NUTRITIONAL PROGRAMMING OF GILTHEAD SEA BREAM (*SPARUS AURATA*) THROUGH BROODSTOCK DIETS FOR A BETTER UTILIZATION OF LOW FISH MEAL AND FISH OIL DIETS BY THE OFFSPRING

Hanlin Xu

PhD Thesis 2020

PhD program in Sustainable Aquaculture and Marine Ecosystems (ACUISEMAR®)

Grupo de Investigación en Acuicultura (GIA®)

Institute of Aquaculture and Sustainable Marine Ecosystems (IU-ECOAQUA®)

Universidad de Las Palmas de Gran Canaria

Supervisors

Prof. Marisol Izquierdo

Prof. Juan Manuel Afonso

UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

D. DANIEL MONTERO VÍTORES COORDINADOR DEL PROGRAMA DE DOCTORADO EN ACUICULTURA SOSTENIBLE Y ECOSISTEMAS MARINOS (ACUISEMAR®) DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha ..... tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada **"NUTRITIONAL PROGRAMMING OF GILTHEAD SEABREAM (*SPARUS AURATA*) THROUGH BROODSTOCK DIETS FOR A BETTER UTILIZATION OF LOW FISH MEAL AND FISH OIL DIETS BY THE OFFSPRING**" presentada por el/la doctorando/a **D. HANLIN XU** y dirigida por la Doctora **Marisol Izquierdo y el Dr. Juan Manuel AFONSO.**

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a…..de.....de dos mil.....

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

ESCUELA DE DOCTORADO

Programa de doctorado ACUICULTURA SOSTENIBLE Y ECOSISTEMAS MARINO (ACUISEMAR®)

Título de la Tesis

NUTRITIONAL PROGRAMMING OF GILTHEAD SEABREAM (*SPARUS AURATA*) THROUGH BROODSTOCK DIETS FOR A BETTER UTILIZATION OF LOW FISH MEAL AND FISH OIL DIETS BY THE OFFSPRING

Tesis Doctoral presentada por D. **HANLIN XU**

Dirigida por la Dra.Dª **MARISOL IZQUIERDO y el Dr. JUAN MANUEL AFONSO**

La Directora,	**El Codirector**	**El Doctorando,**
(firma)	**(firma)**	**(firma)**

Las Palmas de Gran Canaria, a 31 de octubre 20 20

LIST OF CONTENTS

LIST OF ABBREVIATIONS

ALA	α-linolenic acid;
ARA	arachidonic acid
cox2	*cycloxigenase-2*
cpt-1	*carnitin palmitoil transferase 1*
DHA	docosahexaenoic acid
elovl2	*elongase 2*
elovl6	*elongase 6*
EFA	essential fatty acids
EPA	eicosapentaenoic acid
fads2	*fatty acyl desaturase 2*
FAMES	fatty acid methyl esters
FCR	feed conversion ratio
FI	feed intake
FM	fishmeal
FO	fish oil
gh	*growth hormone*
ghr	*growth hormone receptor*
HSI	hepatosomatic index
igf-1	*Insulin-like growth factor 1*
g6p	glucose 6-phosphatase
LA	linoleic acid
LC-PUFA	long-chain polyunsaturated fatty acid
LNA	linoleic acid
LO	linseed oil
lpl	*lipoprotein lipase*
MiRNA	MicroRNA
OA	oleic acid
PA	palmitic acid
PBC	peripheral blood cell
ppar α	*peroxisome proliferator activated receptor α*
RO	rapeseed oil
rpl-27	*ribosomal protein l27*
SGR	specific growth rate
srebp1	*sterol regulatory element-binding transcription factor 1*
tnfα	*tumor necrosis factor α*
tor	*target of rapamycine*
VM	vegetable meal
VO	vegetable oil
VSI	viscerosomatic index

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENT

To anyone who helped me, I cannot be who I am now without your support, especially in this tough time.

‘‘We may gaze up to the mountain’s brow; we may travel along the greater road;’ signifying that although we cannot hope to reach the goal, still we may push on thitherward in spirit’ (A eulogy upon Confucius). I want to quote this old Chinese saying to express my sincere gratitude to my supervisors, Prof. Marisol Izquierdo and Prof. Juan Manuel Afonso. It is always a great honor to be a student of two of the world best scientists in aquaculture. Thank you very much for your time and patience on helping me to design different trials, to conduct different analysis, to participate in different conference, to go to different lab for internship, and to learn more and more on aquaculture. Maybe I will not achieve your height in the future, but you show me the destination I can fight for throughout the rest of my life.

I also want to thank Prof. Sadasivam Kaushik. I am appreciated that I can learn from you, not only the suggestion and comments you gave on my work, but also your broad knowledge in aquaculture.

I want to express my gratitude to Prof. Genciana Terova for allowing me to conduct the analysis on DNA methylation in University of Insubria. Although time was a little bit tense, with the valuable help from Simona, we finally achieved our goal.

I would like to thank Prof. Jorge Fernandes for the supervision during my internship in Nord University on microRNA analysis. I also want to emphasize my thanks to Artem Nedoluzhko for the availability to help me and share with me valuating knowledge, to Joanna and Matina on the guidance of the analysis. I would also like to thank the help and accompany from Prof. Galindo-Villegas, Arun, Ciu, Park, Robabani and other staffs. Thanks to Dr. Robin Mjelle (Norwegian University of Science and Technology) for the crucial help in data analysis.

I am grateful to Prof. Daniel Montero and Prof. Maria Jesus Zamorano for coordinating such a brilliant PhD course, and for your help in keeping my experiments and analysis well-organized.

I also appreciate the help and the guidance from all other professors in Ecoaqua.

Thanks to the technicians of GIA, Andrea, Carmen, Guacimara, Jimena, Marco, Nabil, Ruben and Rayco, for helping me in feeding, weighing, separating and sampling the fish from broodstock to their offspring juveniles, in biochemical, histological and molecular analysis. Thank you for your effort on such a huge amount of work. Thanks to Natalia for your help in the documents and picked me up from the airport the first time when I arrived in Gran Canaria in 2016.

Thanks to my colleagues, Adnane, Álvaro, M., Álvaro, L., David, Marta, Raquel, Reda, Samira, Siva, Suk, Toni and Yiyen, for giving a hand in the samplings, and also for the accompany, which makes me have the feeling of home even I am far away from China

Specially, I want to express my gratitude to my teammate, Serhat and Ferosekhan for the all the help and support along the time of my PhD study. I cannot finish this work without you.

Thanks to all the reviewers and the editors who helped us to improve our manuscript.

Lastly, I want to appreciate the support from my parents and from my fiancée, Yahui. The life is tough, but you make it meaningful.

谨以此文，为水产学科的发展尽一份微薄之力

FUNDING

The researches were partly funded by the EU funded project PerformFISH (H2020-SFS-2016-2017; 727610)

The researches were partly funded by Institute of Sustainable Aquaculture and Marine Ecosystems (IU-ECOAQUA®)

The PhD study was supported by China Scholarship Council

Chapter 1

Introduction

1.1 Fish meal (FM) and fish oil (FO) replacement as a main challenge for development of intensive aquaculture

Fish food is a well-balanced source of minerals and highly digestible proteins for human being, and it accounted for 17% intake of animal protein of global population (FAO, 2020). Besides, it is the main source of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) that are important for fetal development (Helland *et al.*, 2003) and contribute to prevent the cardiovascular diseases (Schmidt *et al.*, 2000) and non-alcoholic fatty liver disease (NAFLD) (Oya *et al.*, 2010), among many other properties for human health. Therefore, fish demand by the consumers is continuously increasing. From 1961 to 2017, the annual growth rate of fish supplement (3.1%) was nearly twice as much as the growth rate of global population (1.6%) and higher than that of all other animal protein food (2.1%). In 2018, global fish production reached 178.5 million tons, of which 45.8% came from aquaculture (FAO, 2020). The global capture fisheries remain relatively stable since late-1980's, with small fluctuation among different years and according to the long-term monitor on accessed marine fish stock, fish resources are continuously decreasing (FAO,2020). Due to this stagnant production of fisheries, aquaculture is taking over the responsibility to provide sufficient safe and sustainable products to satisfy market demands. In 2018, Aquaculture produced 52% of the fish used for human consumption, and from 2001 to 2018, the average annual growth rate of global aquaculture production was 5.3 % annually (FAO, 2020). However, further development of aquaculture is restricted by the limited availability and increasing prices of fish meal (FM) and fish oil (FO), traditional protein and lipid sources in aquafeeds that are mostly derived from capture fisheries. In 2015, the global production of FM and FO was 4.7 million and 856,000 tons, respectively, of which aquaculture consumed 70% FM and 73% FO production (Green, 2018). However, the production of FM and FO is gradually decreasing. For example, FM production was 2 million tons less in 2015 compared with in 1997. Apart from the decrease in supply, the use of FM and

FO in aquafeed raises the concerns about the contribution of aquaculture to overexploitation of fisheries resources and the presence of persistent organic pollutant or heavy metals accumulated in FM or FO (Council, 2011). Therefore, dietary FM and FO need to be replaced by other high quality and nutritious ingredients with a more economical, environmental and socially sustainable production.

Plant, animal byproducts, single cell ingredients or insects meal protein sources are often used to replace the dietary FM (Caballero *et al.*, 2002; Wang *et al.*, 2016; Rosales *et al.*, 2017; Rimoldi *et al.*, 2018). Plant protein sources have the advantage of having a large, stable, and sustainable production, and are widely used to replace dietary FM in aquafeeds. For example, in the diet of rainbow trout (*Oncorhynchus mykiss*) juveniles, up to 66% of FM can be replaced by plant protein sources (Gomes *et al.*, 1995). In the diet of Atlantic salmon (*Salmo salar* L.), up to 27% pea protein concentrate or 22% lupin protein concentrate can be added (Carter and Hauler, 2000). In gilthead seabream (*Sparus aurata*), a mixture of extruded peas and rapeseed meal can replace up to 50% FM without affecting the growth rate (Sitjà-Bobadilla *et al.*, 2005). However, at high levels of replacement, the imbalanced amino acid composition and the anti-nutrient factors present in plant protein sources may impair the growth and digestibility, and negatively affect immune system of the fish (Vergara *et al.*, 1996b; Vergara *et al.*, 1996a; Francis *et al.*, 2001; Caballero *et al.*, 2004; Gómez-Requeni *et al.*, 2004; Castro *et al.*, 2015). Moreover, the phospholipid content in plant protein sources is lower than in FM (Council, 2011). High replacement levels of FM by plant proteins also lead to negative effects on the nutritional value of the fillet including the decrease in n-3 LC-PUFA (De Francesco *et al.*, 2007). These shortages restrict the further use of plant protein sources in aquafeeds.

Compared to plant protein sources, animal byproducts may contain an amino acid composition closer to FM and other interesting nutrients like minerals and phospholipids. However, their nutritional composition differs among ingredients and the policy based on the concern of safety (Council, 2011), restricting their use in aquafeed. Depending on the source and nutritional value, 20- 40% of FM can be replaced by animal byproducts (Oliva-Teles *et al.*, 2015). Single cell ingredients like brewer's yeast have been used in aquaculture since 1990's with the advantage

of high protein contents, high level of nucleotides and the absence of anti-nutrient diets (Oliva-Teles *et al.*, 2015). Meanwhile, they can be used as prebiotics to stimulate the growth of bacteria in the intestinal tract (Ferreira *et al.*, 2010). However, like the animal byproducts, the quality of single cell ingredients varies among different manufacturers. In addition, methionine is a potential limiting amino acid in the use in aquafeed (Oliva-Teles *et al.*, 2015). Insect meal, especially black solider fly larvae, is getting growing interest in recent years (Kroeckel *et al.*, 2012), due to the ability of converting food waste to high quality protein (Newton *et al.*, 1977), but the use is restricted by a limited production.

For certain freshwater species, some vegetable oils can be used as the sole lipid source in diet. However, for mariculture species, long chain polyunsaturated fatty acids (LC-PUFAs), which mainly include DHA, eicosapentaenoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, ARA), are essential for growth and FO is the main commercially competitive source of LC-PUFAs. Oil from oilseed or animal are used in the replacement of dietary FO, but the insufficient LC-PUFAs content in these resources restricts the replacement level, since the complete FO replacement leads to decreased the growth performance, reduced nutritional value of fillet, abnormal liver lipid metabolism, impaired immune system and weakened reproduction performance in high replacement level (Tocher *et al.*, 2002; Menoyo *et al.*, 2004; Izquierdo *et al.*, 2005; Lin and Shiau, 2007; Sink *et al.*, 2010). Compared with the other two types of lipid sources, microalgae oil may contain abundant LC-PUFA, especially docosahexaenoic acid (22:6n-3, DHA) (Ryckebosch *et al.*, 2012). Meanwhile, during process of the oil production, tons of carbon dioxide in atmosphere is fixed by microalgae, making it an environment friendly process (Doughman *et al.*, 2007). However, the use of microalgae oil is restricted by its high price compared with FO (Oliva-Teles *et al.*, 2015).

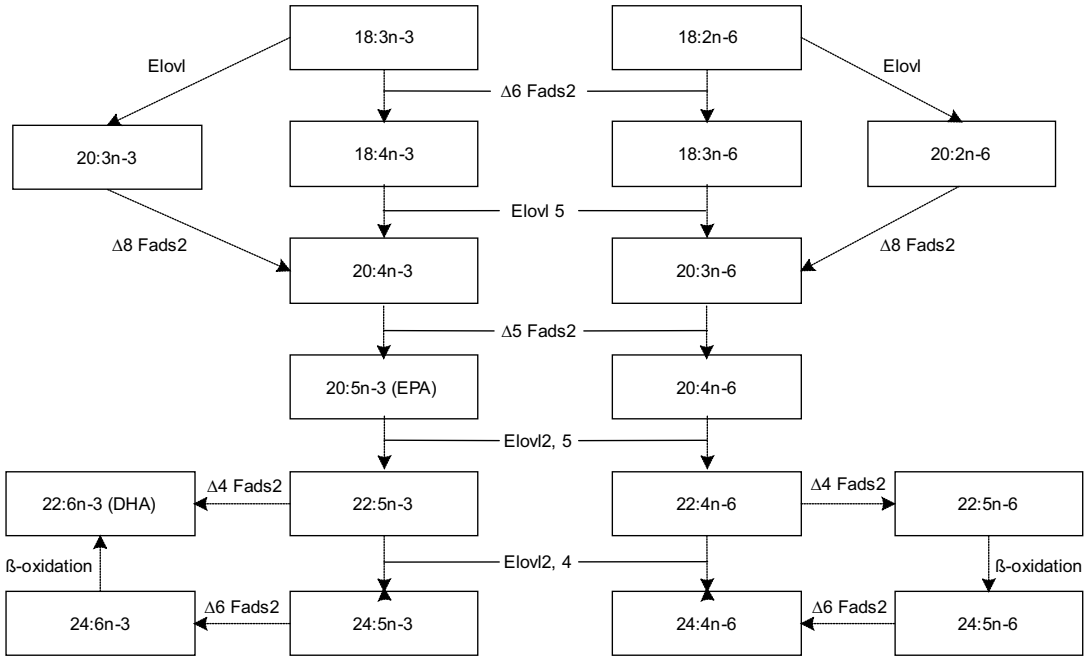
1.2 Importance of LC-PUFA for fish production

LC-PUFAs have many structure and functional roles (Watanabe, 1993; Calder, 2012). For example, they are critical components of cell and organelle membranes (Wassall and Stillwell, 2008) and their derivates, like eicosanoids and docosanoids, participate in the cell signaling procedure (Gill and Valivety, 1997). In fish, LC-PUFA are essential fatty acid that is

compulsory for the growth (Peng *et al.*, 2014a), brain and immune system development and homeostasis maintenance (Menoyo *et al.*, 2004; Torrecillas *et al.*, 2017b).

Like mammals, the ability of synthesize the LC-PUFA *de novo* in fish is absent because they lack n-12 and n-15 desaturases which are only present in some plants or invertebrates. Therefore, either LC-PUFAs or their 18C precursors are required in fish feeds. The biosynthesis of LC-PUFA is catalyzed by a set of desaturases and elongases (Figure 1). ARA is synthetized from linoleic acid (LNA), whereas EPA and DHA are obtained from α-linolenic acid (ALA). Comparing with freshwater fish or euryhaline fish, marine fish have a lower ability to synthesize LC-PUFA from their 18C precursors (Dong *et al.*, 2017). The reason could be partly related to with the environment, since the food for marine fish contains sufficient LC-PUFA, originally produced by phytoplankton, marine fish do not need the enzymes to produce them, losing the capacity of synthesis of LC-PUFA (Sargent *et al.*, 2003).

Figure 1.1 Pathway of >C18 PUFA (polyunsaturated fatty acids) biosynthesis (adapt from Oboh *et al.* (2017) and Bond *et al.* (2016))



Traditionally, FO is the main source of LC-PUFAs in the aquafeeds. Other plant lipid sources used in the replacement of dietary FO lack LC-PUFA but are abundant in the precursors, 18C PUFAs. Therefore, the replacement level of FO for each fish species highly depends on its the

ability of LC-PUFA biosynthesis. Generally, the use of FO by alternative plant lipid sources is higher in diets for freshwater for than in diets for marine species. For example, the content of FO is 0-2% in the diet of Chinese carp diets, in contrast to the 1-15% FO in marine fish species diets (Tacon and Metian, 2008). Therefore, a higher proportion of FO could be replaced if the fish ability to synthetize LC-PUFA is increased.

Different strategies may enhance the ability of fish to synthesize the LC-PUFA, such as the regulation of certain environmental factors. For instance, reduction in salinity may increase the ability of fish to synthesize DHA from ALA and up-regulate the expression of hepatic *fatty acyl desaturase 2* (*fads2*), as it occurs in white-spotted rabbit fish (*Siganus canaliculatus*) (Li *et al.*, 2008) or in puye (Dantagnan *et al.*, 2010). Temperature may regulate n-3 LC-PUFA biosynthesis capacity, as occurs in pound loach (*Misgurnus anguillicaudatus*) that fish raised in 4 °C contained higher EPA/ALA ratio with the increase of genes related with lipid metabolism like *sterol-regulator element binding protein 1* (*srebp1*), *carnitine palmitoyltransferase 1* (*cpt1*) compared to fish raise at 28 °C (Chen *et al.*, 2018).

Insufficient ability of LC-PUFA synthesis is also related with the very limited activity of Fads2 (Li *et al.*, 2014) in marine species. Fads2 is a speed-limited enzyme in LC-PUFA biosynthesis, the function of which is catalyzing the ALA or LNA to 18:4n-3 or 18:3n-6, respectively, and from 24:4n-6 to 24:5n-6 or from 24:5n-3 to 24:6n-3 (Vagner and Santigosa, 2011). For example, the activity of Fads2 in cod (*Gadus morhua* L) is lower than in Atlantic salmon (Tocher *et al.*, 2006). The missing SP-1 binding site of *fads2* in cod compared with Atlantic salmon can be one of the reasons of the activity difference (Zheng *et al.*, 2009). Apart from the difference between species, *fads2* can also be regulated by nutritional factor. Increased *fads2* expression or Fads2 desaturase products are found when fish is fed with FO replacement diet in many species (Bell *et al.*, 2002; Seiliez *et al.*, 2003; Xu *et al.*, 2014; Gregory *et al.*, 2016), so it is possible to use nutritional factor as a tool to produce fish which can better ability of LC-PUFA synthesis.

1.3 Nutritional programing

A common strategy employed by nature that allows the adaptation of the organism metabolism

to the environmental conditions is nutritional programming. Nutritional programming refers to the outcome in an animal that receives a nutritional stimulus at critical periods of its development, like pre- or postnatal stages (Lucas, 1994). A well-known example can be found in honeybees that can become a fertile queen or a sterile worker through the consumption of royal jelly (Kucharski *et al.*, 2008). In mice, malnutrition during both pre- or postnatal periods leads to the nutritional programming of lipid metabolism causing long-term reduction of plasma cholesterol, HDL-cholesterol and triacylglycerol (Lucas *et al.*, 1996). In human mothers exposed to famine, the offspring develop impaired glucose, decreased lung function, increased atherogenic lipid profile, more obesity and three-fold increased coronary heart disease (Painter *et al.*, 2005). More specifically, the supplement of n-3 LC-PUFA during pregnancy and lactation improved the score in K-ABC test (Helland *et al.*, 2003). Similarly, in fish, nutritional stimulus during reproduction by feeding broodstock with different dietary fatty acid profiles, markedly affects lipid metabolism and growth of the progeny (Izquierdo *et al.*, 2015). For instance, feeding gilthead sea bream (*Sparus aurata*) broodstock with diets containing partial replacement of FO by linseed oil (LO), low in n-3 LC-PUFA but high in their ALA precursor, up-regulated *fads2* expression and growth in the progeny (Izquierdo *et al.*, 2015). Moreover, when the 4-month-old progeny were fed a low FM and FO diet, those fish from parents fed partial FO replacement by LO showed improved growth and feed utilization (Izquierdo *et al.*, 2015). Indeed, broodstock feeding exerts a very long-term effect in the progeny and replacement of FO by LO in parental diets, in combination to juvenile feeding with low-FM and low-FO diets, markedly improves 16-month-old offspring growth and feed utilization (Turkmen *et al.*, 2017a). These studies demonstrated that it is possible to improve the ability of marine fish to use low FM and low FO diets by nutritional programing though broodstock feeding. Similarly, exposure to a vegetable-based diet in early life of Atlantic salmon improves growth performance and feed efficiency later in life when fish are fed a low FO and FM diet (Clarkson *et al.*, 2017).

The improved growth performance caused by nutritional programming due to the replacement of dietary FM/FO by alternative protein or lipid sources can be explained by 'Barker hypothesis'

that malnutrition leads to sparing of growth of key tissues and the fetus programmed its metabolism to be thrifty (Edwards, 2017). When facing a malnourishment situation, the fetus limits the growth or function of the organs that are not essential to immediate survival or adopts a state of insulin resistance, crucial glucose or other limited energy supplies for the development of organs essential for the survival like heart and brain (Edwards, 2017). These changes at molecular, cellular and tissue levels, increase the survival of the fetus and later life stages when submitted to a malnourishment situation. For instance, the European sea bass (*Dicentrarchus labrax*) juveniles programmed by a low n-3 LC-PUFA content diet fed from first exogenous feeding to 45-day-old showed a higher DHA content in polar lipid and a higher expression of *fads2* when challenged at day 151-181 with a n-3 PUFA deprived diet (Vagner *et al.*, 2007). These results suggested that fish subjected to low n-3 LC-PUFA in early stages are prepared for the upcoming deprived n-3 LC-PUFA diet. Similarly, the programming with dietary vegetable oil (VO) during spawning season led to molecular adaptations in the larvae progeny by up-regulating lipid metabolism- and health- related genes, such as cycloxigenase-2 (*cox2)* and tumor necrosis factor-alpha (*tnf-a)*, lipoprotein lipase (*lpl*), carnitin palmitoil transferase 1 (*cpt1*) and elongase 6 (*elovl6*) (Turkmen *et al.*, 2017a).

1.4 Epigenetics mechanisms

Epigenetics are "the transmittable changes in gene "off-on" states through modulation of chromatin, which is not brought about by changes in the DNA sequence" (Allis *et al.*, 2008). Epigenetic mechanisms include the DNA methylation, post-transcriptional histone modification, chromatin remodeling and none coding RNA.

DNA methylation is the modification that coverts cytosine by the addition of a methyl group to 5-methylcytocine in DNA template, which is one of the prime epigenetic mechanisms associated with gene repression (Allis *et al.*, 2008). DNA methylation is widely involved in the effects of nutritional programming (Heijmans *et al.*, 2008; Kucharski *et al.*, 2008; Hoile *et al.*, 2013; Niculescu *et al.*, 2013). For instance, DNA methyltransferase3, a gene that regulates the methylation status of CpG region, was involved in the effect of royal jelly on honeybee (Kucharski *et al.*, 2008). Dietary genistein *in utero* at the level of 250 mg/kg enhanced early

establishment of DNA methylation at the CpG sites in the promotor region of A^{vy} *intracisternal A particle* upstream of transcription start site of the *Agouti* gene of A^{vy} mouse and this difference of DNA methylation level led to a change in coat color (Dolinoy *et al.*, 2006). DNA methylation is also associated with the effect of nutritional programming on lipid metabolism. Methylation of the CpG sites in the promoter region of *fads2* of adult offspring are regulated by parental dietary lipid level and a negative correlation is reported between the methylation of a CpG site and the hepatic *fads2* expression (Hoile *et al.*, 2013). The research on the relation of nutritional programming with DNA methylation remains scarce. A global hypomethylation was found in the gilthead seabream fed with soybean meal in early feeding when the soybean meal diet was removed (Perera and Yufera, 2017).

Besides DNA methylation, microRNA (miRNA) which belongs to noncoding RNA with a length between 21-23 nt, in combination with Argonaute protein 2, leads to the decay or transcriptional repression of target mRNA (Best *et al.*, 2018). MiRNA plays important roles in tissue-specific differentiation (Kloosterman *et al.*, 2007; Laudadio *et al.*, 2012; Latimer *et al.*, 2017) and homeostasis of fish (Mennigen *et al.*, 2014). The changes of gene expression regulated by nutritional programming are also associated with the participation of miRNA. In mice, when the parents are fed with a high fat diet, 5.7% of 579 miRNA were regulated in the offspring, including miRNAs related with developmental timing and lipid oxidation (Zhang *et al.*, 2009). Besides, also in mice, maternal protein restriction leads to an increase of miR-375 and the down-regulation in two target genes *Pdl-1* and myotrophin in the 3-month-old offspring mouse, together with the diminished insulin secretion that lead to a glucose intolerance (Dumortier *et al.*, 2014). However, to our knowledge, the information of the connection of miRNA and nutritional programming between generation in fish remains unknown.

1.5. Gilthead sea bream as a model for nutritional programming studies

Gilthead seabream was chosen as the model for nutritional programming studies in this thesis. It is a euryhaline species widely distributed throughout Mediterranean, and from British Isles to Cape Verde including occasionally the Canary Islands. Meanwhile, it is a high prized food in the Mediterranean and eastern Atlantic and is widely cultured in Turkey, Greece, Spain and

Italy. In Spain, gilthead seabream is the second largest aquaculture species with the production of 13.740 tons, occupied 21.2% of total aquaculture production in 2016. In the same year, the production in Europe and worldwide was 160.563 and 185.980 tons respectively (Source: Federation of European Aquaculture Producers and FAO FishStat).

Using fish as a model for nutritional programming studies present several advantages comparing with mammals including:

(1) *Their high batch fecundity* makes it easy to obtain a large sample size with the same genotype.

(2) *Lecithotrophic embryogenesis* and *organogenesis* allows that the embryo receives nutrition only from yolk avoids the effects brought by maternal nutrients, hormone and metabolism during gestation and lactation (Hou and Fuiman, 2019).

Specifically, using gilthead seabream has some advantages such as:

(1) *Being a multi-batch spawner* allows to follow the effect of nutritional programming multiple times during spawning season.

(2) *It is possible to modify the egg fatty acid composition* in egg through broodstock dietary fatty acids profiles in only three weeks (Fernández-Palacios *et al.*, 1995), whereas the spawning season lasts 3 months. Therefore, the nutritional programming can be tested several times during spawning season. In other species, such as salmonids, the vitellogenesis periods are longer and it may take months to change the egg fatty acid profiles (Fernández-Palacios *et al.*, 2011).

(3) *There is previous information* confirming the efficacy of nutritional programing and the optimum timing for this species has been previously determined. These studies show that the response of offspring fed low FM and low FO diets can be regulated by parental programming during spawning season (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017a), whereas programming during early larval stages leads to low survival rate (Turkmen *et al.*, 2017b).

(4) *Nutritional programming with broodstock diets high in vegetable oil (VO)* allows to improve the growth of offspring juvenile challenged with low FM and low FO diet

(Izquierdo *et al.*, 2015) and this programming effects lasts in the offspring juveniles (Turkmen *et al.*, 2017a).

However, a series of shortages constrain the use of gilthead seabream as a model for nutritional programming studies:

(1) *The wide genetic variety among the individuals*, which increases the difficulties on looking into the mechanisms brought by nutritional programming (National Research Council, 2011);

(2) *The sensitivity of embryogenesis to environmental factors* that may interfere with nutritional programming (Hou and Fuiman, 2019).

Finally, despite there is some basic information about the nutritional programming of gilthead sea bream through broodstock diets, there are still some lack of knowledge that is necessary to fill in order design practical nutritional programming protocols for this species, among them:

(1) It is yet unknown if FM replacement in broodstock diets would allow nutritional programming of gilthead seabream.

(2) The epigenetic mechanisms involved in nutritional programming of gilthead seabream are not sufficiently clarified.

(3) There is no clear information about the influence of the broodstock ability to synthetize LC-PUFA on the progeny obtained from this broodstock.

1.6 Objectives

The massive use of the FM and FO in aquatic feed animals, especially in mariculture, restricts the further development of an economical and sustainable aquaculture industry. Dietary FM and FO needs to be replaced by other ingredients. However, FM and FO are the main protein and lipid source of diet. The replacement may lead to unbalances in amino acids, fatty acids, minerals and vitamins, and bring in the anti-nutritional factors, which will negatively affect the growth performance and the health of the fish. The hypothesis of this thesis is that nutritional programming can be a tool to improve the use of low FM and low FO diet of aquaculture fish. However, previous studies were focused on using VO for nutritional programming and the

vegetable oil used contained linseed oil that is easy to be oxidized, so in this study, we aimed to determine:

(1) the effect of FM replacement by vegetable meals (VM) alone or in combination with FO replacement by VO in broodstock diets for gilthead sea bream as a nutritional tool to modify gene expression and improve utilization of low FM and low VO contain diets in the progeny (Chapter 3),

(2) the change of *fads2* expression during embryogenesis and determining the effect of broodstock *fads2* expression and the diet consumed during spawning season on the *fads2* expression in the eggs produced (Chapter 4),

(3) the combined effect of broodstock nutritional programming through FO replacement by RO during the spawning season or broodstock *fads2* expression on the offspring juvenile utilization of low FM and low FO content diet (Chapter 5),

(4) the long-term effect of broodstock nutritional programming through FO replacement by RO during spawning season on the offspring juvenile performance under nutritional challenge test and the modification of hepatic genes and miRNA expression (Chapter 6),

(5) the long-term effect of broodstock *fads2* expression on the offspring juvenile performance under nutritional challenge test and the modification of hepatic genes and miRNA expression (Chapter 7),

(6) the effect of nutritional status in different stages of gilthead sea bream on the performance under nutritional challenge test.

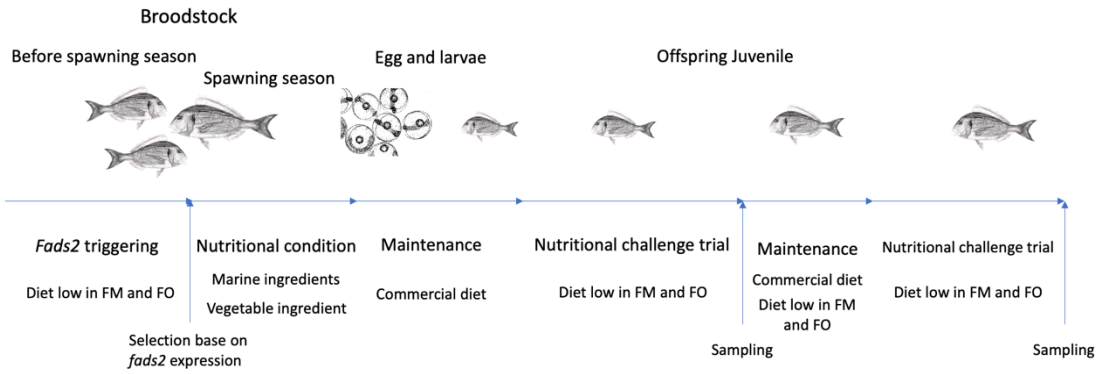
Chapter 2

Materials and methods

2.1 General design

The general design of the trials conducted in this thesis included the nutritional programming of the gilthead seabream broodstock, belonging to the Grupo de Investigacion en Acuicultura (GIA) from the ECOAQUA Institute (ULPGC, Telde, Spain), by feeding with programming diets containing vegetable ingredients during spawning season for at least one month. After this period, eggs were collected, and the offspring were produced separately following common commercial protocols until they reached the desired size in each trial. Then, they were challenged with a diet low in FM and FO for the determination of their growth performance (Figure 2.1).

Figure 2.1 Schematic view of experimental design



2.2 Diet production

2.2.1 Diet in stimulus stage

Three months before the spawning season, the broodstock used in Chapters 4 to 7 were fed with diet contained 5% FM and 3% FO for one month. Soya protein concentrates, corn gluten, wheat gluten and rapeseed meal were used to replace dietary FM and the mixture of RO, linseed oil

(LO) and palm oil (PO) was used to replace dietary FO. Diet was produced by BioMar (Brande, Denmark).

Table 2.1 Diet formulation for broodstock stimulus

Ingredients (%)	
Fish meal[1]	5.0
Blood meal (spray-dried)[2]	7.0
Soya protein concentrate[3]	20.0
Corn gluten meal[4]	22.0
Wheat gluten[4]	5.5
Rapeseed meal[5]	11.3
Wheat[6]	6.9
Fish oil[7]	3.0
Rapeseed oil[5]	5.2
Linseed oil[8]	2.6
Palm oil[4]	5.2
Supplemented ingredients[9]	5.5
Vitamin and mineral premix[10]	0.8
Antioxidant[11]	0.05
Yttrium oxide	0.03
Proximate analysis (% DM)	
Protein	46.6
Lipids	24.0
Ash	5.4

1South-American, Superprime – Feed Service Bremen, Germany,2Daka, Denmark, 3Svane Shipping, Denmark, 4Cargill, Netherlands, 5Emmelev, Denmark, 6Hedegaard, Denmark, 7South American fish oil, LDN Fish Oil, Denmark, 8Ch. Daudruy, France, 9Contains lysine, methionine, monocalcium phosphate, choline, inositol, phospholipids (Emulthin G35). Vilomix (Denmark), Evonik Industries (Germany), Pöhner (Germany), 10Supplied the following vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany), 11BAROX BECP, Ethoxyquin, Vilomix (Denmark)

2.2.2 Broodstock nutritional conditioning diet

In Chapter 3, three isoproteic and isolipidic diets were formulated to contain approximately 55% protein, 20% lipid and 5% ash (% dry weight). Three diets were formulated to contain different replacement level of FM or FO by vegetable ingredient. Diets were produced by BioMar.

In Chapter 4-7, broodstock with high (H) or low (L) *fads2* expression in blood were fed either with a diet rich in FO or rich in RO during the spawning season (Ferosekhan *et al.*, 2020b). Compared with FO diet, diet RO was rich in 18C PUFA, including 18:1n-9, 18:2n-6 and 18:3n-3. Diets were provided by Skretting ARC (Stavanger, Norway).

2.2.3 Challenge diet

For the challenge test in Chapter 3, offspring juveniles from broodstock fed three different diets were challenged by feeding them either a control diet (20% FM and 6% FO) or a challenge diet with low marine-orient materials (5% FM and 3% FO). Diets were isoenergetic, isonitrogenous and contained 45% protein, 22% lipid (% dry weight) and produced by BioMar.

For the challenge tests in Chapter 5-7, offspring juveniles, were fed by diet contained 7.5% FM and 0% FO. Challenge diet was produced by Skretting ARC.

Formulation, proximate contents, and fatty acid composition of challenge diets were described in each chapter.

2.3 Broodstock maintenance

In the verge of spawning season, the gilthead seabream broodfish of the same origin were maintained in a 40 m^3 circular tank with 600% water exchange rate and nature photoperiod. Each broodstock from this pool was pit-tagged (EID Iberica SA-TROVAN, Madrid, Spain) for identification, and its gender determined by a gentle abdominal massage to identify males that were spermiating. Then, the broodfish were distributed with a ratio of 2:1 male:female into the different experimental 1000 L tanks, fed a commercial diet (Europa Turbot 18, Skretting, Spain), and their spawning quality analysed for 1 month to ensure that there were no significant differences among the different broodstock groups. After this period, broodstock were fed the respective nutritional programming diet, broodstock were fed the respective nutritional programming diet, according to the different studies conducted, at the rate of 1% fish biomass. Broodstock were fed two times per day (9:00 and 14:00 h). Seawater temperature was in the range of 18-22 °C and fish were kept under natural photoperiod (approximately 12 h light).

Spontaneously spawned eggs obtain from each experimental broodstock were collected six times per week during the broodstock feeding. Spawning performance was evaluated based on total number of egg produced, number of fertilized eggs, viable egg at 24 hours after spawning, hatched out eggs and the survival rate of larvae 3 days after the hatch (Fernández-Palacios *et al.*, 1995). Firstly, eggs from each spawning were collected into a 5 litres beaker with strong aeration. From this pool of eggs, 3 randomized 5 mL samples were placed in bogorov chamber and counted under binocular microscope for the total number of eggs produced and the number of nonfertilized eggs. Then randomly chosen eggs were transfer to three 96-well microplates (VWR, West Chester, USA) each tank with the density of 1 egg per well in which contained 250 µL of sterilized seawater. These microplates were incubated at 19°C. The egg viability was determined when the egg showed a transparent, perfectly spherical with clear, symmetrically

early cleavages. Total number of hatched eggs and survived larvae was also determined based on the hatching rate and larval survival rate of these samples.

In chapter 4 to 7, before entering spawning season, broodstock were first selected based on their *fads2* expression in peripheral blood. Before the selection, 71 2-year-old males and 114 4-year-old females broodstock were fed a diet contained 5% FM and 3% FO that was low in LC-PUFA but high in 18C fatty acids to stimulus LC-PUFA biosynthesis and the expression of related genes (Table 2.1). After one month of feeding, broodstock were fast for 24 hours and their blood was extracted and centrifuge at 3000 g for 15 min at 4°C. Blood cells were separated for the *fads2* expression analysis. Caudal fin with the size around 0.5×0.2 cm was taken from each breeder and stored in 75% ethanol.

2.4 Egg incubation and sampling for embryogenesis studies

After programming on broodstock for one month that was described in 2.2, eggs were collected from 6 breeders with relatively high *fads2* expression and 6 breeders with relatively low *fads2* expression fed with diet containing 20% FO and 80% rapeseed oil of total lipid, and 3 breeders with relatively low *fads2* expression were fed with diet containing FO as the only lipid source, separately into 500 L tank. Eggs or larvae samples from each spawning were culture separately in 500 L incubators and were taken at the stage of spawning (0 hour post spawning [hps]), morula (4 hps), high blastula (6 hps), gastrula (10 hps), neurula (16 hps), heart appearance (30 hps), hatch (53 hps) and 3 day after hatch (125 hps) according to the description in (Kamacı *et al.*, 2005). Samples were washed by diethyl pyrocarbonate (DEPC) water and taken into *RNAlater* (MilliporeSigma, Darmstadt, Germany), incubated at 4 °C overnight and then stored in -80 °C until analysis.

2.5 Offspring production

2.5.1 Larvae production

Larvae were produced using the protocols established in EcoAqua Institute facilities, following commercial procedures. Fertilized eggs were stocked into 2000 L tanks at a density of 100 eggs/L for larval production. Sand filtered; UV-sterilized seawater flow was progressively

increased from 10 to 40% per h until larvae reached 46 days after hatching (dah). Water was continuously aerated at 125 mL/min; a 1500–3500 lx 12 h single central light (TLD 36 W/54, Philips, Amsterdam, Netherlands) photoperiod was provided and living phytoplankton (*Nannochloropsis* sp.) (250 ± 100 × 10^3 cells/mL) was added to the rearing tanks. In each tank water temperature and pH were daily recorded. Regarding feeding, from 3–32 dah, larvae were fed twice a day with rotifers (*Brachionus plicatilis*) enriched with ORI-GREEN (Skretting) at 5–10 rotifers/mL. From 15–17 dah, larvae were fed *Artemia sp*. enriched with ORI-GREEN (Skretting), which was added three times a day. From 20 dah, larvae were fed commercial weaning diets (Gemma Micro, Skretting).

2.5.2 Juvenile production

After the larval stage, post-larvae were transferred into 8000 L pre-weaning tanks. Fish were fed with corresponding commercial diets of increased diameter depending on fish size. Fish were regularly sized and graded to avoid cannibalism and to select the appropriate feed size. When fish reached around 2 g, they were transferred to 1000 L flow-through tanks in triplicates.

2.6 Offspring challenge test

Once the offspring obtained from different broodstock groups reached desired size, they were selected based on their average weight and randomly distributed into the experimental tanks (250 L) for the challenge trial to determine the potential effect of nutritional programming or broodstock selection for high *fads2* expression.

Offspring from broodstock fed the different diets were challenged by feeding them the corresponding low FM and low FO challenge diet as described in each trial. During the feeding trails, juveniles with initial weight around 2-3g were fed to apparent satiation 4 times per day (8:30, 10:30, 13:30 and 16:30) until fish body weight was triplicated, and juveniles with initial weight more than 50g were fed to satiation 3 times per day (8:30, 11:30, 14:30) for 74 days. Tanks were provided with 200 L/h seawater at 21-23°C and strong aeration. Tanks were illuminated by fluorescent lights placed above the tank with a light intensity of 100 lx, programmed for 12h light photoperiod (from 8 a.m. to 8 p.m.).

2.7 Growth and feed utilization monitoring

Growth, in terms of standard length (cm) and whole-body weight (g), was recorded in the different trials at different periods according to the experimental design, but always at the beginning and at the end of the trial by measuring and weighting all fish individually. Throughout the experiments, feed intake per replicate was recorded. To calculate feed intake, feed delivery was daily determined, and uneaten pellets were collected in a net by opening water outlet 30 min after each meal, dried in an oven for 24 h and weighed. At the end of the trials, productive parameters were calculated including mortality, specific growth rate (SGR), feed conversion ratio (FCR), weight gain (WG), feed intake (FI), biological feed conversion ratio (FCR), hepatosomatic index (HSI), viscerosomatic index (VSI) using the following formulate:

- Mortality (%) = 100 × (nº dead fish/ nº total fish)
- Weight gain (WG, g) = final body weight (BW_1) – initial body weight (BW_0)
- Specific growth rate (SGR, % day^{-1}) = 100* (ln BW_1 − ln BW_0) / nº days, where BW_0: initial body weight (g) and BW_1: final body weight (g)
- Feed intake (FI, g $fish^{-1} day^{-1}$) = Feed delivered / (nº of fish*nº days)
- Biological feed conversion ratio (FCR) = Feed delivered ($t_1 - t_0$) / (Biomass t_1 – Biomass t_0 + Biomass $_{harvested}$ + Biomass $_{lost}$), where t_0: initial biomass (g) and t_1: final biomass (g)
- Hepatosomatic index (HSI) = 100 × Liver weight (g) / Body weight (g)
- Viscerosomatic index (VSI) = 100 × Visceral weight (g) / Body weight (g)

2.8 Sampling procedures

Before sampling, all fish were submitted to 24 h fasting. During samplings fish were caught and introduced into an anaesthetic tank containing clove oil (2ml/100L) (Guinama S.L.U., Spain) to reduce stress and improve handling. Those fish that were meant to be returned to the tanks were recovered in a tank with abundant aeration and water flow until symptoms of recovery appeared (recuperation of verticality and sense of equilibrium, normal movement and

response to external stimuli). At the beginning and end of challenge trials, fish from each tank were weighted individually, and 3-5 fish were painlessly killed by immersion in seawater containing 10 ppm clove oil and stored directed at -80 °C for whole body biochemistry analysis. At the end of the trial, visceral mass and livers of 5 fish per tank were weighted for the calculation of HSI and VSI, and then livers, left epaxial muscle from these 5 fish were collected for biochemical analysis. At the same time, approximately 200 mg liver or muscle from 3-5 fish per tank were pooled and collected into 2 mL microcentrifuge tube (VWR) filled with 1.5mL *RNAlater* (MilliporeSigma) for molecular study. Samples in *RNAlater* were kept at 4 °C overnight and then stored at -80 °C until analysis. Livers of 5 fish per tank were sampled and stock in 4% formalin (VWR) at room temperature for histologic study.

2.9 Biochemical analysis

Samples for biochemical analysis were collected into a plastic bag, avoiding the presence of air, and stored at -80 ºC until analysed. Samples were homogenized immediately prior to analyses.

2.9.1 Moisture

Moisture the samples was determined by measuring the constant weight of the samples at 110°C in the oven according to the description in A.O.A.C (Horwitz, 2002). Briefly, weighting bottles were put into 110 °C to constant weight. Then 0.5-2 g of samples were put into the weighting bottle and their total weight were measured. The weighting bottles with sample were dried at 110°C overnight to constant weight at room temperature.

$$\text{Moisture\%} = 100 \times (\text{Weight}_{\text{sample+ weighting bottle}} - \text{Weight}_{\text{weighting bottle}}) \div (\text{Weight}_{\text{sample+ weighting bottle}} - \text{Weight}_{\text{dried sample+ weighting bottle}})$$

2.9.2 Protein

Protein contents were determined according A.O.A.C. (Horwitz, 2002). After the digestion of the sample (between 200 - 500 mg, depending on sample size from different tissues) with Kjeldahl tablets (Catalyst with 1.5 % $CuSO_4 \cdot 5H_2O$ and 2 % Se) (Panreac, Barcelona, Spain) concentrated sulphuric acid (VWR) at a temperature of 400°C. Then total nitrogen content from

the samples were determined by Kjeltec 8100 (FOSS, Hillerød, Denmark) and back titrated by 0.1mol/L HCl.

$$\text{Protein\%} = 100 \times ((\text{Volume}_{\text{HCl samples}} - \text{Volume}_{\text{HCl blank}}) \times 0.1 \times 14.007 \times 6.25) \div \text{Weight}_{\text{sample}}$$

2.9.3 Lipids

Lipids were extracted with chloroform/methanol (2:1 v/v) (Folch *et al.*, 1957). Approximately 100 mg of samples were weighted and homogenized in an UltraTurrax (T25 digital Ultra Turrax, IKA, Königswinter, Germany) during 5 min in a solution of 5 mL of chloroform: methanol (2:1) with 0.01% of butylated hydroxytoluene (BHT) (VMR). After the homogenization, 5 mL of chloroform: methanol with 0.01% BHT was added to clean the remains on the machine. For increasing the water phase polarity, 0.88% KCl was added and the resulting solution was filtered by gravimetric pressure through glass wool. After centrifugation at 622 ×g during 5 min, the watery and organic phases were separated. Once the watery phase was eliminated, the solvent was dried under nitrogen atmosphere and subsequently total lipids weighted.

$$\text{Lipid\%} = 100 \times (\text{Weight}_{\text{lipid}} \div \text{Weight}_{\text{sample}})$$

2.9.4 Fatty acid composition

In order to measure the fatty acid composition of samples, the lipids extracted from last step were transmethylated to obtain fatty acid methyl esters (FAMES) (Christie, 1982). Maximum 80 mg of total lipids were mixed with 1 mL of toluene with 50 mg/L BHT. The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50°C. Afterwards, FAMES were extracted with hexane:diethyl ether (1:1, v/v) and purified by adsorption chromatography on NH_2 Sep-pack cartridges (Waters S.A., Massachussets, USA). FAMES were separated by Gas-Liquid Chromatography (GLC) (Agilent 7820A, CA, USA) in a Supercolvax-10-fused silica capillary column (length:30 mm, internal diameter: 0.32 mm, Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180°C for the first 10 min, increasing to 215 °C at a rate of 2.5 °C min^{-1} and then held at 215 °C for 10 min, following the conditions described in (Izquierdo, 1989). Fatty acid methyl esters were quantified by Flame ionization

detector (FIED) and identified by comparison with external standards and well-characterized FO (EPA 28, Nippai, Ltd. Tokyo, Japan).

2.9.5 Ash

Ash was measured based on dry ashing method (Horwitz, 2002) by putting 0.5-2 g sample at 600°C overnight (Carbolite, Sheffield, U.K.).

$$\text{Ash\%} = 100 \times (\text{Weight}_{\text{total weight after drying}} - \text{Weight}_{\text{ceramic crucible}}) \div (\text{Weight}_{\text{sample+ceramic crucible}} - \text{Weight}_{\text{ceramic crucible}})$$

2.10 Histological studies

The livers of 5 fish per tank were sampled and stock in 4% formalin. Dehydration of the samples was carried out using a Histokinette 2000 (Leica, Nussloch, Germany) with gradually increasing alcohol grades beginning with 70º and ending with 100º, being the last two steps xylene and paraffin (MilliporeSigma). This process substitutes water and fat from the tissues, allowing the staining of the sample. After embedded in paraffin wax, blocks were made and cut with a Leica microtome (Jung Autocut 2055; Leica) in 4 μm sections, which were placed in slides and stained with haematoxylin and eosin (H&E) (Panreac) (Martoja *et al.*, 1970). Slides were studied and photos were taken by a light microscope (CX41, Olympus, Tokyo, Japan). Area, length of long and short axis of 60 hepatocyte per tank were analysed with *ImagePro plus 6.0* (Media Cybenetics, Rockville, USA).

2.11 Molecular studies

2.11.1 RNA extraction

RNA from 200 mg sample was extracted using TRI Reagent (Sigma, U.S.A) and RNeasy kit (Qiagen, Hilden, Germany). *RNAlater* was removed from the samples before extraction. Egg sample was squeezed in tube for breaking chorion. Then each sample was weighted and 1 mL TRI Reagent with 1 steel bead was added. Then the sample was homogenized in *TissueLyser II* (Qiagen) at 30 Hz for 30 s and centrifuged at 13 000 ×g for 1min. All the liquid phase was transferred to a new 1.5 mL tube and 300 μL of chloroform (MilliporeSigma) was added and

mixed. The mixture was centrifuged at 12 000 ×g for 15 mins. The transparent phase was isolated and mixed with same volume of 70% ethanol. The mixture was loaded to RNeasy mini spin column and washed by RW1 and RPE offered in the kit according to the instruction of manufacturer. Concentrated RNA was eluted in 30 µL of RNase free water. RNA quality was checked by 1.4% agarose electrophoresis and quantity was measured by NanoDrop™ 1000 (ThermoFisher, Waltham, USA.). 1000 ng of RNA per sample was used for cDNA synthesis through *iScript* cDNA synthesis kit (Bio-rad, Hercules, U.S.A.) in *iCycler* (Bio-Rad). The expression of key enzymes related with growth and lipid metabolism were determined using the primers listed in Table 2-8.

2.11.2 Droplet Digital PCR

Droplet Digital PCR was conducted in *QX200™ Droplet Digital™ PCR System* (Bio-rad). The reaction system, with the volume of 20 µL, containing 100 ng of cDNA, primers and *Evagreen SuperMix* (Bio-rad), was loaded to droplet generator to generate the oil droplet and then proceeded in *C1000 TOUCH™ thermal cycler* (Bio-Rad). The cycling condition of PCR was: 95℃ for 5 mins, followed by 40 cycles of 95℃ 30 sec, Tm 1min, and then stabilized the signal at 4℃ for 5 min, 90℃ for 5 min, finally the reaction was hold at 4℃. The PCR amplification of the nucleic acid target in the droplets was read in *QX200 droplet reader* (Bio-Rad). Table 2.4 lists of gene used in the studies. Data was analysis through *QuantaSoft Analysis Pro* (Bio-Rad) and the gene expression was calculated using following formulate:

Gene expression (copies/ µL) $= \frac{\text{concentration of target gene(copies/ µL)}}{\text{concentration of housekeeping gene(copies/ µL)}} \times$ average concentration of housekeeping gene (copies/ µL)

2.11.3 Genomic DNA extraction

Genomic DNA was extracted from 3 liver samples per experimental group (1 sample per tank) using a manual method including RNase I treatment. Briefly 35 mg of tissue was digested in 350 µl of lysis buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl 1% SDS at pH8, containing 8 µl of Proteinase-K [20 mg/ml]) at 55 °C overnight with gentle shaking. 250 µl of

Table 2.2 Primer sequence for Droplet Digital PCR

Gene		Sequence (5'-3')	Tm (°C)	Gene bank No.
β-actin	Forward	GAC CAA CTG GGA TGA CAT GG	61	X89920.1
	Reverse	GCA TAC AGG GAC AGC ACA GC		
rpl27	Forward	ACA ACT CAC TGC CCC ACC AT	61	AY188520
	Reverse	CTT GCC TTT GCC CAG AA CTT		
fads2	Forward	GCA GAG CCA CAG CAG CAG GGA	63	AY055749
	Reverse	CGG CCT GCG CCT GAG CAG TT		
scd1a	Forward	CGG AGG CGG AGG CGT TGG AGA AGA AG	62	XM_030400656.1
	Reverse	AGG GAG ACG GCG TAC AGG GCA CCT ATA TG		
srebp1	Forward	AGG GCT GAC CAC AAC GTC TCC TCT CC	62	XM_030407356.1
	Reverse	GCT GTA CGT GGG ATG TGA TGG TTT GGG		
lpl	Forward	CGT TGC CAA GTT TGT GAC CTG	60	AY495672
	Reverse	AGG GTG TTC TGG TTG TCT GC		
cpt-1ß	Forward	CCA CCA GCC AGA CTC CAC AG	60	DQ866821
	Reverse	CAC CAC CAG CAC CCA CAT ATT TAG		
elovl6	Forward	GTG CTG CTC TAC TCC TGG TA	60	JX975702
	Reverse	ACG GCA TGG ACC AAG TAG T		
ppara	Forward	TCT CTT CAG CCC ACC ATC CC	58	AY590299
	Reverse	ATC CCA GCG TGT CGT CTC C		
igf1	Forward	GTG TGT GGA GAG AGA GGC TT	58	AY996779.2
	Reverse	CTC TTG GCA TGT CTG TGT GG		
g6p	Forward	CGC TGG AGT CAT TAC AGG CGT	62	XM_030399413.1
	Reverse	CAG GTC CAC GCC CAG AAC TC		
cox-2	Forward	GAG TAC TGG AAG CCG AGC AC	61	AM296029
	Reverse	GAT ATC ACT GCC GCC TGA GT		
ghr1	Forward	ACC TGT CAG CCA CCA CAT GA	62	AH014067.4
	Reverse	TCG TGC AGA TCT GGG TCG TA		
ghr2	Forward	GAG TGA ACC CGG CCT GAC AG	62	AH014068.4
	Reverse	GCG GTG GTA TCT GAT TCA TGG T		
tor	Forward	CAG ACT GAC GAG GAT GCT GA	61	XM_030422395.1
	Reverse	AGT TGA GCA GCG GGT CAT AG		

*Complete gene name: *rpl27*: ribosomal protein L27; *fads2*: fatty acid desaturase 2; *scd1*: stearoyl-CoA desaturase-1; *srebp1*:sterol regulatory element binding transcription factor 1; *lpl*: lipoprotein lipase; *cpt-1ß:* carnitine palmitoyltransferase 1ß*;elovl6*: elongation of very long chain fatty acids protein 6; *ppara*: Peroxisome Proliferator Activated Receptor α; *igf-1*: insulin-like growth factor 1; *g6p*: Glucose 6-phosphate; *cox-2*: cyclooxygenase-2; *ghr1*: growth hormone receptor 1; *ghr2*: growth hormone receptor 2; *tor*: mechanistic target of rapamycin kinase.

5 M NaCl was then added to each sample. Samples were vortexed for 30 s at maximum speed, and tubes spun down for 10 min at 12 000 ×g. The supernatant was transferred to new tubes and 500 µL of cold isopropanol was added to each sample. Samples were then centrifuged for 5 min at 13 000 ×g at 4°C. The pellet was twice washed with 75% ethanol, dried, and finally suspended in 100 µL of 5 mM Tris-HCl pH 8.5.

2.11.4 Methylation-specific PCR (MSP)

Analysis of DNA methylation level was performed in Università degli Studi dell'Insubria (Varese, Italy). DNA was firstly treated with sodium bisulfite to convert cytosine to uridine using *EpiTect Bisulfite Kit* (Qiagen). The converted DNA was then amplified by PCR using two pairs of primers, with one pair specific for methylated DNA (M pair) and the other for unmethylated DNA (U pair) (Table 2.5).

The CpG-rich regions of the *fads2* promoter were identified by in silico analysis of gilthead sea bream *fads2* gene sequence (Chromosome 8), by using *MethPrimer* (plus CpG Island

Prediction, http://www.urogene.org/cgi-bin/methprimer2/MethPrimer.cgi). Genomic DNA samples (1.5 µg) from 3 samples per group were acid-catalyzed and converted with bisulfite using the *EpiTect Bisulfite Kit* (Qiagen) and stored at −20 °C until further analysis. Two fragments, one of 215 bp and the other of 232 bp, corresponding to regions of the *fads2* promoter between −676 and −891 (region 1) and between −3977 and −3744 (region 2), respectively (adenine of ATG was numbered +1) were amplified by PCR from 30 ng of bisulfite-treated DNA. The PCR reactions were performed with *Taq DNA polymerase* (Qiagen, Germany) supplied with Q-solution and primer sets described in Table 2.5. Thermocycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94°C for 1 min, 50°C (or 54°C) for 1 min, 72°C for 1 min, and a final extension step of 10 min at 72 °C.

Table 2.3 Sequences of primers used for Methylation-specific PCR (MSP)

Primer name	Nucleotide sequence (5'-3')	Tm
Left M1	TTG GTT TAG AAA TTT TCG AAT GAT C	59.76
Right M1	AAT TTT CGA CTA CTC CTC TAA TCG A	58.94
Left U1	TGG TTT AGA AAT TTT TGA ATG ATT G	58.59
Right U1	ATT TTC AAC TAC TCC TCT AAT CAA A	54.94
Left M2	TAA ATA GGG AGA TAG GTT GTA CGT	55.43
Right M2	TCC ATA TAT TTC CAT AAT TAT CGA A	56.06
Left U2	TAA ATA GGG AGA TAG GTT GTA TGT	53.26
Right U2	TTC CAT ATA TTT CCA TAA TTA TCA AA	55.39

2.11.5 fads2 promoter sequencing

DNA was extracted from caudal fin described in 2.3 using *DNAeasy kit* (Qiagen). 25 mg caudal fin from each broodstock was cut into small pieces and transferred into 1.5 mL tube with 180 µL ATL buffer. Then 20 µL proteinase K was added and mixed by shaking. The mixture was incubated at 56 °C overnight until the sample was digested. After a brief centrifuge, 200 µL buffer AL was added into the mixture and mixed by shaking for 15 s. Then 200 ul of 100% ethanol was immediately added to the sample. After mixing, briefly centrifuge the sample tube to remove the droplets from inside the cap. Then the mixture was carefully pipet to the *DNAeasy column* placed in a 2 ml collection tube and centrifuged at 6000 ×g for 1min. The *DNAeasy column* with sample was then placed into another clean 2 ml collection tube, and 500 µl Buffer AW1 was added, followed by centrifuge at 6000 ×g for 1 min. After centrifuge, the DNAeasy column was place in a new clean 2 mL collection tube and 500 µl Buffer AW2 was added. To

dry the *DNAeasy membrane*, the tube with column was centrifuged at full speed (14,000 rpm) for 3 min. The *DNAeasy column* was placed in a 1.5 mL bead tube. 100 µl Buffer AE was added into the column. After 1 min incubation at room temperature, the tube was centrifuged at 8000 *rpm* for 5 min to elute. The quality of extracted DNA was check by 1% agarose gel electrophoresis, and the quantity was measured in the NanoDrop™ 1000 spectrophotometer (ThermoFisher).

The primers used for *fads2* promoter region sequencing were designed based on Perera *et al.* (2019) (Forward: 5' CTC CTG GAA TTT CCC TCA 3'; Reverse: 5' TTT TCG GCT GCT CCT CTG 3'). PCR was performed using 40 ng/µL extracted DNA with *iTaq Kit* (Intron) according to the instruction of the manufacturer. Thermocycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 34 cycles at 94°C for 20 s, 60°C for 1 min, 72°C for 30 min, and a final extension step of 3 min at 72 °C. The PCR product was quantified in NanoDrop™ 1000 spectrophotometer (ThermoFisher), diluted to 75 ng/µL and sent to Macrogen (Madrid, Spain) for sequencing.

2.11.6 MicroRNA analysis

Analysis of microRNA was performed in Nord University (Bodø, Norway). RNA was extracted from pool of 5 livers per tank with *Direct-zol™ RNA Miniprep kit* (Zymo Research, Irvine, USA). Around 200 mg liver was transfer into a 2 mL *Lysing Matrix D tube* (MP biomedicals, Irvine, USA) with 1 mL *TRI Reagent* (Zymo). Then the tissue was homogenized in *Precellys 24 homogenizer* (Bertin Instruments, Montigny-le-Bretonneux, France) at 4 000 rpm for 10 second. Then the 200 µL mixture was transferred to a new 2 mL tube with 300 µL *TRI Reagent*. 500 µL ethanol was added to sample lysed in *TRI Reagent*. After thoroughly mixing, the mixture was transferred into a *Zymo-Spin™ IICR Column* in a Collection Tube and centrifuge. Afterwards, *Direct-zol™ RNA PreWash*, RNA Wash Buffer and RNase-Free Water was added to the column based on the instruction from manufacturer. The quality and quantity of RNA was checked in *2200 Tapestation* (Agilent, Santa Clara, USA) with *High Sensitivity RNA ScreenTape* (Agilent). 200 ng RNA was used for RNA library preparation using *NEXTFLEX Small RNA-Seq Kit*. After the ligation and first strand synthesis according to the instruction of

manufacturer, purified first strand synthesis product was mixed with universal primer, barcode primer and PCR master mix for amplification in thermal cycler (Bio-rad) according to following condition: 95 °C for 2 min, followed by 17 cycles at 95°C for 20 sec, 60°C for 30 sec, 72°C for 15 sec, and a final extension step of 2 min at 72 °C. Unique Barcode primer was assigned to each sample. PCR product around 160 bp was selected in 6% TBE-PAGE gel (Thermofisher). Afterwards, selected DNA was quantified and qualified in *2200 Tapestation* (Agilent) with *High Sensitivity D1000 ScreenTape* (Agilent). 15 nmol libraries that contained same amount of library from each sample was sequenced in *NextSeq 500* (illumina, San Diego, USA) with *NextSeq 500/550 Kit* (illumina).

2.12 Statistical analysis

Unless otherwise specified, data was shown as mean ± S.D. SPSS 20.0 for Windows (IBM, New York, USA) was used in data analysis. Boxplot graph was used for detecting outliners, Shapiro-Wilk analysis for the normality and Levene's test for the homogeneity of variance for the data. Once these assumptions were met, two-way ANOVA analysis was then used for the analysis of the effect of broodstock *fads2* expression and nutritional programming on the progenies. Then independent-Sample T-test or simple main effect analysis and the pairwise comparison were used to compare the difference between the groups. In the research on effects nutritional programming through dietary vegetable proteins and lipids on broodstock and on embryogenesis, the comparison of the data between different developmental stages was analysed by One-way ANOVA. *Fads2* expression data between groups were analysed by Independent-Samples T-test. Pearson correlation was performed through *SPSS 20.0* (IBM). ClusterW alignment between *fads2* promoter region of each broodstock was performed through *BioEdit 7.2* (Tom Hall). RNA library was demultiplexed based on barcode, then the adapter sequence was move adapters and random nucleotides at 3' and 5' end map trimmed reads to gilthead seabream genome (Genome assembly: GCA_900880675.1). MicroRNAs expression was identified using *miRDeep2*. Reads were counted based on mature miRNA based on all Teleostei species. Differential expression was analyzed using *limma-voom* in R.

Chapter 3

Nutritional intervention through dietary vegetable proteins and lipids to gilthead sea bream (*Sparus aurata*) broodstock affect the offspring utilization on low fishmeal fish oil diet

Hanlin **Xu,** Serhat **Turkmen**, Simona **Rimoldi**, Genciana **Terova**, Maria Jesus **Zamorano**, Juan Manuel **Afonso**, Samira **Sarih** and Hipólito **Fernández-Palacios**, Marisol **Izquierdo**

Published at Aquaculture Journal (https://doi.org/10.1016/j.aquaculture.2019.734402)

Impact factor 2019 (JCR): 3.225

Abstract

Nutritional intervention with vegetable oils (VO) supplemented to fish broodstock enhances progeny ability to utilize low fish meal (FM) and fish oil (FO) diets. Therefore, intervention with vegetable meals (VM), alone or in combination with VO could also be a useful nutritional programming strategy to obtain progenies that are better prepared to use low FM and FO. This study aims to determine the effect of FM replacement by VM alone or in combination with FO replacement by VO as a programming tool. Different broodstocks of gilthead sea bream were fed one of three diets: one high contained 35% FM and 10% FO, another one supplemented with VM in replacement of FM (FM: 15%) or a third one supplemented with both VM and VO in replacement of FM and FO (15% FM and 2.7% FO). At the weight of 3 g, their offspring were challenged with a low FM and FO diet for 45 days. Spawning performance of broodstock and growth performance of offspring juveniles were evaluated. FM replacement by VM in broodstock diets did not affect fish reproductive performance but altered offspring fatty acids profiles, reduced the SGR and feed efficiency of progeny juveniles and down-regulated *fatty acyl desaturase 2*(*fads2*) expression in liver. Combined supplementation of VM and VO in broodstock diets led to a poor reproductive performance, reducing female fecundity and the egg content in eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA). At molecular level, the expression of *fads2* and *target of rapamycine (tor)* genes in the eggs were upregulated, similarly, *fads2* expression was upregulated in liver of juvenile progenies. Nutritional programming by VM supplementation in replacement of FM in gilthead sea bream broodstock negatively affects growth performance of juvenile progenies, whereas the VO supplementation in replacement of FO up-regulates gene expression of key enzymes for PUFA biosynthesis.

Keywords: Nutritional programming, Reproductive performance, Offspring performance, Fatty acyl desaturase, Plant ingredient utilization

3.1 Introduction

Aquatic products are crucial for providing high-quality food and fighting against hunger. However, since the production of fisheries remains stagnant for decades, aquaculture industry is taking the responsibility to increase aquatic food production. In 2014, the consumption of aquatic production from aquaculture had already overpassed that from wild capture (Nations, 2018). The development of aquaculture, especially marine species, is restricted by the usage of fishmeal (FM) and fish oil (FO) since these two important but limited dietary ingredients come from fisheries. Plant resources are frequently used in replacement of dietary FM and FO because of their low price and sustainability. However, high level of FM replacement by VM leads to reduced growth, harmed digestive activity, lipid accumulation in liver and oxidative stress (Sitjà-Bobadilla *et al.*, 2005; Santigosa *et al.*, 2008) and is associated with the down-regulation of protein biosynthesis-related genes in liver (Panserat *et al.*, 2008), such as suppression of rapamycin (*tor*) signal pathway (Xu *et al.*, 2017). Given that the availability of n-3 long chain polyunsaturated fatty acid (LC-PUFA) is limited in vegetable oils, the use of VO in marine teleost feeds can lead to deleterious effects on growth and basic physiological functions (Vergara *et al.*, 1996b; Benitez-Santana *et al.*, 2012; Zuo *et al.*, 2012a; Benitez-Santana *et al.*, 2014) and reproductive performance (Jaya-Ram *et al.*, 2008). Most of marine fish species have the restricted capability for essential fatty acids (EFA) biosynthesis of from their 18C precursors, which can be abundant in vegetable oils (Kanazawa *et al.*, 1979), in relation to a very limited activity of the fatty acyl desaturase 2 (Fads2) (Li *et al.*, 2014). Therefore, combined replacement of both FM and FO by VM and VO in diets for marine fish frequently affects negatively growth performance and health (Torrecillas *et al.*, 2017b; Torrecillas *et al.*, 2017c; Torrecillas *et al.*, 2017a) , affecting not only lipid and protein

---

Abbreviation: FM, fish meal; FO, fish oil; VM, vegetable meal; VO, vegetable oil; fatty acyl desaturase 2, Fads2; eicosapentaenoic acid, EPA; docosahexanoic acid, DHA; target of rapamycine, tor; essential fatty acids, EFA; Fatty acid methyl esters, FAMES; LC-PUFA, long chain polyunsaturated fatty acid; Specific growth rate, SGR; feed conversion ratios (FCR); elovl6, elongation of very long chain fatty acids protein 6; srebp1, sterol regulatory element-binding transcription factor 1; g6pase, glucose 6-phosphatase; Igf-1, Insulin-like growth factor 1

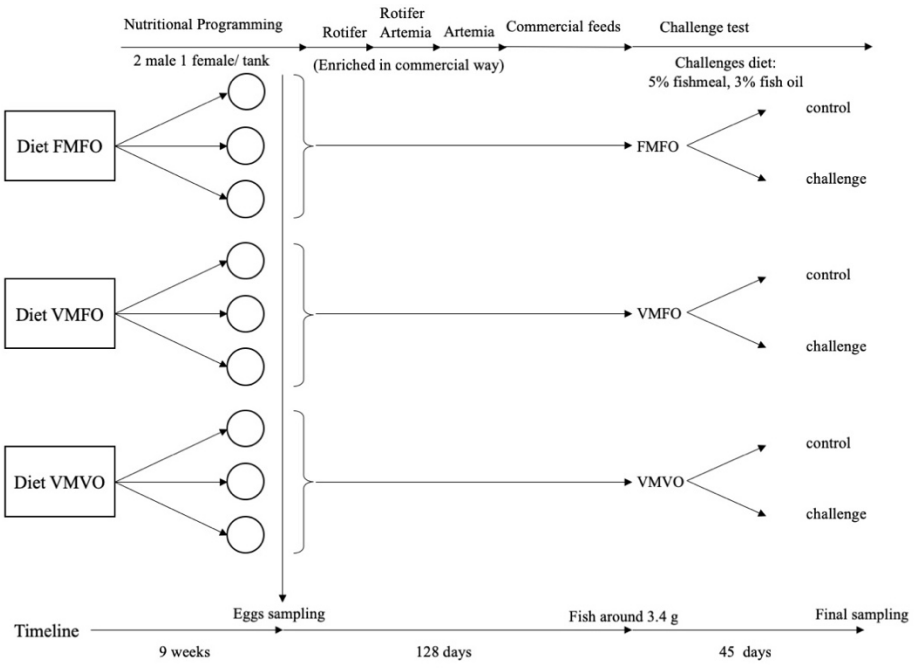
metabolism-related genes, but also glucose metabolism (Panserat *et al.*, 2009). Thus, new strategies are required to overcome the disadvantages derived from FM and FO replacement.

Nutritional programming implies the induction of nutritional stimuli during critical stages of development that lead to permanent changes in function or structure of tissues and organs later in life (Lucas, 1994; 1998), altering cell differentiation and affecting mechanisms of homeostatic control (Gluckman and Hanson, 2004). For example, in rats, protein restriction during pre- and postnatal periods leads to the reduction of plasma cholesterol, HDL-cholesterol and triacylglycerol concentration in adult life (Lucas *et al.*, 1996). It was also shown very early in rats that maternal protein restriction can affect the fatty acid biosynthesis pathway, reducing Fads2 activity in fetal liver (Mercuri *et al.*, 1979). Besides protein, a dietary deficiency in omega-3 fatty acid during gestation and postnatal development restricts the deposition of docosahexanoic acid (DHA) in the retina and cerebral cortex in Rhesus monkeys (*Macaca mulatta*) by the age of 22 months and prolong the dark-adapted electroretinogram after a saturating flash (Neuringer *et al.*, 1986). N-3 LC-PUFA in parental diet of rats also affects the adult offspring aortae (Kelsall *et al.*, 2012) or hepatic *fads2* expression by the modifying methylation status in *fads2* promoter (Hoile *et al.*, 2013).

In fish, parental feeding seems to be very effective for nutritional programming (Geurden *et al.*, 2013; Fang *et al.*, 2014; Izquierdo *et al.*, 2015). When gilthead seabream (*Sparus aurata*) broodstock were fed a diet with 60% replacement of FO by linseed oil, the progenies showed a better ability to utilize low FM and FO diets (Izquierdo *et al.*, 2015). This nutritional programming through parental feeding persisted even after 16 months, when fish were on the verge of reproduction with modulation of hepatic lipid metabolism (Turkmen *et al.*, 2017a). It was shown in rainbow trout that nutritional programming during early feeding with a VM and VO-based diet improves acceptance and utilization of the same diet at later life stages (Geurden *et al.*, 2013). More recently, first exogenous feeding of Atlantic salmon (*Salmo salar*) with a plant-based diet for 3 weeks leads to a higher growth rate and better feed efficiency in 15 weeks fish challenged with 0% FO diet for 6 weeks (Clarkson *et al.*, 2017). However, there are no studies on the potential nutritional programming effect of feeding VM or VM and VO feeds in

gilthead sea bream. In this species, nutritional programming applied at early feeding is constrained by the increase mortality along on-growing periods (Turkmen *et al.*, 2017a) while nutritional stimuli during broodstock feeding seems more effective (Izquierdo *et al.*, 2015). Thus, the main aim of the present study was to determine the effect of FM replacement by vegetable meals (VM) alone or in combination with FO replacement by vegetable oils (VO) in broodstock diets for gilthead sea bream as a nutritional tool to modify gene expression and improve utilization of VM and VO diets in the progeny (Figure 3.1).

Figure 3.1 Schematic view of the experimental design of experiment



FMFO: 35% FM and 9.6% FO; VMFO: 15% FM and 10.9% FO; VMVO:15% FM and 2.7% FO. Control: 20% FM and 6% FO; Challenge: 5% FM and 3% FO

3.2 Material and methods

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

3.2.1 Nutritional intervention in the broodstock

3.2.1.1 Diets

Three isoproteic and isolipidic diets were formulated to contain approximately 55% protein, 20% lipid and 5% ash (% dry weight) (Table 3.1). The amino acid profile and the fatty acid profile is shown in Table 3.1 and 3, respectively. Diet FMFO contained 35% FM and 9.6% FO and diet VMFO contains 15% FM by the replacement with VM, whereas the content of FO was adjusted to maintain the contents in essential fatty acids. Diet VMVO, FM contents were also kept at 15%, while FO was replaced by a combination of VO that kept similar proportions of saturated, monounsaturated, n-3 and n-6 fatty acids, while reducing LC-PUFA and increasing their 18C precursors (Table 3.3). In a previous study, it was shown that this level did not affect the reproductive performance (Izquierdo *et al.*, 2015).

3.2.1.2 Experimental animals and environmental conditions

Gilthead sea bream broodstock (body weight (mean ± S.E.M): 1736.30 ± 440.80 g males, 1757.73 ± 430.03 g females) were randomly distributed into 12 tanks (1000 L) at a 2:1 ratio male:female. Tanks were supplied with 16L/min seawater at a 19.4 ± 0.5 C° and a 7.98 ± 0.10 pH and kept under natural light photoperiod (11-13 h light). During the first 10 spawns brood fish were fed a commercial diet (Skretting ARC, Stavanger, Norway) to ensure that there were no differences in egg production and quality among different broodstock groups. Afterwards, fish were daily fed 1% biomass with the experimental diets in triplicate tanks for 9 weeks (Table 3.1).

3.2.1.3 Egg collection and spawning performance evaluation

Spawning performance and egg quality were controlled 5 times per week for 63 days following procedures described earlier (Fernández-Palacios *et al.*, 2011). Live egg and larvae were counted after 24h, the day of hatch and 3 days after hatch to calculate hatching rate and larvae survival rates.

Table 3.1 Main ingredients, protein, lipid and ash composition of gilthead seabream parental diets during the nutritional stimulus

Ingredients (%)	FMFO	VMFO	VMVO
Fish meal, N. Atlantic[1]	35.0	15.0	15.0
Corn gluten[2]	5.0	10.0	10
Faba beans[1]	5	10	10
Wheat[1]	12.5	8.0	8.0
Wheat gluten[1]	11.4	18.4	18.4
Soy protein concentrate[1]	18.3	25	25
Fish oil, S. American[1]	9.6	10.9	2.7
Linseed oil[3]	0	0	1.2
Palm oil[3]	0	0	2.5
Rapeseed oil[1]	0	0	4.5
Premix[4]	2.8	2.8	2.8
Proximate analysis (% DM)			
Protein	55.2	54.7	54.9
Lipids	19.0	20.8	21.3
Ash	6.5	5.0	6.1
Amino acid composition (%)			
ARG	3.4		3.0
HIS	1.3		1.2
ILE	2.1		2.1
LEU	3.2		3.9
LYS	2.9		2.3
MET	1.1		0.9
CYS	0.6		0.6
PHE	2.4		2.5
TYR	1.8		1.8
THR	1.9		1.7
TRP	0.6		0.6
VAL	2.5		2.4

1 Skretting, Stavanger, Norway;2 Cargill Nordic AS, Charlottenlund, Denmark; 3 AAK AB, Karlshamn, Sweden;4 Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins and minerals; Vitamin and mineral supplementation as estimated to cover requirements according NRC (2011).

In order to determine dietary effects, eggs of same group from the same day were cultured using a common protocol (Izquierdo *et al.*, 2015) for 128 days until fish reached a body weight of 3 g for the nutritional challenge test.

3.2.2 Nutritional challenge of juvenile offspring

3.2.2.1 Diets

Offspring from broodstock fed the different diets were challenged by feeding them either a control diet (20% FM and 6% FO) or a challenge diet (5% FM and 3% FO) (Table 3.2). In the challenge diet, soy protein concentrate, rapeseed meal and wheat were used to replace FM and a mixture of rapeseed, linseed and palm oil was used to replace FO. Accordingly, the challenge diet was lower in LC-PUFA and higher in the precursors 18:3n-3 and 18:2n-6 (Table 3.3). Diets were isoenergetic, isonitrogenous and contained 45% protein, 22% lipid (% dry weight) (Table 3.2).

Table 3.2 Main ingredients, protein, lipid and ash composition of nutritional challenge diets for gilthead seabream juvenile offspring

Ingredients (%)	Control	Challenge
Fish meal[1]	20	5
Blood meal (spray-dried)[2]	5	7
Soya protein concentrate[3]	14.2	20
Corn gluten meal[4]	14	22
Wheat gluten[4]	5	5.5
Rapeseed meal[5]	13	11.3
Wheat[6]	10	6.89
Fish oil[7]	6	3
Rapeseed oil[5]	4	5.2
Linseed oil[8]	2	2.6
Palm oil[4]	4	5.2
Supplemented ingredients[9]	1.98	5.49
Vitamin and mineral premix[10]	0.75	0.75
Antioxidant[11]	0.05	0.05
Yttrium oxide	0.03	0.03
Proximate analysis (% DM)		
Protein	46.2	46.6
Lipids	23.6	24.0
Ash	6.2	5.4

1 South-American, Superprime – Feed Service Bremen, Germany. 2 Daka, Denmark 3 Svane Shipping, Denmark 4 Gargill, Netherlands 5 Emmelev, Denmark 6 Hedegaard, Denmark 7 South American fish oil, LDN Fish Oil, Denmark 8 Daudruy, France 9 Contains lysine, methionine, monocalcium phosphate, choline, inositol, phospholipids (Emulthin G35). Vilomix (Denmark), Evonik Industries (Germany), Pöhner (Germany) 10 Supplied the following vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany) 11 BAROX BECP, Ethoxyquin, Vilomix (Denmark)

3.2.2.2 Experimental conditions

To determine the effect of the nutritional stimulus through the broodstock diet on the ability of the offspring juveniles to utilize low FO and FM diets, six hundred juveniles (mean weight: 3.36 ± 0.48 g), produced from each group of broodstock as described in 2.1.4, were randomly and equally distributed into 6x200 L tanks and fed either the Control or the Challenge diet in triplicate tanks. Fish were fed to apparent satiation 4 times per day (8:30, 10:30, 13:30 and 16:30) for 45 days until fish body weight was triplicated. Tanks were provided with 200 L/h seawater at 23.3 ± 0.3°C and strong aeration under artificial light-controlled photoperiod (12 h light). At the end of the trial, fish were fasted for 24 h and anesthetized with eugenol and methanol (50:50) 10 ppm in sea water before samplings.

3.2.3 Analysis

3.2.3.1 Chemical composition and fatty acid analysis

Diets, spawned eggs and whole body and liver of juveniles were analysed for chemical and fatty acid composition. Moisture, crude protein (CUNIFF, 1995) and crude lipid (Folch *et al.*,

1957) contents of eggs, larvae, juveniles and diets were analysed. Fatty acid methyl esters (FAMES) were obtained by trans-methylation of crude lipids as previously described (Christie, 1982). FAMES were separated using gas chromatography (GC-14A; Shimadzu)

Table 3.3 Fatty acid composition of diets for gilthead seabream broodstock during the nutritional stimulus and juveniles during the nutritional challenge gilthead seabream

Fatty acid	Broodstock			Juvenile	
(% of total fatty acid)	FMFO	VMFO	VMVO	Control diet	Challenge diet
14:0	5.94	5.87	2.21	2.86	1.44
14:1n-7	0.03	0.03	0.01	0.02	0.01
14:1n-5	0.24	0.27	0.08	0.08	0.04
15:0	0.49	0.49	0.19	0.21	0.11
15:1n-5	0.04	0.04	0.01	0.00	0.01
16:0ISO	0.10	0.10	0.03	0.04	0.02
16:0	17.97	18.61	17.00	18.01	17.65
16:1n-7	6.22	6.23	2.21	2.97	1.37
16:1n-5	0.28	0.28	0.09	0.14	0.06
16:2n-6	0.01	0.01	0.00	0.01	0.00
16:2n-4	0.77	0.75	0.25	0.40	0.17
17:0	0.79	0.79	0.25	0.50	0.21
16:3n-4	0.21	0.18	0.11	0.10	0.07
16:3n-3	0.23	0.27	0.07	0.05	0.02
16:3n-1	0.11	0.10	0.01	0.01	0.00
16:4n-3	1.22	1.19	0.40	0.63	0.26
16:4n-1	0.01	0.00	0.00	0.00	0.00
18:0	3.35	3.46	3.18	3.15	2.96
18:1n-9	10.73	11.11	30.83	31.59	39.60
18:1n-7	2.49	2.55	2.02	2.36	1.96
18:1n-5	0.14	0.12	0.07	0.06	0.04
18:2n-9	0.07	0.09	0.02	0.06	0.02
18:2n-6	9.31	12.20	18.82	14.85	18.34
18:2n-4	0.24	0.25	0.09	0.15	0.07
18:3n-6	0.22	0.21	0.07	0.07	0.03
18:3n-4	0.15	0.15	0.05	0.09	0.04
18:3n-3	1.46	1.48	7.84	8.33	9.39
18:3n-1	0.02	0.02	0.02	0.02	0.01
18:4n-3	2.34	2.15	0.78	0.67	0.26
18:4n-1	0.15	0.14	0.05	0.08	0.03
20:0	0.32	0.33	0.43	0.38	0.39
20:1n-9	0.28	0.22	0.12	0.08	0.05
20:1n-7	2.80	1.99	1.60	0.83	0.69
20:1n-5	0.26	0.28	0.10	0.10	0.05
20:2n-9	0.05	0.06	0.02	0.05	0.02
20:2n-6	0.15	0.23	0.11	0.08	0.05
20:3n-9	0.08	0.07	0.03	0.03	0.01
20:3n-6	0.09	0.10	0.03	0.05	0.02
20:4n-6	0.80	0.79	0.27	0.43	0.18
20:3n-3	0.10	0.06	0.03	0.03	0.02
20:4n-3	0.55	0.53	0.18	0.21	0.08
20:5n-3	11.22	10.71	3.72	5.13	2.10
22:1n-11	3.93	2.50	1.65	0.53	0.31
22:1n-9	0.42	0.33	0.27	0.15	0.14
22:4n-6	0.09	0.09	0.03	0.05	0.02
22:5n-6	0.07	0.07	0.02	0.12	0.04
22:5n-3	1.23	1.21	0.40	0.60	0.25
22:6n-3	12.23	11.29	4.21	3.64	1.38
Saturated	28.86	29.55	23.26	25.15	22.78
Mono-unsaturated	27.86	25.95	39.06	38.91	44.33
Poly-unsaturated	43.18	44.40	37.63	35.94	32.88
∑ n-3	30.58	28.89	17.63	19.29	13.76
∑ n-6	10.74	13.70	19.35	15.66	18.68
∑ n-9	11.63	11.88	31.29	31.91	39.82
DHA/EPA	1.09	1.05	1.13	0.71	0.66

following the conditions described previously (Izquierdo, 1990) and identified by comparison with previously characterized standards and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific).

3.2.3.2 RNA extraction and digital PCR

Eggs from broodstock fed with different diets and the liver from the offspring juveniles after the nutritional challenge were kept at 4ºC overnight in 1.5 mL tubes with 5 times of RNAlater and then transferred to -80C° until themolecular analyse. RNA from 100 mg sample was extracted according to TRI Reagent (sigma, USA) instructions and then purified by RNeasy kit (Qiagen, Germany). RNA quality was checked by 1.4% agarose electrophoresis and quantity was measured by NanoDrop 1000 (Thermo, U.S.). cDNA was synthesised from 1 μgof RNA in iCycler (Bio-rad, U.S.), using the iScript cDNA synthesis kit (Bio-Rad, U.S.). The expression of key enzymes of lipid, carbohydrate and protein metabolism (*fads2*, *elongation of very long chain fatty acids protein 6* (*elovl6*), *sterol regulatory element-binding transcription factor 1* (*srebp1*), *glucose 6-phosphatase* (*g6pase*), *tor* and *insulin-like growth factor 1* (*igf-1*)) (Enes *et al.*, 2008; Benedito-Palos *et al.*, 2014; Velez *et al.*, 2014; Betancor *et al.*, 2016; Turkmen *et al.*, 2017a) was determined using the primers listed in Table 3.4. Digital PCR was performed using in QX200™ Droplet Digital™ PCR System (Bio-rad, U.S.). The reaction mixture (20 μL), containing 100 ng of cDNA, primers and Evagreen SuperMix (Bio-rad), was loaded to droplet generator to generate the oil droplets and then proceeded in C1000 TOUCH^{TM} thermal cycler (Bio-Rad, U.S.). The amplification conditions of PCR were: 95 C° for 5 min, followed by 40 cycles at 95C° for 30 sec, elongation at Tm temperature for 1min, and then stabilized the signal at 4 C° for 5 min, 90C° for 5 min, finally the reaction was hold at 4C°. The PCR amplification was read in QX200 droplet reader (Bio-Rad, U.S.) and droplets were counted to provide absolute quantification of target transcript in digital form using QuantaSoft Software (Bio-Rad, U.S.).

Table 3.4 Primers sequence for digital PCR and GeneBank accession numbers and reference articles for sequences of target genes

Gene	Forward	Reverse	GenBank accession
ß-actin	TCT GTC TGG ATC GGA GGC TC	AAG CAT TTG CGG TGG ACG	X89920
fads2	CGA GAG CCA CAG CAG CAG GGA	CGG CCT GCG CCT GAG CAG TT	AY055749
elovl6	GTG CTG CTC TAC TCC TGG TA	ACG GCA TGG ACC AAG TAG T	JX975702
srebp1	AGG GCT GAC CAC AAC GTC TCC TCT CC	GCT GTA CGT GGG ATG TGA TGG TTT GGG	JQ277709
g6pase	CGC TGG AGT CAT TAC AGG CGT	CAG GTC CAC GCC CAG AAC TC	AF151718
tor	CAG ACT GAC GAG GAT GCT GA	AGT TGA GCA GCG GGT CAT AG	unpublished
Igf-1	GTG TGT GGA GAG AGA GGC TT	CTC TTG GCA TGT CTG TGT GG	AY996779.2

3.2.3.3 Genomic DNA extraction

Genomic DNA was extracted from 3 liver samples per experimental group (1 sample per tank) using a manual method including RNase I treatment. Briefly 35 mg of tissue was digested in 350 µl of lysis buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl 1% SDS at pH8, containing 8 µl of Proteinase-K (20 mg/ml)) at 55 °C overnight with gentle shaking. 125 µl of 5 M NaCl was then added to each sample. Samples were vortexed for 30 s at maximum speed and centrifuged for 10 min at 13 000 rpm. The supernatant was transferred to new tubes and 250 µL of cold isopropanol was added to each sample. Samples were then centrifuged for 5 min at 13 000 rpm at 4°C. The pellet was twice washed with 75% ethanol, dried, and finally suspended in 100 µL of 5 mM Tris-HCl pH 8.5.

3.2.3.4 Methylation-specific PCR (MSP)

Genomic DNA (1.5 µg) from three fish per experimental group was firstly acid-catalyzed converted with bisulfite using the EpiTect Bisulfite Kit (Qiagen, Germany) and stored at −20 °C until further analysis. The converted DNA was then amplified by PCR using two pairs of primers, with one pair specific for methylated DNA (M pair) and the other for unmethylated DNA (U pair).

The CpG-rich regions of the *fads2* promoter were identified from sea bream genome (Pauletto *et al.*, 2018) using MethPrimer (plus CpG Island Prediction, http://www.urogene.org/cgi-bin/methprimer2/MethPrimer.cgi Two regions of 215 bp and 232 bp, compromised between −676 and −891 (region 1 or proximal promoter) and between −3977 and −3744 (region 2 or

distal region), respectively (adenine of ATG was numbered +1) were amplified by PCR from 30 ng of bisulfite converted DNA template. The PCR reactions were performed using Taq DNA polymerase (Qiagen, Germany) supplied with Q-solution and primer sets reported in Table 3.5. Thermocycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94°C for 1 min, 50°C (or 54°C) for 1 min, 72°C for 1 min, and a final extension step of 10 min at 72 °C.

Table 3.5 Sequences of primers used for Methylation-specific PCR (MSP)

Primer name	Nucleotide sequence	Tm
Left M1	TTGGTTTAGAAATTTTCGAATGATC	59.76
Right M1	AATTTTCGACTACTCCTCTAATCGA	58.94
Left U1	TGGTTTAGAAATTTTTGAATGATTG	58.59
Right U1	ATTTTCAACTACTCCTCTAATCAAA	54.94
Left M2	TAAATAGGGAGATAGGTTGTACGT	55.43
Right M2	TCCATATATTTCCATAATTATCGAA	56.06
Left U2	TAAATAGGGAGATAGGTTGTATGT	53.26
Right U2	TTCCATATATTTCCATAATTATCAAA	55.39

3.2.4 Data analysis

Results were presented as means ± S.E.M and analyzed by SPSS 20.0 (IBM, America) for Mac. All data were analyzed by Levene test (p >0.05) and, if variances were homogeneous, compared through one-way ANOVA. Data from offspring obtained from broodstock fed the same diet but challenged with one of the two diets for juveniles were compared thought Independent-Samples T-test. Two-way ANOVA was used to test the interaction between the broodstock diet and juvenile challenge diet in the juvenile nutritional challenge experiment. The level of significance was chosen at $p < 0.05$ in Dunkan's multiple range analysis to test the differences among the means.

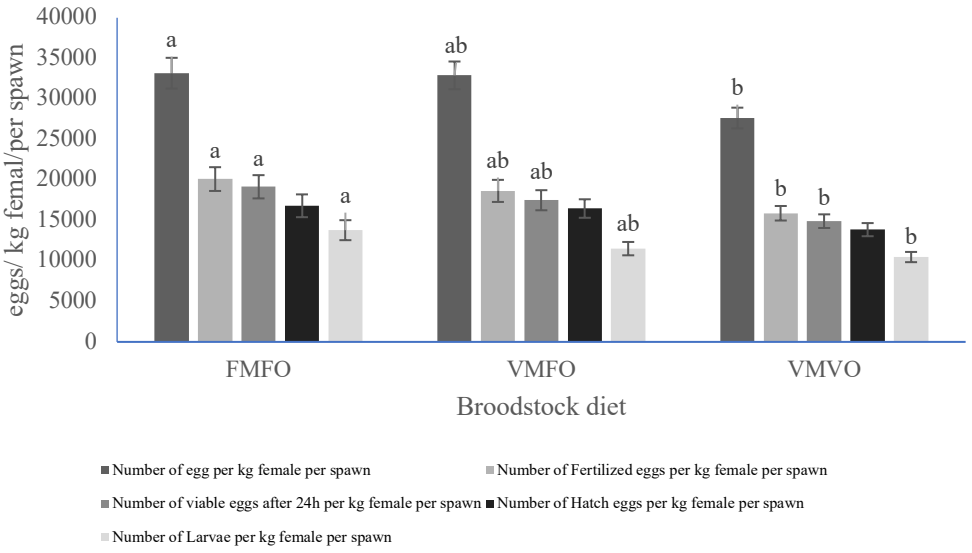
3.3 Results

3.3.1 Nutritional stimulus through broodstock feeding

The experimental diets were well accepted by the different broodstock groups and, accordingly, daily feeding rations were always consumed. Although no significant differences were found in spawning quality of broodstock fed the same diet during the preliminary first 10 spawns (34059 ± 3418 per kg female per spawning, n=10), the spawning performance decreased significantly in the broodstock fed the VMVO diet compared to the FMFO one (Figure 3.2). Thus, all parameters of spawning performance except the number of hatched

eggs were significantly lower for broodstock fed VMVO than for those fed FMFO (Figure 3.2). However, spawning performance in broodstock fed VMFO was intermediate and no significant differences were found with broodstock fed either FMFO or VMVO. No difference was found in the rate of fertilization, viable eggs, hatching and survival (Table 3.6).

Figure 3.2 Spawning performance of broodstock after 63 days feeding diets containing different FM and FO replacement levels by VM and VO



Bars bearing with different letter showed significantly difference by Duncan's test ($p < 0.05$).

Table 3.6 Rate of spawning performance parameter of gilthead seabream broodstock during 63 days fed different diets in spawning season containing different FM and FO replacement levels by VM and VO during the nutritional stimulus

	FMFO		VMFO		VMVO	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
Fertilization rate	63.78	2.75	57.91	3.82	56.63	2.99
Viable rate	59.63	2.72	53.97	3.58	53.65	2.81
Hatching rate	49.65	3.26	50.21	3.36	49.39	2.82
3 dah survival rate	38.20	2.73	38.91	2.88	39.00	2.50

Fertilization rate: number of fertilized eggs per spawn per kg female/ total number of eggs per spawn per kg female; Viable rate: number of viable eggs 24 hours after spawn per spawn per kg female/ total number of eggs per spawn per kg female; Hatching rate: number of hatched eggs per spawn per kg female/ total number of eggs per spawn per kg female; 3 dah survival rate: number of survived larvae 3 days after hatching per spawn per kg female/ total number of eggs per spawn per kg female

There were no significant differences in protein, lipid or ash contents of the egg from broodstock fed the three different diets (Table 3.7). In general, the fatty acid composition of eggs (Table 3.8) reflected fatty acid composition of the diets given to the broodfish (Table

3.3). The substitution of dietary FM by VM significantly (p<0.05) increased 18:2n-6 and 18:2n-9 in agreement with dietary values but reduced the egg contents in 16:0 and 18:1n-9, despite their increase in the diet. Besides, slight (around 10%) reduction in LC-PUFA such as EPA and DHA in VMFO diet did not significantly affected the content of these fatty acids in the egg. Combined substitution of both FM and FO by VM and VO, in agreement with the dietary changes, increased the egg contents in 18:1n-9, 18:2n-6 and 18:3n-3, substrates for Fads2, and reduced saturated (14:0, 15:0, 16:0), monounsaturated (16:1, 16:3, 18:1n-7, 18:1n-5, 20:1, 22:1) and polyunsaturated fatty acids (18:3n-6, 18:3n-4, 18:4n-3, 18:4n-1, 20:3n-9, 20:4n-6, 20:4n-3, 20:5n-3, 22:4n-6, 22:5n-6, 22:5n-3, 22:6n-3). However, the levels of 18:4n-3, 18:2n-9, products of fads2, 20:2n-6 and 20:3n-3, products of elongation from 18:2n-6 and 18:3n-3, significantly (p<0.05) increased in VMVO eggs in comparison to FMFO eggs.

Table 3.7 Chemical composition of the eggs from gilthead seabream broodstock after 63 days fed different diets in spawning season containing different FM and FO replacement levels by VM and VO during the nutritional stimulus

(% in dry matter)	FMFO	VMFO	VMVO
Protein	71.02±3.74	64.54±3.08	66.98±4.58
Lipids	25.64±1.69	25.59±1.32	26.25±1.20
Ash content	16.80±1.74	15.56±3.63	12.17±3.42

No significant difference was observed between the groups.

FM replacement by VM in broodstock diets did not significantly affect *fads2* or *tor* gene expression in VMFO eggs in comparison to FMFO eggs (Figure 3.3). The combined substitution of both FM and FO by VM and VO significantly increased (p< 0.05) the expression of *fads2* gene in VMVO eggs. Besides, *tor* gene expression of the VMVO eggs was significantly higher (p< 0.05) than in VMFO eggs (Figure 3.3).

3.3.2 Nutritional challenge of juvenile offspring

The effect of nutritional programming through broodstock feeding during the spawning season was assessed in the offspring through the juvenile nutritional challenge trial (Table 3.9). Among offspring fed the control diet (20%FM, 6%FO), final weight of VMFO juveniles was lower (p< 0.05) than that from FMFO fish. The specific growth rate (SGR) showed the same tendency (p< 0.05), whereas no significant differences were found in the feed conversion ratios (FCR) (Table 3.9). However, among juveniles fed the challenge diet, the final weight of VMVO

juveniles was lower ($p< 0.05$) than FMFO group and FCR was higher ($p< 0.05$). Regarding SGR, the values from VMVO juveniles and those from VMFO juveniles

Table 3.8 Fatty acid composition of eggs from gilthead seabream broodstock after 63 days feeding diets containing different FM and FO replacement levels by VM and VO during the nutritional stimulus (% total identified fatty acids)

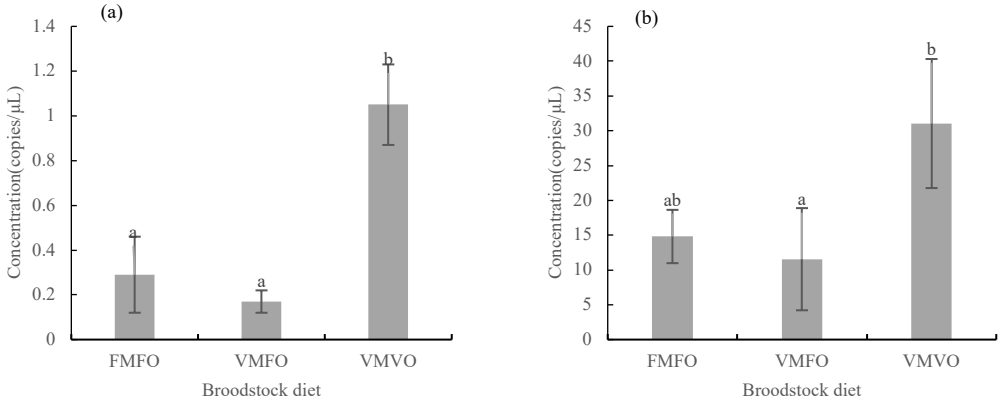
	FMFO		VMFO		VMVO	
Fatty acid (% of total fatty acid)	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
14:0	3.96^{a}	0.06	3.70^{a}	0.09	2.14^{b}	0.09
14:1n-7	0.02	0.00	0.02	0.00	0.01	0.00
14:1n-5	0.17^{a}	0.01	0.18^{a}	0.01	0.08^{b}	0.00
15:0	0.40^{a}	0.01	0.38^{a}	0.01	0.23^{b}	0.01
15:1n-5	0.04^{a}	0.00	0.04^{a}	0.00	0.02^{b}	0.00
16:0ISO	0.08^{a}	0.00	0.08^{a}	0.00	0.04^{b}	0.00
16:0	18.22^{a}	0.21	16.85^{b}	0.34	15.90^{c}	0.19
16:1n-7	6.38^{a}	0.08	6.37^{a}	0.11	3.40^{b}	0.30
16:1n-5	0.13^{a}	0.01	0.12^{a}	0.01	0.08^{b}	0.01
16:2n-4	0.47^{a}	0.02	0.51^{a}	0.01	0.21^{b}	0.01
17:0	0.35^{b}	0.02	0.40^{a}	0.01	0.17^{c}	0.01
16:3n-4	0.27^{a}	0.01	0.24^{b}	0.01	0.19^{c}	0.01
16:3n-3	0.14^{a}	0.01	0.13^{a}	0.01	0.07^{b}	0.00
16:3n-1	0.09	0.01	0.08	0.00	0.07	0.00
16:4n-3	0.06^{b}	0.00	0.05^{b}	0.00	0.09^{a}	0.01
18:0	4.10	0.18	3.98	0.10	3.86	0.29
18:1n-9	17.34^{b}	0.89	16.82^{b}	0.61	28.47^{a}	0.59
18:1n-7	3.18^{a}	0.04	3.08^{a}	0.05	2.33^{b}	0.06
18:1n-5	0.16^{a}	0.01	0.14^{a}	0.00	0.10^{b}	0.02
18:2n-9	0.11^{c}	0.01	0.15^{b}	0.01	0.24^{a}	0.01
18:2n-6	10.03^{c}	0.34	12.62^{b}	0.12	17.84^{a}	0.61
18:2n-4	0.04^{a}	0.01	0.05^{a}	0.02	0.09^{b}	0.02
18:3n-6	0.24^{a}	0.01	0.27^{a}	0.01	0.11^{b}	0.01
18:3n-4	0.20^{a}	0.01	0.23^{a}	0.00	0.11^{b}	0.01
18:3n-3	1.41^{b}	0.06	1.51^{b}	0.14	5.50^{a}	0.43
18:4n-3	1.08^{a}	0.06	1.19^{a}	0.04	0.53^{b}	0.01
18:4n-1	0.20^{a}	0.01	0.22^{a}	0.01	0.12^{b}	0.01
20:0	0.16	0.04	0.12	0.02	0.14	0.02
20:1n-9	0.18^{a}	0.01	0.14^{b}	0.00	0.10^{c}	0.00
20:1n-7	0.83^{a}	0.01	0.66^{b}	0.03	0.70^{b}	0.01
20:1n-5	0.17^{a}	0.01	0.17^{a}	0.00	0.10^{b}	0.00
20:2n-9	0.04	0.00	0.05	0.00	0.07	0.01
20:2n-6	0.25^{b}	0.01	0.28^{b}	0.01	0.40^{a}	0.02
20:3n-9	0.06^{a}	0.00	0.06^{a}	0.00	0.02^{b}	0.00
20:3n-6	0.13	0.00	0.14	0.01	0.15	0.01
20:4n-6	0.93^{a}	0.06	0.93^{a}	0.05	0.45^{b}	0.03
20:3n-3	0.14^{b}	0.01	0.13^{b}	0.01	0.28^{a}	0.02
20:4n-3	0.73^{a}	0.01	0.77^{a}	0.02	0.47^{b}	0.01
20:5n-3	7.25^{a}	0.46	7.30^{a}	0.39	3.26^{b}	0.15
22:1n-11	0.25^{a}	0.02	0.19^{b}	0.01	0.10^{c}	0.01
22:1n-9	0.08^{a}	0.01	0.07^{ab}	0.01	0.06^{b}	0.00
22:4n-6	0.10^{a}	0.02	0.07^{ab}	0.00	0.04^{b}	0.00
22:5n-6	0.12^{a}	0.04	0.04^{ab}	0.00	0.02^{b}	0.00
22:5n-3	2.07^{a}	0.01	2.31^{a}	0.06	1.55^{b}	0.14
22:6n-3	17.61^{a}	0.59	17.12^{a}	0.69	10.05^{b}	1.10

* n=3(one pool of eggs from all the spawns eggs from 3 days), different superscripts for each fatty acid would denote significant differences by Duncan's test ($p < 0.05$)

were ($p< 0.05$) lower than those from FMFO juveniles (Table 3.9). Comparison between juveniles from the same broodstock and fed either Control or Challenge diet showed that juveniles from VMFO broodstock had significantly ($p< 0.05$) lower final body weight and SGR, and higher FCR when fed the Challenge diet than the Control diet (Table 3.9). No significant effects of the control or challenge diet were found for FMFO or VMVO juveniles. Thus, the two-way ANOVA analysis denoted a strong significant ($p< 0.01$) effect of broodstock diet on growth performance and feed utilization, which was even higher than that

of the juvenile diet ($p< 0.05$) (Table 3.9). No interaction between nutritional programming of broodstock and juvenile diets was observed on growth performance of juveniles.

Figure 3.3 Gene expression of *fads2* (a) and *tor* (b) in eggs from the broodstock fed with different diets



fads2: fatty acid desaturase 2. *tor*: rapamycin. Bars bearing with different letter showed significantly difference by Duncan's test ($p < 0.05$).

Table 3.9 Growth performance of juvenile seabream after the nutritional challenge trial

Juvenile diet	Broodstock diet	Weight intial (g)		Weight final (g)		SGR		FCR	
		Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M
Control diet	FMFO	3.36	0.04	11.35^{A}	0.14	2.71^{A}	0.03	1.36	0.03
	VMFO	3.39	0.04	$9.87^{B,*}$	0.15	$2.36^{B,*}$	0.01	1.43	0.03
	VMVO	3.35	0.03	10.07^{AB}	0.68	2.43^{AB}	0.14	1.50	0.08
Challenge diet	FMFO	3.34	0.02	10.64^{a}	0.66	2.56^{a}	0.14	1.37^{a}	0.06
	VMFO	3.39	0.03	9.13^{ab}	0.11	2.19^{b}	0.02	$1.57^{ab,*}$	0.03
	VMVO	3.39	0.04	8.80^{b}	0.46	2.12^{b}	0.12	1.69^{b}	0.09
Two-way ANOVA									
Broodstock diet		N.D.		$p<0.01$		$p<0.01$		$p<0.01$	
Juvenile diet		N.D.		$p<0.05$		$p<0.05$		$p<0.05$	
Broodstock diet* Juvenile diet		N.D.		N.D.		N.D.		N.D.	

SGR, specific growth rate. FCR, Feed convert rate. N.D., $p > 0.05$

Different upper case superscript indicates significant difference among juveniles from different nutritionally stimulated broodstock fed with control diet by Duncan's test ($p < 0.05$). Different low case superscript a, b indicates significant difference ($p < 0.05$) between juvenile from different nutritional stimulated broodstock fed with challenge diet by Duncan's test ($p < 0.05$). Superscript * means the significant difference between the VMFO group juvenile fed with different diet during juvenile challenge trial by independent t-test.

No significant difference was found in lipid content of whole body among the different fish groups (Table 3.10). However, the liver from VMFO juveniles fed with the Challenge diet contained more lipid than that fed with Control diet (Table 3.10). The analysis of the fatty acid composition of liver from juveniles fed the Control diet showed that those from broodstock fed VMFO diets had the lowest contents in 20:2n-6, 20:4n-6, 22:5n-3 and 22:6n-3, final products of elongation and desaturation from 18:2n-6 and 18:3n-3. However, combined replacement of FM and FO in VMVO diets for broodstock significantly increased

the contents in 20:2n-6, 20:3n-6, 20:4n-6, 20:3n-3, 20:4n-3, 22:5n-3 and 22:6n-3 in livers of juveniles fed the control diet. When juveniles were fed the Challenge diet, livers of juveniles from broodstock fed the VMFO diet, showed the highest contents of 18:0 and lowest of 18:2n-9, 18:3n-6, 18:4n-3, products of fads2 activity on 18:1n-9, 18:2n-6 and 18:3n-3. On the contrary, liver of juveniles from broodstock fed VMVO showed significantly higher 14:0, 14:1, 15:0, 17:0, 20:0, products of lipid synthesis, as well as the highest 20:4n-6 and 22:6n-3, end-products of fatty acid desaturation and elongation activity. When comparing the juveniles from the same broodstock but fed with different diets, there was a strong effect of dietary fatty acid profiles with reduction in 14:0, 14:1n-5, 16:1, 16:2n-4, 17:0, 16:3, 16:4, 18:1n-7, 18:1n-5, 20:1, 22:1, 20:4n-6, 20:4n-3, 20:5n-3, 22:5n-6, 22:5n-3 and 22:6n-3, and increase in 18:1n-9, 18:2n-6, 18:3n-6, 18:3n-3. Combined effect of broodstock diet and juvenile diet was found in the composition of 20:2n-6, 20:3n-6, 20:4n-3 and 22:5n-3 through the analysis of two-way ANOVA. The products of Fads2 (18:3n-6, 18:4n-3 and 18:2n-9) were the highest in VMVO group when the fish was fed with challenge diet ($p< 0.05$) (Table 3.11).

Table 3.10 Lipid content of juvenile seabream after the nutritional challenge trial

Juvenile diet	Broodstock diet	Lipid content of whole fish (% of dry weight)		Lipid content of liver (% of dry weight)	
		Mean	S.E.M	Mean	S.E.M
Control diet	FMFO	10.45	0.64	27.40	0.28
	VMFO	9.54	0.25	26.59	1.83
	VMVO	10.68	0.54	25.88	1.32
Challenge diet	FMFO	10.48	0.53	32.24	1.87
	VMFO	10.13	0.38	33.04*	0.57
	VMVO	10.45	0.37	31.56	1.59
Two-way ANOVA					
Broodstock diet		N.D.		N.D.	
Juvenile diet		N.D.		$p<0.01$	
Broodstock diet* Juvenile diet		N.D.		N.D.	

Superscript * means the significant difference between the VMFO group juvenile fed with different diet during juvenile challenge trial by independent t-test.

Molecular studies in juvenile liver showed a significant influence of the nutritional programming with the lowest expression in *fads2* ($p< 0.05$) in offspring from broodstock fed VMFO, regardless of the diets fed to the juveniles. The *fads2* expression of juveniles from VMVO broodstock was as high as the FMFO fish when fed the Control diet. However, when the same fish (VMVO) were fed with the Challenge diet, *fads2* expression was higher than fish from the other two groups ($p<0.05$). Therefore, besides the strong influence of broodstock and

Table 3.11 Fatty acid composition in liver of juvenile seabream after the nutritional challenge trial

Juvenile diet	Control diet						Challenge diet						Two-way ANOVA		
Broodstock diet	FMFO		VMFO		VMVO		FMFO		VMFO		VMVO		*B*	*J*	*B*J*
%	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M			
14:0	1.67*	0.04	1.76*	0.02	1.79*	0.06	1.23b	0.03	1.30ab	0.04	1.37a	0.03	N.D.	$p< 0.01$	N.D.
14:1n-7	0.01	0.00	0.02	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.01	0.00	N.D.	$p< 0.01$	N.D.
14:1n-5	0.05*	0.00	0.05*	0.00	0.06*	0.00	0.03b	0.00	0.03ab	0.00	0.04a	0.00	N.D.	$p< 0.01$	N.D.
15:0	0.14*	0.01	0.13*	0.01	0.15	0.01	0.10b	0.01	0.09b	0.00	0.12a	0.00	0.02	$p< 0.01$	N.D.
15:1n-5	0.02	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.02	0.00	N.D.	N.D.	N.D.
16:0ISO	0.03	0.00	0.03	0.00	0.03	0.00	0.02	0.00	0.02	0.00	0.02	0.00	N.D.	N.D.	N.D.
16:0	14.39	0.77	15.59*	0.52	13.70	0.62	13.48	0.57	13.66	0.50	12.71	0.27	N.D.	$p< 0.01$	N.D.
16:1n-7	3.20*	0.08	3.26*	0.08	3.09*	0.03	2.04	0.08	2.00	0.06	2.02	0.01	N.D.	$p< 0.01$	N.D.
16:1n-5	0.13*	0.01	0.11*	0.01	0.13*	0.00	0.07	0.00	0.06	0.00	0.07	0.00	N.D.	$p< 0.01$	N.D.
16:2n-4	0.18*	0.01	0.18*	0.01	0.19*	0.02	0.09ab	0.01	0.09b	0.00	0.10a	0.00	N.D.	$p< 0.01$	N.D.
17:0	0.24*	0.01	0.23*	0.01	0.25*	0.02	0.12ab	0.01	0.12b	0.00	0.14a	0.01	N.D.	$p< 0.01$	N.D.
16:3n-4	0.18	0.01	0.19*	0.00	0.18*	0.00	0.14	0.00	0.14	0.00	0.14	0.00	N.D.	$p< 0.01$	N.D.
16:3n-3	0.04	0.00	0.05*	0.01	0.06*	0.00	0.03	0.00	0.01	0.01	0.02	0.00	N.D.	$p< 0.01$	N.D.
16:3n-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	N.D.	$p< 0.01$	N.D.
16:4n-3	0.11*	0.00	0.12*	0.01	0.12*	0.01	0.05	0.00	0.05	0.00	0.06	0.00	N.D.	$p< 0.01$	N.D.
18:0	6.08	0.19	7.00	0.16	6.58	0.41	5.84b	0.21	6.55a	0.06	6.22ab	0.18	$p< 0.05$	N.D.	N.D.
18:1n-9	39.11	0.53	39.06	0.18	38.33	0.47	42.68*	0.48	42.75*	0.05	41.60*	0.34	$p< 0.05$	$p< 0.01$	N.D.
18:1n-7	3.00*	0.08	3.01*	0.05	3.07*	0.03	2.37	0.01	2.42	0.03	2.45	0.02	N.D.	$p< 0.01$	N.D.
18:1n-5	0.08	0.01	0.10*	0.00	0.08*	0.01	0.06	0.00	0.05	0.01	0.06	0.00	N.D.	$p< 0.01$	N.D.
18:2n-9	2.11	0.37	1.40	0.15	1.90	0.06	2.27a	0.11	1.68b	0.11	2.34a,*	0.05	$p< 0.05$	N.D.	N.D.
18:2n-6	11.44	0.25	11.22	0.20	11.61	0.73	15.03*	0.67	15.16*	0.18	15.37*	0.21	N.D.	$p< 0.01$	N.D.
18:2n-4	0.17*	0.01	0.18*	0.00	0.19*	0.01	0.09	0.00	0.09	0.00	0.09	0.00	N.D.	$p< 0.01$	N.D.
18:3n-6	1.97	0.35	1.36	0.16	1.94	0.06	2.56a	0.16	2.01b,*	0.12	2.83a,*	0.08	$p< 0.05$	$p< 0.01$	N.D.
18:3n-4	0.13AB,*	0.00	0.12B,*	0.00	0.14A,*	0.01	0.07	0.01	0.07	0.00	0.07	0.01	N.D.	$p< 0.01$	N.D.
18:3n-3	4.46	0.16	4.47	0.05	4.59	0.37	5.46*	0.26	5.55*	0.09	5.58	0.15	N.D.	$p< 0.01$	N.D.
18:4n-3	1.27	0.16	1.07	0.08	1.39	0.05	1.28ab	0.06	1.17b	0.08	1.48a	0.05	$p< 0.05$	N.D.	N.D.
18:4n-1	0.09*	0.00	0.09	0.00	0.09*	0.00	0.04	0.00	0.04	0.00	0.04	0.00	N.D.	$p< 0.01$	N.D.
20:0	0.16AB	0.01	0.17A	0.00	0.15B	0.00	0.15b	0.01	0.17ab	0.00	0.17a	0.01	$p< 0.05$	N.D.	N.D.
20:1n-9	0.11*	0.00	0.10*	0.00	0.11*	0.00	0.06	0.00	0.06	0.00	0.07	0.00	N.D.	$p< 0.01$	N.D.
20:1n-7	0.51	0.02	0.54	0.01	0.55*	0.02	0.45b	0.01	0.49a	0.02	0.48ab	0.01	N.D.	$p< 0.01$	N.D.
20:1n-5	0.10	0.00	0.10*	0.00	0.10*	0.00	0.06	0.00	0.06	0.00	0.07	0.00	N.D.	$p< 0.01$	N.D.
20:2n-9	0.43	0.02	0.32	0.03	0.48	0.07	0.35	0.02	0.32	0.02	0.30	0.00	N.D.	$p< 0.01$	N.D.
20:2n-6	0.17B	0.01	0.18B,*	0.00	0.20A	0.00	0.19	0.01	0.21	0.01	0.20	0.01	$p< 0.05$	$p< 0.01$	N.D.
20:3n-9	0.02*	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.00	N.D.	$p< 0.01$	N.D.
20:3n-6	0.23AB	0.00	0.18B	0.01	0.25A,*	0.03	0.19	0.01	0.18	0.01	0.16	0.01	$p< 0.05$	$p< 0.01$	$p< 0.05$
20:4n-6	0.48AB,*	0.01	0.47B,*	0.01	0.52A,*	0.01	0.20b	0.01	0.22ab	0.00	0.23a	0.00	$p< 0.01$	$p< 0.01$	N.D.
20:3n-3	0.11B	0.01	0.12AB	0.00	0.13A	0.00	0.11	0.00	0.13	0.01	0.12	0.01	$p< 0.05$	N.D.	N.D.
20:4n-3	0.34AB,*	0.02	0.31B,*	0.01	0.38A,*	0.02	0.20	0.01	0.19	0.01	0.18	0.01	N.D.	$p< 0.01$	$p< 0.05$
20:5n-3	3.02*	0.04	2.88*	0.08	3.10*	0.12	1.24	0.04	1.20	0.04	1.20	0.03	N.D.	$p< 0.01$	N.D.
22:1n-11	0.16*	0.01	0.16*	0.01	0.16*	0.01	0.09	0.01	0.09	0.00	0.10	0.01	N.D.	$p< 0.01$	N.D.
22:1n-9	0.21	0.01	0.22	0.01	0.24	0.01	0.19	0.00	0.21	0.01	0.21	0.01	$p< 0.05$	$p< 0.01$	N.D.
22:4n-6	0.05*	0.00	0.04	0.01	0.06*	0.00	0.03	0.00	0.03	0.00	0.0	0.00	N.D.	$p< 0.01$	N.D.
22:5n-6	0.09*	0.00	0.08*	0.00	0.09*	0.01	0.04	0.00	0.03	0.00	0.04	0.00	N.D.	$p< 0.01$	N.D.
22:5n-3	0.81B,*	0.02	0.72C,*	0.02	0.90A,*	0.03	0.30	0.03	0.27	0.01	0.30	0.02	$p< 0.01$	$p< 0.01$	$p< 0.01$
22:6n-3	2.70AB,*	0.03	2.57B,*	0.08	2.90A,*	0.12	0.98	0.11	0.97	0.04	1.12	0.05	$p< 0.05$	$p< 0.01$	N.D.
Δ5 product	3.49*	0.04	3.35*	0.09	3.61*	0.12	1.44	0.05	1.42	0.05	1.43	0.03	N.D.	$p< 0.01$	N.D.
Δ6 product	5.36	0.88	3.83	0.38	5.22	0.10	6.10a	0.28	4.87b	0.32	6.65a,*	0.16	$p< 0.01$	$p< 0.05$	N.D.

Different superscript indicates significant difference ($p < 0.05$) between juvenile from different nutritional stimulated broodstock fed with control diet. Different low case superscript indicates significant difference ($p < 0.05$) between juvenile from different nutritional stimulated broodstock fed with challenge diet. Superscript * means the significant difference between the juvenile from the same broodstock broodstock but fed with different diet during the juvenile challenge trial.

juvenile diet, there was also a combined effect of type of diets in the expression of *fads2* (Table 3.12). Meanwhile, hepatic expression of *elovl6* was significantly affected by different experimental diet of juvenile. When the juveniles were fed with control diet, *elovl6* expression in VMVO group was higher than that in the other two groups(p<0.05), but no difference was observed when the fish were fed with challenge diet.

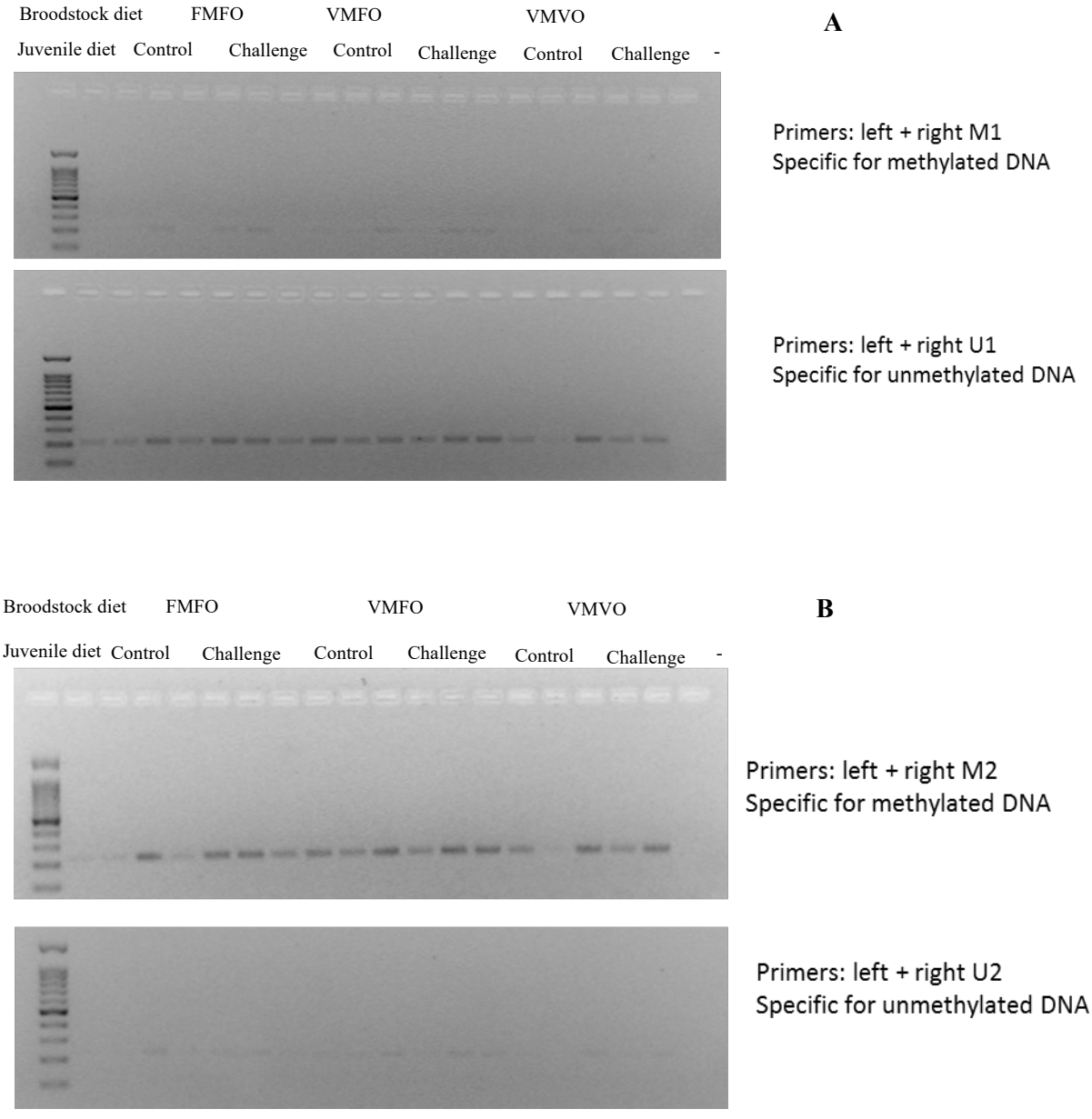
No differences were observed in the expression of hepatic *srebp, g6p*, *tor or igf-1* (Table 3.12). The methylation-specific PCR analysis of two CpG- rich-regions of *fads2* gene showed that proximal promoter was mostly non-methylated while distal region resulted mostly methylated, regardless of the broodstock or the juvenile diet used (Figure 3.4).

Table 3.12 Gene expression in liver of juvenile seabream after the nutritional challenge trial

Juvenile diet	Broodstock diet	*ß-actin*		*fads2*		*elovl6*		*srebp*		*g6p*		*tor*		*igf-1*	
Concentration (copies/μL)		Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M
Control diet	FMFO	2052.12	299.09	108.76^{A}	17.00	38.41^{B}	3.10	13.85	3.31	67.21	23.15	10.89	0.70	26.51	5.42
	VMFO	2275.74	215.24	58.4633^{B}	9.59	34.17^{B}	3.93	10.33	2.41	57.27	15.27	11.71	3.43	27.24	3.16
	VMVO	2060.22	68.48	72.5233^{AB}	10.79	67.13^{A}	7.44	11.16	2.84	47.27	7.46	13.78	2.24	27.25	2.29
Challenge diet	FMFO	2219.37	554.58	105.4333^{b}	8.74	70.78	36.18	17.41	3.75	71.40	3.71	13.94	2.71	13.51	1.94
	VMFO	2174.09	479.35	101.1^{b}	23.01	65.31	18.82	14.15	2.46	50.02	10.14	14.06	5.97	22.25	3.59
	VMVO	2632.78	529.22	$185.91^{a,*}$	16.42	154.18	52.45	16.77	1.02	62.17	21.23	15.52	6.12	21.30	7.93
Two-way ANOVA															
Broodstock diet		N.D.		$p<0.05$		N.D.		N.D.		N.D.		N.D.		N.D.	
Juvenile diet		N.D.		$p<0.01$		$p<0.05$		N.D.		N.D.		N.D.		N.D.	
Broodstock diet* Juvenile diet		N.D.		$p<0.05$		N.D.		N.D.		N.D.		N.D.		N.D.	

Different superscript A, B indicates significant difference ($p < 0.05$) between juvenile from different nutritional stimulated broodstock fed with control diet. Different superscript a, b indicates significant difference ($p < 0.05$) between juvenile from different nutritional stimulated broodstock fed with challenge diet. Superscript * means the significant difference between the VMFO group juvenile fed with different diet during juvenile challenge trial.

Figure 3.4 Electrophoresis of MSP amplicons on 2% agarose gel.



The methylation status of regions of the *fads2* promoter between −676 and −891 (A)and between −3977and −3744 (B)

3.4 Discussion

3.4.1 FM replacement by VM did not affected broodstock performance during nutritional stimulus

In the present trial, replacement of 60% FM in broodstock diets by a mixture of faba beans, wheat gluten and soya protein concentrate did not affect the spawning performance of gilthead sea bream. Up to 50% replacement of fishmeal by cottonseed meal protein does not affect spawn quality in terms of embryo survival in rainbow trout (Rinchard *et al.*, 2003). On the contrary, early studies showed that FM replacement by VM reduced reproductive success, egg size and offspring survival in rainbow trout (Pereira *et al.*, 1998; Lazzarotto *et al.*, 2015). In channel catfish (*Ictalurus punctuatus*), total replacement of FM in broodstock diet decreases female fecundity, egg mass weight and individual egg weight (Sink *et al.*, 2010). Since nutritional status of broodstock affects fecundity, egg fertilization and embryo development (Izquierdo *et al.*, 2001), the spawning performance of this experiment suggest the good palatability of the diets, which were well accepted by the different broodstock groups and may indicate the adequate amino acid profiles and protein digestibility (Kaushik, 1998), allowing to fulfil the needs for amino acids for gametogenesis and maintenance of a high fecundity (Tandler, 1995). These results suggest the good palatability of the diets, which were well accepted by the different broodstock groups and may indicate the adequate amino acid profiles and protein digestibility (Kaushik, 1998), allowing to fulfil the needs for amino acids for gametogenesis and maintenance of a high fecundity (Tandler, 1995). Thus, essential amino acids that are main constituents of vitellogenin chorion proteins that provides energy to the embryo, such as LEU, LYS, ILE or VAL, were similar in VMFO and FMFO diets. TRP and HIS contents, which have also an important effect on reproduction of marine fish, were similar among the diets. On the contrary, the replacement of dietary FM by VM leads to the increase of 18:2n-6 and 18:2n-9, important fatty acids components in eggs (Fernández-Palacios *et al.*, 2011). Nevertheless, LC-PUFA content in the eggs was not affected by the FM replacement by VM.

3.4.2 Negative nutritional programing effect of FM replacement by VM

A clear negative programming effect of FM replacement by VM in broodstock diet on growth of offspring juveniles fed the high FM and FO diet was observed. Moreover, when juveniles were fed the low FM and FO challenge diet, feed utilization was further reduced as denoted by the increase of FCR and the reduction of final body weight and SGR. These results are in agreement with those recently reported in rainbow trout, which indicated that feeding broodstock with a plant-based diet did not improve the offspring ability to adapt to diets high in plant ingredients, but reduced the growth performance (Sink *et al.*, 2010). These authors found a persistent effect of broodstock diets on gene expression in offspring, with an up-regulation of genes involved in carbohydrate and energy metabolism (Lazzarotto *et al.*, 2016). In the present study, the activity of hepatic Fads2 was inhibited, which was reflected in the fatty acids profiles that showed the lowest contents in PUFA products of elongation and desaturation. Besides, the expression of *fads2* also decreased, which means the biosynthesis of LC-PUFA is negatively affected since the capability of biosynthesis of n-3 LC-PUFA from 18C fatty acid is mostly decided by the ability of Fads2 and the elongases (Bell and Tocher, 2009) and in most of the marine fish, Fads2 is considered as the rate-limiting enzyme in this process (Li *et al.*, 2014). Alteration in the methylation status of CpG dinucleotides is indeed one of the major epigenetic mechanism implicated in gene transcriptional regulation. Typically, DNA methylation at the promoter of a gene is presumed to render it silent (Han *et al.*, 2011) by modifying DNA accessibility to the transcriptional machinery. In this study, the methylation-specific PCR analysis of f*ads2* promoter did not show any differences in methylation rate. Regardless of the diet or the selection of broodstock, the CpG regions of *fads2* promoter showed that the proximal promoter was mostly nonmethylated while distal region resulted methylated, which accords to the fact that the most clusters of CpG are being hypomethylated near the transcriptional start site (Costello and Plass, 2001). Previously, the methylation status of *fads2* promoter was investigated in Japanese seabass (*Lateolabrax japonicus*) (Xu *et al.*, 2014). In this study, the effects of different dietary fatty acid profiles, i.e., diet with low or high n-3 LC-PUFA content, were evaluated in juvenile Japanese seabass.

In contrast with our findings, Xu *et al.* (2014) demonstrated that the methylation rate in fads2 gene promoter was significantly regulated by dietary fatty acid profiles and, even more interesting, they found a negative correlation between the promoter methylation rate and expression level of *fads2*. It is also true, that we cannot exclude that other epigenetic mechanisms could be involved in our case, such as indirect mechanisms which lead to histone deacetylation, chromatin condensation and a transcriptionally inactive chromatin structure responsible of observed differences in *fads2* expression.

Overall, the replacement of FM by VM in the broodstock diets leads to a deleterious effect on growth performance of gilthead sea bream offspring juveniles by different factors. Dietary changes in specific amino acids may affect transcription in the offspring in both mammals (Morgane *et al.*, 2002) as well as in fish (Fontagné-Dicharry *et al.*, 2017). For instance, in rainbow trout an increase of 0.5 or 1 g/100g in Met in broodstock diets improved fry survival rate with marked changes in genes expression (Fontagné-Dicharry *et al.*, 2017). Other factors may be involved like unbalanced n-6/n-3 ratios (Fernández-Palacios *et al.*, 1997) correlate to the increase of 18:2n-6 in the egg, or the increase of carbohydrate contents in the VMFO diet that would affect energy metabolism. Thus, FM replacement by VM has been associated to a reduced ability for protein biosynthesis (Panserat *et al.*, 2008). Replacement of FM by VM provoke increase in hepatic lipid contents (Benedito-Palos *et al.*, 2008; Peng *et al.*, 2014a; Torrecillas *et al.*, 2017a) and the negative effect brought by VM in broodstock diet may reflect persistent changes in the offspring. Further studies must be conducted to clarify the mechanisms involved in this nutritional programming effect caused by the replacement of FM by VM conducted in this trial.

3.4.3 Combined replacement of FM and FO by VM and VO affects broodstock spawning performance negatively

Whereas FM replacement by VM did not modify broodstock performance, that of FO by VO in broodstock diets led to a reduction in dietary essential fatty acids, which would be related to the reduced fecundity in terms of eggs produced per female. This can be related to the reduced dietary contents in LC-PUFA which are EFA and act also as precursors of

prostaglandins and leukotrienes involved in reproductive processes, including steroidogenesis, gonadal development and ovulation (Asturiano *et al.*, 2001; Fernández-Palacios *et al.*, 2011). These results are in conformity with the reduction in egg viability found in this species after feeding the broodstock even for only 15 days with soybean oil in substitution of FO (Harel *et al.*, 1994). Moreover, the egg content in EPA and DHA was also reduced when broodstock was fed the combined VMVO diet, in agreement with previous studies (Fernández-Palacios *et al.*, 1995). In turn, reduced contents in n-3 LC-PUFA in eggs would be related to the reduced number of normal eggs and larvae, since they are required by the embryo for construction of membrane and, particularly, contribute to the rapid development of the visual and nerve system (Sargent *et al.*, 2003). Interestingly, LC-PUFA reduction did not equally affect the different fatty acids and, thus, a selective retention was found for DHA, since its content in egg was higher than that in the diet, while the EPA relative content was lower in the egg than in the diet. The replacement of dietary FM and FO led to the enrichment in eggs of 20:2n-6 and 20:3n-3, products of elongation from 18:2n-6 and 18:3n-3, respectively (Stanley-Samuelson *et al.*, 1987), evidencing the increased elongase activity in eggs with the higher content of their precursors by the replacement of fish oil. The stagnant level of another fatty acid, 18:4n-3, in egg after the replacement of fish oil in diet even in the condition of the abundance of the precursor, 18:3n-3, illustrates the restricted ability of fads2 to catalyze the desaturation from 18:3n-3 to 18:4n-3. Meanwhile, the replacement of fish oil increased the content of another product of Fads2, 18:2n-9, meaning that the ability of fads2 is enhanced by dietary FO replacement by VO. The n-3 LC-PUFA, which is high in fish oils, has been found to suppress the activity of the Fads2 in larvae or juveniles of marine fish (Li *et al.*, 2014; Izquierdo *et al.*, 2015), but the evidence in egg is still scarce. Nevertheless, the high expression of *fads2* in eggs shows the high potential of utilization of dietary VO in the offspring. Moreover, *tor* gene was down-regulated with the FO replacement by VO that caused a reduction of LC-PUFA in the egg. In mammals, TOR is implied in several physiological processes that control cell differentiation and proliferation and its function has been found to be regulated by dietary fatty acids through the production

of phosphatidic acid, an intermediate metabolite of phospholipids synthesis (Menon *et al.*, 2017). Thus, n-3 LC-PUFAs are known to suppress TOR complex in mammals (Liu *et al.*, 2016) and, particularly, EPA, but not ARA, decreases the activity of mTOR (Gao *et al.*, 2016). Indeed, in fish, increase in dietary n-3 PUFA, as well as VO replacement by FO, enhances lipoprotein synthesis reducing the accumulation of phosphatidic acid (Vergara *et al.*, 1996a; Caballero *et al.*, 2006; 2007). In the present study, the reduction in dietary and egg LC-PUFA was related to an up-regulation of *tor* gene, suggesting that these fatty acids may not only interfere with *tor* at a post-transcriptional level but also regulate the expression of this gene.

3.4.4 Nutritional programming effect of combined FM and FO replacement by VM and VO

Although juveniles from broodstock fed the VMVO diet showed a low growth performance when they were fed the high FM and FO diet or the challenge diet low in FM and FO, their growth was similar to the progenies from broodstock fed the VMFO diet. These evidences denote that FM replacement by VM, rather than FO replacement by VO, was the cause of this nutritional programming effect as discussed above. FO replacement by VO in broodstock diet markedly affected liver fatty acid profiles, increasing the Fads2 and Elovl6 activity, particularly when fed the low FM and FO challenge diet, as denoted by the increased fatty acid products from both enzymes, as well as the upregulation of *fads2* expression. These results denote the nutritional programming effect of FO replacement by VO in broodstock diets for gilthead sea bream in agreement with previous studies (Turkmen *et al.*, 2017a). These studies showed that FO replacement by linseed oil leads to a persistent up-regulation of *fads2* and *elovl6* in offspring larvae (Izquierdo *et al.*, 2015) and adults (Turkmen *et al.*, 2017a) that was correlated with the increase in conversion rates of related PUFA. Besides, other genes related to immune system or lipid and energy metabolism are also regulated as a result of the nutritional programming by FO replacement by linseed oil (Izquierdo *et al.*, 2015). In mammals, parental feeding with LC-PUFA also lead to differential expression and phenotypes in relation to epigenetic changes (Lillycrop *et al.*, 2005). Thus, in juveniles from broodstock fed the VMVO diet the products of elongation (20:2n-6, 20:3n-3, 20:4n-3 and 22:5n-3) and products of desaturation (20:3n-6, 20:4n-6, and 22:6n-3) were increased. 20:3n-

6 can be elongated from 18:3n-6 and also synthesized from 20:2n-6 through the catalysis of fads2 and then join the process of desaturation to 20:4n-6 as the precursor. The synthesis of 22:6n-3 is the result of desaturation, elongation and ß-oxidation (Schaeffer *et al.*, 2006). Our results showed that nutritional programming by VO in broodstock diets enhanced the capability of elongation in offspring.

In conclusion, the replacement of FM alone or in combination with FO by VM in diets for gilthead sea bream broodstock lead to negative effect on the growth performance of juvenile offspring, whereas the replacement of FO by VO enhanced the fatty acid desaturase and elongase activity, especially when the juveniles were fed a diet with a high replacement of FM and FO by VM and VO.

Acknowledgements

Thanks to Skretting ARC (Stavanger, Norway). and BioMar (Brande, Denmark) for providing the experimental diets. We also want to thank to the reviewers for providing us the very helpful comments and suggestions.

M. I., S. T. and H. X. participated in the design of the experiment. The control of spawning performance was supervised by H. F and S. S. H. X. conducted all feeding experiments, all biological, biochemical and molecular analyses were conducted and evaluated by H. X. and S. T. Molecular biology samples were analysed by H. X with the supervision of M. J. Z and J. M. A. DNA methylation level was anaylsis by H. X, S.R and G.T. M. I. supervised the entire work. The paper was written by H. X. and M. I.

This work was supported by the Institute of Sustainable Aquaculture and Marine Ecosystems and China scholarship council

The authors declare that there are no conflicts of interest. The views expressed in this work are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

Chapter 4

Influence of parental *fatty acid desaturase 2* (*fads2*) expression and diet on gilthead seabream (*Sparus aurata*) offspring *fads2* expression along ontogenesis

Hanlin **Xu,** Shajahan **Ferosekhan**, Serhat **Turkmen**, Juan Manuel **Afonso**, Maria Jesus **Zamorano**, Marisol **Izquierdo**

Minor revisión at *life*

Impact factor (2019): 2.323

Abstract

Previous studies have shown that it is possible to increase the ability of marine fish to produce long-chain polyunsaturated fatty acid from their 18C precursors by nutritional programming or using broodstock with a higher fatty acyl desaturase 2 (*fads2*) expression. However, those studies failed to show the effect of these interventions on the expression of the *fads2* gene in the developing egg. Moreover, there were no studies on the temporal expression of the *fads2* along ontogeny in gilthead sea bream (*Sparus aurata*). In order to determine the changes in expression of *fads2* during ontogeny, gilthead sea bream broodstock with a high (HRO) or low (LRO) *fads2* expression fed a diet previously used for nutritional programming, or a fish oil-based diet (LFO) were allowed to spawn. The samples were taken at the stages of spawning, morula, high blastula, gastrula, neurula, heart beating, hatch and 3 day-old first exogenous feeding larvae to determine *fads2* expression throughout the embryonic development. The results showed the presence of *fads2* mRNA in the just spawned egg, denoting the maternal mRNA transfer to the developing oocyte. Later, *fads2* expression increased after the neurula, from heart beating until 3-day-old larvae, denoting the transition from maternal to embryonic gene expression. Besides, eggs obtained from broodstock with high *fads2* expression showed a high docosahexaenoic acid content, which correlated with the down-regulation of the *fads2* expression found in the developing embryo and larvae. Finally, feeding with the nutritional programming diet with partial replacement of fish oil by rapeseed oil did not affected LC-PUFA contents nor *fads2* expression in gilthead sea bream developing eggs.

Keywords: aquaculture; lipid metabolism; embryogenesis; parental gene expression; parental nutritional status.

4.1 Introduction

Long chain polyunsaturated fatty acids (LC-PUFAs), particularly docosahexaenoic acid (22:6n-3, DHA), eicosapentaenoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, ARA) play relevant structural and functional roles in animal cells (Watanabe, 1993; Calder, 2012). They are critical components of cell and organelle membranes (Wassall and Stillwell, 2008) and their derived molecules, such as eicosanoids or docosanoids, participate in cell signalling processes (Gill and Valivety, 1997). In fish, LC-PUFAs are required for growth (Peng *et al.*, 2014b) or brain and immune system development and maintenance (Menoyo *et al.*, 2004; Izquierdo and Koven, 2011; Torrecillas *et al.*, 2017a), among many other functions. In human nutrition, fish is the most important source of LC-PUFAs, especially DHA and EPA, whereas in farmed fish, fish oil (FO) is the traditional source of LC-PUFAs. However, FO is mostly derived from capture fisheries, which is a limited resource that would restrain the sustainable development of aquaculture. Thus, certain vegetable oils may partly replace fish oil in marine fish diets, since they are more environmentally and economically sustainable (Carvalho *et al.*, 2020). Despite vegetable oils lack LC-PUFAs, they are abundant in their 18-carbon precursors (Izquierdo *et al.*, 2015).

The biosynthesis of LC-PUFA is catalysed by desturases and elongases. Like mammals, fish are unable to synthesize *de novo* LC-PUFA, since they lack n-12 and n-15 desaturases, which only exist in some plants or invertebrates (Pereira *et al.*, 2003). Therefore, either LC-PUFAs or their 18-carbon precursors must be included in diets for farmed fish. Linoleic acid (18:2n-6, LNA) and α-linolenic acid (18:3n-3, ALA) are the main precursors for ARA, and EPA and DHA, respectively. Thus, ARA and EPA are synthesized from 18:2n-6 and 18:3n-3, respectively, through the Δ6 desaturation – elongation and Δ5 desaturation pathway, or the elongation - Δ8 desaturation and Δ5 desaturation pathway (Sprecher, 2000; Lopes-Marques *et al.*, 2018). DHA may be synthesized *in vivo* from EPA through elongation-Δ4 desaturation or elongation-elongation- Δ6 desaturation and ß-oxidation (Sprecher, 2000). Comparing with freshwater fish or euryhaline fish, marine fish have a lower ability to synthesize LC-PUFAs from their 18-carbon precursors (Dong *et al.*, 2017). One of the mechanisms leading to a limited ability from LC-PUFA synthesis in marine fish is the weak activity of these enzymes (Tocher and Ghioni, 1999; Vagner and Santigosa, 2011). For instance, the activity of fatty acyl

desaturase 2 (Fads2) in cod (*Gadus morhua* L.) is lower than in Atlantic salmon (*Salmo salar*) (Tocher *et al.*, 2006). This could be related with the abundance of LC-PUFAs in the marine food web, originated from phytoplankton, which would reduce the need to synthesis these important fatty acids (Izquierdo, 1996; Sargent *et al.*, 2003). Besides, LC-PUFA synthesis capacity of different fish species is also related to genome specificities. For instance, the *fatty acyl desaturase 1* (*fads1*) gene, which codes for Δ5 desaturation, is absent in many telostei such although duplicated *fads2* or Δ5/Δ6 bifunctional Fads2 may be found in those species (Kabeya *et al.*, 2017; Lopes-Marques *et al.*, 2018). Considering the absence of *fads1* in gilthead sea bream(Lopes-Marques *et al.*, 2018), *fads2* is a major rate-limiting enzyme in the LC-PUFA biosynthesis in this species. Indeed, in gilthead sea bream (*Sparus aurata*), Fads2 has both Δ6 and Δ8 desaturase activities (Monroig *et al.*, 2011). On the contrary, Δ5 desaturate activity seems weak in gilthead sea bream, since the addition of ^{14}C-label 18:3n-3 to the hepatocytes culture medium leads to a 41% of the radioactivity recovered in the Δ6 product 18:4n-3, whereas when ^{14}C-labelled 18:4n-3 is added, only 0.7% of the radioactive is recovered from the Δ5 product, EPA (Tocher and Ghioni, 1999).

Large variations in Fads2 activity are found among individuals of the same species in different environmental or nutritional conditions (Izquierdo and Koven, 2011; Ferosekhan *et al.*, 2020b). For instance, in gilthead sea bream, *fads2* level in liver or red blood cells can be up to 6 or 5 times different among individuals of the same gender and kept under the same environmental and nutritional conditions (Ferosekhan *et al.*, 2020a). Besides, feeding a low LC-PUFA diet increases *fads2* level or Fads2 products (Bell *et al.*, 2002; Sargent *et al.*, 2003; Xu *et al.*, 2014; Gregory *et al.*, 2016; Ferosekhan *et al.*, 2020b). Therefore, either individual selection of high *fads2* fish or feeding a low LC-PUFA diet could be used as tools to produce fish with a higher Fads2 activity and, thus, a better ability to synthesize LC-PUFA when fed Vegetable oil. In one hand, the offspring from broodstock with high *fads2* level showed improved growth performance and feed utilization when challenged with a diet low in fishmeal (FM) and FO (Turkmen *et al.*, 2019b). Besides, broodstock with high *fads2* level showed a better reproductive performance, in terms of fecundity and sperm and egg quality (Ferosekhan *et al.*, 2020b). In the other hand, feeding gilthead sea bream broodstock during the spawning season

with a diet low in LC-PUFA diet improves the use of low FM/FO diets in their offspring (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2019b) even up to 16 months (Turkmen *et al.*, 2017a). In rainbow trout (Oncorhynchus mykiss), nutritional condition by complete replacement of dietary FM and FO by vegetable ingredients affects the level of genes related with growth, amino acid and cholesterol metabolism (Lazzarotto *et al.*, 2016) without negative effects for the second generation (Lazzarotto *et al.*, 2015). However, none of those studies were able to show any effect on the fads2 level in eggs due to the large variations obtained (Ferosekhan *et al.*, 2020b), which could be related to the different egg developmental stages in the eggs or the interference with a potential maternal transfer of *fads2* mRNA (Monroig *et al.*, 2009). Despite the *fads2* expression may occur from an early developmental stage in teleost (Monroig *et al.*, 2009; Tanomman *et al.*, 2013; Torres *et al.*, 2020), the temporal expression of *fads2* along ontogenesis of gilthead sea bream has not yet been studied. Moreover, the effect of broodstock *fads2* expression and nutritional status on *fads2* expression along ontogeny has neither been addressed.

Therefore, the aim of the present study was to determine, in one hand, the temporal changes in *fads2* expression along ontogeny and, in the other hand, the influence of broodstock diet and the parental ability to express *fads2*. For that purpose, gilthead sea bream broodstock with different ability to synthesize LC-PUFA were fed either a FO-based diet or a diet previously used for nutritional programming of progeny (Ferosekhan *et al.*, 2020b) and their eggs were sampled along ontogenesis to determine the temporal changes in *fads2* expression.

4.2 Materials and Methods

All the animal experiments were performed according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at Fundación Canaria Parque Científico Tecnológico (FCPCT), University of Las Palmas de Gran Canaria (Las Palmas de Gran Canaria, Spain).

4.2.1 Broodstock fads2 expression

To obtain broodstock with different ability to synthesize LC-PUFA from the precursors LNA and ALA, 3 months prior the spawning season, 185 gilthead sea bream brood fish of 1-2 kg were fed for one month with a diet high in LNA and ALA and low in LC-PUFA promoting the

up-regulation of *fads2* (Ferosekhan *et al.*, 2020b). Then, peripheral blood samples were collected and the *fads2* expression of blood cells was determined. Based on their *fads2* mRNA copy numbers per µl in blood cells, brood fish with the highest *fads2* expression values (H broodstock) or with the lowest ones (L broodstock) were chosen (Table 4.1). To determine the effect of the broodstock *fads2* expression on the *fads2* expression in developing eggs and larvae, 6 brood fish with high *fads2* expression (HRO) and 6 with low expression (LRO) were allocated into 4×1000 L tanks, (2 males and 1 female/tank) and fed a diet containing 1.76% FO and 7.54% rapeseed oil (RO) (Ferosekhan *et al.*, 2020b) (Table 4.2).

Table 4.1 Average *fads2* expression in peripheral blood cells of the broodstock used in present study

Group	Gender	*fads2* expression (copies/µL)
HRO (High *fads2* +RO diet)	Female 1	14.92
	Male 1-1	7.06
	Male 1-2	3.37
	Female 2	12.72
	Male 2-1	4.09
	Male 2-2	3.33
LRO (Low *fads2* +RO diet)	Female 3	1.41
	Male 3-1	1.53
	Male 3-2	1.46
	Female 4	0.49
	Male 4-1	1.74
	Male 4-2	1.66
LFO (Low *fads2* +FO diet)	Female 5	1.11
	Male 5-1	1.26
	Male 5-2	1.27

Table 4.2 Ingredients of the experimental diets used to feed broodstock during the spawning season

Ingredients (%)	FO	RO
Fish meal (North-Atlantic 12C)	59.36	59.36
Squid meal	3.00	3.00
Krill meal	7.00	7.00
Wheat	20.57	20.57
Fish oil (South American)	9.30	1.76
Rapeseed oil	0.00	7.54
Vitamin-mineral premix*	0.50	0.50
L-Histidine HCl	0.27	0.27
Proximate composition		
Crude protein (%DM)	53.4	54.6
Crude lipid (%DM)	18.8	17.3
Ash (%DM)	11.3	11.6
Moisture (%)	7.9	7.3

Vitamin-mineral premix*: vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik (Germany), Deutsche Lanolin Gesellschaft (Germany).

4.2.2 Effect of the broodstock diet

In order to determine the effect of the broodstock diet on the offspring *fads2* expression, another 3 low *fads2* expression broodstock (2 males and 1 female/tank) were fed with a diet containing FO as the only lipid source (LFO) and compared with the previous LRO broodstock. Both experimental diets were isoproteic and isolipidic and manufactured by Skretting ARC (Stavanger, Norway) (Ferosekhan *et al.*, 2020b) (Table 4.2). Compared with FO diet, the replacement level of FO by vegetable oil in RO diet was 80%, a level that affects the offspring growth performance and lipid biosynthesis in response to a diet containing low FM/FO diet (Turkmen *et al.*, 2017a; Turkmen *et al.*, 2019b). The content of LC-PUFA (DHA+EPA+ARA) was 24.11% and 15.42% of total fatty acid in FO diet and in RO diet, respectively (Table 4.3).

Table 4.3 Fatty acid composition of the experimental diets used to feed broodstock during the spawning season

Fatty acid (% of total fatty acid)	FO	RO
14:0	5.04	1.87
14:1n-5	0.15	0.08
15:0	0.46	0.17
16:0 ISO	0.09	0.09
16:0	18.83	9.42
16:1n-7	6.84	2.67
16:1n-5	0.26	0.11
16:2n-4	0.75	0.29
17:0	0.83	0.20
16:3n-4	0.23	0.17
16:3n-1	0.20	0.11
16:3n-3	0.12	0.08
16:4n-3	1.09	0.43
18:0	3.95	2.47
18:1n-9	12.82	31.76
18:1n-7	3.37	3.28
18:1n-5	0.30	0.16
18:2n-9	0.19	0.04
18:2n-6	4.11	11.14
18:2n-4	0.24	0.09
18:3n-6	0.32	0.13
18:3n-4	0.15	0.14
18:3n-3	1.30	4.95
18:4n-3	2.19	1.22
18:4n-1	0.00	0.11

Fatty acid (% of total fatty acid)	FO	RO
20:0	0.47	0.61
20:1n-9	3.77	4.06
20:1n-7	0.31	0.18
20:2n-9	0.06	0.05
20:2n-6	0.20	0.17
20:3n-9	0.07	0.09
20:3n-6	0.12	0.10
20:4n-6	1.04	0.43
20:3n-3	0.15	0.12
20:4n-3	0.57	0.35
20:5n-3	11.96	6.57
22:1n-11	3.73	4.98
22:1n-9	0.51	0.66
22:4n-6	0.17	0.23
22:5n-6	0.43	0.27
22:5n-3	1.40	0.79
22:6n-3	11.11	8.42
Σ Saturates	29.58	14.74
Σ Monoenes	32.12	48.59
Σ n-3	29.89	22.93
Σ n-6	6.38	12.47
Σ n-3 LC-PUFA	25.19	16.25
DHA/EPA	0.93	1.28
DHA/ARA	10.72	19.82
n-3/n-6	4.68	1.84

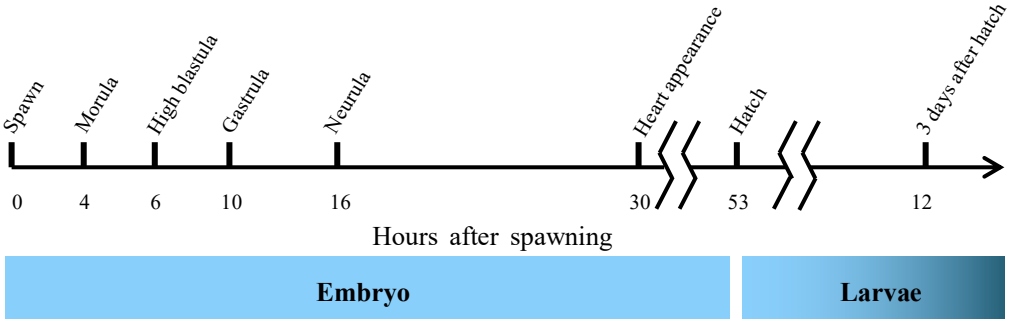
All broodstock groups were manually fed twice per day (9:00 and 14:00 h) until apparently satiation. Seawater temperature was in the range of 18-22 °C (January-April 2018) and fish were kept under natural photoperiod (approximately 12 h light).

4.2.3 Sampling along offspring ontogenesis

At the end of the spawning season, eggs from the same spawning were collected and separately incubated in 500 L tanks. Eggs or larvae samples from each spawn were taken at consecutive

stages of development: just after spawning (0 hour post spawning (hps)), morula (4 hps), high blastula (6 hps), gastrula (10 hps), neurula (16 hps), heart beating (30 hps), hatch (53 hps) and 3 day-old larvae (125 hps) (Kamacı *et al.*, 2005) (Figure 1). Samples were washed by DEPC water, kept overnight in RNAlater (MilliporeSigma, St. Louis, U.S.A.) at 4 °C and then stored at -80 °C until analysis.

Figure 4.1. Timeline of selected point during embryogenesis. The timeline is based on the description of stage during embryogenesis of gilthead sea bream (Kamacı *et al.*, 2005) under experimental condition.



4.2.4 RNA extraction and digital PCR

RNA from 200 mg sample was extracted using TRI Reagent (MilliporeSigma) and RNeasy kit (Qiagen, Hilden, Germany). RNAlater was removed from the samples before extraction. Then each sample was weighted and 1 mL TRI Reagent with 1 steel bead was added. Then the sample was homogenized in TissueLyser II (Qiagen) at 30 Hz for 30 s and centrifuged at 13 000 g for 1min. All the liquid phase was transferred to a new 1.5 mL tube and 300 µL of chloroform (MilliporeSigma) was added and mixed. The mixture was centrifuged at 13 000 g for 15 mins. The transparent phase was isolated and mixed with same volume of 70% ethanol. The mixture was loaded to RNeasy mini spin column and washed by RW1 and RPE offered in the kit according to the instruction of manufacturer. Concentrated RNA was eluted in 30 µL of RNase free water. RNA quality was checked by 1.4% agarose electrophoresis and quantity was measured by NanoDrop 1000 (ThermoFisher, Waltham, USA). 1000 ng of RNA was used per sample for

cDNA synthesis through iScript cDNA synthesis kit (Bio-Rad, Hercules, USA.) in iCycler (Bio-rad). Digital PCR was conducted in QX200™ Droplet Digital™ PCR System (Bio-rad). The reaction system, containing 100 ng of cDNA, *fads2* primer (Forward: 5' GCA GAG CCA CAG CAG CAG GGA 3', Reverse: 5' CGG CCT GCG CCT GAG CAG TT 3', Gene bank No. GQ162822.1) and Evagreen SuperMix (Bio-rad), was loaded to droplet generator to generate the oil droplet and then proceeded in C1000 Touch thermal cycler (Bio-Rad). The cycling condition of PCR was: 95℃ for 5 mins, followed by 40 cycles of 95℃ for 30 sec, 63℃ for 1 min, and then stabilized the signal at 4℃ for 5 min, 90℃ for 5 min, finally the reaction was hold at 4℃. The PCR amplification of the nucleic acid target in the droplets was read in QX200 droplet reader (Bio-Rad).

4.2.5 Lipid and fatty acid analysis

Lipids from diets and 24hps eggs were extracted with chloroform/methanol (2:1 v/v) (Folch *et al.*, 1957) and then transmethylated to obtain fatty acid methyl esters (FAMES) (Christie, 1988). FAMES were then separated by gas liquid chromatography FAMES were extracted with hexane:diethyl ether (1:1, v/v) and purified by adsorption chromatography on NH2 Sep-pack cartridges (Waters, Milford, USA). Afterwards, FAMES were separated by Gas-Liquid Chromatography (GLC) (Agilent 7820A, CA, USA) in a Supercolvax-10-fused silica capillary column (length:30 mm, internal diameter: 0.32 mm, Supelco, U.S.A.) using helium as a carrier gas. Column temperature was 180℃ for the first 10 min, increasing to 215 ℃ at a rate of 2.5 ℃ min^{-1} and then held at 215 ℃ for 10 min (Takeuchi *et al.*, 1990).Peaks were identified by comparison with external standards and well-characterized FO (EPA 28, Nippai, Ltd. Tokyo, Japan).

4.2.6 Data analysis

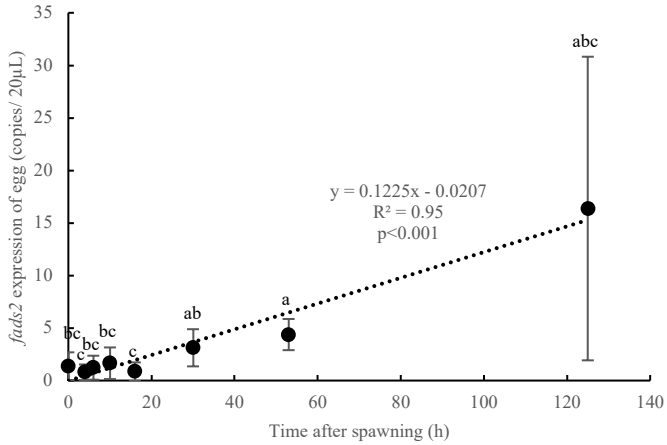
Target cDNA concentration was analyzed by QuantaSoft Analysis Pro tool (Bio-Rad). Results were presented as means ± S.D. and analyzed by SPSS 20.0 (IBM, New York, USA) for Mac. Comparison of the data between different stage within the group were analyzed by One-way ANOVA. *Fads2* expression data between groups were analyzed by Independent-Samples T-test. Linear, exponential and logarithmic regression were performed through SPSS 20.0 (IBM).

4.3 Results

4.3.1 Temporal expression of fads2 along offspring ontogenesis

Analysis of the average *fads2* expression values in eggs and larvae for all experimental groups showed an increase along embryogenesis, following a significant lineal regression (R^2=0.95, p<0.001) with time after spawning (Figure 4.2). Thus, *fads2* expression was registered at spawning (0 hps), remained low during morula (4 hps), blastula (6 hps) and gastrula stages (10 hps) and even decreased until the neurula stage (16 hps), which showed the lowest *fads2* expression values (Figure 4.2). From the heart beating stage (30 hps) onwards *fads2* expression increased, being at this stage significantly (p<0.05) higher than at the previous morula (4 hps) and neurula stages (16 hps), and, at hatching (53 hps), it was also significantly (p<0.05) higher than at the spawning to neurula stages (Figure 4.2). The highest values were observed in the 3-day-old larvae, although the large deviations did not allow to obtain significant differences with the *fads2* at other developmental stages (Figure 4.2).

Figure 4.2 Temporal changes in fatty acid desaturase 2 (*fads2*) mRNA copies along gilthead seabream (*Sparus aurata*) ontogenesis, from 0 h to 125 h after spawning



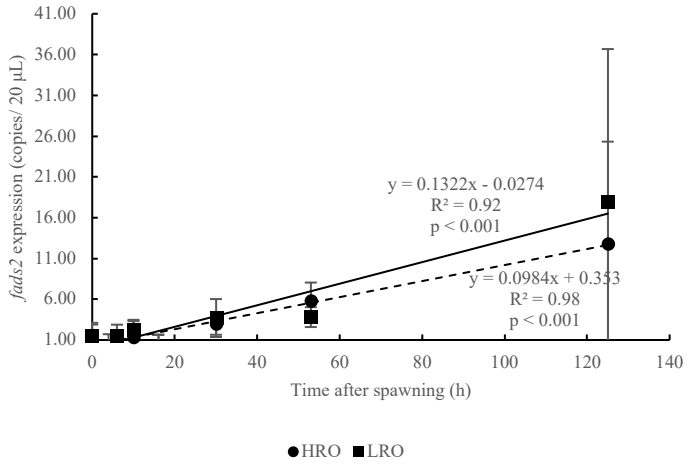
Different letters denote significant differences among developmental stages (p<0.05, n=15). Bars bearing with different letter showed significant difference by one-way ANOVA analysis (p<0.05)

4.3.2 Influence of parental fads2 expression in offspring fads2 expression along ontogenesis

The *fads2* expression in eggs from broodstock with high (HRO) or low *fads2* expression (LRO) also followed significant (p<0.001) lineal regressions (R^2=0.98 and R^2=0.92, respectively) (Figure 4.3). These regression lines crossed at 10 hps and, thus, whereas in the earlier stages of development *fads2* expression tended to be higher for HRO, the later ones tend to be higher for LRO. Nevertheless, due to the large deviations among sampling batches, t-test comparison

between HRO and LRO values at each developmental stage did not showed significant (p>0.05) differences (Figure 4.3).

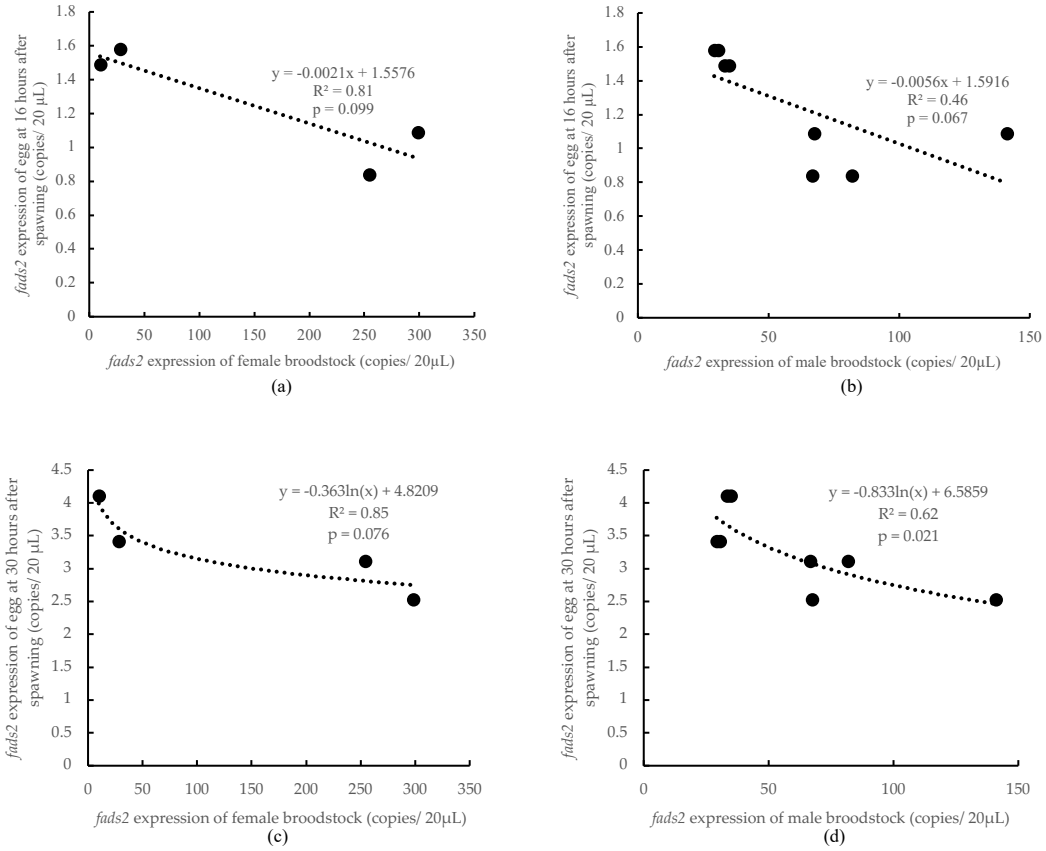
Figure 4.3 Influence of parental *fatty acid desaturase 2* (*fads2*) expression (high HRO or low LRO) on temporal changes in *fads2* mRNA copies along gilthead seabream (*Sparus aurata*) ontogenesis, from 0 h to 125 h after spawning (n=3).



No significantly difference was observed in the comparison using t-test (p>0.05)

The *fads2* expression values in females and males broodstock were in the range of 0.49-14.92 and 1.26-7.06 copies/µL, respectively (Table 4.1). Comparison of broodstock and offspring *fads2* expression by regression analysis showed that *fads2* expression at neurula stage (16 hps) followed an inverse lineal relation with *fads2* expression in broodstock females (R^2=0.81, p=0.099) or males (R^2=0.46, p=0.067) (Figure 4.4). These regressions were higher and more significant at heart beating stage (30 hps), when *fads2* expression in embryos followed an inverse logarithmic relation with *fads2* expression in females (R^2=0.85, p=0.076) or males (R^2=0.62, p=0.021) (Figure 4.5).

Figure 4.4 Relations of fatty acid desaturase 2 (*fads2*) mRNA copies between eggs at neurula stage (16 hps) and female (a) or male (b) (including both HRO and LRO broodstock) or between eggs at heart appearence stage (30 hps) and female (c) or male (d) broodstock



4.3.3 Influence of parental fads2 expression in egg fatty acid profiles

At 24 hps, DHA content in eggs obtained from HRO group was 6.46% higher than that from LRO group (p=0.028). Besides, the ratio between DHA and ARA in the eggs of HRO group was 8.97 higher than in eggs of HFO group(p=0.004). Whereas 22:4n-6 and 22:5n-6, two very long chain n-6 PUFA, in HRO group were significantly lower than in LRO group (p<0.05). Similar differences were also found in the comparison in 18:0, 18:2n-4 and 20:2n-9 content between two groups(p<0.05) (Table 4.4).

Fads2 expression in both females and males broodstock was significantly (p<0.05) and positively related to the DHA contents in the eggs (R^2=0.99, p = 0.004 and R^2=0.68, p=0.012, for females and males, respectively) (Figure 4.6). In comparison, DHA content in eggs was inversely related (R^2=0.86, p=0.074) to *fads2* expression in the eggs at neurula stage (16 hps) (R^2=0.86, p=0.07, Figure 4.7a) and followed a similar trend at heart beating stage (30 hps) (R^2=0.73, p=0.146) (Figure 4.8a). Besides, the ratio 20:4n-3/20:3n-3, an indicator of desaturase

Table 4.4 Fatty acid composition of eggs at 24hps from broodstock fed a RO diet during spawning and showing either high (HRO) or low (LRO) expression of *fads2*

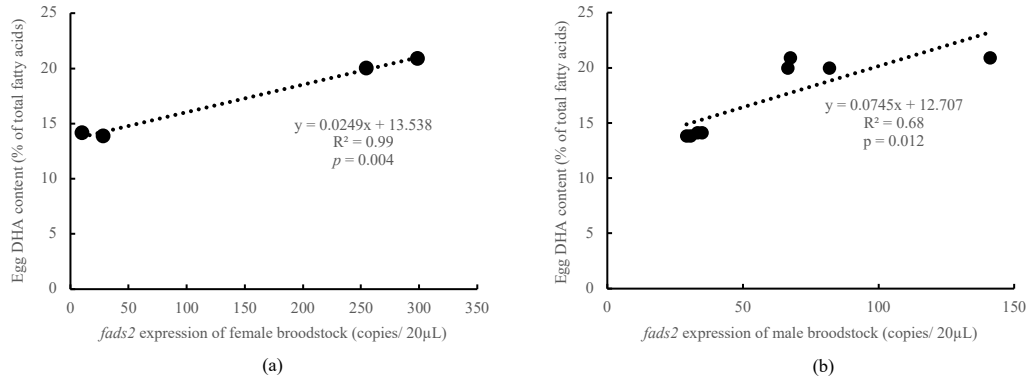
	HRO		LRO	
	Mean	S.D.	Mean	S.D.
14:0	1.12	0.08	4.03	1.94
14:1n-7	0.02	0.01	0.13	0.02
14:1n-5	0.05	0.01	0.18	0.11
15:0	0.17	0.00	0.38	0.11
15:1n-5	0.03	0.01	0.18	0.04
16:0 ISO	0.04	0.00	0.18	0.02
16:0	11.58	0.25	16.17	2.02
16:1n-7	2.70	0.07	3.73	0.52
16:1n-5	0.07	0.02	0.27	0.04
16:2n-4	0.19	0.00	0.33	0.17
17:0	0.14	0.02	0.26	0.09
16:3n-4	0.18	0.01	0.49	0.35
16:3n-3	0.11	0.01	0.40	0.29
16:3n-1	0.10	0.01	0.34	0.30
16:4n-3	0.14	0.03	0.37	0.24
18:0	3.58	0.06	4.05*	0.08
18:1n-9	27.06	1.03	19.69	7.04
18:1n-7	3.09	0.04	4.98	1.90
18.1n-5	0.15	0.01	0.82	0.74
18:2n-9	0.10	0.02	0.33	0.13
18.2n-6	10.81	0.24	8.63	1.76
18:2n-4	0.11	0.01	0.31*	0.03
18:3n-6	0.20	0.04	0.56	0.15
18:3n-4	0.11	0.00	0.36	0.08
18:3n-3	2.92	0.12	2.20	0.53
18:4n-3	0.53	0.01	1.02	0.58
18:4n-1	0.09	0.01	0.20	0.07
20:0	0.15	0.01	0.33	0.08
20:1n-9	0.24	0.01	0.23	0.01
20:1n-7	1.50	0.14	0.77	0.44
20.1n-5	0.19	0.01	0.22	0.06
20:2n-9	0.08	0.01	0.22*	0.02
20:2n-6	0.42	0.06	0.41	0.02
20:3n-9	0.05	0.01	0.24	0.13
20:3n-6	0.17	0.01	0.47	0.26
20:4n-6	0.76	0.03	0.78	0.01
20:3n-3	0.31	0.06	0.37	0.01
20:4n-3	0.65	0.01	0.49	0.10
20:5n-3	5.95	0.10	6.98	3.49
22:1n-11	0.48	0.02	0.35	0.08
22:1n-9	0.20	0.01	0.24	0.02
22:4n-6	0.08	0.02	0.63*	0.07
22:5n-6	0.25	0.04	0.60*	0.04
22:5n-3	2.80	0.09	2.20	0.78
22:6n-3	20.45*	0.64	13.99	0.21
SFA	16.76	0.36	25.38*	0.31
MUFA	35.74	0.82	31.76	5.30
PUFA	47.49	1.20	42.87	4.97
n-3 FA	33.84	0.83	28.01	5.15
n-6 FA	12.67	0.38	12.06	1.42
n-9 FA	27.71	1.04	20.93	6.80
DHA/ARA	26.91*	0.16	17.94	0.05
DHA/EPA	3.44	0.05	2.28	1.11

*denotes the significantly difference between two groups (p<0.05). FA, fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

activity, also followed a significant (R^2=0.93, p=0.036) negative relation with *fads2* expression at neurula stage (16 hps) (Figure 4.7b). On the contrary, 18:2n-9, product of Fads2 on 18:1n-9, contents in eggs were directly related to the *fads2* expression in the egg at heart beating stage (30 hps) (R^2=0.95, p=0.027) (Figure 4.8b), and a similar tendency could be observed at neurula stage (16 hps) (R^2=0.63, p=0.205) (Figure 4.7c). Also, 18:3n-6, product of Fads2 on 18:2n-6,

and the ratio 18:3n-6/ 18:2n-6 were directly related to the *fads2* expression in the egg at neurula stage (16 hps) (p<0.10) (Figure 4.7d) and heart beating stage (30 hps) (p<0.10), respectively.

Figure 4.5 Relations between DHA contents in the egg and *fads2* mRNA copies of female or male broodstock (including both HRO and LRO broodstock)



(a) Relation between DHA content in egg at 24 hours after spawning and *fads2* mRNA copies in female (a) or male (b) broodstock.

Figure 4.6 Relations between *fads2* mRNA copies of egg at 16 hours after spawning with the content of fatty acids in eggs at 24 hours after spawning (including eggs from both HRO and LRO broodstock)

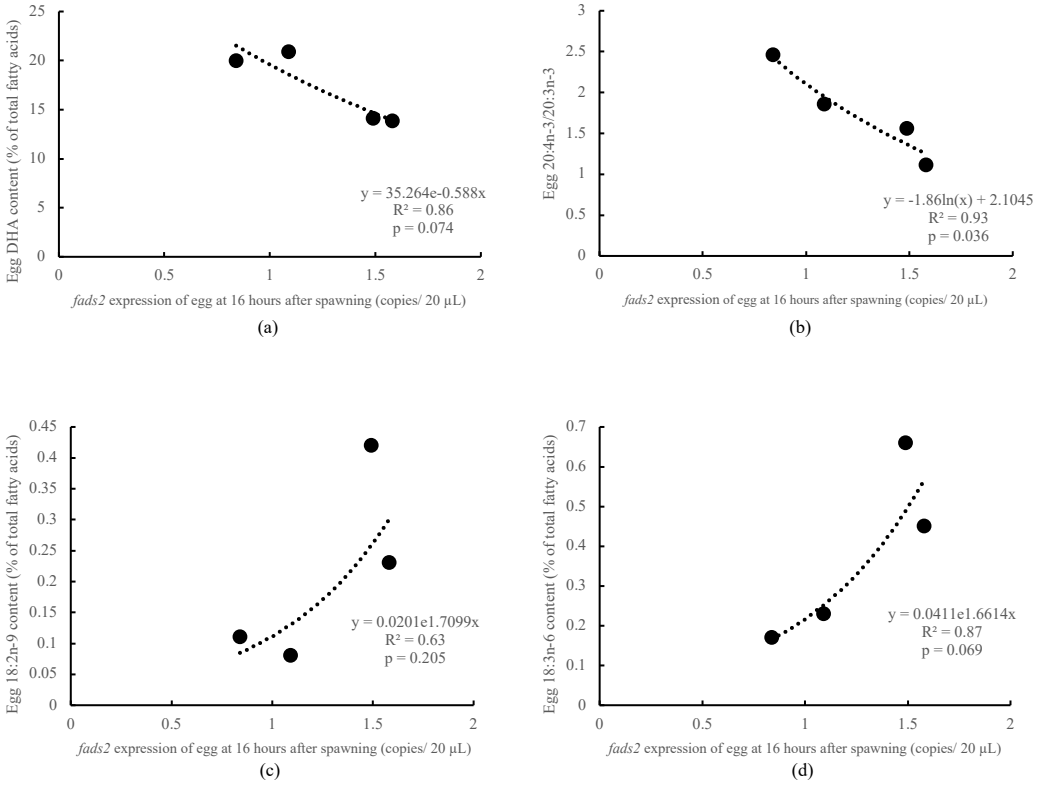
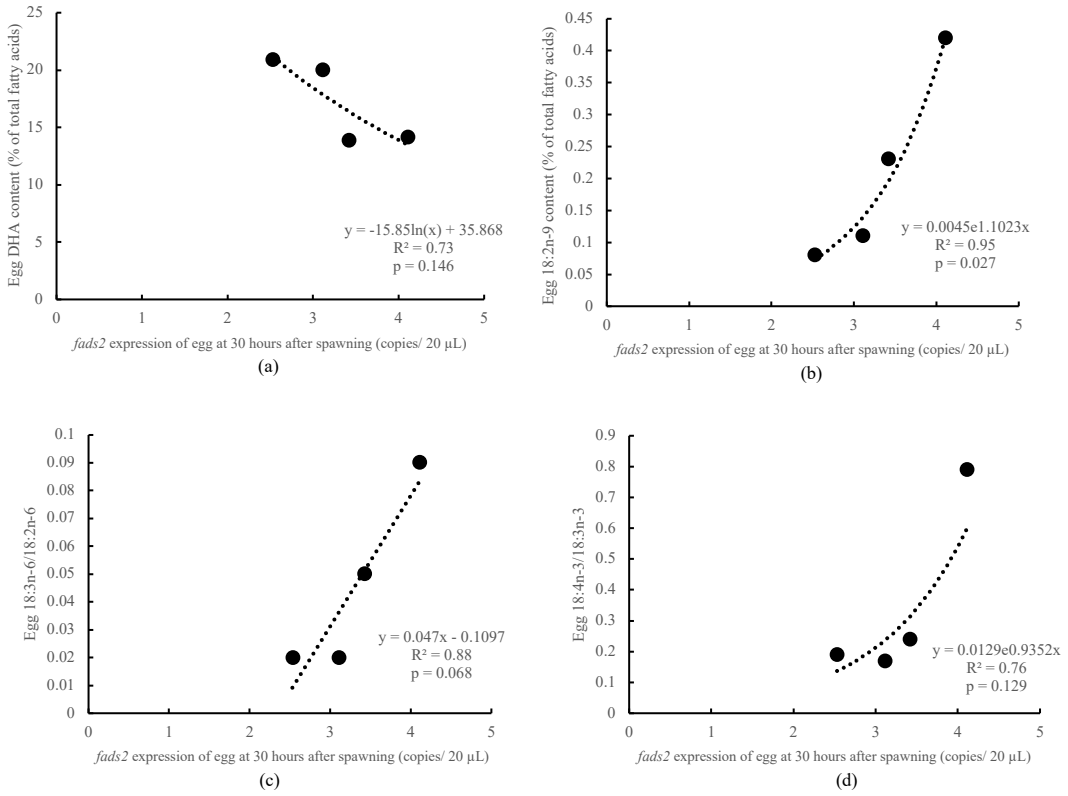


Figure 4.7 Relations between *fads2* mRNA copies of egg at 30 hours after spawning with the content of fatty acids in eggs at 24 hours after spawning (including eggs from both HRO and LRO broodstock).



Relations *fads2* mRNA copies of egg at 30 hours after spawning and the content of DHA (a), ratio 20:4n-3/20:3n-3 (b), 18:2n-9(c) and 18:3n-6 (d) in eggs at 24 hours after spawning

4.3.4 Influence of broodstock diet in offspring fads2 expression along ontogenesis

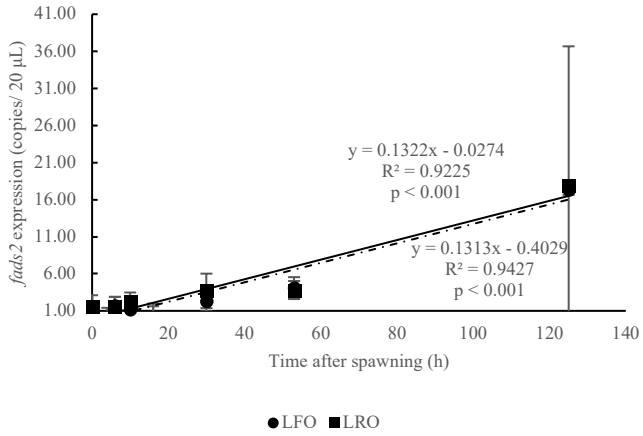
No significant (p>0.05) difference was observed on *fads2* expression along embryogenesis between eggs from broodstock fed with diet rich in FO (LFO broodstock) or RO (LRO broodstock), which followed a similar positive linear regression (p<0.001) (Figure 4.9).

4.4 Discussion

The present study aimed at determining the temporal changes in *fads2* expression along ontogeny of gilthead seabream and the potential influence of broodstock diet and the parental ability to express *fads2*. Overall, the results showed the presence of *fads2* mRNA even in the just spawned egg, denoting the maternal mRNA transfer to the developing oocyte. Then, from the neurula stage onwards *fads2* expression increased denoting the transition from maternal to

embryonic gene expression. Regarding the broodstock effect, eggs obtained from parents with high *fads2* expression showed a high DHA content, together with the down-regulation of *fads2*

Figure 4.8 Changes in *fads2* expression during embryogenesis in eggs and larval offspring from broodstock with low expression of *fads2* and fed either a FO (LFO) or a RO (LRO) diet during spawning



expression. Finally, partial replacement of FO by RO did not affect LC-PUFA contents nor *fads2* expression in gilthead seabream eggs.

4.4.1 Changes of fads2 mRNA in gilthead seabream eggs during ontogeny

The use of gilthead seabream broodstock with a high ability to express *fads2* when fed low FO diets improves reproductive success (Ferosekhan *et al.*, 2020b) and the offspring capacity to use low FO and FM diets (Xu *et al.* in press, Chapter 5). However, their consequences on the *fads2* expression in the offspring, particularly along development of embryo and larvae, have not been yet studied. This is partly due, to the lack of knowledge on the ontogeny of *fads2* expression. The present study has demonstrated that in gilthead seabream *fads2* expression can be detected even in the just spawned egg (0 hps) immediately after fertilization. These results are in agreement with the presence of fatty acid desaturase gene (*fad*) expression in zebrafish zygote (Monroig *et al.*, 2009), and denote a maternal mRNA transfer to the developing oocyte. Despite maternal mRNA transfer would be proved by the presence of transcripts in unfertilized eggs, no differences are found in mRNA levels between unfertilized and 1.5 h post-fertilized zebrafish eggs, denoting that just fertilized or just spawn eggs are useful to study maternal transfer mRNAs (Vergauwen *et al.*, 2018). In fish, maternal mRNAs produced by the mother

based on her genome are incorporated into the developing oocytes at very early stages of oogenesis (Cheung *et al.*, 2018). Despite originally maternal transcripts may be distributed throughout the oocyte cytoplasm, after fertilization most of them are pulled into the blastodisc through cytoplasmic streaming or ooplasmic segregation. There, from mid-blastula on-wards, maternal mRNAs become important components of the protein translation machinery contributing to the proper embryogenesis, and their expression is being regulated by their stability and degradation (Cheung *et al.*, 2018). In seabream, *fads2* mRNA copies were constant during morula (4 hps), blastula (6 hps) and gastrula stages (10 hps) and decreased at the neurula stage (16 hps) suggesting the degradation of the maternal *fads2* mRNA at this stage.

From the neurula stage onwards a significant increase was observed in the seabream *fads2* expression, denoting the transition from maternal to embryonic gene expression. At the neurula stage the embryo has already establish the bases for the development of the principal organs and systems, including neural and circulatory systems, and the increased *fads2* expression may contribute to fulfill the LC-PUFA requirements for organs development (Izquierdo and Koven, 2011). LC-PUFA, and specially DHA, are necessary for brain development and functioning in seabream as in other vertebrates (Benitez-Santana *et al.*, 2014). In agreement, *fad* is particularly expressed in the head area of zebrafish (Monroig *et al.*, 2009). Indeed, *fads2* expression in gilthead seabream was significantly up-regulated at heart beating, when brain development becomes very notorious, and remained increasing during hatching and 3-day-old larvae stages. These results are relatively different from those obtained in zebrafish, where *fad* expression is claimed to increase earlier during embryo development, from 12 h post fertilization onwards (Monroig *et al.*, 2009). Nevertheless, those conclusions in zebrafish are based on comparisons of transcript levels from RT-PCR analyses and have to be made cautiously, as stated by the authors (Monroig *et al.*, 2009), whereas the present study was based on digital PCR determining absolute quantification mRNA copies with 9 replicates for each developmental stage. Indeed, rather than at 12 h, a more evident increase in *fad* expression was observed at 19 h post fertilization (Monroig *et al.*, 2009), coinciding with the late segmentation stage in zebrafish when organogenesis occurs (Karlstrom and Kane, 1996), in agreement with our findings in gilthead seabream. Therefore, a marked up-regulation of the embryo *fads2* expression is found

after the neurula stages, particularly from heart beating, suggesting that the determination of *fads2* expression in the offspring embryo should be conducted from this stage onwards to avoid the potential influence of the maternal mRNA.

4.4.2 Influence of parental fads2 expression in offspring fads2 expression along ontogenesis

The regression lines for the temporal expression of *fads2* along ontogenesis of seabream offspring from high or low *fads2* expressing broodstock crossed at 10 hps, denoting a higher number of mRNA copies in offspring from high *fads2* expressing broodstock. This observation would be in agreement with the maternal transfer of *fads2* mRNA previously discussed. Interestingly, in later developmental stages the *fads2* expression tend to be lower in offspring from broodstock with high *fads2* expression, as it was supported by the significant negative relation between *fads2* expression in broodstock and offspring at neurula or, particularly, heart beating stages. This down-regulation of *fads2* expression in offspring from broodstock with higher *fads2* expression, seemed to be due to their increased DHA content in the eggs in comparison to those from lower *fads2* expression broodstock. Maternal transfer of DHA into fish eggs may occur during endogenous vitellogenesis by the synthesis in the ovaries of lipid vesicles that will lead to the oil globule, or exogenous vitellogenesis by the synthesis of vitellogenin in the liver forming the lipoprotein yolk lipids (Wiegand, 1996; Poupard *et al.*, 2000). In gilthead seabream broodstock *fads2* is expressed in both tissues, but specially in liver, where it increases the production of LC-PUFA, particularly, DHA (Ferosekhan *et al.*, 2020b). Whereas in seabream male broodstock, DHA in liver increases proportionally to the hepatic expression of *fads2*, in the females DHA does not accumulate in the liver (Ferosekhan *et al.*, 2020b), but it is incorporated into vitellogenin and transferred into the developing oocytes. In agreement, in the present study, DHA contents in the eggs were positively correlated to the *fads2* expression in the broodstock, particularly females, denoting the maternal transfer of DHA. DHA is highly demanded during embryogenesis and larval development to sustain growth, being the most abundant fatty acid in eggs of many fish species (Izquierdo, 1996; Izquierdo *et al.*, 2001). DHA is the most potent LC-PUFA resisting membrane packing, greatly regulates membrane fluidity and has a structural function on the cell membrane, directly promoting growth (Izquierdo and Koven, 2011). Besides, it is a precursor of docosanoids, which function

as local hormones (autacoids) with autocrine and paracrine functions targeting cells in the area where they are formed, and are involved in the regulation of a wide array of cellular pathways and cascades, including cell proliferation and differentiation (Izquierdo and Koven, 2011).

In turn, increased DHA contents in the seabream eggs were inversely related to *fads2* expression in embryos at neurula and heart appearance stages, denoting the down regulation of *fads2* expression by DHA as observed along the whole fish life cycle (Izquierdo *et al.*, 2008; Li *et al.*, 2014; Gregory *et al.*, 2016). Indeed, feeding a FO diet with high levels of LC-PUFA, particularly DHA, inhibits *fads2* expression in comparison to a VO diet low in these fatty acids (Vagner *et al.*, 2007; Izquierdo *et al.*, 2008; Li *et al.*, 2014; Xu *et al.*, 2014). In mammals, the presence of LC-PUFA restricts the expression *FADS2* through the E-box like sterol regulatory element (Nara *et al.*, 2002). Studies on compartmental location of fatty acids along fish ontogeny have shown that despite DHA is initially found in the yolk, this fatty acid is preferentially incorporated into structural lipids in the embryo tissues (Jaroszewska and Dabrowski, 2011), where it would down-regulate *fads2* expression. Thus, the higher the *fads2* expression in the broodstock the higher the incorporation of DHA into the eggs, and such increased incorporation of DHA inhibited the *fads2* expression in the eggs.

In agreement, the eggs obtained from low *fads2* parents showed low DHA contents and a high *fads2* expression, associated to the increase Δ6-activity on n-3 and n-6 precursors as denoted by the increase in the ratio of synthesis from 18:3n-6 to 18:2n-6, or from 18:4n-3 to 18:3n-3. Besides, high *fads2* expression in the egg was also associated to a reduction in Δ8-desaturase activity as denoted by the reduction in the ratio of synthesis of 20:4n-3 from 20:3n-3. The Δ8-desaturase activity is an alternative but minor pathway for LC-PUFA biosynthesis, more active when there is a high demand of eicosanoid synthesis (Park *et al.*, 2009; Monroig *et al.*, 2011). The study of this alternate pathway in baker´s yeast (*Saccharomyces cerevisiae*) with a mammals *FADS2* gene showed that the activity of Δ6-desaturase is higher than that of Δ8-desaturase by 7.2-folds on n-6 fatty acids and by 23 folds on n-3 fatty acids (Park *et al.*, 2009). In agreement, the negative correlation of the Δ8 activity and *fads2* expression in gilthead seabream embryos could be related with the activation of Δ6 activity of Fads2 in the embryos with lower DHA content.

4.4.3 Influence of broodstock diet in offspring fads2 expression along ontogenesis

FO has been partly replaced by RO in broodstock diets, in order to induce a nutritional programing in the offspring for a better utilization of low FM and low FO diets (Turkmen *et al.*, 2019b). Since dietary LC-PUFA, high in FO, are required for good spawning quality in fish (Izquierdo *et al.*, 2001), FO content in broodstock diets can be replaced by RO for nutritional programing purposes, but not as much as to reduce spawning quality (Izquierdo *et al.*, 2015). Thus, the diets tested in the present study were sufficient to cause a nutritional programing effect in the offspring (Xu *et al.*, submitted), without negatively affecting broodstock performance (Ferosekhan *et al.*, 2020b). However, their effect in the temporal expression of *fads2* along gilthead seabream ontogenesis had not been studied. In the present study, feeding the same broodstock diets as in previous studies (Ferosekhan *et al.*, 2020b) did not affect *fads2* expression along embryogenesis. Even though the dietary LC-PUFA levels where slightly different, the LC-PUFA contents in the eggs were similar. Despite the egg contents in LC-PUFA are usually correlated to the LC-PUFA levels in broodstock diets, there is a preferential retention of these fatty acids in the eggs, particularly in species with oligolecythic eggs such as gilthead seabream and, therefore, small dietary differences may not be reflected in the fatty acid profiles of the eggs (Fernández-Palacios *et al.*, 1995; Izquierdo *et al.*, 2001). Moreover, the optimum LC-PUFA content of gilthead sea bream broodstock during spawning season is 11.27% of total fatty acid in the diet (Fernández-Palacios *et al.*, 1995) and both broodstock diets in the present study fulfill these requirements. Thus, the similarity of the LC-PUFA content in eggs can explain why there was no difference on the *fads2* expression in eggs from the broodstock fed different lipid sources. These results agree well with the lack of effect of these diets on *fads2* expression in gilthead seabream eggs (Ferosekhan *et al.*, 2020b). However, more extreme diets may affect the expression of LC-PUFA biosynthesis related genes. For instance, in *Solea senegalensis*, feeding broodstock with a diet markedly higher in LC-PUFA suppresses *elovl5* expression in egg (Morais *et al.*, 2014). Further studies with diets containing extreme levels of LC-PUFA should be conducted to understand the effect of broodstock diets on expression of genes related to LC-PUFA biosynthesis.

4.5 Conclusions

In summary, the results of the present study have shown the presence of *fads2* mRNA in the just spawned gilthead seabream egg, denoting the maternal mRNA transfer to the developing oocyte, whereas from the neurula stage onwards *fads2* expression increased denoting the transition from maternal to embryonic gene expression. Besides, eggs obtained from broodstock with high fads2 expression showed a high DHA content, which could be responsible for a down-regulation of fads2 expression in the developing embryo and larvae. Finally, partial replacement of FO by RO did not affect LC-PUFA contents nor *fads2* expression in gilthead seabream eggs.

Author Contributions: Design of the experiment, M. I., S. F., S.T. and H. X.; Feeding experiments, biological, biochemical and molecular analyses S. F. and H. X.; Molecular analysis supervision, M. J. Z and J. M. A.; Manuscript writing, M. I. and H. X.

Funding: This study was funded by the EU funded project PerformFISH, grant number H2020-SFS-2016-2017; 727610) and China scholarship council.

Acknowledgments: The authors acknowledge Skretting ARC (Stavanger, Norway) for providing the experimental diets.

Conflicts of Interest: The authors declare no conflict of interest.

Chapter 5

High broodstock fads2 expression combined with nutritional programing through broodstock diet improves the use of low fishmeal and low fish oil diets in gilthead seabream (*Sparus aurata*) progeny

Hanlin **Xu,** Shajahan **Ferosekhan**, Serhat **Turkmen**, Juan Manuel **Afonso**, Maria Jesus **Zamorano**, Marisol **Izquierdo**

Major Revision at *Aquaculture*

Abstract

One of the factors that limits the replacement of fish meal (FM) and fish oil (FO) by plant ingredients in diets for marine fish, is their lack of long chain-polyunsaturated fatty acids (LC-PUFA). LC-PUFA are essential fatty acids for these fish species, which lack sufficient fatty acyl desaturase 2 (Fads2) activity to synthesize them. Nutritional programing or the use of broodstock with a higher Fads2 activity could improve marine fish ability to synthesize LC-PUFA and their ability to use low FM and FO diets. The aim of this study was to determine the effect of gilthead seabream broodstock with inherently high or inherently low *fads2* gene expression and nutritional programing with broodstock diets rich in FO or rapeseed oil (RO) on the progeny growth performance, liver morphology, biochemical composition and expression of selected genes. Sea bream juveniles (2.31± 0.01 g initial body weight, mean ± SD) obtained from broodstock with either high (H) or low (L) *fads2* expression and fed a broodstock diet based on FO or RO were randomly distributed into 12 x 250 L tanks and nutritionally challenged for 45 days with a diet containing only 7.5% FM and no FO. The highest growth was found in juveniles from broodstock with a high *fads2* expression and fed the RO diet, whereas the lowest growth was obtained in those from broodstock with a low *fads2* expression and fed the RO diet. Juveniles from broodstock with high *fads2* expression showed significantly higher *fads2* expression in liver and increased PUFA contents in liver and muscle. Replacement of FO by RO in broodstock diets led to a significantly increased hepatic 18:3n-6/18:2n-6 ratio and reduction in the viscerosomatic index of the progeny juveniles, the hepatocyte size and the *ghr-1/ghr-2* expression in muscle. Overall, the results showed significant trans-generational effects of both the broodstock *fads2* expression and the type of lipid on the broodstock diet on the metabolism and performance of the juvenile progeny challenged with a diet low in FM and FO.

Keyword: Nutritional programming; plant ingredients utilization; n-3 long-chain PUFA biosynthesis; fatty acyl desaturase 2

5.1 Introduction

Besides being a well-balanced source of minerals and highly digestible proteins, fish food is the main source of n-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA) for people. Therefore, fish demand by the consumers is continuously increasing. Due to the stagnant production of fisheries, aquaculture is taking over the responsibility to provide safe and sustainable fish food to satisfy market demands (FAO, 2020). However, further development of aquaculture is restricted by the limited availability and increasing prices of fishmeal (FM) and fish oil (FO), traditional protein and lipid sources in aquafeeds that are mostly derived from capture fisheries. Therefore, dietary FM and FO need to be replaced by other high quality and nutritious ingredients with a more economically, environmentally and socially sustainable production. Many alternative feedstuffs are used to replace FM and FO in aquafeeds such as plant ingredients, animal byproducts, single cell ingredients or insect meals (Caballero *et al.*, 2002; Wang *et al.*, 2016; Rosales *et al.*, 2017; Rimoldi *et al.*, 2018). However, depending on the type of ingredient and the replacement level, these alternative feedstuffs may lead to malnutritional effects on fish growth, nutrients digestibility, immune system, *etc*. (Vergara *et al.*, 1996b; Vergara *et al.*, 1996a; Caballero *et al.*, 2004; Gómez-Requeni *et al.*, 2004).

One of the factors that restricts the replacement of FM and FO by alternative ingredients is their frequent lack of essential fatty acids. FO is rich in n-3 long chain polyunsaturated fatty acids, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are important components of biomembranes for normal cell functioning (Gorjao *et al.*, 2009). Therefore, they are necessary for the adequate development of brain and larval tissues (Mourente, 2003), immune system functioning (Zuo *et al.*, 2012b), reproductive performance (Fernández-Palacios *et al.*, 2011) and flesh nutritional value (Monge-Ortiz *et al.*, 2018). LC-PUFA can be synthesized from ALA and LNA in vertebrates through a series of desaturation and elongation reactions (Bell and Tocher, 2009), starting from desaturation of oleic acid, α-linolenic acid ALA or linoleic acid LNA to 18:2n-9, 18:4n-3 or 18:3n-6, respectively, by fatty acid desaturase 2 (Fads2) (Vagner and Santigosa, 2011). Marine fish have a limited capacity to synthetize LC-PUFA, what could be related with the abundance of LC-PUFAs in the marine food webs, originated from phytoplankton (Sargent *et al.*, 2003). Since the plant oils used to replace FO

lack n-3 LC-PUFA but are rich in their precursors (ALA and LNA), enhancing the marine fish capacity to synthetize LC-PUFA would facilitate the replacement of FO by plant oils.

Nutritional programming is a common strategy employed by nature that allows an organism to adapt its metabolism to the environmental conditions. Nutritional programming refers to the metabolic consequences of a nutritional stimulus applied at a critical period during life, such as pre- or postnatal stages (Lucas, 1994). A well-known example can be found in honeybees that may become a fertile queen or a sterile worker through the consumption or not of royal jelly (Kucharski *et al.*, 2008). Specific evidences of the regulation of nutritional programming on lipid metabolism include the long-term reduction of plasma cholesterol, high-density lipoprotein cholesterol and triacylglycerol caused by malnutrition during both pre- and postnatal periods in mouse (Lucas *et al.*, 1996). Similarly, in fish, nutritional stimulus during reproduction by feeding broodstock with different dietary fatty acid profiles markedly affects lipid metabolism and growth of the progeny (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2019a). For instance, feeding gilthead sea bream (*Sparus aurata*) broodstock with diets containing partial replacement of FO by linseed oil (LO), low in n-3 LC-PUFA but high in their ALA precursor, up-regulated *fads2* expression and growth in the progeny (Izquierdo *et al.*, 2015). Besides, it also up-regulated other lipid metabolism and health related genes, such as cycloxigenase-2 (*cox2)* and tumor necrosis factor-alpha (*tnf-a)*, lipoprotein lipase (*lpl*), carnitin palmitoil transferase 1 (*cpt1*) or elongase 6 (*elovl6*) (Turkmen *et al.*, 2017a; Turkmen *et al.*, 2019a). Moreover, when the 4-month-old progeny were fed a low FM and FO diet, those fish from parents fed partial FO replacement by LO showed improved growth and feed utilization (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2019a). Indeed, broodstock feeding exerts a very long-term effect in the progeny, and the replacement of FO by LO in parental diets, in combination to juvenile feeding with low-FM and low-FO diets, markedly improves 16-month-old offspring growth and feed utilization (Turkmen *et al.*, 2017a). These studies demonstrated that it is possible to improve the ability of marine fish to use low FM and low FO diets by nutritional programing though broodstock feeding. Similarly, exposure to a vegetable-based diet in early life of Atlantic salmon (*Salmo salar*) improves growth performance and feed efficiency later in life, when fish are challenged with a low FO and FM diet (Clarkson *et al.*, 2017).

Finally, choosing broodstock with higher *fads2* expression could also be an effective way to produce fish better able to use low FO diets. Recent studies have demonstrated that progeny obtained from broodstock with a high expression of *fads2* shows improved growth and feed utilization, particularly when challenged with a low FM and low FO diet (Turkmen *et al.*, 2019a). However, the potential beneficial effect of combining broodstock with a high *fads2* expression and nutritional programming failed to be demonstrated. More recently, we conducted a study aimed to evaluate the reproductive performance of broodstock with different *fads2* expression in combination with broodstock diets to stimulate nutritional programming (Ferosekhan *et al.*, 2020b). In this study, gilthead seabream broodfish inherently having either a high (H) or low (L) *fads2* expression were fed during the spawning season with two diets containing different fatty acid profiles. The results demonstrated that blood *fads2* expression in females, which tended to be higher than in males, is positively related to plasma 17β-estradiol levels, and improved reproductive performance (Ferosekhan *et al.*, 2020b). However, the potential effect of these type of broodstock on progeny performance or *fads2* expression had not been yet determined. Thus, the present study, following the previous one, aimed to determine the combined effect of broodstock with different *fads2* expression and nutritional programing through broodstock diets on the progeny performance. For that purpose, juveniles obtained from the previous study (Ferosekhan *et al.*, 2020b) were fed a low FM and FO diet for 45 days, and their growth, feed utilization, fatty acid composition of different tissues and expression of selected genes were studied.

5.2 Materials and Methods

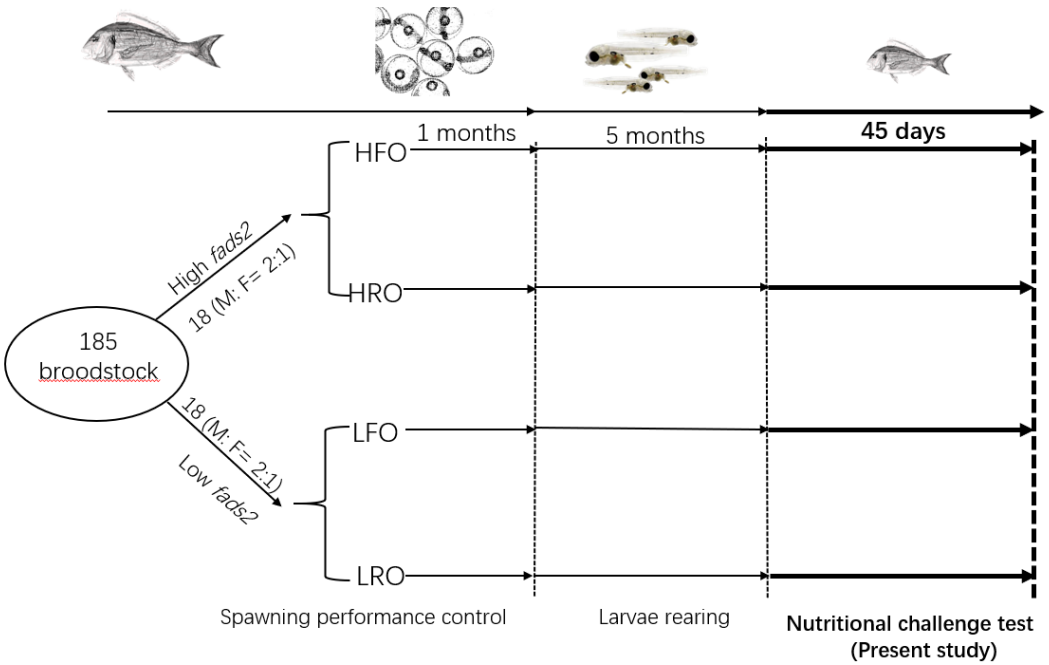
All the experiments were performed according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at Fundación Canaria Parque Científico Tecnológico (FCPCT), University of Las Palmas de Gran Canaria (Las Palmas de Gran Canaria, Spain).

5.2.1 Feeding trial and fish performance

Gilthead sea bream juveniles for this trial were obtained from broodstock with high (H) or low (L) *fads2* expression in blood when fed a low n-3-LC-PUFA diet and fed either with a high fish oil diet (FO) or with a high rapeseed oil diet (RO) during the spawning season (Ferosekhan *et*

al., 2020). Twelve broodstock with relatively high *fads2* expression and 12 broodstock with relatively low *fads2* expression were distributed into twelve 1000 L tanks (6H and 6L) and fed for 1 month with a diet that contained either FO or a mixture of 20% FO and 80% RO. Then eggs from each of the 4 broodstock groups (HFO, HRO, LFO and LRO) were collected in the same day and incubated in different tanks. The offspring obtained was reared under the same culture conditions and commercial diets (Figure 5.1). 300 fish (2.31 ± 0.01 g) from each of the 4 groups were randomly distributed into 3 tanks of 200 L (100 fish/tank, a total of 12 tanks). Tanks were provided with 200 L/h seawater at 23.3 ± 0.3°C and strong aeration, and dissolved oxygen (6.0 ± 0.3 mg/L) was daily determined. Tanks were illuminated by fluorescent lights placed above the tank at an intensity of 100 lx and programmed for a 12h light photoperiod (from 8 a.m. to 8 p.m.).

Figure 5.1 Schematic view of the experimental design of experiment



In order to challenge the fish with a very low FM and FO diet for evaluating their potential ability to cope with low n-3 LC-PUFA, an experimental diet was formulated to contain 7.5% of FM and 0% FO (Table 5.1). Dietary FM was replaced by corn gluten, soybean meal, wheat gluten, soya protein concentrates and faba bean meal in the experimental diets. Poultry oil and RO were used to replace the dietary fish oil. The diet was low in ARA, EPA and DHA and high in oleic acid (OA, 18:1n-9), LNA, palmitic acid (PA, 16:0) and ALA (Table 5.2).

Table 5.1 Ingredients and proximate composition of the challenge diet used to feed the juveniles obtained from broodstock with different *fads2* expression (high H or low L) and fed either a FO or a RO broodstock diet

Ingredient (% d.w.)	
Poultry oil[a]	6.14
Wheat[b]	9.46
Corn gluten[b]	10.00
Soybean meal[b]	5.00
Wheat Gluten[b]	19.18
Soya protein concentrate[b]	30.00
Faba beans[b]	5.00
Fish meal[b]	7.50
Rapeseed oil[b]	7.52
premix vit[c]	0.10
premix min[c]	0.10
Proximate composition (% d.w.)	
Crude protein	53.74
Crude lipid	22.90
Crude ash	4.71

a Poultry oil: Sonac. B.V. (The Netherlands). b Skretting AS (Norway). c Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC (Stavanger, Norway).

Table 5.2 Fatty acid composition (% total fatty acids) of the challenge diet used for juveniles obtained from broodstock with different *fads2* expression (high H or low L) and fed either a FO or a RO broodstock diet

Fatty acid	%
14:0	0.39
14:1n-7	0.04
14:1n-5	0.02
15:0	0.05
15:1n-5	0.02
16:0 ISO	0.04
16:0	9.99
16:1n-7	1.52
16:1n-5	0.01
16:2n-4	0.02
17:0	0.03
16:3n-4	0.08
16:3n-3	0.02
16:3n-1	0.03
16:4n-3	0.05
16:4n-1	0.01

Fatty acid	%
18:0	3.74
18:1n-9	42.03
18:1n-7	2.48
18.1n-5	0.06
18:2n-9	0.03
18.2n-6	25.09
18:2n-4	0.06
18:3n-6	0.16
18:3n-4	0.06
18:3n-3	5.43
18.3n-1	0.02
18:4n-3	0.21
18:4n-1	0.02
20:0	0.55
20:1n-9	0.15
20:1n-7	1.68

Fatty acid	%
20:1n-5	0.07
20:2n-9	0.02
20:2n-6	0.15
20:3n-9	0.03
20:3n-6	0.11
20:4n-6	0.27
20:3n-3	0.10
20:4n-3	0.09
20:5n-3	0.85
22:1n-11	1.33
22:1n-9	0.34
22:4n-6	0.07
22:5n-6	0.20
22:5n-3	0.30
22:6n-3	1.98

Fish were fed the experimental diet until apparent satiation 4 times per day (8:30, 10:30, 13:30 and 16:30) and 6 days per week for 45 days until fish body weight was tripled. Feed delivery was daily calculated, and uneaten pellets were collected in a net by opening the water outlet 30 min after each meal, dried in an oven for 24 h and weighed to estimate feed intake. All fish were weighted individually at day 30 and day 45 of the feeding trial. At the end of the trial, fish were fasted for 24 h, weighed and anesthetized with ethanol diluted clove oil (50:50) before samplings. The following performance parameters on mortality, growth (Weight gain (WG), Specific growth rate (SGR)), feed acceptance (Feed intake (FI)), Biological feed conversion ratio (FCR) (Council, 2011), energy status (hepatosomatic index (HSI)) (Chellappa *et al.*, 1995), lipid deposition (viscerosomatic index (VSI)), were calculated:

Mortality (%) = 100* (nº dead fish/ nº total fish)

Weight gain (g) = final body weight (BW_1) – initial body weight (BW_0)

Specific growth rate (% day^{-1}) = 100* (ln BW_1−ln BW_0) / nº days

Feed intake (FI, g $fish^{-1}$ day^{-1}) = Feed delivered / (nº of fish*nº days)

Biological feed conversion ratio (FCR) = Feed delivered ($t_1 - t_0$)/ (Biomass t_1 – Biomass t_0 + Biomass $_{harvested}$ + Biomass $_{lost}$)

Hepatosomatic index (HSI) = Liver weight (g) / Body weight (g, weight of liver included)

Viscerosomatic index (VSI) = Visceral weight (g) / Body weight (g, viscerosomatic weight included)

5.2.2 Histological study

The livers of 5 fish per tank were sampled and stored in 4% formalin. After embedded in paraffin wax, blocks were made and cut with a Leica microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany) in 4 μm sections, which were placed in slides and stained with haematoxylin and eosin (H&E) (Martoja *et al.*, 1970). Slides were studied and photos were taken by a light microscope (Olympus, Tokyo, Japan). Area, length of long and short axis of 60 hepatocyte per tank were analyzed with ImagePro plus 6.0 (Media Cybernetics, Rockville, USA).

5.2.3 Biochemical analysis

Chemical composition and fatty acid analysis

At the end of the feeding trial, 5 fish per tank were euthanized by immersion in 10 ppm clove oil in methanol (50:50) and sampled to determine lipid content and fatty acid composition of whole-body, liver and muscle. All samples were frozen at -80ºC until analysis. All samples were homogenized immediately prior to analysis. Moisture was determined according to A.O.A.C. (2000) (Horwitz, 2002). Lipids were extracted with chloroform/methanol (2:1 v/v) (Folch *et al.*, 1957) and then transmethylated to obtain the fatty acid methyl esters (FAMES) (Christie, 1982). FAMES were then separated by gas liquid chromatography, quantified by Flame ionization detector, and identified by comparison with external standards and well-characterized FO (EPA 28, Nippai, Ltd. Tokyo, Japan) (Izquierdo, 1989).

5.2.4 RNA extraction and digital PCR

The liver and muscle from the juveniles after the nutritional challenge were kept at 4 ºC overnight in 2.0 mL tubes with 1.5 mL of RNAlater and then transferred to -80℃ until molecular analysis. RNA from 100 mg sample was extracted using TRI Reagent (MilliporeSigma, Darmstadt, Germany) and then purified by RNeasy kit (Qiagen, Hilden, Germany). RNA quality was checked by 1.4% agarose electrophoresis and quantity was measured by NanoDrop 1000 (ThermoFisher, Waltham, U.S.A.). cDNA was synthesized from 1 µg of RNA in iCycler (Bio-rad, Hercules, U.S.A.), using the iScript cDNA synthesis kit (Bio-Rad). The expression of key enzymes related with growth and lipid metabolism were determined using the primers listed in Table 4. Digital PCR was performed as previous described in (Xu *et al.*, 2019) using in QX200™ Droplet Digital™ PCR System (Bio-Rad).

The amplification conditions of PCR were: 95℃ for 5 min, followed by 40 cycles at 95℃ for 30 sec, elongation at Tm temperature for 1min, and then stabilized the signal at 4 ℃ for 5 min, 90℃ for 5 min, finally the reaction was hold at 4℃.

Table 5.3 Primers sequence for digital PCR and GeneBank accession numbers for sequences of target genes

Gene	Forward	Reverse	GenBank accession
rpl-27	ACA ACT CAC TGC CCC ACC AT	CTT GCC TTT GCC CAG AA CTT	AY188520
fads2	CGA GAG CCA CAG CAG CAG GGA	CGG CCT GCG CCT GAG CAG TT	AY055749
elovl6	GTG CTG CTC TAC TCC TGG TA	ACG GCA TGG ACC AAG TAG T	JX975702
Igf-1	GTG TGT GGA GAG AGA GGC TT	CTC TTG GCA TGT CTG TGT GG	AY996779.2
ghr1	ACC TGT CAG CCA CCA CAT GA	TCG TGC AGA TCT GGG TCG TA	AH014067.4
ghr2	GAG TGA ACC CGG CCT GAC AG	GCGGTGGTATCTGATTCATGGT	AH014068.4
cox-2	GAG TAC TGG AAG CCG AGC AC	GAT ATC ACT GCC GCC TGA GT	AM296029

5.2.5 Data analysis

Two-way Anova was used for the comparison of the effects of broodstock *fads2* expression and broodstock diet, as well as their interaction, on the progeny's performance through SPSS 20.0 (IBM, New York, U.S.A.). Main effect analysis was performed for analyzing the effect of broodstock *fads2* expression or broodstock diet on offspring across the other factor. Pairwise comparison was used for the analysis of simple main effect. The main effect and comparison were corrected using Bonferroni procedure through SPSS. Unless special statement, data was shown as mean ± S.D. Shapiro-Wilk analysis was conducted for the normality and Levene's

test for the homogeneity of data variance. Residuals subjected to the standard normal distribution (p>0.05) and the error variance was equal. Gene expression was normalized by hepatic expression of *rpl27* (Park and Crowley, 2005). Peason correlation coefficient was calculated throught SPSS for the analysis of correlation between different results.

5.3 Results

5.3.1 Feeding trial and fish performance

Feeds were well accepted by all fish groups and no significant differences were found in feed intake or mortality rates. Even after only 30 days of feeding, the highest body weight was found in juveniles from broodstock with high *fads2* and fed diet RO (HRO), which was significantly (p<0.05) higher than that of HFO and LRO (Figure 5.2). Thus, at the end of the trial, the largest growth in terms of body weight, weight gain and SGR were found in juveniles from broodstock with high *fads2* and fed RO diet (HRO), whereas the lowest was found in juveniles obtained from broodstock with low *fads2* and fed RO diet (LRO) (Table 5.4). The two-way ANOVA showed a highly significant (p=0.001) interaction between the broodstock *fads2* expression (H, L) and the broodstock diet (LO, FO) on SGR (Table 5.4). According to the simple main effect analysis, SGR was 23% higher in HRO than in LRO juveniles (p=0.002), whereas it was 12% higher in LFO than in HFO juveniles (p=0.029) (Table 5.4). Comparing the juveniles from broodstock with the same *fads2* expression, SGR was 16% higher in HRO than in HFO juveniles (p=0.009), whereas it was 19% higher in LFO than in LRO juveniles (p=0.005). The FCR values followed a similar trend and the two-way ANOVA showed the interaction between the broodstock *fads2* expression and the broodstock diet (p=0.023) on FCR (Table 5.4). The simple main effect analysis showed that the FCR was 17% lower in HRO than in LRO juveniles (p=0.033). No significant differences were observed for HSI (p>0.05). Regarding VSI, increased *fads2* expression in broodstock or FO replacement by RO in broodstock diets significantly reduced VSI in juveniles (p=0.035, p=0.001, respectively) (Table 5.4). Thus, VSI was significantly lower in HRO than in HFO juveniles (p=0.007) and significantly lower in LRO than in LFO juveniles (p=0.014).

Figure 5.2 Body weight of of juveniles obtained from broodstock with different *fads2* expression (high H or low L) and fed either a FO or a RO broodstock diet along the 45 days of feeding the challenge diet

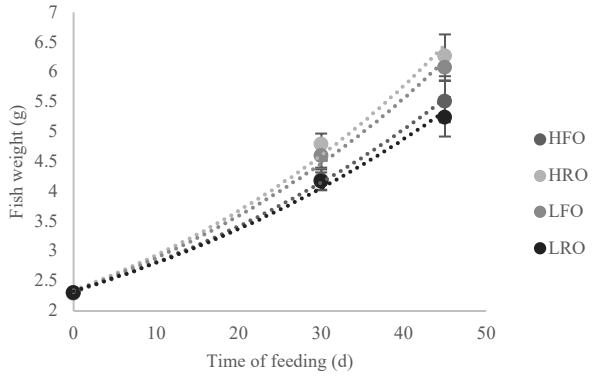


Table 5.4 Growth performance of gilthead seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) and fed either a FO or a RO broodstock diet, after 45 days of feeding the challenge diet

	HFO		HRO		LFO		LRO		Two-way ANOVA		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	*Bfads2*	*Bdiet*	*Bfads2*Bdiet*
Initial weight (g)	2.31	0.01	2.31	0.00	2.30	0.01	2.31	0.01	*n.s.*	*n.s.*	*n.s.*
Weight gain (g)	$^{b}3.20^{B}$	0.35	$^{a}3.98^{I}$	0.03	$^{1}3.78^{A}$	0.23	$^{2}2.94^{II}$	0.33	*n.s.*	*n.s.*	*0.001*
Mortality (%)	5.67	6.43	2.66	0.58	2.66	0.58	3.33	1.15	*n.s.*	*n.s.*	*n.s.*
SGR (%day)	$^{b}1.97^{B}$	0.15	$^{a}2.28^{I}$	0.02	$^{1}2.21^{A}$	0.09	$^{2}1.86^{II}$	0.14	*n.s.*	*n.s.*	*0.001*
Feed intake (g fish^{-1} day^{-1})	0.12	0.01	0.12	0.01	0.12	0.01	0.11	0.01	*n.s.*	*n.s.*	*n.s.*
FCR (%)	1.61	0.11	1.37^{II}	0.06	1.47	0.15	1.64^{i}	0.2	*n.s.*	*n.s.*	*0.023*
HSI (%)	2.31	0.17	2.21	0.25	2.41	0.11	2.22	0.11	*n.s.*	*n.s.*	*n.s.*
VSI (%)	$^{a}7.59$	0.64	$^{b}6.08$	0.62	$^{1}8.25$	0.5	$^{2}6.92$	0.16	*0.035*	*0.001*	*n.s.*

SGR: Specific growth rate; FCR: Feed conversion rate; HSI: Hepatic somatic index; VSI: Visceral somatic index; Bfads2 refers to the effect of the broodstock *fads2* expression (H or L); Bdiet refers to the effect of the broodstock diet (FO or RO).

a, b in front of the value mean there is significant difference between the offspring come from same selection broodstock group (High) but the broodstock were fed with different diet. 1,2 in front of the value mean there is significant difference between the offspring come from same selection broodstock group (Low) but the broodstock were fed with different diet. A, B in the back of the value mean there is significant difference between the offspring come from broodstock fed with diet FO but with different *fads2* expression. I, II in the back of the value mean there is significant difference between the offspring come from broodstock fed with diet VO but with different *fads2* expression.

5.3.2 Histology study

The area, length and width of the hepatocyte of juveniles from broodstock fed with RO diet was 21%, 14% and 8% smaller than that from broodstock fed with FO diet (p_{area}=0.023, p_{length} =0.002, p_{width}=0.031) (Table 5.5). A positive correlation was found between the area of hepatocyte and the HSI of fish (r=0.96, p=0.043). Besides, the hepatocyte width in juveniles from broodstock with high *fads2* expression was 11% shorter than that from broodstock with low *fads2* expression (p=0.011). In the pairwise comparison, the width of HRO hepatocytes was significantly shorter than that of HFO and LRO hepatocytes (p = 0.038 and p = 0.018, respectively).

Table 5.5 Morphometry of hepatocytes from gilthead seabream juveniles obtained from broodstock with different fads2 expression (high H or low L) and fed either a FO or a RO rich diet, after 45 days of feeding the experimental diet

	HFO		HRO		LFO		LRO		Two-way Anova		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	*Bfads2*	*Bdiet*	*Bfads2*Bdiet*
Area (μm^2)	80.45	11.51	65.76	8.29	87.72	10.2	72.88	5.19	*n.s.*	*0.023*	*n.s.*
Long axis (μm)	11.11	0.86	9.53	0.33	11.54	0.51	10.3	0.21	*n.s.*	*0.002*	*n.s.*
Short axis(μm)	6.88	0.46	6.09	0.58	7.41	0.19	7.03	0.17	*0.011*	*0.031*	*n.s.*

n.s. No statistical significance

5.3.3 Biochemical analysis

No significant differences were found in lipid contents of whole fish, liver or muscle (p>0.05) (Table 5.6). Regarding the fatty acid profiles of liver, the two-way ANOVA denoted the increase in hepatic PUFA contents in juveniles from broodstock with high *fads2* expression (p=0.014) and the combined effect with the broodstock diet (p=0.015) (Table 5.7). Thus, the pairwise analysis showed that PUFA contents were higher in LRO than in LFO juveniles (p=0.036). Accordingly, hepatic n-6 PUFA were increased in juveniles from broodstock with high *fads2* expression (p=0.027), with higher values in HFO than in LFO juveniles (p=0.015). Also, hepatic n-3 fatty acids contents were significantly (p=0.017) affected by the interaction of broodstock *fads2* expression and broodstock diets, with higher n-3 PUFA contents in HFO than in LFO (p=0.007) and in HFO than in HRO (p=0.032). The same trend was observed for ARA (p=0.012), with higher contents in HFO than in LFO (p=0.005) and in HFO than in HRO (p=0.009), and for EPA (p=0.036), with higher contents in HFO than in LFO (p=0.038).

Table 5.6 Lipid content of different tissues from gilthead seabream juveniles obtained from broodstock with different fads2 expression (high H or low L) and fed either a FO or a RO broodstock diet, after 45 days of feeding the challenge diet

(% wet weight)	HFO		HRO		LFO		LRO	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Whole fish	10.46	1.23	10.75	1.21	11.07	0.47	11.24	1.55
Liver	32.56	0.31	33.89	2.77	33.47	1.57	35.47	2.31
Muscle	7.25	2.63	7.57	1.10	8.62	0.87	8.08	0.44

No statistical differences were observed

SFA was 21% lower in HFO than LFO (p=0.019). MUFA showed a complementary trend to the PUFA contents, with lower values for HFO than for LFO juveniles (p_{MUFA}=0.007) and lower values for LRO than for LFO juveniles (p_{MUFA}=0.042). Besides, 16:1/16:0 ratios were higher in juveniles from broodstock with higher *fads2* expression than in those from low *fads2*

Table 5.7 Main fatty acids (% total fatty acids) of liver from gilthead seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) and fed either a FO or a RO broodstock diet, after 45 days of feeding the challenge diet

	HFO		HRO		LFO		LRO		Two-way ANOVA		
Name	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Selection	Diet	Selection* Diet
14:0	0.85	0.23	0.93	0.42	^{1}1.10	0.03	^{2}0.69	0.07	*n.s.*	*n.s.*	*n.s.*
14:1n-7	^{a}0.25^{A}	0.11	^{b}0.08	0.08	0.02^{B}	0.00	0.13	0.10	*n.s.*	*n.s.*	*0.024*
14:1n-5	0.24^{A}	0.11	0.08	0.09	0.01^{B}	0.00	0.12	0.10	*n.s.*	*n.s.*	*0.027*
15:0	0.21^{A}	0.60	0.12	0.07	0.07^{B}	0.01	0.16	0.08	*n.s.*	*n.s.*	*0.038*
15:1n-5	^{a} 0.21^{A}	0.09	^{b}0.06	0.06	0.02^{B}	0.00	0.12	0.10	*n.s.*	*n.s.*	*0.017*
16:0 ISO	^{a}0.22^{A}	0.10	^{b}0.06	0.07	0.00^{B}	0.00	0.10	0.08	*n.s.*	*n.s.*	*0.018*
16:0	10.04^{B}	0.25	10.90	2.64	13.66^{A}	0.35	11.24	2.23	*n.s.*	*n.s.*	*n.s.*
16:1n-7	1.86^{B}	0.15	2.03	0.42	2.16^{A}	0.04	1.97	0.25	*n.s.*	*n.s.*	*n.s.*
16:1n-5	0.21^{A}	0.08	0.09	0.03	^{2}0.05^{B}	0.01	^{1}0.16	0.09	*n.s.*	*n.s.*	*0.016*
16:2n-4	^{a}0.23^{A}	0.11	^{b}0.06	0.04	0.02^{B}	0.00	0.12	0.09	*n.s.*	*n.s.*	*0.012*
17:0	^{a}0.27^{A}	0.13	^{b}0.09	0.03	0.05^{B}	0.01	0.11	0.07	*0.048*	*n.s.*	*0.021*
16:3n-4	^{a} 0.32^{A}	0.12	^{b}0.15	0.01	0.13^{B}	0.01	0.18	0.06	*n.s.*	*n.s.*	*0.02*
16:3n-3	0.28^{A}	0.17	0.08	0.07	0.03^{B}	0.01	0.14	0.12	*n.s.*	*n.s.*	*0.044*
16:3n-1	^{a}0.28^{A}	0.17	^{b}0.07	0.08	0.02^{B}	0.00	0.12	0.11	*n.s.*	*n.s.*	*0.036*
16:4n-3	^{a} 0.44^{A}	0.18	^{b}0.11	0.11	0.03^{B}	0.01	0.15	0.13	*0.037*	*n.s.*	*0.035*
18:0	6.06^{B}	0.74	6.29	0.41	^{1}7.45^{A}	0.45	^{2}6.64	0.17	*0.014*	*n.s.*	*n.s.*
18:1n-9	37.46^{B}	3.31	41.67	2.40	43.71^{A}	0.38	40.08	2.19	*n.s.*	*n.s.*	*0.019*
18:1n-7	2.55	0.27	2.77	0.07	2.70	0.04	3.44	0.97	*n.s.*	*n.s.*	*n.s.*
18:1n-5	0.28^{A}	0.10	0.12	0.11	0.07^{B}	0.01	0.11	0.13	*n.s.*	*n.s.*	*n.s.*
18:2n-9	^{b}2.57	0.50	^{a}3.54	0.54	2.85	0.63	3.24	0.10	*n.s.*	*0.042*	*n.s.*
18:2n-6	14.07	1.66	15.44	0.97	14.33	0.79	14.73	1.69	*n.s.*	*n.s.*	*n.s.*
18:2n-4	^{a}0.25^{A}	0.06	^{b}0.09	0.09	0.04^{B}	0.01	0.09	0.12	*n.s.*	*n.s.*	*n.s.*
18:3n-6	^{b}3.38	0.35	^{a}4.45	0.27	^{2}3.11	0.46	^{1}3.96	0.21	*n.s.*	*0.001*	*n.s.*
18:3n-4	0.23^{A}	0.08	0.12	0.07	0.05^{B}	0.01	0.09	0.11	*0.048*	*n.s.*	*n.s.*
18:3n-3	2.37	0.27	2.33	0.24	2.13	0.16	2.24	0.33	*n.s.*	*n.s.*	*n.s.*
18:4n-3	0.99^{A}	0.13	0.95	0.04	^{2}0.68^{B}	0.07	^{1}0.86	0.30	*0.047*	*n.s.*	*0.013*
18:4n-1	^{a}0.31^{A}	0.15	^{b}0.09	0.08	0.03^{B}	0.01	0.18	0.15	*n.s.*	*n.s.*	*0.023*
20:0	^{a}0.36^{A}	0.03	^{b}0.23	0.10	0.22^{B}	0.02	0.27	0.07	*n.s.*	*n.s.*	*0.048*
20:1n-9	0.37^{A}	0.11	0.22	0.09	0.18^{B}	0.02	0.28	0.10	*n.s.*	*n.s.*	*0.034*
20:1n-7	0.86	0.13	0.68	0.23	0.69	0.02	0.76	0.17	*n.s.*	*n.s.*	*n.s.*
20:1n-5	0.13	0.11	0.15	0.11	0.08	0.02	0.15	0.08	*n.s.*	*n.s.*	*n.s.*
20:2n-9	0.61	0.11	0.52	0.28	0.56	0.07	0.40	0.10	*n.s.*	*n.s.*	*n.s.*
20:2n-6	0.47	0.09	0.28	0.13	0.27	0.03	0.33	0.19	*n.s.*	*n.s.*	*n.s.*
20:3n-9	0.50	0.21	0.32	0.20	0.19	0.15	0.03	0.25	*n.s.*	*n.s.*	*n.s.*
20:3n-6	0.35	0.16	0.14	0.21	0.12	0.15	0.27	0.04	*n.s.*	*n.s.*	*n.s.*
20:4n-6	^{a}0.65^{A}	0.24	^{b}0.26	0.10	0.22^{B}	0.01	0.34	0.08	*n.s.*	*n.s.*	*0.012*
20:3n-3	^{a}0.47^{A}	0.17	^{b}0.21	0.20	0.09^{B}	0.02	0.18	0.09	*0.032*	*n.s.*	*n.s.*
20:4n-3	0.44^{A}	0.14	0.20	0.17	0.12^{B}	0.02	0.29	0.24	*n.s.*	*n.s.*	*n.s.*
20:5n-3	0.86^{A}	0.22	0.45	0.22	0.36^{B}	0.05	0.67	0.38	*n.s.*	*n.s.*	*0.037*
22:1n-11	0.55	0.51	0.37	0.24	0.27	0.04	0.45	0.28	*n.s.*	*n.s.*	*n.s.*
22:1n-9	0.38	0.34	0.35	0.21	0.30	0.04	0.37	0.21	*n.s.*	*n.s.*	*n.s.*
22:4n-6	0.91	0.59	0.32	0.47	0.08	0.03	0.50	0.68	*n.s.*	*n.s.*	*n.s.*
22:5n-6	1.26	1.10	0.33	0.40	0.13	0.02	0.42	0.37	*n.s.*	*n.s.*	*n.s.*
22:5n-3	1.18^{A}	0.89	0.31	0.31	0.10^{B}	0.01	0.41	0.33	*n.s.*	*n.s.*	*0.075*
22:6n-3	3.23	1.72	1.88	1.16	1.43	0.03	2.30	0.23	*n.s.*	*n.s.*	*n.s.*
∑SFA	17.78^{B}	0.75	18.55	3.31	^{1}22.55^{A}	0.05	^{2}19.12	2.02	*n.s.*	*n.s.*	*n.s.*
∑MUFA	45.15^{B}	2.86	48.61	1.64	^{1}50.25^{A}	0.50	^{2}48.02	1.06	*n.s.*	*n.s.*	*0.023*
∑PUFA	36.64^{A}	2.80	32.71	4.11	^{2}27.17^{B}	0.06	^{1}32.64	1.88	*0.014*	*n.s.*	*0.015*
∑n-3 FA	^{a}10.25^{A}	2.30	^{b}6.51	2.40	5.02^{B}	0.21	7.38	1.14	*n.s.*	*n.s.*	*0.017*
∑n-6 FA	21.10^{A}	1.01	21.23	1.57	^{2}18.27^{B}	0.39	^{1}20.55	1.19	*0.027*	*n.s.*	*n.s.*
18:1/18:0	0.42	0.01	0.44	0.03	0.36	0.02	0.52	0.16	*n.s.*	*n.s.*	*n.s.*
18:4n-3/18:3n-3	0.42	0.08	0.41	0.04	0.34	0.06	0.45	0.09	*n.s.*	*n.s.*	*n.s.*
18:3n-6/18:2n-6	0.24	0.03	0.29	0.02	0.22	0.05	0.27	0.03	*n.s.*	*0.03*	*n.s.*
20:3n-6/20:2n-6	0.78	0.45	0.37	0.47	0.46	0.56	1.03	0.59	*n.s.*	*n.s.*	*n.s.*
18:2n-9/18:1n-9	0.07	0.01	0.09	0.01	0.07	0.02	0.08	0.01	*n.s.*	*0.03*	*n.s.*
16:1/16:0	0.19^{A}	0.02	0.19	0.01	0.16^{B}	0.01	0.18	0.02	*0.02*	*n.s.*	*n.s.*
18:0/16:0	0.60	0.08	0.59	0.10	0.54	0.04	0.61	0.12	*n.s.*	*n.s.*	*n.s.*
18:1n-7/16:1n-7	1.39	0.24	1.41	0.29	^{2}1.25	0.03	^{1}1.73	0.27	*n.s.*	*n.s.*	*n.s.*
DHA/EPA	4.13	2.55	3.95	0.64	3.97	0.44	4.25	2.35	*n.s.*	*n.s.*	*n.s.*

SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid. Bfads2 refers to the effect of the broodstock *fads2* expression (H or L); Bdiet refers to the effect of the broodstock diet (FO or RO).

a, b in front of the value mean there is significant difference between the offspring come from same selection broodstock group (High) but the broodstock were fed with different diet.1,2 in front of the value mean there is significant difference between the offspring come from same selection broodstock group (Low) but the broodstock were fed with different diet. A, B in the back of the value mean there is significant difference between the offspring come from broodstock fed with diet FO but with different *fads2* expression.

n.s. No statistical significance

expression broodstock (p=0.015). Also, juveniles from broodstock fed with RO diet showed significantly higher 18:3n-6/18:2n:6, 18:2n-9/18:1n-9 ratio than that from broodstock fed with FO diet ($p_{18:3n-6/18:2n:6}$ =0.033, $p_{18:2n-9/18:1n-9}$ =0.033). A negative correlation was found between 18:3n-

6/18:2n:6 and the hepatocyte area (r =-0.999, p=0.001).

Table 5.8 Fatty acid composition of muscle of juveniles obtained from broodstock with different *fads2* expression (high H or low L) and fed either a FO or a RO broodstock diet, after 45 days of feeding the challenge diet

	HFO		HRO		LFO		LRO		Two-way ANOVA		
Name	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	*Selection*	*Diet*	*S* D*
14:0	0.83	0.2	0.72	0.13	0.74	0.06	0.79	0.13	*n.s.*	*n.s.*	*n.s.*
14:1n-7	0.05	0.01	0.05	0.01	0.08	0.05	0.06	0.02	*n.s.*	*n.s.*	*n.s.*
14:1n-5	0.03	0.03	0.02	0.03	0.05	0.06	0.03	0.02	*n.s.*	*n.s.*	*n.s.*
15:0	0.12	0.03	0.10	0.01	0.11	0.04	0.1	0.01	*n.s.*	*n.s.*	*n.s.*
15:1n-5	0.03	0.02	0.04	0.00	0.06	0.04	0.05	0.04	*n.s.*	*n.s.*	*n.s.*
16:0 ISO	0.03	0.03	0.03	0.01	0.05	0.06	0.03	0.03	*n.s.*	*n.s.*	*n.s.*
16:0	10.95	0.97	10.62	1.06	10.91	1.37	11.01	0.49	*n.s.*	*n.s.*	*n.s.*
16:1n-7	2.34	0.23	2.42	0.28	2.31	0.24	2.41	0.05	*n.s.*	*n.s.*	*n.s.*
16:1n-5	0.06	0.04	0.04	0.01	0.08	0.03	0.06	0.02	*n.s.*	*n.s.*	*n.s.*
16:2n-4	0.05	0.02	0.04	0.01	0.07	0.03	0.05	0.00	*n.s.*	*n.s.*	*n.s.*
17:0	0.08	0.03	0.06	0.01	0.09	0.03	0.08	0.01	*n.s.*	*n.s.*	*n.s.*
16:3n-4	0.14	0.03	0.13	0.02	0.15	0.02	0.14	0.02	*n.s.*	*n.s.*	*n.s.*
16:3n-3	0.04	0.03	0.04	0.02	0.06	0.05	0.04	0.02	*n.s.*	*n.s.*	*n.s.*
16:3n-1	0.11	0.02	0.08	0.03	0.1	0.04	0.11	0.03	*n.s.*	*n.s.*	*n.s.*
16:4n-3	0.06	0.01	0.1	0.076	0.12	0.11	0.08	0.02	*n.s.*	*n.s.*	*n.s.*
16:4n-1	0.01	0.02	0.02	0.03	0.00	0.02	0.02	0.00	*n.s.*	*n.s.*	*n.s.*
18:0	4.06	0.18	3.84	0.15	3.98	0.2	4.08	0.13	*n.s.*	*n.s.*	*n.s.*
18:1n-9	38.29	0.99	39.67	1.37	39.63	1.3	38.99	0.73	*n.s.*	*n.s.*	*n.s.*
18:1n-7	2.58	0.06	2.57	0.38	2.55	0.07	2.59	0.04	*n.s.*	*n.s.*	*n.s.*
18.1n-5	0.09	0.03	0.08	0.02	0.14	0.13	0.09	0.02	*n.s.*	*n.s.*	*n.s.*
18:2n-9	1.28	0.23	1.00	0.31	1.09	0.10	1.03	0.13	*n.s.*	*n.s.*	*n.s.*
18.2n-6	21.3	0.65	21.09	0.24	20.39	0.46	20.65	0.22	*0.026*	*n.s.*	*n.s.*
18:2n-4	0.05	0.02	0.06	0.01	0.09	0.09	0.06	0.03	*n.s.*	*n.s.*	*n.s.*
18:3n-6	1.58^{A}	0.16	1.29	0.4	1.19^{B}	0.12	1.24	0.06	*n.s.*	*n.s.*	*n.s.*
18:3n-4	0.09	0.03	0.09	0.03	0.11	0.05	0.10	0.02	*n.s.*	*n.s.*	*n.s.*
18:3n-3	3.88	0.13	4.00	0.03	3.95	0.03	3.89	0.07	*n.s.*	*n.s.*	*n.s.*
18:4n-3	0.5^{A}	0.01	0.46	0.09	0.46^{B}	0.15	0.47	0.04	*n.s.*	*n.s.*	*n.s.*
18:4n-1	0.07	0.07	0.04	0.01	0.09	0.07	0.05	0.00	*n.s.*	*n.s.*	*n.s.*
20:0	0.34	0.04	0.33	0.07	0.42	0.14	0.33	0.01	*n.s.*	*n.s.*	*n.s.*
20:1n-9	0.21	0.01	0.21	0.03	0.24	0.1	0.21	0.01	*n.s.*	*n.s.*	*n.s.*
20:1n-7	1.17	0.04	1.26	0.11	1.32	0.32	1.22	0.03	*n.s.*	*n.s.*	*n.s.*
20.1n-5	0.10	0.02	0.18	0.11	0.18	0.16	0.13	0.02	*n.s.*	*n.s.*	*n.s.*
20:2n-9	0.24^{B}	0.08	0.33	0.07	$^{1}0.46^{A}$	0.03	$^{2}0.3$	0.02	*0.018*	*n.s.*	*0.006*
20:2n-6	0.36	0.01	0.37	0.01	0.45	0.17	0.37	0.01	*n.s.*	*n.s.*	*n.s.*
20:3n-9	0.29	0.07	0.33	0.00	0.34	0.01	0.29	0.04	*n.s.*	*n.s.*	*n.s.*
20:3n-6	0.08	0.04	0.09	0.02	0.08	0.04	0.06	0.06	*n.s.*	*n.s.*	*n.s.*
20:4n-6	0.46	0.06	0.39	0.06	0.36	0.06	0.43	0.06	*n.s.*	*n.s.*	*n.s.*
20:3n-3	0.17	0.08	0.16	0.03	0.16	0.05	0.13	0.02	*n.s.*	*n.s.*	*n.s.*
20:4n-3	0.20	0.03	0.20	0.02	0.24	0.03	0.20	0.01	*n.s.*	*n.s.*	*n.s.*
20:5n-3	1.12	0.12	1.14	0.29	1.03	0.19	1.29	0.18	*n.s.*	*n.s.*	*n.s.*
22:1n-11	0.66	0.02	0.68	0.05	0.79	0.23	0.65	0.00	*n.s.*	*n.s.*	*n.s.*
22:1n-9	0.46	0.02	0.42	0.02	0.54	0.14	0.41	0.06	*n.s.*	*n.s.*	*n.s.*
22:4n-6	0.19	0.07	0.15	0.02	0.24	0.2	0.19	0.06	*n.s.*	*n.s.*	*n.s.*
22:5n-6	0.43	0.13	0.39	0.11	0.40	0.09	0.41	0.07	*n.s.*	*n.s.*	*n.s.*
22:5n-3	0.49	0.60	0.49	0.11	0.55	0.32	0.52	0.08	*n.s.*	*n.s.*	*n.s.*
22:6n-3	4.33	1.2	4.18	1.31	3.56	0.81	4.54	0.82	*n.s.*	*n.s.*	*n.s.*
∑ SFA	16.37	1.16	15.66	1.04	16.25	1.42	16.39	0.50	*n.s.*	*n.s.*	*n.s.*
∑ MUFA	46.04	1.14	47.61	1.44	47.90	0.61	46.85	0.66	*n.s.*	*n.s.*	*n.s.*
∑ PUFA	37.53	1.65	36.66	2.43	35.74	1.75	36.67	0.98	*n.s.*	*n.s.*	*n.s.*
∑ n-3 PUFA	10.79	1.23	10.76	1.76	10.14	1.55	11.16	0.99	*n.s.*	*n.s.*	*n.s.*
∑ n-6 PUFA	24.41^{A}	0.53	23.77	0.39	$^{2}23.09^{B}$	0.03	$^{1}23.35$	0.02	*0.002*	*n.s.*	*0.046*
18:1/18:0	0.64	0.04	0.67	0.04	0.64	0.03	0.64	0.01	*n.s.*	*n.s.*	*n.s.*
18:4n-3/18:3n-3	0.13	0.01	0.12	0.03	0.12	0.00	0.12	0.01	*n.s.*	*n.s.*	*n.s.*
18:3n-6/18:2n-6	0.074	0.008	0.061	0.020	0.058	0.005	0.060	0.003	*n.s.*	*n.s.*	*n.s.*
20:3n-6/20:2n-6	0.23	0.11	0.24	0.06	0.17	0.03	0.16	0.16	*n.s.*	*n.s.*	*n.s.*
18:2n-9/18:1n-9	0.034	0.007	0.025	0.009	0.028	0.002	0.027	0.003	*n.s.*	*n.s.*	*n.s.*
16:1/16:0	$^{b}0.21$	0.01	$^{a}0.23$	0.00	0.21	0.01	0.22	0.01	*n.s.*	*0.039*	*n.s.*
18:0/16:0	0.37	0.04	0.36	0.05	0.37	0.03	0.37	0.03	*n.s.*	*n.s.*	*n.s.*
18:1n-7/16:1n-7	1.11	0.08	1.07	0.13	1.11	0.11	1.08	0.03	*n.s.*	*n.s.*	*n.s.*
DHA/EPA	3.83	0.66	3.63	0.22	3.44	0.16	3.51	0.23	*n.s.*	*n.s.*	*n.s.*

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids, Bfads2 refers to the effect of the broodstock *fads2* expression (H or L); Bdiet refers to the effect of the broodstock diet (FO or RO). a, b in front of the value mean there is significant difference between the offspring come from same selection broodstock group (High) but the broodstock were fed with different diet. 1,2 in front of the value mean there is significant difference between the offspring come from same selection broodstock group (Low) but the broodstock were fed with different diet. A, B in the back of the value mean there is significant difference between the offspring come from broodstock fed with diet FO but with different *fads2* expression.I, II in the back of the value mean there is significant difference between the offspring come from broodstock fed with diet RO diet but with different *fads2* expression. *n.s.* No statistical significance

In muscle, the n-6 fatty acids contents and, particularly, 18:2n-6, were significantly higher in juveniles from high *fads2* expression broodstock than in those from broodstock with low *fads2* expression ($p_{\text{n-6 FA}}$ =0.002; $p_{18:2n-6}$ =0.027). Juveniles from broodstock fed with RO diet were higher in the ratio 16:1n-7/16:0 than those from broodstock fed with FO diet (p=0.039) (Table 5.8).

In whole body, there was also an interaction with broodstock diets (p=0.046) and therefore, n-6 content was significantly higher in HRO than in LRO juveniles (p=0.012), and also higher in LFO than in LRO juveniles (p = 0.031) (Table 5.9). Besides, there was a combined effect of both factors, *fads2* expression and broodstock diet, on EPA (p=0.002), which was significantly higher in HFO than in HRO (p=0.033) or LRO (p=0.022).

Table 5.9 Whole body fatty acid composition of seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) and fed either a FO or a RO broodstock diet, after 45 days of feeding the challenge diet

	HFO		HRO		LFO		LRO		Two-way ANOVA		
Name	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Selection	Diet	Selection* Diet
14:0	0.96	0.39	1.17	0.52	1.20	0.14	1.43	0.38	*n.s.*	*n.s.*	*n.s.*
14:1n-7	0.11	0.10	0.07	0.31	0.08	0.01	0.06	0.02	*n.s.*	*n.s.*	*n.s.*
14:1n-5	0.18	0.26	0.03	0.01	0.06	0.06	0.03	0.01	*n.s.*	*n.s.*	*n.s.*
15:0	0.20	0.14	0.13	0.04	0.12	0.00	0.14	0.02	*n.s.*	*n.s.*	*n.s.*
15:1n-5	0.13	0.14	0.04	0.03	0.06	0.02	0.03	0.01	*n.s.*	*n.s.*	*n.s.*
16:0 ISO	0.13	0.17	0.03	0.03	0.05	0.04	0.02	0.01	*n.s.*	*n.s.*	*n.s.*
16:0	10.25	2.76	11.85	3.00	11.97	0.91	12.94	0.87	*n.s.*	*n.s.*	*n.s.*
16:1n-7	2.46	0.62	2.88	0.59	2.62	0.16	2.99	0.57	*n.s.*	*n.s.*	*n.s.*
16:1n-5	0.11	0.10	0.08	0.03	0.13	0.07	0.04	0.01	*n.s.*	*n.s.*	*n.s.*
16:2n-4	0.34	0.10	0.10	0.03	0.06	0.06	0.10	0.03	*n.s.*	*n.s.*	*n.s.*
17:0	0.12	0.04	0.09	0.03	0.10	0.03	0.11	0.03	*n.s.*	*n.s.*	*n.s.*
16:3n-4	0.15	0.00	0.15	0.01	0.15	0.03	0.15	0.02	*n.s.*	*n.s.*	*n.s.*
16:3n-3	0.10	0.09	0.03	0.02	0.06	0.03	0.04	0.01	*n.s.*	*n.s.*	*n.s.*
16:3n-1	0.16	0.07	0.13	0.05	0.14	0.02	0.12	0.03	*n.s.*	*n.s.*	*n.s.*
16:4n-3	0.24	0.14	0.20	0.07	0.16	0.02	0.14	0.02	*n.s.*	*n.s.*	*n.s.*
16:4n-1	0.09	0.09	0.07	0.04	0.03	0.04	0.02	0.01	*n.s.*	*n.s.*	*n.s.*
18:0	3.91	0.22	3.63	0.42	4.11	0.18	4.17	0.24	*n.s.*	*n.s.*	*n.s.*
18:1n-9	36.88	3.03	37.46	1.97	39.04	1.33	39.24	2.62	*n.s.*	*n.s.*	*n.s.*
18:1n-7	2.67	0.06	5.20	4.63	2.63	0.05	2.61	0.01	*n.s.*	*n.s.*	*n.s.*
18.1n-5	0.15	0.09	0.06	0.05	0.14	0.07	0.07	0.01	*n.s.*	*n.s.*	*n.s.*
18:2n-9	1.29	0.33	1.23	0.19	1.13	0.06	1.20	0.11	*n.s.*	*n.s.*	*n.s.*
18.2n-6	18.49	1.30	20.15	1.58	19.14	0.95	18.3	0.76	*n.s.*	*n.s.*	*n.s.*
18:2n-4	0.13	0.09	0.05	0.03	0.1	0.06	0.07	0.02	*n.s.*	*n.s.*	*n.s.*
18:3n-6	1.44	0.15	1.53	0.08	1.35	0.07	1.40	0.14	*n.s.*	*n.s.*	*n.s.*
18:3n-4	0.21	0.18	0.08	0.03	0.15	0.07	0.08	0.02	*n.s.*	*n.s.*	*n.s.*
18:3n-3	3.59	0.28	3.91	0.26	3.73	0.11	3.51	0.18	*n.s.*	*n.s.*	*n.s.*
18:4n-3	0.74	0.03	0.58	0.05	0.5	0.02	0.59	0.05	*n.s.*	*n.s.*	*n.s.*
18:4n-1	0.16	0.16	0.05	0.04	0.10	0.05	0.04	0.02	*n.s.*	*n.s.*	*n.s.*
20:0	0.42	0.16	0.26	0.06	0.33	0.06	0.30	0.01	*n.s.*	*n.s.*	*n.s.*
20:1n-9	0.33	0.21	0.17	0.05	0.20	0.03	0.19	0.02	*n.s.*	*n.s.*	*n.s.*
20:1n-7	1.62	0.43	1.09	0.18	1.11	0.07	1.20	0.05	*n.s.*	*n.s.*	*n.s.*
20.1n-5	0.31	0.31	0.16	0.06	0.14	0.05	0.11	0.02	*n.s.*	*n.s.*	*n.s.*
20:2n-9	0.39	0.10	0.33	0.04	[1]0.44	0.08	[2]0.26	0.05	*n.s.*	*0.02*	*n.s.*
20:2n-6	0.44	0.17	0.27	0.05	[1]0.38	0.03	[2]0.29	0.02	*n.s.*	*0.035*	*n.s.*
20:3n-9	0.10	0.16	0.03	0.03	0.35	0.08	0.14	0.12	*0.022*	*n.s.*	*n.s.*
20:3n-6	0.35	0.18	0.26[I]	0.05	0.08	0.10	0.09[II]	0.07	*0.009*	*n.s.*	*n.s.*
20:4n-6	0.47	0.17	0.37	0.13	0.37	0.10	0.37	0.04	*n.s.*	*n.s.*	*n.s.*
20:3n-3	0.26	0.26	0.14	0.09	0.20	0.09	0.12	0.03	*n.s.*	*n.s.*	*n.s.*
20:4n-3	0.32	0.15	0.19	0.05	0.23	0.06	0.20	0.06	*n.s.*	*n.s.*	*n.s.*
20:5n-3	1.62	0.37	1.03	0.28	0.98	0.21	1.33	0.23	*n.s.*	*n.s.*	*0.02*
22:1n-11	1.12	0.53	0.57	0.25	0.75	0.15	0.74	0.09	*n.s.*	*n.s.*	*n.s.*
22:1n-9	0.55	0.24	0.36	0.11	0.48	0.08	0.38	0.05	*n.s.*	*n.s.*	*n.s.*
22:4n-6	0.39	0.29	0.15	0.13	0.72	0.93	0.12	0.01	*n.s.*	*n.s.*	*n.s.*
22:5n-6	0.47	0.30	0.27	0.15	0.51	0.39	0.26	0.04	*n.s.*	*n.s.*	*n.s.*
22:5n-3	0.76	0.32	0.37	0.13	0.51	0.25	0.51	0.11	*n.s.*	*n.s.*	*n.s.*
22:6n-3	4.50	1.53	3.13	1.18	3.08	0.59	3.74	0.52	*n.s.*	*n.s.*	*n.s.*

	HFO		HRO		LFO		LRO		Two-way ANOVA		
Name	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Selection	Diet	Selection* Diet
∑SFA	15.86	3.02	17.11	3.98	17.83	1.06	19.10	1.09	*n.s.*	*n.s.*	*n.s.*
∑MUFA	46.40	1.29	48.13	2.26	47.38	0.91	47.67	2.10	*n.s.*	*n.s.*	*n.s.*
∑PUFA	37.46	3.90	34.69	2.57	34.67	1.87	33.18	1.78	*n.s.*	*n.s.*	*n.s.*
∑n-3 FA	12.58	2.80	9.48	1.86	9.45	1.11	10.17	1.08	*n.s.*	*n.s.*	*n.s.*
∑n-6 FA	22.04	0.35	23.00^{I}	1.32	$^{1}22.56$	0.51	$^{2}20.83^{II}$	0.72	*n.s.*	*n.s.*	*n.s.*
18:1/18:0	0.68	0.05	1.55	1.55	0.64	0.04	0.63	0.04	*n.s.*	*n.s.*	*n.s.*
18:4n-3/18:3n-3	0.21	0.10	0.15	0.02	0.13	0.01	0.17	0.01	*n.s.*	*n.s.*	*n.s.*
18:3n-6/18:2n-6	0.078	0.013	0.076	0.007	0.071	0.003	0.077	0.011	*n.s.*	*n.s.*	*n.s.*
20:3n-6/20:2n-6	0.53	0.35	0.48	0.23	0.53	0.23	0.43	0.08	*n.s.*	*n.s.*	*n.s.*
18:2n-9/18:1n-9	0.036	0.012	0.033	0.004	0.029	0.001	0.031	0.002	*n.s.*	*n.s.*	*n.s.*
16:1/16:0	0.24	0.01	0.25	0.01	0.22	0.00	0.23	0.03	*n.s.*	*n.s.*	*n.s.*
18:0/16:0	0.40	0.09	0.31	0.05	0.34	0.02	0.32	0.04	*n.s.*	*n.s.*	*n.s.*
18:1n-7/16:1n-7	1.14	0.31	2.08	2.21	1.01	0.08	0.90	0.18	*n.s.*	*n.s.*	*n.s.*
DHA/EPA	3.02	0.42	2.98	0.39	3.17	0.14	2.85	0.39	*n.s.*	*n.s.*	*n.s.*

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids, Bfads2 refers to the effect of the broodstock *fads2* expression (H or L); Bdiet refers to the effect of the broodstock diet (FO or RO).

[a, b] in front of the value mean there is significant difference between the offspring come from same selection broodstock group (High) but the broodstock were fed with different diet**,** [1,2] in front of the value mean there is significant difference between the offspring come from same selection broodstock group (Low) but the broodstock were fed with different diet**.** [A, B] in the back of the value mean there is significant difference between the offspring come from broodstock fed with diet FO but with different *fads2 e*xpression**.** [I, II] in the back of the value mean there is significant difference between the offspring come from broodstock fed with diet RO diet but with different *fads2* expression**.** n.s. No statistical significance

5.3.4 Gene expression

Hepatic *fads2* expression was 38 % higher in juveniles from broodstock with high *fads2* expression (p =0.044) than in juveniles from broodstock with low *fads2* expression (Table 5.10). Moreover, a significant lineal regression was found between the *fads2* expression in broodstock and in the juvenile progeny (r=0.97, p=0.01). Besides, hepatic *fads2* expression in juveniles was also correlated to the SGR (r=0.580, *p=0.048*). The expression of hepatic *fads2* showed the interaction of broodstock *fads2* expression and broodstock diets (p = 0.049). Simple main effect analysis showed that expression of *elovl6* was 3.69 times higher in HFO than in HRO juveniles (p=0.019). Besides, *elovl6* expression in liver was correlated to the EPA contents in liver lipids (r=0.67, p=0.06). No significant differences were found in the expression of the other genes studied in the liver, including *cox-2*, *igf-1*, *srebp* or *ppara.* Nevertheless, *igf-1* in liver was negatively correlated to the HSI (r=-0.92, p=0.08) and the n-3 fatty acid contents in muscle (r=-0.95, p=0.05). In muscle, the ratio *ghr-1*/ *ghr-2* showed the interaction of the broodstock *fads2* expression and the broodstock diet (p=0.039). Thus, the muscle *ghr1/ghr2* was significantly higher (p=0.04) in HFO juveniles than in HRO ones. Juveniles from broodstock fed with FO diet had a significantly higher *ghr1/ghr2* ratio than those from broodstock fed with RO (p=0.014).

5.4 Discussion

The results of the present study showed the trans-generational effect of both the broodstock

fads2 expression and the type of lipid on the broodstock diet on the metabolism and

Table 5.10 Gene expression of progenies juveniles from blood fads2 selected and nutritional intervened broodstock challenged by low FM and FO diet after 45 days

	HFO		HRO		LFO		LRO		Two-way ANOVA		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	*Bfads2*	*Bdiet*	*Bfads2*Bdiet*
Liver (copies/μL)											
fads2	683.13	177.46	793.87	145.51	491.17	101.10	426.45	105.16	*0.014*	*n.s.*	*n.s.*
cox-2	1.19	0.55	1.65	0.40	1.52	0.88	1.81	1.42	*n.s.*	*n.s.*	*n.s.*
elovl6	^{a}144.52^{A}	17.27	^{b}39.18^{II}	32.52	^{2}69.31^{B}	11.77	119.40^{I}	45.22	*n.s.*	*n.s.*	*0.005*
igf-1	20.79	4.53	22.46	6.24	18.34	1.80	24.65	0.61	*n.s.*	*n.s.*	*n.s.*
srebp	59.91	12.63	60.43	24.52	52.46	18.82	50.86	10.29	*n.s.*	*n.s.*	*n.s.*
ppara	55.43	23.23	74.97	4.84	62.00	22.57	74.07	3.84	*n.s.*	*n.s.*	*n.s.*
Muscle											
ghr-1	14.82	3.55	10.04	1.11	13.33	2.06	13.49	5.33	*n.s.*	*n.s.*	*n.s.*
ghr-2	22.06	3.99	31.65	10.84	23.73	4.54	25.61	9.81	*n.s.*	*n.s.*	*n.s.*
ghr-1/ghr-2	^{a}0.67	0.10	^{b}0.35	0.14	0.56	0.02	0.53	0.10	*n.s.*	*0.014*	*0.039*

Bfads2 refers to the effect of the broodstock *fads2* expression (H or L); Bdiet refers to the effect of the broodstock diet (FO or RO).

a, b in front of the value mean there is significant difference between the offspring come from same selection broodstock group (High) but the broodstock were fed with different diet. I, II in the back of the value mean there is significant difference between the offspring come broodstock fed with diet RO diet but with different *fads2* expression

performance of the juvenile progeny challenged with a diet low in FM and FO.

In marine fish, the limited ability to synthesize LC-PUFAs, due to insufficient expression of key genes such as *fads2*, constrains their capacity to use low FM and FO diets. This gene codes for the enzyme delta-6 desaturase, which initiates the synthesis of LC-PUFA from the 18-carbon atom fatty acid precursors. In the present study, high *fads2* expression in broodstock markedly increased hepatic *fads2* expression in juveniles, affecting fatty acid composition of different tissues, and reducing VSI (p<0.05). In agreement, PUFA contents in liver of juveniles from high *fads2* broodstock significantly increased due to the elevation of n-6 PUFA contents, particularly ARA, the end product of n-6 PUFA biosynthesis initiated by Fads2 on 18:2n-6. In muscle, n-6 PUFA were also significantly increased. This was due in one hand to the increase in 18:2n-6, which was higher than in liver since this fatty acid is preferentially incorporated into PL in this species (Caballero *et al.*, 2006), and muscle has a proportionally larger content of PL (Izquierdo *et al.*, 2005). Indeed, this is one of the reasons for the persistence of 18:2n-6 in muscle of seabream fed vegetable oils, even after several months after being fed with a "wash out" diet containing low levels of 18:2n-6 (Izquierdo *et al.*, 2005). On the other hand, a 25% increase in the ARA contents in muscle of juveniles from broodstock with high *fads2* expression contributed to the increase in n-6 PUFA, in agreement with the higher *fads2* expression in these juveniles. All these results agree well with the increase in n-6 PUFA found in muscle of chicken expressing high *fads2* (Boschetti *et al.*, 2016). The lack of effect on the n-3 PUFA contents in liver, muscle or whole body would be related to the low 18:3n-3 content

in the diet fed to the juveniles, in comparison to the high dietary 18:2n-6 content. Besides, the high *fads2* expression in the broodstock was also associated with a significant (p<0.05) reduction in juveniles VSI. These results agree well with the tendency to a higher *fads2* expression found in livers of gilthead seabream juveniles from parents with high *fads2* expression (Turkmen *et al.*, 2019b). However, in such study no significant differences were observed in *fads2* expression, what could be related to the shorter duration of the trial, since fish only doubled their weight, and the larger initial weight of the juveniles (20g). In comparison, the present study was conducted until fish from all experimental groups had at least tripled their initial weight.

Therefore, to our knowledge, this is the first study that shows a degree of inheritance in the ability to express *fads2* in fish, and particularly in gilthead seabream. Even though it could seem obvious that the progeny from a high *fads2* expression broodstock should have a high *fads2* expression, heritability of the *fads2* expression has been suggested to be very low in fish. Moreover, most studies have focused on the potential heritability of DHA and EPA content in muscle but have not directly targeted *fads2* expression heritability. For instance, in salmonids, a moderate cross-validation accuracy for selection for DHA and EPA has been recently demonstrated, opening the possibilities for selection of these traits (Horn *et al.*, 2018). However, fish contents in DHA and EPA are regulated by a number of factors apart from *fads2* expression. In gilthead seabream, preliminary studies with a reduced but representative number of genetically characterized fish suggest an estimation of the *fads2* expression heritability in this species of 0.08±0.20 (Afonso and Izquierdo, unpublished data), in agreement with the high correlation between *fads2* expression in broodstock and juveniles in the present study. These increased *fads2* expression in the progeny of high *fads2* broodstock, in comparison to the low *fads2* broodstock, could be related to genetic or epigenetic factors. For instance, in humans, various genotypes are responsible for differences in *FADS2* expression and delta-6 desaturase activity (Howard *et al.*, 2014), which are affected by single nucleotide polymorphisms (Schaeffer *et al.*, 2006; Xie and Innis, 2008). Besides, DNA methylation of enhancer regions of the FADS are associated to delta-6 desaturase activity in humans (Howard *et al.*, 2014). Increased methylation of specific CpG sites in the promoter region of *fads2* has been also found

in the progeny of gilthead seabream from broodstock with low *fads2* expression (Turkmen *et al.*, 2019b). Nevertheless, it cannot be dismissed that those differences on *fads2* expression or the products of Fads2 activity could be related to or mediated by other factors associated to the selection of high *fads2* expression broodstock. For instance, in the seabream females used in the present study, *fads2* expression was positively related to plasma 17β-estradiol levels (Ferosekhan *et al.*, 2020b). Also in female rats, estradiol increases DHA tissue contents through the up-regulation of Δ6 desaturase gene (Kitson *et al.*, 2013). Nevertheless, having seabream a proterandry development of gonads, aromatase activity to produce 17β-estradiol would be extremely low until the fish reach at least 300g. Since the juveniles in the present study weighted only few grams, this steroid hormone would not likely be the cause of the *fads2* up-regulation.

Feeding broodstock with the RO diet significantly (p<0.05) increased the hepatic 18:3n-6/18:2n-6 and 18:2n-9/18:1n-9 ratios, indicators of Fads2 activity (Vagner and Santigosa, 2011), in the juvenile offspring. These results denote a significant nutritional programming effect of the broodstock diet on the juvenile progeny, especially on promoting the Fads2 activity. These results are in agreement with previous studies where the seabream juvenile progeny of broodstock fed a linseed oil rich diet showed increased contents of Fads2 products in comparison to those from broodstock fed a FO diet (Turkmen *et al.*, 2019a). Similarly, in red drum (*Sciaenops ocellatus*) nutritional programming by essential fatty acids increase Fads2 products in the 21-day-old larvae progeny challenged with a low LC-PUFA diet (Fuiman and Perez, 2015). In the present study, the increase in these hepatic indicators of Fads2 activity, together with the lack of a significant effect on the *fads2* expression, suggest that a post-transcriptional factor could mediate this nutritional programming effect. This hypothesis is in agreement with the down-regulation of microRNAs related with lipid metabolism in offspring of mice fed a high fat diet from conception to lactation, in comparison to those fed a chow diet (Zhang *et al.*, 2009). Interestingly, this enhancement of Fads2 activity was not observed in previous studies when broodstock was fed a diet high in LNA but not sufficiently low in LC-PUFA (Turkmen *et al.*, 2019a). In such study, the difference in the n-3 LC-PUFA content between the control FO and the high VO broodstock diets was only of 2.6 %, in comparison to

the 8.1% difference between the broodstock diets of the present study (Ferosekhan *et al.*, 2020b). Thus, the high n-3 LC-PUFA content of the previous study (Turkmen *et al.*, 2019a) could have prevented the success in the nutritional programming. In agreement with this hypothesis, in Atlantic Salmon the VO diet used to effectively induce a nutritional programming effect was 22.3% lower in n-3 LC-PUFA than the control FO diet (Clarkson *et al.*, 2017).

Feeding broodstock a diet with partial replacement of FO by RO led to a marked ($p<0.001$) reduction in the VSI of juveniles and the size of hepatocytes, which was significantly correlated to the HSI ($r = 0.96$, $p<0.05$). However, the liver lipid contents were not affected, suggesting that the reduced hepatocyte size would be rather related to the catabolism of glycogen than lipid, since hepatocytes are energy reservoirs in these type of fish species (Chellappa *et al.*, 1995). Besides, HSI was negatively correlated with the expression of liver *igf-1*. However, no significant differences were found in *igf-1* expression values, what could have been related to the low number of copies/µL obtained. *Igf-1* is preferentially expressed in the liver, by stimulation of growth hormone (GH), and promotes systemic body growth, although is not always directly correlated to fish growth (Beckman, 2011). *Igf-1* is regulated by different factors including fish nutritional status (Pérez-Sánchez *et al.*, 2018) and it is down-regulated in European sea bass fed low LC-PUFA diets (Escobar-Aguirre *et al.*, 2020). As in the later study, in the present one, it is possible that the very low LC-PUFA contents in the diet for juveniles would have also inhibited *igf-1*expression, leading to the low number of copies/µL obtained. Nevertheless, the juveniles from broodstock fed the RO diet seemed to be more resilient to this inhibition, since they showed *igf-1*expression values that were 20% higher than those of juveniles from broodstock fed the FO diet.

In juveniles coming from broodstock with high *fads2* expression, FO replacement by RO in the broodstock diets, led to a significant ($p<0.05$) reduction in the *ghr-1*/*ghr-2* ratio in muscle as well as a significant ($p<0.01$) increase in SGR. The growth hormone receptors (Ghrs) are main components of the somatotropic axis that mediate the action of Gh (Sakamoto *et al.*, 1993). Doubled Ghrs are actively transcribed in fish (Saera-Vila *et al.*, 2007) and their expression can be regulated by nutrition and season, among other factors (Pérez-Sánchez *et al.*, 2018). For

instance, in gilthead sea bream, *ghr-1* and *ghr-2* have a protective and/or growth promoting action and *ghr-2* expression in muscle is up-regulated when the fish are fast or fed with a low FO diet (Benedito-Palos *et al.*, 2007; Saera-Vila *et al.*, 2007). In comparison with *ghr-2*, *ghr-1* is more actively transcribed in liver and adipose tissue than in muscle of gilthead sea bream and remains stable in muscle when fish are fast or fed with different lipid sources (Saera-Vila *et al.*, 2005; Benedito-Palos *et al.*, 2007). Thus, the ratio between *ghr-2* and *ghr-1* in muscle could be used to evaluate the ability of muscle maintenance and growth in gilthead sea bream (Benedito-Palos *et al.*, 2007). In the present study, the ratio of *ghr-1*/*ghr-2* expressions in muscle was significantly reduced in juveniles coming from broodstock with high *fads2* expression and fed the RO diet instead of the FO diet. These results demonstrate the significant effect of nutritional programming on *ghr-1*/*ghr-2* expression in muscle and support their value as a mechanism of growth regulation to confront poor nutritional conditions previously suggested (Pérez-Sánchez *et al.*, 2018). This increased SGR found in juveniles coming from broodstock with high *fads2* expression and fed the RO diet instead of FO is in agreement with other studies regarding the nutritional programming effect of feeding plant ingredients to broodstock or first feeding fish. For instance, 70% replacement of dietary fish oil by a combination of vegetable oils on diets for broodstock gilthead seabream promotes growth performance in offspring juveniles (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017a; Turkmen *et al.*, 2019a; Turkmen *et al.*, 2019b). Among freshwater fish, feeding plant-based diets in early life stages improves growth later in life (Geurden *et al.*, 2013; Clarkson *et al.*, 2017). In general, the plasticity of the fish during the developmental stages of rapid growth facilitates the regulation of metabolism by nutritional factors. Specifically, ingestion, digestion, absorption and biosynthesis pathways can be regulated by nutritional programming (reviewed in (Hou and Fuiman, 2019)). However, in marine fish such as European sea bass, feeding larvae with low dietary LC-PUFA from first exogenous feeding for 39 days does not improve growth performance or fatty acid biosynthesis capacity when fish is later challenged with a low n-3 LC-PUFA diet (Vagner *et al.*, 2009). Similarly, nutritional programming was not successful in gilthead seabream when larvae were fed a low n-3 LC-PUFA diet (Turkmen *et al.*, 2017b). In both species, and generally in marine fish, larval stages are weak and very sensitive to low

dietary n-3 LC-PUFA levels, what constrains the use of these nutrients for nutritional programming during larval development. The mechanisms underlying these nutritional programming effects are not yet clearly understood. In fish, the development of embryos relies on nutrients deposited in the yolk sac, which depend on the maternal intake of nutrients during and before oogenesis. In addition, evidence suggests a high maternal (Rauwerda *et al.*, 2016) and paternal (Otero-Ferrer *et al.*, 2020) influence on the transcriptome of the developing embryos and the transfer of gene expression regulating RNAs, lncRNA and miRNA (Sullivan *et al.*, 2015) or specific proteins (Lubzens *et al.*, 2017).

Despite the increased growth found in juveniles coming from broodstock with high *fads2* expression and fed RO diet instead of FO, the ARA and n-3 PUFA contents in the liver were significantly reduced. Since, none of these fatty acids, neither DHA, were significantly reduced in whole body or muscle, their lower levels in liver suggest the mobilization of these PUFAs from liver to other tissues to support such increased growth. Only EPA levels in whole body of juveniles from broodstock with high *fads2* expression was reduced when FO was replaced by RO in the broodstock diets. Besides, expression of *elovl6* in liver of juveniles from broodstock with high *fads2* expression, was down regulated by the replacement of FO by RO in broodstock diets, denoting a strong nutritional programming effect. Indeed, hepatic *elovl6* expression was inversely correlated to SGR (r=-0.62, p=0.05). These results are in agreement with our previous studies in gilthead seabream where broodstock nutrition induced a strong nutritional programming effect on the expression of certain energy and lipid metabolism-related genes such as *elovl6* (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017a). In those studies, *elovl6* in the progeny was down-regulated by the FO replacement by vegetable oils in the broodstock diet, in agreement with the improved utilization of dietary lipids and carbohydrates found in *Elovl6* disrupted mice models (Matsuzaka and Shimano, 2009).

In conclusion, the results of the present study have shown that it was possible to up-regulate the *fads2* expression of juvenile gilthead seabream by using broodstock with inherently high *fads2* expression, what led to increased PUFA contents in liver and muscle. Besides, nutritional programming through FO replacement by RO in broodstock diets increased the Fads2 activity (based on the ratio of fatty acid products and substrates for Fads2), reduced VSI, hepatocyte

size and expression of *elovl6* in liver and *ghr-1/ghr-2* in muscle. Moreover, the combination of both broodstock with high *fads2* expression and nutritional programing with RO allowed producing gilthead seabream juveniles that showed a faster growth when challenged with a low FM and low FO diet. Further studies are being conducted to better understand the regulation of nutritional programming through broodstock nutrition in gilthead seabream.

Acknowledgements

This study was supported by the EU funded project PerformFISH (H2020-SFS-2016-2017; 727610) and China scholarship council. The authors acknowledge Skretting ARC (Stavanger, Norway) for providing the experimental diets.

M. I., S. T., S. F and H. X. participated in the design of the experiment. Feeding experiments, biological, biochemical and molecular analyses were conducted and evaluated by H. X. Molecular biology samples were analysed by H. X with the supervision of M. J. Z and J. M. A. M. I. supervised the entire work. The paper was written by H. X. and M. I.

The authors declare that there are no conflicts of interest

Chapter 6

High *fads2* expression in gilthead seabream broodstock improves offspring use of low fish meal and low fish oil diets: growth, fatty acid metabolism and *fads2* promoter sequencing

Status: To be submitted.

Abstract

One of the factors that limits the replacement of fish meal (FM) and fish oil (FO) by plant ingredients in diets for marine fish is their limited capacity to express *fatty acyl desaturase 2* gene (*fads2*) and synthetize long chain-polyunsaturated fatty acids (LC-PUFA). The aim of this study was to determine if the inherent *fads2* expression in gilthead seabream broodstock had long term effects on offspring ability to use low FM and FO diets. For that purpose, growth, fatty acid profiles, expression of selected genes and *fads2* promotor sequencing were studied in offspring juveniles obtained from broodstock with either high (H) or low (L) *fads2* expression and nutritionally challenged with a 7.5% FM and 0% FO diet. The results showed a 22% improvement in final body weight in offspring of broodstock with a higher *fads2* expression when offspring was fed from 2.3 to over 100 g with diets containing low FM and low FO levels. Besides Juveniles from broodstock with higher *fads2* expression showed significantly higher 22:6n-3, 22:4n-6 and 22:5n-3 content and the downregulation of *fads2* in liver. No differences were observed in sequencing alignment in the *fads2* promoter region among fish with different *fads2* expression. Overall, the study demonstrated the long-term trans-generational effects of the broodstock *fads2* expression on growth and the fatty acid metabolism of the offspring fed with low FM and FO diets.

Keywords: Parental gene expression; plant ingredients utilization; n-3 long-chain PUFA biosynthesis; fatty acyl desaturase 2

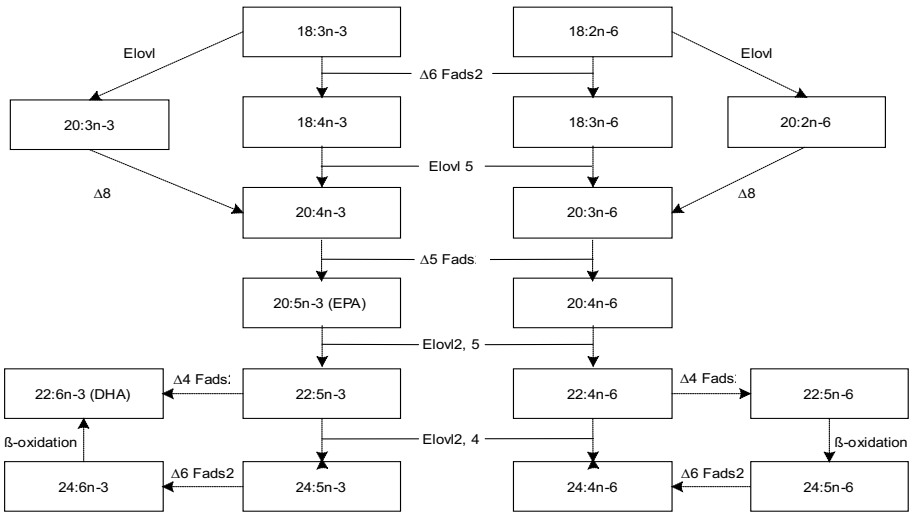
6.1 Introduction

Fish is a main source of n-3 long chain polyunsaturated fatty acids (LC-PUFAs) for consumers, with a well-balanced content of minerals and highly digestible proteins, what contributes to the continuous increase in the demands of these product (FAO, 2020). Due to the stagnant production of fisheries, aquaculture is taking over the responsibility to provide safe and sustainable fish food to satisfy market requests (FAO, 2020). However, the further development of aquaculture is limited by the high demand, restricted availability and increasing prices of fishmeal (FM) and fish oil (FO), traditional protein and lipid sources in aquafeeds (Hardy and Barrows, 2002). For instance, in 2015, aquaculture industry used over 70% of the FM and FO produced worldwide (Green, 2018). These ingredients are mostly derived from capture fisheries (Hardy and Barrows, 2002) and, therefore, their production is stagnant or even decreased (Green, 2018). Besides this limitation in availability, the use of FM and FO in aquafeed raises the concerns on the overexploitation of fisheries (Council, 2011). Therefore, dietary FM and FO need to be replaced by other high quality and nutritious ingredients with a more economical, environmental and social sustainability. Many alternative ingredients are used to replace FM and FO in aquafeeds such as plant ingredients, animal byproducts, single cell ingredients or insect meals (Caballero *et al.*, 2002; Wang *et al.*, 2016; Rosales *et al.*, 2017; Rimoldi *et al.*, 2018; Carvalho *et al.*, 2020). However, depending on the type of ingredient and the replacement level, these alternative ingredients may lead to malnutritional effects on fish growth, nutrients digestibility, immune system, *etc*. (Vergara *et al.*, 1996b; Vergara *et al.*, 1996a; Caballero *et al.*, 2004; Gómez-Requeni *et al.*, 2004).

One of the main problems encountered when FM and FO are replaced by plant ingredients in diets for marine fish, is their low capacity to synthesis LC-PUFA, essential for these fish species, and high in FM and FO but low in plant ingredients. LC-PUFAs, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are critical for the adequate development of brain and larval tissues (Mourente, 2003), immune system functioning (Zuo *et al.*, 2012b), reproductive performance (Fernández-Palacios *et al.*, 2011) and fillet nutritional value for the consumer (Monge-Ortiz *et al.*, 2018). The biosynthesis of LC-PUFA is catalysed by fatty acid desaturases (fads) and elongases (elovl), being Fads2 and Elovl6 major rate-limiting enzymes in the LC-PUFA biosynthesis pathways in several marine fish species (Monroig *et al.*, 2011;

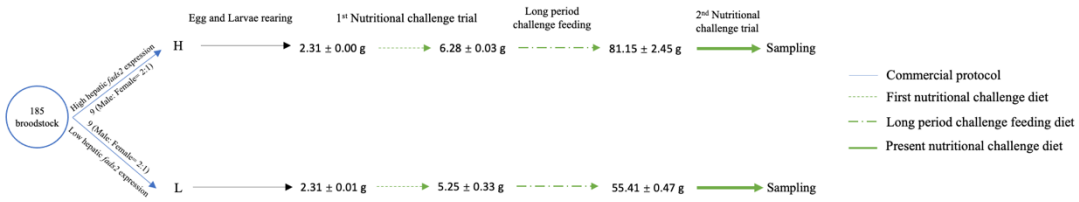
Kabeya *et al.*, 2014). Besides, *fads2* expression can be modulated through the diet (Izquierdo *et al.*, 2008), suggesting that LC-PUFA biosynthesis could be enhanced through diet modulation for a better utilization of plant ingredients. Therefore, recent research has focused on increasing the ability of the different fish species to use diets low in FM and FO. For instance, inclusion of key genes on LC-PUFA synthesis could potentially enhance this pathway improving the use of vegetable oils, such as the transgenesis of the salmonid elovl2 gene to nibe croaker (*Nibea mitsukurii*) (Kabeya *et al.*, 2014). However, the use of transgenics often arises safety concerns and restriction policies. In addition, selective breeding can be used to produce fish with a higher ability to use low FM and FO diet. For instance, the selection of rainbow trout with higher growth and survival when fed with diets based on vegetable ingredients to replace FM and FO for three generations, effectively improved growth performance when fish were fed with a 100% plant-based (Callet *et al.*, 2017). In gilthead seabream, selecting broodstock with a higher expression of *fads2* may improve the ability to use a low FM and FO diet in the progeny (Turkmen *et al.*, 2019b). Although *fads2* is highly expressed in the liver of gilthead sea bream (Benedito-Palos *et al.*, 2014), determination of this gene expression in peripheral blood cells (PBCs) allows the selection of fish with different *fads2* expression trough a non-invasive method. Besides, a highly positive correlation was found in *fads2* expression between liver and PBCs (Ferosekhan *et al.*, 2020a).

Figure 6.1 Pathways of >C18 PUFA (polyunsaturated fatty acids) biosynthesis (Bond *et al.*, 2016 and Oboh *et al.*, 2017)



Our previous studies have found that broodstock with higher *fads2* expression had a higher reproductive performance in terms of fecundity and quality of sperm and egg (Ferosekhan *et al.*, 2020b). Besides, blood *fads2* expression in gilthead sea bream broodstock females, which tended to be higher than in males, was positively related to plasma oestrogen levels (Ferosekhan *et al.*, 2020b). Moreover, the offspring produced from broodstock with high blood *fads2* expression showed a higher growth when, from 2 g to 6 g, they were challenged with a diet containing 7.5% FM and no FO (Xu *et al.*, 2020, chapter 5). Furthermore, these juveniles from broodstock with high *fads2* expression showed a significantly higher fads2 expression in liver and increased PUFA content in liver and muscle (Xu *et al.*, 2020, chapter 5). However, the persistence of this positive effect on growth and LC-PUFA biosynthesis or the potential mechanism involved have not been yet study. Therefore, the aim of this study was to determine the potential long-term effect of broodstock *fads2* expression on growth performance, tissue fatty acid profiles and expression of selected genes in offspring challenged with a low FM/FO content diet. Besides, the sequence of alignment of the promoter region in *fads2* was studied.

Figure 6.2 Schematic view of the fish nutritional background and experimental design



6.2 Material and Methods

All the animal experiments were performed according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at Fundación Canaria Parque Científico Tecnológico (FCPCT) of University of Las Palmas de Gran Canaria (Canary Islands, Spain) (OEBA-ULPGC-20/2018).

6.2.1 Feeding trial and fish performance

To produce these juveniles, eggs from each of the 2 broodstock groups with high or low *fads2* expression (H, L) and fed a low FO diet (Ferosekhan *et al.*, 2020b) were collected in the same

day and separately incubated and larvae and juvenile reared under the same conditions and commercial diets (Figure 6.2). When they reached 2.3 g, they were challenged with a plant-based diet with 7.5% of FM and 0% FO (Hanlin *et al*., in press) until their body weight reached at least 5 g (6.28±0.03 and 5.25±0.33 g for H and L fish, respectively). Afterwards, fish were fed with a plant-based diet containing only 5% FM and 3% FO until their body weight reached at least 50g. Then, 60 fish from the remaining 130 fish in each group were randomly sampled and distributed into 3 tanks of 250 L (20 fish/tank) (85.1 ± 2.5 g and 55.4 ± 0.5 g for H and L fish, respectively). Tanks were provided with 250 L/h seawater at 21.0 ± 0.8ºC and strong aeration, and dissolved oxygen was daily determined (6.2 ± 0.1 mg/L). Tanks were illuminated by fluorescent lights placed above the tank at an intensity of 100 lx, programmed for a 12h light photoperiod (from 8 a.m. to 8 p.m.).

In order to challenge the fish with a very low FM and FO diet for stimulating their potential ability to cope with low n-3 LC-PUFA diet, an experimental diet was formulated to contain 7.5% of FM and 0% FO (Table 6.1). Corn gluten, soybean meal, wheat gluten, soya protein concentrates and faba bean meal were used to replace the dietary fishmeal. Poultry oil and rapeseed oil were used to replace the dietary fish oil. The diet was low in ARA, EPA and DHA and high in oleic acid (OA, 18:1n-9), LA, palmitic acid (PA, 16:0) and ALA (Table 6.1).

Fish were fed the experimental diet until apparent satiation 3 times per day (8:30, 11:30, 14:30) and 6 days per week for 74 days. Feed delivery was daily calculated, and uneaten pellets were collected in a net by opening the water outlet 30 min after each meal. Then, the collected pellets were dried in an oven for 24 h and weighed to estimate feed intake. All fish were weighted individually every two weeks during the feeding trial. At the end of the trial, fish were fasted for 24 h, weighed and anesthetized with ethanol diluted clove oil (50:50) before samplings. The following performance parameters were calculated:

- Mortality (%) = 100 × (nº dead fish/ nº total fish)
- Weight gain (WG, g) = final body weight (BW_1) – initial body weight (BW_0)
- Specific growth rate (SGR, % day^{-1}) = 100* (ln BW_1 – ln BW_0) / nº days, where BW_0: initial body weight (g) and BW_1: final body weight (g)
- Feed intake (FI, g $fish^{-1}$ day^{-1}) = Feed delivered / (nº of fish*nº days)

- Biological feed conversion ratio (FCR) = Feed delivered ($t_i - t_0$) / (Biomass t_i – Biomass t_0 + Biomass $_{harvested}$ + Biomass $_{lost}$), where t_0: initial biomass (g) and t_1: final biomass (g)
- Hepatosomatic index (HSI) = 100 × Liver weight (g) / Body weight (g)
- Viscerosomatic index (VSI) = 100 × Visceral weight (g) / Body weight (g)

Table 6.1 Ingredients, proximate composition and main fatty acids content of the challenge diet fed to the one-year-old juveniles obtained from broodstock with different *fads2* expression

Ingredient (% of dry weight.)	
Poultry oil[a]	6.14
Wheat[b]	9.46
Corn gluten[b]	10.00
Soybean meal[b]	5.00
Wheat gluten[b]	19.18
Soya protein concentrate[b]	30.00
Faba beans[b]	5.00
Fish meal[b]	7.50
Rapeseed oil[b]	7.52
Vitamin premix[c]	0.10
Mineral premix[c]	0.10
Proximate composition (% of dry weight)	
Crude protein	53.74
Crude lipid	22.90
Crude ash	4.71
Fatty acid profile (% of total fatty acids)	
16:0	7.79
18:0	3.43
18:1n-9	41.74
18:2n-6	24.58
18:3n-3	10.20
20:4n-6	0.23
20:5n-3	0.87
22:6n-3	1.98

a Poultry oil: Sonac. B.V. The Netherlands.

b Skretting AS (Norway).

c Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC (Stavanger, Norway).

6.2.2 Biochemical composition and fatty acid analysis

At the end of the feeding trial, 5 fish per tank were painlessly killed at the end of the procedure by immersion in 10 ppm clove oil and methanol (50:50) and sampled to determine proximate composition of whole-body, liver and muscle. All samples were frozen at -80ºC until analysis. All samples were homogenized immediately prior to analysis. Moisture and protein contents were determined according A.O.A.C. (Horwitz, 2002). Lipids were extracted with chloroform/methanol (2:1 v/v) (Folch *et al.*, 1957) and then transmethylated to obtain fatty acid methyl esters (FAMES) (Christie, 1988). FAMES were then separated by gas liquid

chromatography (Polaris QTRACETM Ultra; ThermoFisher, Waltham, U.S.A.) under conditions previously described (Izquierdo, 1989).

6.2.3 RNA extraction and digital PCR

The liver and muscle from the juveniles after the nutritional challenge were kept at 4 ºC overnight in 2.0 mL tubes with 1.5 mL of *RNAlater* and then transferred to -80ºC until molecular analyse. RNA from 100 mg sample was extracted using *TRI Reagent* (MilliporeSigma, Darmstadt, Germany) and then purified by *RNeasy kit* (Qiagen, Hilden, Germany). RNA quality was checked by 1.4% agarose electrophoresis and quantity was measured by *NanoDrop*^{TM} *1000* (ThermoFisher). cDNA was synthesized from 1 µg of RNA in *iCycler* (Bio-rad, Hercules, U.S.A.), using the *iScript cDNA synthesis kit* (Bio-Rad). The expression of genes related with growth (*insulin grow factor 1* [*igf1*]), lipid and energy metabolism (*fads2*; *glucose 6-phosphate* [*g6p*]; *sterol regulatory element-binding protein 1* [*srebp1*]; *lipoprotein lipase*, [*lpl*]; *carnitine palmitoyltransferase Iß* [*cpt-1ß*]; *elongation of very long chain fatty acids protein 6* [*elovl6*]; *peroxisome proliferator activated receptor α* [*ppar-α*]) were determined using the primers listed in Table 6.2. Digital PCR was performed as previously described in (Xu *et al.*, 2019) using in QX200™ Droplet Digital™ PCR System (Bio-rad). The amplification conditions of PCR were: 95 ℃ for 5 min, followed by 40 cycles at 95℃ for 30 sec, elongation at Tm temperature for 1min, and then stabilized the signal at 4 ℃ for 5 min, 90℃ for 5 min, finally the reaction was hold at 4℃.

Table 6.2 Primers sequence for digital PCR and GeneBank accession numbers for sequences of target genes

Gene		Sequence (5'-3')	Tm (℃)	Gene bank No.
β-actin	Forward	GAC CAA CTG GGA TGA CAT GG	61	X89920.1
	Reverse	GCA TAC AGG GAC AGC ACA GC		
fads2	Forward	GCA GAG CCA CAG CAG CAG GGA	63	AY055749
	Reverse	CGG CCT GCG CCT GAG CAG TT		
g6p	Forward	CGC TGG AGT CAT TAC AGG CGT	62	XM_030399413.1
	Reverse	CAG GTC CAC GCC CAG AAC TC		
srebp	Forward	AGG GCT GAC CAC AAC GTC TCC TCT CC	62	XM_030407356.1
	Reverse	GCT GTA CGT GGG ATG TGA TGG TTT GGG		
lpl	Forward	CGT TGC CAA GTT TGT GAC CTG	60	AY495672
	Reverse	AGG GTG TTC TGG TTG TCT GC		
cpt-1ß	Forward	CCA CCA GCC AGA CTC CAC AG	60	DQ866821
	Reverse	CAC CAC CAG CAC CCA CAT ATT TAG		
elovl6	Forward	GTG CTG CTC TAC TCC TGG TA	60	JX975702
	Reverse	ACG GCA TGG ACC AAG TAG T		
ppar-α	Forward	TCT CTT CAG CCC ACC ATC CC	58	AY590299
	Reverse	ATC CCA GCG TGT CGT CTC C		
igf1	Forward	GTG TGT GGA GAG AGA GGC TT	58	AY996779.2
	Reverse	CTC TTG GCA TGT CTG TGT GG		

6.2.4 fads2 promoter sequencing

DNA was extracted from 25 mg caudal fin from broodstock of experimental juveniles with *DNAeasy kit* (Qiagen) according to the instruction from manufacturer. The quality of extracted DNA was check by 1% agarose gel electrophoresis, and the quantity was measured in the *NanoDrop™ 1000 spectrophotometer* (ThermoFisher). The primers used for *fads2* promoter region sequencing were designed based on Perera *et al.* (2019) (Forward: 5' CTC CTG GAA TTT CCC TCA 3'; Reverse: 5' TTT TCG GCT GCT CCT CTG 3'). PCR was performed using 40 ng/µL extracted DNA with *iTaq Kit* (Intronbio, Seongnam, Republic of Korea) according to the instruction of the manufacturer. Thermocycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 34 cycles at 94°C for 20 s, 60°C for 1 min, 72°C for 30 min, and a final extension step of 3 min at 72 °C. The PCR product was quantified in *NanoDrop™ 1000 spectrophotometer* (ThermoFisher), diluted to 75 ng/µL and sent to Macrogen (Madrid, Spain) for sequencing.

6.2.5 Data analysis

Data was shown in mean ± S.D. Independent sample t-test was used in the comparison between two experimental group through SPSS 20.0 (IBM, U.S.A.). Gene expression was normalized by hepatic expression of *rpl27* according to the method described in Park and Crowley (2005). *ClusterW* alignment between *fads2* promoter region of each broodstock was performed through *BioEdit 7.2* (Tom Hall).

6.3 Results

6.3.1 Growth performance

Feeds were well accepted by all fish groups and no significant differences were found in feed intake or mortality rates. During both nutritional challenge trials (the 1st and the 2nd) feeding the fish with low FM and FO diets, the offspring from broodstock with a high *fads2* expression (H) showed a significantly (p <0.05) higher body weight than those from broodstock with a low *fads2* expression (L) (Figure 6.3). Thus, fish body weight along the two feeding trials followed a highly correlated (R^2 >0.90) and significant (p <0.001) potential relation with a higher slope for offspring from broodstock with a high *fads2* expression. Therefore, at the end of the second nutritional challenge trial, the whole body weight of fish from broodstock with high *fads2*

expression (H) was 22% higher (p <0.001) than that of fish from broodstock with low *fads2* expression (L) (Table 6.3). SGR values measured along both nutritional challenge trials followed a highly correlated (R^2 >0.90) and significant (p <0.05) logarithmic inverse relation with fish body weight, with SGR values for young fish during the first nutritional challenge being significantly higher for H fish (Figure 6.4). However, the SGR and body weight relation curves were closer as fish were getting older. Therefore, during the second nutritional challenge trial the SGR of L fish, that were smaller than H fish, was significantly higher (p=0.002) (Table 6.3). No significant (p >0.05) differences were observed for FCR, HSI or VSI, during the second nutritional challenge (Table 6.3).

Figure 6.3 Changes of body weight of the fish from broodstock with high (H) or low (L) *fads2* expression along the nutritional challenge study

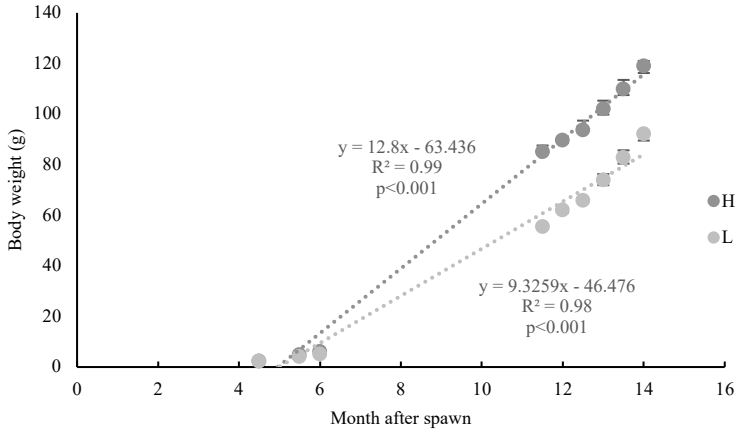


Figure 6.4 Relation between body weight and specific growth rate (SGR) of the fish from broodstock with high (H) or low (L) *fads2* expression along the nutritional challenge study

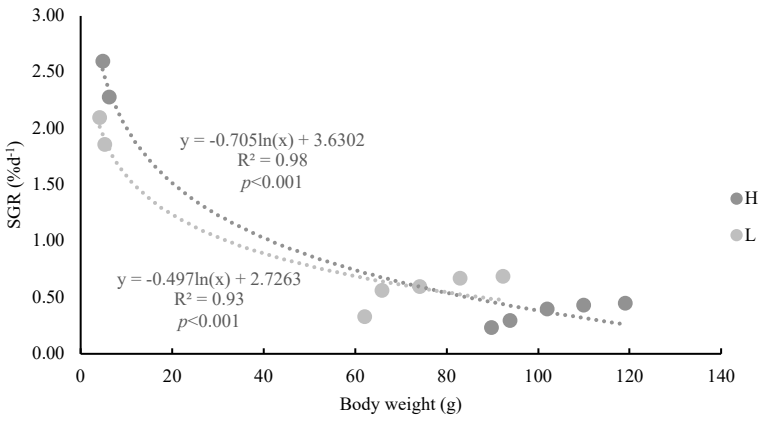


Table 6.3 Growth performance of gilthead seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) after 74 days of feeding the challenge diet

	H		L	
	Mean	S.D.	Mean	S.D.
Final weight of 1st challenge experiment (g) [a]	6.28*	0.03	5.25	0.33
Initial weight (g)	85.15*	2.45	55.41	0.47
Final weight (g)	118.72*	2.02	92.26	2.79
Weight gain (g)	33.58	0.63	36.85	3.25
SGR (% day^{-1})	0.45	0.17	0.69*	0.05
Feed intake (g)	1.24	0.02	1.18	0.10
FCR	2.59	0.18	2.38	0.24
HSI	1.28	0.22	1.31	0.10
VSI	3.68	0.46	3.98	0.34

Abbreviations: SGR: Specific growth rate; FCR: Feed conversion rate; HSI: Hepatic somatic index; VSI: Visceral somatic index.

a Final weight of fish after nutrition challenge test by diet contained 7.5% FM and 0% FO from approximately 2-6 g.

* Denotes significant differences between the two fish groups.

6.3.2 Biochemical analysis

No significant (p>0.05) difference was observed between L and H fish in protein, lipid and ash contents of whole fish, liver or muscle (Table 6.4). Regarding the fatty acid composition in the liver, DHA content was significantly (p<0.05) higher in H group than in L group (Table 6.5). Besides, 20:0, 20:1n-7, 22:4n-6 and 22:5n-3 were significantly higher in the liver of H fish than in L fish (p<0.05). Liver of L fish contained higher 16:3n-4, 18:2n-9,18:3n-6 and 18:4n-3 than liver of H fish (p<0.05). Comparing the fatty acids ratios, L fish showed a higher ratio of the desaturated products of 18C fatty acids to their precursors, including 18:4n-3/18:3n-3, 18:3n-6/18:2n-6 and 18:2n-9/18:1n-9. Besides, hepatic DHA was directly correlated with EPA (r=0.92, p<0.01) and 22:5n-3 (r=0.95, p<0.01), but adversely correlated with the 18:3n-6 to 18:2n-6 ratio (r=-0.917, p=0.01), the 20:3n-6 to 20:2n-6 ratio (r=-0.862, p=0.027) and the 18:2n-9 to 18:1n-9 ratio (r=-0.850, p=0.032).

Table 6.4 Proximate composition of liver, muscle and whole fish samples from gilthead seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) after 74 days of feeding the challenge diet

	H		L	
%	Mean	S.D.	Mean	S.D.
Liver				
Lipid	14.13	3.77	17.10	6.22
Protein	12.13	1.06	12.75	0.97
Ash	1.14	0.39	1.16	0.30
Muscle				
Lipid	1.96	0.71	3.50	3.28
Protein	20.95	0.11	20.48	0.41
Ash	1.56	0.06	1.52	0.14
Whole fish				
Lipid	10.36	1.88	10.38	1.60
Protein	16.36	0.49	16.74	0.38
Ash	4.37	0.96	3.44	0.39

Table 6.5 Fatty acid composition in liver of gilthead seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) after 74 days of feeding the challenge diet

	H		L	
Fatty acid (%)	Mean	S.D.	Mean	S.D.
14:0	0.31	0.11	0.48	0.16
14:1n-7	0.03	0.03	0.01	0.00
14:1n-5	0.03	0.03	0.01	0.00
15:0	0.05	0.02	0.06	0.02
15:1n-5	0.02	0.02	0.01	0.00
16:0ISO	0.03	0.03	0.01	0.00
16:0	7.19	1.24	8.20	1.46
16:1n-7	1.21	0.12	1.41	0.23
16:1n-5	0.04	0.01	0.04	0.01
16:2n-4	0.03	0.02	0.02	0.01
17:0	0.04	0.01	0.05	0.02
16:3n-4	0.10	0.01	0.12*	0.01
16:3n-3	0.03	0.02	0.02	0.01
16:3n-1	0.02	0.02	0.02	0.01
16:4n-3	0.05	0.02	0.03	0.01
16:4n-1	0.00	0.01	0.00	0.00
18:0	4.94	0.71	5.27	0.47
18:1n-9	37.01	1.89	39.97	2.16
18:1n-7	2.56	0.08	2.68	0.14
18:1n-5	0.05	0.04	0.06	0.00
18:2n-9	0.91	0.22	1.69*	0.06
18:2n-6	17.82	0.70	18.16	0.18
18:2n-4	0.04	0.02	0.04	0.00
18:3n-6	1.54	0.18	2.56*	0.09
18:3n-4	0.08	0.01	0.06	0.01
18:3n-3	5.32	0.28	5.25	0.15
18:3n-1	0.02	0.02	0.01	0.00
18:4n-3	0.64	0.06	0.95*	0.04
18:4n-1	0.04	0.03	0.02	0.01
20:0	0.25*	0.03	0.19	0.01
20:1n-9	0.19	0.02	0.16	0.01
20:1n-7	1.64*	0.24	1.15	0.15
20:1n-5	0.12	0.03	0.08	0.01
20:2n-9	1.16	0.09	1.21	0.21
20:2n-6	1.05	0.20	0.72	0.11
20:3n-9	0.02	0.03	0.01	0.01
20:3n-6	0.75	0.09	0.70	0.07
20:4n-6	0.62	0.08	0.46	0.14
20:3n-3	0.51	0.12	0.35	0.06
20:4n-3	0.51	0.09	0.42	0.06
20:5n-3	1.47	0.21	0.97	0.34
22:1n-11	0.55	0.18	0.30	0.06
22:1n-9	0.95	0.19	0.59	0.20
22:4n-6	0.13*	0.03	0.08	0.01
22:5n-6	0.14	0.03	0.08	0.03
22:5n-3	1.13*	0.29	0.58	0.12
22:6n-3	8.62*	2.07	4.77	1.01
∑SFA	12.81	1.98	14.26	1.82
∑MUFA	44.41	1.42	46.46	2.47
∑PUFA	42.78	3.39	39.28	0.74
∑n-3 FA	18.30	2.89	13.34	1.15
∑n-6 FA	22.05	0.80	22.76	0.20
18:4n-3/18:3n-3	0.12	0.02	0.18*	0.00
18:3n-6/18:2n-6	0.09	0.01	0.14*	0.01
16:1/16:0	0.17	0.02	0.17	0.02
18:1/18:0	7.56	0.68	7.63	0.86
20:3n-6/20:2n-6	0.72	0.06	0.98*	0.05
18:2n-9/18:1n-9	0.02	0.01	0.04*	0.01
18:1n-7/16:1n-7	2.13	0.14	1.94	0.39
22:6n-3/20:5n-3	5.80	0.64	5.09	0.74

* Denotes significant differences between the two fish groups.

Regarding the muscle fatty acid profiles (Table 6.6), L fish showed 0.07% and 0.11% higher contents of the fads2 products 18:2n-9 and 18:3n-6, respectively, than H fish. No differences were found in the fatty acid profiles of whole fish between the two experimental fish groups (Table 6.7).

Table 6.6 Fatty acid composition of muscle of gilthead seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) after 74 days of feeding the challenge diet

	H		L	
Fatty acid (%)	Mean	S.D.	Mean	S.D.
14:0	0.28	0.12	0.28	0.08
14:1n-7	0.02	0.01	0.03	0.03
14:1n-5	0.03	0.01	0.04	0.03
15:0	0.06	0.02	0.07	0.03
15:1n-5	0.03	0.02	0.02	0.02
16:0ISO	0.03	0.02	0.05	0.05
16:0	6.55	0.57	6.14	1.01
16:1n-7	1.16	0.35	1.09	0.38
16:1n-5	0.06	0.05	0.08	0.08
16:2n-4	0.08	0.03	0.08	0.04
17:0	0.09	0.01	0.08	0.02
16:3n-4	0.13	0.05	0.13	0.05
16:3n-3	0.07	0.03	0.06	0.03
16:3n-1	0.21	0.07	0.23	0.17
16:4n-3	0.10	0.03	0.09	0.02
16:4n-1	0.05	0.02	0.05	0.03
18:0	3.98	0.73	3.94	0.69
18:1n-9	25.61	3.21	27.11	6.95
18:1n-7	2.19	0.16	2.21	0.27
18:1n-5	0.07	0.02	0.09	0.03
18:2n-9	0.35	0.04	0.42*	0.02
18:2n-6	15.77	0.23	16.40	2.20
18:2n-4	0.09	0.03	0.08	0.02
18:3n-6	0.55	0.03	0.66*	0.02
18:3n-4	0.12	0.03	0.10	0.02
18:3n-3	4.56	0.58	5.04	1.29
18:3n-1	0.05	0.04	0.03	0.02
18:4n-3	0.48	0.13	0.49	0.11
18:4n-1	0.06	0.02	0.06	0.03
20:0	0.35	0.02	0.38	0.08
20:1n-9	0.27	0.05	0.30	0.08
20:1n-7	2.06	0.27	2.07	0.56
20:1n-5	0.13	0.01	0.13	0.03
20:2n-9	0.55	0.07	0.51	0.05
20:2n-6	0.74	0.05	0.70	0.03
20:3n-9	0.04	0.01	0.02	0.02
20:3n-6	0.62	0.15	0.62	0.17
20:4n-6	0.87	0.24	0.82	0.41
20:3n-3	0.38	0.03	0.37	0.05
20:4n-3	0.66	0.05	0.62	0.03
20:5n-3	4.93	0.41	4.35	1.60
22:1n-11	1.67	0.54	1.74	0.52
22:1n-9	0.82	0.17	1.13	0.55
22:4n-6	0.26	0.04	0.29	0.06
22:5n-6	0.50	0.10	0.51	0.31
22:5n-3	2.96	0.21	2.65	0.61
22:6n-3	19.37	4.11	17.64	8.40
∑SFA	11.34	0.35	10.96	0.99
∑MUFA	34.12	4.72	36.04	8.29
∑PUFA	54.54	4.70	53.00	8.29
∑n-3 FA	33.51	4.01	31.30	9.24
∑n-6 FA	19.30	0.73	19.98	1.37
18:4n-3/18:3n-3	0.10	0.01	0.09	0.01
18:3n-6/18:2n-6	0.04	0.01	0.04	0.00
16:1/16:0	0.17	0.04	0.18	0.04
18:1/18:0	6.66	1.80	7.24	3.27
20:3n-6/20:2n-6	0.84	0.16	0.88	0.25
18:2n-9/18:1n-9	0.01	0.00	0.02	0.01
18:1n-7/16:1n-7	2.01	0.58	2.12	0.48
22:6n-3/20:5n-3	3.90	0.52	3.92	0.65

* Denotes significant differences between the two fish groups.

Table 6.7 Whole body fatty acid composition of gilthead seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) after 74 days of feeding the challenge diet

	H		L	
Fatty acid (%)	Mean	S.D.	Mean	S.D.
14:0	0.66	0.59	0.33	0.14
14:1n-7	0.07	0.09	0.09	0.10
14:1n-5	0.08	0.11	0.11	0.13
15:0	0.13	0.06	0.11	0.09
15:1n-5	0.07	0.11	0.08	0.07
16:0ISO	0.07	0.09	0.09	0.06
16:0	8.53	2.86	5.56	0.37
16:1n-7	2.17	0.74	1.30	0.16
16:1n-5	0.18	0.22	0.20	0.22
16:2n-4	0.19	0.10	0.20	0.21
17:0	0.17	0.06	0.17	0.12
16:3n-4	0.19	0.08	0.30	0.33
16:3n-3	0.16	0.19	0.22	0.25
16:3n-1	0.18	0.18	0.26	0.26
16:4n-3	0.24	0.19	0.30	0.31
18:0	2.97	0.46	2.87	0.33
18:1n-9	33.28	6.31	31.24	3.17
18:1n-7	2.68	0.13	2.62	0.29
18:1n-5	0.16	0.14	0.23	0.22
18:2n-9	0.51	0.09	0.57	0.14
18:2n-6	16.95	2.72	15.95	1.68
18:2n-4	0.14	0.12	0.06	0.05
18:3n-6	0.60	0.15	0.74	0.03
18:3n-4	0.19	0.14	0.19	0.11
18:3n-3	5.80	0.65	5.94	0.50
18:3n-1	0.09	0.13	0.12	0.13

	H		L	
Fatty acid (%)	Mean	S.D.	Mean	S.D.
18:4n-3	0.65	0.03	0.63	0.07
18:4n-1	0.14	0.15	0.16	0.13
20:0	0.42	0.24	0.57	0.17
20:1n-9	0.38	0.16	0.44	0.07
20:1n-7	2.25	0.66	2.60	0.05
20:1n-5	0.21	0.18	0.29	0.21
20:2n-9	0.62	0.23	0.81	0.16
20:2n-6	0.63	0.26	0.86	0.21
20:3n-9	0.07	0.10	0.13	0.10
20:3n-6	0.38	0.14	0.55	0.15
20:4n-6	0.38	0.14	0.46	0.07
20:3n-3	0.37	0.21	0.49	0.12
20:4n-3	0.63	0.29	0.75	0.13
20:5n-3	2.44	0.57	2.73	0.18
22:1n-11	1.88	1.03	2.53	0.23
22:1n-9	0.83	0.46	1.34	0.25
22:4n-6	0.35	0.43	0.35	0.12

	H		L	
Fatty acid (%)	Mean	S.D.	Mean	S.D.
22:5n-6	0.51	0.67	0.37	0.17
22:5n-3	1.90	1.20	2.32	0.06
22:6n-3	8.47	4.97	11.77	0.11
∑SFA	12.88	3.47	9.62	0.53
∑MUFA	44.25	3.75	43.08	1.29
∑PUFA	42.79	7.03	47.22	1.13
∑n-3 FA	20.65	6.95	25.14	0.44
∑n-6 FA	19.82	1.23	19.28	0.99
18:4n-3/18:3n-3	0.11	0.01	0.11	0.02
18:3n-6/18:2n-6	0.04	0.01	0.05	0.01
18:1/18:0	11.15	0.45	10.88	0.18
20:3n-6/20:2n-6	0.62	0.03	0.64	0.05
18:2n-9/18:1n-9	0.02	0.00	0.02	0.01
16:1/16:0	0.25	0.00	0.23	0.02
18:1n-7/16:1n-7	1.38	0.64	2.04	0.24
22:6n-3/20:5n-3	3.30	1.13	4.33	0.31

6.3.3 Gene expression

Hepatic *fads2* expression was significantly higher in juveniles from broodstock with low *fads2* expression, showing in average a 64% higher value than those for fish from broodstock with high *fads2* expression (p<0.05) (Table 6.8). Similarly, hepatic expression of *srebp* and *elovl6* was slightly higher in L fish than in H fish (p_{srebp}=0.088 and p_{elovl6}=0.101). No significant differences were observed in hepatic expression of *g6p*, *lpl*, *cpt-1ß*, *ppara* and *igf-1* (p>0.1).

Table 6.8 Hepatic gene expression of gilthead seabream juveniles obtained from broodstock with different *fads2* expression (high H or low L) after 74 days of feeding the diet contained 7.5% FM and no FO

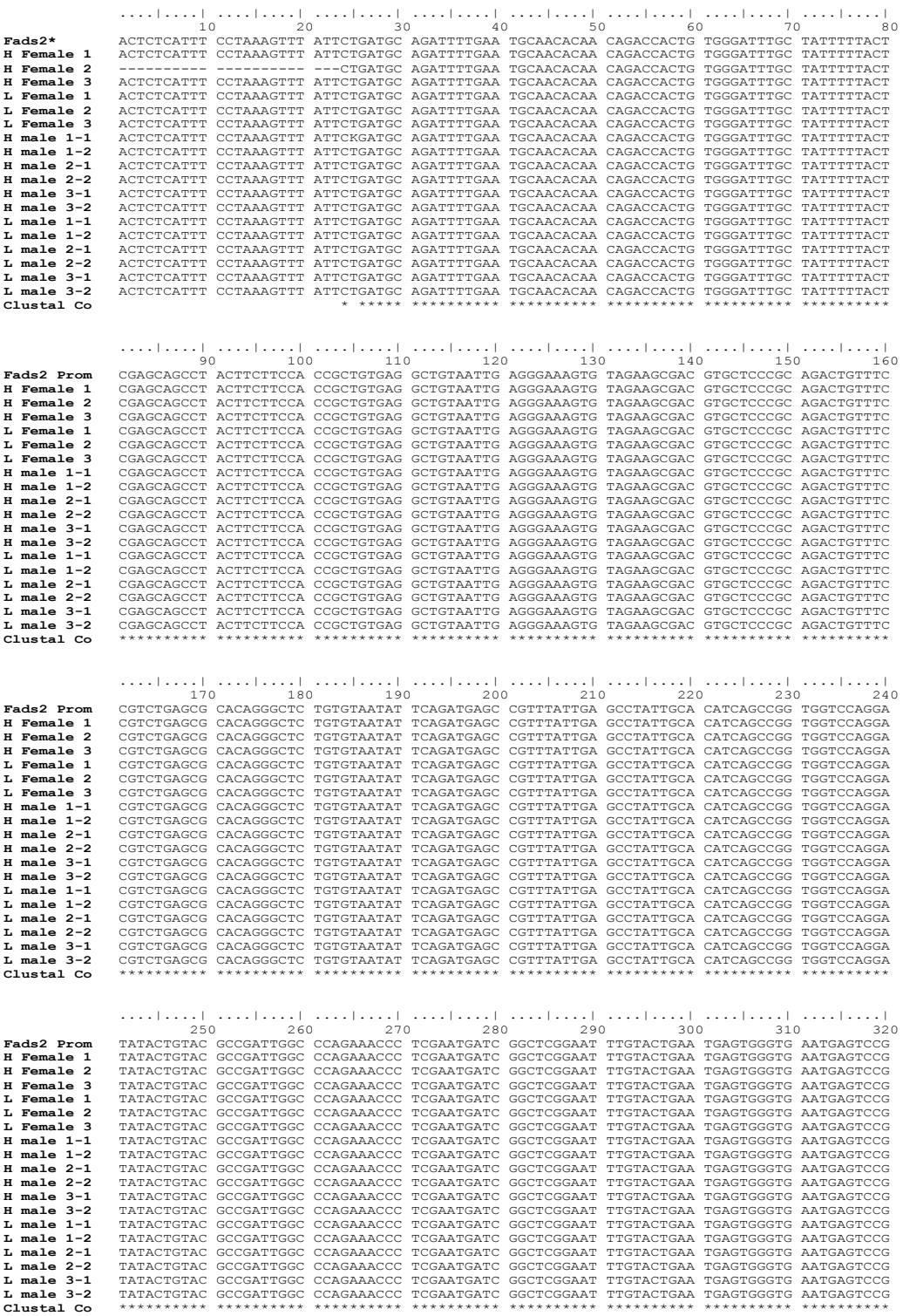
	H		L	
(Copies/µL)	Mean	S.D.	Mean	S.D.
fads2	56.89	20.97	144.98**	6.37
g6p	52.46	14.61	72.16	20.05
srebp	27.06	0.40	58.30*	17.26
lpl	128.57	30.86	156.01	32.45
cpt-1ß	16.13	10.81	21.22	6.36
elovl6	39.11	12.13	78.10*	29.41
ppara	38.75	12.02	45.66	11.55
igf1	195.78	32.43	285.42	197.88

* and ** denote significant differences between the two fish groups (p<0.1 and p<0.05, respectively).

6.3.4 fads2 promoter sequence alignment

No difference was observed in the alignment of the *fads2* promoter region sequence studied between broodstock with different *fads2* expression (Figure 6.3).

Figure 6.5 Alignment of *fads2* promoter region sequence of broodstock with inherently high (H) or low(L) *fads2* expression



* *fads2* promoter region of gilthead seabream(Perera *et al.*, 2019).

6.4 Discussion

The present study has shown the long-term effects of broodstock *fads2* expression on growth performance and fatty acid profiles of sea bream fed a low FM and low FO diet from 2 to 100 g, offspring from broodstock with a high *fads2* expression showing a higher body weight. Thus, at the end of the trial, offspring from high *fads2* broodstock was 22 % larger in body weight than those from low *fads2* broodstock, despite both fish had been fed the same feeds since they were 2.31 g. These results agree well with the higher growth rates found in young sea bream fingerlings (3-month-old) obtained from high *fads2* broodstock in previous studies (Turkmen *et al.*, 2020) (Turkmen *et al*., 2020; Xu *et al*., in press, Chapter 5) and confirm the persistence of this growth improvement even when fish had reached 100 g. This better growth performance in offspring from high *fads2* fish was more pronounced in younger fish from the first nutritional challenge trial than in older fish from the second nutritional challenge trial. Thus, despite the SGR in juveniles with a few grams of body weight was markedly higher in offspring from high *fads2* broodstock, as the offspring increased in body weight the SGR values were getting closer. In fact, at the end of the second nutritional challenge trial, SGR was higher in offspring from low *fads2* fish, what seems to be related to their lower body weight. These results suggest a stronger effect of the broodstock *fads2* expression on growth of offspring during younger stages, when fish have a higher sensitivity to low FM and FO diets. In agreement, FM/FO reduction from 23/15.6 to 3/3.6 in diets for gilthead sea bream significantly reduced growth in 15-20 g fish, but did not affected growth when the fish reached 100-300g (Benedito-Palos *et al.*, 2016). Similarly, FO replacement by linseed oil in diets for gilthead sea bream reduced growth in a higher extend in young juveniles of 15 g than in larger fish of 85-400g (Izquierdo *et al.*, 2003; Izquierdo *et al.*, 2005), what could be related to the higher LC-PUFA requirements in younger fish (Izquierdo *et al.*, 2005). Despite not confirmed yet in fish, in chicken, the expression of the fatty acid transport protein Slc27 shows an age-related pattern (Wang *et al.*, 2011).

The higher body weight found in offspring from high *fads2* broodstock agreed well with the higher incorporation of DHA into the different body tissues, particularly in liver, since this fatty acid is a very active promoter of growth in fish. Besides, the eggs from high *fads2* broodstock also showed a higher content in DHA (Ferosekhan *et al.*, 2020b). DHA is one of the most

abundant fatty acids in fish eggs (Izquierdo *et al.*, 2001) and its incorporation in cell membranes affects not only membrane structure and function but also intercellular interaction, receptor expression, nutrient transport and signal transduction, all of which affect cell growth (Izquierdo and Koven, 2011). Indeed, DHA more than any other fatty acid promotes fish growth (Watanabe *et al.*, 1989; Watanabe and Kiron, 1994). In agreement, in mammals, n-3 LC-PUFA levels during the prenatal stage lead to persistent life-time effects (Li *et al.*, 2006), and supplementing DHA during mid-pregnancy favor a larger body mass index in children up to 6-year-old (Bergmann *et al.*, 2012). Thus, the higher final body weight found in juveniles from broodstock with high *fads2* expression in the present trial could be also associated with the importance of DHA for fish development and growth (Fernández-Palacios *et al.*, 2011). Besides a higher content of DHA, offspring from high *fads2* expression broodstock showed a higher hepatic accumulation of 22:5n-3 and 22:4n-6, late products of elongation and desaturation from 18:3n-3 and 18:2n-6, respectively, and indicators of Elovl2 activity. These results suggested a better utilization of dietary lipids in offspring from high *fads2* expression broodstock, in comparison to those from low *fads2* expression broodstock, what would contribute to the faster growth of the earlier offspring as discussed before. In agreement, at the end of the first nutritional challenge trial, hepatic *fads2* was significantly up-regulated in offspring from high *fads2* broodstock, in comparison to those from low *fads2* broodstock (Xu *et al.*, in press, Chapter 5). Interestingly, after the second nutritional challenge trial in the present study, hepatic *fads2* expression values were lower in offspring from broodstock with high *fads2* expression. This was in agreement with the lower liver contents in 18:3n-6 and 18:4n-3, direct products from Δ6 desaturation activity coded by *fads2*, in comparison to the offspring from low *fads2* broodstock. Such reduced Δ6 desaturation activity and down-regulation of *fads2* in liver of offspring from high *fads2* expression broodstock could be due to the inhibitory action of the high hepatic contents in DHA, 22:4n-6 and 22:5n-3, final products of LC-PUFA synthesis. Different studies have demonstrated the inhibitory effect of LC-PUFA on *fads2* expression or Δ6 desaturation activity. For instance, feeding gilthead sea bream with a FO based diet down-regulated *fads2* expression and inhibited Fads2 activity in comparison with fish fed with a LC-PUFA free diet (Seiliez *et al.*, 2003). Similar results are found in larvae

of this species, where feeding a FO based diet led to a six-fold down-regulation of *fads2* expression in comparison to larvae fed a vegetable oil diet (Izquierdo *et al.*, 2008). In mammals, LC-PUFA suppress *Fads2* expression regulating Srebp by inhibition of liver-X receptor and reduction of the Srebp1 mRNA stability and the nuclear form Srebp1 (Nakamura and Nara, 2003). In fish, although the mechanisms involved are not clear, LC-PUFA may regulate *fads2* through Srebp since a binding region of Srebp is found in the promoter region of *fads2* (Perera *et al.*, 2019) and Srebp may increase the activity of the *fads2* promoter (Dong *et al.*, 2017). In agreement, in the present study, a mild ($p<0.1$) decreased in *srebp* was found in offspring from high *fads2* expression broodstock. Therefore, the lower expression of *fads2* found in offspring from high *fads2* expression broodstock seems to be associated to the higher liver content in the LC-PUFAs DHA, 22:4n-6 and 22: 5n-3.

The molecular mechanisms behind the different accumulation of DHA among different fish individuals are not clear yet. For instance, in Asian seabass (*Lates calcarifer*) different quantitative trait loci (QTL) have been associated to different LC-PUFA contents in muscle (Xia *et al.*, 2014). More recently, molecular markers associated to the DHA/DPA ratio located on chromosome 19, close to the candidate gene *elovl2*, have been proposed for Atlantic Salmon (Horn *et al.*, 2020). In the present study, despite an increased Elovl2 activity in offspring of high *fads2* broodstock, the DHA/DPA ratios were similar between offspring of different broodstock, suggesting that markers different than those proposed for Atlantic salmon may be responsible for these differences in growth and fatty acid profiles. Our previous studies have shown that among sea bream broodstock some individuals show 23 and even 47 times a higher expression of *fads2* than others (Ferosekhan *et al.*, 2020b; Turkmen *et al.*, 2020). In gilthead sea bream, as in mammals, we have recently showed an increased methylation of specific CpG sites in the promoter region of *fads2* in offspring from broodstock with low *fads2* expression (Turkmen *et al.*, 2019b). Besides, in mammals, different FADS2 activities are also linked to different *FADS2* genotypes. For instance, low *FADS1* and *FADS2* expression, as well as a lower level of 22:5n-3 and EPA, have been associated with the minor allele (T) of the SNP site *rs174570* (Schaeffer *et al.*, 2006), whereas SNPs from *rs174545* to *rs174570* are associated with arachidonic acid in serum and erythrocyte membranes (Malerba *et al.*, 2008). However,

studies on potential polymorphisms in gilthead sea bream *fads2* had not been yet addressed. In the present study, we have analyzed *fads2* promoter sequence alignment designing primers based on Perera *et al.* (2019) and no differences were observed in the *fads2* promoter region studied among fish with different *fads2* expression. Finally, differences in DHA content in tissues have been also associated to the preferential mitochondrial beta-oxidation of EPA over DHA (Madsen *et al.*, 1999; Izquierdo and Koven, 2011; Horn *et al.*, 2019). However, in the present study, EPA was not lower in offspring of high *fads2* fish in comparison with those from low *fads2* fish. In agreement, neither expression of *cpt1* nor *ppara*, indicators of beta-oxidation in mitochondria and peroxisomes, respectively, were significantly different between offspring from the two type of broodstocks.

Although offspring from high *fads2* expression had higher hepatic 22:4n-6, 22:5n-3 and DHA composition, they showed a lower content of 18C fatty acid products from Fads2 activity and a low *fads2* expression. This could be related with the multifunction of *fads2* in fish. According to the research on hepatocyte of gilthead sea bream, over 35% radioactivity is detected in 18:3n-6 when 0.35μM ^{14}C-label 18:2n-6 was added into the medium and almost 41% radioactivity is detected in 18:4n-3 when 0.35 μM ^{14}C-label 18:3n-3 is added into the medium (Tocher and Ghioni, 1999), but only 0.7% radioactive Δ5 product, EPA, is detected when 0.1 μM U-^{14}C 18:4n-3 or 20:4n-3 is added to the medium (Tocher and Ghioni, 1999). These results illustrate that gilthead sea bream has a high Δ6 activity but the restriction factor of LC-PUFA biosynthesis in gilthead seabream is the lack of Δ5 desaturation activity. Although in mammals, Δ5 desaturation is catalysed by *fads1* gene (Glaser *et al.*, 2010), in fish, for instance, in zebrafish (*Danio rerio*), protein translated from gene similar to mammalian *fads2* also has the function of Δ5 desaturation (Hastings *et al.*, 2001). Although it is still not clear due to the extremely low Δ5 desaturation, gilthead sea bream desaturase clone shows residual Δ5 activity (Mourente and Tocher, 1993; Mourente and Tocher, 1994; Tocher and Ghioni, 1999). A positive correlation between hepatic DHA content and the products of EPA, the product of Δ5 desaturation, suggested that the higher content of hepatic LC-PUFA in offspring from broodstock with higher *fads2* expression may be related to an enhanced Δ5 activity.

In summary, the results of the present study showed the long-term effects of broodstock *fads2* expression on growth performance of the offspring fed low FM and low FO diets. The offspring from broodstock with a high *fads2* expression showed a larger body weight, which was more pronounced in younger fish. In relation to the increased body weight, the offspring from broodstock with a high *fads2* expression showed a high incorporation of DHA, particularly in the liver. This higher DHA, together with the increase in 22:5n-3 and 22:4n-6, late products of elongation and desaturation, would be responsible for the downregulation of hepatic *fads2*, possibly mediated by the downregulation in *srebp*. No differences were observed in the *fads2* promoter region sequence alignment studied among fish with different *fads2* expression.

Acknowledgements

This study was supported by the EU funded project PerformFISH (H2020-SFS-2016-2017; 727610) and China scholarship council. The authors acknowledge Skretting ARC (Stavanger, Norway) for providing the experimental diets.

Chapter 7

Nutritional programming through broodstock diet persistently improves the use of low fishmeal and fish oil diets in gilthead sea bream (*Sparus aurata*) juveniles

Status: To be submitted

Abstract

One of the factors that limits the replacement of fishmeal (FM) and fish oil (FO) by plant ingredients in the diets for marine fish is their ability of synthesizing long-chain polyunsaturated fatty acid (LC-PUFA). Nutritional programming by feeding gilthead sea bream broodstock a diet rich in rapeseed oil (RO) during spawning season can enhance the LC-PUFA biosynthesis of their offspring when they are challenged by low FM and low FO diet. However, the potential persistence of this effect it has not been sufficiently studied. The aim of this study was to determine if nutritional programming using RO rich diet for broodstock had long term effect on offspring ability to use low FM and FO diet. For that purpose, growth, fatty acid profiles, expression of selected genes, *fads2* promoter sequencing and microRNA libraries were studied in juveniles obtained from broodstock fed a diet contained 100% FO (BFO) or 80% RO, 20%FO (BRO) in lipids and nutritional challenged with a 7.5% FM and 0% FO diet. The results showed a 27% improvement in final body weight in the offspring from broodstock fed with RO diet than fish from broodstock fed with FO diet. Besides, fish from broodstock fed with RO diet had lower HSI, hepatic lipid content and smaller size of hepatocyte (p<0.05), and around two times higher content of LC-PUFA, including arachidonic acid, 22:5n-6 and DHA in muscle, than that from broodstock fed with FO diet. Moreover, a higher activity of Δ5 desaturase was observed based on the higher ratio of 20:4n-6/20:3n-6 and 20:5n-3/20:4n-3 in the muscle of fish from broodstock fed with RO diet. No differences were observed in sequencing alignment in the *fads2* promoter region among broodstock programmed by different diets, nor in the microRNA libraries in the offspring juveniles. Overall, the study demonstrated the long-term nutritional programming effect on broodstock on the growth, liver histological structure and lipid metabolism of the offspring fed with low in FM and FO diet.

Keywords: aquaculture, lipid metabolism, fishmeal replacement, fish oil replacement, nutritional programming

7.1 Introduction

Fish food is a well-balanced source of minerals and highly digestible proteins for human being. Besides, it is the main source of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) that are important for fetal development (Helland *et al.*, 2003) and contribute to prevent cardiovascular diseases (Schmidt *et al.*, 2000) and non-alcoholic fatty liver disease (NAFLD) (Oya *et al.*, 2010), among many other properties for human health. Therefore, fish demand by the consumers is continuously increasing. However, global capture fisheries remain relatively stable since late-1980's (FAO, 2020). Due to this stagnant production of fisheries, aquaculture is taking over the responsibility to provide sufficient safe and sustainable products to satisfy market demands. In 2018, Aquaculture produced 52% of the fish used for human consumption (FAO, 2020). However, further development of aquaculture is limited by the high demand, restricted availability and increasing prices of fishmeal (FM) and fish oil (FO), traditional protein and lipid sources in aquafeeds. For instance, in 2015, aquaculture used over 70% FM and FO produced worldwide (Green, 2018). Both FM and FO are mostly derived from capture fisheries (Hardy and Barrows, 2002), and, therefore, their production is stagnant or even decreased (Green, 2018). Besides this limitation, the use of FM and FO in aquafeed raises concerns about overexploitation of fisheries resources (Council, 2011). Therefore, dietary FM and FO need to be replaced by other high quality and nutritious ingredients with a more economical, environmental and social sustainability. Many alternative ingredients are used to replace FM and FO like in aquafeeds such as plant ingredients, animal by-products, single cell ingredients or insect meals (Caballero *et al.*, 2002; Wang *et al.*, 2016; Rosales *et al.*, 2017; Rimoldi *et al.*, 2018). However, depending on the type of ingredient and the replacement level, these alternative ingredients may lead to malnutritional effects on fish growth, nutrients digestibility, immune system, *etc*. (Vergara *et al.*, 1996b; Caballero *et al.*, 2004; Gómez-Requeni *et al.*, 2004; Carvalho *et al.*, 2019).

One of the factors that restricts the replacement of FM and FO by alternative ingredients is their frequent lack of essential fatty acids. N-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA), especially docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA), are important components of biomembranes that determine normal cell functioning. Therefore, they are necessary for the normal development of brain and tissues (Mourente, 2003),

functioning of immune system (Zuo *et al.*, 2012b), affecting the reproduction performance (Fernández-Palacios *et al.*, 2011) and affect the nutritional value of fillet for the consumers (Monge-Ortiz *et al.*, 2018). Therefore, either LC-PUFAs or their 18C precursors are required in fish feeds. For certain freshwater species, some vegetable oils, which are rich in 18C precursors of LC-PUFA, can be used as the sole dietary lipid source (Sargent *et al.*, 2003). However, due to the limited LC-PUFA biosynthesis ability of some strict carnivore freshwater species or marine species, LC-PUFAs are essential in their diet. So far, FO is the main commercially competitive source of LC-PUFAs. Oil from oilseed or animal are used in the replacement of dietary FO, but the insufficient LC-PUFAs content in these resources restricts the replacement level, since the complete FO replacement leads to the decreased growth performance, reduced nutritional value of fillet, abnormal liver lipid metabolism, impaired immune system and weakened reproduction performance (Tocher *et al.*, 2002; Menoyo *et al.*, 2004; Izquierdo *et al.*, 2005; Lin and Shiau, 2007; Sink *et al.*, 2010).

The biosynthesis pathway of LC-PUFA is catalyzed by a set of desaturases and elongases (Figure 6.1). For instance, AA is synthetized from linoleic acid (18:2n-6, LNA), and EPA and DHA are obtained from α-linolenic acid (18:3n-3, ALA). Partly due to the abundant LC-PUFA in the marine food web, originally from phytoplankton (Sargent *et al.*, 2003), marine fish have a lower ability to synthesize LC-PUFA from their 18C precursors (Dong *et al.*, 2017). This restricted ability is related with the deficiency in the activity of one or more enzymes like fatty acyl desaturase 2 (Fads2) (Tocher and Ghioni, 1999; Sargent *et al.*, 2003). For instance, the activity of Fads2 in cod (*Gadus morhua* L) is lower than in Atlantic salmon (Tocher *et al.*, 2006). Fads2 is the first enzyme in LC-PUFA biosynthesis pathway with the function of catalyzing the ALA or LNA to 18:4n-3 or 18:3n-6 and also participate in the synthesis of DHA from EPA (Vagner and Santigosa, 2011). In addition, desaturase translated from *fads2* shows activities on desaturation at Δ5 and Δ8 position of fatty acids (Mourente and Tocher, 1993; Mourente and Tocher, 1994; Tocher and Ghioni, 1999; Monroig *et al.*, 2011). Since ingredients used to replace FO are rich in LC-PUFA precursors, such as ALA and LNA, the enhancement of LC-PUFA biosynthesis ability would facilitate a larger replacement of FO by vegetable oils.

A common strategy employed by nature that allows the adaptation of the organism metabolism to the environmental conditions is nutritional programming. Nutritional programming referred to the outcome on an animal that receives a nutritional stimulus at a critical period in life, such as pre- or postnatal stages (Lucas, 1994). A general protocol for the establishment of nutritional programming includes: 1) experimental animals are exposed to a nutritional stimulating factor in a specific period; 2) the animals or their offspring are fed with a 'normal' diet for several weeks or month; 3) the animals are challenged with an experimental diet and their response is studied (Hou and Fuiman, 2019). In fish, nutritional stimulus during reproduction by feeding broodstock with different dietary fatty acid profiles markedly affects lipid metabolism and growth of the progeny. For instance, feeding gilthead sea bream (*Sparus aurata*) broodstock with diets containing partial replacement of FO by linseed oil, low in n-3 LC-PUFA but high in their ALA precursor, up-regulated *fads2* expression and growth in the progeny (Izquierdo *et al.*, 2015). Besides, it also up-regulated other lipid metabolism and health related genes, such as *cycloxigenase-2* (*cox2)* and *tumor necrosis factor-alpha* (*tnf-a)*, *lipoprotein lipase* (*lpl*), *carnitin palmitoil transferase 1* (*cpt1*) or *elongase 6* (*elovl6*) (Turkmen *et al.*, 2017a; Turkmen *et al.*, 2019b). Indeed, broodstock feeding exerts a very long-term effect in the progeny, and replacement of FO by LO in parental diets, in combination to juvenile feeding with low-FM and low-FO diets, markedly improves 16-month-old offspring growth and feed utilization (Turkmen *et al.*, 2017a). These studies demonstrated that it is possible to improve the ability of marine fish to use low FM and low FO diets by nutritional programming though broodstock feeding. Similarly, exposure to a vegetable-based diet in early life of Atlantic salmon (*Salmo salar*), zebrafish or brown trout (*Salmo trutta*) improves growth performance and feed efficiency later in life when fish are fed a low FO and FM diet (Clarkson *et al.*, 2017; Michl *et al.*, 2017; Martínez-Llorens *et al.*, 2020). However, other studies in European seabass (*Dicentrarchus labrax*) or Siberian sturgeon (*Acipenser baerii* Brandt) did not found such nutritional programming effect (Vagner *et al.*, 2009; Luo *et al.*, 2019).

Previous studies have shown interactions on the 16-month-old offspring between nutritional programming through broodstock diet and nutritional challenge in early life stages (Turkmen *et al.*, 2017a). However, the sole long-term effect of broodstock nutritional programming on

offspring growth performance or fatty acid composition on the offspring were not demonstrated. More recently, we conducted a study aimed to evaluate the reproductive performance of broodstock with different broodstock diets to stimulate nutritional programming (Ferosekhan *et al.*, 2020b). In this study, gilthead seabream breeders were fed during the spawning season with diets containing 9.3% FO or a programming diet which contained 1.8% FO and 7.5 % rapeseed oil (RO). No significantly difference was observed on the spawning performance of between broodstock with higher inherently *fads2* expression fed with FO diet or programming diet (Ferosekhan *et al.*, 2020b). Afterwards, the offspring from broodstock with high *fads2* expression fed with programming diet showed a better growth performance when challenged with a diet low in FM and FO for 45 days (Xu *et al*. in press, Chapter 5). Following this previous study, the present one aimed to determine the long-term effect of nutritional programming through broodstock diets on the offspring response to nutritional challenge test. Growth performance, tissue fatty acid profiles, expression of selected genes and microRNA were studied in offspring from broodstock fed two different programming diet and challenged with a low FM/FO content challenged diet.

7.2 Material and Methods

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

7.2.1 Feeding trial and fish performance

To produce these juveniles, eggs from each of the two broodstock groups programmed with a diet rich in RO diet (1.76% FO and 7.54% RO) (BRO) or just fed with FO diet (9.3% FO) (BFO) (Ferosekhan *et al.*, 2020b) were collected in the same day and separately incubated. Offspring larvae and juveniles were reared under the same conditions and fed with commercial marine based diets until they were over 50g. Afterwards, 60 fish from the remaining 130 fish in each group were randomly sampled (79.0±0.05 g and 110.8±2.0 g for BFO and BRO fish, respectively) and distributed into three tanks of 250 L (20 fish/tank). Tanks were provided with 250 L/h seawater at 21.0 ± 0.8°C and strong aeration, and dissolved oxygen (6.2 ± 0.1 mg/L)

was daily determined. Tanks were illuminated by fluorescent lights placed above the tank at an intensity of 100 lx, programmed for a 12h light photoperiod (from 8 a.m. to 8 p.m.).

In order to challenge the fish with a very low FM and FO diet for triggering their potential ability to cope with low n-3 LC-PUFA, an experimental diet was formulated to contain 7.5% of FM and 0% FO (Table 7.1). Corn gluten, soybean meal, wheat gluten, soya protein concentrates and faba bean meal were used to replace the dietary fishmeal. Poultry oil and rapeseed oil were used to replace the dietary fish oil. The diet was low in ARA, EPA and DHA and high in oleic acid (OA, 18:1n-9), LA, palmitic acid (PA, 16:0) and ALA (Table 7.1).

Table 7.1 Ingredients, proximate composition and main fatty acids content of the challenge diet fed to the one-year-old juveniles obtained from broodstock with fed with diet rich in FO or RO during spawning season

Ingredient (% of dry weight.)	
Poultry oil[a]	6.14
Wheat[b]	9.46
Corn gluten[b]	10.00
Soybean meal[b]	5.00
Wheat gluten[b]	19.18
Soya protein concentrate[b]	30.00
Faba beans[b]	5.00
Fish meal[b]	7.50
Rapeseed oil[b]	7.52
Vitamin premix vit[c]	0.10
Mineral premix[c]	0.10
Proximate composition (% of dry weight)	
Crude protein	53.74
Crude lipid	22.90
Crude ash	4.71
Fatty acid profile (% of total fatty acids)	
16:0	7.79
18:0	3.43
18:1n-9	41.74
18:2n-6	24.58
18:3n-3	10.20
20:4n-6	0.23
20:5n-3	0.87
22:6n-3	1.98

a Poultry oil: Sonac. B.V. The Netherlands. b Skretting AS (Norway). c Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC (Stavanger, Norway).

Fish were fed the experimental diet until apparent satiation three times per day (8:30, 11:30, 14:30) and six days per week for 74 days. Feed delivery was daily calculated, and uneaten pellets were collected in a net by opening the water outlet 30 min after each meal. Then, the collected pellet were dried in an oven for 24 h and weighed to estimate feed intake. All fish were weighed individually every two weeks during the feeding trial. At the end of the trial, fish were fasted for 24 h, weighed and anesthetized with ethanol diluted clove oil (50:50) before samplings. The following performance parameters on mortality, growth (Weight gain (WG), Specific growth rate (SGR)), feed acceptance (Feed intake (FI)), Biological feed conversion

ratio (FCR) (National Research Council, 2011), energy status (hepatosomatic index (HSI)) (Chellappa *et al.*, 1995), lipid deposition (viscerosomatic index (VSI)), were calculated:

- Mortality (%) = 100 × (nº dead fish/ nº total fish)
- Weight gain (WG, g) = final body weight (BW_1) – initial body weight (BW_0)
- Specific growth rate (SGR, % day^{-1}) = 100* (ln BW_1 − ln BW_0) / nº days, where BW_0: initial body weight (g) and BW_1: final body weight (g)
- Feed intake (FI, g $fish^{-1}$ day^{-1}) = Feed delivered / (nº of fish*nº days)
- Biological feed conversion ratio (FCR) = Feed delivered ($t_1 - t_0$) / (Biomass t_1 – Biomass t_0 + Biomass $_{harvested}$ + Biomass $_{lost}$), where t_0: initial biomass (g) and t_1: final biomass (g)
- Hepatosomatic index (HSI) = 100 × Liver weight (g) / Body weight (g)
- Viscerosomatic index (VSI) = 100 × Visceral weight (g) / Body weight (g)

7.2.2 Histological study

The livers of five fish per tank were sampled and stock in 4% formalin. After embedded in paraffin wax, blocks were made and cut with a Leica microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany) in 4 μm sections, which were placed in slides and stained with haematoxylin and eosin (H&E) (Martoja *et al.*, 1970). Slides were studied and photos were taken by a light microscope (Olympus, Tokyo, Japan). Area, length of long and short axis of 60 hepatocyte per tank were analysed with *ImagePro plus 6.0* (Media Cybernetics, Rockville, USA).

7.2.3 Biochemical composition and fatty acid analysis

At the end of the feeding trial, five fish per tank were painlessly killed at the end of the procedure by immersion in 10 ppm clove oil and methanol (50:50) and sampled to determine proximate composition of whole-body, liver and muscle. All samples were frozen at -80ºC until analysis. All samples were homogenized immediately prior to analysis. Moisture and protein contents were determined according A.O.A.C. (2000) (Horwitz, 2002). Lipids were extracted with chloroform/methanol (2:1 v/v) (Folch *et al.*, 1957) and then transmethylated to obtain fatty acid methyl esters (FAMES) (Christie, 1988). FAMES were then separated by gas chromatography (Polaris QTRACETM Ultra; ThermoFisher, Waltham, USA) under the conditions previously described (Izquierdo, 1989).

7.2.4 RNA extraction and digital PCR

The liver and muscle from the juveniles after the nutritional challenge were kept at 4 ºC overnight in 2.0 mL tubes with 1.5 mL of *RNAlater* and then transferred to -80℃ until molecular analyze. RNA from 100 mg sample was extracted using *TRI Reagent* (MilliporeSigma, Darmstadt, Germany) and then purified by *RNeasy kit* (Qiagen, Hilden, Germany). RNA quality was checked by 1.4% agarose electrophoresis and quantity was measured by *NanoDrop™ 1000* (ThermoFisher). cDNA was synthesized from 1 μg of RNA in *iCycler* (Bio-rad, Hercules, U.S.A.), using the *iScript cDNA synthesis kit* (Bio-rad). The expression of genes related to growth (*insulin grow factor 1*, *igf1*), lipid and energy metabolism (*fads2*; *glucose 6-phosphate*, *g6p*; *sterol regulatory element-binding protein 1*, *srebp1*; *lipoprotein lipase*, *lpl*; *carnitine palmitoyltransferase 1ß*, *cpt-1ß*; *elongation of very long chain fatty acids protein 6*, *elovl6*; *peroxisome proliferator activated receptor α*; *pparα*) were determined using the primers listed in Table 7.2. Digital PCR was performed as previous described (Xu *et al.*, 2019) using in *QX200™ Droplet Digital™ PCR System* (Bio-rad). The amplification conditions of PCR were: 95 ℃ for 5 min, followed by 40 cycles at 95℃ for 30 sec, elongation at Tm temperature for 1min, and then stabilized the signal at 4 ℃ for 5 min, 90℃ for 5 min, finally the reaction was hold at 4℃.

Table 7.2 Primers sequence for digital PCR and GeneBank accession numbers for sequences of target genes

Gene		Sequence (5'-3')	Tm (℃)	Gene bank No.
β-actin	Forward	GAC CAA CTG GGA TGA CAT GG	61	X89920.1
	Reverse	GCA TAC AGG GAC AGC ACA GC		
fads2	Forward	GCA GAG CCA CAG CAG CAG GGA	63	AY055749
	Reverse	CGG CCT GCG CCT GAG CAG TT		
g6p	Forward	CGC TGG AGT CAT TAC AGG CGT	62	XM_030399413.1
	Reverse	CAG GTC CAC GCC CAG AAC TC		
srebp	Forward	AGG GCT GAC CAC AAC GTC TCC TCT CC	62	XM_030407356.1
	Reverse	GCT GTA CGT GGG ATG TGA TGG TTT GGG		
lpl	Forward	CGT TGC CAA GTT TGT GAC CTG	60	AY495672
	Reverse	AGG GTG TTC TGG TTG TCT GC		
cpt-1ß	Forward	CCA CCA GCC AGA CTC CAC AG	60	DQ866821
	Reverse	CAC CAC CAG CAC CCA CAT ATT TAG		
elovl6	Forward	GTG CTG CTC TAC TCC TGG TA	60	JX975702
	Reverse	ACG GCA TGG ACC AAG TAG T		
ppara	Forward	TCT CTT CAG CCC ACC ATC CC	58	AY590299
	Reverse	ATC CCA GCG TGT CGT CTC C		
igf1	Forward	GTG TGT GGA GAG AGA GGC TT	58	AY996779.2
	Reverse	CTC TTG GCA TGT CTG TGT GG		

7.2.5 fads2 promoter sequencing

DNA was extracted from 25 mg caudal fin of broodstock used in present study with *DNAeasy kit* (Qiagen) according to the instruction from manufacturer. The quality of extracted DNA was check by 1% agarose gel electrophoresis, and the quantity was measured in the *NanoDrop*™ *1000 spectrophotometer* (Thermo). The primers used for *fads2* promoter region sequencing were designed based on Perera *et al.* (2019) (Forward: 5' CTC CTG GAA TTT CCC TCA 3'; Reverse: 5' TTT TCG GCT GCT CCT CTG 3'). PCR was performed using 40 ng/µL extracted DNA with *iTaq Kit* (Intron) according to the instruction of the manufacturer. Thermocycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 34 cycles at 94°C for 20 s, 60°C for 1 min, 72°C for 30 min, and a final extension step of 3 min at 72 °C. The PCR product was quantified in *NanoDrop*™ *1000 spectrophotometer* (Thermo), diluted to 75 ng/µL and sent to Macrogen (Madrid, Spain) for sequencing.

7.2.6 MicroRNA analysis

RNA was extracted from 200 mg pool of 5 livers per tank with *Direct-zol*™ *RNA Miniprep kit* (Zymo Research, Irvine, USA) according to the instruction of manufacturer. The quality and quantity of RNA was checked in *2200 Tapestation* (Agilent, Santa Clara, USA) with *High Sensitivity RNA ScreenTape* (Agilent). 200 ng RNA was used for RNA library preparation using *NEXTFLEX Small RNA-Seq Kit*. After the ligation and first strand synthesis according to the instruction of manufacturer, purified first strand synthesis product was mixed with universal primer, barcode primer and PCR master mix for amplification in thermal cycler (Bio-rad) according to following condition: 95 °C for 2 min, followed by 17 cycles at 95°C for 20 sec, 60°C for 30 sec, 72°C for 15 sec, and a final extension step of 2 min at 72 °C. Unique Barcode primer was assigned to each sample. PCR product around 160 bp was selected in 6% TBE-PAGE gel (Thermofisher). After the selection, library was quantified and qualified in *2200 Tapestation* (Agilent) with *High Sensitivity D1000 ScreenTape* (Agilent). 15 nmol libraries that contained same amount of library from each sample was sequenced in *NextSeq 500* (illumina, San Diego, USA) with *NextSeq 500/550 Kit* (illumina).

7.2.6 Data analysis

Data was shown in mean ± S.D. Independent t-test was used in the comparison between two experimental group through *SPSS 20.0* (IBM, U.S.A.). Gene expression was normalized by hepatic expression of *β-actin* according to the method decribed in Park and Crowley (2005). ClusterW alignment between *fads2* promoter region of each broodstock was performed through *BioEdit 7.2* (Tom Hall). RNA library was demultiplexed based on barcode, then the adapter sequence was move adapters and random nucleotides at 3' and 5' end map trimmed reads to gilthead seabream genome (Genome assembly: GCA_900880675.1). MicroRNAs expression was identified using *miRDeep2*. Reads were counted based on mature miRNA based on all Teleostei species. Differential expression was analyzed using *limma-voom* in R.

7.3 Results

7.3.1 Growth performance

No mortality was observed in two groups. After 74 days of challenge test, the final average weight of fish from broodstock fed with RO diet (BRO) was 153.7± 1.2 g, which was 27% higher (p <0.05) than fish from broodstock fed with FO diet (BFO) (Table 7.3). Whereas SGR of BRO fish was 0.2 % day^{-1} lower than BFO fish (p<0.05). Besides, fish from BRO group had a lower feed intake and HSI than fish from BFO group (p<0.05).

Table 7.3 Growth performance of one-year-old gilthead seabream juveniles obtained from broodstock fed FO or RO diet during spawning season, after 74 days of feeding the challenge diet

	BFO		BRO	
	Mean	S.D.	Mean	S.D.
Initial weight (g)	79.0	0.5	110.8*	2.0
Final weight (g)	121.0	2.0	153.7*	1.2
Weight gain (g)	41.8	2.7	42.5	1.4
Mortality (%)	0	0	0	0
SGR (%day^{-1})	0.6*	<0.1	0.4	<0.1
Feed intake (g fish^{-1} day^{-1})	1.3*	<0.1	1.1	0.1
FCR	2.3	0.2	2.0	0.2
HSI (%)	1.3*	<0.1	1.2	<0.1
VSI (%)	3.6	0.6	3.1	0.2

Abbreviations: SGR: Specific growth rate; FCR: Feed conversion rate; HSI: Hepatic somatic index; VSI: Visceral somatic index;

* Denotes significant differences (p <0.05) between the two fish groups.

7.3.2 Histology study

Hepatocytes of liver from BFO fish were significantly bigger than those from BRO (p<0.05), in which the length of long axis, short axis and the area of hepatocyte were 16.7%, 15% and

38% higher, respectively (Table 7.4). However, no significant differences were observed in the size of nucleus between fish of two experimental group.

Table 7.4 Morphometry of hepatocytes from gilthead seabream juveniles obtained from broodstock fed FO or RO diet during spawning season, after 74 days of feeding the challenge diet

	BFO		BRO	
	Mean	S.D.	Mean	S.D.
Long axis (μm)	10.42*	0.16	8.93	0.07
Short axis (μm)	6.67*	0.11	5.81	0.10
Area (μm^2)	62.38*	2.43	45.16	2.96
Nuclear	6.56	0.43	6.34	0.51

7.3.3 Biochemical analysis

Lipid content in the liver of BFO group was 3.79% higher than that of BRO group (p<0.05). No significant differences were found in the protein, lipid and ash content of whole fish and muscle between two fish groups (p>0.05) (Table 7.5).

Table 7.5 Proximate composition of liver, muscle and whole fish samples from gilthead seabream juveniles obtained from broodstock fed FO or RO diet during spawning season, after 74 days of feeding the challenge diet

		BFO		BRO	
% wet weight		Mean	S.D.	Mean	S.D.
Liver					
	Lipid	14.31*	0.92	10.52	0.79
	Protein	12.97	1.48	12.56	0.86
	Ash	1.04	0.24	1.12	0.28
Muscle					
	Lipid	5.21	4.34	1.40	0.31
	Protein	21.12	0.17	20.81	0.21
	Ash	1.47	0.19	1.47	0.26
Whole fish					
	Lipid	10.01	1.16	10.75	1.61
	Protein	16.35	0.27	16.60	0.43
	Ash	3.87	0.48	4.29	0.95

* Denotes significant differences (p <0.05) between the two fish groups.

Regarding the fatty acid profiles, hepatic 20:4n-6 content in BRO fish was significantly higher than that in BFO fish (Table 7.6). In muscle, BFO fish showed significantly higher (p<0.05) contents of 18C fatty acid than BRO, including 18:1n-9, 18:2n-6, 18:3n-3 and 18:4n-3 (Table 7.7). Whereas BRO fish had higher (p<0.05) content of very long chain PUFA (C>20) like 20:4n-6 and 20:5n-6, especially DHA, which was more than two times higher in the muscle of BRO fish than that in BFO fish. Besides, BRO fish had higher PUFA, n-3 fatty acid but BFO had higher MUFA and n-6 fatty acid content in muscle (p<0.05). The muscle of BRO fish was

significantly higher in the ratio between products and precursors of desaturation, which include 20:4n-6/20:3n-6, 20:5n-3/20:4n-3 and 22:6n-3/20:5n-3. No differences were found between in the fatty acid profiles of whole fish between the two experimental fish groups (Table 7.8).

Table 7.6 Hepatic fatty acid profile of gilthead seabream juveniles obtained from broodstock fed FO or RO diet during spawning season, after 74 days of feeding the challenge diet

	BFO		BRO	
Fatty acid(%)	Mean	S.D.	Mean	S.D.
14:0	0.43	0.06	0.38	0.06
14:1n-7	0.01	0.00	0.01	0.01
14:1n-5	0.01	0.01	0.01	0.00
15:0	0.06	0.01	0.06	0.01
15:1n-5	0.01	0.00	0.01	0.00
16:0ISO	0.01	0.00	0.01	0.00
16:0	7.35	0.71	7.52	0.13
16:1n-7	1.49	0.05	1.46	0.13
16:1n-5	0.04	0.00	0.03	0.01
16:2n-4	0.04	0.01	0.03	0.01
17:0	0.06	0.01	0.05	0.01
16:3n-4	0.12	0.01	0.12	0.01
16:3n-3	0.04	0.01	0.03	0.01
16:3n-1	0.01	0.01	0.03	0.02
16:4n-3	0.05	0.01	0.06	0.02
16:4n-1	0.00	0.00	0.00	0.01
18:0	4.34	0.43	4.25	0.33
18:1n-9	35.37	2.39	33.95	0.58
18:1n-7	2.55	0.06	2.60	0.06
18:1n-5	0.09	0.01	0.08	0.01
18:2n-9	0.78	0.15	0.67	0.05
18:2n-6	16.44	1.34	17.69	0.27
18:2n-4	0.06	0.01	0.06	0.01
18:3n-6	1.33	0.27	1.41	0.09
18:3n-4	0.08	0.01	0.08	0.01
18:3n-3	4.92	0.41	5.30	0.24
18.3n-1	0.01	0.01	0.01	0.01
18:4n-3	0.63	0.09	0.68	0.07
18:4n-1	0.04	0.01	0.03	0.01
20:0	0.22	0.01	0.21	0.02
20:1n-9	0.30	0.06	0.25	0.02
20:1n-7	2.02	0.12	1.83	0.08
20:1n-5	0.12	0.02	0.10	0.01
20:2n-9	1.00	0.08	0.94	0.09
20:2n-6	1.00	0.06	1.10	0.13
20:3n-9	0.01	0.01	0.01	0.01
20:3n-6	0.66	0.08	0.71	0.02
20:4n-6	0.63	0.04	0.79*	0.02
20:3n-3	0.51	0.04	0.56	0.05
20:4n-3	0.64	0.10	0.62	0.02
20:5n-3	1.99	0.37	2.05	0.17
22:1n-11	1.08	0.23	0.82	0.14
22:1n-9	0.84	0.07	0.86	0.04
22:4n-6	0.14	0.03	0.13	0.01
22:5n-6	0.16	0.05	0.16	0.01
22:5n-3	1.75	0.73	1.63	0.01
22:6n-3	10.56	4.02	10.63	0.25
∑SFA	12.47	1.19	12.48	0.36
∑MUFA	43.92	2.07	42.00	0.46
∑PUFA	43.60	3.18	45.52	0.61
∑n-3 FA	21.09	4.75	21.55	0.49
∑n-6 FA	20.36	1.44	21.98	0.20
18:4n-3/18:3n-3	0.13	0.01	0.13	0.01
18:3n-6/18:2n-6	0.08	0.01	0.08	0.00
20:3n-6/20:2n-6	0.66	0.07	0.65	0.06
18:2n-9/18:1n-9	0.02	0.01	0.02	0.00
16:1n-7/16:0	0.20	0.02	0.19	0.02
18:1n-9/18:0	8.17	0.33	8.02	0.59
20:4n-6/20:3n-6	0.96	0.14	1.12	0.03
20:5n-3/20:4n-3	3.12	0.19	3.29	0.20
22:6n-3/20:5n-3	5.20	1.09	5.22	0.50

* Denotes significant differences (p <0.05) between the two fish groups.

Table 7.7 Fatty acid composition of muscle of gilthead seabream juveniles obtained from broodstock fed FO or RO diet during spawning season, after 74 days of feeding the challenge diet

	BFO		BRO	
Fatty acid (%)	Mean	S.D.	Mean	S.D.
14:0	0.50	0.12	0.37	0.19
14:1n-7	0.01	0.01	0.06	0.06
14:1n-5	0.02	0.00	0.08	0.09
15:0	0.08	0.02	0.15	0.14
15:1n-5	0.01	0.01	0.06	0.07
16:0ISO	0.01	0.01	0.07	0.09
16:0	7.17	1.48	7.23	1.60
16:1n-7	1.78*	0.21	1.16	0.26
16:1n-5	0.04	0.01	0.09	0.09
16:2n-4	0.10	0.01	0.10	0.06
17:0	0.09	0.02	0.09	0.03
16:3n-4	0.13	0.01	0.12	0.04
16:3n-3	0.04	0.01	0.13	0.13
16:3n-1	0.09	0.06	0.26	0.10
16:4n-3	0.09	0.01	0.17	0.10
16:4n-1	0.02	0.01	0.07	0.06
18:0	3.31	0.83	4.06	0.48
18:1n-9	31.30*	3.44	20.32	3.68
18:1n-7	2.55*	0.08	2.10	0.25
18:1n-5	0.10	0.01	0.16	0.14
18:2n-9	0.28	0.01	0.18	0.06
18:2n-6	15.55*	0.85	12.30	1.46
18:2n-4	0.08	0.01	0.15	0.12
18:3n-6	0.46	0.00	0.43	0.12
18:3n-4	0.13	0.01	0.15	0.11
18:3n-3	5.15*	0.70	3.35	0.53
18:3n-1	0.01	0.01	0.08	0.08
18:4n-3	0.61*	0.08	0.44	0.06

	BFO		BRO	
Fatty acid (%)	Mean	S.D.	Mean	S.D.
18:4n-1	0.06	0.01	0.09	0.05
20:0	0.34	0.07	0.35	0.08
20:1n-9	0.49	0.12	0.33	0.06
20:1n-7	3.02	0.59	2.28	0.49
20:1n-5	0.14	0.04	0.19	0.06
20:2n-9	0.50*	0.10	0.31	0.04
20:2n-6	0.80	0.06	0.81	0.06
20:3n-9	0.01	0.01	0.06	0.06
20:3n-6	0.41	0.04	0.50	0.06
20:4n-6	0.54	0.23	1.15*	0.20
20:3n-3	0.43	0.05	0.39	0.00
20:4n-3	0.76	0.11	0.69	0.04
20:5n-3	3.72	0.90	5.51	0.67
22:1n-11	2.61	0.93	1.74	0.59
22:1n-9	1.00	0.33	0.85	0.01
22:4n-6	0.23	0.07	0.31	0.10
22:5n-6	0.28	0.09	0.65*	0.16
22:5n-3	2.49	0.41	3.51	0.55
22:6n-3	12.47	3.72	26.34*	5.40
∑SFA	11.49	2.27	12.33	2.49
∑MUFA	43.07*	4.88	29.41	4.79
∑PUFA	45.44	3.71	58.27*	5.01
∑n-3 FA	25.76	4.17	40.54*	5.85
∑n-6 FA	18.28*	0.56	16.15	0.97
18:4n-3/18:3n-3	0.12	0.01	0.13	0.02
18:3n-6/18:2n-6	0.03	0.00	0.04	0.01
20:3n-6/20:2n-6	0.52	0.06	0.62	0.06
16:1n-7/16:0	0.25*	0.05	0.16	0.03
18:1n-9/18:0	9.96	3.11	5.09	1.34
20:4n-6/20:3n-6	1.31	0.45	2.29*	0.27
20:5n-3/20:4n-3	4.95	1.42	7.98*	1.14
22:6n-3/20:5n-3	3.32	0.19	4.76*	0.58

* Denotes significant differences (p <0.05) between the two fish groups.

Table 7.8 Fatty acid composition of whole body of gilthead seabream juveniles obtained from from broodstock fed FO or RO diet during spawning season, after 74 days of feeding the challenge diet

	BFO		BRO	
Fatty acid(%)	Mean	S.D.	Mean	S.D.
14:0	0.56	0.25	0.49	0.17
14:1n-7	0.02	0.02	0.10	0.12
14:1n-5	0.03	0.02	0.14	0.17
15:0	0.09	0.02	0.14	0.07
15:1n-5	0.03	0.02	0.10	0.11
16:0ISO	0.03	0.02	0.16	0.21
16:0	7.12	0.78	6.08	2.70
16:1n-7	2.18	0.33	1.82	1.06
16:1n-5	0.04	0.01	0.19	0.21
16:2n-4	0.13	0.01	0.23	0.17
17:0	0.12	0.02	0.21	0.18
16:3n-4	0.18	0.03	0.26	0.15
16:3n-3	0.08	0.00	0.22	0.23
16:3n-1	0.09	0.02	0.26	0.27
16:4n-3	0.14	0.02	0.26	0.17
18:0	2.89	0.11	2.36	0.41
18:1n-9	34.21	4.74	27.35	7.16
18:1n-7	2.79	0.28	2.42	0.45
18:1n-5	0.12	0.01	0.17	0.18
18:2n-9	0.30	0.04	0.27	0.08
18:2n-6	16.10	2.61	13.03	3.27
18:2n-4	0.11	0.01	0.18	0.10
18:3n-6	0.47	0.05	0.47	0.07
18:3n-4	0.16	0.03	0.21	0.08
18:3n-3	5.43	0.89	4.54	0.81
18:3n-1	0.03	0.02	0.16	0.21
18:4n-3	0.71	0.05	0.69	0.08
18:4n-1	0.09	0.03	0.21	0.16
20:0	0.33	0.09	0.46	0.18
20:1n-9	0.48	0.12	0.54	0.10
20:1n-7	2.98	0.58	3.37	0.64
20:1n-5	0.18	0.03	0.25	0.09
20:2n-9	0.46	0.07	0.59	0.25
20:2n-6	0.72	0.15	0.92	0.27
20:3n-9	0.04	0.02	0.16	0.15
20:3n-6	0.32	0.04	0.45	0.15
20:4n-6	0.37	0.04	0.59	0.27
20:3n-3	0.40	0.11	0.50	0.16
20:4n-3	0.69	0.12	0.96	0.30
20:5n-3	2.85	0.63	3.69	0.73
22:1n-11	2.59	1.22	3.92	1.60
22:1n-9	0.94	0.42	1.39	0.60
22:4n-6	0.25	0.18	0.48	0.37
22:5n-6	0.25	0.14	0.52	0.39
22:5n-3	2.24	1.09	3.59	1.55
22:6n-3	9.66	4.31	14.93	6.32
∑SFA	11.11	0.57	9.75	3.06
∑MUFA	46.60	2.89	41.75	5.30
∑PUFA	42.26	3.42	48.34	7.73
∑n-3 FA	22.21	5.36	29.38	8.46
∑n-6 FA	18.47	2.10	16.46	1.86
18:4n-3/18:3n-3	0.13	0.02	0.16	0.03
18:3n-6/18:2n-6	0.03	0.00	0.04	0.02
20:3n-6/20:2n-6	0.45	0.05	0.49	0.06
16:1n-7/16:0	0.31	0.02	0.29	0.04
18:1n-9/18:0	11.82	1.23	11.48	1.10
20:4n-6/20:3n-6	1.14	0.07	1.28	0.14
20:5n-3/20:4n-3	4.13	0.23	3.95	0.52
22:6n-3/20:5n-3	3.29	0.71	3.91	1.07

* Denotes significant differences (p <0.05) between the two fish groups.

7.3.4 Gene expression

No significantly differences were found in the expression of the selected genes between the experimental fish groups (Table 7.9).

Table 7.9 Hepatic gene expression of gilthead seabream juveniles obtained from from broodstock fed FO or RO diet during spawning season, after 74 days of feeding the challenge diet

	BFO		BRO	
copies/µL	Mean	S.D.	Mean	S.D.
fads2	104.37	33.07	59.80	17.33
cox-2	4.37	4.46	5.60	5.62
g6p	84.14	43.50	87.75	39.87
srebp	39.29	6.10	40.23	16.35
lpl	139.31	35.14	173.27	52.27
cpt-1ß	14.40	4.01	21.61	8.78
elovl6	70.32	37.06	47.13	11.08
ppara	41.86	12.00	47.31	5.61
igf1	276.44	120.18	324.50	111.94

7.3.5 fads2 promoter sequence alignment

No difference was observed in the alignment of the *fads2* promoter region sequence studied between broodstock fed with different nutritional programming diet (Figure 7.1).

Figure 7.1 Alignment of fads2 promoter region sequence of broodstock fed with diet rich in FO or RO.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50         60         70         80         90        100        110
Fads2*          ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
FO Female 1     ACTCTCATTT CCTAAAGTTT ATTCTGATGS AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
FO Male 1-1     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
FO Male 1-2     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
FO Female 2     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
FO Male 2-1     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Female 1     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Male 1-1     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Male 1-2     ACTCTCATTT CCTAAAGTTT ATTCKGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Female 2     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Male 2-1     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Male 2-2     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Female 3     ---------- ---------- ---CTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Male 3-1     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
RO Male 3-2     ACTCTCATTT CCTAAAGTTT ATTCTGATGC AGATTTTGAA TGCAACACAA CAGACCACTG TGGGATTTGC TATTTTTACT CGAGCAGCCT ACTTCTTCCA CCGCTGTGAG
ClustalW                              * ****  ********** ********** ********** ********** ********** ********** ********** **********
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       120        130        140        150        160        170        180        190        200        210        220
Fads2*          GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
FO Female 1     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
FO Male 1-1     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
FO Male 1-2     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
FO Female 2     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
FO Male 2-1     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Female 1     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Male 1-1     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Male 1-2     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Female 2     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Male 2-1     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Male 2-2     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Female 3     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Male 3-1     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
RO Male 3-2     GCTGTAATTG AGGGAAAGTG TAGAAGCGAC GTGCTCCCGC AGACTGTTTC CGTCTGAGCG CACAGGGCTC TGTGTAATAT TCAGATGAGC CGTTTATTGA GCCTATTGCA
ClustalW        ********** ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       230        240        250        260        270        280        290        300        310        320        330
Fads2*          CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
FO Female 1     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
FO Male 1-1     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
FO Male 1-2     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
FO Female 2     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
FO Male 2-1     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Female 1     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Male 1-1     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Male 1-2     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Female 2     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Male 2-1     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Male 2-2     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Female 3     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Male 3-1     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
RO Male 3-2     CATCAGCCGG TGGTCCAGGA TATACTGTAC GCCGATTGGC CCAGAAACCC TCGAATGATC GGCTCGGAAT TTGTACTGAA TGAGTGGGTG AATGAGTCCG TGAACACACT
ClustalW        ********** ********** ********** ********** ********** ********** ********** ********** ********** ********** **********


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       340        350        360        370        380        390        400        410        420        430        440
Fads2*          GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
```

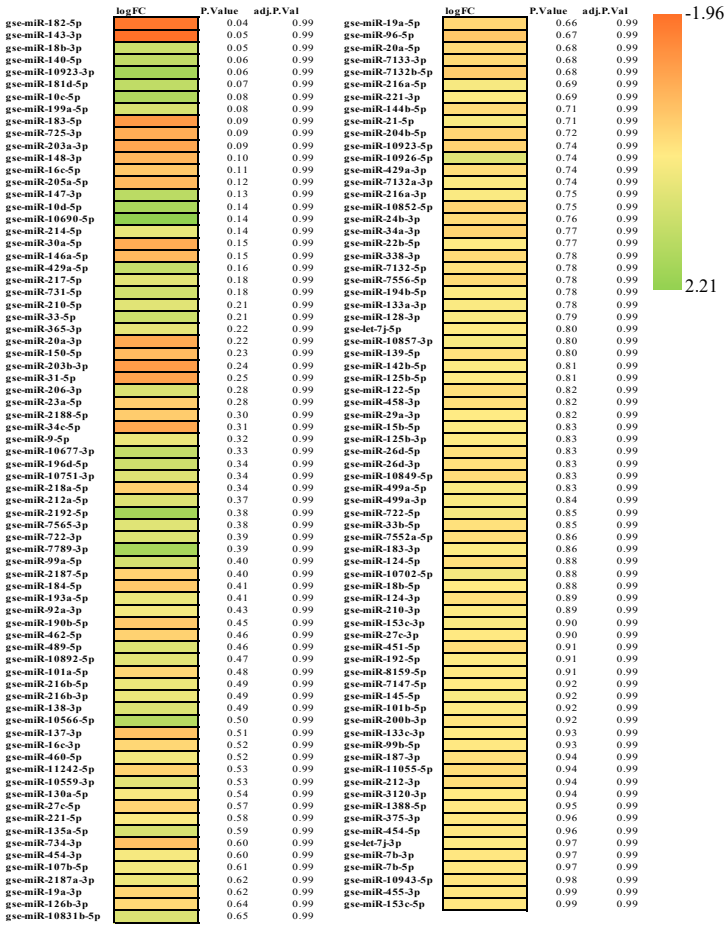
```
FO Female 1   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
FO Male 1-1   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
FO Male 1-2   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
FO Female 2   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
FO Male 2-1   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Female 1   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Male 1-1   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Male 1-2   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Female 2   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Male 2-1   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Male 2-2   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Female 3   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Male 3-1   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
RO Male 3-2   GGGGGCAGGA CAGAGGATGA ATTTTGGGGA TGTGCACGAT TTAAAATGGA CAGTGAACGC ATCGGGGGCG CATAAAAGTG AGGCGGTGTG TTAATGTGAG TGAAAGAAAA
ClustalW      ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
              ....|....| ..
                      450
Fads2*        ACAACCCGAT CA
FO Female 1   ACAACCCGAT CA
FO Male 1-1   ACAACCCGAT CA
FO Male 1-2   ACAACCCGAT CA
FO Female 2   ACAACCCGAT CA
FO Male 2-1   ACAACCCGAT CA
RO Female 1   ACAACCCGAT CA
RO Male 1-1   ACAACCCGAT CA
RO Male 1-2   ACAACCCGAT CA
RO Female 2   ACAACCCGAT CA
RO Male 2-1   ACAACCCGAT CA
RO Male 2-2   ACAACCCGAT CA
RO Female 3   ACAACCCGA- --
RO Male 3-1   ACAACCCGAT CA
RO Male 3-2   ACAACCCGAT CA
ClustalW      *********
```

* *fads2* promoter region of gilthead sea bream (Perera *et al.*, 2019).

7.3.6 MicroRNA libraries analysis

No significant difference was observed between BFO fish and RO fish on microRNA expression (adjusted p value>0.05) (Figure 7.2).

Figure 7.2 Heat map of the comparison of hepatic microRNA expression between one-year-old juveniles from broodstock fed with diet rich in FO or in RO.



7.4 Discussion

The present study was designed to determine the effect of nutritional programming by replacing dietary FO by RO during spawning season of broodstock on growth performance and lipid biosynthesis of one-year-old offspring when challenged with diet contain low FM and FO. The results showed nutritional programming by feeding broodstock with FO replacement diet by RO during spawning season led to significant differences in lipid metabolism in the offspring. According to the previous study on gilthead seabream, nutritional programming by feeding broodstock with diet contained 8.0% linseed oil promotes the growth performance of offspring when challenged with low FM and low FO diet (Izquierdo *et al.*, 2015). Similarly, nutritional programming by RO also lead to an increased growth performance when the offspring is challenged between 2 to 10 g (Hanlin *et al*. in press, Chapter 5). However, such promoting effect could not be found when the offspring reached around 16-month-old (Turkmen *et al.*, 2017a). In present study, even though offspring from broodstock programmed with diet contained RO had higher final weight, the growth rate of broodstock fed with FO-based diet was higher, which seems to be related to their lower body weight. In the research on other species, the effects of nutritional programming, especially long-term effects, remain uncertain. For example, a positive effect of three weeks nutritional programming by feeding vegetable-based diet since first exogenous feeding leads to a 24% higher growth rate in the challenge experiment of Atlantic salmon 15 weeks after the nutritional programming (Clarkson *et al.*, 2017). Similar phenomenon also exists in rainbow trout in the nutritional challenge test when the fish is around 8 months old (Balasubramanian *et al.*, 2016). However, such nutritional programming effect on growth performance was not observed in European sea bass and Siberian sturgeon in certain studies (Vagner *et al.*, 2009; Luo *et al.*, 2019). Thus, further long-term investigation is required to investigate the effect of nutritional programming on growth.

Although effect on growth performance is not confirmed in present study, nutritional programming led to a difference in the structure of liver. Under the premise that no difference was observed on the size of the nucleus of hepatocyte between two experimental group, offspring of broodstock programmed by RO contained diet had lower HSI, hepatic lipid content and smaller size of hepatocyte. These results denote a significant nutritional programming by RO in broodstock diet during spawning season on the hepatic lipid deposition. Although

nutritional programming by different fatty acid is proved to regulate the hepatic lipid metabolism in fish (Vagner *et al.*, 2007; Vagner *et al.*, 2009; Geurden *et al.*, 2013; Izquierdo *et al.*, 2015; Clarkson *et al.*, 2017), little information is available in the change of hepatic lipid deposition. A possible explanation for this might be that nutritional programming by RO on broodstock promotes the use of low FM and low FO diet of their offspring by up-regulating lipid metabolism. Nutritional programming during early larval stage of Atlantic salmon by vegetable ingredients up-regulates the expression of genes that participate in the pathways related with intermediary metabolism such as oxidative phosphorylation, TCA cycle and fatty acid metabolism (Vera *et al.*, 2017). However, the present study was unable to demonstrate the metabolism of nutritional programming on hepatic fatty acid metabolism since no difference on expression of genes related with lipid metabolism was observed between two experimental group, which might due to the large variation within the group. Besides, no difference was observed in the sequence of promoter region of *fads2* gene of broodstock nor in the hepatic microRNA expression. Further study is needed for the verification of specific microRNA expression by RT-PCR.

Another important finding was on the fatty acid composition in muscle. Muscle of the offspring from broodstock programmed by RO contained diet had around two times higher content of LC-PUFA, including arachidonic acid, 22:5n-6 and DHA, than that from broodstock fed with FO diet during spawning season. These results show a possibility on using nutritional programming as a tool to produce fish that can accumulate higher LC-PUFA in muscle when fish are fed with diet contains high level of vegetable ingredients. Besides, the higher ratio of 20:4n-6/20:3n-6 and 20:5n-3/20:4n-3 in the muscle of offspring from RO programmed fish showed a higher activity of Δ5 desaturase, which is a restricted enzyme in the LC-PUFA biosynthesis of gilthead seabream (Tocher and Ghioni, 1999). Whereas the muscle of offspring from broodstock fed with FO diet contained higher amounts of precursors of LC-PUFA such as 18:1n-9, LNA and ALA. These results are partly be related to a lower ability of LC-PUFA biosynthesis. In mammal, Δ5 desaturase is translated from *Fads1* (Cho *et al.*, 1999). Whereas in zebrafish (*Danio rerio*), protein translated from gene similar to mammalian *Fads2* has the function of Δ5 desaturation (Hastings *et al.*, 2001). Although it is still not clear due to the

extremely low Δ5 desaturation, the gilthead sea bream desaturase clone shows residual Δ5 activity (Mourente and Tocher, 1993; Mourente and Tocher, 1994; Tocher and Ghioni, 1999). Gene expression of *fads2* also exists in muscle of gilthead seabream (Seiliez *et al.*, 2003), which suggests that the increased LC-PUFA content in muscle of offspring from RO programmed broodstock may be related with the higher Δ5 activity. However, although parental dietary fatty acid can affect the Δ5 expression (Missotten *et al.*, 2009), specific mechanism of nutritional programming on muscle remains unknown in fish. Thus, the effect of nutritional programming on muscle fatty acid deposition is also important issue for future research.

In conclusion, this study has shown a long-term existence of nutritional programming effect by feeding broodstock with RO contained diet during spawning season on their offspring when challenged with diet low in FM and FO. Offspring from RO programmed broodstock had lower HSI, hepatic lipid content and smaller size of hepatocyte. Besides, a higher content and a higher biosynthesis activity of LC-PUFA was observed in muscle of the offspring from RO programmed fish, compared with offspring from broodstock fed with FO contained diet. However, the effect of nutritional programming on the growth performance remained uncertain. Further research needs to examine the mechanism on the LC-PUFA accumulation in muscle which may contribute to the production of better-quality fish products along with decrease use of marine ingredients in diet.

Acknowledgement

This study was supported by the EU funded project PerformFISH (H2020-SFS-2016-2017; 727610) and China scholarship council. The authors acknowledge Skretting ARC (Stavanger, Norway) for providing the experimental diets.

Chapter 8

Conclusions

1. When challenged with a diet low in fish meal (FM) and fish oil (FO), an increased growth performance was observed in the offspring juveniles from broodstock with higher *fads2* expression and fed with a diet rich in rapeseed oil (RO) during the spawning season (Chapter 5). This improved growth performance in offspring from broodstock with a high *fads2* expression was persistent in a long-term challenge trial, even until fish overpassed 100 g and was in the verge of male and gonad maturation (Chapter 6). However, the replacement of 50% dietary FM alone or combined with 70% FO in broodstock diets during spawning season led to a decrease on growth performance of offspring juveniles (Chapter 3).
2. When challenged with a diet low in FM and FO in early life stages, offspring from broodstock nutritionally programmed by 80% replacement of dietary FO by vegetable oil during spawning season had increased LC-PUFA content in fatty acid profiles (Chapter 3, 5), reduced viscerosomatic index (Chapter 5) and hepatic lipid accumulation, increased expression of *elovl6* in liver and *ghr-1/ghr-2* in muscle (Chapter 5) than offspring from broodstock fed with a diet high in FO.
3. In Chapter 7, a long-term effect of nutritional programming was observed even when fish reached 100 g, in the verge of male gonadal maturation. Thus, in the muscle of the offspring from RO programmed broodstock, the content and biosynthesis activity of LC-PUFA was higher, compared with offspring from broodstock fed with FO contained diet.
4. An up-regulation the *fads2* expression, as well as the PUFA content in liver and muscle was observed in juvenile gilthead seabream from broodstock with inherently high *fads2* expression, when challenged by a diet low in FM and FO in early life stages. However, in the long-term challenge test, a down-regulation of *fads2* was observed in offspring from broodstock with higher *fads2* expression, together with a high incorporation of DHA, particularly in the liver. This higher DHA, together with the increase in 22:5n-3 and 22:4n-

6, late products of elongation and desaturation, would be responsible for the down-regulation of hepatic *fads2*, possibly mediated by the downregulation in *srebp*.

5. The results on the changes of *fads2* during ontogeny showed the presence of *fads2* mRNA in the just spawned gilthead seabream egg, denoting the maternal mRNA transfer to the developing oocyte, whereas from the neurula stage onwards *fads2* expression increased denoting the transition from maternal to embryonic gene expression. Besides, eggs obtained from broodstock with high *fads2* expression showed a high DHA content, which could be responsible for a down-regulation of *fads2* expression in the developing embryo and larvae. Finally, partial replacement of FO by RO did not affect LC-PUFA contents nor *fads2* expression in gilthead seabream eggs (Chapter 4).
6. The changes led by broodstock nutritional programming or *fads2* expression were not due to the sequence of *fads2* promoter region.

Overall, nutritional programming on broodstock by the replacement of FO by vegetable oil, especially RO, increased the ability LC-PUFA biosynthesis in offspring when challenged with diets containing low FM and FO. Such effect was observed in both early life stages and long-term nutritional challenge tests. Besides, the thesis firstly provides the evidence on the trans-generation effect of broodstock *fads2* expression on changes during ontogeny and use of diet low in FM and FO on offspring. Thus, strategies of combining broodstock with higher *fads2* expression and nutritional programming by vegetable oil can be applied to increase the use of low FM and FO diets in the offspring. However, further studies are needed for the mechanism in molecular level since no differences was observed in gene sequence of broodstock, offspring *fads2* CpG level and miRNA expression.

Chapter 9

Resumen ampliado en español

9.1 La sustitución de la harina y el aceite de pescado como principal desafío para el desarrollo de la acuicultura intensiva

El alimento para peces es una fuente equilibrada de minerales y proteínas altamente digeribles para el ser humano, y representó el 17% de la ingesta de proteína animal de la población mundial (FAO, 2020). Además, es la principal fuente de ácidos grasos poliinsaturados de cadena larga n-3 (n-3 LC-PUFA) que son importantes para el desarrollo fetal (Helland *et al.*, 2003) y contribuyen a prevenir las enfermedades cardiovasculares (Schmidt *et al.*, 2000) y la enfermedad de hígado graso no alcohólico (NAFLD) (Oya *et al.*, 2010), entre muchas otras propiedades para la salud humana. Por lo tanto, la demanda de pescado por parte de los consumidores aumenta continuamente. De 1961 a 2017, la tasa de crecimiento anual del consumo de pescado (3,1%) fue casi el doble de la tasa de crecimiento de la población mundial (1,6%) y superior a la de todos los demás alimentos de proteína animal (2,1%). En 2018, la producción mundial de pescado alcanzó los 178,5 millones de toneladas, de las cuales el 45,8% procedía de la acuicultura (FAO, 2020). La pesca de captura mundial se mantiene relativamente estable desde finales de los años ochenta, con pequeñas fluctuaciones entre los distintos años y, según el seguimiento a largo plazo de la población de peces marinos a la que se accede, los recursos pesqueros disminuyen continuamente (FAO, 2020). Debido a este estancamiento de la producción pesquera, la acuicultura está asumiendo la responsabilidad de proporcionar suficientes productos inocuos y sostenibles para satisfacer las demandas del mercado. En 2018, la acuicultura produjo el 52% del pescado utilizado para el consumo humano y, de 2001 a 2018, la tasa media de crecimiento anual de la producción acuícola mundial fue del 5,3% anual (FAO, 2020). Sin embargo, el desarrollo ulterior de la acuicultura se ve restringido por la limitada disponibilidad y el aumento de los precios de la harina y el aceite de pescado, fuentes tradicionales de proteínas y lípidos en los piensos para peces, que en su mayoría se derivan de la pesca de captura. En 2015, la producción mundial de harina de pescado y aceite de pescado fue de 4,7 millones y 856.000 toneladas, respectivamente, de las cuales la acuicultura consumió

el 70% de la producción de harina de pescado y el 73% de aceite de pescado (Green, 2018). Sin embargo, la producción de FM y FO está disminuyendo gradualmente. Por ejemplo, la producción de FM fue de 2 millones de toneladas menos en 2015 en comparación con la de 1997. Aparte de la disminución de la oferta, el uso de la FM y la FO en los alimentos para peces suscita la preocupación por la contribución de la acuicultura a la sobreexplotación de los recursos pesqueros y la presencia de contaminantes orgánicos persistentes o metales pesados acumulados en la FM o la FO (Council, 2011). Por lo tanto, es necesario sustituir la FM y la FO en la dieta por otros ingredientes de alta calidad y nutritivos con una producción más económica, ambiental y socialmente sostenible.

Los subproductos vegetales y animales, los ingredientes unicelulares o las fuentes de proteínas de las harinas de los insectos se utilizan a menudo para reemplazar la FM de las dietas para peces (Caballero *et al.*, 2002; Wang *et al.*, 2016; Rosales *et al.*, 2017; Rimoldi *et al.*, 2018). Las fuentes de proteínas vegetales tienen la ventaja de tener una producción grande, estable y sostenible, y se utilizan ampliamente para sustituir la FM dietética en los alimentos acuícolas. Por ejemplo, en la dieta de los alevines de trucha arco iris (*Oncorhynchus mykiss*), hasta el 66% de la FM puede sustituirse por fuentes de proteínas vegetales (Gomes *et al.*, 1995). En la dieta del salmón del Atlántico (*Salmo salar* L.), se puede añadir hasta un 27% de concentrado de proteína de guisante o un 22% de concentrado de proteína de altramuz (Carter and Hauler, 2000). En la dorada (*Sparus aurata*), una mezcla de guisantes extruidos y harina de colza puede sustituir hasta el 50% de la FM sin afectar la tasa de crecimiento (Sitjà-Bobadilla *et al.*, 2005). Sin embargo, a niveles altos de sustitución, la composición desequilibrada de aminoácidos y los factores anti-nutricionales presentes en las fuentes de proteínas vegetales pueden perjudicar el crecimiento y la utilización digestiva del alimento, y afectar negativamente al sistema inmunitario de los peces (Vergara *et al.*, 1996b; Vergara *et al.*, 1996a; Francis *et al.*, 2001; Caballero *et al.*, 2004; Gómez-Requeni *et al.*, 2004; Castro *et al.*, 2015). Además, el contenido de fosfolípidos en las fuentes de proteínas vegetales es menor que en la FM (Council, 2011). Los altos niveles de sustitución de la FM por proteínas vegetales también producen efectos negativos en el valor nutritivo del filete, incluida la disminución de los AGP-CL-3 (De

Francesco *et al.*, 2007). Esta escasez restringe el uso de fuentes de proteínas vegetales en los alimentos para peces.

En comparación con las fuentes de proteínas vegetales, los subproductos animales pueden contener una composición de aminoácidos más cercana a la FM y otros nutrientes interesantes como minerales y fosfolípidos. Sin embargo, su composición nutricional difiere entre los ingredientes y la política basada en la preocupación por la seguridad (Council, 2011), restringiendo su uso en los alimentos para peces. Dependiendo de la fuente y el valor nutricional, entre el 20 y el 40% de la FM puede ser reemplazada por subproductos animales (Oliva-Teles *et al.*, 2015). Los ingredientes unicelulares como la levadura de cerveza se han utilizado en la acuicultura desde la década de 1990, con la ventaja de su alto contenido en proteínas, su alto nivel de nucleótidos y la ausencia de anti-nutrientes (Oliva-Teles *et al.*, 2015). Mientras tanto, pueden ser utilizados como prebióticos para estimular el crecimiento de las bacterias en el tracto intestinal (Ferreira *et al.*, 2010). Sin embargo, al igual que los subproductos animales, la calidad de los ingredientes de las células individuales varía entre los diferentes fabricantes. La harina de insectos, especialmente las larvas de la mosca solitaria negra, está recibiendo un creciente interés en los últimos años (Kroeckel *et al.*, 2012), debido a la capacidad de convertir los residuos de alimentos en proteína de alta calidad (Newton *et al.*, 1977), pero su uso está restringido por una producción limitada.

En el caso de ciertas especies de agua dulce, algunos aceites vegetales pueden utilizarse como única fuente de lípidos en la dieta. Sin embargo, para las especies marinas, los ácidos grasos poliinsaturados de cadena larga (LC-PUFAs), que incluyen principalmente DHA, ácido eicosapentaenoico (20:5n-3, EPA) y ácido araquidónico (20:4n-6, ARA), son esenciales para el crecimiento y el FO es la principal fuente comercialmente competitiva de LC-PUFAs. El aceite de semillas oleaginosas o la grasa animal se utiliza en la sustitución del FO dietético, pero el insuficiente contenido de LC-PUFAs en estos ingredientes restringe el nivel de sustitución, ya que la sustitución muy alta del FO conduce a una disminución del rendimiento de crecimiento, un reducido valor nutritivo del filete, un metabolismo anormal de los lípidos del hígado, un sistema inmunológico deteriorado y un rendimiento de reproducción debilitado (Tocher *et al.*, 2002; Menoyo *et al.*, 2004; Izquierdo *et al.*, 2005; Lin and Shiau, 2007; Sink *et*

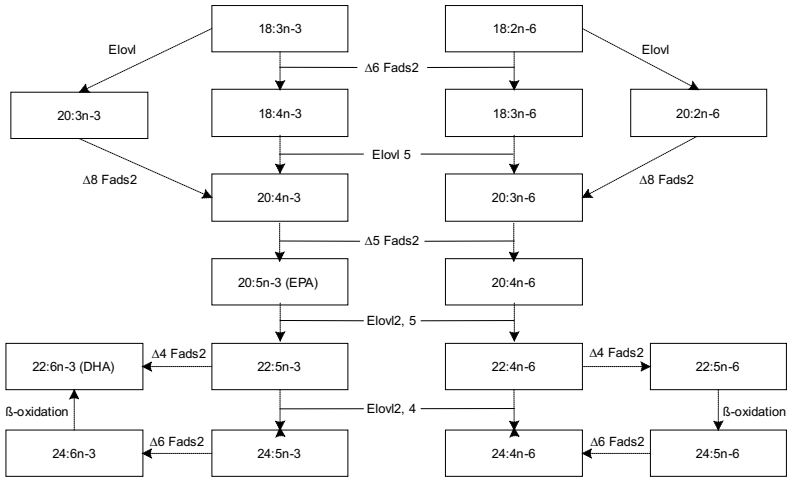
al., 2010). En comparación con los otros dos tipos de fuentes de lípidos, el aceite de microalgas puede contener abundante LC-PUFA, especialmente ácido docosahexaenoico (22:6n-3, DHA) (Ryckebosch *et al.*, 2012). Mientras tanto, durante el proceso de la producción de petróleo, toneladas de dióxido de carbono en la atmósfera son fijadas por las microalgas, lo que lo convierte en un proceso respetuoso con el medio ambiente (Doughman *et al.*, 2007). Sin embargo, el uso del aceite de microalgas está restringido por su alto precio en comparación con el FO (Oliva-Teles *et al.*, 2015).

9.2 Importancia de la LC-PUFA para la producción pesquera

Los LC-PUFAs tienen muchas funciones estructurales y funcionales (Watanabe, 1993; Calder, 2012). Por ejemplo, son componentes críticos de las membranas celulares y orgánicas (Wassall and Stillwell, 2008) y sus derivados, como los eicosanoides y los docosanoides, participan en el procedimiento de señalización celular (Gill and Valivety, 1997). En el pescado, los LC-PUFAs son ácidos grasos esenciales que son obligatorios para el crecimiento (Peng *et al.*, 2014a), desarrollo y mantenimiento del cerebro y del sistema inmunológico (Menoyo *et al.*, 2004; Torrecillas *et al.*, 2017b).

Al igual que los mamíferos, la capacidad de sintetizar el LC-PUFA *de novo* en los peces está ausente porque carecen de desaturasas n-12 y n-15 que sólo están presentes en algunas plantas o invertebrados. Por lo tanto, es necesario incluir en pienso para peces o bien los LC-PUFAs o sus precursores 18C. La biosíntesis de los LC-PUFAs a partir de los ácidos grasos de 18C es catalizada por un conjunto de desaturasas y elongasas (Figura 1). El ARA se sintetiza a partir del ácido linoleico (LNA), mientras que el EPA y el DHA se obtienen a partir del ácido α-linolénico (ALA). En comparación con los peces de agua dulce o los peces eurihalinos, los peces marinos tienen una menor capacidad de sintetizar LC-PUFA a partir de sus precursores 18C (Dong *et al.*, 2017). La razón podría estar relacionada en parte con el medio ambiente, ya que el alimento para peces marinos contiene suficiente LC-PUFA, originalmente producido por el fitoplancton, y por ellos los peces marinos no necesitan las enzimas para producirlas (Sargent *et al.*, 2003).

Figura 9.1 Ruta de biosíntesis de >C18 PUFA (ácidos grasos poliinsaturados) (modificado según Oboh et al. (2017) y Bond et al. (2016))



Tradicionalmente, el FO es la principal fuente de LC-PUFAs en los en los piensos para animales acuáticos. Otras fuentes de lípidos vegetales utilizadas en la sustitución del FO dietético carecen de LC-PUFA pero son abundantes en los precursores, los PUFAs 18C. Por lo tanto, el nivel de reemplazo de FO para cada especie de peces depende en gran medida de su capacidad de biosíntesis de LC-PUFA. En general, el reemplazo de FO por fuentes alternativas de lípidos vegetales es mayor en las dietas para peces de agua dulce que en las dietas para especies marinas. Por ejemplo, el contenido de FO es de 0-2% en la dieta de las carpas chinas, en contraste con el 1-15% de FO en las dietas de las especies de peces marinos (Tacon and Metian, 2008). Sin embargo, una mayor proporción de FO podría ser reemplazada si se aumenta la capacidad de los peces para sintetizar LC-PUFA.

Diferentes estrategias pueden mejorar la capacidad de los peces para sintetizar el LC-PUFA, como la regulación de ciertos factores ambientales. Por ejemplo, la reducción de la salinidad puede aumentar la capacidad de los peces para sintetizar DHA a partir de ALA e incrementar la expresión del gen de la *acido graso desaturasa 2* (*fads2*) en la grasa hepática, como ocurre en el pez con manchas blancas (*Siganus canaliculatus*) (Li *et al.*, 2008) o en puye (Dantagnan *et al.*, 2010). La temperatura puede regular la capacidad de biosíntesis de n-3 LC-PUFA, como ocurre en el *Misgurnus anguillicaudatus* donde los peces criados a 4 °C contenían una mayor relación EPA/ALA con el aumento de genes relacionados con el metabolismo de los lípidos

como la proteína de unión del *elemento regulador de esteroles 1* (*srebp1)* o la *carnitina palmitoiltransferasa 1* (*cpt1*) en comparación con los peces criados a 28 °C (Chen *et al.*, 2018). La insuficiente capacidad de síntesis de LC-PUFA también está relacionada con la muy limitada actividad de Fads2 (Li *et al.*, 2014) en las especies marinas. Fads2 es una enzima de velocidad limitada en la biosíntesis de LC-PUFA, cuya función es catalizar el ALA o LNA a 18:4n-3 o 18:3n-6, respectivamente, y de 24:4n-6 a 24:5n-6 o de 24:5n-3 a 24:6n-3 (Vagner and Santigosa, 2011). Por ejemplo, la actividad de Fads2 en el bacalao (*Gadus morhua* L) es menor que en el salmón del Atlántico (Tocher *et al.*, 2006). La falta del sitio de unión SP-1 de las *fads2* en el bacalao en comparación con el salmón del Atlántico puede ser una de las razones de la diferencia de actividad (Zheng *et al.*, 2009). Aparte de la diferencia entre especies, *las fads2* también pueden ser reguladas por el factor nutricional. El aumento de la expresión de las *fads2* o los productos de la desaturasa de las Fads2 se encuentra cuando se alimenta a los peces con una dieta de reemplazo de FO en muchas especies (Bell *et al.*, 2002; Seiliez *et al.*, 2003; Xu *et al.*, 2014; Gregory *et al.*, 2016) por lo que es posible utilizar el factor nutricional como una herramienta para producir peces que pueden mejorar la capacidad de síntesis de LC-PUFA.

9.3 Programación nutricional

Una estrategia común empleada por la naturaleza que permite la adaptación del metabolismo del organismo a las condiciones ambientales es la programación nutricional. La programación nutricional se refiere al resultado en un animal que recibe un estímulo nutricional en ciertos períodos críticos de su desarrollo, como las etapas pre o postnatales (Lucas, 1994). Un ejemplo bien conocido es el de las abejas melíferas que pueden convertirse en una reina fértil o en una obrera estéril mediante el consumo o no de jalea real (Kucharski *et al.*, 2008). En los ratones, la malnutrición durante los períodos pre o postnatal conduce a la programación nutricional del metabolismo de los lípidos, lo que provoca una reducción a largo plazo del colesterol plasmático, el colesterol HDL y el triacilglicerol (Lucas *et al.*, 1996). En las madres humanas expuestas a la hambruna, la descendencia desarrolla un deterioro de la utilización de la glucosa, una disminución de la función pulmonar, un aumento del perfil lipídico aterogénico, más obesidad y un aumento triple del riesgo de la enfermedad coronaria (Painter *et al.*, 2005). Más específicamente, el suplemento de n-3 LC-PUFA durante el embarazo y la lactancia mejoró la

puntuación en la prueba K-ABC (Helland *et al.*, 2003). De manera similar, en los peces, el estímulo nutricional durante la reproducción mediante la alimentación de reproductores con diferentes perfiles de ácidos grasos en la dieta, afecta notablemente al metabolismo de los lípidos y al crecimiento de la progenie (Izquierdo *et al.*, 2015). Por ejemplo, la alimentación de los reproductores de dorada (*Sparus aurata*) con dietas que contienen una sustitución parcial de FO por aceite de linaza (LO), bajo en LC-PUFA n-3 pero alto en su precursor de ALA, afetar la expresión de *fads2* y el crecimiento de la progenie (Izquierdo *et al.*, 2015). Además, cuando la progenie de 4 meses fue alimentada con una dieta baja en FM y FO, los peces de los padres alimentados con reemplazo parcial de FO por LO mostraron una mejora en el crecimiento y la utilización del alimento (Izquierdo *et al.*, 2015). De hecho, la alimentación de los reproductores ejerce un efecto a muy largo plazo en la progenie y la sustitución de FO por LO en las dietas parentales, en combinación con la alimentación de los juveniles con dietas de bajo contenido de FM y bajo contenido de FO, mejora notablemente el crecimiento y la utilización de la alimentación de las crías de 16 meses (Turkmen *et al.*, 2017a). Estos estudios demostraron que es posible mejorar la capacidad de los peces marinos para utilizar dietas con bajo contenido de FM y FO mediante programas nutricionales de alimentación de los patres. De manera similar, la exposición a una dieta de base vegetal en los primeros años de vida del salmón del Atlántico mejora el rendimiento del crecimiento y la eficiencia de la alimentación en etapas posteriores de la vida, cuando los peces se alimentan con una dieta baja en FO y FM (Clarkson *et al.*, 2017) . La mejora en el crecimiento causada por la programación nutricional debido a la sustitución de la dieta FM/FO por fuentes alternativas de proteínas o lípidos puede explicarse por la 'hipótesis Barker' de que la malnutrición conduce a la preservación del crecimiento de los tejidos clave y el feto programó su metabolismo para ser ahorrativo (Edwards, 2017). Cuando se enfrenta a una situación de desnutrición, el feto limita el crecimiento o la función de los órganos que no son esenciales para la supervivencia inmediata o adopta un estado de resistencia a la insulina, glucosa crucial u otros suministros de energía limitados para el desarrollo de órganos esenciales para la supervivencia como el corazón y el cerebro (Edwards, 2017). Estos cambios a nivel molecular, celular y tisular aumentan la supervivencia del feto y las etapas posteriores de la vida cuando se somete a una situación de desnutrición. Por ejemplo, los juveniles de lubina

europea (*Dicentrarchus labrax*) programados por una dieta de bajo contenido en n-3 LC-PUFA, alimentados desde la primera alimentación exógena hasta los 45 días de edad, mostraron un mayor contenido de DHA en los lípidos polares y una mayor expresión de las *fads2* cuando se les desafió en el día 151-181 con una dieta carente de n-3 PUFA (Vagner *et al.*, 2007). De manera similar, la programación con aceite vegetal dietético (VO) durante la temporada de desove dio lugar a adaptaciones moleculares en la progenie de las larvas mediante la mejora de la regulación del metabolismo de los *lípidos* y los genes relacionados con la salud, como la cicloxigenasa-2 (*cox2)* y el factor de necrosis tumoral alfa (*tnf-a)*, la lipoproteína lipasa (*lpl*), la carnitina palmitoil transferasa 1 (*cpt1)* y la elongasa 6 (*elovl6*) (Turkmen *et al.*, 2017a).

9.4 Mecanismos epigenéticos

La epigenética son "los cambios transmisibles en los estados "off-on" de los genes mediante la modulación de la cromatina, que no se produce por cambios en la secuencia del ADN" (Allis *et al.*, 2008). Los mecanismos epigenéticos incluyen la metilación del ADN, la modificación de la histona, la remodelación de la cromatina y la ausencia de codificación del ARN.

La metilación del ADN es la modificación que cubre la citosina mediante la adición de un grupo metilo a la 5-metilcitocina en la plantilla de ADN, que es uno de los principales mecanismos epigenéticos asociados con la inhibición de la expresión de los genes (Allis *et al.*, 2008). La metilación del ADN está ampliamente implicada en los efectos de la programación (Heijmans *et al.*, 2008; Kucharski *et al.*, 2008; Hoile *et al.*, 2013; Niculescu *et al.*, 2013). Por ejemplo, la ADN metiltransferasa3 que regula el estado de metilación de la región CpG, está implicada en el efecto de la jalea real en la abeja melífera (Kucharski *et al.*, 2008). La genisteína dietética *en* el *útero* a un nivel de 250 mg/kg potenció el establecimiento temprano de la metilación del ADN en los sitios de la CpG en la región promotora de la *partícula intracisterna A de* A^{vy} (*IAP*), antes del sitio de inicio de la transcripción del gen *Agouti* del ratón $A^{vy,}$ y esta diferencia de nivel de metilación del ADN provocó un cambio en el color del pelaje (Dolinoy *et al.*, 2006). La metilación del ADN también se asocia con el efecto de la programación nutricional en el metabolismo de los lípidos. La metilación de los sitios CpG en la región promotora de las *fads2* de la descendencia adulta está regulada por el nivel de lípidos de la dieta de los padres y se informa de una correlación negativa entre la metilación de un sitio CpG y la expresión de la

fads2 (Hoile *et al.*, 2013). La investigación sobre la relación de la programación nutricional con la metilación del ADN sigue siendo escasa. Se encontró una hipometilación global en la dorada alimentada con harina de soja en la alimentación temprana cuando se eliminó la dieta de harina de soja (Perera and Yufera, 2017).

Además de la metilación del ADN, el microARN (miRNA), que pertenece a un ARN no codificante con una longitud entre 21-23 nt, en combinación con la proteína Argonauta 2, conduce al decaimiento o a la represión transcripcional del ARNm objetivo (Best *et al.*, 2018). El ARNm desempeña importantes funciones en la diferenciación específica de los tejidos (Kloosterman *et al.*, 2007; Laudadio *et al.*, 2012; Latimer *et al.*, 2017) y en la homeostasis de los peces (Mennigen *et al.*, 2014). Los cambios de la expresión génica regulados por la programación nutricional también están asociados a la participación de miARN. En los ratones, cuando los padres son alimentados con una dieta alta en grasas, el 5,7% de los 579 miARN fueron regulados en la descendencia, incluyendo miARN relacionados con el tiempo de desarrollo y la oxidación de lípidos (Zhang *et al.*, 2009). Además, también en los ratones, la restricción de las proteínas maternas provocó un aumento de miR-375 y la regulación a la baja de dos genes diana, el *Pdl-1* y la miotrofina, en el ratón de tres meses de edad, junto con la disminución de la secreción de insulina que provoca una intolerancia a la glucosa (Dumortier *et al.*, 2014). Sin embargo, hasta donde sabemos, la información de la conexión del miARN y la programación nutricional entre generaciones en los peces sigue siendo desconocida.

9.5 La dorada como modelo para los estudios de programación nutricional

La dorada fue elegida para los estudios de programación nutricional en esta tesis. Es una especie eurialina ampliamente distribuida por todo el Mediterráneo, y desde las Islas Británicas hasta Cabo Verde, incluyendo ocasionalmente las Islas Canarias. Mientras tanto, es un alimento muy apreciado en el Mediterráneo y el Atlántico oriental y se cultiva ampliamente en Turquía, Grecia, España e Italia. En España, la dorada es la segunda especie de acuicultura más importante, con una producción de 13.740 toneladas, y ocupó el 21,2% de la producción acuícola total en 2016. Ese mismo año, la producción en Europa y en todo el mundo fue de 160.563 y 185.980 toneladas, respectivamente (Fuente: Federación de Productores Europeos de Acuicultura y FAO FishStat).

El uso del pescado como modelo para los estudios de programación nutricional presenta varias ventajas en comparación con los mamíferos, entre ellas:

(1) *Su alta fecundidad que facilita la obtención de* un gran tamaño de muestra con el mismo genotipo.

(2) La embriogénesis y *organogénesis lecitrotrófica* permite que el embrión reciba la nutrición sólo del vitelo, evitando los efectos que en mamíferos traen consigo el aporte de los nutrientes maternos, la hormona y el metabolismo durante la gestación y la lactancia (Hou and Fuiman, 2019).

Específicamente, el uso de la dorada tiene algunas ventajas como:

(1) *Ser un desovador múltiple* permite seguir el efecto de la programación nutricional varias veces durante la temporada de desove.

(2) *Es posible modificar la composición de los ácidos grasos del huevo* a través de los perfiles de los ácidos grasos de la dieta de los reproductores en sólo tres semanas (Fernández-Palacios *et al.*, 1995), mientras que la temporada de desove dura 3 meses. Por lo tanto, la programación nutricional puede ser probada varias veces durante la temporada de desove. En otras especies, como los salmónidos, los períodos de vitelogénesis son más largos y puede llevar meses cambiar los perfiles de ácidos grasos del huevo (Fernández-Palacios *et al.*, 2011).

(3) Existe *información previa que confirma* la eficacia de la programación nutricional y se ha determinado previamente el momento óptimo para esta especie. Estos estudios muestran que la respuesta de las crías alimentadas con dietas bajas en FM y FO puede regularse mediante la programación de los padres durante la temporada de desove (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017a), mientras que la programación durante las primeras etapas larvarias conduce a una baja tasa de supervivencia (Turkmen *et al.*, 2017b).

(4) La *programación nutricional con dietas para reproductores con alto contenido de aceite vegetal (VO)* permite mejorar el crecimiento de las crías de los jóvenes desafiados con una dieta baja en FM y baja en FO (Izquierdo *et al.*, 2015) y este efecto de programación persiste en la progenie (Turkmen *et al.*, 2017a).

Sin embargo, una serie de carencias limitan el uso de la dorada como modelo para los estudios de programación nutricional:

(1) *La amplia variedad genética entre los individuos*, lo que aumenta las dificultades para investigar los mecanismos que trae la programación nutricional (Council, 2011);

(2) *La sensibilidad de la embriogénesis a los factores ambientales* que pueden interferir con la programación nutricional (Hou and Fuiman, 2019).

Por último, a pesar de que existe cierta información básica sobre la programación nutricional de la dorada mediante las dietas de los reproductores, todavía hay cierta falta de conocimientos que es necesario llenar para poder diseñar protocolos prácticos de programación nutricional para esta especie, entre ellos:

(1) Aún se desconoce si el reemplazo de la FM en las dietas de los reproductores permitiría la programación nutricional de la dorada.

(2) Los mecanismos epigenéticos que intervienen en la programación nutricional de la dorada no están suficientemente aclarados.

(3) No hay información clara sobre la influencia de la capacidad de los reproductores para sintetizar LC-PUFA en la progenie obtenida de estos reproductores.

9.6 Objetivos

El uso masivo de la FM y la FO en los animales de alimentación acuática, especialmente en la maricultura, restringe el desarrollo ulterior de una industria acuícola económica y sostenible. La FM y la FO dietéticas deben ser reemplazadas por otros ingredientes. Sin embargo, la fauna y flora silvestres son la principal fuente de proteínas y lípidos de la dieta. La sustitución puede conducir a desequilibrios en los aminoácidos, los ácidos grasos, los minerales y las vitaminas, e introducir los factores antinutricionales, que afectarán negativamente al crecimiento y la salud de los peces. La hipótesis de esta tesis es que la programación nutricional puede ser un instrumento para mejorar el uso de la dieta con baja FM y bajo FO de peces de acuicultura. Sin embargo, estudios anteriores se centraron en el uso de VO para la programación nutricional y el aceite vegetal utilizado contenía aceite de linaza que es fácil de oxidar, por lo que en este estudio, nos propusimos determinar:

(1) el efecto de la sustitución de la FM por harinas vegetales (VM), solas o en combinación

con la sustitución del FO por VO, en las dietas para reproductores de dorada como herramienta nutricional para modificar la expresión de los genes y mejorar la utilización de dietas con bajo contenido de FM y VO en la progenie (Capítulo 3);

(2) el cambio de la expresión de *las fads2* durante la embriogénesis y la determinación del efecto de la expresión de las *fads2 de* los reproductores y la dieta consumida durante la temporada de desove en la expresión de las *fads2 en* los huevos producidos (Capítulo 4).

(3) el efecto combinado de la programación nutricional de los reproductores mediante la sustitución de FO por RO durante la temporada de desove o la expresión de *fads2* de los reproductores en la utilización por parte de los jóvenes de una dieta de bajo contenido en FM y FO (capítulo 5);

(4) el efecto a largo plazo de la programación nutricional de los reproductores mediante la sustitución de FO por RO durante la temporada de desove en el rendimiento de los juveniles de la progenie mediante la prueba de desafío nutricional y la modificación de los genes hepáticos y la expresión de miARN (Capítulo 6)

(5) el efecto a largo plazo de la expresión de *fads2* en los reproductores en el rendimiento de los jóvenes de la progenie en la prueba de desafío nutricional y la modificación de los genes hepáticos y la expresión del miARN (Capítulo 7)

(6) el efecto del estado nutricional en las diferentes etapas de la dorada en el rendimiento en la prueba de desafío nutricional.

9.4 Resúmens de los estudios

9.4.1 Capítulo 3. La intervención nutricional a través de las proteínas y lípidos vegetales en la cría de la dorada (Sparus aurata) afecta a la utilización de las crías en la dieta baja en aceite de pescado.

La intervención nutricional con VO suplementados a los reproductores de peces mejora la capacidad de la progenie para utilizar dietas bajas en FM y FO. Por lo tanto, la intervención con harinas vegetales (VM), sola o en combinación con VO también podría ser una estrategia de programación nutricional útil para obtener progenies que estén mejor preparadas para utilizar dietas bajas en FM y FO. Este estudio tuvo como objetivo determinar el efecto de la sustitución de la FM por la VM, sola o en combinación con la sustitución de FO por VO como herramienta de programación. Diferentes lotes de reproductores de dorada fueron alimentados

con una de estas tres dietas: una control con 35% de FM y 10% de FO, otra con VM en substitución de la FM (FM: 15%) y una tercera con VM y VO en substitución de FM y FO (15% de FM y 2,7% de FO). Cuando la descendencia alcanzó un peso de 3 g fue desafiada con una dieta baja en FM y FO durante 45 días. Se evaluó el rendimiento de desove de los reproductores y el rendimiento de crecimiento de las crías jóvenes. La sustitución de la FM por la VM en las dietas de los reproductores no afectó al rendimiento reproductivo de los peces, pero alteró los perfiles de ácidos grasos de las crías, redujo el SGR y la eficiencia alimentaria de los jóvenes de la progenie y disminuyó la expresión de *fads2* en el hígado. La suplementación combinada de VM y VO en las dietas de los reproductores dio lugar a un rendimiento reproductivo deficiente, reduciendo la fecundidad en las hembras y el contenido del ácido eicosapentaenoico (EPA) y el ácido docosahexanoico (DHA) en los huevos. A nivel molecular, la expresión de las *fads2* y el gen del receptor de la *rampamicina (tor)* en los huevos se elevaron. De manera similar, la expresión del *fads2* se elevó en el hígado de la progenie. La programación nutricional mediante el suplemento de VM en sustitución de la FM en las crías de dorada afectó negativamente al rendimiento del crecimiento de la progenie, mientras que el suplemento de VO en sustitución de la FO aumenta la expresión génica de las enzimas clave para la biosíntesis de los LC-PUFA.

9.4.2 Capítulo 4. Influencia de la dieta y la expresión de fads2 en los reproductores en la expresión temporal de fads2 de la progenie a lo largo de la ontogénesis de la dorada (Sparus aurata)

Estudios anteriores han demostrado que es posible aumentar la capacidad de los peces marinos para producir LC-PUFA a partir de sus precursores 18C mediante la programación nutricional o utilizando reproductores con una mayor expresión de *fads2*. Sin embargo, esos estudios no lograron demostrar el efecto de esas intervenciones en la expresión del gen *fads2* en el huevo en desarrollo. Además, no hubo estudios sobre la expresión temporal de la *fads2* a lo largo de la ontogenia en la dorada (*Sparus aurata*). Para determinar los cambios durante la ontogenia en la expresión de *fads2*, se permitió que desovaran los reproductores de dorada con una expresión de *fads2* alta (HRO) o baja (LRO) alimentados con una dieta previamente utilizada para la programación nutricional, o una dieta basada en aceite de pescado (LFO). Las muestras se tomaron en las etapas de desove, mórula, blástula alta, gastrula, neurula, latido del corazón,

eclosión y primeras larvas exógenas de 3 días de edad que se alimentan para determinar la expresión de *las fads2* a lo largo del desarrollo embrionario. Los resultados mostraron la presencia de ARNm de *fads2* en el huevo recién desovado, lo que denota la transferencia de ARNm materno al ovocito en desarrollo. Más tarde, la expresión de *fads2* aumentó después de la neurona, desde el latido del corazón hasta las larvas de 3 días de edad, lo que denota la transición de la expresión génica materna a la embrionaria. Además, los huevos obtenidos de reproductores con alta expresión de *fads2* mostraron un alto contenido de DHA, que se correlacionó con la disminución de la expresión de *fads2* en el embrión y las larvas en desarrollo. Por último, la alimentación con la dieta de programación nutricional con sustitución parcial del aceite de pescado por aceite de colza no afectó al contenido de LC-PUFA ni a la expresión de las fads2 en los huevos en desarrollo de la dorada.

9.4.3 Capítulo 5. La alta expresión de las modas de los reproductores2 combinada con la programación nutricional a través de la dieta de los reproductores mejora el uso de dietas bajas en harina y aceite de pescado en la progenie de la dorada (Sparus aurata)

Uno de los factores que limita la sustitución de la harina de pescado (FM) y el aceite de pescado (FO) por ingredientes vegetales en las dietas para peces marinos, es su falta de ácidos grasos poliinsaturados de cadena larga (LC-PUFA). Los LC-PUFA son ácidos grasos esenciales para estas especies de peces, que carecen de suficiente actividad de la desaturasa 2 de los ácidos grasos (Fads2) para sintetizarlos. La programación nutricional o el uso de reproductores con una mayor actividad de Fads2 podría mejorar la capacidad de los peces marinos para sintetizar LC-PUFA y su capacidad para usar dietas bajas en FM y FO. El objetivo de este estudio fue determinar el efecto de las poblaciones de dorada con una expresión génica de *fads2* inherentemente alta o baja y la programación nutricional con dietas de poblaciones de cría ricas en FO o aceite de colza (RO) en el rendimiento del crecimiento de la progenie, la morfología del hígado, la composición bioquímica y la expresión de los genes seleccionados. Los juveniles de dorada (2,31± 0,01 g de peso corporal inicial, media ± SD) obtenidos de reproductores con expresión de *fads2* alta (H) o baja (L) y alimentados con una dieta de reproductores basada en FO u RO se distribuyeron al azar en tanques de 12 x 250 L y fueron sometidos a un desafío nutricional durante 45 días con una dieta que contenía sólo un 7,5% de FM y ningún FO. El crecimiento más alto se obtuvo en los juveniles de reproductores con una alta expresión de

fads2 y alimentados con la dieta RO, mientras que el crecimiento más bajo se obtuvo en los de reproductores con una baja expresión de *fads2* y alimentados con la dieta RO. Los jóvenes procedentes de reproductores con una alta expresión de *fads2* mostraron una expresión de *fads2* significativamente mayor en el hígado y un mayor contenido de AGPI en el hígado y el músculo. La sustitución de FO por RO en las dietas de los reproductores condujo a un aumento significativo de la proporción hepática 18:3n-6/18:2n-6 y a la reducción del índice viscerosomático de los jóvenes de la progenie, el tamaño de los hepatocitos y la expresión de *ghr-1/ghr-2* en el músculo. En general, los resultados mostraron efectos trans-generacionales significativos tanto de la expresión de *fads2* de los reproductores como del tipo de lípido en la dieta de los reproductores sobre el metabolismo y el rendimiento de la progenie juvenil desafiada con una dieta baja en FM y FO.

9.4.4 Capítulo 6. La alta expresión de las fads2 en los reproductores de dorada mejora la utilización por parte de las crías de dietas bajas en harina y aceite de pescado: crecimiento, metabolismo de los ácidos grasos y secuenciación de los promotores de las fads2

Uno de los factores que limita la sustitución de la harina y el aceite de pescado por ingredientes vegetales en las dietas para peces marinos es su limitada capacidad para expresar el gen de la desaturasa de los ácidos grasos *2* (*fads2*) y para sintetizar los ácidos grasos poliinsaturados de cadena larga (LC-PUFA). El objetivo de este estudio era determinar si la expresión inherente de las *fads2* en los reproductores de dorada tenía efectos a largo plazo en la capacidad de las crías para utilizar dietas bajas en FM y FO. Para ello, se estudiaron el crecimiento, los perfiles de los ácidos grasos, la expresión de genes seleccionados y la secuenciación del promotor de las *fads2* en los juveniles de las crías obtenidas de reproductores con una expresión de *fads2* alta (H) o baja (L) y con un desafío nutricional con una dieta de 7,5% de FM y 0% de FO. Los resultados mostraron una mejora del 22% en el peso corporal final de las crías de los reproductores con una mayor expresión de *fads2* cuando las crías fueron alimentadas de 2,3 a más de 100 g con dietas que contenían bajos niveles de FM y bajos niveles de FO. Además, los jóvenes de reproductores con mayor expresión de *fads2* mostraron un contenido significativamente mayor de 22:6n-3, 22:4n-6 y 22:5n-3 y la disminución de *fads2* en el hígado. No se observaron diferencias en la alineación de la secuenciación en la región promotora de *fads2* entre los peces con diferente expresión de *fads2*. En general, el estudio demostró los

efectos transgeneracionales a largo plazo de la expresión de las fads2 de los reproductores en el crecimiento y el metabolismo de los ácidos grasos de las crías alimentadas con dietas bajas en FM y FO.

9.4.5 Capítulo 7. La programación nutricional mediante la dieta de los reproductores mejora persistentemente el uso de dietas bajas en harina y aceite de pescado en los juveniles de dorada (Sparus aurata)

Uno de los factores que limita la sustitución de la harina y el aceite de pescado por ingredientes vegetales en la dieta de los peces marinos es su capacidad de sintetizar los ácidos grasos poliinsaturados de cadena larga (LC-PUFA). La programación nutricional mediante la alimentación de los reproductores de dorada con una dieta rica en aceite de colza durante la temporada de desove puede mejorar la biosíntesis de LC-PUFA de sus crías cuando se ven desafiados por una dieta baja en FM y FO. Sin embargo, no se ha estudiado suficientemente la posible persistencia de este efecto. El objetivo de este estudio fue determinar si la programación nutricional que utiliza la dieta rica en RO en los reproductores tenía un efecto a largo plazo en la capacidad de las crías para utilizar la dieta baja en FM y FO. Para ello, se estudiaron el crecimiento, los perfiles de ácidos grasos, la expresión de genes seleccionados, la secuenciación de promotores de *fads2* y las bibliotecas de microARN en jóvenes obtenidos de reproductores alimentados con una dieta que contenía 100% FO (BFO) o 80% RO, 20%FO (BRO) en lípidos y que presentaba un desafío nutricional con una dieta de 7,5% FM y 0% FO. Los resultados mostraron una mejora del 27% en el peso corporal final de las crías de los reproductores alimentados con la dieta RO que los peces de los reproductores alimentados con la dieta FO. Además, los peces de los reproductores alimentados con la dieta RO tenían menor HSI, contenido de lípidos hepáticos y menor tamaño de hepatocitos ($p<0,05$), y alrededor de dos veces mayor contenido de LC-PUFA, incluyendo ácido araquidónico, 22:5n-6 y DHA en el músculo, que los de los reproductores alimentados con la dieta FO. Además, se observó una mayor actividad de Δ5 desaturasa basada en la mayor proporción de 20:4n-6/20:3n-6 y 20:5n-3/20:4n-3 en el músculo de los peces de los reproductores alimentados con dieta RO. No se observaron diferencias en la alineación de la secuencia en la región promotora de las *fads2* entre los reproductores programados por diferentes dietas, ni en las bibliotecas de microARN en los juveniles de las crías. En general, el estudio demostró el efecto de la programación nutricional

a largo plazo en los reproductores sobre el crecimiento, la estructura histológica del hígado y el metabolismo de los lípidos de las crías alimentadas con una dieta baja en FM y FO.

9.5 Conclusions

1. Cuando se se enfrentan a una dieta baja en harina de pescado (FM) y aceite de pescado (FO), se observó un mayor rendimiento de crecimiento en los jóvenes descendientes de reproductores con mayor expresión de *fads2* y alimentados con una dieta rica en aceite de colza (RO) durante la temporada de desove (capítulo 5). Además, las crías de los reproductores con una alta expresión de *fads2* mostraron un mayor peso corporal en el ensayo de desafío a largo plazo (capítulo 6). Sin embargo, la sustitución del 50% de la FM dietética sola o en combinación con el 70% de FO en la dieta de los reproductores durante la temporada de desove condujo a una disminución del rendimiento de crecimiento de las crías juveniles (Capítulo 3).
2. Cuando se desafió con una dieta baja en FM y FO en las primeras etapas de la vida, la descendencia de los reproductores programados nutricionalmente por el reemplazo del 80% de FO dietético por aceite vegetal durante la temporada de desove había aumentado la capacidad de biosíntesis de LC-PUFA en términos de los cambios en los perfiles de ácidos grasos (Capítulo 3, 5). Ademas se redujo el índice viscerosomático (Capítulo 5) y la acumulación de lípidos hepáticos, la expresión de *elovl6 en el* hígado y *ghr-1/ghr-2* en el músculo (Capítulo 5).
3. En el capítulo 7, se observó un efecto a largo plazo de la programación nutricional de que en el músculo de la descendencia de los reproductores programados con RO, el contenido y la actividad de biosíntesis de LC-PUFA era mayor, en comparación con la descendencia de los reproductores alimentados con una dieta con contenido de FO.
4. Se observó un aumento de la expresión *de fads2,* así como del contenido de LC-PUFA en el hígado y el músculo en la dorada juvenil de reproductores con una expresión de *fads2* inherentemente alta, cuando se le impuso una dieta baja en FM y FO en las primeras etapas de la vida. Además, se observó una disminución de la expresión *fads2* en las crías de reproductores con mayor expresión de *fads2,* junto con una alta incorporación de DHA, en particular en el hígado. Este mayor DHA, junto con el aumento de 22:5n-3 y 22:4n-6,

productos tardíos de la elongación y la desaturación, sería responsable de la regulación a la baja de la expresión hepática de *fads2*, posiblemente mediada por la regulación a la baja en el *srebp*.

5. Los resultados sobre los cambios de *las fads2* durante la ontogenia mostraron la presencia de ARNm de *fads2* en el huevo de dorada recién desovado, lo que denota la transferencia de ARNm materno al ovocito en desarrollo, mientras que a partir de la etapa de neurula la expresión de *fads2* aumentó, lo que denota la transición de la expresión génica materna a la embrionaria. Además, los huevos obtenidos de reproductores con alta expresión de *fads2* mostraron un alto contenido de DHA, lo que podría ser responsable de una regulación a la baja de la expresión de *fads2* en el embrión y las larvas en desarrollo. Por último, la sustitución parcial de FO por RO no afectó al contenido de LC-PUFA ni a la expresión de las *fads2* en los huevos de dorada (Capítulo 4).
6. Los cambios liderados por la programación nutricional de los reproductores o la expresión de *las fads2* no se debieron a la secuencia de la región promotora de *las fads2*.

En resumen, la programación nutricional en los reproductores mediante la sustitución de FO por aceite vegetal, especialmente RO, aumentó la capacidad de biosíntesis de LC-PUFA en la descendencia cuando se desafió con dietas que contenían bajo FM y FO. Ese efecto se observó tanto en las primeras etapas de la vida como en la prueba de desafío a largo plazo. Además, la tesis proporciona en primer lugar las pruebas sobre el efecto transgeneracional de la expresión de *fads2* en los cambios durante la ontogenia y el uso de dietas bajas en FM y FO de la descendencia. Así pues, pueden aplicarse estrategias de combinación de la elección de reproductores con una mayor expresión de las *fads2* y la programación nutricional por medio del aceite vegetal para aumentar el uso de dietas bajas en FM y FO. Sin embargo, es necesario realizar más estudios sobre el mecanismo a nivel molecular, ya que no se observaron diferencias en la secuencia genética de los reproductores, el nivel de CpG de las *fads2* de la descendencia y la expresión de miARN.

Reference

Allis, C.D., Caparros, M.-L., Jenuwein, T., Reinberg, D., 2008. Epigenetics, 2 ed. Cold Spring Harbor Laboratory Press, New York, USA.

Asturiano, J.F., Sorbera, L.A., Carrillo, M., Zanuy, S., Ramos, J., Navarro, J.C., Bromage, N., 2001. Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture. 194, 173-190.

Balasubramanian, M.N., Panserat, S., Dupont-Nivet, M., Quillet, E., Montfort, J., Le Cam, A., Medale, F., Kaushik, S.J., Geurden, I., 2016. Molecular pathways associated with the nutritional programming of plant-based diet acceptance in rainbow trout following an early feeding exposure. BMC Genomics. 17, 449.

Beckman, B.R., 2011. Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. Gen. Comp. Endocrinol. 170, 233-252.

Bell, J.G., Henderson, R.J., Tocher, D.R., McGhee, F., Dick, J.R., Porter, A., Smullen, R.P., Sargent, J.R., 2002. Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism. J. Nutr. 132, 222-230.

Bell, M.V., Tocher, D.R., 2009. Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: general pathways and new directions, Lipids in Aquatic Ecosystems, Springer, New York, USA. pp. 211-236.

Benedito-Palos, L., Ballester-Lozano, G., Perez-Sanchez, J., 2014. Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). Gene. 547, 34-42.

Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J.-A., Kaushik, S., Pérez-Sánchez, J., 2007. Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): Networking of systemic and local components of GH/IGF axis. Aquaculture. 267, 199-212.

Benedito-Palos, L., Navarro, J.C., Sitja-Bobadilla, A., Bell, J.G., Kaushik, S., Perez-Sanchez, J., 2008. High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty acid profiles and histological alterations of target tissues. Br. J. Nutr. 100, 992-1003.

Benedito-Palos, L., Ballester-Lozano, G.F., Simó, P., Karalazos, V., Ortiz, Á., Calduch-Giner, J., Pérez-Sánchez, J., 2016. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). Aquaculture. 454, 8-18.

Benitez-Santana, T., Juarez-Carrillo, E., Betancor, M.B., Torrecillas, S., Caballero, M.J., Izquierdo, M.S., 2012. Increased Mauthner cell activity and escaping behaviour in seabream fed long-chain PUFA. Br. J. Nutr. 107, 295-301.

Benitez-Santana, T., Atalah, E., Betancor, M.B., Caballero, M.J., Hernandez-Cruz, C.M., Izquierdo, M., 2014. DHA but not EPA, enhances sound induced escape behavior and Mauthner cells activity in *Sparus aurata*. Physiol. Behav. 124, 65-71.

Bergmann, R.L., Bergmann, K.E., Richter, R., Haschke-Becher, E., Henrich, W., Dudenhausen, J.W., 2012. Does docosahexaenoic acid (DHA) status in pregnancy have any impact on postnatal growth? Six-year follow-up of a prospective randomized double-blind monocenter study on low-dose DHA supplements. J. Perinat. Med. 40, 677-684.

Best, C., Ikert, H., Kostyniuk, D.J., Craig, P.M., Navarro-Martin, L., Marandel, L., Mennigen, J.A., 2018. Epigenetics in teleost fish: From molecular mechanisms to physiological phenotypes. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 224, 210-244.

Betancor, M.B., Sprague, M., Montero, D., Usher, S., Sayanova, O., Campbell, P.J., Napier, J.A., Caballero, M.J., Izquierdo, M., Tocher, D.R., 2016. Replacement of Marine Fish Oil with de novo Omega-3 Oils from Transgenic Camelina sativa in Feeds for Gilthead Sea Bream (*Sparus aurata* L.). Lipids. 51, 1171-1191.

Bond, L.M., Miyazaki, M., O'Neill, L.M., Ding, F., Ntambi, J.M., 2016. Fatty Acid Desaturation and Elongation in Mammals, Biochemistry of Lipids, Lipoproteins and Membranes, pp. 185-208.

Boschetti, E., Bordoni, A., Meluzzi, A., Castellini, C., Dal Bosco, A., Sirri, F., 2016. Fatty acid composition of chicken breast meat is dependent on genotype-related variation of FADS1 and FADS2 gene expression and desaturating activity. Animal. 10, 700-708.

Caballero, M.J., Gallardo, G., Robaina, L., Montero, D., Fernandez, A., Izquierdo, M., 2006. Vegetable lipid sources affect in vitro biosynthesis of triacylglycerols and phospholipids in the intestine of sea bream (*Sparus aurata*). Br. J. Nutr. 95, 448-454.

Caballero, M.J., Izquierdo, M.S., Kjorsvik, E., Fernandez, A.J., Rosenlund, G., 2004. Histological alterations in the liver of sea bream, *Sparus aurata* L., caused by short- or long-term feeding with vegetable oils. Recovery of normal morphology after feeding fish oil as the sole lipid source. J. Fish. Dis. 27, 531-541.

Caballero, M.J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M., Izquierdo, M.S., 2002. Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 214, 253-271.

Caballero, M.J., Torstensen, B.E., Robaina, L., Montero, D., Izquierdo, M., 2007. Vegetable oils affect the composition of lipoproteins in sea bream (*Sparus aurata*). Br. J. Nutr. 96. 830-839

Calder, P.C., 2012. Mechanisms of action of (n-3) fatty acids. J. Nutr. 142, 592S-599S.

Callet, T., Medale, F., Larroquet, L., Surget, A., Aguirre, P., Kerneis, T., Labbe, L., Quillet, E., Geurden, I., Skiba-Cassy, S., Dupont-Nivet, M., 2017. Successful selection of rainbow trout (*Oncorhynchus mykiss*) on their ability to grow with a diet completely devoid of fishmeal and fish oil, and correlated changes in nutritional traits. PLoS ONE. 12, e0186705.

Carter, C.G., Hauler, R.C., 2000. Fish meal replacement by plant meals in extruded feeds for Atlantic salmon, *Salmo salar* L. Aquaculture. 185, 299-311.

Carvalho, M., Castro, P., Montero, D., Peres, H., Acosta, F., Fontanillas, R., Rosenlund, G., Robaina, L., Izquierdo, M., 2019. Essential fatty acid deficiency increases hepatic non-

infectious granulomatosis incidence in meagre (*Argyrosomus regius*, Asso 1801) fingerlings. Aquaculture. 505, 393-404.

Carvalho, M., Montero, D., Rosenlund, G., Fontanillas, R., Ginés, R., Izquierdo, M., 2020. Effective complete replacement of fish oil by combining poultry and microalgae oils in practical diets for gilthead sea bream (*Sparus aurata*) fingerlings. Aquaculture. 529: 735696.

Castro, C., Corraze, G., Panserat, S., Oliva-Teles, A., 2015. Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. Aquac. Nutr. 21, 592-603.

Chellappa S., Huntingford F. A., Strang R. H. C., Thomson, R.Y., 1995. Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. J. Fish Biol, 47(5): 775-787.

Chen, J., Cui, Y., Yan, J., Jiang, J., Cao, X., Gao, J., 2018. Molecular characterization of *elongase of very long-chain fatty acids 6* (*elovl6*) genes in *Misgurnus anguillicaudatus* and their potential roles in adaptation to cold temperature. Gene. 666, 134-144.

Cheung, C.T., Pasquier, J., Bouleau, A., Nguyen, T., Chesnel, F., Guiguen, Y., Bobe, J., 2018. Double maternal-effect: duplicated nucleoplasmin 2 genes, *npm2a* and *npm2b*, with essential but distinct functions are shared by fish and tetrapods. BMC Evol. Biol. 18, 167.

Cho, H.P., Nakamura, M., Clarke, S.D., 1999. Cloning, expression, and fatty acid regulation of the human delta-5 desaturase. J. Biol. Chem. 274, 37335-37339.

Christie, W., 1988. Equivalent chain-lengths of methyl ester derivatives of fatty acids on gas chromatography A reappraisal. J. Chromatogr. A. 447, 305-314.

Christie, W.W., 1982. A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. J. Lipid. Res. 23, 1072-1075.

Clarkson, M., Migaud, H., Metochis, C., Vera, L.M., Leeming, D., Tocher, D.R., Taylor, J.F., 2017. Early nutritional intervention can improve utilisation of vegetable-based diets in diploid and triploid Atlantic salmon (*Salmo salar* L.). Br. J. Nutr. 118, 17-29.

Costello, J.F., Plass, C., 2001. Methylation matters. Journal of medical genetics. 38, 285-303.

National Research Council, 2011. Nutrient requirements of fish and shrimp. 1st ed, National academies press, Washington D.C., USA.

CUNIFF, P.E., 1995. Official Methods of Analysis of AOAC International: Agricultural Chemicals; Contaminants; Drugs. AOAC International.

Dantagnan, P., Bórquez, A., Hernández, A., Izquierdo, M., 2010. Effect of EPA/DHA ratios on the growth and survival of *Galaxias maculatus* (Jenyns, 1842) larvae reared under different salinity regimes. Aquac. Res. 41, e239-e244.

De Francesco, M., Parisi, G., Pérez-Sanchez, J., Gómez-Réqueni, P., Medale, F., Kaushik, S.J., Mecatti, M., Poli, B.M., 2007. Effect of high-level fish meal replacement by plant proteins in gilthead sea bream (*Sparus aurata*) on growth and body/fillet quality traits. Aquac. Nutr. 13, 361-372.

Dolinoy, D.C., Weidman, J.R., Waterland, R.A., Jirtle, R.L., 2006. Maternal genistein alters coat color and protects A^{vy} mouse offspring from obesity by modifying the fetal epigenome. Environ. Health Perspect. 114, 567-572.

Dong, X., Tan, P., Cai, Z., Xu, H., Li, J., Ren, W., Xu, H., Zuo, R., Zhou, J., Mai, K., Ai, Q., 2017. Regulation of FADS2 transcription by SREBP-1 and PPAR-alpha influences LC-PUFA biosynthesis in fish. Sci. Rep. 7, 40024.

Doughman, S.D., Krupanidhi, S., Sanjeevi, C.B., 2007. Omega-3 fatty acids for nutrition and medicine: considering microalgae oil as a vegetarian source of EPA and DHA. Curr. Diabetes Rev. 3, 198-203.

Dumortier, O., Hinault, C., Gautier, N., Patouraux, S., Casamento, V., Van Obberghen, E., 2014. Maternal protein restriction leads to pancreatic failure in offspring: role of misexpressed microRNA-375. Diabetes. 63, 3416-3427.

Edwards, M., 2017. The Barker Hypothesis. in: Preedy, V., Patel, V.B. (Eds.), Handbook of Famine, Starvation, and Nutrient Deprivation: From Biology to Policy. Springer, Berlin, Germany. pp. 1-21.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008. Hepatic glucokinase and glucose-6-phosphatase responses to dietary glucose and starch in gilthead sea bream (*Sparus aurata*) juveniles reared at two temperatures. Comp. Bio. chem. Physiol. A Mol. Integr. Physiol. 149, 80-86.

Escobar-Aguirre, S., Felip, A., Mazón, M.J., Ballester-Lozano, G., Pérez-Sánchez, J., Björsson, B.T., Zanuy, S., Carrillo, M., 2020. Long-term feeding of a maintenance ration affects the release of Igf-1 and leptin, and delays maturation in a male teleost fish, *Dicentrarchus labrax* L. Aquaculture. 527.

Fang, L., Liang, X.F., Zhou, Y., Guo, X.Z., He, Y., Yi, T.L., Liu, L.W., Yuan, X.C., Tao, Y.X., 2014. Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, *Danio rerio*. Br. J. Nutr. 111, 808-818.

FAO. 2018. The State of World Fisheries and Aquaculture 2018. Meeting the sustainable development goals. Rome

FAO. 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome.

Fernández-Palacios, H., Norberg, B., Izquierdo, M., Hamre, K., 2011. Effects of broodstock diet on eggs and larvae. In Larval fish nutrition, 1st ed, John Wiley & Sons, Hoboken, USA. pp. 151-181.

Fernández-Palacios, H., Izquierdo, M.S., Robaina, L., Valencia, A., Salhi, M., Vergara, J., 1995. Effect of n-3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L.). Aquaculture. 132, 325-337.

Fernández-Palacios, H., Izquierdo, M., Robaina, L., Valencia, A., Salhi, M., Montero, D., 1997. The effect of dietary protein and lipid from squid and fish meals on egg quality of broodstock for gilthead seabream (*Sparus aurata*). Aquaculture. 148, 233-246.

Ferosekhan, S., Turkmen, S., Xu, H., Afonso, J.M., Zamorano, M.J., Kaushik, S., Izquierdo, M., 2020a. The relationship between the expression of *fatty acyl desaturase 2* (*fads2*) gene in peripheral blood cells (pbcs) and liver in gilthead seabream, *Sparus aurata* broodstock fed a low n-3 LC-PUFA diet. Life. 10, 117.

Ferosekhan, S., Xu, H., Turkmen, S., Gómez, A., Afonso, J.M., Fontanillas, R., Rosenlund, G., Kaushik, S., Izquierdo, M., 2020b. Reproductive performance of gilthead seabream (*Sparus aurata*) broodstock showing different expression of *fatty acyl desaturase 2* and fed two dietary fatty acid profiles. Sci. Rep. 10, 15547.

Ferreira, I.M.P.L.V.O., Pinho, O., Vieira, E., Tavarela, J.G., 2010. Brewer's Saccharomyces yeast biomass: characteristics and potential applications. Trends Food Sci. Technol. 21, 77-84.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497-509.

Fontagné-Dicharry, S., Alami-Durante, H., Aragão, C., Kaushik, S.J., Geurden, I., 2017. Parental and early-feeding effects of dietary methionine in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 469, 16-27.

Francis, G., Makkar, H.P.S., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 199, 197-227.

Fuiman, L.A., Perez, K.O., 2015. Metabolic programming mediated by an essential fatty acid alters body composition and survival skills of a marine fish. Proc. Biol. Sci. 282.

Gao, B., Han, Y.H., Wang, L., Lin, Y.J., Sun, Z., Lu, W.G., Hu, Y.Q., Li, J.Q., Lin, X.S., Liu, B.H., Jie, Q., Yang, L., Luo, Z.J., 2016. Eicosapentaenoic acid attenuates dexamethasome-induced apoptosis by inducing adaptive autophagy via GPR120 in murine bone marrow-derived mesenchymal stem cells. Cell Death Dis. 7, e2235.

Geurden, I., Borchert, P., Balasubramanian, M.N., Schrama, J.W., Dupont-Nivet, M., Quillet, E., Kaushik, S.J., Panserat, S., Medale, F., 2013. The positive impact of the early-feeding of a plant-based diet on its future acceptance and utilisation in rainbow trout. PLoS ONE. 8, e83162.

Gill, I., Valivety, R., 1997. Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications. Trends Biotechnol. 15, 401-409.

Glaser, C., Heinrich, J., Koletzko, B., 2010. Role of FADS1 and FADS2 polymorphisms in polyunsaturated fatty acid metabolism. Metabolism. 59, 993-999.

Gluckman, P.D., Hanson, M.A., 2004. Living with the past: evolution, development, and patterns of disease. Science. 305, 1733-1736.

Gomes, E.F., Rema, P., Kaushik, S.J., 1995. Replacement of fish meal by plant proteins in the diet of rainbow trout (*Oncorhynchus mykiss*): digestibility and growth performance. Aquaculture. 130, 177-186.

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Médale, F., Martin, S.A.M., Houlihan, D.F., Kaushik, S., Pérez-Sánchez, J., 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture. 232, 493-510.

Gorjao, R., Azevedo-Martins, A.K., Rodrigues, H.G., Abdulkader, F., Arcisio-Miranda, M., Procopio, J., Curi, R., 2009. Comparative effects of DHA and EPA on cell function. Pharmacol. Ther. 122, 56-64.

Green, K., 2018. Fishmeal and fish oil facts and figures. Seafish. 12, 1-33.

Gregory, M.K., Collins, R.O., Tocher, D.R., James, M.J., Turchini, G.M., 2016. Nutritional regulation of long-chain PUFA biosynthetic genes in rainbow trout (*Oncorhynchus mykiss*). Br. J. Nutr. 115, 1721-1729.

Han, H., Cortez, C.C., Yang, X., Nichols, P.W., Jones, P.A., Liang, G., 2011. DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. Hum. Mol. Genet. 20, 4299-4310.

Hardy, R.W., Barrows, F.T., 2002. Diet formulation and manufacture. In Fish nutrition, 3rd ed. Elsevier, Amsterdam, Netherlands. pp. 505-600.

Harel, M., Tandler, A., Kissil, G.W., Applebaum, S.W., 1994. The kinetics of nutrient incorporation into body tissues of gilthead seabream (*Sparus aurata*) females and the subsequent effects on egg composition and egg quality. Br. J. Nutr. 72, 45-58.

Hastings, N., Agaba, M., Tocher, D.R., Leaver, M.J., Dick, J.R., Sargent, J.R., Teale, A.J., 2001. A vertebrate fatty acid desaturase with Delta 5 and Delta 6 activities. Proc. Natl. Acad. Sci. 98, 14304-14309.

Heijmans, B.T., Tobi, E.W., Stein, A.D., Putter, H., Blauw, G.J., Susser, E.S., Slagboom, P.E., Lumey, L.H., 2008. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc. Natl. Acad. Sci. 105, 17046-17049.

Helland, I.B., Smith, L., Saarem, K., Saugstad, O.D., Drevon, C.A., 2003. Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. Pediatrics. 111, e39-44.

Hoile, S.P., Irvine, N.A., Kelsall, C.J., Sibbons, C., Feunteun, A., Collister, A., Torrens, C., Calder, P.C., Hanson, M.A., Lillycrop, K.A., Burdge, G.C., 2013. Maternal fat intake in rats alters 20:4n-6 and 22:6n-3 status and the epigenetic regulation of Fads2 in offspring liver. J. Nutr. Biochem. 24, 1213-1220.

Horn, S.S., Ruyter, B., Meuwissen, T.H.E., Hillestad, B., Sonesson, A.K., 2018. Genetic effects of fatty acid composition in muscle of Atlantic salmon. Genet. Sel. Evol. 50, 23.

Horn, S.S., Ruyter, B., Meuwissen, T.H.E., Moghadam, H., Hillestad, B., Sonesson, A.K., 2020. GWAS identifies genetic variants associated with omega-3 fatty acid composition of Atlantic salmon fillets. Aquaculture. 514.

Horn, S.S., Sonesson, A.K., Krasnov, A., Moghadam, H., Hillestad, B., Meuwissen, T.H.E., Ruyter, B., 2019. Individual differences in EPA and DHA content of Atlantic salmon are associated with gene expression of key metabolic processes. Sci. Rep. 9, 3889.

Horwitz, W., 2002. Official methods of analysis of AOAC International. AOAC International, Gaithersburg, Gaithersburg, USA.

Hou, Z., Fuiman, L.A., 2019. Nutritional programming in fishes: insights from mammalian studies. Rev. Fish Biol. Fish. 30, 67-92.

Howard, T.D., Mathias, R.A., Seeds, M.C., Herrington, D.M., Hixson, J.E., Shimmin, L.C., Hawkins, G.A., Sellers, M., Ainsworth, H.C., Sergeant, S., Miller, L.R., Chilton, F.H., 2014. DNA methylation in an enhancer region of the FADS cluster is associated with FADS activity in human liver. PLoS ONE. 9, e97510.

Izquierdo, M., 1989. Optimal EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*), Proc. Third Intn. Symp. on Feeding and Nutr. in Fish, pp. 221-232.

Izquierdo, M., Koven, W., 2011. Digestive development and nutrient requirements. Larval fish nutrition, 47-81.

Izquierdo, M.S., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red sea bream (*Pagrus major*). The Current Status of Fish Nutrition in Aquaculture, 221-232.

Izquierdo, M.S., 1996. Essential fatty acid requirements of cultured marine fish larvae. Aquac. Nutr. 2, 183-191.

Izquierdo, M.S., Fernández-Palacios, H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture. 197, 25-42.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L., Rosenlund, G., 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. Aquac. Nutr. 9, 397-407.

Izquierdo, M.S., Montero, D., Robaina, L., Caballero, M.J., Rosenlund, G., Ginés, R., 2005. Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. Aquaculture. 250, 431-444.

Izquierdo, M.S., Robaina, L., Juarez-Carrillo, E., Oliva, V., Hernandez-Cruz, C.M., Afonso, J.M., 2008. Regulation of growth, fatty acid composition and delta 6 desaturase expression by dietary lipids in gilthead seabream larvae (*Sparus aurata*). Fish Physiol. Biochem. 34, 117-127.

Izquierdo, M.S., Turkmen, S., Montero, D., Zamorano, M.J., Afonso, J.M., Karalazos, V., Fernández-Palacios, H., 2015. Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream. Aquaculture. 449, 18-26.

Jaroszewska, M., Dabrowski, K., 2011. Utilization of yolk: transition from endogenous to exogenous nutrition in fish. In Larval fish nutrition, 1st ed, John Wiley & Sons, Hoboken, USA. 183-218.

Jaya-Ram, A., Kuah, M.-K., Lim, P.-S., Kolkovski, S., Shu-Chien, A.C., 2008. Influence of dietary HUFA levels on reproductive performance, tissue fatty acid profile and desaturase and elongase mRNAs expression in female zebrafish *Danio rerio*. Aquaculture. 277, 275-281.

Kabeya, N., Yoshizaki, G., Tocher, D.R., Monroig, Ó., 2017. Diversification of Fads2 in finfish species: Implications for aquaculture. Avances en Nutrición Acuicola.

Kabeya, N., Takeuchi, Y., Yamamoto, Y., Yazawa, R., Haga, Y., Satoh, S., Yoshizaki, G., 2014. Modification of the n-3 HUFA biosynthetic pathway by transgenesis in a marine teleost, nibe croaker. J. Biotechnol. 172, 46-54.

Kamacı, H.O., Saka, Ş., Fırat, K., 2005. The Cleavage and Embryonic Phase of Gilthead Sea Bream (*Sparus aurata Linnaeus*, 1758) Eggs. Su Ürünleri Dergisi. 22, 205-209.

Kanazawa, A., Teshima, S., Ono, K., 1979. Relationship between essential fatty acid requirements of aquatic animals and the capacity for bioconversion of linolenic acid to highly unsaturated fatty acids. Comp. Biochem. Physiol. B. 63, 295-298.

Karlstrom, R.O., Kane, D.A., 1996. A flipbook of zebrafish embryogenesis. Development. 123, 461.

Kaushik, S.J., 1998. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Aquat. Living Resour. 11, 355-358.

Kelsall, C.J., Hoile, S.P., Irvine, N.A., Masoodi, M., Torrens, C., Lillycrop, K.A., Calder, P.C., Clough, G.F., Hanson, M.A., Burdge, G.C., 2012. Vascular dysfunction induced in offspring by maternal dietary fat involves altered arterial polyunsaturated fatty acid biosynthesis. PLoS ONE. 7, e34492.

Kitson, A.P., Marks, K.A., Shaw, B., Mutch, D.M., Stark, K.D., 2013. Treatment of ovariectomized rats with 17beta-estradiol increases hepatic delta-6 desaturase enzyme expression and docosahexaenoic acid levels in hepatic and plasma phospholipids. Prostaglandins Leukot. Essent. Fatty Acids. 89, 81-88.

Kloosterman, W.P., Lagendijk, A.K., Ketting, R.F., Moulton, J.D., Plasterk, R.H., 2007. Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. PLoS Biol. 5, e203.

Kroeckel, S., Harjes, A.G.E., Roth, I., Katz, H., Wuertz, S., Susenbeth, A., Schulz, C., 2012. When a turbot catches a fly: Evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute — Growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). Aquaculture. 364-365, 345-352.

Kucharski, R., Maleszka, J., Foret, S., Maleszka, R., 2008. Nutritional control of reproductive status in honeybees via DNA methylation. Science. 319, 1827-1830.

Latimer, M., Sabin, N., Le Cam, A., Seiliez, I., Biga, P., Gabillard, J.C., 2017. miR-210 expression is associated with methionine-induced differentiation of trout satellite cells. J. Exp. Biol. 220, 2932-2938.

Laudadio, I., Manfroid, I., Achouri, Y., Schmidt, D., Wilson, M.D., Cordi, S., Thorrez, L., Knoops, L., Jacquemin, P., Schuit, F., Pierreux, C.E., Odom, D.T., Peers, B., Lemaigre, F.P., 2012. A feedback loop between the liver-enriched transcription factor network and miR-122 controls hepatocyte differentiation. Gastroenterology. 142, 119-129.

Lazzarotto, V., Corraze, G., Larroquet, L., Mazurais, D., Medale, F., 2016. Does broodstock nutritional history affect the response of progeny to different first-feeding diets? A whole-body transcriptomic study of rainbow trout alevins. Br. J. Nutr. 115, 2079-2092.

Lazzarotto, V., Corraze, G., Leprevost, A., Quillet, E., Dupont-Nivet, M., Medale, F., 2015. Three-year breeding cycle of rainbow trout (*Oncorhynchus mykiss*) fed a plant-based diet, totally free of marine resources: consequences for reproduction, fatty acid composition and progeny survival. PLoS ONE. 10, e0117609.

Li, D., Weisinger, H.S., Weisinger, R.S., Mathai, M., Armitage, J.A., Vingrys, A.J., Sinclair, A.J., 2006. Omega 6 to omega 3 fatty acid imbalance early in life leads to persistent reductions in DHA levels in glycerophospholipids in rat hypothalamus even after long-term omega 3 fatty acid repletion. Prostaglandins Leukot. Essent. Fatty Acids. 74, 391-399.

Li, S., Mai, K., Xu, W., Yuan, Y., Zhang, Y., Ai, Q., 2014. Characterization, mRNA expression and regulation of Δ6 fatty acyl desaturase (FADS2) by dietary n−3 long chain polyunsaturated fatty acid (LC-PUFA) levels in grouper larvae (*Epinephelus coioides*). Aquaculture. 434, 212-219.

Li, Y., Hu, C., Zheng, Y., Xia, X., Xu, W., Wang, S., Chen, W., Sun, Z., Huang, J., 2008. The effects of dietary fatty acids on liver fatty acid composition and Δ6-desaturase expression differ with ambient salinities in Siganus canaliculatus. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 151, 183-190.

Lillycrop, K.A., Phillips, E.S., Jackson, A.A., Hanson, M.A., Burdge, G.C., 2005. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J. Nutr. 135, 1382-1386.

Lin, Y.-H., Shiau, S.-Y., 2007. Effects of dietary blend of fish oil with corn oil on growth and non-specific immune responses of grouper, *Epinephelus malabaricus*. Aquac. Nutr. 13, 137-144.

Liu, J., Xu, M., Zhao, Y., Ao, C., Wu, Y., Chen, Z., Wang, B., Bai, X., Li, M., Hu, W., 2016. n-3 polyunsaturated fatty acids abrogate mTORC1/2 signaling and inhibit adrenocortical carcinoma growth *in vitro* and *in vivo*. Oncol. Rep. 35, 3514-3522.

Lopes-Marques, M., Kabeya, N., Qian, Y., Ruivo, R., Santos, M.M., Venkatesh, B., Tocher, D.R., Castro, L.F.C., Monroig, O., 2018. Retention of fatty acyl desaturase 1 (fads1) in Elopomorpha and Cyclostomata provides novel insights into the evolution of long-chain polyunsaturated fatty acid biosynthesis in vertebrates. BMC Evol. Biol. 18, 157.

Lubzens, E., Labbé, C., Ciereszko, A., 2017. Special issue on Recent advances in fish gametes and embryo research. Celebrating 40 years of aquaculture. Elsevier. Amsterdam, Netherlands.

Lucas, A., 1994. Role of nutritional programming in determining adult morbidity. Arch. Dis. Child. 71, 288-290.

Lucas, A., 1998. Programming by early nutrition: an experimental approach. J. Nutr. 128, 401S-406S.

Lucas, A., Baker, B.A., Desai, M., Hales, C.N., 1996. Nutrition in pregnant or lactating rats programs lipid metabolism in the offspring. Br. J. Nutr. 76, 605-612.

Luo, L., Wei, H., Ai, L., Liang, X., Wu, X., Xing, W., Chen, P., Xue, M., 2019. Effects of early long-chain n-3 HUFA programming on growth, antioxidant response and lipid metabolism of Siberian sturgeon (*Acipenser baerii* Brandt). Aquaculture. 509, 96-103.

Madsen, L., Rustan, A.C., Vaagenes, H., Berge, K., Dyroy, E., Berge, R.K., 1999. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. Lipids. 34, 951-963.

Malerba, G., Schaeffer, L., Xumerle, L., Klopp, N., Trabetti, E., Biscuola, M., Cavallari, U., Galavotti, R., Martinelli, N., Guarini, P., Girelli, D., Olivieri, O., Corrocher, R., Heinrich, J., Pignatti, P.F., Illig, T., 2008. SNPs of the FADS gene cluster are associated with polyunsaturated fatty acids in a cohort of patients with cardiovascular disease. Lipids. 43, 289-299.

Martínez-Llorens, S., Molinari, G.S., McCracken, V.J., Wojno, M., Rimoldi, S., Terova, G., Kwasek, K., 2020. Can intestinal absorption of dietary protein be improved through early exposure to plant-based diet? PLoS ONE. 15, e0228758.

Martoja, R., Martoja-Pierson, M., GrassÈ, P.P., Estart´s Moncanut, M., Durfort Coll, M., 1970. Técnicas de histología animal. Toray-Masson, Barcelona, Spain.

Matsuzaka, T., Shimano, H., 2009. Elovl6: a new player in fatty acid metabolism and insulin sensitivity. J. Mol. Med. 87, 379-384.

Mennigen, J.A., Martyniuk, C.J., Seiliez, I., Panserat, S., Skiba-Cassy, S., 2014. Metabolic consequences of microRNA-122 inhibition in rainbow trout, *Oncorhynchus mykiss*. BMC Genomics. 15, 70.

Menon, D., Salloum, D., Bernfeld, E., Gorodetsky, E., Akselrod, A., Frias, M.A., Sudderth, J., Chen, P.H., DeBerardinis, R., Foster, D.A., 2017. Lipid sensing by mTOR complexes via de novo synthesis of phosphatidic acid. J. Biol. Chem. 292, 6303-6311.

Menoyo, D., Izquierdo, M.S., Robaina, L., Gines, R., Lopez-Bote, C.J., Bautista, J.M., 2004. Adaptation of lipid metabolism, tissue composition and flesh quality in gilthead sea bream (*Sparus aurata*) to the replacement of dietary fish oil by linseed and soyabean oils. Br. J. Nutr. 92, 41-52.

Mercuri, O., de Tomas, M.E., Itarte, H., 1979. Prenatal protein depletion and Delta9, Delta6 and Delta5 desaturases in the rat. Lipids. 14, 822-825.

Michl, S.C., Weis, B., Hutchings, J.A., Schulz, C., 2017. Plastic responses by wild brown trout (*Salmo trutta*) to plant-based diets. Aquaculture. 476, 19-28.

Missotten, J., De Smet, S., Raes, K., Doran, O., 2009. Effect of supplementation of the maternal diet with fish oil or linseed oil on fatty-acid composition and expression of Delta5- and Delta6-desaturase in tissues of female piglets. Animal. 3, 1196-1204.

Monge-Ortiz, R., Tomás-Vidal, A., Rodriguez-Barreto, D., Martínez-Llorens, S., Pérez, J.A., Jover-Cerdá, M., Lorenzo, A., 2018. Replacement of fish oil with vegetable oil blends in feeds for greater amberjack (*Seriola dumerili*) juveniles: Effect on growth performance, feed efficiency, tissue fatty acid composition and flesh nutritional value. Aquac. Nutr. 24, 605-615.

Monroig, O., Li, Y., Tocher, D.R., 2011. Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 159, 206-213.

Monroig, O., Rotllant, J., Sanchez, E., Cerda-Reverter, J.M., Tocher, D.R., 2009. Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis. Biochim. Biophys. Acta. 1791, 1093-1101.

Morais, S., Mendes, A.C., Castanheira, M.F., Coutinho, J., Bandarra, N., Dias, J., Conceição, L.E.C., Pousão-Ferreira, P., 2014. New formulated diets for *Solea senegalensis* broodstock: Effects of parental nutrition on biosynthesis of long-chain polyunsaturated fatty acids and performance of early larval stages and juvenile fish. Aquaculture. 432, 374-382.

Morgane, P.J., Mokler, D.J., Galler, J.R., 2002. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci. Biobehav. Rev. 26, 471-483.

Mourente, G., 2003. Accumulation of DHA (docosahexaenoic acid; 22: 6n-3) in larval and juvenile fish brain. The Big Fish Bang. Proceedings of the 26th Annual Larval Fish Conference, 239-248.

Mourente, G., Tocher, D.R., 1993. Incorporation and metabolism of (14)C-labelled polyunsaturated fatty acids in juvenile gilthead sea bream *Sparus aurata* L. *in vivo*. Fish Physiol Biochem. 10, 443-453.

Mourente, G., Tocher, D.R., 1994. In vivo metabolism of [1-14C]linolenic acid (18:3(n − 3)) and [1-14C]eicosapentaenoic acid (20:5(n − 3)) in a marine fish: Time-course of the desaturation/elongation pathway. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism. 1212, 109-118.

Nakamura, M.T., Nara, T.Y., 2003. Essential fatty acid synthesis and its regulation in mammals. Prostaglandins Leukot. Essent. Fatty Acids. 68, 145-150.

Nara, T.Y., He, W.S., Tang, C., Clarke, S.D., Nakamura, M.T., 2002. The E-box like sterol regulatory element mediates the suppression of human Δ-6 desaturase gene by highly unsaturated fatty acids. Biochem. Biophys. Res. Commun.. 296, 111-117.

Neuringer, M., Connor, W.E., Lin, D.S., Barstad, L., Luck, S., 1986. Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys. Proc. Natl. Acad. Sci. 83, 4021-4025.

Newton, G., Booram, C., Barker, R., Hale, O., 1977. Dried Hermetia illucens larvae meal as a supplement for swine. J. Anim. Sci. Biotechnol.. 44, 395-400.

Niculescu, M.D., Lupu, D.S., Craciunescu, C.N., 2013. Perinatal manipulation of alpha-linolenic acid intake induces epigenetic changes in maternal and offspring livers. FASEB J. 27, 350-358.

Oboh, A., Kabeya, N., Carmona-Antonanzas, G., Castro, L.F.C., Dick, J.R., Tocher, D.R., Monroig, O., 2017. Two alternative pathways for docosahexaenoic acid (DHA, 22:6n-3) biosynthesis are widespread among teleost fish. Sci. Rep. 7, 3889.

Oliva-Teles, A., Enes, P., Peres, H., 2015. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish, Feed and Feeding Practices in Aquaculture, pp. 203-233.

Otero-Ferrer, F., Lattekivi, F., Ord, J., Reimann, E., Koks, S., Izquierdo, M., Holt, W.V., Fazeli, A., 2020. Time-critical influences of gestational diet in a seahorse model of male pregnancy. J. Exp. Biol. 223.

Oya, J., Nakagami, T., Sasaki, S., Jimba, S., Murakami, K., Kasahara, T., Wasada, T., Sekiguchi, H., Hasegawa, M., Endo, Y., Iwamoto, Y., 2010. Intake of n-3 polyunsaturated fatty acids and non-alcoholic fatty liver disease: a cross-sectional study in Japanese men and women. Eur. J. Clin. Nutr. 64, 1179-1185.

Painter, R.C., Roseboom, T.J., Bleker, O.P., 2005. Prenatal exposure to the Dutch famine and disease in later life: an overview. Reprod. Toxicol. 20, 345-352.

Panserat, S., Kolditz, C., Richard, N., Plagnes-Juan, E., Piumi, F., Esquerre, D., Medale, F., Corraze, G., Kaushik, S., 2008. Hepatic gene expression profiles in juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets. Br. J. Nutr. 100, 953-967.

Panserat, S., Hortopan, G.A., Plagnes-Juan, E., Kolditz, C., Lansard, M., Skiba-Cassy, S., Esquerré, D., Geurden, I., Médale, F., Kaushik, S., Corraze, G., 2009. Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. Aquaculture. 294, 123-131.

Park, J.W., Crowley, D.E., 2005. Normalization of soil DNA extraction for accurate quantification of target genes by real-time PCR and DGGE. Biotechniques. 38, 579-586.

Park, W.J., Kothapalli, K.S., Lawrence, P., Tyburczy, C., Brenna, J.T., 2009. An alternate pathway to long-chain polyunsaturates: the FADS2 gene product Delta8-desaturates 20:2n-6 and 20:3n-3. J. Lipid. Res. 50, 1195-1202.

Pauletto, M., Manousaki, T., Ferraresso, S., Babbucci, M., Tsakogiannis, A., Louro, B., Vitulo, N., Quoc, V.H., Carraro, R., Bertotto, D., Franch, R., Maroso, F., Aslam, M.L., Sonesson, A.K., Simionati, B., Malacrida, G., Cestaro, A., Caberlotto, S., Sarropoulou, E., Mylonas, C.C., Power, D.M., Patarnello, T., Canario, A.V.M., Tsigenopoulos, C., Bargelloni, L., 2018. Genomic analysis of *Sparus aurata* reveals the evolutionary dynamics of sex-biased genes in a sequential hermaphrodite fish. Commun. Biol. 1, 119.

Peng, M., Xu, W., Mai, K., Zhou, H., Zhang, Y., Liufu, Z., Zhang, K., Ai, Q., 2014a. Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. Aquaculture. 433, 442-449.

Peng, S., Yue, Y., Gao, Q., Shi, Z., Yin, F., Wang, J., 2014b. Influence of dietary n-3 LC-PUFA on growth, nutritional composition and immune function in marine fish *Sebastiscus marmoratus*. Chin. J. Oceanol. Limnol.. 32, 1000-1008.

Pereira, J.O.B., Reis-Henriques, M.A., Sanchez, J.L., Costa, J.M., 1998. Effect of protein source on the reproductive performance of female rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac. Res. 29, 751-760.

Pereira, S.L., Leonard, A.E., Mukerji, P., 2003. Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. Prostaglandins Leukot. Essent. Fatty Acids. 68, 97-106.

Perera, E., Yufera, M., 2017. Effects of soybean meal on digestive enzymes activity, expression of inflammation-related genes, and chromatin modifications in marine fish (*Sparus aurata* L.) larvae. Fish Physiol. Biochem. 43, 563-578.

Perera, E., Turkmen, S., Simo-Mirabet, P., Zamorano, M.J., Xu, H., Naya-Catala, F., Izquierdo, M., Perez-Sanchez, J., 2019. Stearoyl-CoA desaturase (scd1a) is epigenetically regulated by broodstock nutrition in gilthead sea bream (*Sparus aurata*). Epigenetics, 1-18.

Pérez-Sánchez, J., Calduch-Giner, J.A., Simó-Mirabet, P., 2018. Updates on the cross talk between growth, endocrine factors, nutrients and environmental factors in marine farmed fish.

Poupard, G., Andre, M., Durliat, M., Ballagny, C., Boeuf, G., Babin, P.J., 2000. Apolipoprotein E gene expression correlates with endogenous lipid nutrition and yolk syncytial layer lipoprotein synthesis during fish development. Cell Tissue Res. 300, 251-261.

Rauwerda, H., Wackers, P., Pagano, J.F., de Jong, M., Ensink, W., Dekker, R., Nehrdich, U., Spaink, H.P., Jonker, M., Breit, T.M., 2016. Mother-specific signature in the maternal transcriptome composition of mature, unfertilized zebrafish eggs. PLoS ONE. 11, e0147151.

Rimoldi, S., Terova, G., Ascione, C., Giannico, R., Brambilla, F., 2018. Next generation sequencing for gut microbiome characterization in rainbow trout (*Oncorhynchus mykiss*) fed animal by-product meals as an alternative to fishmeal protein sources. PLoS ONE. 13, e0193652.

Rinchard, J., Lee, K.J., Czesny, S., Ciereszko, A., Dabrowski, K., 2003. Effect of feeding cottonseed meal-containing diets to broodstock rainbow trout and their impact on the growth of their progenies. Aquaculture. 227, 77-87.

Rosales, M., Castillo, S., Pohlenz, C., Gatlin, D.M., 2017. Evaluation of dried yeast and threonine fermentation biomass as partial fish meal replacements in the diet of red drum *Sciaenops ocellatus*. Anim. Feed Sci. Technol. 232, 190-197.

Rosjo, C., Berg, T., Manum, K., Gjoen, T., Magnusson, S., Thomassen, M.S., 1994. Effects of temperature and dietary n-3 and n-6 fatty acids on endocytic processes in isolated rainbow trout (*Oncorhynchus mykiss*, Walbaum) hepatocytes. Fish Physiol. Biochem. 13, 119-132.

Ryckebosch, E., Bruneel, C., Muylaert, K., Foubert, I., 2012. Microalgae as an alternative source of omega-3 long chain polyunsaturated fatty acids. Lipid Technology. 24, 128-130.

Saera-Vila, A., Calduch-Giner, J.A., Perez-Sanchez, J., 2005. Duplication of growth hormone receptor (GHR) in fish genome: gene organization and transcriptional regulation of GHR type I and II in gilthead sea bream (*Sparus aurata*). Gen. Comp. Endocrinol. 142, 193-203.

Saera-Vila, A., Calduch-Giner, J.A., Perez-Sanchez, J., 2007. Co-expression of IGFs and GH receptors (GHRs) in gilthead sea bream (*Sparus aurata* L.): sequence analysis of the GHR-flanking region. J. Endocrinol. 194, 361-372.

Sakamoto, T., McCormick, S.D., Hirano, T., 1993. Osmoregulatory actions of growth hormone and its mode of action in salmonids: A review. Fish Physiol. Biochem. 11, 155-164.

Santigosa, E., Sánchez, J., Médale, F., Kaushik, S., Pérez-Sánchez, J., Gallardo, M.A., 2008. Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. Aquaculture. 282, 68-74.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2003. The lipids. In Fish nutrition. Elsevier, Amsterdam, Netherlands. pp. 181-257.

Schaeffer, L., Gohlke, H., Muller, M., Heid, I.M., Palmer, L.J., Kompauer, I., Demmelmair, H., Illig, T., Koletzko, B., Heinrich, J., 2006. Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. Hum. Mol. Genet. 15, 1745-1756.

Schmidt, E.B., Skou, H.A., Christensen, J.H., Dyerberg, J., 2000. N-3 fatty acids from fish and coronary artery disease: implications for public health. Public Health Nutr. 3, 91-98.

Seiliez, I., Panserat, S., Corraze, G., Kaushik, S., Bergot, P., 2003. Cloning and nutritional regulation of a Delta6-desaturase-like enzyme in the marine teleost gilthead seabream (*Sparus aurata*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 135, 449-460.

Sink, T.D., Lochmann, R.T., Pohlenz, C., Buentello, A., Gatlin, D., 2010. Effects of dietary protein source and protein–lipid source interaction on channel catfish (*Ictalurus punctatus*) egg biochemical composition, egg production and quality, and fry hatching percentage and performance. Aquaculture. 298, 251-259.

Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sánchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture. 249, 387-400.

Sprecher, H., 2000. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim Biophys Acta Mol Cell Biol Lipids. 1486, 219-231.

Stanley-Samuelson, D.W., Jurenka, R.A., Loher, W., Blomquist, G.J., 1987. Metabolism of polyunsaturated fatty acids by larvae of the waxmoth, *Galleria mellonella*. Arch. Insect Biochem. Physiol. 6, 141-149.

Sullivan, C.V., Chapman, R.W., Reading, B.J., Anderson, P.E., 2015. Transcriptomics of mRNA and egg quality in farmed fish: Some recent developments and future directions. Gen. Comp. Endocrinol. 221, 23-30.

Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture. 285, 146-158.

Takeuchi, T., Toyota, M., Satoh, S., Watanabe, T., 1990. Requirement of juvenile red seabream Pagrus major for eicosapentaenoic and docosahexaenoic acids. Nippon Suisan Gakkaishi. 56, 1263-1269.

Tandler, A., 1995. Broodstock and larval nutrition in gilthead seabream Sparus aurata : New findings on its mode of involvement in improving growth, survival and swimbladder inflation. Isr. J. Aquacult. 47, 95-111.

Tanomman, S., Ketudat-Cairns, M., Jangprai, A., Boonanuntanasarn, S., 2013. Characterization of fatty acid delta-6 desaturase gene in Nile tilapia and heterogenous expression in *Saccharomyces cerevisiae*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 166, 148-156.

Tocher, D.R., Ghioni, C., 1999. Fatty acid metabolism in marine fish: low activity of fatty acyl delta5 desaturation in gilthead sea bream (*Sparus aurata*) cells. Lipids. 34, 433-440.

Tocher, D.R., Fonseca-Madrigal, J., Bell, J.G., Dick, J.R., Henderson, R.J., Sargent, J.R., 2002. Effects of diets containing linseed oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes in Atlantic salmon (*Salmo salar*). Fish Physiol. Biochem.. 26, 157-170.

Tocher, D. R., Zheng, X., Schlechtriem, C., Hastings, N., Dick, J.R., Teale, A.J., 2006. Highly unsaturated fatty acid synthesis in marine fish: Cloning, functional characterization, and nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua* L.). Lipids. 41(11), 1003-1016

Torrecillas, S., Robaina, L., Caballero, M.J., Montero, D., Calandra, G., Mompel, D., Karalazos, V., Kaushik, S., Izquierdo, M.S., 2017a. Combined replacement of fishmeal and fish oil in European sea bass (*Dicentrarchus labrax*): Production performance, tissue composition and liver morphology. Aquaculture. 474, 101-112.

Torrecillas, S., Caballero, M.J., Mompel, D., Montero, D., Zamorano, M.J., Robaina, L., Rivero-Ramirez, F., Karalazos, V., Kaushik, S., Izquierdo, M., 2017b. Disease resistance and response against Vibrio anguillarum intestinal infection in European seabass (*Dicentrarchus labrax*) fed low fish meal and fish oil diets. Fish Shellfish Immunol. 67, 302-311.

Torrecillas, S., Mompel, D., Caballero, M.J., Montero, D., Merrifield, D., Rodiles, A., Robaina, L., Zamorano, M.J., Karalazos, V., Kaushik, S., Izquierdo, M., 2017c. Effect of fishmeal and fish oil replacement by vegetable meals and oils on gut health of European sea bass (*Dicentrarchus labrax*). Aquaculture. 468, 386-398.

Torres, M., Navarro, J.C., Varó, I., Agulleiro, M.J., Morais, S., Monroig, Ó., Hontoria, F., 2020. Expression of genes related to long-chain (C18–22) and very long-chain (>C24) fatty acid biosynthesis in gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) larvae: investigating early ontogeny and nutritional regulation. Aquaculture. 520.

Turkmen, S., Zamorano, M.J., Fernandez-Palacios, H., Hernandez-Cruz, C.M., Montero, D., Robaina, L., Izquierdo, M., 2017a. Parental nutritional programming and a reminder during juvenile stage affect growth, lipid metabolism and utilisation in later

developmental stages of a marine teleost, the gilthead sea bream (*Sparus aurata*). Br. J. Nutr. 118, 500-512.

Turkmen, S., Hernandez-Cruz, C.M., Zamorano, M.J., Fernandez-Palacios, H., Montero, D., Afonso, J.M., Izquierdo, M., 2019a. Long-chain PUFA profiles in parental diets induce long-term effects on growth, fatty acid profiles, expression of *fatty acid desaturase 2* and selected immune system-related genes in the offspring of gilthead seabream. Br. J. Nutr. 122, 25-38.

Turkmen, S., Perera, E., Zamorano, M.J., Simo-Mirabet, P., Xu, H., Perez-Sanchez, J., Izquierdo, M., 2019b. Effects of dietary lipid composition and fatty acid desaturase 2 expression in broodstock gilthead sea bream on lipid metabolism-related genes and methylation of the *fads2* gene promoter in their offspring. Int. J. Mol. Sci. 20.

Turkmen, S., Zamorano, M.J., Xu, H., Fernandez-Palacios, H., Robaina, L., Kaushik, S., Izquierdo, M., 2020. Parental LC-PUFA biosynthesis capacity and nutritional intervention with ALA affect performance of *Sparus aurata* progeny. J Exp Biol.

Turkmen, S., Castro, P.L., Caballero, M.J., Hernández-Cruz, C.M., Saleh, R., Zamorano, M.J., Regidor, J., Izquierdo, M., 2017b. Nutritional stimuli of gilthead seabream (*Sparus aurata*) larvae by dietary fatty acids: effects on larval performance, gene expression and neurogenesis. Aquac. Res. 48, 202-213.

Vagner, M., Santigosa, E., 2011. Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: A review. Aquaculture. 315, 131-143.

Vagner, M., Zambonino Infante, J.L., Robin, J.H., Person-Le Ruyet, J., 2007. Is it possible to influence European sea bass (*Dicentrarchus labrax*) juvenile metabolism by a nutritional conditioning during larval stage? Aquaculture. 267, 165-174.

Vagner, M., Robin, J.H., Zambonino-Infante, J.L., Tocher, D.R., Person-Le Ruyet, J., 2009. Ontogenic effects of early feeding of sea bass (*Dicentrarchus labrax*) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. Br. J. Nutr. 101, 1452-1462.

Velez, E.J., Lutfi, E., Jimenez-Amilburu, V., Riera-Codina, M., Capilla, E., Navarro, I., Gutierrez, J., 2014. IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes. Gen. Comp. Endocrinol. 205, 296-304.

Vera, L.M., Metochis, C., Taylor, J.F., Clarkson, M., Skjaerven, K.H., Migaud, H., Tocher, D.R., 2017. Early nutritional programming affects liver transcriptome in diploid and triploid Atlantic salmon, *Salmo salar*. BMC Genomics. 18, 886.

Vergara, J., Manuel,, Robainà, L., Izquierdo, M., Higuera, M.D.L., 1996a. Protein sparing effect of lipids in diets for fingerlings of gilthead sea bream. Fisheries science. 62, 624-628.

Vergara, J., Manuel,, Fernández-Palacios, H., Robainà, L., Jauncey, K., Higuera, M.D.L., Izquierdo, M., 1996b. The effects of varying dietary protein level on the growth, feed efficiency, protein utilization and body composition of gilthead sea bream fry. Fisheries science. 62, 620-623.

Vergauwen, L., Cavallin, J.E., Ankley, G.T., Bars, C., Gabriels, I.J., Michiels, E.D.G., Fitzpatrick, K.R., Periz-Stanacev, J., Randolph, E.C., Robinson, S.L., Saari, T.W.,

Schroeder, A.L., Stinckens, E., Swintek, J., Van Cruchten, S.J., Verbueken, E., Villeneuve, D.L., Knapen, D., 2018. Gene transcription ontogeny of hypothalamic-pituitary-thyroid axis development in early-life stage fathead minnow and zebrafish. Gen. Comp. Endocrinol. 266, 87-100.

Wang, Q., He, G., Mai, K., Xu, W., Zhou, H., 2016. Fishmeal replacement by mixed plant proteins and maggot meal on growth performance, target of rapamycin signalling and metabolism in juvenile turbot (*Scophthalmus maximus* L.). Aquac. Nutr. 22, 752-758.

Wang, Y., Zhu, Q., Zhao, X.L., Yao, Y.G., Liu, Y.P., 2011. Age-related expression profile of the SLC27A1 gene in chicken tissues. Mol. Biol. Rep. 38, 5139-5145.

Wassall, S.R., Stillwell, W., 2008. Docosahexaenoic acid domains: the ultimate non-raft membrane domain. Chem. Phys. Lipids. 153, 57-63.

Watanabe, T., 1993. Importance of Docosahexaenoic Acid in Marine Larval Fish. J World Aquac Soc. 24, 152-161.

Watanabe, T., Kiron, V., 1994. Prospects in larval fish dietetics. Aquaculture. 124, 223-251.

Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S., Kitajima, C., 1989. Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabream. Nippon Suisan Gakkaishi. 55, 1635-1640.

Wiegand, M.D., 1996. Utilization of yolk fatty acids by goldfish embryos and larvae. Fish Physiol. Biochem. 15, 21-27.

Xia, J., Lin, G., He, X., Yunping, B., Liu, P., Liu, F., Sun, F., Tu, R., Yue, G., 2014. Mapping quantitative trait loci for omega-3 fatty acids in Asian seabass. Mar Biotechnol. 16, 1-9.

Xie, L., Innis, S.M., 2008. Genetic variants of the *FADS1 FADS2* gene cluster are associated with altered (n-6) and (n-3) essential fatty acids in plasma and erythrocyte phospholipids in women during pregnancy and in breast milk during lactation. J. Nutr. 138, 2222-2228.

Xu, D., He, G., Mai, K., Wang, Q., Li, M., Zhou, H., Xu, W., Song, F., 2017. Effect of fish meal replacement by plant protein blend on amino acid concentration, transportation and metabolism in juvenile turbot (*Scophthalmus maximus* L.). Aquac. Nutr. 23, 1169-1178.

Xu, H., Dong, X., Ai, Q., Mai, K., Xu, W., Zhang, Y., Zuo, R., 2014. Regulation of tissue LC-PUFA contents, delta6 fatty acyl desaturase (*fads2*) gene expression and the methylation of the putative *fads2* gene promoter by different dietary fatty acid profiles in Japanese seabass (*Lateolabrax japonicus*). PLoS ONE. 9, e87726.

Xu, H., Turkmen, S., Rimoldi, S., Terova, G., Zamorano, M.J., Afonso, J.M., Sarih, S., Fernández-Palacios, H., Izquierdo, M., 2019. Nutritional intervention through dietary vegetable proteins and lipids to gilthead sea bream (*Sparus aurata*) broodstock affects the offspring utilization of a low fishmeal/fish oil diet. Aquaculture. 513.

Zhang, J., Zhang, F., Didelot, X., Bruce, K.D., Cagampang, F.R., Vatish, M., Hanson, M., Lehnert, H., Ceriello, A., Byrne, C.D., 2009. Maternal high fat diet during pregnancy and lactation alters hepatic expression of insulin like growth factor-2 and key microRNAs in the adult offspring. BMC Genomics. 10, 478.

Zheng, X., Leaver, M.J., Tocher, D.R., 2009. Long-chain polyunsaturated fatty acid synthesis in fish: Comparative analysis of Atlantic salmon (*Salmo salar* L.) and Atlantic cod

(*Gadus morhua* L.) Delta6 fatty acyl desaturase gene promoters. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 154, 255-263.

Zuo, R., Ai, Q., Mai, K., Xu, W., Wang, J., Xu, H., Liufu, Z., Zhang, Y., 2012a. Effects of dietary docosahexaenoic to eicosapentaenoic acid ratio (DHA/EPA) on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (*Cryptocaryon irritans*). Aquaculture. 334-337, 101-109.

Zuo, R., Ai, Q., Mai, K., Xu, W., Wang, J., Xu, H., Liufu, Z., Zhang, Y., 2012b. Effects of dietary n-3 highly unsaturated fatty acids on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (*Cryptocaryon irritans*). Fish Shellfish Immunol. 32, 249-258.

Annex

SCI Publications

1. **Xu, H.**, Turkmen, S., Rimoldi, S., Terova, G., Zamorano, M.J., Afonso, J.M., Sarih, S., Fernández-Palacios, H., Izquierdo, M., 2019. Nutritional intervention through dietary vegetable proteins and lipids to gilthead sea bream (*Sparus aurata*) broodstock affect the offspring utilization on low fishmeal fish oil diet. Aquaculture. 513, 734402.
2. **Xu, H.**, Ferosekhan, S., Turkmen, S., Zamorano, M.J., Afonso, J.M., Izquierdo, M., Influence of parental *fatty acid desaturase 2* (*fads2*) expression and diet on gilthead seabream (*Sparus aurata*) offspring *fads2* expression along ontogenesis. Life, in press

Collaborative works in SCI publications

1. Ferosekhan, S., Turkmen, S., **Xu, H.**, Afonso, J.M., Zamorano, M.J., Kaushik, S., Izquierdo, M., 2020a. The Relationship between the Expression of Fatty Acyl Desaturase 2 (*fads2*) Gene in Peripheral Blood Cells (PBCs) and Liver in Gilthead Seabream, Sparus aurata Broodstock Fed a Low n-3 LC-PUFA Diet. Life. 10, 117.
2. Ferosekhan, S., **Xu, H.**, Turkmen, S., Gómez, A., Afonso, J.M., Fontanillas, R., Rosenlund, G., Kaushik, S., Izquierdo, M., 2020b. Reproductive performance of gilthead seabream (*Sparus aurata*) broodstock showing different expression of fatty acyl desaturase 2 and fed two dietary fatty acid profiles. Sci. Rep. 10, 15547.
3. Perera, E., Turkmen, S., Simo-Mirabet, P., Zamorano, M.J., **Xu, H.**, Naya-Catala, F., Izquierdo, M., Perez-Sanchez, J., 2019. Stearoyl-CoA desaturase (scd1a) is epigenetically regulated by broodstock nutrition in gilthead sea bream (*Sparus aurata*). Epigenetics, 1-18.
4. Turkmen, S., Perera, E., Zamorano, M.J., Simo-Mirabet, P., **Xu, H.**, Perez-Sanchez, J., Izquierdo, M., 2019. Effects of dietary lipid composition and *fatty acid desaturase 2* expression in broodstock gilthead sea bream on lipid metabolism-related genes and methylation of the *fads2* gene promoter in their offspring. Int. J. Mol. Sci. 20, 6250.
5. Turkmen, S., Zamorano, M.J., **Xu, H.**, Fernandez-Palacios, H., Robaina, L., Kaushik, S., Izquierdo, M., 2020. Parental LC-PUFA biosynthesis capacity and nutritional intervention with ALA affect performance of *Sparus aurata* progeny. J Exp Biol.

Oral presentation at international conference

1. **Xu, H.**, Turkmen, S., Ferosekhan, S., Zamorano, M.J., Afonso, J.M., Izquierdo, M., 2018. Effect of low fishmeal and fish oil diets on nutritionally programmed juvenile gilthead sea bream growth performance, liver fatty acid composition and gene expression. 18th International Symposium on Fish Nutrition and Feeding, Las Palmas, Spain.
2. **Xu, H.,** 2019. Effect of broodstock nutritional programming on the utility of low fishmeal and low fish oil diet in offspring. Reti symposium 2019, Las Palmas, Spain.
3. **Xu. H.**, Rimoldi, S., Ferosekhan, S., Turkmen, S., Afonso, J.M., Zamorano, M.J., Montero, D., Izquierdo, M., 2019. Improved use of low fm and low fo diets in gilthead seabream (*Sparus aurata*) juveniles obtained by combined broodstock selection and nutritional programing. Aquaculture Europe 2019, Berlin, Germany.

4. **Xu. H.**, Ferosekhan, S., Turkmen, S., Afonso, J.M., Montero, D., Izquierdo, M., 2020. The effect of the broodstock nutritional background on the growth of gilthead sea bream (*Sparus aurata*) juveniles fed with low fish meal and low fish oil content diet. Epimar, online.