill Oil-KO) for 71 days.

Fatty acid (mg/g)	Diet	
	FO	KO
16:0	7.07 ± 0.25	6.86 ± 0.53
18:0	2.58 ± 0.08	2.45 ± 0.14
18:1n-9 (OA)	11.44 ± 0.47[a]	9.79 ± 0.79[b]
18:2n-6 (LA)	7.40 ± 0.17	6.92 ± 0.34
18:3n-3 (LNA)	1.57 ± 0.07	1.55 ± 0.12
20:4n-6 (ARA)	0.44 ± 0.00[a]	0.19 ± 0.01[b]
20:5n-3 (EPA)	3.44 ± 0.13[a]	1.58 ± 0.02[b]
22:6n-3 (DHA)	4.87 ± 0.03[a]	2.87 ± 0.04[b]
Σ SFA	10.92 ± 0.37	10.96 ± 0.89
Σ MUFA	14.34 ± 0.60	13.82 ± 1.08
Σ n-3	11.46 ± 0.24[a]	7.09 ± 0.35[b]
Σ n-6	8.18 ± 0.15[a]	7.23 ± 0.35[b]
n-3/n-6	1.40 ± 0.01[a]	0.99 ± 0.03[b]
EPA + DHA	8.31 ± 0.12[a]	4.45 ± 0.03[b]

Data are expressed as mean ± standard deviation (mean ± SD); n = 3, per each treatment. Different letters indicate significant differences ($p < 0.05$) between treatments.

Liver FA composition clearly reflected the dietary FA patterns and, thus, the lower levels of EPA and DHA in the diet (KO) decreased (up 2-fold) the levels of these n-3 LC-PUFA in the hepatic tissue (**Table 4.4**). Liver of fish fed FO diet showed significantly ($p = 0.000$; $p = 0.000$, respectively) higher (up to 5-fold) EPA and DHA than liver of fish fed KO diet. Likewise, ARA content in liver of fish fed KO diet showed significantly ($p = 0000$) lower levels than liver of fish fed FO diet.

As in muscle, 16:0 was the most dominant SFA in the liver of all meagre juveniles, although there were no significant ($p = 0.138$) differences, regardless of diet. Nevertheless, fish fed KO diet had significantly ($p = 0.014$) higher (190.88 ± 15.92 mg/g) content of SFA in liver than those fish fed FO diet (140.92 ± 13.36 mg/g). Concerning to stearic acid (18:0), this SFA was significantly ($p = 000$) higher in fish fed KO diet, compared to those fed FO diet.

As observed in muscle, among the MUFA, OA (18:1n-9) was the most dominant in liver, although there were no significant ($p = 0.826$) differences between dietary treatments. Additionally, between LC-PUFA, LA was the dominant FA, although no significant ($p = 0.096$) differences were found between fish fed different diets.

The concentration of total n-3 was significantly ($p = 0.003$) lower (26.12 ± 1.64 mg/g) in liver of fish fed KO diet compared to those fed FO diet (26.12 ± 1.64 mg/g). Although there were no significant ($p = 0.068$) differences, the total n-6 content had lower values in liver from fish fed KO diet than in fish fed FO diet (**Table 4.4**). **Appendixes II** and **III** contain details of the FA composition obtained from meagre muscle and liver, respectively.

Table 4.4. Composition in main fatty acids (mg/g total fatty acids) in liver of meagre (*A. regius*) juveniles fed different diets (Fish oil-FO or Krill Oil-KO), for 71 days.

Fatty acid (mg/g)	Diet	
	FO	KO
16:0	80.00 ± 10.24	95.94 ± 10.84
18:0	43.79 ± 0.57^{b}	69.47 ± 4.48^{a}
18:1n-9 (OA)	187.58 ± 22.32	184.47 ± 5.37
18:2n-6 (LA)	81.43 ± 14.77	62.68 ± 2.29
18:3n-3 (LNA)	17.76 ± 2.73	15.05 ± 0.69
20:4n-6 (ARA)	1.48 ± 0.18^{a}	0.03 ± 0.05^{b}
20:5n-3 (EPA)	16.43 ± 2.18^{a}	3.18 ± 0.16^{b}
22:6n-3 (DHA)	9.42 ± 1.64^{a}	1.97 ± 0.10^{b}
Σ SFA	140.92 ± 13.36^{b}	190.88 ± 15.92^{a}
Σ MUFA	242.11 ± 28.52	265.53 ± 8.09
Σ n-3	54.22 ± 7.21^{a}	26.12 ± 1.64^{b}
Σ n-6	85.71 ± 14.99	63.96 ± 2.46
n-3/n-6	0.64 ± 0.04^{a}	0.41 ± 0.01^{b}
EPA + DHA	25.85 ± 3.57^{a}	5.16 ± 0.26^{b}

Data are expressed as mean ± standard deviation (mean ± SD); n = 3, per each treatment. Different letters indicate significant differences ($p < 0.05$) between treatments.

4.4. Histological analysis

Histological analysis of cross-section of hepatic (**Figure 4.1**) tissue revealed that no signs of granulomatosis nor necrosis was found in meagre juveniles fed diets with different lipid source. Regardless of diet, all meagre juveniles exhibited alterations of the normal histological structure of liver, an apparent steatosis with intense lipid accumulation. The liver of fish fed KO diet with lower levels of EPA and DHA (21 and 11 mg/g of total FA, respectively) presented a severe steatosis (**Figure 4.1 KO and KO'**). Contrarily, the liver of fish fed FO diet with higher levels of EPA and DHA (71 and 36 mg/g of total FA, respectively) exhibited a lower degree of steatosis (**Figure 4.1 FO and FO'**). The integrity of the hepatocytes was also

affected; swelling and nuclei displacement from central position in the cell to the periphery were evident in all samples (**Figure 4.1**).

Concerning to diameter of the hepatocytes, this measure was significantly (p = 0.000) higher in fish fed KO diet (6.98 ± 0.93 µm) compared with the diameter of the hepatocytes of fish fed FO diet (4.86 ± 0.81 µm).

Hepatocytes exhibited a polygonal shape-like, disposed along sinusoids. Scattered regions of pancreatic tissue surrounded by hepatic tissue were also observed (**Figure 4.1**).

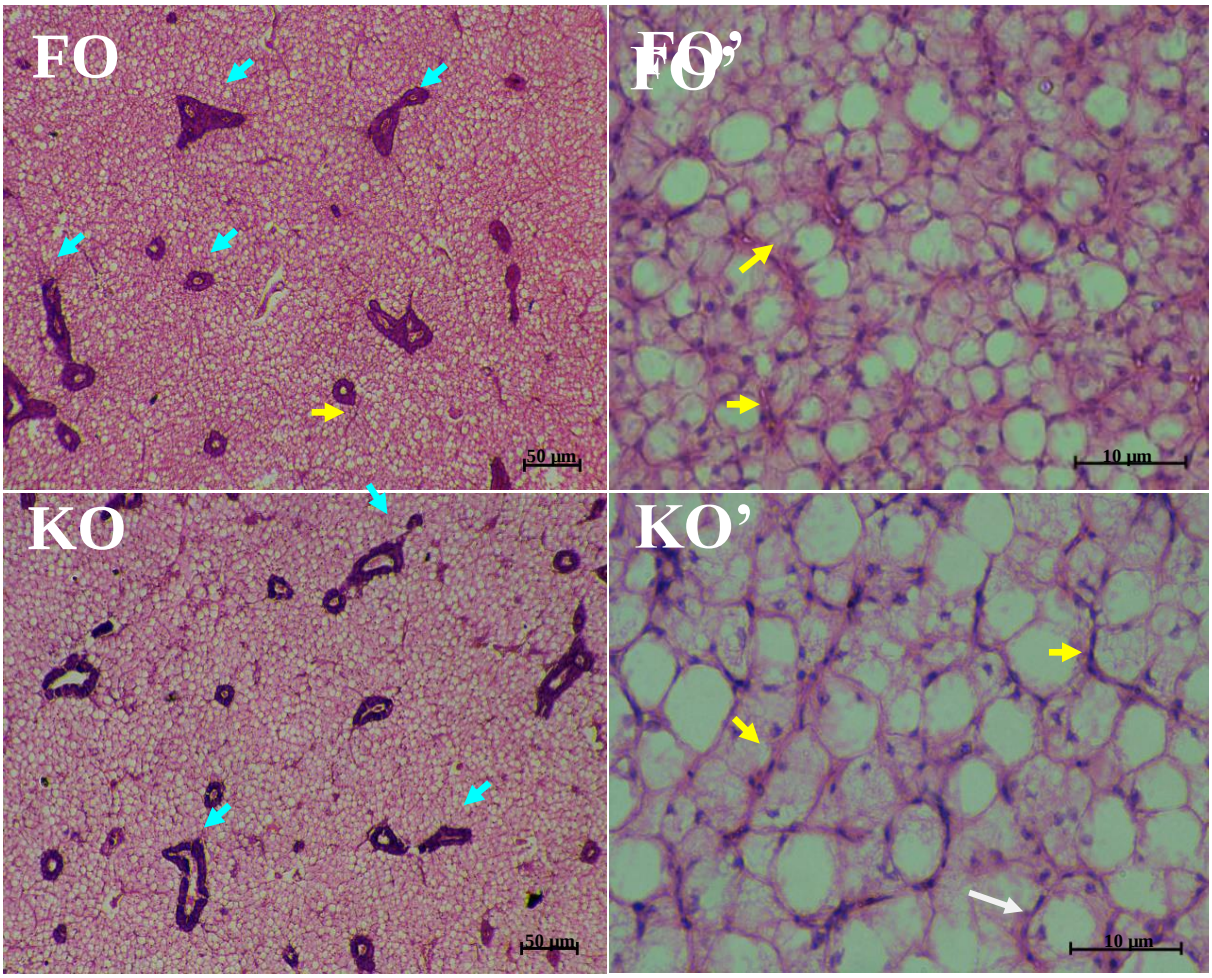

Figure 4.1. Light microphotographs of cross-sections in the liver of meagre (*A. regius*) fed for 71 days diets with different lipid source (Fish Oil–FO and Krill Oil–KO). (**FO**) liver morphology of fish fed FO diet, 100x magnification; (**FO'**) liver morphology of fish fed FO diet, 400x magnification; (**KO**) liver morphology of fish fed KO diet, 100x magnification; (**KO'**) liver morphology of fish fed KO diet, 400x magnification. Blue arrows indicates pancreatic tissue; yellow arrows signal the sinusoids; white arrow indicates nucleus laterally displaced; H&E staine; scale bars are 50 µm and 10 µm.

5. DISCUSSION

Meagre (*Argyrosomus regius*) is a species of marine carnivorous fish that have a high demand for protein and lipids, with FM and FO as the main ingredients included in its diet. Currently, replacement of higher percentages of FM by plant proteins can be achieved in diets containing FO as the main lipid source, without affecting growth performance. However, FO has been over-exploited, and KO appeared as an alternative source of n-3 LC-PUFA, since KO is known to be a rich source of these FA (namely, EPA and DHA), which is crucial for the normal growth of marine fish. The alteration of the dietary FA profile, for instance, through the replacement of FO by KO in plant-based diets may modify the contents of n-3 LC-PUFA, affecting fish growth.

In the present study, the effect of FO replacement by KO in plant-based diets, and consequently variation in EPA and DHA levels, were assessed on growth performance, feed utilization, hepatosomatic index, whole-body composition, and liver histology of meagre juveniles. To our knowledge, this is the first time the effect of FO replacement by KO in plant-based diets was assessed in meagre juveniles. Thus, no direct comparison was made between experimental results and similar research.

5.1. Effect of FO replacement by KO in plant-based diets

The replacement of FO by KO in the diet significantly decreased (more than three-fold) dietary levels of EPA and DHA.

FO and KO are both abundant in n-3 LC-PUFA, but the relative content of the main FA species and molecular composition are different. How these differences affect the lipid composition in animals fed with these oils has not been clarified (Skorve *et al.,* 2015). Contrarily to what was expected, KO diet showed lower values of EPA and DHA than FO diet. According to Ulven *et al*. (2011) and Salini *et al*. (2016) the DHA content of KO is similar to that of oily fish such as rainbow trout (*Salmo gairdneri*) and wild coho salmon (*Oncorhynchus kisutch*), but the EPA content is higher (Tou *et al.,* 2007).

5.2. Growth performance

Fish fed the control diet (FO), with higher levels of EPA and DHA, exhibited similar values of growth performance compared to other studies carried out with the same species (Kotzamanis *et al*., 2018; Saavedra *et al*., 2020), but higher than those reported by Estévez *et*

al. (2011), Emre *et al.* (2016), and Ruiz *et al*. (2019). This allowed to validate the rearing conditions applied in this experiment.

In the present trial, fish fed the KO diet, with reduced levels of EPA and DHA (0.36 and 0.19% in DM, respectively), showed significantly poorer growth performance when compared to fish fed FO diet with greater levels of those EFA (1.18 and 0.60% in DM of EPA and DHA, respectively). A tendency of reduced growth was also observed by Emre *et al*. (2016) in meagre juveniles when the dietary levels of EPA and DHA were reduced. Likewise, a similar pattern was observed for other marine species such as Japanese flounder *Paralichthys olivaceus* (Kim *et al*., 2002), and silvery-black porgy *Sparidentex hasta* (Hossain *et al*., 2017). Therefore, the reason why fish fed FO diet exhibited greater growth performance than those fed KO diet could be mainly attributed to the low dietary levels of EPA and DHA. Both n-3 LC-PUFA are crucial for the normal growth (Kjær *et al.*, 2008) since they play important roles in many biological functions [e.g., influencing the cell membrane fluidity, being second messenger and having antiviral, antifungal and antiparasitic actions (Das, 2006)]. Consequently, the deficiency of dietary n-3 LC-PUFA may lead to downgrade of the growth performance (Koueta *et al.*, 2002) and the immune capacity of marine fish (Zuo *et al*., 2012).

Additionally, marine fish generally exhibit a good growth when EPA and DHA are supplied at a combined rate of between 0.80% and 2.00% (NCR, 2011). In the present study, the combined absolute dietary level of EPA and DHA in FO and KO diets were 1.78% and 0.55%, respectively. Thus, contrarily to FO diet, KO diet is not within the reported requirement levels for marine fish species. Moreover, the level of total n-3 LC-PUFA in KO diet was 0.60%, whereas in the FO diet was 1.98%, which agrees with the value proposed by Carvalho *et al*. (2018) in meagre fingerlings – 2.10%.

The declining of fish growth, due to reduction of dietary EPA and DHA in this study, can be also related to the limited capacity of meagre to elongate and desaturate FA, as reported in other marine finfish such as juvenile gilthead seabream (Mourente and Tocher, 1993; Fountoulaki *et al*., 2009), juvenile black seabream *Acanthopagrus schlegeli* (Peng *et al*., 2008), rock bream, *Oplegnathus fasciatus* (Hong *et al*., 2016). Contrarily, Simões *et al*. (2017) did not observed any effect of reducing dietary levels of EPA and DHA on the growth performance of meagre juveniles.

5.3. Feed utilization

Considering the results for feed utilization, fish fed lower dietary levels of EPA and DHA (KO diet) exhibited higher feed conversion ratio (FCR) and daily feed intake (DFI) than fish fed higher levels of those EFA (FO diet). Nevertheless, fish fed KO showed a FCR value of 1.09 ± 0.05, meaning that fish were able to efficiently convert feed into growth. The FCR results from both experimental diets are a positive result, considering that the FCR values previously recorded for meagre juveniles were in the range of 3.13–0.70 (Chatzifotis *et al*., 2010, 2012; Emre *et al*., 2016; Estévez *et al*., 2011; FAO, 2020-2021; Khalil *et al*., 2019; Kotzamanis *et al*., 2018; Lozano *et al*., 2017; Mansour *et al*., 2017; Matias *et al*., 2020; Monfort, 2010; Ruiz *et al*., 2019; Saavedra *et al*., 2020; Velazco-Vargas *et al*., 2013). The results obtained in the present study agree with those achieved by Fountoulaki *et al.* (2009), who noticed a significant increase of FCR and feed intake (FI) values in gilthead seabream fed lower levels of EPA and DHA. Similar FCR results were found by Hossain *et al*. (2012) in juvenile silver pomfrets, *Pampus argenteus.* Contrarily, Carvalho *et al*. (2018) did not observe any effect of decreasing dietary levels of EPA and DHA in the FCR and FI values of meagre fingerlings. Likewise, Hossain *et al*. (2017) and Peng *et al*. (2008) did not notice any impact of decreasing dietary levels of EPA and DHA in FCR values when tested in other marine carnivorous fish such as silvery-black porgy and juvenile black seabream, respectively. On the other hand, juvenile cobia (*Rachycentron canadum*) (Trushenski *et al*., 2012), meagre (Emre *et al*., 2016), tilapia (*Oreochromis niloticus*) (Peng *et al*., 2016), and gilthead seabream (Torno *et al*., 2019) fed lower levels of EPA and DHA in the diet displayed significantly higher FCR values than those fed higher levels of those EFA, although no significant differences were observed in DFI values. Similarly, Takeuchi *et al*. (1990) and Kim *et al*. (2002) did not find any effect in the FI of red seabream and juvenile Japanese flounder, respectively, when decreasing dietary levels of EPA and DHA. Differently, Torrecillas *et al.* (2017), observed a significant decrease in FI of European seabass when fed reduced levels of EPA and DHA, like those used in this study. Trushenski *et al*. (2011) also noticed that juvenile cobia fed lower levels of EPA and DHA had a significant reduction of FI, although no significant differences were observed in FCR values.

In this study, the fact that fish fed lower dietary levels of EPA and DHA (KO diet) exhibited higher FCR and DFI than those fed higher levels of EFA can be related to the lack of n-3 LC-PUFA in the KO diet. This ω-3 deficiency may lead the fish to increase their DFI to reach the EFA levels required by this species for normal growth. Consequently, the same fish obtained an increase in FCR. A study by Trushenski *et al*. (2012) showed that cobia juveniles

fed high levels of DHA had improved FCR values, regardless of dietary levels of EPA. In addition, the authors noticed that fish fed the highest levels of EPA, but low levels of DHA, displayed the worst FCR values (Trushenski *et al.,* 2012). This suggests that DHA can be essential for normal fish growth, more than EPA.

5.4. Biochemical composition

Considering fish whole-body composition, it is affected by the composition of feed ingested (Orban *et al*., 2007). Thus, in the present study, the composition of meagre juveniles was analyzed since this parameter is known to be influenced by endogenous and exogenous factors. The levels of protein and ash are primarily related to the size of the fish (endogenous factor), while the lipid content is associated with exogenous factor such as diet (Shearer, 1994). In the current study, the results obtained showed that the protein content was not affected by the decreased dietary levels of EPA and DHA. This result was consistent with the study of Emre *et al*. (2016) and Carvalho *et al*. (2018), where no significant differences were observed in the protein content of meagre juveniles fed dietary levels of EPA and DHA like those used in the present study. This result agrees with other studies performed in other marine fish species such as black seabream (Peng *et al*., 2008), gilthead seabream juveniles (Benedito-Palos *et al.,* 2007; Fountoulaki *et al.,* 2009; Torno *et al*., 2019), and silvery-black porgy (Hossain *et al*., 2017), European seabass (Skalli and Robin, 2004) and Japanese seabass *Lateolabrax japonicus* (Xu *et al.* 2017), where no significant differences were observed in body protein content when providing diets with lower levels of EPA and DHA.

Like protein, there was no effect of reducing dietary levels of EPA and DHA in the ash content. This result agrees with those of Emre *et al*. (2016), Simões *et al*. (2017) and Carvalho *et al*. (2018), who did not report significant differences in ash content of meagre juveniles fed levels of EPA and DHA similar to those used in the present study. Likewise, Benedito-Palos *et al.* (2007) and Fountoulaki *et al*. (2009) did not report significant differences in ash content of gilthead seabream juveniles, when the levels of EPA and DHA were reduced in the diet. Similar results were obtained by Peng *et al.* (2008) in juvenile black seabream, Hossain *et al.* (2017) in silvery-black porgy, and Hossain *et al*. (2012) in silver promfrets. Contrary to these results, Torno *et al*. (2019) found a significant increase in ash content of gilthead seabream fed lower levels of EPA and DHA compared to those fed higher contents.

Also, the lipid content was not affected by the reduced levels of EPA and DHA in the diet. Similar, Benedito-Palos *et al.* (2007) and Fountoulaki *et al*. (2009) did not observe any effect of reducing dietary levels of EPA and DHA in gilthead seabream juveniles, as Peng *et*

al. (2008) in black seabream, and Emre *et al*. (2016), Simões *et al.* (2017) and Carvalho *et al*. (2018) in meagre juveniles. Contrasting results were reported by Hossain *et al*. (2017), who found significantly lowest lipid content in silvery-black porgy fed lower levels of EPA and DHA. This result agrees with a study by Hossain *et al*. (2012) in juvenile silver promfret and another by Torno *et al*. (2019) in gilthead seabream juveniles. Differently, Xu *et al*. (2017) noticed a significant increase of body lipid content in Japanese seabass fed lower levels of EPA and DHA, compared to those fed higher levels.

Gross energy values in whole body were not affected by the dietary levels of EPA and DHA. This result is similar to those of Benedito-Palos *et al.* (2007) and Torno *et al*. (2019), who reported no significant differences in the gross energy content of gilthead seabream, regardless the dietary levels of EPA and DHA ingested. Peng *et al*. (2008) obtained an identical result in other marine fish species such as black seabream. Contrasting results were reported in meagre juveniles by Simões *et al.* (2017), where fish fed higher dietary levels of EPA and DHA displayed significantly lower values of body gross energy.

The content of dry matter was the only parameter affected by the reduction of EPA and DHA in the diet, where fish fed lower levels of those EFA had significantly lower DM content, contrarily to respective diets. Contrary to this result, a study by Hossain *et al.* (2017) showed that silvery-black porgy fed lower dietary levels of EPA and DHA had significantly higher values of dry matter, like Hossain *et al*. (2012) in juvenile silver promfret. On the other hand, Emre *et al*. (2016), Simões *et al.* (2017) and Carvalho *et al*. (2018), did not observe any effect in dry matter content of meagre juveniles when decreasing dietary levels of EPA and DHA. Similarly, Benedito-Palos *et al.* (2007) and Torno *et al*. (2019) did not register any effect in such gilthead seabream juveniles, as Peng *et al*. (2008) in black seabream and Xu *et al*. (2017) in Japanese seabass.

5.5. Muscle and liver fatty acid composition

It is well known that FA composition of fish generally reflects by the dietary FA composition, as already displayed in other marine fish species (Bell *et al*., 1994; Cardinal *et al*., 2011; Foutoulaki *et al*., 2009; Glencross and Rutherford, 2011; Grigorakis *et al*., 2002; Hamza *et al.*, 2008; Ibeas *et al.*, 1994; Kim *et al.*, 2002; Li *et al*., 2015; Martínez-Llorens *et al*., 2007; Montero *et al*., 2005; Sargent *et al*., 1995; Senso *et al*., 2007; Villalta *et al*., 2005; Yildiz *et al*., 2008). Similarly, a dependence of body FA composition on dietary FA has been reported in previous studies on meagre (Carvalho *et al*., 2019; Fountoulaki *et al*., 2017; Mesa *et al*., 2014; Grigorakis *et al*., 2011; Piccolo *et al*., 2008; Poli *et al*., 2003). Inclusion of KO in fish diets

modifies the body FA profiles, and this effect is more evident in marine fish species with a limited capacity to convert C_{18} FA into LC-PUFA (Watanabe, 1982). Moreover, FA composition is obviously influenced by that of its constituent lipid classes. Whereas lipid-rich tissues are likely to have TG as their principal lipid, PL generally predominate in those of low lipid content. Consequently, comparisons between different fish and tissues of the FA compositions of their total lipids are of limited values since differences in lipid content and lipid class composition will influence the observed FA pattern (Henderson and Tocher, 1987).

5.5.1. Muscle

In general, FA composition of muscle reflects the FA diet composition, showing the high sensitivity of this tissue to dietary FA modifications, as observed in this and other marine fish such as gilthead seabream (Benedito-Palos *et al*., 2008; Magalhães *et al*., 2020) and European seabass (Izquierdo *et al*., 2003). Furthermore, all FA were accumulated in the muscle, at a lesser extent than its respective dietary concentrations suggesting utilization of all FA for growth. On the other hand, the reduction in n-3 LC-PUFA in muscle tissue is an effect of dietary EFA deficiencies (Watanabe, 1982) which was documented in the present study. Similarly, this effect has been described for different species including sunshine bass (*Morone chrysops* x *Morone saxatilis*) (Nematipour and Gatlin III, 1993), gilthead seabream (Ibeas *et al*., 1994), and eel (*Anguilla anguilla*) (Agradi *et al.*, 1995).

In comparison to EPA, DHA content was higher in muscle, regardless the diet, although much lower than dietary concentration. This might be explained by the preferential mitochondrial β-oxidation of EPA (Frøylland *et al*., 2000; Izquierdo *et al.*, 2003; Madsen *et al.*, 1998; Montero *et al*., 2005; Regost *et al.*, 2003) due to the complex catabolism of DHA (Bell *et al*., 2001). Fountoulaki *et al*. (2009) suggests that it occurs when dietary levels decrease, possibly to meet the requirements for tissue membrane composition and function. Furthermore, the pronounced decrease of EPA in muscle suggests a preferred utilization of this FA for energy or for eicosanoids production (Mourente and Bell, 2006), which agrees with previous studies (Fountoulaki *et al.*, 2009; Izquierdo *et al*., 2003; Regost *et al*., 2003). According to Izquierdo (2005) and Sargent *et al*. (1995), DHA is the major component in cell membranes, maintaining their integrity and being component of PL. This FA is necessary for reproduction, neural development, growth, survival, flat fish metamorphosis and disease prevention (Izquierdo, 2005; Sargent *et al.*, 1995). However, EPA is particularly important for growth in early life stages (Watanabe *et al.*, 1989), for broodstock fertility (Fernández-Palacios *et al.*, 1995) and it is precursor of proteinoids and leukotrienes along with ARA (Izquierdo, 2005).

Palmitic acid (16:0), oleic acid (OA; 18:1n-9) and linoleic acid (LA; 18:2n-6) were found to be the most abundant saturated (SFA), monounsaturated (MUFA) and n-6 long-chain polyunsaturated fatty acids (LC-PUFA), respectively, in both fish fed the FO or KO diet, reflecting dietary concentrations of those FA in each diet.

Concerning to 18:2n-6, the possibility that this FA was catabolized as an energy source should be considered, as FA requirements are more evident in early life stages of fish growth, as reported for Simões *et al.* (2017) in meagre juveniles.

The reduction in OA in muscle tissue, compared to dietary levels, was unexpected and do not agree with previous studies in this and other species. According to Geay *et al*. (2011) fish and other vertebrates can produce 18:1n-9 from 18:0 FA by the action of stearoyl-CoA (Δ9) desaturase in response to n-3 LC-PUFA deficiency. Watanabe (1982) previously observed that decreased dietary EFA (i.e., EPA and DHA) may produce an increase in OA in lipid from muscle, a symptom of EFA deficiencies. Also, the increase in n-9 LC-PUFA, particularly 18:1n-9, has been described for different marine fish species, such as red seabream (Izquierdo *et al*., 1989; Takeuchi *et al*., 1990), grey mullet (*Mugil cephalus*) (Argyropoulou *et al*., 1992), Japanese flounder (Izquierdo *et al.*, 1992), grouper (*Epinephelus malabaricus*) (Wu *et al.*, 2002), gilthead seabream (Ibeas *et al*. 1994; Magalhães *et al*., 2020), and European seabass (Mourente and Bell, 2006) as a good indicator of EFA deficiencies. This increase has been related to the reduced Δ6 desaturase activity found in marine fish (Mourente and Tocher, 1994). However, in the present study this increase was not recorded. This result could be explained by a wide range of factors: meagre juveniles have a possible limited capacity of producing stearoyl-CoA (Δ9) desaturase enzyme, which is required to produce 18:1n-9 from 18:0, however insufficient to cover meagre OA requirements for growth in early life stages; possible utilization of OA for energy production, as mentioned before, since this FA is considered one of the most important lipid energy sources in situations that impose a high demand such as larval development (Izquierdo, 1996) or starvation (Greene and Selivonchicks, 1987); and probable production of 20:1n-9 (gondoic acid) from 18:1n-9.

In the present study, apparent retention of chain-elongation products of OA, mainly 20:1n-9, in meagre juveniles, regardless the diet, suggests an endogenous biosynthesis of 20:1n-9 from C_{18} through FA elongase 5 (*Elovl5*). Although, this possible limited capacity seems to be insufficient to cover meagre n-3 LC-PUFA requirements (**Appendix II**), since fish fed FO or KO diets showed clear symptoms of EFA deficiency, as reduced growth (Carvalho *et al*., 2018). The present result agrees with an *in vitro* study that provided evidence that meagre

expresses at least one fatty acyl desaturase (*Fads2*) and one elongase (*Elovl5*) involved in the endogenous production of LC-PUFA (Monroig *et al*., 2013). The same ability to biosynthesize LC-PUFA from C_{18}-PUFA precursors was reported recently in this species (Campoverde and Estevez, 2017; Carvalho *et al.*, 2019; Khalil *et al.*, 2018), as well as in other marine fish such as gilthead seabream (Mourente and Tocher, 1993), Japanese flounder (Izquierdo *et al.*, 1992; Kim and Lee, 2004; Lee *et al*., 2003) or turbot (Owen *et al*., 1975).

Nevertheless, FA profile on fish can vary depending on dietary lipid sources, season, water temperature, salinity, and some other factors (Codier *et al*., 2002; Saavedra *et al.*, 2017).

5.5.2. Liver

FA profile modulation is faster observed in the liver, since this organ is the main site of lipid metabolism, as well as of its regulation (Parolini *et al*., 2014), therefore, major changes in FA composition were expected in this tissue with the different oil source used in each diet.

The results obtained in liver tissue support the theory that the diet composition directly interferes in animal body composition (Visentainer *et al*., 2005) especially for liver that mirrors the feeds FA contents.

Similar to muscle, all FA were accumulated in the liver at a lesser extent than its respective dietary concentrations suggesting utilization of all FA for growth.

The FA composition in the liver of meagre was affected by the oil source, mainly stearic acid (18:0), ARA, EPA and DHA. Fish fed the lowest dietary concentration of 18:0 (KO diet) exhibited higher content of 18:0 in the liver tissue compared to fish fed higher levels of that SFA (FO diet). This result may suggest that fish fed KO diet revealed a higher deficiency of n-3 LC-PUFA than those fed FO diet, and, therefore, they showed a higher requirement to produce n-3 LC-PUFA to meet the FA requirements for normal growth.

Regarding to MUFA levels in liver tissue, the lower abundance of MUFA in liver lipids in comparison to the dietary levels, could be also denoting an increase in lipolysis activity, since MUFA such as 18:1n-9, 20:1n-9 and 22:1n-11 are preferred substrates for mitochondrial β-oxidation in fish (Henderson and Sargent 1985; Sargent *et al*., 1989). The relative reduction in SFA, compared to dietary concentrations, also suggests an inhibition of *de novo* lipogenesis, due to increased lipid accumulation in meagre fed diets with FO or KO. Such effect was earlier observed by Torrecillas *et al.* (2017) in European seabass through the replacement of FO by VO.

The results of the present study showed that FO replacement by KO in diets had a significant impact on the levels of 18:1n-9 in liver of meagre juveniles. However, this result

does not agree with those of a recent study on meagre juveniles (Simões *et al.,* 2017), as in other marine fish species such as gilthead seabream (Ibeas *et al.,* 1994), and European seabass (Mourente and Bell, 2006), where it was reported that low dietary levels of EPA and DHA significantly improved the content of 18:1n-9 in liver. According to these authors, the retention of OA may be related to energy storage, as this FA is the preferred substrate for mitochondrial β-oxidation in fish (Mourente and Bell, 2006; Torstensen *et al*., 2000). Izquierdo *et al*. (2003) also suggested that OA seemed to be well utilized in both tissues (liver and muscle), typically for a good energy source. However, in the present study this increase was not recorded. This result could be explained by the same reasons mentioned above to explain the reduction of OA in muscle tissue.

Concerning to EFA, all fish registered a reduction in n-3 LC-PUFA in liver tissue, an effect of dietary EFA deficiencies (Watanabe, 1982) – a result observed before in muscle tissue. Furthermore, significantly higher contents of ARA, EPA, and DHA were observed in liver FA composition of fish fed FO diet than those of fish fed the KO diet. These results also suggest that meagre juveniles have a limited capacity to convert 20:4n-6 from 18:2n-6 and 20:5n-3 or 22:6n-3 from 18:3n-3. These findings agree with other results showing that the conversion rates of 18:2n-6 to 20:4n-6 and 18:3n-3 to 20:5n-3 or 22:6n-3 are negligible or nonexistent in other marine fish (Kim *et al*., 2002; Lee, 2001). Therefore, ARA, EPA and DHA are EFA for marine species (Izquierdo, 1996; Kanazawa, 1997).

The different utilization in muscle of 16:0, 18:1n-9, 18:2n-6, and 18:3n-3, comparing to liver, may be related to the lower capacity of β-oxidation of the two latter FA (Izquierdo *et al*., 2003). According to Frøyland *et al*. (2000) and Montero *et al.* (2005), the difference between liver and muscle can be related to a higher peroxisomal β-oxidation than mitochondrial β-oxidation activity in liver, whereas in muscle mitochondrial β-oxidation dominates. Likewise, Henderson (1996) mentioned that SFA and MUFA are preferred over LC-PUFA for energy production in fish by the mitochondrial system. This may explain the lower content of SFA, namely 14:0, 16:0 and 18: in muscle compared to liver. This result may be related to lipid class composition, with liver having less TG and more PL than muscle (Henderson and Tocher, 1987; Sargent *et al.,* 1989). Additionally, 16:0 is an important component of PL (Fountoulaki *et al*., 2009).

5.6. HSI

HSI provides an indication on the status of the energy reserve and metabolic activity of an animal (Chellappa *et al.*, 1995; Pyle *et al.*, 2005). Usually, fish with poor growth have a smaller liver with less energy reserve in this organ (Hossain *et al*., 2017). In the present study, fish fed diet containing low levels of EPA and DHA (KO diet) displayed higher values of HSI than those fed higher values of those EFA (FO diet). This finding is similar to that obtained for this species by Carvalho *et al*. (2019), who reported that meagre fed lower levels of EPA and DHA had higher values of HSI compared to those individuals fed increased levels of those EFA. These results denote an imbalance in dietary FA, which is a general symptom of dietary EFA deficiency (namely, EPA and DHA) in this fish (Carvalho *et al*., 2019), related with an increase in triacylglycerols (TG) (Watanabe, 1982). A simultaneous increasing of HSI was also found in other species such as gilthead seabream (Fountoulaki *et al*., 2009; Ibeas *et al.*, 1994; Kalogeropoulos *et al*., 1992; Montero *et al*., 2001), red sea bream juveniles (Takeuchi *et al*., 1990), turbot (Peng *et al*., 2014), Japanese seabass (Xu *et al.*, 2017), juvenile cobia (Trushenski *et al*., 2012), and Atlantic salmon (Kjaer *et al*., 2008) fed EFA-deficient diets, or in consequence of the replacement of FO by VO.

Lipid rich in n-3 LC-PUFA is reported to inhibit the fat synthesis and to decrease the accumulation of these FA in the liver, particularly, and whole body of fish (Cao *et al.*, 1997; Ribeiro *et al*., 2008), which may be due to an impaired FA metabolism as described for other vertebrates (Fukazawa *et al*., 1971; Montero *et al*., 2001; Olsvik *et al*., 2019; Watanabe, 1982). Likewise, a deficiency in EPA and DHA increases hepatic lipid deposits in many species, such as juvenile red seabream (Takeuchi *et al*., 1990) and European seabass (Lemaire *et al*., 1991). This occurs since the lack of n-3 LC-PUFA, namely EPA and DHA, may affect the adequate PL content of cell membranes, prejudicing the transport of FA 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; Ibeas *et al*., 1994), resulting in abnormal lipid accumulation in liver. This supports our observation that meagre juveniles fed the KO diet displayed higher HSI than fish fed KO diet because its high percentage of SFA can promote lipid accumulation in liver. A similar result was reported by Peng *et al*. (2016) in tilapia.

On the contrary, Emre *et al*. (2016) and Hossain *et al*. (2017) found that meagre and silvery-black porgy, respectively, fed a diet with low levels of EPA and DHA exhibited lower values of HSI compared to those fed higher levels of those EFA. On the other hand, Ribeiro *et al*. (2015) did not observe any effect in HSI when dietary FM and FO were replaced by

vegetable derived ingredients in diets for meagre juveniles. Similar results were reported in other species such as gilthead seabream (Benedito-Palos *et al*., 2007; Torno *et al.*, 2019), juvenile Japanese flounder (Kim *et al.*, 2002), black seabream (Peng *et al*., 2008), juvenile cobia (Trushenski *et al.*, 2011), European seabass (Torrecillas *et al*., 2017), and Japanese seabass (Xu *et al*., 2017). Ji *et al*. (2011) also found no significant differences in HSI values in juvenile grass carp (*Ctenopharyngodon idellus*) fed diets with levels of EPA and DHA like those used in the present study.

5.7. Steatosis

Likewise, hepatic steatosis have been frequently used as an indicator of EFA deficiency further than HSI and lipid content (Hong *et al*., 2016; Verreth *et al*., 1994). 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 of FA occurs, contributing to the formation of free radicals, release of inflammatory cytokines and causing aggression on the hepatic tissue (Benedito-Palos *et al*., 2008; Caballero *et al*., 2004).

In the present study, histological examinations revealed that regardless the dietary levels of EPA and DHA, no sign of necrosis was detected in the liver of meagre. However, clear signs of steatosis were observed in all fish, showing increased lipid deposition in hepatocytes and consequent nuclear displacement. Meagre fed the lowest percentage of EPA and DHA in the diet (KO diet; 0.60% n-3 LC-PUFA) were the most compromised with livers being more steatotic, while such effect was also evident in fish fed higher levels of EPA and DHA in the diet (FO diet; 1.98% n-3 LC-PUFA), but at a lower extent.

According to Berge *et al.* (1999), reduction in dietary n-3 LC-PUFA has been shown to increase hepatic lipid vacuoles by increasing TG synthesis in rats. This accumulation could be due to an impaired lipoprotein synthesis in liver as described also for other vertebrates (Fukuwaza *et al.*, 1971). Svegliati-Baroni *et al.* (2006) showed that the supplement of n-3 LC-PUFA could strikingly decrease the lipid accumulation in liver of rats. Therefore, hepatocytes of fish fed EFA-deficient diet (KO diet) showed a severe steatosis and migrated nuclei due to lipid accumulation. Similar results were obtained in other species such as gilthead seabream (Montero *et al*., 2001; Caballero *et al.*, 1999, 2004), red seabream (Takeuchi *et al*., 1990), European seabass (Lemaire *et al*., 1991), Japanese seabass (Xu *et al*., 2017), african sharptooth catfish (*Clarias gariepinus*) (Verreth *et al*., 1994) and other marine teleost species (Spisni *et al.*,1998), associated to high dietary lipid levels, use of artificial diets, quality of dietary oils used, and increase of dietary inclusion of VO. All these possible steatotic agents are associated

to an imbalance in FA metabolism and are frequently associated to deficient levels of EFA (Montero *et al*., 2001; Verreth *et al*., 1994), and lack of selected lipotropic factors such as lecithin, choline, or creatinine (Caballero *et al*., 2004). On the other hand, Vergara *et al.* (1996) suggested that a consequence of feeding close to or at satiation may be an increase in body and/or liver lipid content, while Kaushik (1997) mentioned that in most marine fish, high lipid reserves in the liver appear to be an adaptive mechanism to periods of low trophic activities at low water temperatures. Wassef *et al*. (2007) also suggested that nuclear displacement to hepatocyte's periphery and accumulation of lipid droplets in the liver of fish fed VO may be only a reflection of liver adaptation to an increment in dietary lipid content, as previously observed by Caballero *et al*. (1999) in gilthead seabream.

According to Ribeiro *et al*. (2015) in meagre, the present result can also be explained by the fact that dietary lipid content induces important metabolic alterations that resulted in lipid accumulation in liver and/or it might be related with insulin resistance as previously observed by Albalat *et al*. (2006) in trout. However, fatty liver does not necessarily mean it will develop lipoid liver disease (Ribeiro *et al*., 2015). Caballero *et al*. (2004) previously noticed that morphological alterations found in the livers of gilthead seabream are reversible when the fish are re-fed with a balanced diet, denoting the non-pathological character of these liver alterations.

The reason why meagre fed the lowest percentage of EPA and DHA in the diet (KO diet) revealed a more steatotic liver than fish fed FO diet could be also attributed to the levels of DHA and OA in diets. According to Spisni *et al.* (1998), hepatic steatosis is also associated to low DHA content and excessive level of OA in diets. Indeed, in the present study, fish fed KO diet, ingested the highest OA/DHA ratio (25.54), compared to fish fed the FO diet (8.69). Contrary to mammals and freshwater species, where OA is well tolerated, in marine species an excessive content in this FA is detrimental (Spisni *et al*., 1998). Thus, maintaining a normal OA/DHA or OA/n-3 LC-PUFA ratio is also important (Spisni *et al*., 1998). In fact, when one FA is the predominance in diets, it could increase the proportion of itself in the polar lipid but decrease the proportion of another (Sargent *et al*. 1999).

Furthermore, EPA is considered a major hypotriglyceridemic component of FO as it reduces lipid vacuoles in liver since it increases the area surface of both mitochondria and peroxisomes (Caballero *et al*., 2004; Frøyland *et al*., 1997; Berge *et al*., 1999; Totland *et al*., 2000) and decreases the FA availability for synthesis (Berge *et al*., 1999). Therefore, not only the reduction of EFA in diets but also the type of non-essential FA included in the diet seems to affect the hepatic morphology (Caballero *et al.*, 2004).

Concerning to hepatocyte diameter, meagre fed KO diet showed a significant higher hepatocyte diameter compared to fish fed higher levels of EPA and DHA - FO. This result corroborates the data discussed previously and agree with a study by Torrecillas *et al*. (2017) in European seabass, where the FO substitution by VO led to increased hepatocellular size due to reduction in LC-PUFA dietary content.

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6. CONCLUSIONS AND FUTURE PRESPECTIVES

The present study was accomplished to test the feasibility of FO replacement by another lipid source of n-3 LC-PUFA suggested as more sustainable, such as KO, in practical diets for meagre juveniles. Results showed that replacing FO by KO in fish diets significantly decreased dietary levels of EPA and DHA. Consequently, deficiency in n-3 LC-PUFA in KO diet led to a decrease in growth performance once these FA play important roles in many biological functions. Furthermore, meagre might have active Elovl5, but their activities were insufficient to produce DHA and EPA from LC-PUFA precursors to sustain growth, at least under the experimental conditions tested. Further studies would be needed to conclude concerning elongation and desaturation capacity in meagre juveniles. The present results also revealed that lack of ω-3 in KO diet induced an increase of FCR and DFI in fish fed that diet, since fish probably needed to ingest more feed to reach the EFA levels required for normal growth. Therefore, the substitution of FO by KO in diets for meagre juveniles should be taken with caution, by ensuring the adequate levels of n-3 LC-PUFA to sustain the normal growth and health condition of fish. Additional studies should be performed to investigate the requirements of n-3 LC-PUFA, namely, EPA and DHA. The results of the present study also suggested that low dietary levels of EPA and DHA significantly reduced the levels of 18:1n-9 in the muscle and the FA composition in the liver of meagre juveniles. Furthermore, fish fed the lowest percentage of EPA and DHA in the diet (KO) were the most compromised, with livers being more steatotic, even with only 71 days of feeding. This result suggests a possible relation between EFA-deficiency, due to the complete replacement of dietary FO by KO, and incidence of steatosis in meagre juveniles. For this reason, further research is needed to illustrate the mechanism on how n-3 LC- PUFA regulates the lipid deposition and FA composition.

In summary, fish fed FO diet showed better growth performance, improved FCR and DFI values, and liver with reduced lipid deposition.

The current study suggests that although KO could be a suitable energy source improving the cost effectiveness of the diet, our results suggest that FO cannot be totally replaced by KO without affecting growth performance, feed utilization, muscle and liver FA composition, and liver histology of meagre juveniles, considering the experimental conditions used in this study. This study recommend that more research is required to test the effect of FO replacement by KO at different levels in plant-based diets in meagre juveniles, and answer to the remain questions: what level of KO should be included in plant-based diets to replace FO and guarantee the optimal growth as well as the nutrition status of fish? It will be feasible the supplementation

of n-3 LC-PUFA in plant-based diets for meagre juveniles through the inclusion of a different sustainable and economic lipid source? In this sense it would be possible to eliminate the main issue that aquaculture has been faced for over 20 years - the EPA and DHA as two of the first limiting FA in plant-based diets for marine carnivorous fish.

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8. APPENDICES

Appendix I - Fatty acid composition (mg/g total fatty acids) of experimental diets for meagre (*A. regius*) juveniles.

Fatty acid (mg/g)	Diets	
	FO	KO
14:0	41	103
15:0	2	3
16:0	168	205
16:1n-7	42	73
17:0	1	1
17:1n-7	6	6
18:0	31	23
18:1n-9	313	281
18:1n-7	20	46
18:2n-6	133	104
18:3n-6	1	5
18:3n-3	53	50
18:4n-3	12	27
20:1n-11	7	8
20:2n-6	2	1
20:3n-6	1	1
20:3n-3	1	1
20:4n-6	4	
20:4n-3	3	1
20:5n-3 (EPA)	71	21
22:0	3	1
22:1n-11	3	
22:1n-9	1	3
22:2n-6	1	
22:5n-3	8	1
22:6n-3 (DHA)	36	11
24:1n-9	2	
Σ SFA	243	335
Σ MUFA	394	428
Σ n-3	184	113
Σ n-3 LC-PUFA[b]	119	35
Σ n-6 LC-PUFA[a]	8	2
Σ n-9	316	284

DHA/EPA	0.51	0.52
EPA + DHA	107	32
Σ n-3 LC-PUFA (% DM)[c]	1.98	0.60
EPA (% DM)[d]	1.18	3.6
DHA (% DM)[e]	0.60	1.9

Fatty acids with missing values were those whose peak were detected but contents were lower than 0.5 mg/g.

Appendix II - Fatty acid composition (mg/g total fatty acids) of muscle of meagre (*A. regius*) juveniles.

Fatty acid (mg/g)	Diets		
	FO	B	KO
14:0	0.65 ± 0.01[b]		1.23 ± 0.20[a]
15:0	0.09 ± 0.00		0.09 ± 0.01
15:0 isobr.	0.00 ± 0.01		0.01 ± 0.02
16:0	7.07 ± 0.25		6.86 ± 0.53
16:0 ante-iso	0.00 ± 0.00		0.04 ± 0.07
16:1n-9	0.14 ± 0.01		0.12 ± 0.05
16:1n-7	1.11 ± 0.06[b]		1.83 ± 0.18[a]
16:2n-4	0.18 ± 0.00[b]		0.66 ± 0.05[a]
16:3n-4	0.06 ± 0.00		0.07 ± 0.03
16:3n-3	0.21 ± 0.01		0.21 ± 0.02
16:4n-3	0.13 ± 0.00[a]		0.11 ± 0.01[b]
17:0	0.11 ± 0.00[a]		0.05 ± 0.02[b]
17:0 isobr	0.06 ± 0.00		0.03 ± 0.03
17:1	0.01 ± 0.02		0.03 ± 0.03
18:0	2.58 ± 0.08		2.45 ± 0.14
18:1n-9	11.44 ± 0.47[a]		9.79 ± 0.79[b]
18:1n-7	1.04 ± 0.03[b]		1.61 ± 0.08[a]
18:1n-5	0.02 ± 0.01		0.04 ± 0.02
18:2n-6	7.40 ± 0.17		6.92 ± 0.34
18:3n-6	0.08 ± 0.00[a]		0.01 ± 0.01[b]
18:3n-4	0.06 ± 0.02		0.02 ± 0.02
18:3n-3	1.57 ± 0.07		1..55 ± 0.12
18:4n-3	0.20 ± 0.01[b]		0.37 ± 0.03[a]
19:0	0.08 ± 0.00		0.07 ± 0.03
19:0 isobr	0.03 ± 0.01		0.01 ± 0.02
20:0	0.12 ± 0.00[a]		0.07 ± 0.03[b]
20:1n-11	0.01 ± 0.01		0.00 ± 0.01
20:1n-9	0.41 ± 0.02[a]		0.30 ± 0.02[b]
20:1n-7	0.06 ± 0.01		0.05 ± 0.02
20:2n-6	0.09 ± 0.00		0.06 ± 0.02

20:3n-3	0.03 ± 0.01	0.02 ± 0.02
20:4n-6	0.44 ± 0.00[a]	0.19 ± 0.01[b]
20:4n-3	0.15 ± 0.01[a]	0.12 ± 0.01[b]
20:5n-3	3.44 ± 0.13[a]	1.58 ± 0.02[b]
22:0	0.06 ± 0.00	0.03 ± 0.03
22:1n-11	0.04 ± 0.01	0.01 ± 0.02
22:1n-9	0.07 ± 0.00	0.04 ± 0.04
22:5n-3	0.77 ± 0.00[a]	0.24 ± 0.00[b]
22:6n-3	4.87 ± 0.03[a]	2.87 ± 0.04[b]
24:0	0.06 ± 0.00	0.02 ± 0.02
Σ SFA	10.92 ± 0.37	10.96 ± 0.89
Σ MUFA	14.34 ± 0.60	13.82 ± 1.08
Σ n-3	11.46 ± 0.24[a]	7.09 ± 0.35[b]
Σ n-6	8.18 ± 0.15[a]	723 ± 0.35[b]
n-3/n-6	1.40 ± 0.01[a]	0.99 ± 0.03[b]
EPA + DHA	8.31 ± 0.12[a]	4.45 ± 0.03[b]

Appendix III - Fatty acid composition (mg/g total fatty acids) of liver of meagre (*A. regius*) juveniles.

Fatty acid (mg/g)	Diets	
	FO	KO
14:0	7.63 ± 2.11[b]	15.21 ± 2.10[a]
15:0	1.05 ± 0.26	1.00 ± 0.09
15:0 ante-iso	0.00 ± 0.00	0.04 ± 0.06
15:0 isobr.	0.30 ± 0.07	0.45 ± 0.17
16:0	80.00 ± 10.24	95.94 ± 10.84
16:0 ante-iso	0.08 ± 0.13	0.17 ± 0.16
16:1n-9	3.83 ± 0.41	4.77 ± 0.52
16:1n-7	23.94 ± 2.67[b]	38.32 ± 2.38[a]
16:2n-4	1.81 ± 0.35[b]	10.57 ± 0.67[a]
16:3n-4	0.92 ± 0.16[b]	1.50 ± 0.05[a]
16:3n-3	0.06 ± 0.07	0.14 ± 0.24
16:4n-3	0.23 ± 0.09	0.11 ± 0.09
17:0	1.73 ± 0.29[a]	1.25 ± 0.04[b]
17:0 isobr	0.86 ± 0.09	0.82 ± 0.02
17:1	0.17 ± 0.06[b]	0.98 ± 0.19[a]
18:0	43.79 ± 0.57[b]	69.47 ± 4.48[a]
18:1n-9	187.58 ± 22.32	184.47 ± 5.37
18:1n-7	15.26 ± 3.68[b]	25.40 ± 0.17[a]

18:1n-5	0.36 ± 0.03[b]	1.02 ± 0.01[a]
18:2n-6	81.43 ± 14.77	62.68 ± 2.29
18:3n-6	1.02 ± 0.10[a]	0.22 ± 0.21[b]
18:3n-4	0.69 ± 0.06[a]	0.09 ± 0.16[b]
18:3n-3	17.76 ± 2.73	15.05 ± 0.69
18:4n-3	2.31 ± 0.30[b]	4.15 ± 0.17[a]
19:0	1.79 ± 0.09[b]	2.31 ± 0.10[a]
19:0 isobr	1.27 ± 0.18[b]	2.04 ± 0.29[a]
20:0	1.28 ± 0.05	1.32 ± 0.05
20:1n-11	0.14 ± 0.02	0.10 ± 0.17
20:1n-9	7.59 ± 0.27[a]	6.14 ± 0.10[b]
20:1n-7	0.67 ± 0.05[b]	1.10 ± 0.02[a]
20:2n-6	1.69 ± 0.11[a]	1.03 ± 0.04[b]
20:3n-3	0.67 ± 0.07[a]	0.24 ± 0.24[b]
20:4n-6	1.48 ± 0.18[a]	0.03 ± 0.05[b]
20:4n-3	1.69 ± 0.14[a]	1.22 ± 0.09[b]
20:5n-3	16.43 ± 2.18[a]	3.18 ± 0.16[b]
22:0	0.81 ± 0.06	0.78 ± 0.07
22:1n-11	0.60 ± 0.38[b]	1.66 ± 0.10[a]
22:1n-9	1.18 ± 0.34[a]	0.17 ± 0.17[b]
22:5n-3	4.55 ± 0.65[a]	0.06 ± 0.10[b]
22:6n-3	9.42 ± 1.64[a]	1.97 ± 0.10[b]
24:0	0.32 ± 0.16	0.07 ± 0.12
24:1n-9	0.78 ± 0.36[b]	1.41 ± 0.07[a]
Σ SFA	140.92 ± 13.36[b]	190.88 ± 15.92[a]
Σ MUFA	242.11 ± 28.52	265.53 ± 8.09
Σ n-3	54.22 ± 7.21[a]	26.12 ± 1.64[b]
Σ n-6	85.71 ± 14.99	63.96 ± 2.46
n-3/n-6	0.64 ± 0.04[a]	0.41 ± 0.01[b]
EPA + DHA	25.85 ± 3.57[a]	5.16 ± 0.26[b]

Appendix IV – Staining procedure – Hematoxylin-eosin method – displayed as steps of immersions in different reagent and corresponding times.

SL No.	Solution	Duration	Process
1	Xylol	15 minutes	Clearing
2	Xylol	6 x 3/5 seconds	
3	100% alcohol	6 x 3/5 seconds	Dehydration
4	100% alcohol	6 x 3/5 seconds	
5	96% alcohol	6 x 3/5 seconds	
6	70% alcohol	6 x 3/5 seconds	
7	50% alcohol	6 x 3/5 seconds	
8	Distilled water	2 minutes	
9	Hematoxylin	10 minutes	
10	Wash in tap or running water	5 minutes	Staining
11	Acid alcohol	2 minutes	
12	Wash in tap or running water	5 minutes	
13	96% alcohol	3 minutes	Hydration
14	Eosin	15 seconds	
15	Wash in tap or running water	until the water is clear	
16	Wash in tap or running water	30 seconds	
17	96% alcohol	6 x 3/5 seconds	
18	96% alcohol	6 x 3/5 seconds	Rehydration
19	100% alcohol	6 x 3/5 seconds	
20	100% alcohol	6 x 3/5 seconds	
21	Xylol	25 minutes	Clearing
22	Xylol	until mounting	